Guide for Practical Biochemistry

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Albert Berghuis



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Permissions

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PREFACE

Readers will get a clear picture of the developments in the area through the book "Laboratory Manual for Practical Biochemistry." It summarizes recent findings and offers a framework for future investigation in the field.

The scientific field of biochemistry is concerned with the chemical and physical-chemical elements and activities that take place inside living things. A great diversity of biochemical events that may be understood using different biochemical tools shape living systems. To ensure that readers have a thorough knowledge of the topic, every attempt has been made to clarify these biochemical approaches throughout the book simply and clearly. Unlike other basic science subjects like Chemistry, Zoology and Botany, Biochemistry practical generally requires a variety of chemicals and expensive equipment. The fact that this work addresses the practical applications of several biochemical procedures for life science undergraduate and graduate students is one of its features. It covers all subjects pertinent to this area. The information in this article is simply a combination of data and analyses from many sources. Some of the most popular experimental techniques in biochemistry are introduced here. Additionally, some of the instruments typically used in biochemistry will be known to readers. This book will be helpful to researchers and students in the subject.

I am highly obliged to my friends and family for their valuable support in finishing this book. Also, appreciate the continued support by the publisher to achieve this goal.

-Author

1

The Effect of Curcumin (as Meriva) on Absolute Lymphocyte Count (ALC), NK Cells and T Cell Populations in Patients with Stage 0/1 Chronic Lymphocytic Leukemia

Terry Golombick[1*], Terrence H. Diamond[1], Arumugam Manoharan[2], Rajeev Ramakrishna[2]

[1]Department of Endocrinology, St George Hospital, Sydney, Australia

[2]Southern Sydney Haematology, University of Wollongong, Wollongong, Australia

Abstract

Purpose: To determine the effect of curcumin (as Meriva) on absolute lymphocyte count (ALC), T cell populations and NK cells in patients with Rai stage 0/1 chronic lymphocytic leukemia (CLL) over a period of six months. Experimental Design: Twenty-one patients with significant lymphocytosis (>20 × 10^9 lymphocytes/L) and stage 0/1 CLL were recruited into the study and received oral curcumin 2 grams/day. Blood samples were collected at baseline and at 2 monthly intervals for six months for full blood count, leukocyte surface antigens, liver function, serum biochemistry, immunoglobulins, CRP and ESR. A positive biologic response was defined as a reduction in the ALC of more than 20% from pre-treatment levels. Results: Four patients (20%) demonstrated more than 20% decrease in ALC, three of whom had lower ALC at the end of the study as compared to baseline. The decrease in ALC was accompanied by an increase in CD4, CD8 and NK cells. No demonstrable response was seen in seventeen patients (80%) who exhibited stable, fluctuating or increasing ALC during the study. Conclusion: A subgroup of stage 0/1 CLL patients may be responsive to curcumin therapy. The beneficial response appears to be immunomodulated.

Keywords

Stage 0/1 Chronic Lymphocytic Leukemia, Curcumin

1. Introduction

Chronic lymphocytic leukemia (CLL) is the most common type of leukemia in the Western world [1]. It involves mature-appearing defective neoplastic lymphocytes (almost always B cells) with an abnormally long life span. The peripheral blood, bone marrow, spleen, and lymph nodes undergo leukemic infiltration. Nonspecific symptoms include fatigue and malaise and are usually attributable to the anemia. Clinical signs include lymphadenopathy, splenomegaly and hepatomegaly. Diagnosis is confirmed by examination of peripheral smear and bone marrow aspirate. Patients with CLL have been shown to have elevated T cell (CD3, CD4 and CD8) and NK cell populations [2]. A higher number of T and NK cells have been associated with delayed disease progression and time to treatment [3].

Unlike most malignant disorders in which early diagnosis increases the probability of a patient cure, treatment of unselected early-stage CLL patients with chemotherapy is associated with increased toxicity and no increase in survival [4] [5]. There is no published intervention that alters the course of disease for patients with early stage CLL *i.e.* Stage 0 or 1 disease (Rai staging system) [6]. These patients are usually monitored and commence therapeutic intervention if there is disease progression. This approach may often lead to patient's anxiety. These patients represent an excellent cohort to evaluate the efficacy of nutraceutical agents (with a favourable toxicity) for disease prevention or delay in disease progression (PD).

Nutraceutical agents such as epigallocatechin gallate (EGCG) derived from green tea, and curcumin, derived from turmeric, have been proposed as health-promoting substances that may reduce cancer risk. Phase 1 and 2 studies using oral green tea extract (EGCG) [7]-[9] have shown that patients with low grade CLL with/out lymphadenopathy demonstrate durable declines in ALC, suggesting an anti-cancer responsiveness.

Curcumin has been shown to be a potent cytotoxic agent for primary CLL B cells by inhibiting specific pro-survival pathways such as STAT3, AKT and NF-kB [10]. Moreover, curcumin also suppresses the expression of the anti-apoptotic proteins Mcl-1 and X-linked inhibitor of apoptosis protein (XIAP), and up-regulates the pro-apoptotic protein BIM. A combination of green tea extract and curcumin selectively upregulates STAT3 protein and its phosphorylation status when administered in co-culture of CLL B cells with stromal cells, while curcumin alone is able to overcome the stromal-induced protection when administered with EGCG in a sequential fashion [11]. These data suggest a potential therapeutic role for curcumin in the treatment of stage 0/1 CLL.

We administered curcumin as Meriva, a standardized mixture of natural curcuminoids and lecithin in a 1:2 ratio [12] [13], to 21 individuals with stage 0/1 CLL to determine 1) its effect on ALC, and 2) whether any observed effect could be mediated by the numbers of CD4, CD8 and NK cells. Our previous data has demonstrated the safety and efficacy of this compound in MGUS/SMM patients [14]-[17]. Meriva was chosen for this study based on its increased absorption and bioavailability.

2. Materials and Methods

Twenty-one patients with significant lymphocytosis (>20 × 10^9 lymphocytes/L) and Rai stage 0 (early CLL and lymphocytosis) or stage 1 (CLL and lymphadenopathy) were recruited. Blood was collected at 6 and 3 months prior to commencement of therapy, at 2 monthly intervals for a period of six months during therapy and at 3 months after completion of therapy. Patients were administered oral curcumin (as Meriva) 2 grams/daily in a divided dose. Blood samples were collected for full blood count, leukocyte surface antigens, liver function, serum biochemistry, immunoglobulins, CRP and ESR at each visit. A positive biologic response was defined as more than 20% reduction in the ALC from the pre-treatment (baseline) levels.

The protocol was reviewed and approved by the South Eastern Sydney and Illawarra ethics committee. All patients provided written informed consent before study enrolment in accordance with the Declaration of Helsinki.

3. Results

There were 14 men and 7 women with mean age of 68 (±12.8) years and mean baseline ALC of 30.1 × 10^9 cells/L (normal 1.0 - 4.0 × 10^9/L). **Table 1** and **Table 2** show the mean baseline clinical and immunological data. Twenty-one patients completed 2 months of the study, 3 withdrew at month 2 and a further 2 at month 4. Most patients in this study had elevated baseline populations of CD4 (mean 2.2 × 10^9/L, normal 0.7 - 2.1 × 10^9/L), CD8 (mean 2 × 10^9/L, normal 0.2 - 0.9 × 10^9/L) and NK (mean 1.1 × 10^9/L, normal 0.05 - 0.6 × 10^9/L) cells and low IgM values (mean 0.3 g/L, normal 0.48 - 3.04 g/L).

There were four patients (20%) who demonstrated a positive biologic response *i.e.* >20% reduction in ALC during the course of the study (**Table 3**). Their pre-study, during study and post-study ALC values are shown in **Table 3**. Three of the four had a lower ALC at the end of the 6 month study period. All four patients showed a relapse and an increase in ALC at three months post-study drug withdrawal. The decrease in ALC was accompanied by an increase in the CD4 count in all 4 patients (100%) after 2 months of curcumin therapy, and an increase in CD8 and NK cells in 2 (50%) patients after 2 months of curcumin therapy. The elevated CD4, CD8 and NK cell counts returned to baseline values at 6 months in all patients. The mean CD4, CD8 and NK cell populations at baseline and during the study are shown in **Table 4**.

Seventeen patients (80%) failed to show a positive response to curcumin therapy. They showed stable, fluctuating or increases in ALC during the course of the study (**Table 5**). The pre-study, during study and post-study ALC values of these patients are shown in **Table 5**. Among the non-responders, 6 (35%) showed an increase in CD4 count after 2 months of curcumin therapy, 4 (23%) showed an increase in CD8 count and 4 (23%) showed an increase in NK cell count after 2 months of curcumin therapy.

Table 1. Mean baseline clinical and haematological data in all patients.

	Age	WCC ($4 - 11 \times 10^9$/L)	ALC ($1 - 4 \times 10^9$/L)	Hb (130 - 180 g/L)	Pltlts ($150 - 450 \times 10^9$/L)	LDH (120 - 250 U/L)	ALT (5 - 40 U/L)	Hct (0.39 - 0.54)
Mean ± SD	68 ± 12.8	35.0 ± 9.9	30.1 ± 10.1	142.3 ± 12.7	185.9 ± 49.7	196.7 ± 34.2	27.1 ± 14.9	0.43 ± 0.03

Table 2. Mean baseline immunological data in all patients.

	IgG (6.2 - 14.4 g/L)	IgA (0.6 - 3.96 g/L)	IgM (0.48 - 3 g/L)	CD3 (0.7 - 2.1 $\times 10^9$/L)	%CD3	CD4 (0.4 - 1.4 $\times 10^9$/L)	%CD4	CD8 (0.2 - 0.9 $\times 10^9$/L)	%CD8	CD 4/8 (1 - 3)	NK (0.05 - 0.6 $\times 10^9$/L)	% NK	CD19 (0.05 - 0.5 $\times 10^9$/L)	%CD19
Mean ± SD	8.8 ± 2.8	1.3 ± 0.9	0.3 ± 0.2	4.7 ± 2.3	17 ± 9.3	2.3 ± 1.2	8.2 ± 4.5	2 ± 1.4	7 ± 5.2	1.5 ± 0.7	1.1 ± 0.7	3.9 ± 2.6	24.5 ± 10.1	79.7 ± 9.9

Table 3. ALC counts of responding patients at 6 months and 3 months pre-study, during study and 3 months post-study.

Patient No.	−6 (n = 4)	−3 (n = 4)	0 (n = 4)	2 (n = 4)	4 (n = 3)	6 (n = 3)	+3 (n = 3)
1	24.9	22.8	26	18.2			20.2
4	32	34.4	43.9	42.3	34.3	41.3	44.3
9	48.5	43.9	49.9	30.2	31.1	36.7	43.2
11	18.3	29.5	38.3	38.7	24.7	40.6	
Mean ± SD	30.9 ± 12.9	32.7 ± 8.9	39.5 ± 10.2	32.3 ± 10.7	30 ± 4.9	39.5 ± 2.5	35.9 ± 13.6

Table 4. CD4, CD8 and NK cell counts (Mean ± SD) in responding and non-responding patients.

	Baseline	2 months	p-value	4 months	p-value	6 months	p-value
CD4 resp.	1.38 ± 0.5 (n = 4)	2.93 ± 1.6 (n = 4)	0.1	1.11 ± 0.4 (n = 3)	0.1	1.2 ± 0.3 (n = 3)	0.1
CD4 non-resp.	2.43 ± 0.9 (n = 17)	2.37 ± 0.9 (n = 17)	0.8	2.07 ± 0.8 (n = 15)	0.2	2.17 ± 0.8 (n = 13)	0.2
CD8 resp.	2 ± 1.5 (n = 4)	2.68 ± 1.9 (n = 4)	0.3	1.8 ± 1.2 (n = 3)	0.3	1.4 ± 0.3 (n = 3)	0.3
CD8 non-resp.	2.03 ± 1.4 (n = 17)	1.61 ± 0.9 (n = 17)	0.1	1.63 ± 0.9 (n = 15)	0.9	1.5 ± 0.7 (n = 13)	0.3
NK resp.	1.11 ± 0.3 (n = 4)	1.46 ± 0.7 (n = 4)	0.3	0.69 ± 0.5 (n = 3)	0.3	0.83 ± 0.3 (n = 3)	0.3
NK non-resp.	1.06 ± 0.8 (n = 17)	1.09 ± 0.7 (n = 17)	0.9	1 ± 0.6 (n = 15)	0.3	1.2 ± 0.9 (n = 13)	0.3

Table 5. ALC counts of non-responding patients at 6 months and 3 months pre-study, during study and 3 months post-study.

Patient No.	−6 (n = 17)	−3 (n = 17)	0 (n = 17)	2 (n = 17)	4 (n = 15)	6 (n = 13)	+3 (n = 15)
2	34.3	31.1	30.7	40.2	30.6	51.5	30
3	50.9	47.4	44.1	45.8	49.3	55.7	63.6
5	23.3	25.4	25.9	25.3	23.4	27.0	32.3
6	32.9	37.2	23.9	24.1	28.4	23.3	33.4
7	13.8	18.3	14.6	17.9			12.8
8	30.2	37.9	33.9	30.4	36.2		36.5
10	17.8	17.2	21.7	18.6	19		
12	43.9	43.9	38.3	40			
13	20.7	23.2	20.2	23.2	19.8	27.4	25.8
14	21.5	25.7	18.9	29.9	26.7	24.5	27.9
15	21.8	17.4	23.8	23.7	28.2	29.7	37.9
16	16.1	22.9	25.3	30.6	35	38	36.5
17	20.4	25.7	29.3	25.9	24.2	39.3	47.7
18	41.2	32	44.8	45.2	56.5	54	54.7
19	41.2	45.5	35.8	40.3	35.9	35.8	51.8
20	17.6	20.8	18.9	21	19.5	19.3	20.2
21	20.9	18.7	23.1	19.9	23.7	23.9	24.9
Mean ± SD	27.6 ± 11.2	28.8 ± 10.2	27.8 ± 8.9	29.5 ± 9.4	30.4 ± 10.8	34.6 ± 12.4	35.7 ± 13.7

Responding patients (*i.e.* >20% decrease in ALC) show a greater increase in T cell and NK cell response (increase in CD4, CD8 and NK cells with R = 0.81, 0.8 and 0.7 respectively) as compared to the non-responders (R = 0.09, 0.05 and −0.2 respectively).

4. Discussion

Our study results suggest that curcumin may be beneficial in a small subset of early CLL patients due to its anti-tumour activity. The decrease in ALC count seen in 4 (20%) of these patients corresponded with an increase in CD4 T cells, a similar finding to Varalakshami [18] in their mouse model. An increase in CD8 T cell and NK cells was also seen in 2 of the responders and 4 of the non-responders, perhaps due to curcumin therapy. Immunomulation of CD4 T cell, CD8 T cell and NK cell subsets has been found in numerous studies on curcumin therapy [18]-[21].

There is a marked clinical heterogeneity in patients with CLL that is associated with a heterogeneous array of genetic and molecular defects [3]. The complexity of this malignancy is further increased by the interaction of leukaemia cells with the microenvironment. Leukaemia cells closely interact with accessory and immune cells that regulate their trafficking, survival and proliferation. Patients with leukemia have been shown to have elevated T and NK cell populations and the relative numbers of T and NK cells at diagnosis has been associated with clinical outcome. A study by Huergo-Zapigo [3] has shown that CLL patients with a relative CD8 count > 0.03 showed significantly longer time to treatment than those with lower numbers of CD8 T cells, suggesting that CD8 may modify disease progression. This suggests that the non malignant immune system may play an important role in the pathogenesis and progression of this malignancy and that the expansion of T cells observed in patients with CLL may be due, in part, to the expansion of anti-tumor T cells [2]. A possible role for curcu-

min therapy may be further expansion of these anti-tumor T cells. Our results suggests that a decrease in the absolute lymphocyte count of responding patients may be due to an augmentation of the T cell response.

The expected pattern for the majority of patients with early CLL is a rising ALC and progressive lymphadenopathy. Fluctuations in ALC can be expected in a subset of patients with CLL. Our data supports these findings as shown by the ALC fluctuations seen in the non-responding patients (80%).

Limitations of this study include the small number of patients and the number of patients who dropped out of the study. Among the 5 patients that did not complete the 6 month study, 2 withdrew due to gastro-intestinal disturbance caused by the curcumin. No other toxicity was noted in the remaining patients.

Curcumin has a high hydrolytic instability at physiological pH and an inherently low intestinal absorption. It is therefore poorly bioavailable, with only conjugates being generally detectable in plasma even after dosages as high as 12 g/day. Curcumin is sparingly soluble in both water and lipophilic organic solvents, but has polar groups that make it a good candidate for phospholipid complexation. A novel curcumin formulation was developed under the brand name Meriva by combining a standardized mixture of natural curcuminoids and lecithin in 1:2 ratio, with 2 parts of microcrystalline cellulose being added to improve the physical state. The relative absorption of a standardized curcuminoid mixture and its corresponding lecithin formulation (Meriva) was investigated in a randomized, double-blind, cross-over human study and total curcuminoid absorption was about 29-fold higher for Meriva than for its corresponding unformulated curcuminoid-like mixture [12] [13]. Thus Meriva was chosen for this study solving the issue of megadoses even though curcumin had been found to be safe even at doses as high as 12 g/day. There is safety data with a number of patients treated with curcumin for as long as 10 years at a dose of 6 g/day (Golombick *et al.* Unpublished Data).

Although current prognostic indicators [ZAP-70, CD38, *IGHV* mutation status, fluorescence *in situ* hybridization (FISH)] are able to identify early stage CLL patients at high risk of rapid disease progression, these tests are not routinely performed in our laboratory and were not performed as part of their work-up.

5. Conclusion

In conclusion, the results of this 6-month study suggest that a small percentage of early CLL patients may derive benefit from curcumin therapy by decreasing their ALC, perhaps through stimulating an immune response by increasing CD4, CD8 and NK cells. CD8 cytotoxic T lymphocytes are involved in antigen-specific tumor destruction and CD4 T cells are essential for helping the CD8 T cell-dependent tumor eradication. A number of nutraceutical agents have been shown to have an immunostimulatory effect and combining their action with curcumin may be synergistic. The analysis of the number of different subsets of T cells should be taken with caution since the functionality of these subsets may be diverse. Follow-up activation studies are suggested. Our results suggest that a larger study is warranted.

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2

Uranium Metabolism Associated with Ontogenetic Growth of Birds—Case Studies with Broilers and Ducks

Joao Dias de Toledo Arruda-Neto[1,2], Guacyara Tenorio Cavalcante[1], Guilherme de Paula Nogueira[3], Tulio Eduardo Rodrigues[1], Luiz Eduardo Correa Fonseca[3], Mitiko Saiki[4], Godofredo Camara Genofre[2,5]

[1]Physics Institute, University of São Paulo, São Paulo, Brazil
[2]CEPESq/UniÍtalo—Italy-Brazilian University Center, São Paulo, Brazil
[3]School of Veterinary Medicine, São Paulo State University/UNESP, Araçatuba, Brazil
[4]Institute for Energy and Nuclear Research, IPEN/CNEN-SP, São Paulo, Brazil
[5]Institute for Biomedical Sciences, University of São Paulo, São Paulo, Brazil

Abstract

Cobb broilers and domestic ducks, both one-day-old, were treated using ration doped with 20 ppm of uranyl nitrate. Uranium concentrations in the tibia (μg-U/g-bone) were measured by neutron activation analysis as function of the animals' age, from the neonatal period to maturity. Results show that Uranium and Calcium qualitatively follow the same metabolic pathway, and that adult ducks incorporate on average ten times more Uranium than broilers. Data interpretation shows that the Uranium clearance rate in broilers is substantially higher than that in ducks, suggesting that metabolic characteristics favoring Calcium retention in bone may hinder the elimination of Uranium in ducks. The need for further comparative biochemistry studies between Galliformes and Anseriformes is addressed.

Keywords

Cobb Broilers, Domestic Ducks, Uranium-Doped-Ration, Uranium-Content in Bones, U/Ca Metabolism, Uranium Residence Times

1. Introduction

Uranium, as well as strontium is usually classified as a "familiar element", as a result of the existence of readily

available natural sources. The interest in uranium metabolism stems from the fact that it is a ubiquitous and a potentially hazardous contaminant although it is a non-essential element. Since uranium is a trace constituent of rock phosphate, it is present in fertilizers and livestock feed supplements. Despite dicalcium phosphate presents concentrations of uranium as high as 200 ppm [1], it is extensively used in broilers diet [2]-[4].

Following uptake through the gastrointestinal tract, uranium is mostly deposited in the skeleton. In fact, bone is one of the most important biological accumulators of uranium [5]. It would appear that Ca^{++} and uranyl ions UO_2^{++} follow the same metabolic path. It has been argued that the uranyl ion apparently exchanges with Ca^{++} at the surfaces of bone mineral crystals, the distribution of uranium among different bones and bone parts being similar to that of calcium, and uranium becomes diffusely distributed in bone volume. Movement of calcium in and out of bone is mediated by several distinct processes in which it may be presumed that uranium also participates. The nature of these processes is such that the residence time of an atom of uranium or other bone seeking element in bone can be a substantial fraction of a lifetime. The matter of uranium was also addressed before in the ICRP Publication 69 (1995) [6].

Although it is commonly said that uranium mimics calcium [7] [8], whether U and Ca qualitatively follow the same metabolic pathway, or if there are quantitative differences in how the body process these two elements has not been investigated as yet.

As in the case of other metals such as Cd, Pb, Si, Sr, etc. for example, uranium is also subject to stochastic processes of loss from circulation via excretion, redeposition in bone, or soft tissue uptake [6]. The latter usually accounts for only a small fraction of the loss out of the circulation. However, from a radiological viewpoint it may constitute a significant fact for consumers of poultry meat and products.

1.1. Uranium Metabolism in Birds—Preliminary Observations in Broilers

More recently, a preliminary experiment with Cobb broilers was carried out at this Laboratory, starting with seven-day-old birds fed with ration doped with uranyl nitrate up to adulthood [9]. Uranium concentration in the tibia was measured by neutron activation analysis. Quite surprisingly, results showed that the concentration of uranium (μg-U/g-bone) decreased along the animal life period, at variance with results from mammals for example, meaning that the skeleton of broilers grows at a faster rate than the corresponding accumulation of uranium. This finding was tentatively interpreted as an interplay between two metabolic peculiarities, associated both with U transfer (uptake), and U removal (clearance) from the bones. A counter-case study to prove this hypothesis would be the performing of a similar experiment with a bird exhibiting rather distinct metabolic characteristics, thus "favoring" retention of U and reducing the clearance rate as a consequence.

1.2. Metabolic Characteristics of Broilers and Ducks

A quite informative article dealing with respiratory, circulatory and metabolic aspects of Tufted Ducks was published almost three decades ago [10]. The correlation of basal rate of metabolism with several factors was worked out in birds by McNab [11] with conclusions still valid nowadays. In the same vein, it was concluded elsewhere that the principal source of variation in avian basal rates, other than body mass, is taxonomic affiliation [12].

In ducks, for instance, the hemoglobin affinity for oxygen allows rapid adjustments of oxygen binding and release since the process is far less demanding in energy than an increase in cardiac output. This adaptation has been attributed to changes in the primary structure of globin chains, modulating oxygen uptake and delivery to the tissues [13] [14]. Moreover, metabolic demand of migratory birds, as in ducks, is associated with intense muscular activity performed during flight; thus, enormous muscle activity requires a large storage of calcium (Ca^{++}) as a key element in order to maintain the necessary demand of fast and intense muscle contractions. Whatsmore, ducks have a predisposition for storing calcium (primarily in the bones, as the main source of the element) to be used over long periods of time, such as migratory flight. Such metabolic peculiarity might be conserved in domestic ducks. Additionally, there must be mechanisms most likely minimizing the loss of bone calcium to ensure calcium supply during shortages of calcium sources, in a way that is more effectively than in birds not subject to the same demand.

1.3. The Goal and the Rationale of This Work

Information and reasoning led us to conjecture that the uranyl (UO_2^{++}), following the pathway of calcium meta-

bolism, would be stored in larger quantities in ducks, due to a metabolic and genetic susceptibility associated with the group.

Since ontogenetic development is fuelled by metabolism, a decision was made to perform a detailed measurement of uranium concentrations in bones of broilers and wild ducks fed with uranyl nitrate doped ration, through a period of time starting from the first day of the animal's life through to the completion of its ontogenetic growth.

In this sense, this experiment would**,** for the first time**,** allow the sensing of information on the pathway characteristics of U in birds with different metabolic characteristics, such as broilers and ducks. This is a rather sensitive issue given the corresponding radiobiological implications to the animals and to their consumers.

2. Materials and Methods

2.1. Animals

Around seventy one-day-old Cobb broilers and seventy one-day-old domestic ducks (Common Name: Duck-Peking) were housed in 06 boxes 1.5 × 3.0 = 4.5 m^2 with wood shavings as bedding, and in each box there were 23 animals, The animals were fed *ad libitum* in infant tubular feeders with a capacity of 5.0 kg during the first 10 days; after this period specific tubular feeders for broiler, with a capacity of 30 kg, were used. The water was initially provided in infant drinkers with a capacity of 3 liters, which were later replaced by bell drinkers.

The animals were separated into two groups, each receiving the following food supplements:

***Group*-1** (control animals-broilers and ducks): basic food with specific formulation for each of the birds and for each distinct life period, following commercial procedures, was supplied and monitored (see **Table 1**).

Table 1. Percent composition and calculated values of starter and growing diets (according to [15]).

Ingredients (%)	Broiler Starter (1 - 21 days)	Broiler Growing (22 - 42 days)	Ducks Starter	Ducks growing
Maize, ground	60.089	63.079	56.847	76.735
Soybean meal	26.517	25.429	31.863	16.576
Soybean oil	2.136	3.561	1.154	
Meat and bone meal	9.05	6.742	6.452	4.839
Limestone	0.064	0.294		0.195
Salt	0.350	0.360	0.350	0.350
Vitamin premix[1]	0.100	0.100	0.1	0.1
Micromineral premix[2]	0.100	0.100	0.1	0.1
DL-Methionine	0.203	0.176	0.026	0.015
L-Lysine HCL	0.142	0.122		
Kaolin-inert	1.249	0.037	3.108	1.090
Total	100	100	100	100
Calculated composition				
ME (kcal/kg)	3054.064	3163.120	2901.283	3087.408
Crude Protein (%)	21.25	19.939	22.016	16.009
Methionine + Cistine (%)	0.844	0.791	0.700	0.55
Lysine (%)	1.189	1.099	1.160	0.749
Calcium (%)	0.971	0.837	0.709	0.60
Available phosphorus (%)	0.545	0.43	0.422	0.331

[1]NUTREMIX-(Vitamin premix). Composition per kg of product: Initial: Vit.A, 10,500.00 UI; Vit.D3, 2,500,000.00 UI; Vit.E, 15.000 mg; Vit.K3, 12.500 mg; Vit.B12, 19.000 mcg; Vit.B1, 1000 mg; Vit.B2, 7500 mg; Piridoxine 1250 mg; Biotine, 100 mg; Folic acid, 450 mg; Pantotenic acid, 9500 mg; Niacin, 40.000 mg; Antioxidant, 50 mg. Growing: Vit.A, 9,000.00 UI; VitD3, 2,000,000.00 UI; Vit.E, 10.000 mg; Vit.K3, 10.500 mg; Vit.B12, 18.000 mcg; Vit.B1, 800 mg; Vit.B2, 6000 mg; Piridoxine 1000 mg; Biotine, 80 mg; Folic acid, 400 mg; Pantotenic acid, 8000 mg; Niacin, 37.000 mg; Antioxidant, 50 mg.

***Group*-2** (doped animals-broilers and ducks): the same basic food of *Group*-1 but doped with 20 ppm of uranyl nitrate, from now on referred to as *U-doped food*.

Feeding (as well as water) was monitored and average accumulated consumptions were estimated. The animals were tagged with individual rings and weighed weekly and afterwards sacrificed. The animals were also clinically checked throughout experiment duration.

Starting with two-day-old birds, two animals per group were euthanized at the following sequence of ages (in days):

Ducks (control and doped animals)—2, 7, 9 and 15. After the 15th day the animals were sacrificed at intervals of 6 or 7 days, *i.e.*, from the 15th to the 78th day.

Broilers—from 2 to 14-day-old doped animals at intervals of one day (2, 4, 6, 8, and 10-day-old for the control), and from 14 to 70 at intervals of 6 or 7 days (control and doped animals).

Three one-day-old ducks and broilers were sacrificed for the purpose of obtaining *base values*, that is, residual uranium transferred to the animals during the pre- and neonatal phases.

The animals were euthanized according to the Guide of Care and Use of Experimental Animals (Canadian Council on Animal Care, vol. 2, 1984), receiving adequate care with appropriate veterinary supervision. The tibiae were immediately removed and frozen at −20˚C for *a posteriori* processing and analysis.

2.2. Sample Preparation

The bones were individualized in porcelain melting pots, weighed and maintained for 12 hours inside an oven at 120˚C leading to water evaporation. Next, the material was kept on a hot plate at 180˚C for 8 hours for carbonization. After this, the melting pots were inserted into an oven at 600˚C until conversion of the material into ashes, diluted into 2% nitric acid.

Approximately 100 mg of bone ash solutions from each animal was weighed and sealed in polyethylene involucres. Standard aliquots of U and Ca solutions, with exact known concentrations, were pipetted onto 2 cm^2 pieces of Whatman n˚. 40 filter paper and dried in a dessicator. Sets constituted by bone samples and standards were irradiated in the IPEN research nuclear reactor (IEA-R1, 2MW) for 8 h at a thermal neutron flux of approximately 10^{12} $n{\cdot}cm^{-2}{\cdot}s^{-1}$.

A solution of the reference bone sample IAEA-A12 was also prepared, so as to check the analytical accuracy of the method.

Additionally, three samples of ration (food) diluted with 2% nitric acid were similarly analyzed to determine their possible residual Uranium content.

2.3. Data Taking

2.3.1. Uranium

Samples of bones, ration and standards, all in triplicate, were analyzed by means of conventional gamma-spectrometry procedures [16] [17], using a high resolution 75 cm^3—high purity Ge detector operated with a 671 ORTEC amplifier in pile-up rejection mode. For the quantification of uranium 2 hour counting runs were used, thus allowing the determination of the three main gamma lines following the decay of ^{239}Np (formed from $^{238}U + n \rightarrow {}^{239}U \rightarrow {}^{239}Np$): 106 keV, 228 keV and 278 keV.

2.3.2. Calcium

About 150 mg of bone samples, weighed and heat-sealed in polyethylene bags, were irradiated at the IEA-R1 nuclear reactor together of synthetic standards of Ca. The Ca standard was previously prepared by pipetting a known aliquot of Ca standard solution provided from SpexCertiprep Chemical (USA) onto a sheet of Whatman No. 40 filter paper. The amount of Ca used was 1000 µg. After drying at room temperature in a dessicator, the filter sheets were placed in clean polyethylene bags, and irradiated for sixteen hours under thermal neutron fluxes of about 10^{12} $n{\cdot}cm^{-2}{\cdot}s^{-1}$. Following an adequate decay time of one week, the irradiated samples and Ca standard were measured using an HPGe detector (Model GX2020) coupled to an integrated signal processor (Model 1510), both from Canberra. The resolution (FWHM) of the system was 0.90 keV for the 122 keV gamma-ray peak of ^{57}Co and 1.87 keV for the 1332 keV gamma-ray of ^{60}Co. Calcium was identified by measuring the ^{47}Ca gamma lines of 159.38 and 1297.09 keV and half life of 4.54 d. The elemental concentration was calculated by comparative method.

2.4. Statistical Evaluation

It should be emphasized that each data point corresponds to an average taken over the 3 gamma ray decay lines of ^{239}Np measured in samples of two animals; therefore it is the average of 6 experimental values for uranium concentrations. All error bars showing up in the figures represent external standard deviations. In reality, since data averaging was the only data handling procedure, calculation of external standard deviations of the averaged values was carried out, simply a conventional parametric statistics in the normal model [18]. Because of the one to two orders of magnitude difference between Uranium concentrations in bones of broilers and ducks, it would seem rather redundant applying statistical tests as e.g. Tukey's or ANOVA.

3. Results

There were transitory treatment effects ($P < 0.05$) in body weight change; attributed to the normal fluctuations in weight within any group of birds and therefore not considered biologically significant or U-induced.

Measurement of uranium concentrations in the ration samples provided an average of 0.28 ± 0.03 ppm. Uranium concentrations in bones of control animals up to 14-day-old were 0.04 ± 0.02 ppm and 0.12 ± 0.5 ppm, for broilers and ducks respectively, and 0.03 ± 0.01 ppm and 0.15 ± 0.6 ppm for 14-day-older broilers and ducks respectively.

In fact, Uranium concentrations measured in control animals were low, from one to two orders of magnitude smaller than the concentrations in the animals fed with uranyl nitrate. This is additional evidence that commercially available ration used contains low levels of uranium as well.

Results for the concentrations of Uranium in bones of broilers and ducks, as function of age, expressed as micrograms of U per gram of bone (wet samples), represented by C(μg-U/g-bone) are shown in **Figure 1**, while the insert in **Figure 1** displays results for the fraction of calcium in bones of broilers, expressed as mg-Ca/mg-bone. Within experimental uncertainties, this calcium data set is a flat and structureless function of the animal age, averaging at 0.25 ± 0.04 (mg-Ca/mg-bone) or (25 ± 4)% in the whole age interval. Results for calcium in ducks (not shown) behave similarly, except that the average fraction is higher: 0.38 ± 0.05 (mg-Ca/mg-bone), or (38 ± 5)%.

Results for the restricted life period of 1 - 14 days (neonatal and very young animals) are shown in **Figure 2**, and plotted in the insert is the ratio C(ducks)/C(broilers), where C(ducks) and C(broilers) are the concentrations of Uranium in bones of ducks and broilers respectively (the horizontal lines in the insert represent average quantities at the corresponding age-interval).

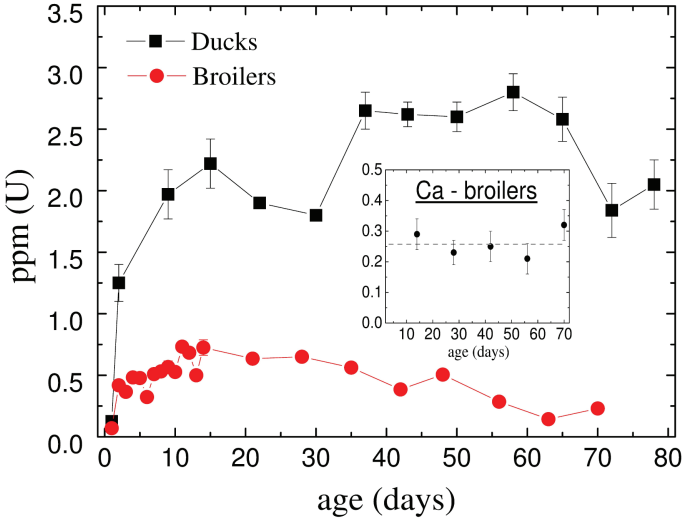


Figure 1. It shows results for the concentrations of Uranium in bones of broilers and ducks, as function of age, expressed as micrograms of U per gram of bone (wet samples), represented by C(μg-U/g-bone). Insert: results for the fraction of calcium in bones of broilers, expressed as mg-Ca/mg-bone.

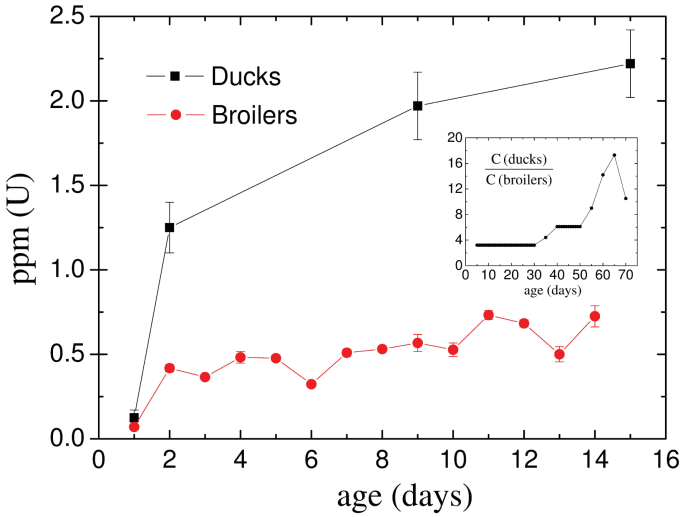


Figure 2. Results for the restricted life period of 1 - 15 days (neonatal and very young animals). Insert: ratio C(ducks)/C(broilers), where C(ducks) and C(broilers) are the concentrations of Uranium in bones of ducks and broilers, respectively. The horizontal lines represent average quantities of the corresponding age-interval.

4. Discussion

4.1. Concentration of Uranium–Deepening of Its Meaning

The experimental results obtained in this work (incorporation of Uranium in bones) are expressed in terms of *concentration of Uranium*, a quantity defined by

$$C_{\mathrm{U}} = m_{\mathrm{U}} / m_{\mathrm{bone}} \tag{1}$$

where m_{U} is the mass of Uranium measured in a bone sample with mass m_{bone}. This being a normalized quantity, that is, it expresses the amount of Uranium mass per bone mass unit. In our case it is micrograms of Uranium (μg-U) per milligram of bone (mg-bone), which is equivalent to parts per million of Uranium (ppm-U).

The concentrations of Uranium in bones of broilers and ducks were measured as function of age, spanning a life period from birth to maturity, that is, from the early stages of skeleton development. It is noted that,

a) when C_{U} is constant, or slowly varying with time, it means that the incorporation of U increases at a rate similar to skeleton growth rate. In other words, m_U and m_{bone} (see Equation (1)) are directly proportional quantities;

b) when C_{U} increases (decreases) with time, it means that the U incorporation rate is higher (smaller) than skeletal growth rate.

The present results (**Figure 1** and **Figure 2**) are discussed by splitting the life period of the animals into *life-period-1* (from 1 to 20-day-old) and *life-period*-2 (20-day-older). Uptake of Uranium is fairly distinct in these two life periods. In fact, it is very likely that young animals (*life-period*-1) are often in positive uranium balance (between uptake and excretion) due to a *build up of uranium* in the growing skeleton, *vis-à-vis* older animals (*life-period*-2) [5].

4.2. Life-Period-1: Earlier Life Stages–Rapidly Growing Skeleton

Broilers—The concentrations of Uranium (C_{U}) in bone of neonatal and young broilers are nearly constant and/or slowly varying with time in *life-period*-1, as shown in **Figure 2** (full circles points). Therefore (see Equation (1)) $C_U = m_U / m_{\mathrm{bone}} \approx k$ (constant), indicating that the amount of U incorporated and the skeleton (bones) mass of younger broilers increases at the same rate. This is what happens with Calcium in bones too as clearly indicated in **Figure 1**—insert, where it is plotted concentrations of Calcium in bone, that is, $C_{\mathrm{Ca}} = m_{\mathrm{Ca}} / m_{\mathrm{bone}} \approx k$ (constant). Therefore, the masses of both Uranium and Calcium measured in bone increases proportionally to the skeleton mass, suggesting that U and Ca follow the same metabolic pathway at least for neonatal and young broilers. Overall, this is compelling evidence for the Uranium Calcium-mimicry hypothesis.

Ducks—A simple visual inspection of **Figure 1** reveals that ducks have a strong affinity for Uranium, and that such affinity is quantitatively quite distinct according to the life period. In the case of neonatal and young ducks, *life-period*-1 (**Figure 2**), Uranium concentration appreciably increases with time, for instance, nearly duplicating from t = 2 d, 1.25 (μg-U/g-bone), to t = 15 d, 2.25 (μg-U/g-bone). It seems that the uptake biokinetics in ducks in early life stage is more *avid* for Uranium compared to Calcium.

Overall, ducks incorporate three times more Uranium than broilers do in *life-period*-1 (see **Figure 2**). This result is consistent with the higher predisposition of ducks to store Calcium in bones, as discussed in the introduction and in accordance with our results: 0.25 ± 0.04 and 0.38 ± 0.05 (mg-Ca/mg-bone) for broilers and ducks respectively.

4.3. Life-Period-2: Young to Mature Animals

Broilers—The Uranium concentration (C_U) in bones of broilers 20-day-older decreases all along *life-period*-2 (**Figure 1**). It is known from Equation (1) that $C_U = m_U / m_{bone}$; thus, a decreasing C_U could imply that the Uranium incorporation rate is slower than skeleton growth rate, and/or that Uranium clearance is involved. The latter possibility was tentatively addressed by us elsewhere [9]. In fact, Uranium concentration drops by a factor of 3, approximately, from 0.75 (μg-U/g-bone) at t = 20 d to 0.2 (μg-U/g-bone) at t = 70 d.

Ducks—The ratio C(ducks)/C(broilers) plotted in the insert in **Figure 2** reveals that Uranium incorporation in ducks, relative to broilers, increased steeply from 3 (observed in *life-period*-1) to 17 at the age of 65 days. Interestingly, the results of this study show that mature ducks incorporate on average ten times more Uranium than broilers; a puzzlingly finding indicating that duck affinity for Uranium is much higher in adult individuals and, proportionally, much higher than for Calcium.

Biokinetics issues as clearance and slower or faster Uranium incorporation rates could be appraised by means of *residence times* via a simple *Two Compartments Biokinetics Approach* for uptake.

4.4. Uranium Uptake through Two Compartments

Let one consider that m_0 milligrams of Uranium administered to the birds move to a first compartment (skeleton), and then to a second compartment (kidneys, for instance), but do not return back to compartment # 1 (the excretion process). A more complete biokinetics approach using e.g. a Multiple Compartments Model, beyond the scope of this *Discussion*, is to be found in our earlier publications [19].

After an elapsed time t (s) Uranium mass incorporated into compartment # 1 is m_1(t), and the incorporation rate, r_1 (mg/s), is given by

$$r_1 = -(\lambda_1 + \lambda) \cdot m_1 \tag{2}$$

where $\lambda\ (s^{-1})$ is the Uranium radioactive decay constant and $\lambda_1\ (s^{-1})$ is the biological "disappearance" rate of Uranium from compartment # 1.

Uranium disappearing in compartment # 1 reappears in compartment # 2. Likewise, if m_2 (t) is the Uranium mass in compartment # 2, its accumulation rate, r_2 (mg/s), is given by

$$r_2 = +\lambda_1 \cdot m_1 - (\lambda + \lambda_2) \cdot m_2 \tag{3}$$

where $\lambda_2\ (s^{-1})$ is the biological removal rate of Uranium from compartment # 2.

Considering that r_1 and r_2 are time derivatives of m_1 and m_2, respectively, the differential Equations (2) and (3) provide straightforward solutions for m_1 and m_2 as, e.g.,

$$m_1(t) = m_0 \cdot \exp\left[-(\lambda_1 + \lambda)t\right] \tag{4}$$

Likewise, one deduces the *residence times* of Uranium in compartments # 1 (skeleton) and # 2 (kidneys), t_1 and t_2 respectively, and given by

$$t_1 = (\lambda_1 + \lambda)^{-1} \tag{5}$$

and

$$t_2 = \lambda_1 \left[(\lambda_1 + \lambda) \cdot (\lambda_2 + \lambda)\right]^{-1} \tag{6}$$

The radioactive decay constant is defined as $\lambda = \tau^{-1}$, where τ is the mean life of the radionuclide. In the case of Uranium the order of magnitude of τ is hundreds of millions of years which here, for estimate purposes, render $\lambda \approx 0$ and *residence times*

$$t_1 = \lambda_1^{-1} \quad \text{and} \quad t_2 = \lambda_2^{-1} \tag{7}$$

In fact, *residence time* t_1 in this approach is the time by which 100% of the total ingested Uranium moves out of compartment # 1 (bones), while $\ln 2 \cdot \lambda_1^{-1} = 0.69 \cdot \lambda_1^{-1} = 0.69 \cdot t_1$ is the time by which 50% of the total ingested Uranium disappears from the bones.

Results showing that mature ducks incorporate on average ten times more Uranium than broilers strongly would indicate that

$$t_1(\text{ducks}) \gg t_1(\text{broilers}) \rightarrow \lambda_1(\text{ducks}) \ll \lambda_1(\text{broilers}) \tag{8}$$

Since the parameter λ_1 is a measure of the Uranium clearance rate in bones (or, as defined above, the biological "disappearance" from compartment # 1), the smaller λ_1 is, the bigger the retention of Uranium in bones. Thus, Uranium clearance rate in broilers is substantially higher than in ducks.

In fact, metabolic properties intended to retain calcium in bone, probably hinder the elimination of uranyl in the body of ducks. Differences in enzymes and transport proteins playing a role in cellular calcium are likely to affect retention and elimination of uranyl in the body of birds; indicating that future studies in comparative biochemistry between the two groups can be explored in the discovery of new forms of metabolic elimination of uranyl, an issue high on the agenda of radiation protection.

5. Conclusions

1) The amount of Uranium present in bones and the bone masses of neonatal and young broilers increase**s** at the same rate, indicating that U and Ca qualitatively follow the *same metabolic pathway.*
2) Mature ducks incorporate on average ten times more Uranium than broilers.
3) Conclusion # 2 and a two compartments biokinetics approach suggest that *Uranium clearance rate* in broilers is much higher than in ducks.
4) Conclusion # 3 supports the possibility that metabolic characteristics favoring Calcium retention in bone may hinder the elimination of Uranium in ducks.
5) Overall, all these conclusions point to the need for future comparative biochemistry studies between Galliformes and Anseriformes.

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3

Twenty-Year Reform of Teaching Biochemistry and Molecular Biology at Fourth Military Medical University in China

Jing Zhao*, Yan Li*, Bo Yan*, Lintao Jia, Xia Li, Lifeng Wang, Xinping Liu, Libo Yao#

Department of Biochemistry and Molecular Biology, Fourth Military Medical University, Xi'an, China

Abstract

Higher medical education in China is dually challenged by inadequate understanding of career motivation among high school graduates and by insufficient self-directed learning capability as a result of long-term exam-based education. Biochemistry and Molecular Biology is one of the most essential basic courses at Fourth Military Medical University (FMMU), but students usually feel torn to deal with it. We have been making efforts to improve teaching efficiency for over twenty years. Our teaching reform involves design of students-centered experimental course, reorganization of theoretical course and attempt to evoke critical thinking in class. A few tips are shared with regards to how to make the course of Biochemistry and Molecular Biology more rewarding.

Keywords

Twenty-Year Teaching Reform; Fourth Military Medical University; Chinese Medical Students; Exam-Based Education; Critical Thinking

1. Introduction

Biochemistry and Molecular Biology has witnessed an array of fundamental breakthroughs in life science over hundreds of years. It seems like a trunk highway leading us to a subtle microworld of cells. In China, higher education in life science refers to Biochemistry and Molecular Biology as one of the most essential required classes. According to distinct training objectives for different majors, teaching activities on Biochemistry and

Molecular Biology have been directed to either a biological lane or a medical lane. As the teachers at Fourth Military Medical University (FMMU), it is truly important for us to think over how the course of Biochemistry and Molecular Biology can contribute to preparing students for their future careers in medicine [1]-[4]. The purpose of this paper is to give a brief historic review on the three phases of our teaching reform in the last two decades and share a few tips in our efforts to improve teaching efficiency. This retrospective analysis would be of significance in not only helping us to summarize the gain and the loss in the long run but also inviting more attention on evolvement of teaching modes among teachers in other medical universities.

2. Student Background and Teaching Rationales at FMMU

2.1. Background of Chinese Medical Students

There is a huge difference in entrance to medical schools between China and the United States, as a result of fundamental difference in higher medical education system. Unlike students in the US who have got bachelor's degree before starting medical schools, Chinese medical students are senior high school graduates. They have got used to exam-driven learning for fifteen years throughout kindergarten, primary school and high school. Homework seldom challenges them to search for information out of the scope of textbooks and to come up with personal opinions. Under the severe competition of entering a desired school and hunting for a good job, too much attention is paid to tests and scores which decide students' future. It is rooted in their minds that high score tips are to copy as exactly as what teachers tell in class, and that different opinions are risky and should be avoided.

In addition, due to limited life experience and knowledge, a considerable number of Chinese medical students made career choice according to their parents' suggestion with even little interest in medicine. It is misleading to emphasize financial and social benefit of being doctors instead of understanding real needs of patients. This job-and-goal mismatch causes lack of sense of responsibility and loss of motivation of active learning and independent thinking while schooling.

In brief, passive learning and aimlessness among students are the two major barriers that our higher medical education has to face.

2.2. Rationales for Teaching Biochemistry and Molecular Biology at FMMU

To meet student background, we have some rationales at FMMU to teach Biochemistry and Molecular Biology based on decades of teaching feedback. First, it is much easier for medical students to recall physiological function of biochemical pathways than structural details of thousands of intermediates in cells. Second, we would like to help students understand better pathological roles that biochemical reactions may well play upon a particular disease context [5] [6]. Third, it really matters to ensure that students develop an integrative view on biochemical network characteristic of homeostasis and dynamic adaptation, which will benefit them for sure when dealing with real patients [7] [8]. Taken together, our course design of Biochemistry and Molecular Biology is friendly, functional and critical.

3. Twenty-Year Teaching Reform of Biochemistry and Molecular Biology at FMMU

We have gone through over-20-year teaching reform on the course setting of Biochemistry and Molecular Biology. Basically, there are three phases in which our efforts are made on design of students-determined experimental course, reorganization of theoretical course and cultivation of critical thinking, respectively (**Figure 1**).

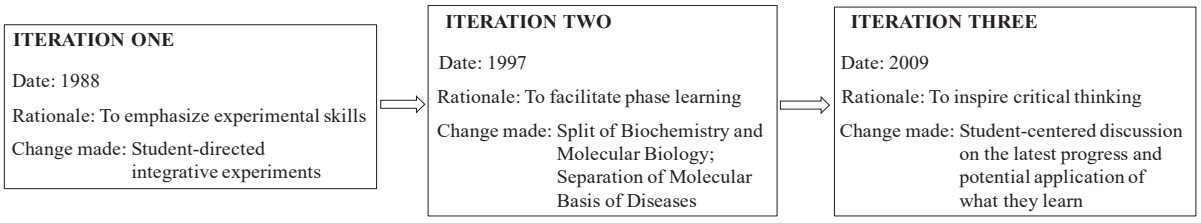


Figure 1. Three iterations of teaching biochemistry and molecular biology at FMMU.

3.1. First Iteration in Experimental Course Setting

The first round of our teaching reform was traced back to the late 1980s when integrative experiments were encouraged. Students were assigned a particular project based on teaching plan, which usually required comprehensive knowledge and skills of several disciplines. With only limited help from teachers, students were responsible for designing workflow of experiments independently, carrying out the whole process from reagent preparation to readout judgment, and giving rise to a reasonable conclusion and explanation. The students-centered experimental course did a very good job on enabling them to make their own choice on how to solve a problem [9]. At that time, this idea of integrative experiments was also adopted by other courses like physiology and immunology, which turned out to be a heavy burden to students. Since the late 1990s, students-directed integrative experiments have converged on a special course and only allowed to carry out at Teaching Experimental Center. As a result, separate experiments of Biochemistry and Molecular Biology have been geared to the basics of proteins and nucleic acids (**Table 1**).

3.2. Second Iteration in Theoretical Course Setting

In the second phase of our teaching reform in the late 1990s, we primarily focused on optimization of theoretical course. The reason for us to do so was due to explosively increasing new concepts and technologies that made the textbook of Biochemistry and Molecular Biology thicker and thicker. Students kept complaining how nervous they were when final exam approached and how depressed when they had too much to go over at a time. In their eyes, Biochemistry and Molecular Biology seemed like a nightmare.

To relieve the pressure, one step we took was to separate Molecular Biology from Biochemistry, leading to two independent courses with two separate tests at the end of each course. The split of Biochemistry and Molecular Biology potentiated two-phased study and made it easier to attain phase goal. Students benefited from the course separation not only by feeling stress relieved but also by giving better performance in exams. As compared with the average grade of 260 students on the original Biochemistry and Molecular biology course from 1994 to 1996, the average grade of 420 students on all the split courses from 2005 to 2007 tended to rise a little bit (**Figure 2**), with variation expressed as SD bars.

The other improvement we made was to reorganize disease-related molecular biology in 2008. It was of note that there existed some novel topics such as oncogenes, tumor suppressor genes, gene diagnosis and gene therapy in modern molecular biology teaching. Students started to learn Biochemistry and Molecular Biology at 3rd

Table 1. The experimental course of biochemistry and molecular biology at FMMU.

Modules	Experiment Topics	Class Hours
Proteins	Denaturation, coagulation and precipitation of proteins	3
	Purification, quantification and functional identification of proteins	12
Nucleic acids	Separation, purification and quantification of DNA and RNA from tissues	3
	Molecular cloning of genes of interest into prokaryotic expression vectors	12

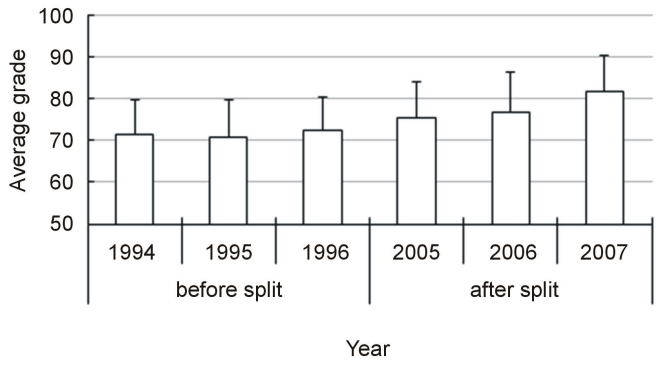


Figure 2. Grade comparison before and after the split of biochemistry and molecular biology at FMMU.

semester right after anatomy and cell biology, when they did not touch physiology, pathology and immunology which were scheduled at 4th semester. Inadequate background knowledge of basic medicine made students feel rather confused about those clinic-relevant parts. Given this awkward situation, we boldly put the molecular basis of diseases backward until 5th semester, prior to the start of clinic medical courses. **Table 2** shows the detailed lectures arranged for the disease section, which have been better received by senior students according to the feedback after class.

3.3. Third Iteration in Evoking Critical Thinking

The third phase of our teaching reform has been initiated by educational internationization since 2009. As an old saying goes, imagination is more important than knowledge. Unfortunately, traditional test-oriented education denies disagreement with authorities and almost kills students' imagination. With more training opportunities abroad for FMMU teaching faculty, we come to realize that it is essential and urgent to inspire students to think critically [10]. For this purpose, we begin trying harder to teach students how to fish rather than give them a fish.

Take the teaching plan on metabolism for example. We have got inflexible ways to interest students in the metabolic field (**Table 3**). Among those, discussion class is the most popular. Students are encouraged to present their personal perspectives with PowerPoint Slides within 7 minutes, with around 12 speeches in each class. The audience of other students is welcomed to ask questions and minidiscussion may occur in the middle [11] [12]. Teachers serve as facilitator instead of guider in every aspect of discussion class including group assignment, topic choice, PowerPoint preparation, oral presentation, brainstorming discussion and decision on winner list.

Out of several rounds of discussion class every year, it appears how to select right topics is a key to successful discussion. Initially students talked about whatever they were interested in the Biochemistry course and the audience hardly figured out the interrelationship of those talks, which resulted in inefficient communication. Then we narrowed discussion topics down to metabolism-based diseases. As a matter of fact, it did work better but students tended to introduce common symptom-treatment disease axis without deep thinking of the underlying molecular basis. This led us to a rethinking of topic choice on cancer cell metabolism, given a rapid advance in extensive mechanistic studies in this field. Students were divided into several groups and looked at cancer metabolism features from different point of views on carbohydrate metabolism, lipid metabolism, amino acid metabolism, oncogenes and tumor suppressor genes, respectively. In each group, there was a leader who showed a

Table 2. The section of molecular basis of diseases at 5th semester.

Chapters	Lecture Topics	Class Hours
1	Genes and diseases	2
2	Molecular basis of proliferative diseases	2
3	Molecular basis of infectious diseases	2
4	Molecular basis of inflammation	2
5	Molecular basis of cardiovascular diseases	2
6	Molecular basis of stress-related diseases	2
7	Gene diagnosis and gene therapy	2

Table 3. Teaching organization on metabolism.

Types	Objectives	Activities	Time
Lectures	The basics of metabolic pathways	Teacher-oriented talk, with open questions for students	In class
Discussion class	Latest research progress and application of metabolism	Student-oriented talk, with little help from teachers	In class
Case analysis	Metabolism-relevant diseases	Student-oriented task, to answer questions raised in the network course	After class
Homework	Integrative view of metabolic network	Student-oriented task, to draw crosstalks of metabolism	After class

whole picture of the section and each group member gave a detailed talk on one point of the picture. The reorganized discussion class was a big success. Students updated themselves with a lot of the latest researches and got more insight on how to acquire knowledge outside the class and how to think critically [13] [14].

4. Tips for Improving Teaching Efficiency

Our twenty-year reform has satisfactorily improved teaching efficiency. The latest feedback survey indicates that 53% students consider Biochemistry and Molecular Biology as their favorite course and that 37% like some chapters because they like teaching styles of particular teachers.

We have a few tips to make Biochemistry and Molecular Biology education more rewarding. Most importantly, try to make the class as interesting as possible. The most effective way to interest students is to make them feel this course truly useful in both daily life and future medical career. For example, when it comes to amino acid metabolism, we ask some questions "is an egg-and-milk-only breakfast sufficient or not?" "Why are some people milk-sick?" "What is the reason for someone to add melamine to food proteins?" "How can metabolic abnormality of proteins cause diseases like hyperammonemia, megaloblastic anemia and albinism?" Such questions are beneficial for arousing students' passion to learn.

Next, treat students as active thinkers rather than passive recipients [15]. There is a tremendous unknown world beyond textbooks and we would love to learn together with students. We respect every question they raise and sing high praise for their independent thinking. We listen to what they get out of updated resources and encourage them to think over both contribution and limitation of the present studies. The intimate cooperation between teachers and students brings out the best of students.

5. Conclusions

In our twenty-year teaching efforts, Biochemistry and Molecular Biology has been structurally optimized and functionally oriented to meet the needs of medical students. The current course setting includes two required theoretical courses (Biochemistry, Molecular Biology), one required experimental course (manipulation of proteins and nucleic acids), one selective theoretical course (molecular basis of diseases), and students feel much easier to adapt themselves to this step-by-step learning as showed by 92% satisfaction (593 out of 645) with the present teaching calendar last year. More importantly, the current teaching modes are more flexible and effective by adding student-centered discussion class, case analysis in network course and homework on particular projects to traditional lectures, and as a result students are more interested and motivated in active learning as indicated by 90% positive feedback (53% preference for the whole course and 37% for certain chapters) among 645 students.

Despite these encouraging progresses, there are still some issues that need further attention. Especially for discussion classes, we still lack detailed evaluation criteria for both students' performance and teachers' guidance. Also we have to think over how to integrate Biochemistry and Molecular Biology into a bigger picture of Cellular and Molecular Basis of Life, enabling a better mechanistic understanding of the relevance of molecular events to cell behaviors and of basic medicine to clinical diseases. More advanced approaches such as problem-based learning would also be considered in the future teaching activities in order to further improve students' self-learning abilities [16]-[18].

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4

Malpighian Tubules in Larvae of *Diatraea saccharalis* (Lepidoptera; Crambidae): A Morphological Comparison between Non-Parasitized and Parasitized by *Cotesia flavipes* (Hymenoptera; Braconidae)

Gislei Maria Rigoni[1], Helio Conte[2]

[1]Departamento de Ciências Biológicas, Universidade Estadual do Centro Oeste (UNICENTRO), Guarapuava, Brazil

[2]Departamento de Biotecnologia, Genética e Biologia Celular, Universidade Estadual de Maringá (UEM), Maringá, Brazil

Abstract

In *Diatraea saccharalis* larvae, the Malpighian tubules are found along the digestive tube, extending from the middle mesenteric region to the end of the posterior intestine, where they come in contact with the rectum to form the cryptonephridium. Scanning and transmission electron microscopy of non-parasitized and parasitized larvae by *Cotesia flavipes* have indicated that the tubules consist of secretory and reabsorption cells. In parasitized larvae, the occurrence of hemocytes and teratocytes around the tubules is indicative of their role in immunological defense; however, they were not observed in non-parasitized larvae. At day 9 of parasitism, the mitochondria-containing vacuoles and myelin-like figures show signs of degeneration. The results of this study have confirmed that *C. flavipes* manipulates the physiology and biochemistry of *D. saccharalis* because the Malpighian tubules of the parasitized larvae remain active until the parasitoid completes its pupal stage and is released from the host organism.

Keywords

Endoparasitism, Sugarcane Borer, Morphology, *Cotesia flavipes*, Biological Control

1. Introduction

Malpighian tubules serve as the main excretory organs of insects. Although they differ among species and various developmental stages of the same species, the tubules are generally long, narrow, and consist of a single layer of epithelial cells [1]-[5]. The tubules are connected to the intestines through a transition region that runs from the mesentery to the proctodeum; they then connect to the hemocoel and deliver fluids to the hemolymph [6]. Besides the excretion of metabolites from the degradation of proteins and purines, these organs are responsible for the maintenance of insect homeostasis, intestinal pH, regulation and secretion of calcium [7], immunological defense, and clearance of toxic substances [8]. During hibernation in crickets, the Malpighian tubules provide minerals for the maintenance of vital processes, which are stored in the form of spherites [9]. In ants, the Malpighian tubules supply metabolites that support symbiotic microorganisms that thrive in the ileum [10], as well as regulate lipid production [11].

The different cell types in insect Malpighian tubules are indicative of its wide range of functions. However, its basic function involves the transport of fluids from the hemolymph to the lumen. In Lepidoptera, the Malpighian tubules consist of two main cell types: the reabsorption cells and the primary or secretory cells. The reabsorption cells vary in terms of quantity and shape of its microvilli, mitochondria, and basal invaginations [2]. The primary cells, which possess secretory functions, are characterized by profound basal invaginations that resemble labyrinths or a profusion of microvilli at the apical region, which comes in contact with the lumen. Mitochondria are located in the basal and apical regions, as well as in the interior of the microvilli. According to Martoja and Ballan-Dufrançais [12], microvilli increase tissue permeability to solutes. Moreover, paracellular transport allows the transfer of fluids across cells that contain septa and into the lumen [13]. Tubules with cells containing the fewest number of microvilli, the thickest basal membranes, and adherent tracheoles are capable of rapidly transporting fluids from the hemolymph [14].

The internal osmolarity of the tubules varies between hypoosmotic and hyperosmotic, which reflects the rate of excretion and reabsorption of water and solutes [13]. A previous study on tubules reported on the presence of aquaporins within the membranes of epithelial cells [15], which are involved in H^+-ATPase-mediated active transport that catalyzes the exchange of sodium, potassium or both, and the excretion of organic compounds such as uric acid, alkaloids, and glycosides [16].

The physiology and structure of insect Malpighian tubules can be altered by the presence of parasites, which include viruses, bacteria, protozoans, nematodes, and entomophagous insects. In baculovirus-infected *Anticarsia gemmatalis*, the Malpighian tubules undergo modifications such as the reorganization of the microvilli, alterations in the cytoplasm, and distortion of the basal membranes [17]. A reduction in the number of apical mitochondria as well as alterations within the microvilli has been observed in the Malpighian tubules of *Aedes taeniorhynchus* parasitized by nematodes [18]. In *Triatoma infestans* parasitized by the protozoan *Trypanosoma*, Malpighian tubules show smaller tracheoles, cellular edema, and a reduction in the number of interdigitations, mitochondria, and microvilli [19].

Cells of the Malpighian tubules of *Diatraea saccharalis* parasitized by the xenobiont endoparasite *Cotesia flavipes* have never been studied. However, in intestinal cells, mitochondrial vacuoles have been observed [20], together with the accumulation of spherites [21] and the presence of inorganic compounds in the fat bodies [22].

D. saccharalis is of great economic importance to the sugar-alcohol industry because of the considerable damages it has caused in sugarcane plantations [23]. Despite numerous attempts, there is currently no insecticide that can efficiently control this insect, and therefore, in this case, chemical control is not as efficient as biological control [24] [25], which highlights the need for morphological and functional studies that will address these concerns.

2. Material and Methods

Larvae of *D. saccharalis* and adult *C. flavipes* were obtained from the Laboratory of Entomology of the Santa Terezinha sugar plant, Iguatemi district, Maringá/Paraná/Brazil (23˚23'54"S - 52˚05'36"W). The third instar larvae of *D. saccharalis* were exposed to recently fecundated *C. flavipes* females to facilitate egg laying and parasitism. The insects were maintained in glass tubes, fed on a artificial diet [26], and kept in a 24˚C incubator at a relative humidity of 64% ± 10% and a photoperiod of 14 h. Third and fifth instar non-parasitized larvae of *D. saccharalis* were also used as controls, and growth was monitored using the same conditions.

D. saccharalis larvae were cold anesthetized and dissected in an insect salt solution (0.1 M NaCl, 0.1 M

Na_2HPO_4, and 0.1 M KH_2PO_4) under a stereoscopic microscope. The specimens used in this study included 10 insects at day 5 of parasitism, 10 insects at day 9 of parasitism, and 10 non-parasitized insects. The Malpighian tubules were separated into the rectal portion and the ampullar region.

2.1. Scanning Electron Microscopy

The tissues were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) for 48 h. Post-fixation was performed using osmium tetroxide and the tissues were dehydrated across an increasing gradient of acetone solutions (30% to 100%). To complete the dehydration process, the tissues were subjected to Balzers CPD/030 critical point drying. Immediately after drying, the tissues were placed on aluminum stubs lined with double-sided tape. They were then transferred to the Balzers Union MED 010 sputtering coater and covered with a 10-nm layer of gold. The pieces were examined and photographed using a TM 3000 scanning electron microscope at the Imaging Laboratory of the Departamento de Química of the Universidade Estadual do Centro Oeste Guarapuava/Paraná/Brazil.

2.2. Transmission Electron Microscopy

D. saccharalis larvae were cold anesthetized and dissected in the insect salt solution (0.1 M NaCl, 0.1 M Na_2HPO_4, and 0.1 M KH_2PO_4) under a stereoscopic microscope. The specimens used in the study included insects at day 5 of parasitism, 10 insects at day 9 of parasitism, and 10 non-parasitized insects. The Malpighian tubules were separated into the rectal portion and the ampullar region. The tissues were fixed in 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.4). Post-fixation was performed using 1% osmium tetroxide using the same buffer for 1 h and staining was conducted using 2% aqueous uranyl acetate solution at room temperature overnight. Dehydration was performed using a series of ethanol solutions of increasing concentrations (50% to 100%), followed by a solution of ethanol:acetone (1:1) and acetone P.A., both for 10 min. The tissues were then embedded in a mixture of resin and acetone for 7 h at 4˚C and in pure resin (Araldite®) for 24 h at 4˚C. The resin blocks were sectioned using an ultra-microtome and then stained with uranyl acetate and lead citrate. Analysis and imaging were performed using the transmission electron microscope JEOL JEM-1400 at the Centro de Microscopia do Complexo de Centrais de Apoio à Pesquisa (COMCAP) of the Universidade Estadual de Maringá/Paraná/Brazil and Leo 906 at the Laboratório de Microscopia e Microanálise/Ibilce-Unesp/São José do Rio Preto/São Paulo/Brazil. The vouchers and data from this work were deposited in the Entomological Collection of the Laboratório de Biotecnologia, Genética e Biologia Celular of the Universidade Estadual de Maringá, Pr, Brazil.

3. Results

The ampullar and rectal regions of the Malpighian tubules of *D. saccharalis* and those parasitized by *C. flavipes* for 5 and 9 days showed the following characteristics;

3.1. Non-Parasitized Larvae

The insertion sites of the tubules in the intestine occurred at the transition region between the mesentery and the proctodeum through two saccular ampullae, which were positioned laterally and symmetrically in the intestinal muscles. The tubules diverged from the ampullae and continued toward the anterior mesentery, devoid of tracheoles. The cells that were part of this structure were arranged in the form of a bead collar with centralized nuclei (**Figure 1(a)**).

The ampullar region contained cells with a basal lamina, numerous labyrinth-like invaginations in the plasma membrane, and centrally positioned nuclei. Electron-dense mitochondria were observed in the basal and apical regions, with the apical surface containing microvilli that were in contact with the lumen (**Figure 2(e)**).

The Malpighian tubules in the rectal region lacked hemocytes and showed numerous tracheoles (**Figure 2(a)**). The tracheoles allowed the Malpighian tubules to attach to each other, as well as to the perinephral membrane and the rectum, thus forming the cryptonephridium (**Figure 2(b)**). In this region, two types of cells constitute the tubules; one cell type contains a small cytoplasm and few mitochondria, which characterizes absorption cells. The typical primary or secretory cells were characterized by a large cytoplasm, microvilli, and extensive and well-developed basal invaginations (**Figure 3(b)**).

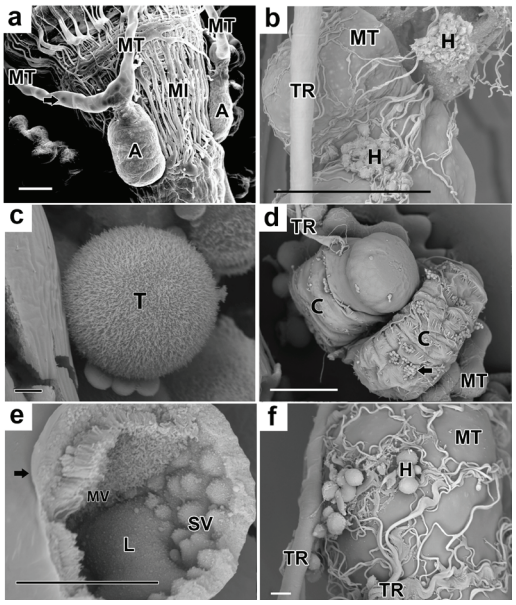


Figure 1. Scanning electron microscopy of Malpighian tubules of *Diatraea saccharalis* larvae. (a) Non-parasitized ampulla; (MT) Malpighian tubules, (arrow) nucleus, (A) ampulla, (IM) intestinal muscle. Bar = 200 µm. (b) MT at day 5 of parasitism with the formation of hemocytes clusters; (MT) Malpighian tubules, (TR) tracheoles, and (H) hemocytes. Bar = 100 µm. (c) Rectum at day 9 of parasitism (T) detail of a teratocyte. Bar = 5 µm. (d) Ampulla at day 9 of parasitism; (C) *Cotesia flavipes* larva; (MT) Malpighian tubules, (TR) tracheoles, (arrow) hemocytes attached to the parasitoid. Bar = 200 µm. (e) Broken rectum at day 9 of parasitism (arrow) basal lamina; (MV) microvilli, (L) lumen, (SV) secretory vesicles. Bar = 30 µm. (f) Ampulla at day 9 of parasitism (TR) tracheoles, (MT) Malpighian tubules, (H) hemocytes. Bar = 50 µm.

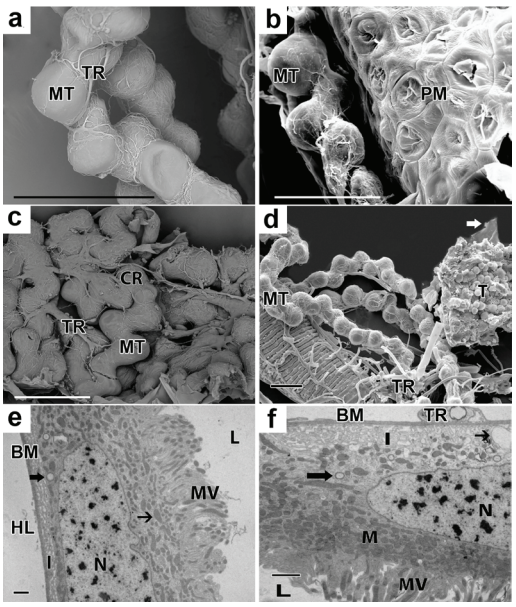


Figure 2. Scanning electron microscopy of *Diatraea saccharalis* larvae. (a) Non-parasitized rectum region; (MT) Malpighian tubules, (TR) tracheoles. Bar = 300 µm. (b) Non-parasitized rectum (MT) Malpighian tubules, and (PM) perinephral membrane. Bar = 200 µm. (c) Rectum at day 5 of parasitism; (CR) cryptonephridium, (MT) Malpighian tubules, and (TR) tracheoles. Bar = 500 µm. (d) Rectum at day 9 of parasitism; (MT) Malpighian tubules, (TR) tracheoles, (arrow) perinephral membrane, (T) teratocytes. Bar = 500 µm. Transmission electron microscopy of Malpighian tubules of *Diatraea saccharalis* larvae. (e) Non-parasitized ampulla; (BM) basal lamina, (I) basal invaginations, (arrrow) electron-lucent vesicles, (N) nucleus, (HL) hemolymph, (L) lumen, (fine arrow) mitochondria, and (MV) microvilli. Bar = 1 µm. (f) Ampulla at day 9 of parasitism; (BM) basal lamina, (I) basal invaginations, (arrow) electron-lucent vesicles, (fine arrow) vacuole, (N) nucleus, (TR) tracheoles, (L) lumen, (M) mitochondria, and (MV) microvilli. Bar = 1 µm.

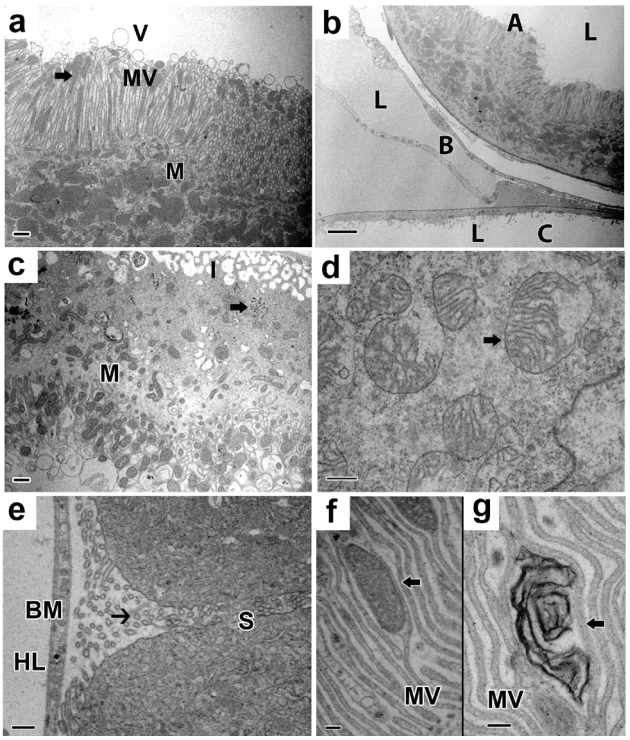


Figure 3. Transmission electron microscopy of Malpighian tubules of *Diatraea saccharalis* larvae. (a) Non-parasitized rectal region; (M) electron-dense mitochondria, (MV) micovilli, (arrow) mitochondria inside microvilli, and (V) electron-lucent vesicles released in the lumen. Bar = 0.5 µm. (b) Non-parasitized rectum, two types of cells forming the Malpighian tubules (A) Secretory, (B) and (C) reabsorption cells, (L) lumen. Bar = 2 µm. (c) Rectum at day 5 of parasitism; (I) basal invaginations, (arrow) glycogen granules. (M) mitochondria, Bar = 0.2 µm. (d) Rectum at day 9 of parasitism; (arrow) vacuole-containing mitochondria. Bar = 1 µm. (e) Rectum at day 9 of parasitism; (BM) double-layered basal lamina, (HL) hemolymph, (S) intercellular space, and (arrow) globular and stretched vesicles. Bar = 0.5 µm. (f) Rectum at day 5 of parasitism; (MV) stick-shaped microvilli, (arrow) mitochondria. Bar = 0.2 µm. (g) Rectum at day 5 of parasitism; (arrow) myelin body, (MV) stick-shaped microvilli. Bar = 0.2 µm.

3.2. Parasitized Larvae

The parasitized larvae showed a significantly higher number of hemocytes and teratocytes attached to the Malpighian tubules (**Figure 1(b)**, **Figure 1(c)**, and **Figure 1(f)**). In the broken tubules, tracheoles, basal membranes, microvilli, and secretory vesicles were observed (**Figure 1(e)**). At day 9 of parasitism, the *C. flavipes* larvae were attached to the Malpighian tubules and hemocytes were observed on the parasitoid (**Figure 1(d)**). Tracheoles were attached to the basal lamina, whereas labyrinth-like basal invaginations, electron-lucent vacuoles, polymorphic mitochondria across the cytoplasm, and centrally positioned nuclei were evident (**Figure 2(f)**). In the Malpighian tubules of larvae at day 5 of parasitism, the perinephral membrane and the cryptonephridium formed a compact structure in the rectal region (**Figure 2(c)**). Moreover, vesicles in the lumen, basal invaginations, and glycogen granules spread across the cytoplasm were observed (**Figure 3(c)**). The cells of the Malpighian tubules from days 5 and 9 of the parasitized larvae showed myelin bodies and vacuole-containing mitochondria (**Figure 3(d)**). At day 9 of parasitism, teratocytes were observed between the perinephral membrane and the Malpighian tubules, which formed the cryptonephridium (**Figure 2(d)**). The presence of an intercellular space where globular and stretched vesicles could be found suggested the occurrence of paracellular transport (**Figure 3(e)**). Stick-shaped microvilli were also observed, with some containing mitochondria and myelin bodies (**Figure 3(f)** and **Figure 3(g)**).

4. Discussion

Similar to the majority of the Lepidoptera, the Malpighian tubules of *D. saccharalis* larvae have been extensively described [2] [27]. The cryptonephridium, which can be found at the end of the rectum, is responsible for

the reabsorption of water and salts, thus allowing the maintenance of homeostasis [2] [28] [29]. The fusion of two tubules could be observed on the wall of the rectum [30].

The larvae of *D. saccharalis* have Malpighian tubules that were comprised of two types of cells that can be found in both the rectal and the ampullar regions: primary or secretory cells and reabsorption cells. The primary cells have centrally positioned nuclei, labyrinth-like and well-developed basal invaginations containing numerous mitochondria, and microvilli in the apical region that come in contact with the lumen. Mitochondria can also be found within the microvilli. This characteristic of the primary or secretory cells has been described for various insect orders, including Coleoptera [31], Díptera [32] [33], Hemiptera [34] and Hymenoptera [6].

The cytoplasm of the primary cells contained electron-dense vesicles, vacuoles, and vesicles protruding into the lumen with material from the hemolymph. Myelin bodies could be observed in the microvilli of parasitized cells, which have resulted from the elimination of cellular residues or organelles [35]. However, mineral formations such as those described in other organs [9] [19] [21] [36] were not observed in the Malpighian tubules of *D. saccharalis*.

Maddrell [7] previously suggested that the main function of the Malpighian tubules was to transport fluids from the hemolymph to the lumen. In addition to passive transport, it has also been suggested that Malpighian tubules are involved in H^+-ATPase-mediated active transport [16], which is in agreement with the high number of mitochondria and tracheoles observed in *D. saccharalis*. No tracheoles were found attached to the *D. saccharalis* Malpighian tubules; however, glycogen deposits were observed in the cytoplasm, and mitochondria could be found inside the microvilli. These polymorphic, electron-dense organelles were observed in larval cells up to day 5 of parasitism. In some cells, mitochondria showed vacuoles and loss of the matrix, which indicated degeneration, as described by [20] for the mid-intestinal cells of *D. saccharalis* parasitized by the same parasitoid.

Blunn [36] previously described different trans-epithelial transport mechanisms, namely through the cytoplasm, with and without vesicles, or through the intercellular spaces. In the rectal region of *D. saccharalis* at day 9 of parasitism, inter-membrane spaces were observed, which indicated that paracellular movement of small vesicles played a role in the excretion of toxins, as suggested by [13] for other insects. The paracellular transport observed in different species was undetected in resting cells [37]-[39].

In addition to fluid transport from the hemolymph to the tubules and later to the rectum, the Malpighian tubules are also responsible for the reabsorption of water and salts for homeostasis maintenance. This is the role of the reabsorption cells, which showed smaller cytoplasm, fewer microvilli, and larger lumen, thus allowing the rapid passage of fluids [11] [28] [31]. In *D. saccharalis*, the reabsorption cells in the ampullar and the rectal regions, where the cryptonephridium is located, showed an increase in reabsorption capacity [40].

Koinobiontparasites lead to alterations in the host hemolymph composition, the endocrine system, and the immunological system. However, the maintenance of the integrity of the host organs is paramount for the life cycle of the parasitoid [41] [42]. In *D. saccharalis* parasitized by *C. flavipes*, no morphological alterations were detected in the Malpighian tubules and the ampullae, except for the presence of hemocytes and teratocytes. These data confirm that *C. flavipes*, which is a koinobiont, gregarious endoparasite with regulatory activities, is capable of manipulating the host's physiology for its benefit [43]-[45]. The activity of the parasitoid may be influenced by a decrease in the number of hemocytes, alterations in the diet, host metamorphosis, non-encapsulation, and nodulation [46].

The manipulation of the host physiology and biochemistry occurs through venom secretion, ovarian proteins, and symbiotic viruses such as the polydnavirus that is injected together with the eggs during oviposition. As a surviving strategy inside the host, the parasitoids release cells derived from the extra-embryonic tissue or the teratocytes [47]-[49]. *D. saccharalis* larvae parasitized by *C. flavipes* were compared with non-parasitized larvae and an initial increase in the number of hemocytes adhering to the Malpighian tubules, the intestine, and the parasitoid bodies was observed. According [50], the host immune response and the number of hemocytes decreased as the parasitoid developed. Nunes and Gregório [51] showed that *C. flavipes* induces ultra-structural modifications in one type of *D. saccharalis* hemocyte, the oenocyte, which can affect the defense mechanisms of the host due to the production/activation of the prophenoloxidase system.

In *D. saccharalis*, teratocytes were observed mainly in the rectal region at day 9 of parasitism. The number of teratocytes can change depending on the extent of degeneration and on the nutrient consumption by the parasitoid [52], as these cells contribute to the maintenance of the endoparasite [53].

No polydnaviruses were observed in the Malpighian tubules of parasitized *D. saccharalis* larvae, possibly

because this organ was not a target of this virus [17], whereas they were detected in *A. gemmatalis* parasitized by a baculovirus and in *Bombyx mori* infected by a nucleopolyedrovirus [53].

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5

Role of Fungal Enzymes in the Biochemistry of Egyptian Ras Cheese during Ripening Period

Husain A. El-Fadaly[1], Sherif M. El-Kadi[1*], Mohamed N. Hamad[2], Abdelhady A. Habib[1]

[1]Agricultural Microbiology Department, Faculty of Agriculture, Damietta University, Damietta, Egypt

[2]Dairy Department, Faculty of Agriculture, Damietta University, Damietta, Egypt

Abstract

Six fungal genera including thirteen species isolated from Ras cheese samples were tested for assimilation of lactose, skim and fat milk hydrolysis. All fungal strains gave negative results in lactose assimilation except *A. nidulans* which produced acid without gas after 24 hr and produced a soluble red pigment after 10 days of incubation. All tested fungal strains showed positive results for protein hydrolysis on modified Czapek agar (MCA) medium by skim milk addition. The presence of casein in the cultural medium caused a strong growth comparing with the growth on control. Changing of growth resulting from milk casein addition was in the highest values in the cultures of *Rhizopus stolonifer*, *A. flavus* and *A. niger* being 87.81, 58.32 and 41.58 mm, respectively and the lowest values were *A. nidulans* and *Geotrichum candidum* being 7.09 and 13.71 mm. All strains gave positive results for lipolysis except *Aspergillus nidulans* on plates containing modified Czapek agar (MCA) medium by milk fat addition. The fungal growth diameter was varied from 9.17 to 30.67 mm in the case of *A. niger* and *A. alliaceus*, respectively. The presence of milk fat in the cultural medium caused a changing of growth in the case of *A. glaucus*; *A. alliaceus* and *A. flavus* being 28.11, 27.42 and 25.19 mm, respectively and *A. niger*, *Rhizopus stolonifer* and *A. flavipes* achieved lowest values being 5.62, 10.80 and 11.82 mm, respectively. Moisture percentage was gradually decreased during storage period (3 months at 15.5°C) from 31.29% in the first month to 30.25% in the third month. Fat percentage was 24.05% in fresh Ras cheese wheels, which increased to 25.89% after salting period (45 day). During ripening period the values of fat in Ras cheese wheels were 30.53%, 35.98% and 32.38% after the 1st, 2nd and 3rd month, respectively. The highest values of total nitrogen, soluble nitrogen and non-protein nitrogen percentages of Ras cheese wheels were 4.18%, 43.97% and 18.98%, respectively. These values tended to increase with advanced storage.

Keywords

Fungal Enzymes, Glycolysis, Proteolysis, Lipolysis, Egyptian Ras Cheese (Romy), Ripening Rooms

1. Introduction

Cheese ripening is a very complex biochemical process by which the rubbery or elastic curd is converted into a smooth-bodied and fully flavoured cheese. Flavour and texture are considered as the two main criteria in determining the acceptability of aged cheese. The time that is required to develop characteristic flavour and texture varies from a few weeks for soft cheeses up to three years for very hard varieties. During this period, cheeses attain their own characteristics through a multitude of chemical, microbiological and biochemical changes whereby protein, fat and residual lactose are broken down to primary products which are further degraded to secondary products [1]-[3].

Microorganisms are an essential component of all natural cheese varieties and play important roles during both cheese manufacture and ripening. Cheese is a very involved microbial ecosystem, and a very complex microflora develops in most cheese varieties. Microorganisms, present in cheese throughout ripening, play a significant role in the ripening process, and selection of suitable strains would enable the cheese maker to control or modify flavour development. However, due to the complexity of the flora and the interactions which occur between individual components of it and the cheese environment, strain selection for flavour improvement is not always very obvious. The advent of molecular techniques to study cheese microflora will lead to a major increase in our understanding of this ecosystem and this knowledge will be harnessed to control cheese ripening [3] [4].

During the ripening of cheese, three major biochemical events occur (glycolysis, lipolysis and proteolysis), each of which is involved in flavour formation. The latter is the most important and also the most complex. Glycolysis is the conversion of lactose to lactic acid and is due to the growth of starter bacteria, and the lactate produced gives the freshly made cheese its overall acidic taste. They can also produce diacetyl, acetate and acetaldehyde, which are important compounds in flavour formation in fresh cheeses; diacetyl is also an important flavour compound in hard cheeses. Lipolysis results in hydrolysis of the milk fat and production of glycerol and free fatty acids, many of which, particularly the short-chain ones, have strong characteristic flavour. The fatty acids can be further metabolized to methyl ketones and fat also acts as a solvent for many of the flavour compounds produced in the cheese. The sources of proteinase in cheese are milk itself, chymosin (rennet), starter lactic acid bacteria, nonstarter lactic acid bacteria, and the secondary microorganisms (micrococci, yeasts and moulds). Milk proteinase is plasmin and is significant in cheese when the chymosin is inactivated during cooking of the cheese [1] [5] [6].

Fungi are important in the ripening of a range of cheeses. Mould ripened cheeses are divided into two groups: those ripened due to the presence of *Pencillium roqueforti* which grows and forms blue veins within the cheese, such as Roquefort, Gorgonzola, Stilton and Danish blue, and those ripened with *P. camemberti* which grows on the surface of the cheese, such as Camembert and Brie. Moulds are associated with a range of other cheese varieties also; however, the moulds and their impact on ripening in these cheeses are less well understood [7]. The dry cheeses are placed in natural caves in the production area, where ripening takes place at a nearly constant relative humidity (90% - 95%) and temperature (9˚C - 12˚C). Under these conditions, *Penicillium roqueforti* enters the cheese and develops in the matrix, providing the final product with its characteristic appearance. Microbiologically, this cheese offers a complex habitat in which prokaryotic and eukaryotic populations interact and develop throughout manufacturing and ripening [8]. The moulds have more complex enzymatic systems than bacteria and their enzymes contribute in cheese maturing of the cheese, *i.e*., to proteolysis and lipolysis which are more extensive in these cheeses [9]. Many authors published a lot of papers about the accelerating of cheese ripening including the use of enzymes like proteinases, peptidases, and lipases. These approaches have shown that the addition of free enzymes to milk or cheese led to uncontrolled biochemical reactions [10].

Ras cheese (Romy), the main traditional hard cheese in Egypt, is manufactured in a high proportion under artisan conditions from raw cow's or mixture of cow's and buffalo's milk without using starter cultures and marketed when it has a queried sharp flavour closed to kefalotyic cheese after 3 to 6 months [11] [12]. The fungal growth of the surface of Ras cheese looks like a white felt after a few weeks of storage such as *Geotrichum candidum*, *Aspergillus ochraceus*, *A. alliaceus*, *A. oryzae*, *A. niger*, *A. nidulans*, *Emericella nidulans*, *A. flavus*, *A.*

glaucus, *Penicillium* sp., *Mucor* sp. and *Rhizopus stolonifer* [13] [14]. Also, the surface of the French cheeses is covered by a complex fungal flora containing *Penicillium*, *Mucor*, *Cladosporium*, *Geotrichum*, *Epicoccum* and *Sporotrichum*, while *Penicillium* and *Mucor* have been reported on the surface of the Italian cheese Taleggio and Geotrichum on that of Robiola [7]. Interior or surface mould-ripened cheeses have different appearances and the high biochemical activities of these moulds produce the typical aroma and taste [3] [4]. On the other hand, mould growth on Jarlsberg and Norvegia (the two most commonly produced and consumed semi-hard cheeses in Norway) can periodically be a quality problem. Mould growth can then be observed on the cheese during ripening, during storage at the factory or during retail distribution. The cheeses become spoiled due to the visible mould colonies on the surface and the off-flavours they produce. The mould growth may also represent a health risk because of the possibility of mycotoxin production by some mould species [15].

This work aims to study the role of fungal enzymes in the three major biochemical events: glycolysis, proteolysis and lipolysis during ripening period of Egyptian Ras cheese (Romy).

2. Materials and Methods

2.1. The Fungal Strains

Six fungal genera including thirteen species were isolated and identified in Agric. Microbiology Dept., Fac. of Agric., Damietta University, Damietta, Egypt as following: *Geotrichum candidum*; *Aspergillus ochraceus*; *A. alliaceus*; *A. oryzae*; *A. niger*; *A. nidulans*; *Emericella nidulans*; *A. flavus*; *A. glaucus*; *Penicillium* sp.; *Mucor* sp.; *Rhizopus stolonifer* and *A. flavipes* [13] [14].

2.2. Maintenance of Fungal Strains

The fungal strains were maintained on potato dextrose agar (PDA) medium slants [16] at 5˚C till use. Before use, the fungal strains were subcultured on new slants of PDA and incubated at 25˚C for 5 days.

2.3. Fungal Spores Photographing

Using a microscope (Bio-4o2-B Microtech, made in Japan) and a digital microscope eye piece (640 × 480) (10× WF made in China). The fungal spores were photographed and the concentrations were calculated.

2.4. Spores Suspension of the Tested Fungal Strains

Spores suspensions were prepared as described by Osman [17]. Fungi were grown on PDA slant at 25˚C for 10 days. The spores were harvested in sterilized tap water. 10 ml of sterilized saline solution (0.09% NaCl) was added to the slants and the spores were loosened by gently brushing with a sterile inoculating loop. A vortex mixer (VM-300 power: 220 VAC, 50 Hz, 0.16 A/made in Taiwan-associated with Cannic, Inc., USA) was used for one min. to remove all spores from slant [18]. Spores count was performed in a Hematocytometer slide and the following equation was used for fungal spores count:

$$\text{Spores count/ml} = \text{mean of spores count in 10 squares} \times \text{slid factor} \times \text{dilution ratio.}$$

where slid factor = 2.5×10^6 Hematocytometer slide (model Buerker MOM BUDA pest).

2.5. Lactose Assimilation

Lactose fermentation was done on a medium containing the following composition (g/L): peptone, 5.0; beef extract, 3.0; lactose, 5.0; bromothymol blue, 0.03 ml; and the pH was adjusted to 7.0. Each test tube was supplemented with Durham's fermentation tube. Sterilization was done in Arnold sterilizer at 100˚C for 60 min for three successive days. All tubes were incubated with approximately 1×10^6 spores per tubes. The tubes were incubated in a digital incubator (Switc, MPM Instruments s.r.l., Bernareggio/made in Italy) at 25˚C. The developed color was observed during the incubation period (10 days), the production of acid was recognized by the change in color from blue to yellow and the production of gas was noted in Durham's tubes [19].

2.6. Qualitative Caseinase-Lipase Activity

Casein and milk fat hydrolysis were done on two different cultivation media. The first one was modified potato

dextrose agar (MPDA) medium and the second was modified Czapek agar (MCA) medium [13] [14]. The measurement was carried out using modified potato dextrose agar (MPDA) medium, one ml of sterilized skim milk or milk fat was added to PDA medium. All plates were spot inoculation by tested fungal strains. After incubation at 25˚C, the plates of casein and milk fat were flooded with hydrochloric acid (10%) or cupper sulfate (10%), respectively [20]. The results were recorded by measuring the diameter of growth and clear zone using a digital vernier caliper, Made in Jiangsu China [14]. In case of modified Czapek agar (MCA) medium, the chemical composition (g/L) was K2HPO4, 1; Czapek concentrate, 10 ml and agar agar, 15. The pH was adjusted to 5.2, sterilized by autoclaving at 121˚C for 15 min [21]. A modification was occurred in this medium to be suitable for casein and fat hydrolysis. One ml of sterilized skim milk or fat of milk was added to every Petri dish. Skim milk and fat of milk were separately sterilized in Arnold sterilizer at 100˚C for 60 min for three successive days and this medium was used for testing the isolated fungi for proteolysis and lipolysis, respectively [14].

The chemical composition of Czapek concentrate was as follow (g/100ml): $NaNO_3$, 30; KCl, 5; $MgSO_4 \cdot 7H_2O$, 5 and $FeSO_4 \cdot 7H_2O$, 0.1 Czapek concentrate will keep indefinitely without sterilization. The precipitate of $Fe(OH)_3$ which forms in time can be resuspended by shaking before use [21].

2.7. Ras Cheese Wheels Manufacturing and Its Chemical Analysis

Ras Cheese was manufactured in El-Ghazy laboratory, Elsawalem, Damietta, Egypt. The procedure suggested by Yossef [22] and Abou-Donia [23] for making Ras cheese was adopted. After completely salting process (dry salting for forty-five day in salting rooms), the Ras cheese wheels were stored at about 15˚C for one month in a commercial ripening room, then the Ras cheese wheels were transferred in a digital incubator at 60˚F (15.5˚C) for another two months, thereafter samples were taken after one, two and three months. Ras Cheese was analyzed for fat, soluble nitrogen (SN) and non-protein nitrogen contents (NPN) according to Ling [24].

3. Results and Discussion

3.1. Adjustment of Fungal Spores Count

Spores of all the tested fungal strains were counted by a Hemocytometer slide (**Figure 1**). It was observed that, *Aspergillus nidulans* was the highest value of spores count being 60×10^6 spore/ml, but *Mucor* sp. was the lowest number being 7×10^6 spore/ml (**Table 1**). Spores suspension were prepared to give approximately 1×10^6 spores per ml using the data presented in **Table 1**, by adding the volume of solution A (spore suspension) to the volume of solution B (saline solution) for adjustment the fungal spores count to give a final concentration of 1×10^6 spores/ml using a micropipette (Micro Volume pipettor-Accumax A Made in China), thus the count of spores became fixed in the following experiments.

3.2. Efficiency of the Fungal Strains for Lactose Assimilation (Glycolysis)

Most lactose in milk is lost in whey as lactose or lactate during cheese manufacture. However, low levels of lactose remain in the curd at the end of manufacturing (0.8% - 1.0%). Residual lactose is metabolized quickly to lactate (Glycolysis) during the early stages of ripening at a rate largely determined by temperature and the salt-in-moisture (S/M) levels of the curd [25] [26]. **Figure 2** showed that, all fungal strains gave surface growth, but negative results in lactose assimilation except *A. nidulans* (F6) which produced acid without gas after 24 hr. This observation was explained by two theories, the fungal deacidification and the formation of alcohols. *P. camemberti* metabolises the lactate to CO_2 and H_2O, which results in deacidification of the cheese surface within three weeks.

The outer part of Camembert undergoes considerable modification of texture and the curd, which is firm and brittle at the beginning of ripening, later becomes soft. The surface flora establishes a pH gradient from the surface (basic) to the interior (acidic) due to consumption of lactic acid and NH_3 production. The increase in pH and breakdown of α_{s1}-casein by rennet are responsible for the softening of the curd, which gradually extends towards the center, and is visible in a cross-section of the cheese. The production of methyl ketones by *P. roqueforti* are inhibitory to further mould growth, and may be a factor in preventing excessive mould development in blue veined cheese. The strong reducing conditions in hard cheeses may favour the formation of alcohols from aldehydes. The findings of [1] [4] [7] [14] [27] may explain why the acid formation decreased.

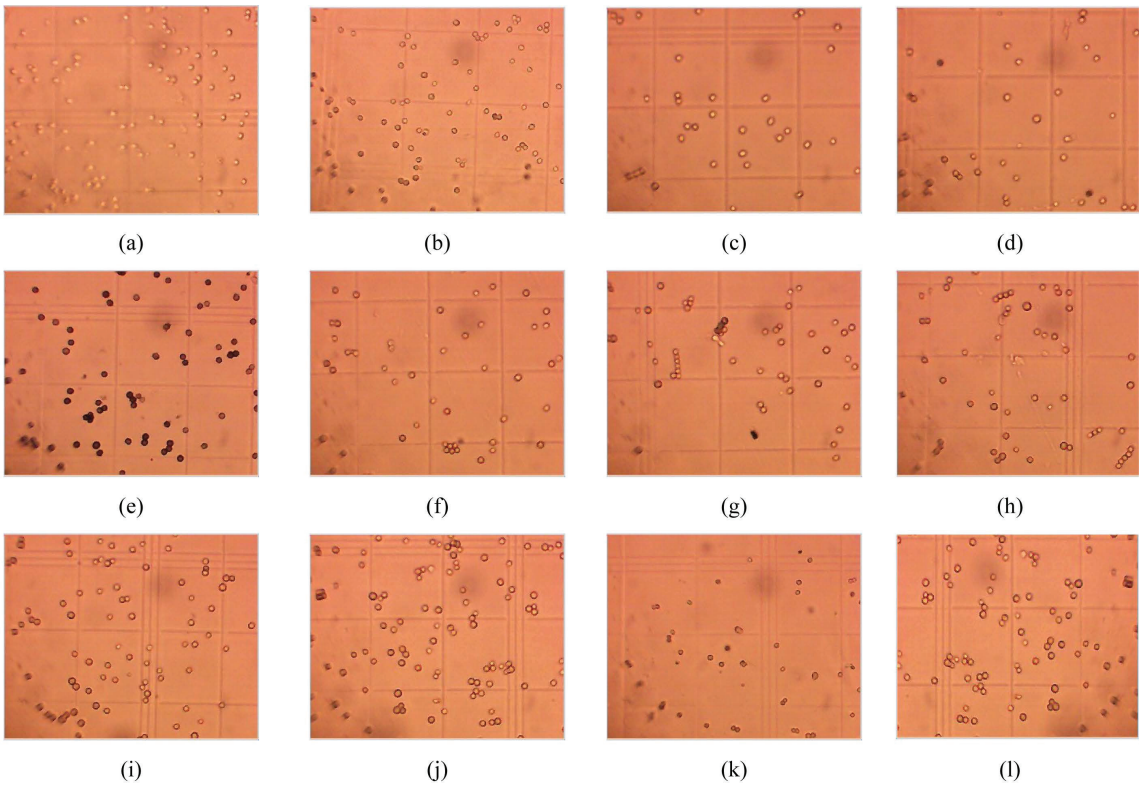


Figure 1. Fungal spores counting using a Hemocytometer slide under light microscope using a digital microscope eye piece with a magnification 400×: (a) *Geotrichum candidum*; (b) *Aspergillus ochraceus*; (c) *A. alliaceus*; (d) *A. oryzae*; (e) *A. niger*; (f) *A. nidulans*; (g) *Emericella nidulans*; (h) *A. flavus*; (i) *A. glaucus*; (j) *Penicillium* sp.; (k) *Rhizopus stolonifer* and (l) *A. flavipes*.

Table 1. Spores suspension counts of fungal strains.

Fungal strains	Spores count ($\times 10^6$/ml) (spore suspension)	Solution A (ml) (spore suspension)	Solution B (ml) (saline solution)
Geotrichum candidum	20.50	0.049	0.951
Aspergillus ochraceus	20.00	0.050	0.950
Aspergillus alliaceus	9.50	0.015	0.895
Aspergillus oryzae	19.00	0.053	0.947
Aspergillus niger	22.00	0.045	0.955
Aspergillus nidulans	60.00	0.017	0.983
Emericella nidulans	17.50	0.057	0.943
Aspergillus flavus	14.00	0.071	0.929
Aspergillus glaucus	9.00	0.012	0.888
Penicillium sp.	27.50	0.036	0.964
Mucor sp.	7.00	0.029	0.857
Rhizopus stolonifer	12.00	0.083	0.917
Aspergillus flavipes	26.00	0.038	0.962

The volume of solution A (spore suspension) was added to the volume of solution B (saline solution) for adjustment the fungal spores count to give a final 1×10^6 spores/ml using a micropipette (Micro Volume pipettor-Accumax A made in China).

All fungal strains did not give gas in the Durham's fermentation tube, but small air pebbles were observed under the fungal growth in case of *Geotrichum candidum*, *Aspergillus ochraceus*, *Emericella nidulans*, *Penicillium* sp., *Mucor* sp. and *Aspergillus flavipes* (**Figure 2**). It was also observed that, *A. nidulans* could produce a soluble red pigment which dissolved in the medium after 10 days of incubation. The finding which reported by Kure *et al*. [28] could explain the formation of gas where *G. candidum* is able to grow in environments with high levels of CO_2.

As Jarlsberg cheese releases higher amounts of carbon dioxide than Norvegia cheese, Jarlsberg cheese may provide an environment that selects for *G. candidum*. Similar results were obtained by Kure and Skaar [15] who reported that, *P. roqueforti* was capable of growth in an atmosphere with high levels of carbon dioxide. The growth was unaffected, or slightly stimulated, by high levels of CO_2, especially when levels of O_2 were low. This species is also resistant to weak acid preservatives. This may explain why *P. roqueforti* var. *roqueforti* is the dominant species with 39.5% of the total strains.

3.3. Efficiency of the Fungal Strains for Proteolysis

All fungal strains were tested for proteolysis on tow cultivation media. The first one was done on plates of modified PDA medium and incubated at 25˚C for 4 days. It was found that, fungal growth was very strong and caused a very rapid clear zone and was not suitable for enzymes activity evaluation. Thus the further experiments were controlled and examined every day on the second medium of modified Czapek agar (MCA). Also, all tested strains were inoculated on plates containing Czapek agar (CA) medium without skim milk addition as a control. Results of proteolysis were presented in **Table 2**. All tested fungal strains showed positive results. The fungal growth diameters were corresponding to clear zone diameter and it was varied between 16.40 to 94.53 mm in the cultures of *Aspergillus nidulans* and *Rhizopus stolonifer*, respectively. Also, *Geotrichum candidum*, *A. alliaceus*, *A. oryzae* and *E. nidulans* achieved the following values 21.76, 35.97, 26.71 and 31.87, respectively (**Figure 3**).

The presence of casein in the cultural medium caused a strong growth comparing with the growth on control. Changing of growth resulting from casein milk addition was in the highest values in the cultures of *Rhizopus stolonifer*, *A. flavus* and *A. niger* being 87.81, 58.32 and 41.58 mm, respectively. On the other hand, the lowest values were achieved by *A. nidulans* and *Geotrichum candidum* being 7.09 and 13.71 mm. Florez *et al*. [29] reported that, *Geotrichum candidum* is commonly found in mould-ripened cheese varieties. The strains consume lactate and produce several enzymes that break down proteins and fats. These processes involved in the softening of the cheese matrix and in the formation of aroma components (e.g., alcohols, methyl ketones, esters and various sulphides) in mature cheeses. *P. roqueforti* was also found in similar numbers of *G. candidum* suggesting that they also play a significant role in maturation of Cabrales cheeses.

Proteolysis is the most important event and the most complex for cheese texture by hydrolyzing the *para*-casein matrix which gives cheese its structure and by increasing the water binding capacity of the curd (*i.e.* to the new α-carboxylic and α-amino groups produced on cleavage of peptide bonds) [2] [5] [30]. However, the major role of proteolysis in cheese flavour is in the production of amino acids which act as precursors for a range of catabolic reactions which produce many important volatile flavour compounds. Small peptides produced from



Figure 2. Efficiency of the fungal strains for lactose assimilation (glycolysis).

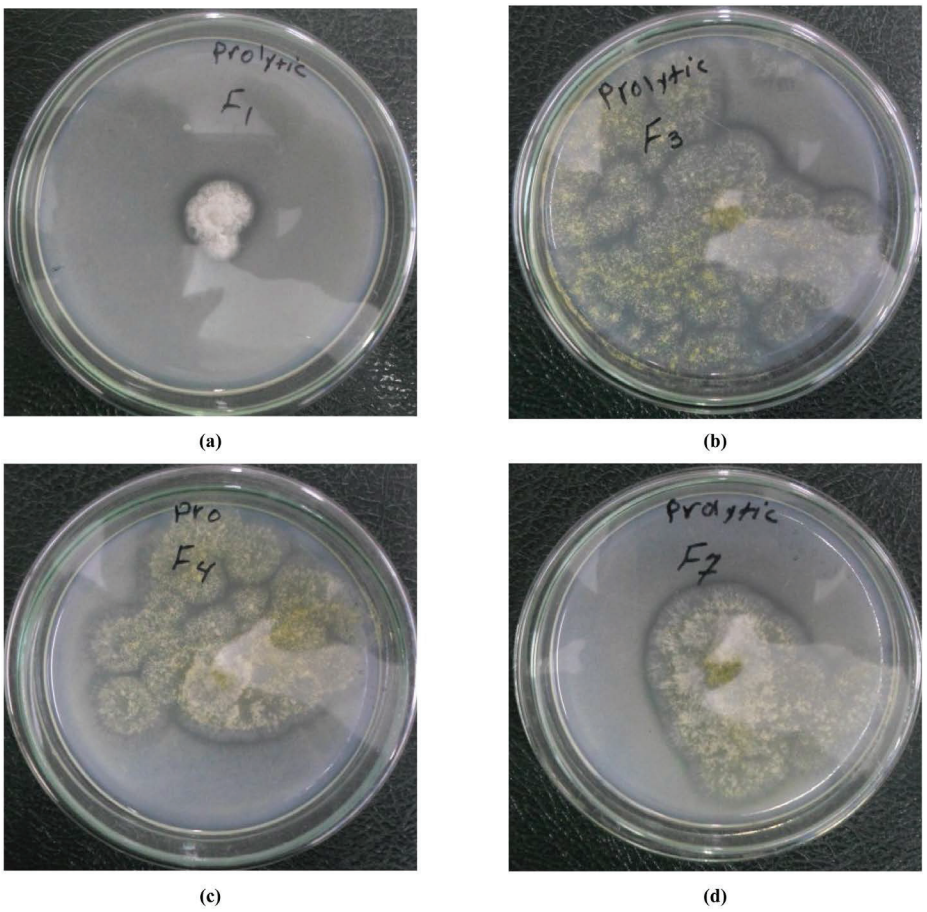


Figure 3. A positive result of casein proteolysis using (a) *Geotrichum candidum*; (b) *A. alliaceus*; (c) *A. oryzae* and (d) *E. nidulans* grown on a modified Czapek agar (MCA) medium.

Table 2. Efficiency of the fungal strains for proteolysis.

Fungal strains	Fungal growth diameter (mm) on CA medium as a control	Fungal growth diameter (mm) on MCA medium	Changing of growth* resulting from milk casein addition (mm)	Clear zone diameter (mm)
Geotrichum candidum	2.53	16.24	13.71	21.76
Aspergillus ochraceus	2.82	33.81	30.99	37.57
Aspergillus alliaceus	3.25	32.44	29.19	35.97
Aspergillus oryzae	3.75	22.86	19.11	26.71
Aspergillus niger	3.55	45.13	41.58	53.06
Aspergillus nidulans	3.58	10.67	7.09	16.40
Emericella nidulans	2.57	28.54	25.97	31.87
Aspergillus flavus	2.57	60.89	58.32	65.31
Aspergillus glaucus	1.24	33.20	31.96	39.79
Penicillium sp.	0.95	21.33	20.38	25.59
Mucor sp.	2.53	34.12	31.59	39.70
Rhizopus stolonifer	4.75	92.56	87.81	94.53
Aspergillus flavipes	1.02	30.90	29.88	35.41

*Changing of growth (mm) = growth diameter on MCA medium (mm) − growth diameter on CA medium as a control (mm).

hydrolysis of casein, can be broken down into short chain amino acids, peptides, amines, alcohols and sulphur containing compounds, which contribute to flavour formation, by proteinases and peptidases [3] [26] [27] [31] [32]. This observation is in agreement with the results of those [13] [14] [33] who reported that, during the aging of Kefalograviera, Cheddar, Domiati and Feta cheeses, the protein matrix was converted to a smoother structure and softening occurs. These changes are probably due to proteolysis of α_{s1}-casein, mainly by residual coagulant.

3.4. Efficiency of the Fungal Strains for Lipolysis

Lipids in foods may undergo hydrolytic or oxidative degradation. However, in cheese, oxidative changes are very limited due to the low oxidation/reduction potential (about −250 mV). However, triglycerides in all cheese varieties undergo hydrolysis by the action of lipases, which result in the liberation of fatty acids in cheese during ripening [25] [26].

After Ras cheese manufactured and completely salting process, the Ras cheese wheels were stored at about 15˚C for one month in a commercial ripening room, then the Ras cheese wheels were transferred in a digital incubator at 60˚F (15.5˚C) for another two months, thereafter samples were taken after one, two and three months. The storage of Ras cheese at this temperature accelerates lipolysis. Similar results were obtained by Mahony *et al*. [34] who studied the effect of temperature from 4˚C to 12˚C for ripened of commercial Cheddar cheeses for a total of 270 days. They found that, the levels of total and individual free fatty acids increased with increasing ripening temperature and progressive ripening time.

Increasing ripening temperature and time resulted in increases in the levels of short-(C4:0-C8:0), medium-(C10:0-C14:0) and long-(C16:0-C18:3) chain of free fatty acids. The results also suggested that the use of higher temperatures during the early stages of ripening (1 to 60 day) was most effective at accelerating lipolysis. Cheddar cheese ripened at low temperature (4˚C) did not attain the flavour and aroma characteristics typical of mature Cheddar cheese.

All the tested fungal strains were inoculated on plates containing Czapek agar (MCA) medium with milk fat addition as a control and incubated at 25˚C. Also, all tested species were inoculated on plates containing Czapek agar (CA) medium without milk fat addition as a control. Data in **Table 3** and **Figure 4** showed that, all strains gave positive results for lipolysis except *Aspergillus nidulans*. The fungal growth diameter was varied between 9.17 to 30.67 mm in the case of *A. niger* and *A. alliaceus*, respectively. The presence of fat milk in the cultural medium caused a changing of growth in the case of *A. glaucus*; *A. alliaceus* and *A. flavus* being 28.11; 27.42 and 25.19 mm, respectively. On the other hand *A. niger*, *Rhizopus stolonifer* and *A. flavipes* achieved lowest values being 5.62, 10.80 and 11.82 mm, respectively.

Results showed also that fungus *A. nidulans* gave negative reaction, but the changing of growth resulting from milk fat addition was 17.70 mm when compared with the value of control (3.58 mm). It may be due to the nutrients of milk fat addition such as fat and protein. Similar results were obtained by Hayaloglu and Kirbag [9] who reported that, *G. candidum* affects the cheese biochemistry during ripening. It releases lipases and proteases into the cheese matrix.

Lipolysis is particularly extensive in hard Italian cheese and blue mould chesses and is essential to correct flavour development in these cheeses. Extensive lipolysis is considered undesirable in other types of cheese varieties such as Cheddar, Gouda and Swiss cheeses; high levels of fatty acids in these cheeses lead to rancidity. However, low concentrations of free fatty acids contribute to the flavour of these cheeses, particularly when they are correctly balanced with the products of proteolysis or other reactions [27]. However, the most lipolytic organisms associated with cheese are *Penicillium* spp., which grow on or in mould-ripened varieties. *Penicillium roqueforti*, which causes the extensive lipolysis in blue cheese, possesses two lipases with pH optima of 7.5 - 8 and 9 - 9.5, respectively. *Penicillium camemberti* produces an extracellular lipase that is optimally active on tributyrin at pH 9 and 35˚C [14] [25] [26].

Malek *et al*. [35] isolated 35 strains of *Enterococcus faecium* isolated from two Egyptian cheeses (“Ras” and “Domiati”) in order to determine their ability for acidification, proteolysis and lipolysis activities, autolysis, texture, flavour development, antibiotic resistance and *β*-haemolytic activity. They demonstrate that, some indigenous strains of *E. faecium* displayed interesting technological properties for cheese manufacture, together with good safety characteristics. They could be useful for the manufacture of typical products in Egypt and Middle East.

Table 3. Efficiency of the fungal strains for lipolysis.

Fungal species	Fungal growth diameter (mm) on CA medium as a control	Lipolysis on MCA medium		
		Test result	Growth diameter (mm)	Changing of growth* resulting from milk fat addition (mm)
Geotrichum candidum	2.53	+	16.95	14.42
Aspergillus ochraceus	2.82	+	21.76	18.94
Aspergillus alliaceus	3.25	+	30.67	27.42
Aspergillus oryzae	3.75	+	18.81	15.00
Aspergillus niger	3.55	+	9.17	5.62
Aspergillus nidulans	3.58	-	21.28	17.70
Emericella nidulans	2.57	+	30.23	17.66
Aspergillus flavus	2.57	+	27.76	25.19
Aspergillus glaucus	1.24	+	29.35	28.11
Penicillium sp.	0.95	+	23.95	23.00
Mucor sp.	2.53	+	19.20	16.76
Rhizopus stolonifer	4.75	+	15.55	10.80
Aspergillus flavipes	1.02	+	12.84	11.82

*Changing of growth (mm) = growth diameter on MCA medium (mm) − growth diameter on CA medium as a control (mm).

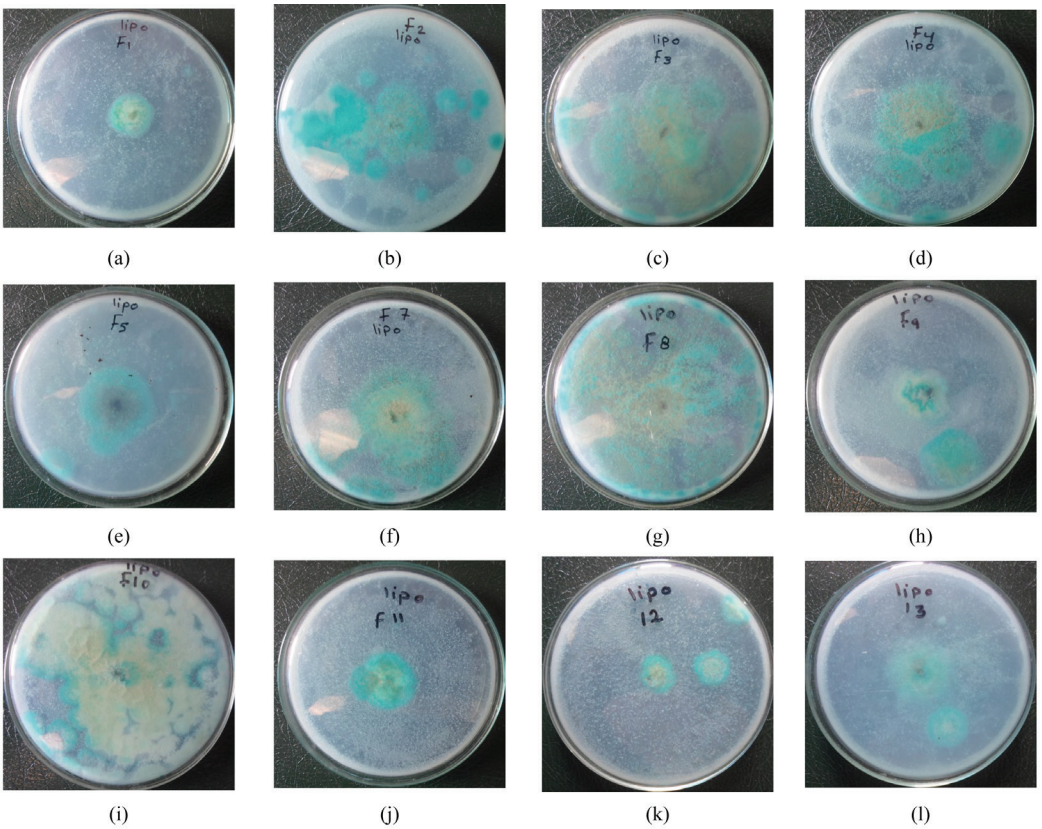


Figure 4. A positive result of lipolysis using fungal species grown on a modified Czapek agar (MCA) medium; (a) *Geotrichum candidum*; (b) *Aspergillus ochraceus*; (c) *A. alliaceus*; (d) *A. oryzae*; (e) *A. niger*; (f) *Emericella nidulans*; (g) *A. flavus*; (h) *A. glaucus*; (i) *Penicillium* sp.; (j) *Mucor* sp.; (k) *Rhizopus stolonifer* and (l) *A. flavipes*.

3.5. Chemical Composition of Ras Cheese during Storage

The moisture percentage (M%) was 38.86% in fresh Ras cheese wheels (**Table 4**), this value decreased to 34.67% after salting (after 45 day). This value gradually decreased during storage period (3 months at 15.5 C) from 31.29% in the first month to 30.25% in the third month. Dry matter percentage (DM%) was corresponded to moisture and took a reserve pattern of moisture. The total solids gradually increased towards the end of storage period. These findings are in agreement with those Kheadr *et al*. [2]; Tejada *et al*. [36]; Abdalla *et al*. [37]; Hamid and Abdelrahman [38]; Habib [14] and El-Fadaly, *et al*. [39] who reported that, the total solids contents of cheese increased during storage period. This increase was due to continuous loss of moisture from the crud a result of lactic acid development which caused crud contraction. However, the decrease in dry matter in the third month was possibly due to proteolytic effect of microorganisms on the protein and composed volatile components such as ammonia and volatile fatty acids into the ripening rooms.

Fat percentage was 24.05% in fresh Ras cheese wheels, this value increased to 25.89% after salting (after 45 day), this increase was due the lost of water and the increasing of dry matter. During ripening period the values of fat in Ras cheese wheels were 30.53%, 35.98% and 32.38% after the 1st, 2nd and 3rd month. The increasing of fat percentage was due to the fungal enzymes activities specially lipase (**Table 2**). These results are in accordance with the findings of Kheadr *et al*. [2]; Habib [14] and El-Fadaly, *et al*. [39] and in disagreement with those Hamid and Abdelrahman [38] and Abdalla *et al*. [37] who reported the decrease in fat content during storage period.

During cheese ripening the lost of weight and the decrease in dry matter was occurred. This decrease was due to the complete oxidation of lactate to CO_2 and H_2O and deamination of amino acids to the corresponding ketoacid and NH_3 [14]. In addition, volatile compounds which composed during ripening period also caused the decrease in dry matter. This compounds such as; hydrogen sulphide and methanethiol (resulting from decomposition of sulphur amino acids); ketones; methyl ketones; aldehydes; primary alcohols (e.g. ethanol); other aliphatic primary alcohols (e.g. 1-butanol, 1-pentanol and 1-hexanol); branched-chain primary alcohols (e.g. 2-methyl-1-butanol, 2-methyl-1-propanol 3-methyl-1-butanol and 3-Methyl-1-butanol) secondary alcohols (e.g. 2-propanol and 2-butanol); butanone, diacetyl, phenol (a major flavour compound in surface-ripened cheeses) and free fatty acids containing four carbons or more can originate from the lipolysis of milk fat or from breakdown of amino acids [1].

The highest value of F/DM% was 46.42% after 3 months of storage. The highest values of total nitrogen (TN%), soluble nitrogen (SN%) and non-protein nitrogen (NPN%) percentages of Ras cheese wheels were 4.18%; 43.97% and 18.98%, respectively. These values tended to increase with advanced storage. This increase was due to the fungal enzymes activities specially casinease and protease (**Table 3**). Awad *et al*. [40] reported the chemical composition of Ras cheese and they found that, moisture was % 47.46, fat was % 36.00 and protein was % 26.78. Dabiza and El-Deib [11] reported the chemical analysis of traditional Ras cheese and they found that, total nitrogen, soluble nitrogen and SN/TN were 16.01, 7.31 and 7.4, respectively.

Similar trends were obtained by El-Soda *et al.* [41]; Hassan *et al.* [42]; Mohedano *et al*. [43]; Osman and Abbas [44]; Abou-Donia [23]; Awad *et al*. [45]; Osman [46]; Habib [14] and El-Fadaly, *et al*. [13]. The proteolysis indices expressed as SN/TN and NPN/TN. The values of both were gradually increased during ripening period. The role of lipolysis and proteolysis in improving quality of Ras cheese was demonstrated by Hattem *et*

Table 4. Chemical composition of Ras cheese during storage.

Storage periods	Chemical examination of Ras cheese wheels						
	M%	DM%	F%	F/DM%	TN%	SN%	NPN%
Fresh	38.86	61.14	24.05	39.33	3.15	9.99	4.98
After salting	34.76	65.24	25.89	39.69	3.22	12.01	7.96
After 1 month	31.29	68.71	30.53	44.43	3.33	16.98	9.99
After 2 month	30.59	69.41	35.98	45.31	3.50	30.00	14.98
After 3 month	30.25	69.75	32.38	46.42	4.18	43.97	18.98

Where: M% = Moisture percentage; DM% = Dry Matter percentage; F% = Fat percentage; F/DM% = Fat on Dry Matter percentage; TN% = Total Nitrogen percentage; SN% = Soluble Nitrogen percentage and NPN% = Non-Protein Nitrogen percentage.

al. [12]. This role could be considered from the values of TVFA, SN/TN and NPN/TN which were greatly correlated with the sensorial properties of the mature cheese.

4. Conclusion

It could be concluded that, all 13 fungal strains gave negative results in lactose assimilation except *A. nidulans* which produced acid without gas after 24 hr and produced a soluble red pigment after 10 days of incubation at 25˚C. All tested fungal strains showed positive results for casein hydrolysis and the presence of casein in the cultural medium caused a strong growth. Also, all strains gave positive results for lipolysis except *Aspergillus nidulans*. The chemical composition of Ras cheese during storage confirmed the fungal enzyme activities, where the fat percentage value increased after salting and during ripening period to reach 35.98 after two months. Also, total nitrogen, soluble nitrogen and non-protein nitrogen percentages reached to the highest values with advanced storage.

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6

Modifying Polyacrylamide Background Color for the Nitroblue Tetrazolium-Based Superoxide Dismutase Staining Assay

Robert Louis Bertrand, Michael Okechukwu Eze*

Department of Chemistry, University of Winnipeg, Winnipeg, Canada

Abstract

The reduction of nitroblue tetrazolium by superoxide radicals generated from photo-reactive riboflavin has been in use for more than four decades to detect superoxide dismutase (SOD) on non-denaturing polyacrylamide gels. SOD research in medicine and biochemistry has warranted the development of multiple assay variants to overcome specific experimental constraints or to combine the SOD assay with other enzyme assays. Fine-tuning reagent concentrations to effectively visualize bands continue to be a major research obstacle in assay development. Herein we describe a straightforward technique to reliably adjust the background color of polyacrylamide gels without compromising assay efficacy. Low micromolar to low millimolar concentrations of yellow riboflavin can be mixed with the blue of reduced nitroblue tetrazolium to controllably produce blue, purple, yellow-brown, or yellow gel backgrounds. The advantage of this technique is that the assay is not modified by the introduction of new reagents. Quantitative reliability of these alternative stains was assessed by plotting determined band intensity values against known enzyme loads. The correlation (R^2) values of trial averages were compared against the average correlation of the standard 0.028 mM riboflavin solution using pooled standard deviation and Student's T-test at 95% confidence. Assay sensitivity was assessed by comparing lowest possible visible enzyme load of the experimental stains with the 0.028 mM riboflavin standard. No difference in the quantitative reliability was found in any riboflavin concentration. The minimum reliable sensitivity of the assay was found to be 10 ng for each concentration of riboflavin. This technique has already been employed to analyze SOD protein expression levels in extracts of *Escherichia coli* (Bertrand *et al*., *Med Hypotheses* 2012; 78:130-133, 2012; Bertrand & Eze, *Adv. Enz. Res.*, 1: 132-141, 2013).

Keywords

Superoxide Dismutase, Riboflavin, Gel Electrophoresis, Assays

1. Introduction

Superoxide dismutase (SOD) is the antioxidant enzyme responsible for eliminating superoxide radicals, ubiquitous in nearly all forms of life. SOD is essential for mitigating cellular oxidative damage: Its absence or abnormal expression has been associated with multiple severe pathologies, including neonatal cardiomyopathy [1], hepatocellular carcinoma [2], age-associated muscular atrophy [3], amyotrophic lateral sclerosis [4], down syndrome [5], hypertension [6], acute respiratory distress syndrome (ARDS) [7], and chronic obstructive pulmonary disease (COPD) [8]. The medical and biochemical relevance of SOD has thus warranted the development of multiple enzyme assays over the decades. The nitroblue tetrazolium (NBT)—based SOD assay, originally developed by Beauchamp and Fridovich in 1971 [9], operates through the reduction of NBT by superoxide radicals generated by photo-reactive riboflavin. As SOD efficiently removes superoxide, achromatic bands form on a uniformly blue background when the technique is applied to non-denaturing polyacrylamide gels. There have since been published multiple variations of this assay in response to specific experimental requirements. For example, some variations employ a lower concentration of NBT [10] [11] this study] because the original concentration prescribed [9] would be prohibitively expensive for routine analysis and with larger staining solutions. Another variation of the NBT-based assay includes an assay for the simultaneous detection of catalase on the same gel [10]. Other variations include modest changes in the riboflavin concentration [12], inclusion of ethylenediaminetetraacetate (EDTA) [11] (which promotes superoxide generation via the light-dependent reduction of free flavins and by chelating trace metals that inhibit superoxide generation [13] [14]), and a simplified methodology that combines NBT and riboflavin application together as a single solution [11]. A significant research obstacle in the development of these assays is the extensive fine-tuning of reagents required to achieve effective band-background contrast and/or to visualize multiple enzymes (e.g., See reference [10]). A straightforward technique for changing the background color for enzyme visualization would therefore be advantageous to assay developers.

We have observed that the background color of non-denaturing polyacrylamide gels during staining can be changed to a variety of colors by exploiting the natural yellow color of riboflavin. This yellow superoxide donor mixes with the blue of NBT to produce green. In lesser concentrations this will darken the background to blue or purple. Alternatively, higher concentrations of riboflavin can be employed to overpower the darkening effect to produce a brilliant yellow background. The accuracy of quantification and the minimum sensitivity were not found to be affected within the range examined (0.028 - 33.22 mM riboflavin). The advantage to this simple technique is that it does not introduce new reagents to the staining solution, simplifying quality control testing during development of future SOD assay variants or refinement of existing ones. Unlike NBT, riboflavin is an inexpensive reagent that can be used liberally or sparsely as needed to achieve effective contrast. Hence, modifying riboflavin concentration is an economical means of exploiting the natural colors of riboflavin and NBT. Both high- and low-riboflavin variants of this assay have already been applied experimentally to elucidate SOD protein expression levels in *Escherichia coli*, and all three isozymes of *E. coli* SOD (iron, manganese, and copper-zinc isoforms) were detectable on polyacrylamide gels [15] [16].

2. Methods

2.1. Materials and Methods

Five, 10, 20, 40, and 80 nanograms of *E. coli* SOD (Sigma-Aldrich; St. Louis, MO, USA) were electrophoresed in 7.5% polyacrylamide gels, and then assayed by the methods of Britigan and colleagues [11] with exception to the concentration of riboflavin, as described: A 50 mM phosphate buffer solution (pH 7.8) containing 0.25 mM NBT, 1 mM EDTA, 28 mM tetramethylethylenediamine (TEMED), and 0.028 - 33.22 mM of riboflavin was applied to gels in darkness for 45 minutes, stirring occasionally, followed by destaining in darkness with phosphate buffer for 45 minutes. Gels were developed by illumination with fluorescent room lighting, photographed with a white overlay, and the bands quantified with a gel analyzer (RaminVakili, Vakili Gel Analysis Unit, Dept. of Chem., University of Winnipeg). A stain containing 0.028 mM riboflavin, the original concentration prescribed by Beauchamp and Fridovich [9], was used throughout as a standard solution.

2.2. Statistical Analysis and Interpretation

Quantitative accuracy was assessed by plotting band intensity values against known quantities of SOD. The

correlation (R^2) values were then determined as an average of three or four replications for each riboflavin concentration examined (33.22, 4.15, 0.52 mM), and challenged against a 0.028 mM average via pooled standard deviation and Student's T-test at 95% confidence. To mitigate the influence of random error on results, the same five protein preparations were used throughout all trials. Thus, any deviation from perfect linearity in the original preparation would be common to all trials; and therefore, differences between data sets would more greatly reflect the inherent effects of each riboflavin stain on quantitation (if any). Whether riboflavin affected assay sensitivity was assessed by comparing the minimum reliably detectable and measurable enzyme load of the three experimental concentrations with the 0.028 mM standard.

3. Results and Discussion

All concentrations of riboflavin examined were equally capable of accurately presenting enzyme loads for quantitation (**Table 1**). There was no change in minimum quantifiable enzyme load from the 0.028 mM standard, which was determined to be 10 ng of SOD throughout (**Figure 1**). Five nanograms of SOD can be seen in some staining applications, but is unreliably detectable and quantifiable at all concentrations of riboflavin tested, including the 0.028 mM standard. Five nanogram SOD loads were thus omitted from calculations. It is remarkable that the minimum reliable sensitivity obtained in the present study (10 ng) is somewhat better than 16 ng originally reported by Beauchamp and Fridovich [9]. These results demonstrate that even dramatic changes in riboflavin concentration do not affect assay sensitivity or band quantitation.

We have observed that as riboflavin concentration increases, the development time decreased: 33.22 mM of riboflavin required 10 - 15 min., 0.028 mM riboflavin required 30 - 60 min., and 4.15 and 0.52 mM solutions required an intermediate period of time. The time required to develop the standard 0.028 mM applications differs greatly from those reported by other authors, which is usually around 5 to 15 min. [9] [10]. This discrepancy is explainable by their reliance on direct intense light sources to enhance the rate of the light-activated reaction,

Table 1. Average correlates (R^2) of SOD activity bands.

Riboflavin (mM)	No. of trials	Average Corr. (R^2)	Standard dev. (s)	Significance (95%)
33.22	3	0.9967	3.35×10^{-3}	None
4.15	3	0.9993	9.74×10^{-4}	None
0.52	4	0.9976	1.37×10^{-3}	None
0.028	4	0.9986	1.28×10^{-3}	N/A

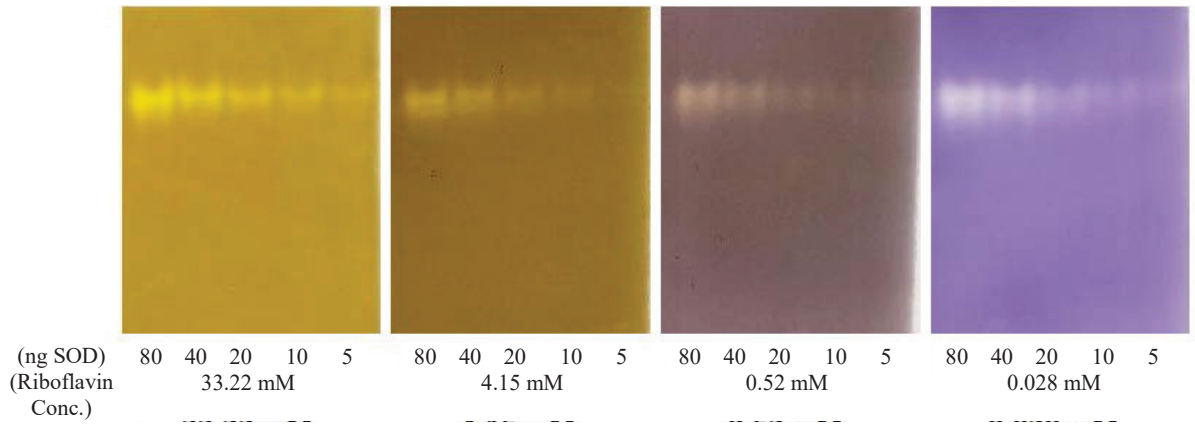


Figure 1. Typical staining results of purified *Escherichia coli* superoxide dismutase (SOD), methodology as described in "Methods". Far right was produced by application of standard riboflavin staining mix (0.028 mM), the concentration originally employed by Beauchamp and Fridovich [9]. The original reliable sensitivity reported by Beauchamp and Fridovich is 16 ng of SOD. A minimum reliable sensitivity of 10 ng was achieved in the present study. Five ng is unreliably detectable and quantifiable in each of the four applications, and cannot therefore be used as a metric of comparison for the relative efficacies of each concentration of riboflavin for visualizing SOD.

as opposed to fluorescent room lighting used in the present study. Bands produced by highly concentrated applications of riboflavin have the tendency to fade after 30 min., and for this reason it is recommended that regular room lighting be used during development to provide economy of time and effort. It is worthy to note that, regardless of the riboflavin concentration used, as gels develop under light, they darken. By slowing the reaction down with indirect light sources, the researcher is enabled to take multiple photographs at a range of color shades throughout development, and choose the photo with the best band presentation. We have personally found that relying on room lighting alone is better for generating high quality photos suitable for publication. It is equally notable that despite the poor aqueous solubility of riboflavin (0.225 mM), even colloidal solutions of riboflavin (e.g., 33.22 mM) were capable of staining gels uniformly and reliably. As the quantity of riboflavin is the only changing variable within each staining application, the variance in color seen in the gel backgrounds must be caused by changes in the quantity of riboflavin employed (**Figure 1**). It appears, therefore, that the modest solubility of riboflavin does not limit the possible colors produced. It is advised that gels stained with such copious riboflavin be briefly rinsed before being allowed to sit in destaining solution.

4. Conclusion

We have demonstrated that by changing the quantity of riboflavin used in the staining solution of the superoxide dismutase assay, one can adjust the background color of the polyacrylamide gel. No new reagents were introduced in order to achieve this effect. Statistical analysis showed that changing the riboflavin concentration does not compromise the reliability of quantitation of superoxide dismutase (**Table 1**). The minimum reliable sensitivity was shown in all applications, including the 0.028 mM riboflavin application originally used by Beauchamp and Fridovich [9], to be 10 ng of superoxide dismutase (**Figure 1**), consistent with 16 ng originally reported by the authors [9]. Five nanograms of SOD were unreliably detectable and quantifiable. Continued research on superoxide dismutase requires the development of variants of this assay in order to overcome experimental constraints or to combine the assay with other enzyme assays for simultaneous visualization of more than one enzyme. This technique will simplify the fine-tuning of reagent concentrations in order to visualize SOD activity bands fortuitous for assay development.

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7

Ameliorative Effects of *Spirulina platensis* on Deltamethrin-Induced Biochemical Alterations and Oxidative Stress in the African Catfish; *Clarias gariepinus*

Heba S. Hamed

Department of Zoology, Faculty of Women for Arts, Science & Education, Ain Shams University, Cairo, Egypt

Abstract

The present study was carried out to determine LC_{50} of deltamethrin (DM) in the African catfish; *Clarias gariepinus*, investigate its effects on the biochemical parameters and the antioxidant status of various tissues, and assess the protective role of *Spirulina platensis* (SP) against DM induced toxicity in *C. gariepinus*. The fish were distributed into seven groups of 10 fish/each for 30 days as follows: Group 1, control; Group 2, orally administered saline; Group 3, oral SP (150 mg/kg b.w.); Group 4, exposed to 5.19 µg/l DM; Group 5, exposed to 5.19 µg/l DM and orally administered SP (150 mg/kg b.w.); Group 6, exposed to 12.97 µg/l DM; and Group 7, exposed to 12.97 µg/l DM and orally administered SP (150 mg/kg b.w.). Results revealed marked elevation in serum AST, ALT, ALP, creatinine, urea and uric acid. Significant decrease in serum total protein and albumin was recorded. At the same time, the level of malondialdehyde (MDA) was significantly increased in liver, kidney and gills. While, tissue level of GSH and, SOD, CAT and GSH-Px activities were reduced. Treatment with SP improved the biochemical parameters and attenuated the DM induced oxidative damage. Therefore, this study confirmed the protective role of SP as an antioxidant against the toxicity of DM in catfish.

Keywords

***Clarias gariepinus*, Deltamethrin, Biochemistry, Oxidative Stress, Spirulina**

1. Introduction

The contamination of waterways, channels, and lakes with concoction substances of anthropogenic starting point

may have unfriendly outcomes; the waters get to be unacceptable for drinking and other family purposes, watering system, and fish cultivation; furthermore the animal communities living in them may endure genuinely [1] [2]. Pesticides have turned into an inexorably serious wellspring of substance contamination of the earth because of their broad utilization in agriculture which can influence the freshwater fauna, especially fish [3]. Pyrethroids are synthetic compounds that mimic the pyrethrins that are detached from chrysanthemum blooms [4]. They have emerged as good substitutes for organochlorine, organophosphate and carbamate insecticides due to their high bio-efficacy, biodegradable and lower toxicity to mammals and birds [5]. They definitely enter the aquatic environment by methods such as harvest dusting, plantation and forest spraying [6]. Deltamethrin (DM) ((S) α-cyano-3-phenoxybenzyl-(1R)-cis-3-(2.2-dibromovinyl)-2,2 dimethyl cyclopropane carboxylate) is a type II pyrethroid that has found wide acceptability [7], in farming, forestry, agriculture, against nuisances on animals and vermin on wooden materials and plants around the world.

In most recent couple of decades, DM has generally been used as home and garden insecticides connected broadly for the control of mosquitoes and in the treatment of ectoparasitic illness. Pyrethroids, including DM are practically non-harmful to birds but highly toxic to fish and aquatic invertebrates because they are metabolized and wiped out essentially slower in fish than mammals or fowls [8]. Therefore, the toxicity of DM in fishes is basically ascribed to its high rate of gill absorption because of its lipophilicity and inadequacy in the fish enzyme system to hydrolyze pyrethroids [9]. Biochemical profiles of blood are studied and considered as essential wellbeing markers that give important information about the internal environment of the organism [10]-[12], and frequently used when fish physiology is connected to analyze and comprehend the toxicological effects of external stressors and harmful substances [13]. Oxidative stress and reactive oxygen species (ROS) mediated toxicity have long been considered as the responsible mechanisms for DM-prompted organ injury in mammals [14] and some fish [7]. Thus, oxidative anxiety occurs when ROS overpower the cellular defenses, causing damage to proteins, membranes, and DNA [15] [16], and is characterized as an interruption of the pro-antioxidant balance, which leads to potential damage. In all organisms, the main anti-oxidative enzymes that serve to recover ROS are superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT) and other low molecular weight scroungers, for example, reduced glutathione (GSH) [17] [18]. Therefore, several studies demonstrated that changes in the levels of antioxidant enzyme activities can be used as possible biomarkers in different aquatic beings [19]. *Spirulina platensis* (SP) is a filamentous microalgae fitting in with the class of cyanobacteria with characteristic photosynthetic capacity [20] which became one of the most commonly used microalgae in aqua feeds as it is a rich wellspring of protein, vitamins, minerals, crucial unsaturated fats, key amino acids and pigments such as carotenoids that have strong antioxidant and anti-inflammatory activities [21] [22]. Recently, SP has gained more attention in fish not only for its growth-promoting and immunomodulatory effects but also for its antioxidant potential [23]. The African catfish, *Clarias gariepinus* was selected in this study as it is distributed throughout Africa [24]. Such a species is of commercial significance because of its high development rate, high stocking-thickness limits, high buyer worthiness and high imperviousness to poor water quality and oxygen depletion [25] [26]. A few records have demonstrated that Clarias fishery contributes around 17% of the yearly fish production from all fisheries areas [27]. Moreover, the African catfish has been utilized as a part of key investigates and considered as a brilliant model for toxicological studies [28]. The objectives of the present study were three folds: 1) to determine LC_{50} of DM in *C. gariepinus*, 2) to investigate the effect of sublethal DM concentrations on blood biochemical parameters in catfish manifested by oxidative stress path ways in different fish tissues and 3) to evaluate the protective role of SP against DM toxicity in *C. gariepinus*.

2. Materials and Methods

2.1. Chemicals

Deltamethrin was purchased as a commercial product (Butox®) 50 mg/ml from El-Naser Pharmaceutical Co. (Cairo-Egypt). Pure *Spirulina platensis* powder was obtained from (Herba Force, UK). All biochemical kits (AST, ALT, ALP, creatinine, urea, uric acid, total protein, albumin, MDA, GSH, SOD, CAT and GSH-Px) were purchased from Bio-Diagnostic Co. (Dokki, Cairo, Egypt).

2.2. Fish Maintenance

A total number of 350 alive catfish *C. gariepinus* (average weight 500 ± 10 g., length 41 ± 3 cm) were obtained from Abassa fish farm, El-Sharkya governorate, Egypt. Fish were transferred to the laboratory in 100 liters well

aerated fiberglass tanks. Fish were treated with (0.5% w/v) potassium permanganate solution for 1 min to remove dermal adherents. The fish were acclimatized for 2 weeks in identical glass aquaria measuring (80 × 40 × 40 cm) having 80 liter of dechlorinated aerated tap water under laboratory conditions (at temperature 25˚C ± 2˚C and natural photoperiod 12 h). The water parameters were as follows: pH 7.5 ± 0.3, temperature 28˚C ± 1˚C, dissolved oxygen 6.5 ± 0.4 mg/l, alkalinity 122 mg/l and hardness 152 mg/l $CaCO_3$. Fish were fed on commercial ration at ratio of 3% of b.w./day.

2.3. Experimental Design

2.3.1. Determination 96 h LC_{50} of Deltamethrin

The half lethal concentration (LC_{50}) of DM was determined with definitive test by the static renewal bioassay method of Litchfield and Wilcoxon [29]. Briefly, five groups each of eight fish were exposed to various concentrations of DM (10.0025, 20.005, 40.01, 80.02 and 160.04 µg/l), plus the control group.

2.3.2. Sublethal Exposures

After acclimation, the fish were divided into seven groups, each group containing 10 fish, and received the following treatments for 30 days: Fish in Group 1 acted as control group. Fish in Group 2 administered saline orally. Fish in Group 3 administered SP (150 mg/kg b.w.) orally. Fish in Group 4 exposed to 5.19 µg/l DM. Fish in Group 5 exposed to 5.19 µg/l DM and administered SP orally (150 mg/kg b.w.). Fish in Group 6 exposed to 12.97 µg/l DM and fish in Group 7 exposed to 12.97 µg/l DM and administered SP orally (150 mg/kg b.w.) as well as three replicates of control and experimental groups. To keep a constant DM concentration, fish were transferred to freshly prepared toxicant solution every 48 h.

2.3.3. Collection of Blood Samples

At the end of the experiment, blood was collected from the caudal vessels of the individual fish after anesthetization with 0.02% benzocaine solution. Blood samples were allowed to clot at room temperature, then centrifuged at 3000 rpm., at 4˚C for 15 minutes and sera were separated for the determination of biochemical parameters.

2.3.4. Biochemical Analysis

Serum AST and ALT were estimated by the method of Reitman and Frankel [30]. ALP was determined using an enzymatic colorimetric method according to Tietz *et al.* [31]. Creatinine was measured according to Larsen [32], urea according to Coulombe and Favreau [33] and uric acid according to Whitehead *et al.* [34]. Serum total protein and albumin were measured according the method of Lowry *et al.* [35] and Doumas *et al.* [36], respectively.

2.3.5. Tissue MDA and Antioxidant Biomarkers

Samples from liver, kidney and gill tissues were homogenized in cold phosphate buffer saline (0.1 M pH 7.4) using a Potter-Elvejhem glass/Teflon homogenizer. Then, this homogenate was filtered and centrifuged at 1600 rpm. at 4˚C for 10 min; the supernatant was stored at −20˚C until analysis. This supernatant (20%) was used for the determination of MDA according to the method of Mihara and Uchiyama [37]. GSH concentration was determined using the method of Beutler *et al.* [38]. The activity of SOD was determined by the method described by Nishikimi *et al.* [39]. Determination of CAT activity was according to the method of Aebi [40]. GSH-Px activity was determined according to Paglia and Valentine [41].

2.3.6. Statistical Analysis

Data were statistically analyzed using analysis of variance, one way “ANOVA”, and Duncan’s multiple range test to evaluate comparison between means at $P < 0.05$ (SPSS, 2004).

3. Results

3.1. LC_{50}

C. gariepinus were exposed to different concentrations of DM for 96 h. The results revealed that the 96 h-LC_{50} was 51.89 µg/l (**Table 1**) and therefore, 1/10 and 1/4 of the median lethal concentrations (5.19 and 12.97 µg/l) were used for chronic toxicity exposure.

Table 1. Half lethal concentration (LC_{50})/96 h of DM for *C. gariepinus*.

DM Conc. (µg/l)	No. of alive fish	No. of dead fish	a	b	a × b
0.00	8	0	0.00	0.00	0.00
10.0025	6	2	10.0025	1	10.0025
20.005	5	3	10.0025	2.5	25.00625
40.01	4	4	20.005	3.5	70.0175
80.02	2	6	40.01	5	200.05
160.04	0	8	80.02	7	560.14
					$\sum a \times b = 865.22$

Half lethal concentration of DM = Highest conc. – $\sum a \times b$ /n. LC_{50} = 160.04 - 865.22/8 = 51.89 µg/l. Where: a: Constant factor of difference between groups. b: Mean value of dead fish between each two successive groups. n: Number of fish in each group.

3.2. Biochemical Parameters

In the present study, *C. gariepinus* fish exposed to both concentrations of DM showed a significant increase in the level of serum liver enzymes AST, ALT and ALP compared to the control group (**Table 2**). The combined treatment of DM and SP resulted in significant improvement in AST, ALT and ALP levels. Similarly, serum creatinine, urea and uric acid levels were also significantly elevated with DM toxicity (**Table 2**). Co-treatment of SP significantly ($P \leq 0.05$) reversed serum renal parameters to nearly normal values. Contrary to these results, a marked reduction in total protein and albumin levels were observed in the DM-treated groups compared to the control fish (**Table 2**). Simultaneous treatment of SP significantly ($P \leq 0.05$) increased these levels compared to that of DM-intoxicated group. No significant difference in the other biochemical parameters was observed between fish supplemented SP and the control group.

Table 2. Serum biochemical parameters in control and experimental groups.

Experimental groups / Parameters	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7
AST µ/l	33.98 ± 0.55^{d}	33.91 ± 0.21^{d}	34.34 ± 0.21^{d}	39.32 ± 0.42^{b}	32.70 ± 0.44^{d}	41.99 ± 1.21^{a}	37.38 ± 0.48^{c}
ALT µ/l	18.22 ± 0.22^{c}	17.25 ± 0.15^{c}	18.48 ± 0.41^{c}	22.85 ± 0.25^{b}	18.02 ± 0.57^{c}	28.53 ± 0.76^{a}	22.15 ± 0.08^{b}
ALP µ/l	48.20 ± 0.68^{a}	46.37 ± 0.60^{a}	47.44 ± 0.99^{a}	39.33 ± 0.88^{c}	43.75 ± 0.94^{b}	32.50 ± 0.81^{d}	41.89 ± 0.45^{b}
Creatinine mg/dl	0.36 ± 0.01^{d}	0.35 ± 0.01^{d}	0.37 ± 0.01^{d}	0.48 ± 0.01^{b}	0.39 ± 0.01^{c}	0.53 ± 0.02^{a}	0.46 ± 0.003^{b}
Urea mg/dl	22.75 ± 0.43^{d}	22.16 ± 0.37^{d}	22.52 ± 0.53^{d}	28.01 ± 0.37^{b}	22.10 ± 0.25^{d}	29.30 ± 0.22^{a}	24.41 ± 0.09^{c}
Uric acid mg/dl	22.22 ± 0.21^{d}	22.61 ± 0.52^{d}	23.10 ± 0.23^{d}	26.55 ± 0.64^{b}	23.00 ± 0.27^{d}	28.88 ± 0.41^{a}	24.67 ± 0.24^{c}
Total protein g/dl	4.21 ± 0.22^{a}	4.37 ± 0.25^{a}	4.42 ± 0.24^{a}	2.72 ± 0.18^{b}	3.98 ± 0.11^{a}	1.85 ± 0.14^{c}	2.93 ± 0.19^{b}
Albumin g/dl	1.90 ± 0.02^{a}	1.88 ± 0.01^{ab}	1.93 ± 0.02^{a}	1.12 ± 0.04^{d}	1.80 ± 0.04^{b}	1.06 ± 0.02^{d}	1.43 ± 0.03^{c}

Data are represented as means ± SE (n = 10). Values with different superscript letter in the same row for each parameter are significantly different ($P < 0.05$).

3.3. Tissue MDA and Antioxidant Biomarkers

MDA levels were significantly increased in the tissues namely, liver, kidney and gill of DM-exposed fish when compared to non-treated ones. Treatment with SP significantly ($P \leq 0.05$) reduced MDA level in comparison to DM-exposed fish. SP alone had no significant effects in the liver, kidney, and gill MDA levels (**Figure 1**). Hepatic, renal and gill tissue of GSH level showed a significant depletion in the groups administered DM compared to the control group. Combination of DM and SP significantly ($P \leq 0.05$) enhanced tissue GSH level compared

to the DM-treated fish. There were no significant differences between enzyme activity of the group that was administered SP alone and the control group (**Figure 2**). The activity of SOD, CAT and GSH-Px were significantly decreased in the liver, kidney and gill tissue samples of the groups treated only with DM. On the other hand, Tissues from fish treated with SP showed significant ($P \leq 0.05$) improvement in the activity of SOD, CAT and GSH-Px than the DM-treated groups. Fish administrated SP exhibited no significant difference in the tissue SOD, CAT and GSH-Px levels compared to the control group (**Figures 3-5**).

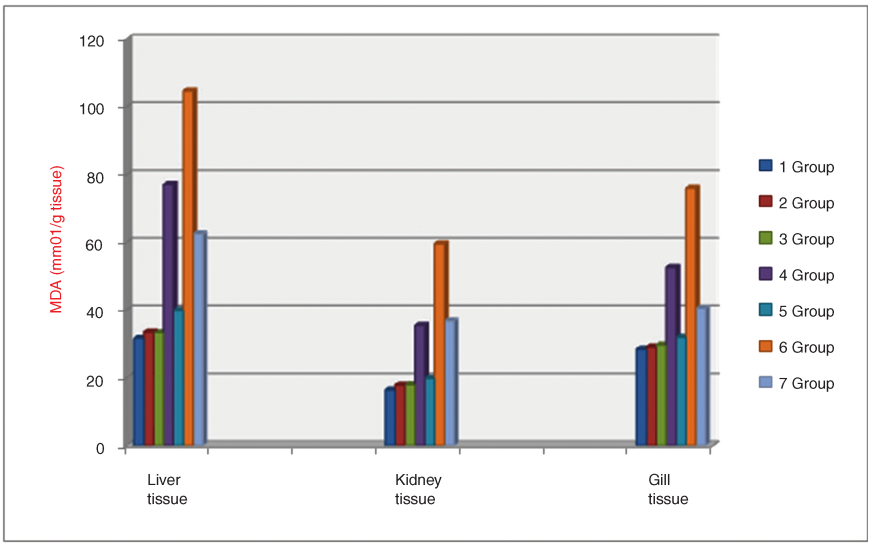


Figure 1. Showing liver, kidney and gill MDA levels (nmol/g tissue) in *C. gariepinus* exposed to 1/10 and 1/4 LC_{50} of DM and treated with SP (150 mg/kg b.w.) for 30 days. Group 1, control, Group 2, orally administered saline, Group 3, orally administered SP (150 mg/kg b.w.), Group 4, exposed to 5.19 µg/l DM, Group 5, exposed to 5.19 µg/l DM and orally administered SP (150 mg/kg b.w.), Group 6, exposed to 12.97 µg/l DM, Group 7, exposed to 12.97 µg/l DM and orally administered SP (150 mg/kg b.w.).

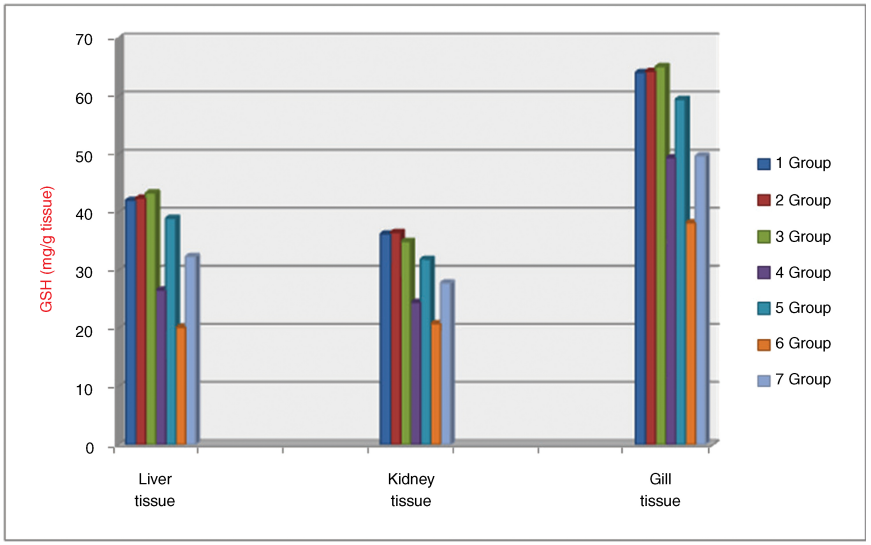


Figure 2. Showing liver, kidney and gill GSH levels (mg/g tissue) in *C. gariepinus* exposed to 1/10 and 1/4 LC_{50} of DM and treated with SP (150 mg/kg b.w.) for 30 days. Group 1, control, Group 2, orally administered saline, Group 3, orally administered SP (150 mg/kg b.w.), Group 4, exposed to 5.19 µg/l DM, Group 5, exposed to 5.19 µg/l DM and orally administered SP (150 mg/kg b.w.), Group 6, exposed to 12.97 µg/l DM, Group 7, exposed to 12.97 µg/l DM and orally administered SP (150 mg/kg b.w.).

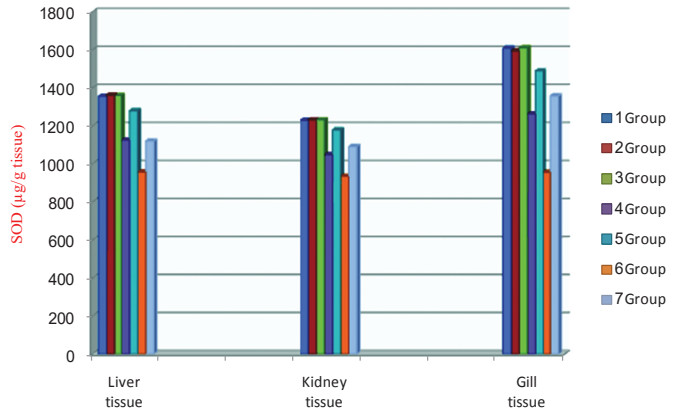


Figure 3. Showing liver, kidney and gill SOD levels (µg/g tissue) in *C. gariepinus* exposed to 1/10 and 1/4 LC_{50} of DM and treated with SP (150 mg/kg b.w.) for 30 days. Group 1, control, Group 2, orally administered saline, Group 3, orally administered SP (150 mg/kg b.w.), Group 4, exposed to 5.19 µg/l DM, Group 5, exposed to 5.19 µg/l DM and orally administered SP (150 mg/kg b.w.), Group 6, exposed to 12.97 µg/l DM, Group 7, exposed to 12.97 µg/l DM and orally administered SP (150 mg/kg b.w.).

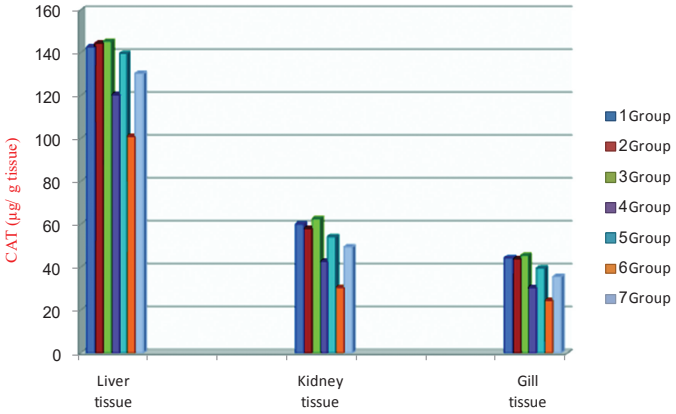


Figure 4. Showing liver, kidney and gill CAT levels (µg/g tissue) in *C. gariepinus* exposed to 1/10 and 1/4 LC_{50} of DM and treated with SP (150 mg/kg b.w.) for 30 days. Group 1, control, Group 2, orally administered saline, Group 3, orally administered SP (150 mg/kg b.w.), Group 4, exposed to 5.19 µg/l DM, Group 5, exposed to 5.19 µg/l DM and orally administered SP (150 mg/kg b.w.), Group 6, exposed to 12.97 µg/l DM, Group 7, exposed to 12.97 µg/l DM and orally administered SP (150 mg/kg b.w.).

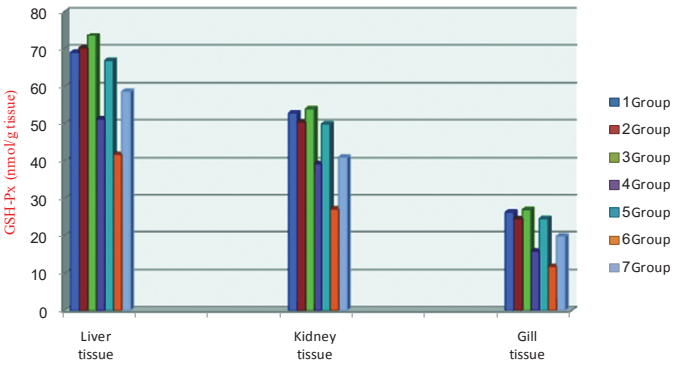


Figure 5. Showing liver, kidney and gill GSH-Px level (nmol/g tissue) in *C. gariepinus* exposed to 1/10 and 1/4 LC_{50} of DM and treated with SP (150 mg/kg b.w.) for 30 days. Group 1, control, Group 2, orally administered saline, Group 3, orally administered SP (150 mg/kg b.w.), Group 4, exposed to 5.19 µg/l DM, Group 5, exposed to 5.19 µg/l DM and orally administered SP (150 mg/kg b.w.), Group 6, exposed to 12.97 µg/l DM, Group 7, exposed to 12.97 µg/l DM and orally administered SP (150 mg/kg b.w.).

4. Discussion

The96 h LC_{50} value of DM in *C. gariepinus* was found as 51.89µg/l in the present study; hence, DM is considered to be highly toxic to fish. The current results are relevant with its potential risk and in good agreement with Datta and Kaviraj [42] who reported 96 h LC_{50} value of DM on catfish as 40.01 µg/l. The determined 96 h LC_{50} of DM was 0.38 mg/l for Indian major carp, *Labeo rohita* (*L. rohita*) [43], 0.0142 mg/l for the freshwater fish, *Puntius chrysopterus* (*P. chrysopterus*) [44], 0.7 µg/l for rainbow trout, *Oncorhynchus mykiss* (*O. mykiss*) [2], 14.6 µg/l for *Oreochromis niloticus* (*O. niloticus*) (monosex type) [45]. Studying the biochemical parameters of exposed fish, may be among the more sensitive indicators of early changes due to the hazardous exposure to insecticides [46]. The tested catfish exposed to DM pesticide showed a significantly ($P < 0.05$) increased serum AST, ALT and ALP activities. The correlation between elevation of hepatic enzymes with liver damage and dysfunction was reported in many studies [23] [45] [47] [48]. Therefore, measurement of transaminases and phosphatase activities in blood plasma of fish can be used as indicator for pesticide toxicity [49]. The increase in renal parameters could be explained by the changes in kidney tissue through the shrinkage of glomeruli and the breakdown of Bowman's capsules of the exposed fish [23] [45]-[47]. Also, the elevation in creatinine level may result from oxidative damage [50]. Proteins are the most essential macromolecules in living beings, which play a vital role in architecture and physiology of the cell and in cellular metabolism [46]. The reduction of total serum protein and albumin at both concentrations of DM could be attributed to adjustment of the fish to its new environmental circumstances as a result of stress response [51]. Similar results were recorded after exposed *C. gariepinus* to diazinon and DM, respectively [46] [47]. In contrary, Velíšek *et al.* [52] estimated a significant increase in total protein and albumin in rainbow trout. The findings of this study suggested that SP provided a marked normalization of the serum biochemical parameters. Oxidative stress in aquatic organisms, particularly fish, has a great importance for environmental and aquatic toxicology. Because oxidative stress is induced by many chemicals, including some pesticides, these pollutants may stimulate ROS and alteration in antioxidant systems [53]. It is well documented that DM may prompt oxidative stress [7] [54]. In the current study, exposure of the fish to DM at both concentrations induced an increase in MDA level of the hepatic, renal and gill tissues. Previously, DM was reported to prompt an increase in MDA levels in *Cyprinus carpio* (*C. carpio*) [3], *O. niloticus* [23] and *C. gariepinus* [47]. The increase in MDA level following DM exposure may be attributed to an excessive production of ROS [55]. However, the simultaneous treatment with SP decreased the levels of MDA in the different tissues of DM-treated fish, which may be due to the antioxidant active constituents found in SP such as C-phy- cocyanins, β-carotene, minerals, vitamins, proteins, carbohydrates and lipids [56]. Chronic exposure to DM decreased hepatic, renal and gill non-enzymatic antioxidant, GSH levels compared to the control group. Previous investigations have reported the reduction of GSH by pesticides such as DM [23] and endosulfan [57]. The decrease in GSH level in the tissues of exposed fish may be due to its exploitation to encounter the prevalent oxidative stress under the influence of ROS generated from DM oxidative stress. Also, the depletion in GSH levels as an adaptive response to the increment in LPO levels, demonstrating severe oxidative stress [58]. As it is known that the antioxidant enzymes CAT, SOD and GSH-Px are the first line of defense against oxidative stress which convert superoxide anions (O_2^-) into H_2O_2 and then into H_2O and O_2 [53] [59]. This view was in agreement with Abdelkhalek *et al.* [23] who recorded significant decrease in SOD, CAT and GSH-Px levels in liver, kidney and gill tissues of tilapia fish upon exposure to DM and Hamed [60] who documented marked depletion in the hepatic SOD and CAT levels of catfish upon exposure to malathion. This decrease in the SOD activity may be the result of excessive free radical production, such as the superoxide anion and hydrogen peroxide, direct damage of its protein structure by pesticide or a direct action of pesticide on the synthesis of the enzyme [55]. Interestingly, administration of SP increased the activity of CAT, SOD and GSH-Px; this increase could be attributed to scavenging free radicals or inhibiting lipid peroxidation and superoxide radical formation.

5. Conclusion

According to the present study, we can conclude that DM treatment to *C. gariepinus* caused fluctuations of serum biochemical assays and oxidative damage in different tissues which are sensitive tools for evaluating the effects of DM for monitoring pollution in aquatic medium including fish. On the other hand, SP could be a helpful supplementation for improving the physiological status of fish treated with DM by enhancing their serum biochemical alterations and anti-oxidative capacity.

Conflict of Interest

This research received no specific grant from any funding agency in the public or commercial sectors.

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8

A 180-Day Subchronic Oral Toxicity Study of Total Flavones of *E. leptorrhizum* Stearn in Rats

Ting Liu, Lianqiang Hui, Chunyu Cao*, Ran Hao, Shuangrong Gao, Rong He, Baoqiang Dai, Lifang Wang

Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences, Beijing, China

Abstract

A subchronic oral toxicity was conducted to evaluate the safety of total flavones of *E. leptorrhizum* Stearn in Sprague-Dawley rats. The test article was administered once daily by gavage in male and female rats at dose levels of 24, 48, and 96 mg/kg body weight/day for 180 days. 90 and 180 days after administration, ten and tweedy animals (each half of male and female) of each group were tested. 28 days after withdrawal, five male and female rats were tested. There were no significant toxicological changes shown in daily clinical signs, body weight, food consumption, hematology parameters, blood biochemistry, organ weights and histopathological examination except leukocyte differential count. It was concluded that the no-observed-effect level (NOEL) for total flavones of *E. leptorrhizum* Stearn was >96 mg/kg in SD rats.

Keywords

Total Flavones of *E. leptorrhizum* Stearn, Safety, Subchronic Toxicity

1. Introduction

Herba epimedium is dried aerial parts of *E. brevicornum Maxim.* or *E. sagittatum Maxim.* or *E. pubescens Maxim.* or *E. wushanense T. S. Ying* or *E koreanum Nakai*de [1]. It was earliest recorded about 2000 years ago in the "Shen Nong's herbal classic", which is China's earliest existing medical monograph. Modern pharmacology studies prove that Epimedium can regulate immune function, increase male reproductive function, delay renal failure [2]-[6]. Therefore, herba epimedium is widely used as food additives in health products. The ex-

*Corresponding author.

traction and active ingredients are an important source of healthy foods and medicine. Many research was done on epimedium extract and active ingredient, more than twenty related patents have been approved by the US Patent and Trademark Office and the World Intellectual Property Organization [7]-[11].

The pharmacological activity of icariin is most explicit, it has multiple pharmacological effects, such as anti-tumor, enhance immune function, improve cardiovascular function, endocrine regulation, prevention and treatment of osteoporosis, anti-aging, etc. [12]. Epimendin C is also from epimedium, having a chemical structure and pharmacological activity similar to Icariin, such as anti-tumor, enhance immune function, against osteoporosis, etc. [13]-[16], therefore, expected to become the new food additives.

Icariin is used as a control standard in Chinese Pharmacopoeia [1], but very little research has been done on epimendin C, a flavonoids in epimedium. Some researchers believe that epimendin C has definite pharmacological activity, and it is another important active ingredient in epimedium. Epimendin C should be used as an important indicator to control epimedium quality [2] [3]. *E. leptorrhizum* Stearn grow mostly in Guizhou region, which is widely distributed locally. Only little amount of *E. leptorrhizum* Stearn is used as medicine because icariin content is lower than the criteria in Chinese Pharmacopoeia, while the content of epimendin C is higher. Wei Deng [17] reported that significant improvement was observed when *E. leptorrhizum* Stearn applied on osteoporosis model mice induced by dexamethasone. Our research also showed that the total flavones of *E. leptorrhizum* Stearn could promote hFOB1.19 osteoblast proliferation and ALP secretion [18]. It indicated that the promotion of osteoblast activity by total flavones of *E. leptorrhizum* Stearn maybe a pathway of prevention and treatment of osteoporosis.

Epimedium is a traditional Chinese medicine, and no adverse reactions were reported from clinical practice. In animal studies, the median lethal dose (LD_{50}) of epimedium water extract is more than 80 g/kg, and all the results including mouse bone marrow micronucleus test, Ames test, TK Gene mutation test were negative [19]. The total flavones of Epimedium 1.0, 2.0 and 4.0 g/kg showed no distinct toxicity, taken orally once a day, continuous for 12 weeks [20]. MTD of acute toxicity test in mice was 1440 times of Human clinical dose, it showed a very mild toxicity [21]. The herb used for above research is not from *E. leptorrhizum* Stearn. There are no animals and no clinically relevant reports about the safety of *E. leptorrhizum* Stearn whether raw material or extract. It is likely to be useful as an alternative medicine as epimedium if it can be confirmed to be safe.

A 180-day repeated oral dose toxicity study in rats was conducted to evaluate the safety of excessive intake of total flavones of *E. leptorrhizum* Stearn. The present study was conducted in compliance with technical specifications for examination and assessment of health food as set forth in Ministry of Health of the People's Republic of China, issued 2003, which meet OECD guidelines.

2. Materials and Methods

2.1. Test Article

E. leptorrhizum Stearn which was provided by Guizhou Tongjitang pharmaceutical CO. LTD. Add 20 times of 70% ethanol into *E. leptorrhizum* Stearn, reflux 3 times, each time for 1 hour, combine extracts, recycling to concentrated liquid. Add distilled water (1:1) into concentrated liquid, allow the solid to settle, discard the precipitation, oncentrate the supernatant. Dry the concentrated extraction under vacuum condition at 80˚C, crushing, that is flavonoids part of *E. leptorrhizum* Stearn (YYH-C), which content 4.96 mg/g of flavonoids, 1.17% of Icariin and 8.87% of Epimendin C [22]. YYH-C was dissolved with distilled water to 2.4, 4.8, and 9.6 mg/ml to provide administered doses of 24, 48, and 96 mg/kg/day.

2.2. Aminals

Male and female Sprague-Dawley rats at 5 weeks of age purchased from Beijing Weitonglihua experimental animal technique Co. Ltd (Licence number: SCXK (Jing) 2012-0001.). The animals were acclimatized to housing conditions for a period of one week, and the treatment started at 6 weeks of age to health animals. The animals were kept in a room maintained at a temperature 23˚C ± 3˚C and a relative humidity of 40% - 70% with 12 hours light and 12 hours dark cycle and with approximately 15 times air change per hour. Animals were bred in bottom-meshed stainless steel cages, with filtrated tap water and the fixed-formula rat granula feed. This experiment was approved by the Animal Care & Welfare Committee of Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences.

2.3. Grouping and Administration

The rats were randomly divided into four groups: a control group and three treatment groups of YYH-C 24, 48, 96 mg/kg. Each group consisted of 20 males and 20 females. The test articles were administered daily by gavage to rats for 180 days at dose of 24, 48, and 96 mg/kg, and the control group rats were given the same volume of distilled water. Individual dose volume of 10 ml/kg was calculated according to the latest measured body weight.

2.4. Observation and Clinical Examination

2.4.1. General Conditions, Body Weight, and Food Consumption

General conditions of rats such as behavior, gait and general activity, appearance, hair luster, stool and urine secretion and so on were observed before and after dosing each day. The body weights of all rats and the remaining granules in each crib were weighed once a week during study under the condition of no fasting.

2.4.2. Hematology and Serum Biochemistry

After the treatment, all animals were fasted overnight and then anesthetized by phenobarbital sodium, and blood samples were collected from the abdominal aorta. The following parameters for hematology analysis using anticoagulated whole blood with EDTA 2 K were measured: red blood cell count (RBC), white blood cell count (WBC), hemoglobin (Hb), haematocrit (Ht), blood platelet number (PLT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), leukocyte differential count, and reticulocytes(Ret) were analyzed by using an automatic blood cell analyser (Siemens ADVIA-2120，made in Germany). The following parameters for blood biochemical analysis using serum were measured: total protein (TP), albumin (ALB), aspartate aminotransferase (AST), alanine amiotransferase (ALT), alkaline phosphatase (ALP), γ-glutamyl transpeptidase (γ-GT), creatine kinase (CK), urea nitrogen (UREA), creatinine (CRE), glucose (GLU), total bilirubin (T-bil), total cholesterol (T-cho), triglyceride (TG) were determined by automatic biochemistry analyzer (TOSHIBA 40FR, made in Japan), and Na^+, K^+, Cl^- were determined by electrolytes analyzer (Easy Lyte PLUS Na/K/Cl NALYZER, made in America).

2.4.3. Histomorphological Assessment

The animals were sacrificed by exsanguinations from the abdominal aorta. At necropsy, the brain, thymus, lung with bronchi, heart, liver, spleen, kidney, adrenal gland, testicle, epididymis, ovary and uterus were removed and weighted. The relative organ weights were calculated using the animals, fasted body weights. In addition, pituitary, the spinal cord, thyroid glands, salivary glands, optic nerve, eyes, stomach, pancreas, small intestine (duodenum, jejunum and ileum), large intestine (cecum, colon and rectum), urinary bladder, epididymis, prostate and bone marrow were also removed. All organs and tissues were examined grossly and then fixed in 10% buffered formalin solution. Histomorphological assessment was first performed on all tissues of control group and the highest dose group. If any treatment-related changes appeared at the highest dose group, the relavant tissues from the lower dose groups were then examined. The organs and tissues were routinely processed for paraffin-embedding, sliced, HE (Haematoxylin & Eosin) stained, and then histomorphological observation was performed under microscopes.

2.5. Statistical Analysis

Data of males and females in each group were separately calculated, and expressed as mean ± SD (standard deviation). Variance in data for body weights, food consumption, hematology, serum biochemistry, and relative organ weights was checked for homogeneity and analyzed with one-way analysis of variance (ANOVA). If the variance was homogeneous, the data were analylyzed by LSD test, if not, the Tamhane's T2 test was applied. Probability values of $P < 0.05$ were considered significant.

3. Result

3.1. In-Life Parameters

No deaths and no clinical signs to the treatment were observed during the administration period.

Body weights (**Figure 1** & **Figure 2**) and food consumption (**Figure 3** & **Figure 4**) in both sexes in each

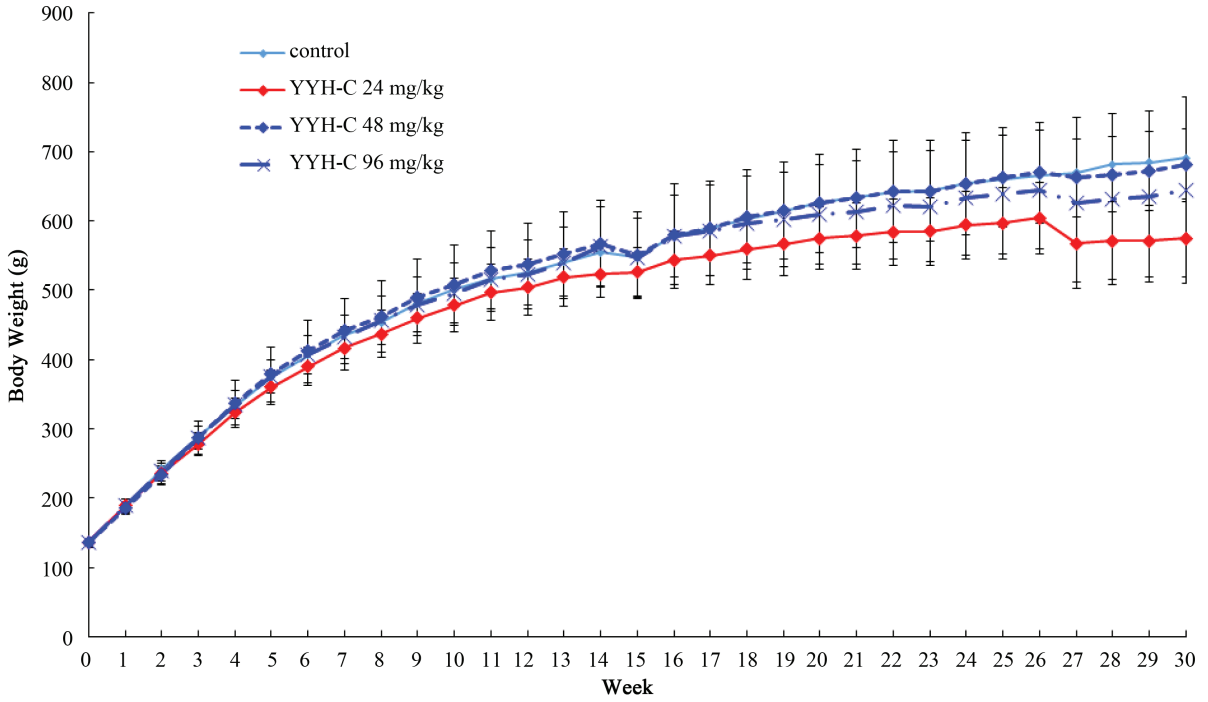


Figure 1. Mean body weight of male rats.

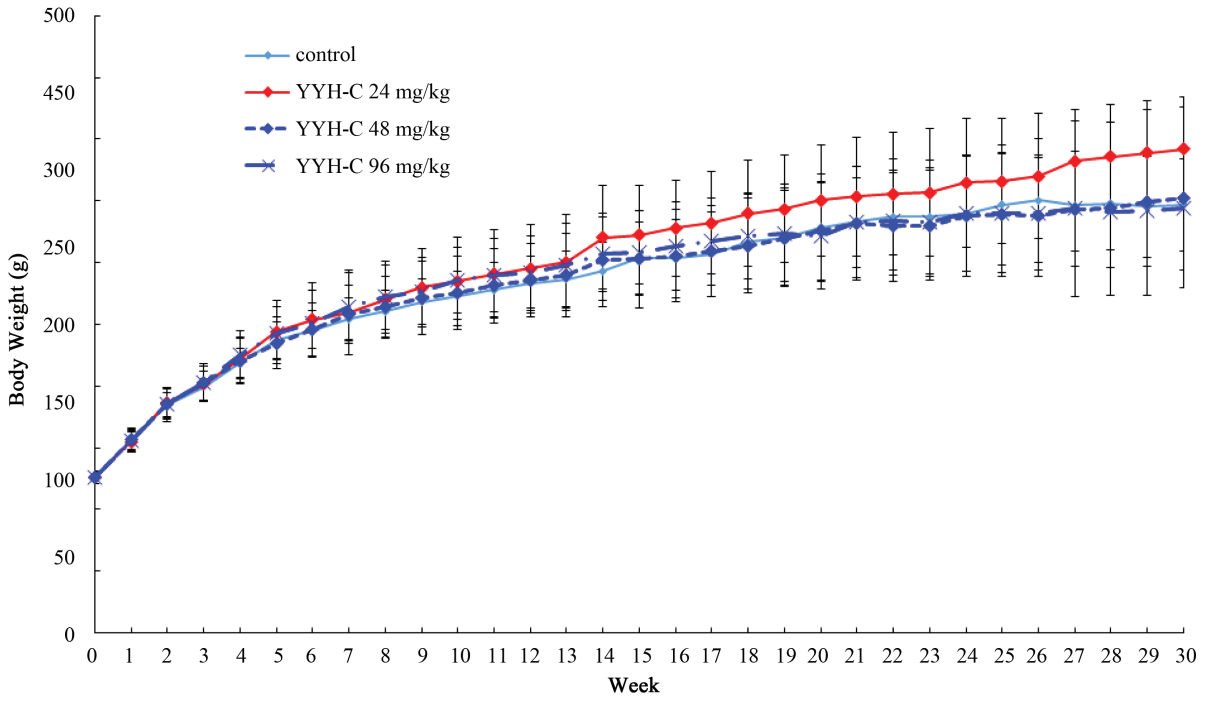


Figure 2. Mean body weight of female rats.

treatment group were comparable to those in the control group. Male rats of 24 mg/kg group of YYH-C decreased significantly on food consumptions in 20 - 23 weeks, as well as 96 mg/kg dose group in 21 week ($P < 0.05$, $P < 0.01$). In 25 - 26 week, 24 mg/kg group of YYH-C reduced significantly compared with the control group both on body weights and food consumptions ($P < 0.05$). The rest of the time points of the remaining treatment group no statistically difference was observed.

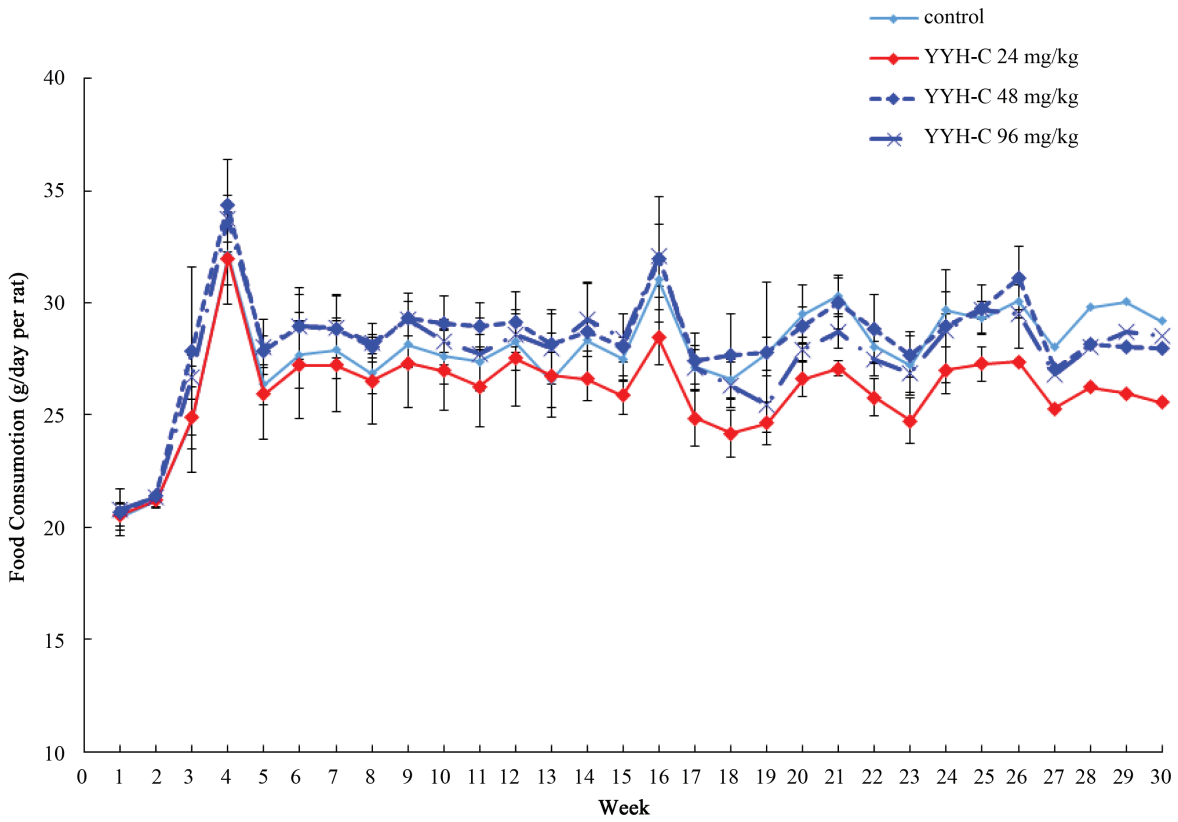


Figure 3. Mean food consumption of male rats.

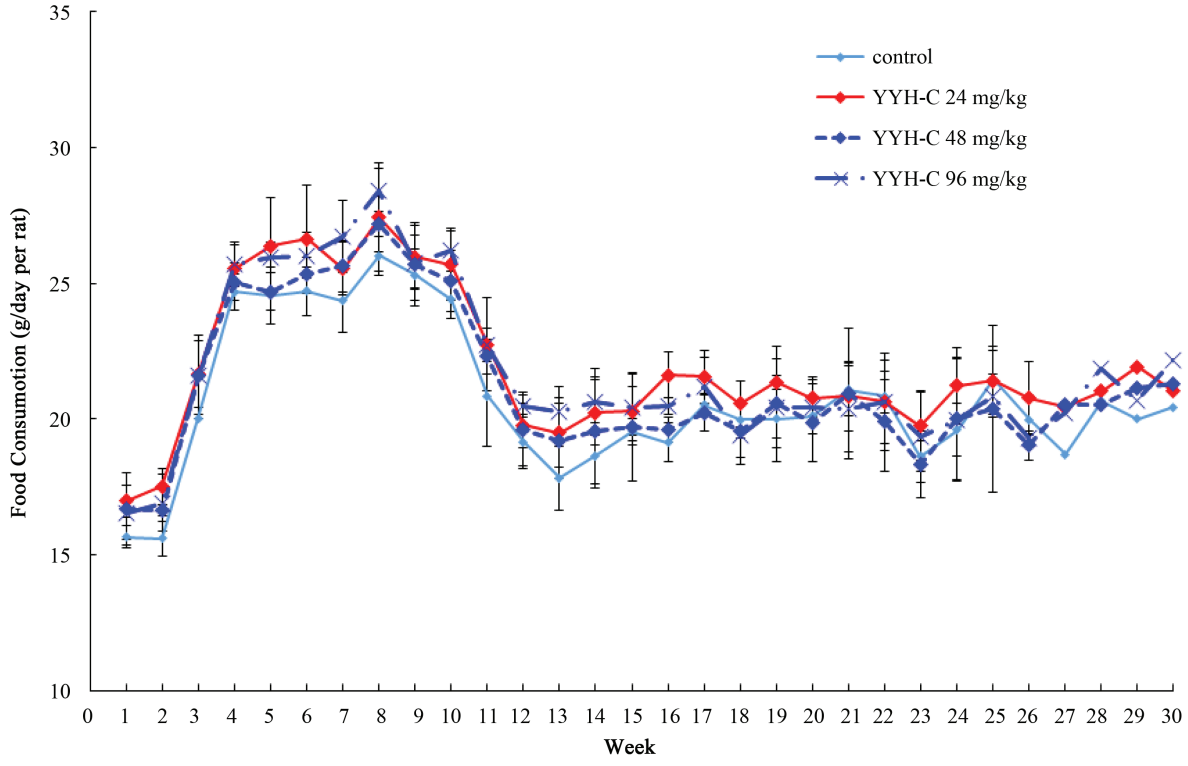


Figure 4. Mean food consumption of female rats.

3.2. Hematology Parameters

Hematology data are shown in **Tables 1-3**.

A dose-depended obvious decrease of LYM% and increase of MID% were seen in male rats of all three dose groups after 90 days administration ($P < 0.05$, $P < 0.01$), moreover, decrease of LYM% and increase of MID% were also seen after 180 days administration with no obviously dose-depended manner ($P > 0.05$). However, an obvious increase of LYM% and decrease of MID% were seen in female rats of 48, 96 mg/kg dose groups after 180 days administration ($P < 0.05$, $P < 0.01$, $P < 0.001$).

Though the results showed an obvious decrease of RBC, Ht , Hb of female rats in 48 mg/kg group, and an obvious increase of Hb in 96 mg/kg groups after 90 days administration; an obvious increase of PLT of male rats both in 24 and 96 mg/kg groups, and an obvious decrease of Ret of female rats in all treatment groups after 180 days administration; an obvious increase of RBC, Ht, Hb and obvious decrease of PLT of female rats in all treatment groups after 28 days withdrawal ($P < 0.05$, $P < 0.01$), no toxicological significance were indicated

Table 1. Hematological examination of rats after 90 days administration (n = 5).

Sex	Parameters	Control group	YYH-C 24 mg/kg		YYH-C 48 mg/kg		YYH-C 96 mg/kg	
♂	RBC (10^{12}/L)	7.67 ± 0.35	7.72 ± 0.25		7.42 ± 0.29		7.46 ± 0.22	
	WBC (10^9/L)	4.94 ± 1.25	4.50 ± 0.70		4.90 ± 1.72		4.19 ± 1.28	
	Hb (g/L)	14.2 ± 0.6	14.0 ± 0.4		13.7 ± 0.6		13.5 ± 0.2	
	Ht (%)	39.6 ± 1.3	39.2 ± 1.1		38.3 ± 1.1		38.7 ± 0.7	
	PLT (10^9/L)	1061 ± 82	1148 ± 192		1065 ± 37		1017 ± 133	
	MCV (fL)	51.7 ± 0.8	50.8 ± 1.2		51.7 ± 1.5		51.9 ± 0.9	
	MCH (pg)	18.5 ± 0.2	18.1 ± 0.5		18.5 ± 0.5		18.2 ± 0.4	
	MCHC (g/L)	35.8 ± 0.5	35.7 ± 0.2		35.7 ± 0.5		34.9 ± 0.2	
	LYM%	85.4 ± 2.4	77.8 ± 6.4	*	76.1 ± 3.4	*	73.0 ± 6.9	**
	MID%	9.74 ± 4.94	18.04 ± 5.99	*	20.10 ± 2.39	**	22.68 ± 7.30	**
	GRN%	1.74 ± 1.03	2.34 ± 1.28		1.84 ± 1.06		2.26 ± 1.12	
	MONO%	2.82 ± 3.74	1.66 ± 0.37		1.60 ± 0.38		1.76 ± 0.37	
	RET‰	2.77 ± 0.27	2.85 ± 0.26		2.69 ± 0.35		2.67 ± 0.51	
♀	RBC (10^{12}/L)	7.07 ± 0.25	7.02 ± 0.15		6.71 ± 0.23	**	7.19 ± 0.06	
	WBC (10^9/L)	1.66 ± 0.15	3.43 ± 0.97		2.60 ± 0.25		2.78 ± 0.86	
	Hb (g/L)	13.3 ± 0.5	13.3 ± 0.3		12.8 ± 0.4	*	13.8 ± 0.2	*
	Ht (%)	37.1 ± 1.4	36.5 ± 0.6		35.6 ± 0.7	*	38.1 ± 0.5	
	PLT (10^9/L)	1055 ± 134	1,131 ± 114		949 ± 157		1057 ± 67	
	MCV (fL)	52.4 ± 1.0	52.1 ± 1.3		53.0 ± 2.5		53.0 ± 1.0	
	MCH (pg)	18.8 ± 0.3	18.9 ± 0.5		19.0 ± 1.0		19.2 ± 0.4	
	MCHC (g/L)	35.8 ± 0.3	36.3 ± 0.4		35.9 ± 0.6		36.2 ± 0.7	
	LYM%	77.1 ± 4.8	80.3 ± 6.3		78.3 ± 5.4		79.1 ± 7.2	
	MID%	18.5 ± 4.0	16.2 ± 5.8		17.7 ± 5.0		16.7 ± 6.7	
	GRN%	2.68 ± 0.91	1.88 ± 0.69		1.88 ± 0.58		2.02 ± 0.38	
	MONO%	1.36 ± 0.59	1.36 ± 0.53		1.58 ± 0.29		1.58 ± 0.66	
	RET‰	2.82 ± 0.49	2.81 ± 0.53		2.44 ± 0.49		2.62 ± 0.35	

Values are presented as mean ± standard variation. *Significantly different from the controls at levels of $P < 0.05$. **Significantly different from the controls at levels of $P < 0.01$.

Table 2. Hematological examination of rats after 180 days administration (n = 10).

Sex	Parameters	Control group	YYH-C 24 mg/kg		YYH-C 48 mg/kg		YYH-C 96 mg/kg	
♂	RBC (10^{12}/L)	7.92 ± 0.41	8.04 ± 0.23		7.74 ± 0.30		7.63 ± 0.38	
	WBC (10^9/L)	4.90 ± 1.13	4.50 ± 2.08		5.23 ± 1.59		4.63 ± 1.12	
	Hb (g/L)	14.1 ± 0.7	14.3 ± 0.7		14.3 ± 0.5		13.9 ± 0.7	
	Ht (%)	38.9 ± 1.6	38.8 ± 1.9		38.2 ± 1.4		37.4 ± 1.9	
	PLT (10^9/L)	1040 ± 149	1206 ± 150	**	1158 ± 122		1183 ± 104	*
	MCV (fL)	49.2 ± 1.7	48.3 ± 1.7		49.3 ± 1.7		49.0 ± 1.1	
	MCH (pg)	17.8 ± 1.2	17.8 ± 0.6		18.4 ± 0.6		18.2 ± 0.5	
	MCHC (g/L)	36.3 ± 2.1	36.8 ± 0.4		37.4 ± 0.2		37.1 ± 0.4	
	LYM%	72.8 ± 6.1	66.3 ± 12.5		60.1 ± 14.2		69.0 ± 6.4	
	MID%	22.14 ± 5.62	27.71 ± 12.45		33.64 ± 13.76		26.60 ± 5.88	
	GRN%	2.19 ± 0.65	3.39 ± 0.97		3.56 ± 2.80		1.86 ± 0.50	
	MONO%	2.57 ± 1.00	2.35 ± 0.73		2.46 ± 0.83		2.37 ± 0.73	
	RET‰	2.70 ± 0.59	2.31 ± 0.46		2.74 ± 0.30		2.76 ± 0.39	
♀	RBC (10^{12}/L)	6.90 ± 0.33	7.30 ± 0.19		7.23 ± 0.35		7.10 ± 0.42	
	WBC (10^9/L)	2.23 ± 0.55	2.20 ± 0.75		2.66 ± 0.78		2.49 ± 0.49	
	Hb (g/L)	13.5 ± 0.6	14.0 ± 0.4		14.0 ± 0.6		13.7 ± 0.8	
	Ht (%)	36.0 ± 1.4	37.3 ± 1.2		37.1 ± 1.8		36.6 ± 2.3	
	PLT (10^9/L)	1001 ± 126	959 ± 107		1025 ± 112		1090 ± 114	
	MCV (fL)	52.2 ± 1.7	51.1 ± 1.9		51.3 ± 1.4		51.6 ± 0.7	
	MCH (pg)	19.6 ± 0.5	19.2 ± 0.6		19.4 ± 0.6		19.3 ± 0.3	
	MCHC (g/L)	37.5 ± 0.5	37.5 ± 0.6		37.8 ± 0.3		37.4 ± 0.4	
	LYM%	71.7 ± 7.8	69.6 ± 7.9		83.1 ± 3.1	***	81.3 ± 8.1	**
	MID%	22.7 ± 8.1	24.7 ± 7.9		13.0 ± 3.2	**	14.4 ± 8.2	*
	GRN%	2.48 ± 0.89	2.68 ± 1.66		1.87 ± 0.46		1.65 ± 0.83	
	MONO%	2.56 ± 0.76	2.58 ± 0.79		1.72 ± 0.35		2.28 ± 1.25	
	RET‰	2.34 ± 0.52	1.94 ± 0.28	*	1.89 ± 0.35	*	1.91 ± 0.29	*

Values are presented as mean ± standard variation. *Significantly different from the controls at levels of $P < 0.05$. **Significantly different from the controls at levels of $P < 0.01$. ***Significantly different from the controls at levels of $P < 0.001$.

as the values of all above were fluctuated within the scopes of historical data of our laboratory and no obvious dose effect relationship. No abnormalities were seen in other hematology parameters of male and female rats in three YYH-C groups.

3.3. Blood Biochemistry

Serum biochemistry data are shown in **Tables 4-6**.

An obvious increase of UREA level after 90 days administration and GGT content after 180 days administration were seen in female rats of all three dose groups ($P < 0.05$, $P < 0.01$), the content of ALB of female rats in 48, 96 mg/kg groups decreased significantly after 180 days administration ($P < 0.05$), moreover, the content of ALP of female rats in 24, 48 mg/kg groups increase significantly after 28 days withdrawal ($P < 0.05$, $P < 0.001$).

The serum Cl^- level showed an obviously increase of male rats in all treatment groups and decrease of female rats in 24, 48 mg/kg groups after 90 days administration ($P < 0.05$, $P < 0.01$), The serum K^+ level decreased significantly of male rats in 96 mg/kg group and increased of female rats in 48mg/kg groups after 180 days admin-

Table 3. Hematological examination of rats after 28 days withdrawal (n = 5).

Sex	Parameters	Control group	YYH-C 24 mg/kg		YYH-C 48 mg/kg		YYH-C 96 mg/kg	
♂	RBC (10^{12}/L)	7.86 ± 0.41	8.20 ± 0.22		7.63 ± 0.50		8.12 ± 0.27	
	WBC (10^9/L)	6.07 ± 1.66	4.65 ± 1.21		4.51 ± 0.81		4.32 ± 0.88	
	Hb (g/L)	14.1 ± 0.8	14.6 ± 0.5		13.8 ± 0.3		14.4 ± 0.6	
	Ht (%)	37.9 ± 1.9	39.3 ± 1.4		37.2 ± 1.1		39.0 ± 1.3	
	PLT (10^9/L)	1,130 ± 82	1,168 ± 141		1,070 ± 25		1,161 ± 75	
	MCV (fL)	48.3 ± 1.7	48.0 ± 1.0		48.8 ± 2.0		48.0 ± 0.3	
	MCH (pg)	17.9 ± 0.7	17.7 ± 0.5		18.2 ± 1.0		17.7 ± 0.2	
	MCHC (g/L)	37.2 ± 0.3	37.0 ± 0.4		37.2 ± 0.6		37.0 ± 0.3	
	LYM%	76.2 ± 6.0	66.2 ± 8.0		66.4 ± 6.6		68.0 ± 7.9	
	MID%	19.24 ± 5.24	28.16 ± 6.93		27.72 ± 6.63		25.72 ± 6.98	
	GRN%	2.10 ± 0.42	2.52 ± 0.75		3.12 ± 0.65		2.70 ± 0.66	
	MONO%	2.28 ± 0.83	2.96 ± 0.73		2.56 ± 1.02		3.42 ± 0.83	
	RET‰	2.66 ± 0.49	2.41 ± 0.53		2.68 ± 0.64		2.53 ± 0.53	
♀	RBC (10^{12}/L)	6.72 ± 0.38	7.14 ± 0.12	*	7.29 ± 0.24	**	7.19 ± 0.29	*
	WBC (10^9/L)	2.11 ± 0.76	2.27 ± 0.53		2.45 ± 0.58		1.97 ± 0.68	
	HGB (g/L)	12.9 ± 0.7	13.9 ± 0.4	**	14.1 ± 0.5	**	13.8 ± 0.5	*
	HCT (%)	34.4 ± 2.1	37.3 ± 1.2	**	37.7 ± 1.6	**	36.6 ± 1.3	*
	PLT (10^9/L)	1097 ± 58	964 ± 87	*	910 ± 81	**	947 ± 106	*
	MCV (fL)	51.2 ± 0.7	52.3 ± 1.5		51.7 ± 1.3		50.9 ± 0.6	
	MCH (pg)	19.2 ± 0.4	19.5 ± 0.6		19.4 ± 0.4		19.1 ± 0.5	
	MCHC (g/L)	37.5 ± 0.3	37.3 ± 0.4		37.6 ± 0.4		37.6 ± 0.6	
	LYM%	70.0 ± 7.8	73.0 ± 8.4		70.2 ± 11.0		70.7 ± 8.5	
	MID%	24.1 ± 7.4	21.0 ± 6.4		23.8 ± 9.7		23.2 ± 7.5	
	GRN%	2.70 ± 0.69	2.72 ± 1.23		2.56 ± 0.92		2.54 ± 0.82	
	MONO%	2.84 ± 0.81	2.90 ± 1.03		3.02 ± 1.08		3.18 ± 1.29	
	RET‰	2.22 ± 0.34	2.31 ± 0.30		2.49 ± 0.91		2.11 ± 0.43	

Values are presented as mean ± standard variation. *Significantly different from the controls at levels of $P < 0.05$. **Significantly different from the controls at levels of $P < 0.01$.

istration ($P < 0.05$), but the change had no physiological significance because the value of these group was fluctuated in a tight range and within the scopes of historical data of our laboratory.

Some other indicators, including the content of AST, UREA, CK of male rats and CRE of female rats after 90 days administration, GLU, CK concentration of male rats and T-bil of female rats after 180 days administration, the content of T-bil, TG of male rats after 28 days withdrawal, were considered as no toxicological significant as the changes mentioned above were decreased compared with the control group.

3.4. Organ Weights

An obvious increase of the relative organ weight of spleen after 90 days administration in female rats of 48mg/kg dose groups ($P < 0.01$) and the relative organ weight of adrenal after 180 days administration in male rats of all treatment groups ($P < 0.05$, $P < 0.01$, $P < 0.001$), however, the changes mentioned above weren't showed a dose-depended relationship, the mean of the relative organ weight of spleen was 0.193, 0.199, 0.250,

Table 4. Blood biochemistry analysis of rats after 90 days administration (n = 5).

Sex	Parameters	Control group	YYH-C 24 mg/kg		YYH-C 48 mg/kg		YYH-C 96 mg/kg	
♂	TP (g/L)	57.7 ± 2.2	57.2 ± 1.7		54.6 ± 2.9		56.6 ± 2.1	
	ALB (g/L)	34.1 ± 1.2	33.9 ± 1.4		32.4 ± 1.8		33.8 ± 1.1	
	AST (U/L)	122 ± 24	123 ± 25		85 ± 17	*	76 ± 12	**
	ALT (U/L)	34 ± 3	40 ± 4		33 ± 3		35 ± 11	
	ALP (U/L)	104 ± 12	108 ± 24		109 ± 18		114 ± 24	
	γ-GT (U/L)	0.75 ± 0.23	0.71 ± 0.17		0.72 ± 0.21		0.72 ± 0.20	
	UREA (mmol/L)	7.8 ± 1.4	5.7 ± 0.8	**	5.9 ± 0.9	**	6.2 ± 0.7	*
	CRE (μmol/L)	33.4 ± 5.9	25.6 ± 2.9		28.2 ± 0.8		25.6 ± 4.2	
	GLU (mmol/L)	7.8 ± 0.6	7.4 ± 0.8		7.3 ± 0.4		6.9 ± 0.4	
	T-bil (μmol/L)	3.8 ± 0.8	3.6 ± 1.2		2.4 ± 0.8		3.2 ± 0.9	
	T-cho (mmol/L)	1.5 ± 0.20	1.6 ± 0.2		1.6 ± 0.2		1.6 ± 0.4	
	TG (mmol/L)	0.3 ± 0.0	0.4 ± 0.2		0.4 ± 0.2		0.4 ± 0.2	
	CK (U/L)	523 ± 163	496 ± 205		210 ± 95		114 ± 66	*
	Na^+ (mmol/L)	146.2 ± 2.3	147.7 ± 1.0		148.2 ± 0.9		149.5 ± 0.5	
	K^+ (mmol/L)	4.6 ± 0.3	4.523 ± 0.3		4.4 ± 0.3		4.1 ± 0.4	
	Cl^- (mmol/L)	106.9 ± 0.9	108.8 ± 1.2	*	108.9 ± 1.2	*	109.7 ± 1.4	**
♀	TP (g/L)	65.1 ± 2.3	63.8 ± 3.0		65.5 ± 6.4		66.8 ± 2.4	
	ALB (g/L)	40.4 ± 1.7	39.6 ± 2.4		40.5 ± 4.1		41.7 ± 1.6	
	AST (U/L)	108 ± 9	92 ± 16		102 ± 13		117 ± 17	
	ALT (U/L)	30 ± 5	30 ± 3		38 ± 7		38 ± 9	
	ALP (U/L)	55 ± 16	80 ± 27		44 ± 6		51 ± 8	
	γ-GT (U/L)	0.72 ± 0.18	0.8548 ± 0.18		0.76 ± 0.23		1.04 ± 0.33	
	UREA (mmol/L)	6.3 ± 0.6	7.5 ± 0.7	*	7.2 ± 0.9		8.0 ± 0.6	**
	CRE (μmol/L)	29.8 ± 3.0	30.0 ± 2.0		24.6 ± 3.5	**	30.4 ± 2.3	
	GLU (mmol/L)	6.7 ± 0.7	7.2 ± 0.6		6.9 ± 0.6		6.9 ± 0.3	
	T-bil (μmol/L)	2.8 ± 0.8	1.8 ± 0.4		2.7 ± 1.3		3.5 ± 0.7	
	T-cho (mmol/L)	2.2 ± 0.4	2.1 ± 0.6		2.3 ± 0.4		2.1 ± 0.4	
	TG (mmol/L)	0.3 ± 0.1	0.2 ± 0.0		0.4 ± 0.2		0.3 ± 0.1	
	CK (U/L)	399 ± 98	298 ± 86		399 ± 116		422 ± 196	
	Na^+ (mmol/L)	148.4 ± 0.8	146.9 ± 1.2		147.7 ± 1.3		147.8 ± 0.5	
	K^+ (mmol/L)	4.0 ± 0.1	4.1 ± 0.1		4.1 ± 0.2		4.1 ± 0.1	
	Cl^- (mmol/L)	111.0 ± 1.5	108.3 ± 1.1	**	108.7 ± 1.8	*	110.0 ± 1.0	

Values are presented as mean ± standard variation. *Significantly different from the controls at levels of $P < 0.05$. **Significantly different from the controls at levels of $P < 0.01$.

and 0.196, the mean of the relative organ weight of adrenal was 0.009, 0.013, 0.015, and 0.013 (the control group and YYH-C 24, 48, 96 mg/kg group respectively). There were no significant differences in relative organ weights between the other treatment compared with the control groups in both sexes (data not shown).

Table 5. Blood biochemistry analysis of rats after 180 days administration (n = 10).

Sex	Parameters	Control group	YYH-C 24 mg/kg		YYH-C 48 mg/kg		YYH-C 96 mg/kg	
♂	TP (g/L)	60.6 ± 3.2	59.1 ± 2.7		58.5 ± 2.9		59.8 ± 3.4	
	ALB (g/L)	35.3 ± 1.9	34.6 ± 1.4		34.6 ± 1.9		34.9 ± 1.9	
	AST (U/L)	172 ± 119	145 ± 35		121 ± 22		140 ± 116	
	ALT (U/L)	59 ± 44	52 ± 15		47 ± 9		73 ± 81	
	ALP (U/L)	89 ± 19	94 ± 14		92 ± 18		86 ± 15	
	γ-GT (U/L)	0.70 ± 0.70	0.81 ± 0.25		0.865 ± 0.27		0.76 ± 0.41	
	UREA (mmol/L)	6.7 ± 1.0	6.6 ± 1.1		6.1 ± 0.6		6.3 ± 1.0	
	CRE (μmol/L)	31.8 ± 4.6	29.4 ± 4.2		29.3 ± 1.4		28.6 ± 3.8	
	GLU (mmol/L)	8.3 ± 1.0	7.9 ± 0.9		7.0 ± 0.4	*	7.4 ± 0.7	
	T-bil (μmol/L)	4.8 ± 1.3	4.3 ± 1.7		4.4 ± 1.1		4.1 ± 1.3	
	T-cho (mmol/L)	1.9 ± 0.4	1.9 ± 0.4		1.8 ± 0.4		2.0 ± 0.2	
	TG (mmol/L)	0.5 ± 0.1	0.4 ± 0.3		0.5 ± 0.2		0.5 ± 0.2	
	CK (U/L)	527 ± 179	489 ± 144		371 ± 187	*	167 ± 143	***
	Na^+ (mmol/L)	146.0 ± 2.2	146.6 ± 1.3		146.5 ± 1.2		147.0 ± 1.0	
	K^+ (mmol/L)	4.9 ± 0.3	5.0 ± 0.3		4.8 ± 0.3		4.5 ± 0.4	*
	Cl^- (mmol/L)	108.4 ± 1.4	108.2 ± 1.3		107.8 ± 1.2		107.9 ± 1.4	
♀	TP (g/L)	67.7 ± 3.2	68.2 ± 4.8		65.2 ± 3.6		65.7 ± 3.0	
	ALB (g/L)	41.4 ± 2.4	41.0 ± 2.3		39.2 ± 2.2	*	39.1 ± 1.8	*
	AST (U/L)	113 ± 25	98 ± 13		106 ± 27		90 ± 20	
	ALT (U/L)	40 ± 8	35 ± 8		34 ± 9		38 ± 6	
	ALP (U/L)	35 ± 17	35 ± 9		34 ± 6		48 ± 21	
	γ-GT (U/L)	0.55 ± 0.28	0.80 ± 0.19	*	0.86 ± 0.16	**	0.90 ± 0.21	**
	UREA (mmol/L)	6.1 ± 0.7	6.1 ± 0.7		6.3 ± 1.4		6.6 ± 0.9	
	CRE (μmol/L)	29.4 ± 4.5	29.7 ± 1.9		30.4 ± 4.8		29.4 ± 2.8	
	GLU (mmol/L)	6.7 ± 0.6	7.3 ± 0.9		6.9 ± 0.6		7.3 ± 0.4	
	T-bil (μmol/L)	5.0 ± 1.7	5.9 ± 1.6		3.6 ± 1.3	*	4.2 ± 1.4	
	T-cho (mmol/L)	2.2 ± 0.5	1.8 ± 0.6		2.1 ± 0.6		2.2 ± 0.6	
	TG (mmol/L)	0.6 ± 0.26	0.66 ± 0.3		0.4 ± 0.2		0.4 ± 0.2	
	CK (U/L)	411 ± 164	319 ± 84		357 ± 195		238 ± 154	
	Na^+ (mmol/L)	145.0 ± 1.7	146.1 ± 1.0		146.5 ± 1.8		146.6 ± 2.5	
	K^+ (mmol/L)	4.2 ± 0.2	4.3 ± 0.2		4.4 ± 0.3	*	4.1 ± 0.3	
	Cl^- (mmol/L)	106.7 ± 1.9	106.8 ± 1.3		106.1 ± 0.8		107.2 ± 1.3	

Values are presented as mean ± standard variation. *Significantly different from the controls at levels of $P < 0.05$. **Significantly different from the controls at levels of $P < 0.01$. ***Significantly different from the controls at levels of $P < 0.001$.

3.5. Histopathology

Histopathology pictures are shown in **Appendix**.

The histopathological examination revealed no findings related to the treatment. The results of histomorphological examination indicated that oral administration of at the doses up to 96 mg/kg for successive 180 days did not cause toxicologically histomorphological damage in main organs.

Table 6. Blood biochemistry analysis of rats after 28 days withdrawal (n = 5).

Sex	Parameters	Control group	YYH-C 24 mg/kg		YYH-C 48 mg/kg		YYH-C 96 mg/kg	
♂	TP (g/L)	58.7 ± 2.3	57.5 ± 1.2		57.4 ± 1.5		59.8 ± 2.3	
	ALB (g/L)	34.8 ± 1.3	34.3 ± 0.7		34.8 ± 1.4		35.9 ± 1.6	
	AST (U/L)	145 ± 45	148 ± 13		145 ± 37		158 ± 104	
	ALT (U/L)	40 ± 9	43 ± 7		69 ± 18		85 ± 77	
	ALP (U/L)	62 ± 14	80 ± 8	*	93 ± 15	***	76 ± 10	
	γ-GT (U/L)	1.09 ± 0.619	1.149 ± 0.26		1.39 ± 0.72		1.42 ± 0.71	
	UREA (mmol/L)	6.0 ± 0.4	6.2 ± 0.2		6.3 ± 0.6		5.9 ± 0.8	
	CRE (μmol/L)	29.8 ± 2.9	28.6 ± 1.8		30.8 ± 2.6		27.8 ± 3.4	
	GLU (mmol/L)	8.5 ± 0.7	7.5 ± 0.5		9.8 ± 1.6		8.6 ± 0.4	
	T-bil (μmol/L)	3.6 ± 0.4	2.3 ± 0.3	**	2.2 ± 1.0	**	2.2 ± 0.8	**
	T-cho (mmol/L)	2.1 ± 0.4	1.9 ± 0.4		2.1 ± 0.6		2.0 ± 0.2	
	TG (mmol/L)	0.7 ± 0.3	0.3 ± 0.1	**	0.5 ± 0.2	*	0.5 ± 0.2	*
	CK (U/L)	598 ± 252	613 ± 155		278 ± 46		242 ± 59	
	Na^+ (mmol/L)	144.7 ± 1.5	145.9 ± 2.1		147.4 ± 1.6		146.5 ± 1.9	
	K^+ (mmol/L)	5.0 ± 0.3	4.9 ± 0.2		4.8 ± 0.2		4.6 ± 0.1	
	Cl^- (mmol/L)	106.4 ± 1.4	107.6 ± 0.3		107.9 ± 1.2		107.9 ± 1.2	
♀	TP (g/L)	65.4 ± 3.1	69.9 ± 6.4		61.6 ± 2.7		66.9 ± 6.1	
	ALB (g/L)	38.4 ± 2.2	43.0 ± 4.0		38.2 ± 2.4		40.6 ± 4.1	
	AST (U/L)	89 ± 21	172 ± 121		93 ± 13		80 ± 15	
	ALT (U/L)	40 ± 11	71 ± 62		37 ± 12		39.± 12	
	ALP (U/L)	36 ± 9	32 ± 11		44 ± 15		39 ± 10	
	γ-GT (U/L)	0.80 ± 0.10	1.15 ± 0.44		1.21 ± 0.16		0.98 ± 0.30	
	UREA (mmol/L)	6.7 ± 0.7	5.8 ± 0.9		6.8 ± 1.0		7.1 ± 1.0	
	CRE (μmol/L)	29.6 ± 2.9	23.4 ± 3.3		28.6 ± 1.9		31.0 ± 7.2	
	GLU (mmol/L)	7.3 ± 0.7	7.7 ± 0.4		7.7 ± 0.5		7.8 ± 0.7	
	T-bil (μmol/L)	3.1 ± 1.3	6.4 ± 4.1		2.9 ± 1.2		5.2 ± 6.4	
	T-cho (mmol/L)	2.3 ± 0.7	2.7 ± 0.6		1.9 ± 0.2		1.9 ± 1.2	
	TG (mmol/L)	0.4 ± 0.2	1.0 ± 0.5		0.5 ± 0.2		0.8 ± 0.9	
	CK (U/L)	251 ± 180	275 ± 169		206 ± 69		153 ± 100	
	Na^+ (mmol/L)	145.0 ± 1.1	145.6 ± 0.6		143.8 ± 4.3		146.1 ± 1.3	
	K^+ (mmol/L)	4.2 ± 0.2	4.2 ± 0.2		4.1 ± 0.2		4.2 ± 0.3	
	Cl^- (mmol/L)	107.6 ± 1.3	108.5 ± 2.0		108.3 ± 0.6		107.8 ± 1.4	

Values are presented as mean ± standard variation. *Significantly different from the controls at levels of $P < 0.05$. **Significantly different from the controls at levels of $P < 0.01$. ***Significantly different from the controls at levels of $P < 0.001$.

4. Discussion

The present study was conducted to evaluate the safety of the total flavones of *E. leptorrhizum* Stearn administered once daily gavage to male and female Sprague-Dawley rats at dose levels of 24, 48, and 96 mg/kg/day for 180 days. Throughout the administration and recovery time, no deaths and no clinical signs were observed. No treatment-related changes were found in body weight, food consumption, hematology parameters, blood biochemistry, organ weights and histopathological examination in male and female in each treatment group.

Our study found that taking YYH-C for a long time didn't inhibit the growth of animal body weight, but could delay the growth rate for a long time. Changes in food consumption and body weight were corresponding in male rat, taking a long time for the test materials, could lead to reduced food intake, and showed the time-related changes in degree. No above mentioned effect were found both in body weight and food consumption in female.

A dose-depended obvious decrease of LYM% and increase of MID% were seen in male rats of all three dose groups after 90 days administration, moreover, decrease of LYM% and increase of MID% were also seen after 180 days administration with no obviously dose-depended manner. However, an obvious increase of LYM% and decrease of MID% were seen in female rats of 48, 96 mg/kg dose groups after 180 days administration. Some other indicators which showed an obvious changes, including hematology parameters, blood biochemistry and organ weights, were considered as no toxicological significant as the values were within the normal ranges recorded in the experimental facility and no obvious dose effect relationship. It is concluded that the administration of YYH-C at levels up to 96 mg/kg/day for 180 days is well-tolerated for both genders without obvious signs and toxicity. However, leukocyte differential count should be observed carefully.

In Chinese Pharmacopoeia (2010), total flavonoid (calculated by Icariin) in Epimedium should be no less than 5.0% on a dry basis. Icariin content is 1.17% in YYH-C which extract from *E. leptorrhizum* Stearn, it is far from the criteria in Chinese Pharmacopoeia; Epimendin C content is 8.87%, it is the main composition. Even though Icariin may be safe as a food additive, no studies have been performed systematically to evaluate the effects of long-term intake of YYH-C. The reason why we conducted this study was to confirm the safety of excessive intake of individual YYH-C because during YYH-C efficacy assessment, we found YYH-C have the same efficacy activity as Icariin to prevent osteoporosis. The present study showed that the no-observed-effect level for YYH-C was 96 mg/kg/day for both genders. This dose is 100 times of clinical dose, therefore it is preliminary considered as a safe food additive.

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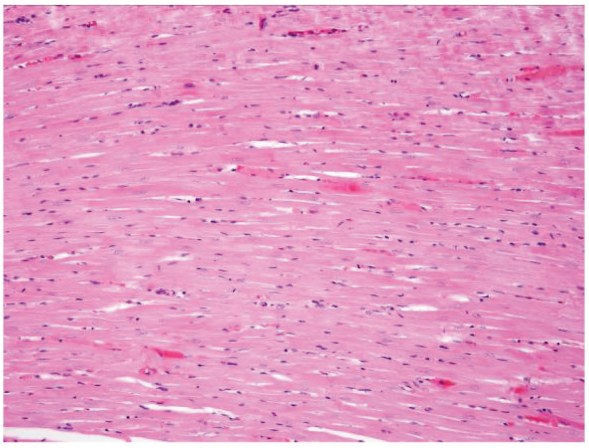
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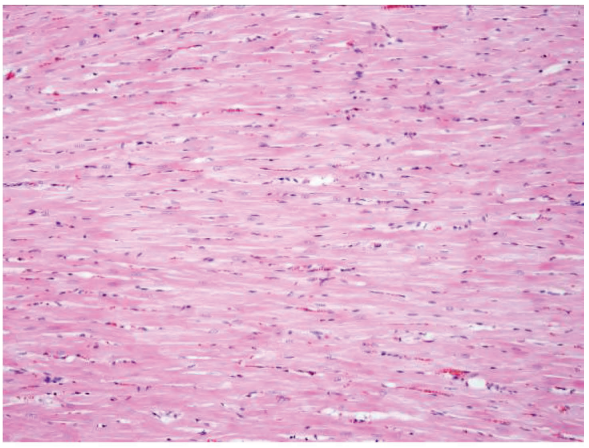
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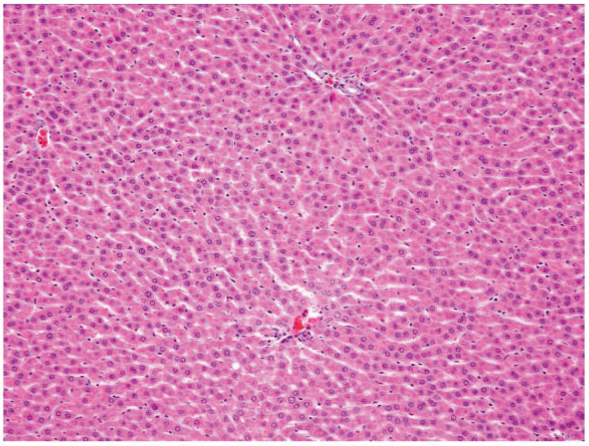
Appendix (Histopathology Pictures after 180 Days Administration, HE × 200)



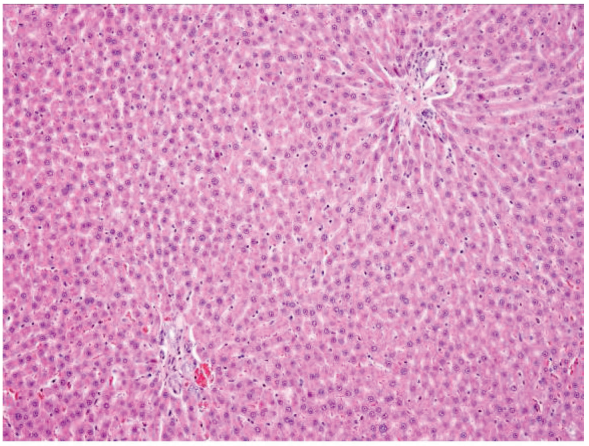
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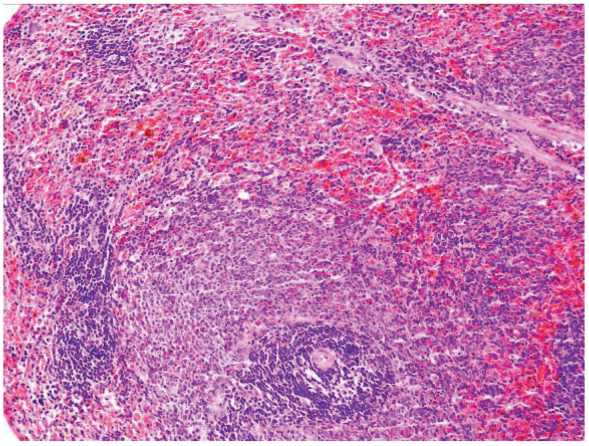
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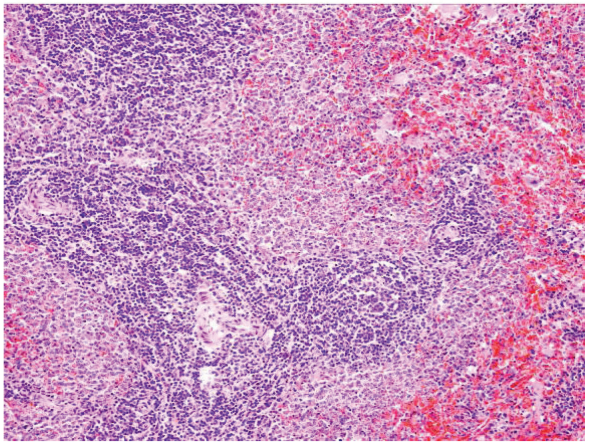
control group: Liver



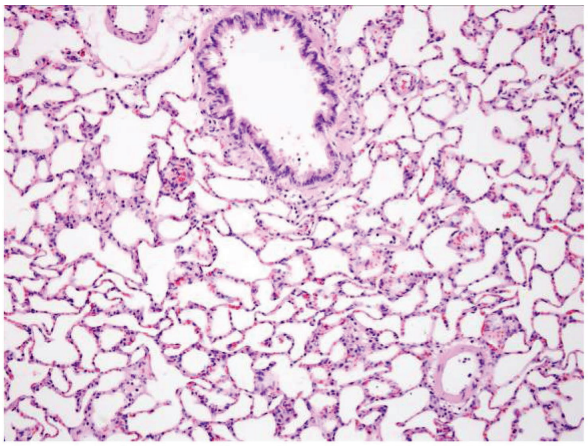
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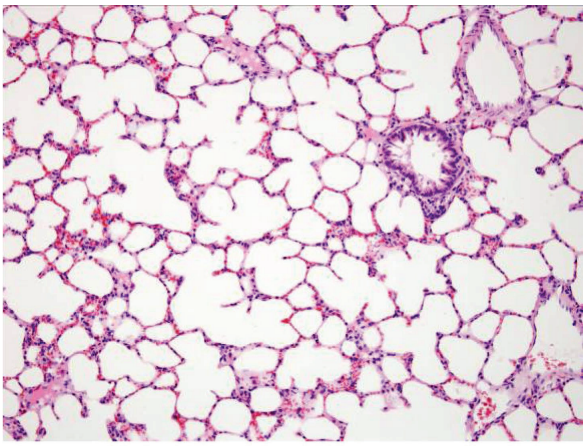
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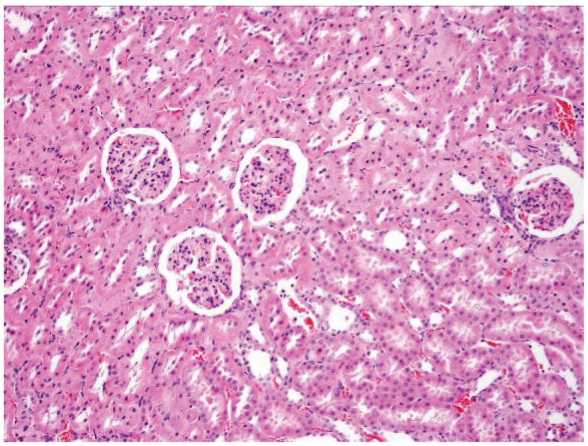
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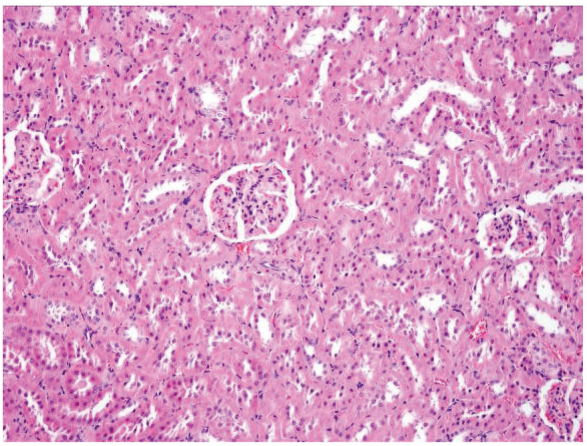
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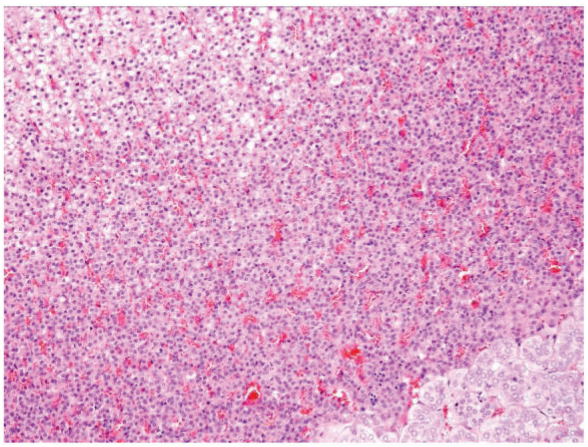
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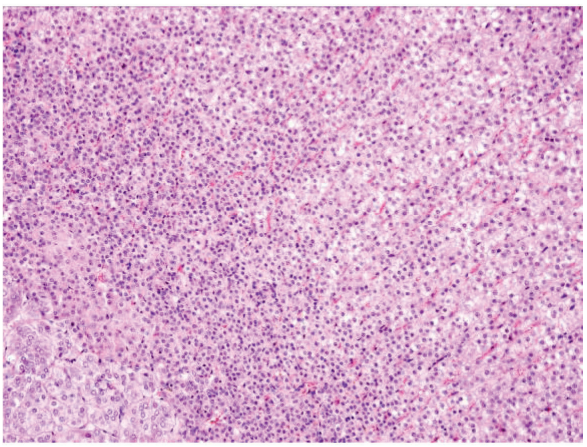
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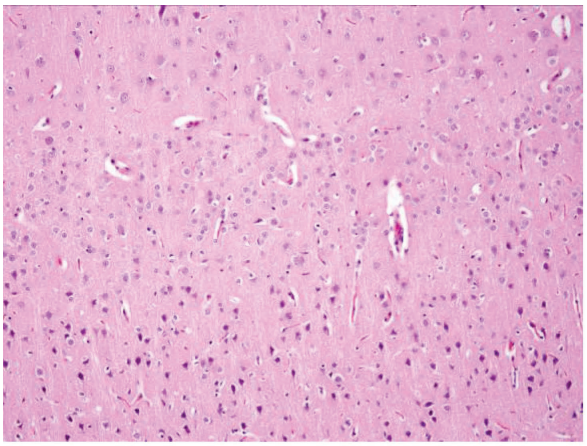
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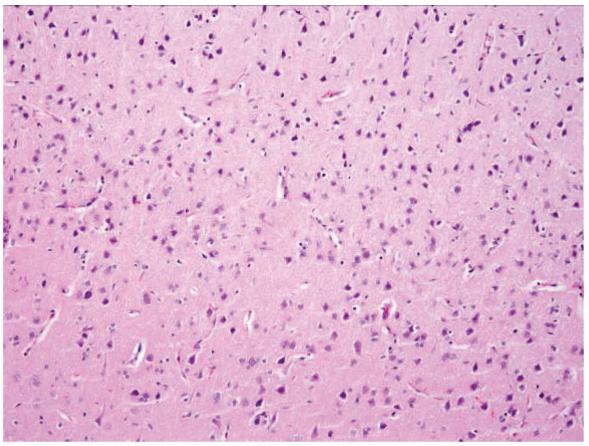
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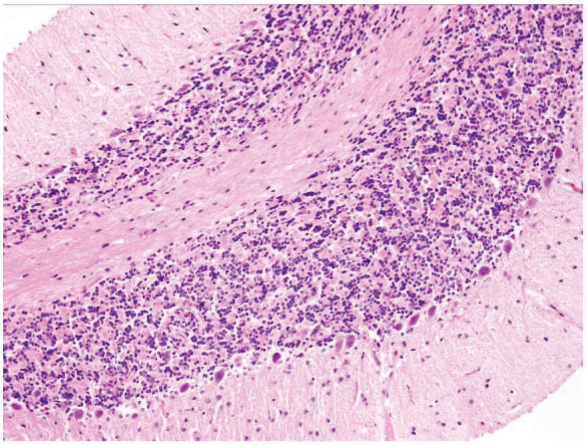
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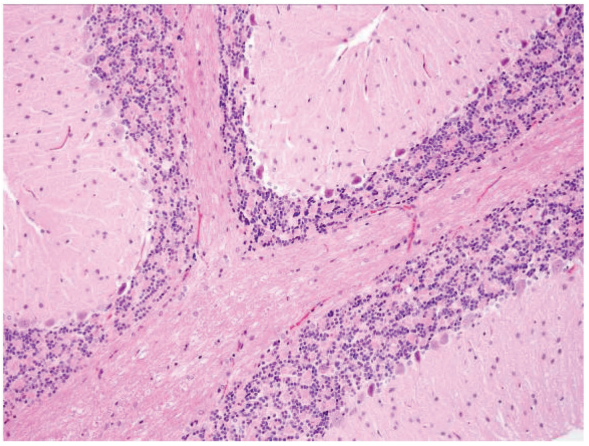
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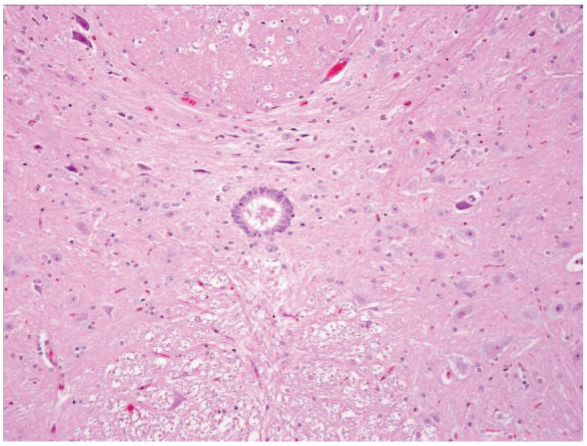
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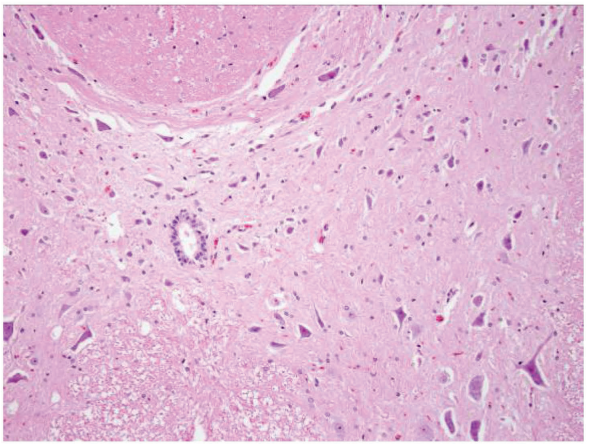
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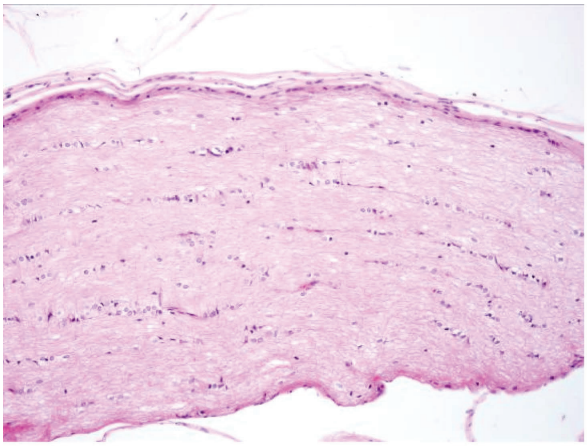
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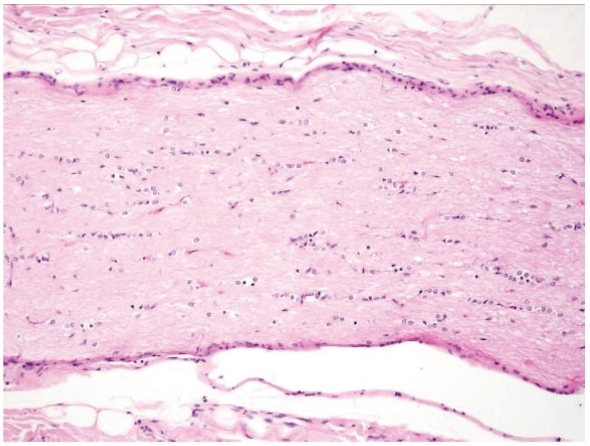
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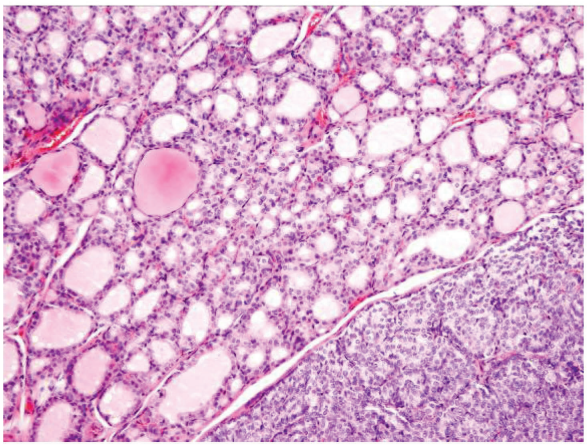
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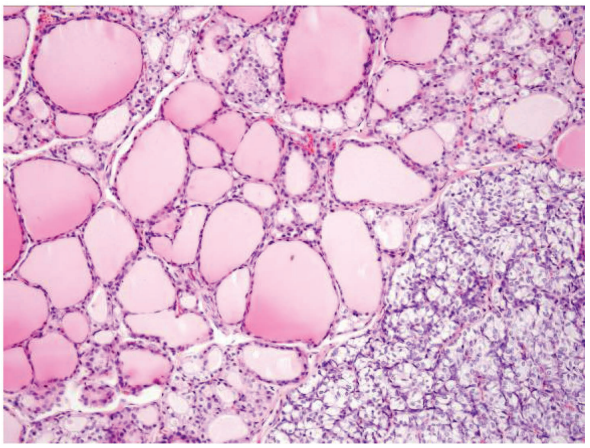
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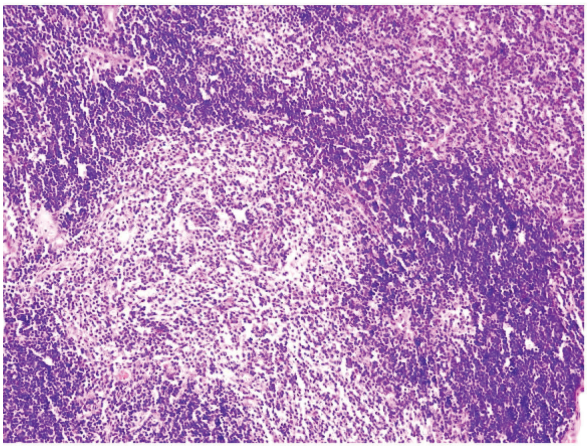
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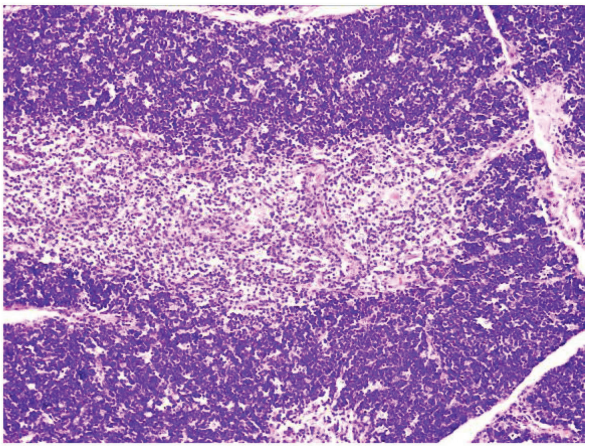
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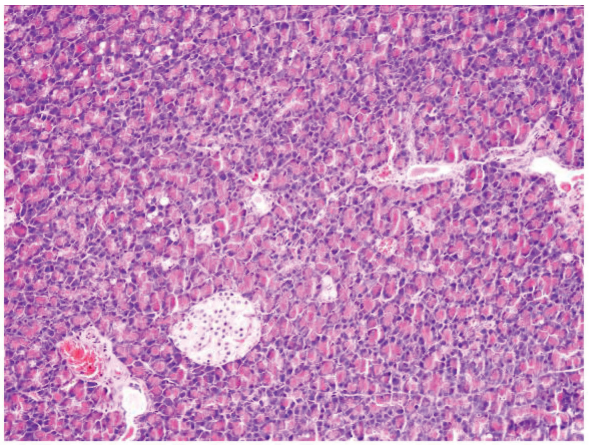
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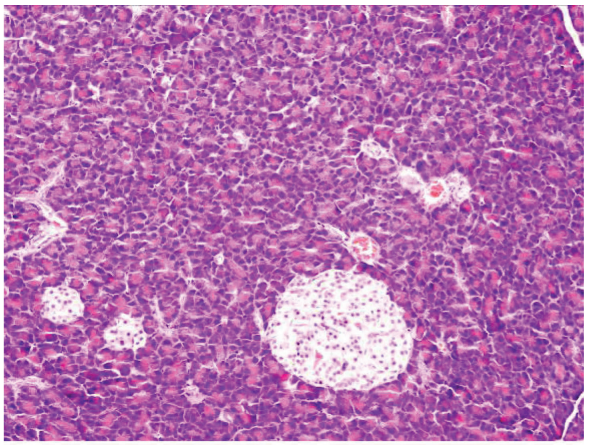
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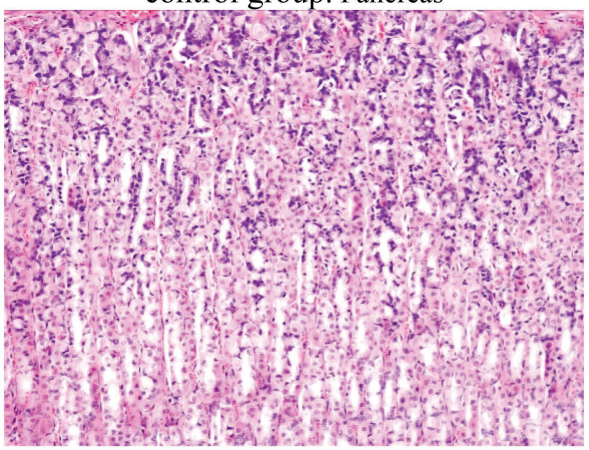
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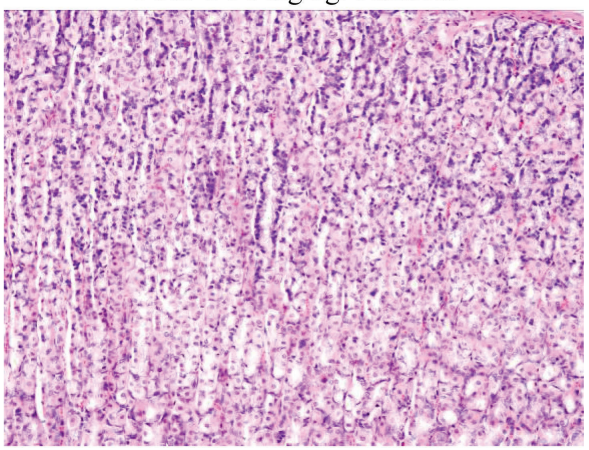
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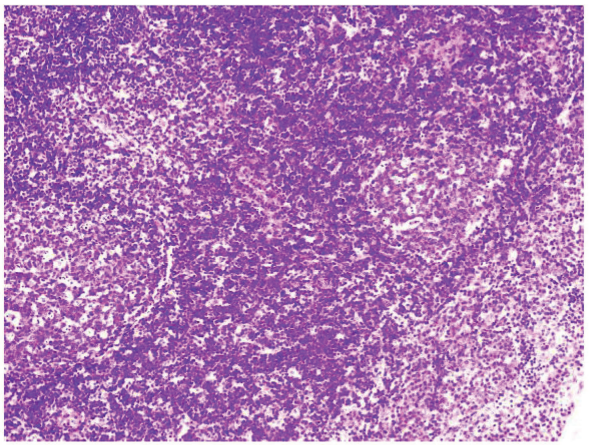
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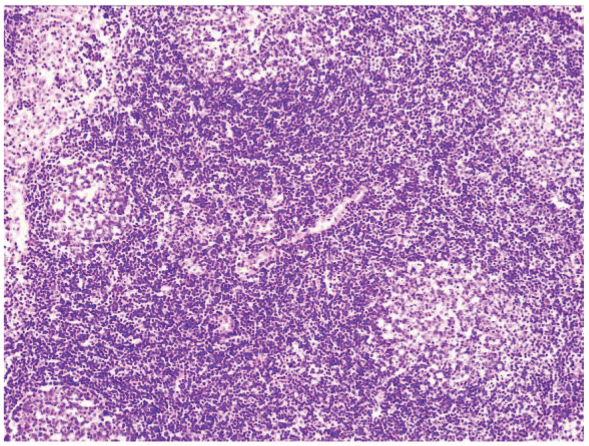
control group: Stomach



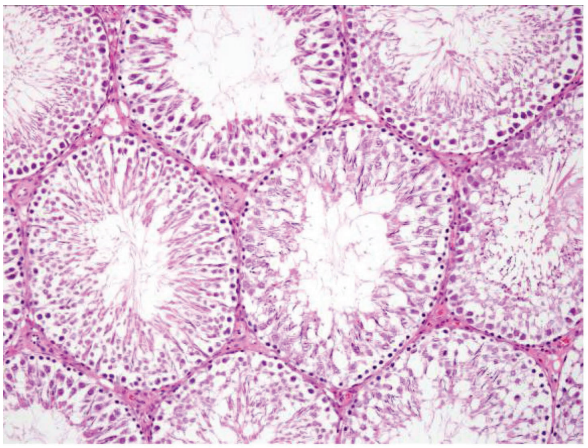
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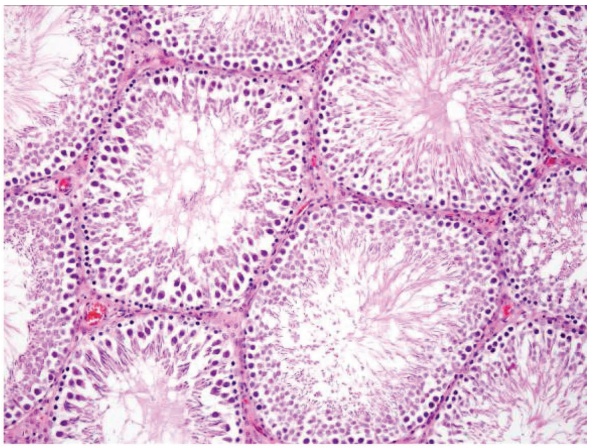
control group: Mesenteric lymph nodes



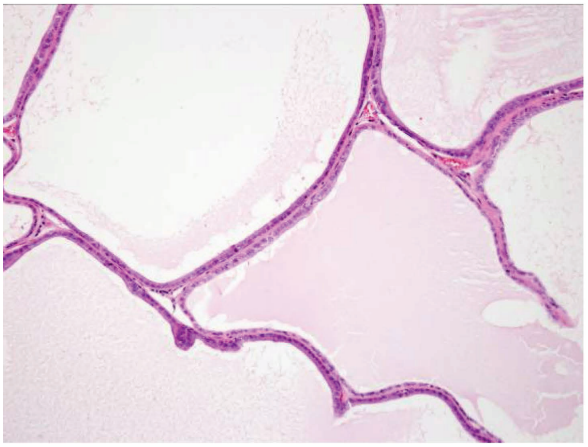
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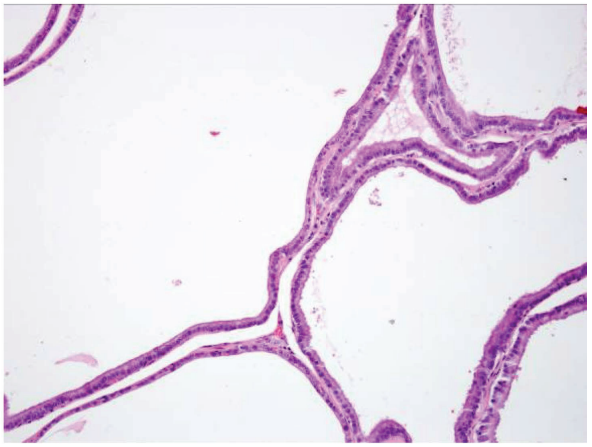
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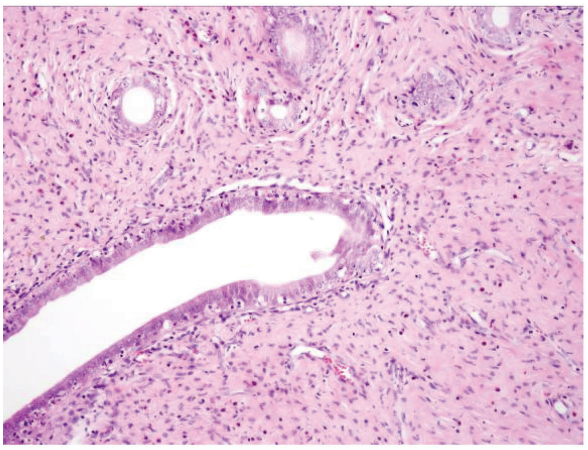
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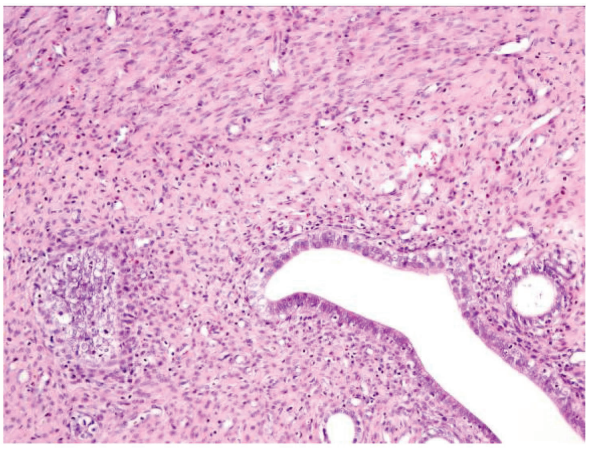
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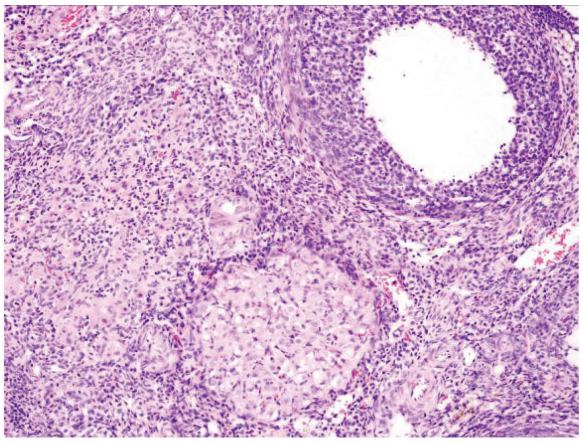
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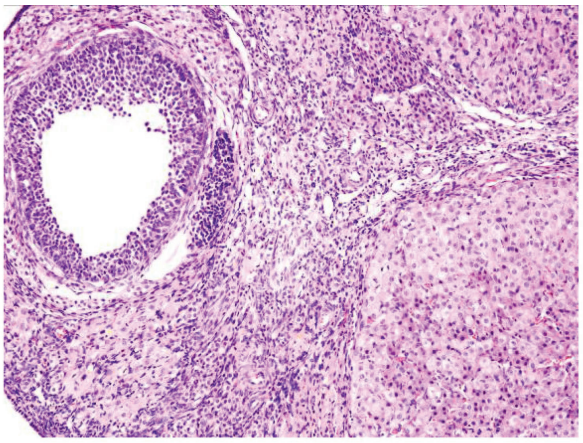
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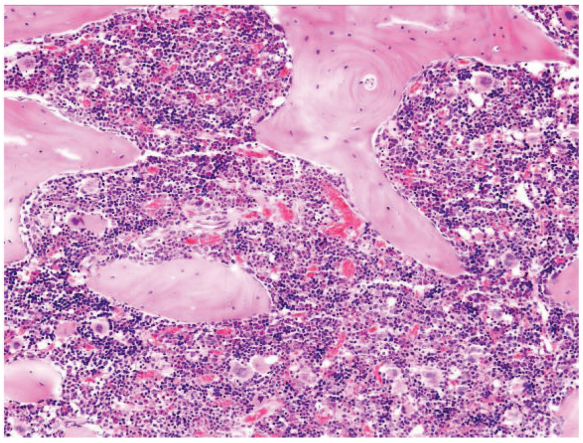
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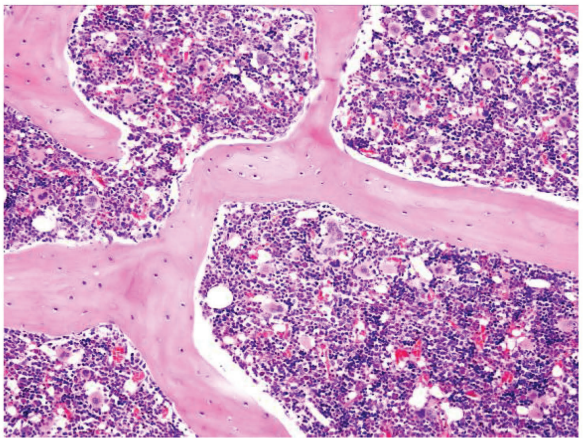
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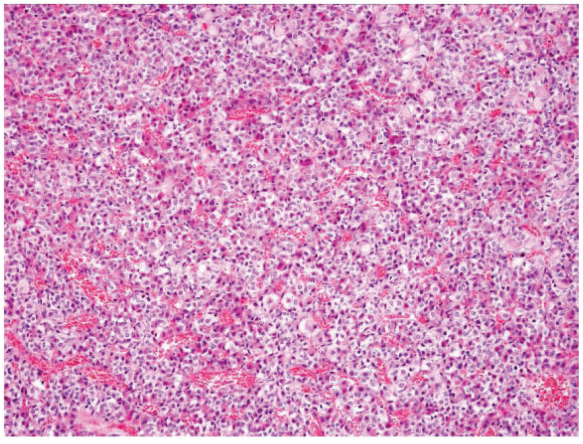
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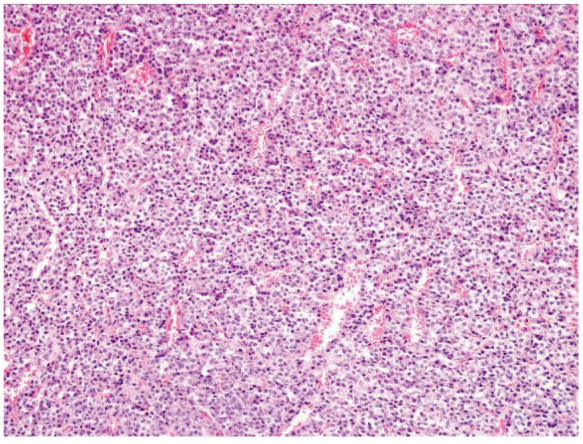
control group: The sternum



YYH-C 96 mg/kg: The sternum



control group: The pituitary



YYH-C 96 mg/kg: The pituitary

9

Melatonin and Circadian Rhythm: Aging, Cancer, and Mechanism

Peter Kovacic[1*], Ratnasamy Somanathan[1,2]

[1]Department of Chemistry and Biochemistry, San Diego State University, San Diego, USA

[2]Centro de Graduados e Investigación del Instituto Tecnológico de Tijuana, Tijuana, Mexico

Abstract

The circadian clock is involved with many aspects of biological processes in animals and plants. Melatonin is a pineal hormone which is known to play a widespread role in the biochemistry of the circadian clock. The present review addresses the clock and melatonin in relation to aging and cancer, which are important aspects of medicine and biochemistry. The literature contains a large variety of associations of these areas with melatonin and circadian rhythm. Mechanistic aspects are addressed with emphasis on the unifying theme based on electron transfer-free radicals-oxidative stress.

Keywords

Melatonin, Circadian Clock, Aging, Cancer, Mechanism

1. Introduction

This review presents a multifaceted approach to melatonin and circadian rhythm in relation to cancer and aging. The circadian clock, which likely evolved 2.5 billion years ago, has been the object of attention in science and the media [1]. The clock, which tracks time in 24 hour cycles, is controlled by genes in cycling feedback loops. An important feature is metabolic efficiency which is promoted by timing activities with day-night rhythms. The machinery plays a role not only in sleep cycles, but also in health and disease. Adverse medical events that relate to rhythm dysfunction are cancer, heart disease, addiction, diabetes and obesity.

This review deals with cancer, aging, and circadian rhythm [2] which are important aspects of medicine and biochemistry, in relation to melatonin. Genes play a fundamental role in many areas of human biology. Mechanistic aspects of the various topics are also treated. The role of melatonin, a pineal hormone, is addressed. The preponderance of bioactive substances, usually as their metabolites, incorporates ET functionalities. We believe that it plays an important role in physiological responses. The main groups include quinones (or phenolic pre-

*Corresponding author.

cursors), metal complexes (or complexors), aromatic nitro compounds (or reduced hydroxylamine and nitroso derivatives), and conjugated imines (or iminium species). *In vivo* redox cycling with oxygen can occur, giving rise to oxidative stress (OS) through generation of reactive oxygen species (ROS), such as hydrogen peroxide, hydroperoxides, alkyl peroxides, and diverse radicals (hydroxyl, alkoxyl, hydroperoxyl, and superoxide). In some cases, ET results in involvement with normal electrical effects (e.g., in respiration or neurochemistry). Generally, active entities possessing ET groups display reduction potentials in the physiologically responsive range, (*i.e.*, more positive than about -0.5 V). Hence, ET *in vivo* can occur resulting in production of ROS which can be beneficial in cell signaling at low concentrations, but produce toxic results at higher levels. Electron donors consist of phenols, N-hetercycles or disulfides in proteins which can produce relatively stable radical cations. ET, ROS, and OS have been increasingly implicated in the mode of action of drugs and toxins.

There is a plethora of experimental evidence supporting the ET-ROS theoretical framework. The evidence includes the generation of the common ROS, lipid peroxidation, degradation products of oxidation, depletion of Ass, effect of exogenous AOs, and DNA oxidation and cleavage products, as well as electrochemical data. This comprehensive, unifying mechanism is in consistent with the frequent observations that many ET substances display a variety of activities (e.g., multiple-drug properties), as well as toxic effects.

It is important to recognize that mode of action in the biodomain is often multifaceted. In addition to the ET-ROS-OS approach, other aspects may pertain, such as enzyme inhibition, allosteric effects, receptor binding, metabolism and physical factors.

2. Melatonin

Melatonin is an internal factor affecting the circadian clock. In all vertebrates investigated so far, melatonin is produced in the pineal body and resynchronizes the SCN by providing information about light/darkness from the retinohypothalmic tract. Melatonin is also essential for regulation of rhythmic functions in peripheral target tissues of the clock. It peaks during darkness and lowers during the day; several reviews have appeared over the years on this subject [3]-[5]. Though this indole hormone was first reported from the pineal gland, it was also found in retina, Harderian gland and gastro-intestinal tracts in vertebrates, as well as in a wide variety of organisms, ranging from invertebrates to plants [5]. One of the characteristic features of the tryptophan derivative molecule is that its synthesis takes place during darkness in a light-dark cycle, irrespective of the habit of the animal. Therefore, it is often described as “hormone of darkness” or a “chronobiotic molecule”. In all vertebrates investigated so far, melatonin is primarily synthesized within the pinealocytes of the pineal gland during the night, regardless of the diurnal or nocturnal locomotor activity of the animals [5].

Biosynthesis of melatonin is a four-step phenomenon [3]. First, its precursor L-tryptophan is taken from the circulation (blood) into pinealocyte and converted to 5-hydroxytryptophan by tryptophan-5-monooxygenase/hydrolase and further decarboxylated by aromatic amino acid carboxylase to form 5-hydroxytryptamine or serotonin. Seratonin is acetylated (N-acylation) to form N-acetylseratonin by serotonin-N-acetyltransferace. Finally, N-acetylseratonin is methylated by hydroxyindole-O-methyltransferase to form melatonin [5] (**Scheme 1**).

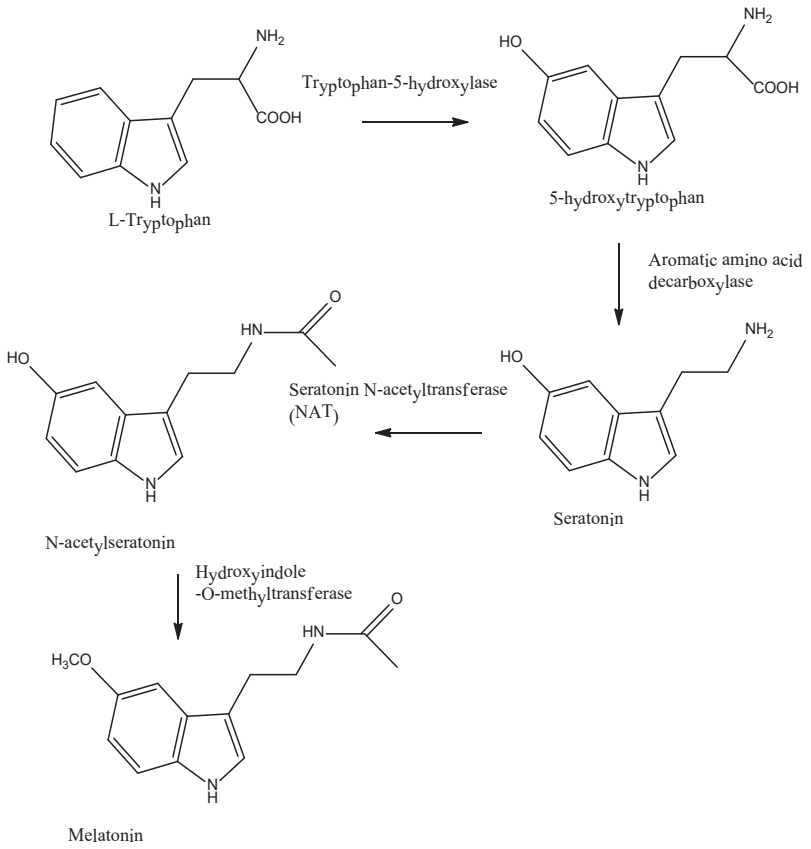
The rhythm of melatonin synthesis is generated by interacting networks of circadian clock genes located in the SCN, circadian oscillator/master pacemaker of the hypothalamus in brain, which is considered as the major central rhythm-generating system or “clock” in mammals [6] [7]. The SCN clock is set to a 24 h day by the natural light-dark cycle via retinal light input which then sends circadian signals over a neural pathway including sympathetic nerve terminals that project from the superior cervical ganglia (SCG) to the pineal gland and thereby driving rhythmic melatonin synthesis [4] [5]. The main photoreceptor pigment for circadian timing appears to be melanopsin in the retinal ganglion cells [8]. SCN is the major regulatory site of serotonin N-acetyl transferase (NAT) activity, which is the key enzyme in the synthesis of melatonin from tryptophan. Melatonin, through various receptors (MT1, MT2 and MT3) induces a differential influence on clock genes [4] [5]. In humans, the pars tuberalis and SCN clock genes expression pattern show 24 h rhythmicity. The synthesis of melatonin is driven by the SCN to convey photoperiodic information to the pars tuberalis, which in turn influences the clock genes PER1 and Cry 1 within the pars tuberalis, translating the melatonin signal to control body rhythm [4] [5].

Melatonin involvement with circadian rhythm and other aspect is related to recent articles [9] [10].

Structure activity relationship (SAR) studies were carried out on melatonin [11]. Data indicate the crucial role played by the methoxy substituent. Various investigations were carried out on metabolism with rat and human subjects [12]-[15]. The principal products are 6-hydroxymelatonin (**Figure 1**) and N-acetylseratonin (**Figure 2**)

from O-demethylation. Hydroxylation occurred to a greater extent [12]. Both reactions have extensive precedent. These findings provide a base for application of the unifying theme of ET-ROS-OS. O-Demethylation of the hydroxylated metabolite should yield a catechol (**Figure 3**) which is commonly oxidized to an ET o-quinone (**Figure 4**). N-Acetylserotonin might conceivably be oxidized to the ET iminoquinone (**Figure 5**). Analogy for **Figure 5** can be found from metabolism of psilocybin [16].

Representative examples are provided from the literature dealing with AO properties of melatonin [17]-[21].



Scheme 1. Biosynthesis of melatonin from tryptophan.

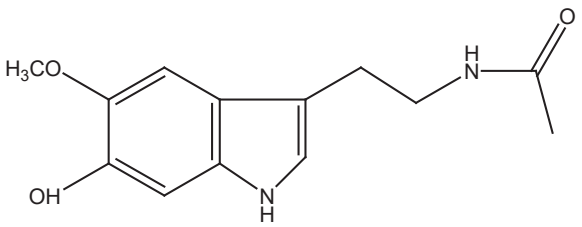


Figure 1. 6-hydroxymelatonin.

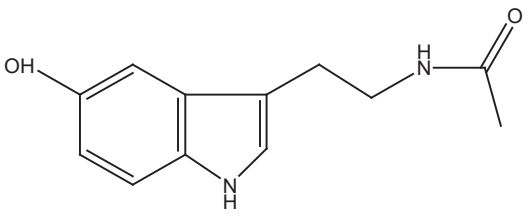


Figure 2. N-acetylserotonin.

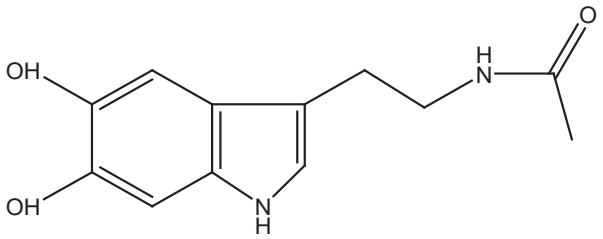


Figure 3. Catechol derivative of melatonin.

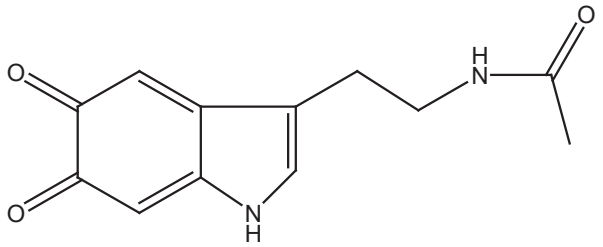


Figure 4. o-Q-uinone derivative of melatonin.

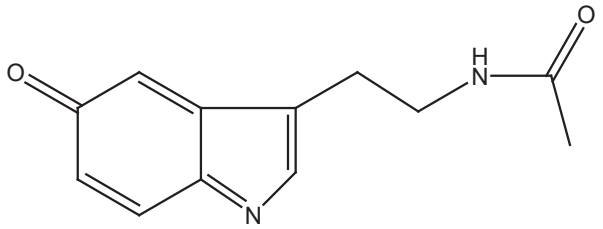


Figure 5. Iminoquinone derivative of N-acetylserotonin.

In relation to mechanism, a suggestion involves the indole nitrogen, presumably acting via the indole NH group-pin terminating ROS [22]. Another possibility entails participation of the N-acetylseratonin metabolite. Phenols comprise a well-known class of AOs, e.g., vitamin E, which operate by way of electron or hydrogen atom donation [23]. Electrical effects, which are common in the brain, might be a result of participation, in part, of the postulated ET iminoquinone and o-quinone.

3. Cancer

Over the decades, many factors have been found to be involved in the biochemistry of cancer, including electron transfer, reactive oxygen species and oxidative stress [24]-[26], which are addressed in other articles. This section provides a general approach involving many factors linking circadian rhythm and cancer.

Circadian rhythms show universally a 24-hour oscillation pattern, in metabolic physiological and behavioral functions of almost all species, due to a fundamental adaptation to rotation of earth around its own axis [27]. Molecular mechanisms of generation of circadian rhythms organize a biochemical network in suprachiasmatic nucleus and peripheral tissues, building cell autonomous clock pacemakers. Rhythmicity is observed in transcriptional expression of a wide range of clock-controlled genes which regulate a variety of normal cell functions like cell division and proliferation. Desynchrony of this rhythmicity seems to be implicated in several pathologic conditions, including tumorigenesis and progression of cancer. In 2007, the International Agency for Research in cancer categorized shift work that involves circadian desynchrony, as being probably carcinogenic to humans. A review discusses the potential relation between the disruption of normal circadian rhythms with the genetic driving machinery of cancer. Elucidation of the role of clockwork disruption, like the exposure to light-at-night and sleep disruption, in cancer biology, could be important in the development of new targeted anticancer therapies, in optimization of individualized chronotherapy and in modification of lighting environment in working environments.

Circadian rhythms are commonly disrupted in women undergoing chemotherapy for breast cancer [28]. Bright light improves and strengthens circadian rhythms. A randomized controlled study examined the effect of morning administration of bright light therapy on circadian rhythms in women undergoing chemotherapy for breast cancer [28]. It was hypothesized that women receiving bright light therapy would exhibit more robust rhythms than women exposed to dim light. The results suggest that morning administration of bright light may protect women from experiencing circadian rhythm deterioration during chemotherapy.

Breast cancer is the worldwide leading cause of cancer incidence among women [29]. Night shift work exposure has been recently considered one of the significant breast cancer risk factors in industrialized countries. The mechanisms by which this works exposure may be responsible for cancer development is still discussed. In the last 15 years, many authors have paid attention to the relationship between night shift work and breast cancer risk. Both the circadian system alteration and the melatonin output reduction, related to the exposure to light-at-night during night shift work, remain the most valid hypotheses on the casual relation of shift work and breast cancer. Overall, the results suggest that there is an association between night shift work and breast cancer development in western countries. However, further studies are needed to confirm such association and to understand which biomolecular mechanisms may be involved in the pathogenesis of cancer diagnosed in patients with night shift work exposure.

Disruption of the circadian rhythm has been hypothesized to increase cancer risk, either because of direct disruption of the molecular machinery generating circadian rhythms or because of disruption of parameters controlled by the clock, such as melatonin levels or sleep duration [30]. Data are sparse about potential effects of circadian disruption on the risk of prostate cancer. A review systematically examines available data evaluating the effects of light at night, sleep patterns, and night shift work on prostate cancer risk.

Circadian clocks are endogenous molecular time-keeping systems that underlie daily fluctuations in multiple physiological and biochemical processes [31]. It is now well recognized that dysfunction of the circadian system may be associated with a heightened incidence of cancer. A review presents evidence supporting the important role played by the circadian clock in the development of cancer and the therapeutic efficacy of anticancer agents. A number of circadian clock genes have been identified. Continued research should increase understanding of the role of circadian clocks which could, ultimately, reduce the incidence of cancer in people with disrupted sleep-wake cycles, such as shift workers and flight attendants, and provide optimal chronopharmacology for cancer treatment.

Hormone secretion, metabolism, and the cell cycles are under rhythmic control [32]. Lack of rhythmic control has been predicted to lead to uncontrolled proliferation and cancer. Consistent with the prediction are findings that circadian disruption by dim light at night or chronic jet lag accelerates tumor growth in desynchronized animals. Circadian controlled factors have been implicated in controlling tumor growth in these animals. Recent attention has focused on the signaling pathways activated by the circadian controlled factors because these pathways hold potential for the development of novel strategies for cancer prevention and treatment.

An investigation involves comparison of circadian characteristics for cytotoxic lymphocytes in cell lung cancer patients versus controls [33]. Timed circadian administration (chronotherapy) in immunotherapy and other cancer treatments may improve efficacy due to persistent circadian entrainment of healthy tissues.

A review article discusses recent work on the melatonin-mediated circadian regulation and integration of molecular, dietary, and metabolic signaling mechanisms involved in human breast cancer growth and the consequences of circadian disruption by exposure to light at night [34]. Experimental evidence in rats and humans indicates that light at night-induced circadian disruption of the nocturnal melatonin signal activates human breast cancer growth, metabolism, and signaling which provides the strongest mechanistic support for population and ecological studies demonstrating elevated breast cancer risk in night shift workers and other individuals increasingly exposed to light at night.

Little is known about the relationship between sleep parameters and fatigue in patients at the initiation of radiation therapy [35]. A significant percentage of oncology patients experience significant disturbances in sleep-wake circadian activity rhythms at the initiation of radiation therapy. The disturbances occur in both sleep initiation and sleep maintenance. Epidemiologic studies show high incidence of cancer in shift workers [36]. The finding suggests that circadian disruption induces the progression of malignant tumors via a Wnt signaling pathway.

Cell proliferation in all rapidly renewing mammalian tissues follows a circadian rhythm that is often disrupted in advanced-stage tumors [37]. Epidemiologic studies have revealed a clear link between disruption of circadian

rhythms and cancer development in humans. Mice lacking certain circadian genes are deficient in cell cycle regulation and Per2 mutant mice are cancer-prone. However, it remains unclear how circadian rhythm in cell proliferation is generated *in vivo* and why disruption of circadian rhythm may lead to tumorigenesis. Tumor suppression *in vivo* is a clock-controlled physiological function. The central circadian clock paces extracellular mitogenic signals that drive peripheral clock-controlled expression of key cell cycle and tumor suppressor genes to generate a circadian rhythm in cell proliferation. Frequent disruption of circadian rhythm is an important tumor promoting factor.

Prostate cancer is a major age-related malignancy as increasing age correlates with increased risk for developing this neoplasm [38]. Similarly, alterations in circadian rhythms have also been associated with the aging population and cancer risk. The pineal hormone melatonin is known to regulate circadian rhythms, which is under the control of a core set of genes. The data suggest that melatonin should be thoroughly investigated as an agent for the management of prostate cancers and other age-related malignancies.

Circadian disruption accelerates cancer progression, whereas circadian reinforcement could halt it [39]. Mice with pancreatic adenocarcinoma were synchronized and fed ad libitum or with meal timing. The reinforcement of the host circadian timing system with meal timing translated into cancer growth inhibition. Targeting circadian clocks represents a novel potential challenge for cancer therapeutics.

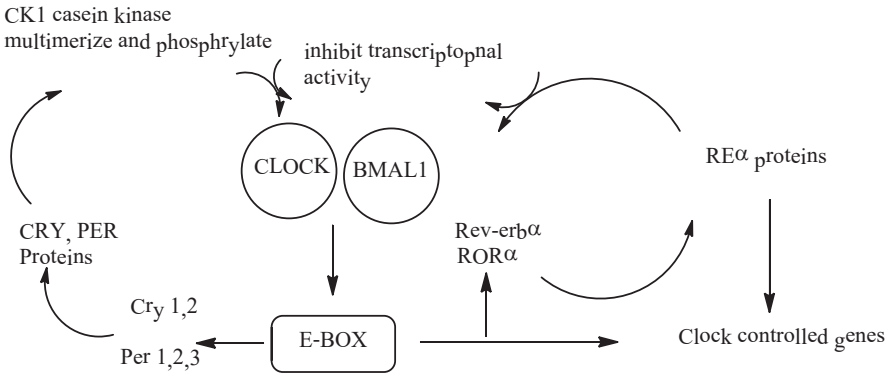
Breast cancer incidence increases rapidly as societies industrialize. Many changes occur during the industrialization process, one of which is dramatic alteration in the lighted environment from a sun-based system to an electricity-based system [40]. Increasingly, the natural dark period at night is being seriously eroded for the bulk of humanity. Based on the fact that light during the night can suppress melatonin, and also disrupt the circadian rhythm, it was proposed in 1987 that increasing use of electricity to light the night accounts in part for the rising risk of breast cancer globally. Predictions from the theory include: non-day shift work increases risk, blindness lowers risk, long sleep duration lowers risk, and population level community nighttime light level co-distributes with breast cancer incidence. Thus far, studies of these predictions are consistent in support of the theory. A new avenue of research has been on function of circadian genes and whether these are related to breast cancer risk.

The circadian clock is an endogenous time keeping system shared by most organisms [41]. In mammals, a master pacemaker in the hypothalamus orchestrates temporal alignment of behavior and physiology by transmitting daily signals to multiple clocks in peripheral tissues. Disruption of this communication has a profound effect on human health and has been linked to diverse pathogenic conditions, including cancer. At the center of the molecular circadian machinery is a set of clock genes generating rhythmic oscillations on a cellular level. In the past several years, research from different fields has revealed the complexity and ubiquitous nature of circadian regulation, uncovering intriguing associations between clock components and cellular pathways implicated in tumorigenesis. A review discusses the emerging role of circadian genes in hematological and hormone-related malignancies. New insights suggest that manipulating circadian biology as a way to fight cancer, as well as other life threatening diseases, is within the realm of possibility.

Some reports have indicated that the core clock gene, PER2, regulates the cell cycle, immune systems and neural functions (**Scheme 2**) [42]. To understand the effects of PER2 on tumor growth *in vivo*, stable transformants of murine sarcoma cell line expressing different levels of PER2 were established. The growth of stable PER2 transformants *in vivo* was significantly and dose-dependently suppressed according to the amount of PER2 expressed, indicating that PERs plays a role in the growth suppression of sarcoma cells. The results suggest that sarcoma cells overexpressing PER2 suppress tumors *in vivo* by changing the nature of tumor cell adhesion.

The circadian timing is composed of molecular clocks, which drive 24 hour changes in xenobiotic metabolism and detoxification, cell cycle events, DNA repair, apoptosis, and angiogenesis [43]. The cellular circadian clocks are coordinated by endogenous physiological rhythms, so that they tick in synchrony in the host tissues that can be damaged by anticancer agents. As a result, circadian timing can modify the tolerability of anticancer medications. Improved efficacy is also seen when drugs are given near their respective times of best tolerability, due to a) inherently poor circadian entrainment of tumors and b) persistent circadian entrainment of healthy tissues. Conversely, host clocks are disrupted whenever anticancer drugs are administered at their most toxic time. On the other hand, circadian disruption accelerates experimental and clinical cancer processes. Gender, circadian physiology, clock genes, and cell cycle critically affect outcome on cancer chronotherapeutics.

A disruption of the circadian timing system, as identified by monitoring maker biorhythms, is common in



Scheme 2. A schematic model of molecular clockwork of the circadian clock in mammals.

cancer patients [44]. A body of evidence paves the path for innovative therapeutic approaches targeting the circadian timing system in an effort to diminish constitutional symptoms induced by cancer and some anticancer treatments.

Practical circadian therapy for the cancer patient involves 3 spheres of intervention, namely improving lifestyle, optimizing internal biochemical milieu, and adjusting treatment times [45]. The potential value of improving overall circadian functioning is shown in the work in which pronounced rest-activity rhythms were associated with better survival in colorectal cancer patients receiving chronomodulated chemotherapy. Lifestyle interventions that may improve circadian functioning involve diet, physical activity, and mind-body therapies. A diet that is ant-inflammatory and has appropriate carbohydrate intake, as well as regular meal timing, encourages normal circadian cycles. Adequate daytime physical activity encourages restful sleep, and morning light exposure during exercise may entrain melatonin rhythms. Meditation and other mind-body therapies can reduce anxiety and depression that may disrupt sleep. Aspects of the biochemical milieu that specifically disrupt circadian functioning are inflammation and stress hormones. Inflammation and cytokine disruption can be addressed with diet, herbs, and other natural substances. Chronomodulation of chemotherapy is discussed. Integrating all the above treatment modalities has the potential to improve both the quality of life and disease outcomes in cancer patients.

In industrialized countries, certain types of cancer, most notably breast and prostate, are more frequent than in poorly developed nations [46]. This high cancer frequency is not explained by any of the conventional causes. Within the past decade, numerous reports have appeared that link light at night with an elevated cancer risk. The three major consequences of light-mediated perturbation of circadian timing are sleep deprivation, chronodisruption, and melatonin suppression. Each of these individually or in combination may contribute to the reported rise in certain types of cancer. In the article, the potential mechanisms underlying the basis of the elevated cancer risk are briefly discussed. Finally, if cancer is a consequence of excessive nighttime light, it is likely that other diseases/conditions may also be exaggerated by the widespread use of light after darkness onset.

The circadian timing system coordinated by the suprachiasmatic nuclei (SCN) of the hypothalamus regulates daily rhythms of behavior, physiology, as well as cellular metabolism and proliferation [47]. Altered circadian rhythms predict for poor survival of cancer patients. An increased incidence of several cancers has been reported in flight attendants and in shift workers. The results support the role of circadian timing system in cancer progression and call for the development of therapeutic strategies aimed at preventing or treating circadian clock dysfunction.

Sirtuins appear to play a critical role in the process of carcinogenesis, especially in age-related neoplasm [48]. Similarly, alterations in circadian rhythms, as well as production of the pineal hormone melatonin, have been linked to aging and cancer risk. Melatonin has been found to act as a differentiating agent in some cancer cells and to lower their invasive and metastatic status. In addition, melatonin synthesis and release occur in a circadian rhythm fashion which has been linked to the core circadian machinery genes. Recent studies have linked sirtuins to the circadian rhythm machinery through direct deacetylation activity, as well as through other means. In this review, evidence is provided for a possible connection between sirtuins, melatonin, and the circadian rhythm circuitry and their implications in aging, chronomodulation, and cancer.

Circadian rhythms govern a remarkable variety of metabolic and physiological functions [49]. Accumulating

evidence indicates that the disruption of circadian rhythms might be directly linked to cancer. Several molecular gears constituting the clock machinery have been found to establish functional interplays with regulators of the cell cycle, and alterations in clock function could lead to aberrant cellular proliferation. Connections between the circadian clock and cellular metabolism have been identified that are regulated by chromatin remodeling. This suggests that abnormal metabolism in cancer could also be a consequence of a disrupted circadian clock. Therefore, a comprehensive understanding of the molecular links that connect the circadian clock to the cell cycle and metabolism could provide therapeutic benefit against certain human neoplasm.

The first whole genome inspections of mutations in human colon and breast cancer have observed specific retained clock gene mutations [50]. Single nucleotide polymorphism within the gene of clock, clock-controlled, and melatonin pathways have been found to confer excess cancer risk or protection from cancer. Experimental studies have shown specific core clock genes (PER2 and PER1) are tumor suppressors because their genetic absence doubles tumor numbers; and decreasing their expression in cancer cells doubles cancer growth rate, whereas their overexpression decreases cancer growth rate and diminishes tumor numbers. Experimental interference with circadian clock function increases cancer growth rate, and clinical circadian disruption is associated with higher cancer incidence, faster cancer progression, and shorter cancer patient survival. Patients with advanced lung cancer suffering greater circadian activity/sleep cycle disruption undergo greater interference with function, greater anxiety and depression, poorer nighttime sleep, greater daytime fatigue, and poorer quality of life than comparable patients who maintain good circadian integration. Screening for small molecules in targeting the circadian clock to stabilize its phase and enhance its turn is likely to help prevent and control human cancer. Cellular proliferation and the expression of cell cycle regulators are also controlled by the circadian clock [51]. The data suggest PER1 has tumor-suppressor function that diminishes cancer proliferation and tumor growth, but only at specific times of day.

A study examined patterns of circadian activity rhythms and their relationship with fatigue. Anxiety/depression, and demographic/medical variable were investigated in women receiving breast cancer adjuvant therapy treatments [52]. Disruption patterns of circadian activity rhythms were prevalent and associated with distressing fatigue and depressive symptoms during chemotherapy and at recovery. The intervention resulted in more robust rhythms. The possibility of a portion of the breast cancer burden might be explained by the introduction and increasing use of electricity to light the night was suggested >20 years ago [53]. The theory is based on nocturnal light-induced disruption of circadian rhythms, notably reduction of melatonin synthesis. The basic understanding of phototransduction for the circadian system and of the molecular genetics of circadian rhythm generation are both advancing rapidly, and will provide for the development of lighting technologies at home and work that minimizes circadian disruption, while maintaining visual efficiency and aesthetics.

A report concludes that the expression pattern of circadian genes might be a biomarker for the prognosis of breast cancer [54]. A study dealt with modulation of circadian rhythm of DNA synthesis in tumor cells by inhibiting platelet-derived growth factor signaling [55]. PER2, a core circadian clock gene, has tumor suppressor properties and is mutated or down regulated in human breast cancers [56]. The expression of this gene was manuplated *in vitro* and *in vivo* to more fully understand how the PER2 clock gene product affects growth. PER2 and perhaps other clock genes represent a class of potential therapeutic targets whose manipulation will modulate cancer growth and cancer cell proliferation.

The circadian timing system controls drug metabolism and cellular proliferation over the 24 hour day through molecular clocks in each cell [57]. These cellular clocks are coordinated by a hypothalamic pacemaker, the suprachiasmetic nuclei, that generate or controls circadian physiology. The circadian timing system plays a role in cancer processes and their treatments through the downregulation of malignant growth and the generation of large and predictable 24 hour changes in toxicity and efficacy of anti-cancer drugs. The tight interactions between circadian clocks, cell division cycle and pharmacology pathways have supported sinusoidal circadian-based delivery of cancer treatments. Such chronotherapeutics have been mostly implemented in patients with metastatic colorectal cancer, the second most common cause of death from cancer. Stochastic and deterministic models of the interactions between circadian clock, cell cycle and pharmacology confirmed the poor therapeutic value of both constant-rate and wrongly timed chronomodulated infusions.

The circadian clock cell cycle interacts at the level of genes, proteins, and biochemical signals [58]. The disruption or reinforcement of the host circadian timing system accelerates or slows down cancer growth through modifications of host and tumor circadian clocks. Thus, cancer cells not only display mutation of cell cycle genes, but also exhibit severe defects in clock gene expression levels or 24 hour patterns, which can in turn favor

abnormal proliferation. Mathematical models show that the therapeutic index of chemotherapeutic drugs can be optimized through distinct delivery profiles, depending on the initial host/tumor status and variability in circadian entrainment and/or cell cycle length.

Proper circadian regulation is essential for the well being of the organism, and disruption of circadian rhythms is associated with pathological conditions including cancer [59]. In mammals, the core clock genes, PER1 and PER2, are key regulators of circadian rhythms, both in the central clock, in the hypothalamus and in peripheral tissues (**Scheme 2**). Findings revealed molecular links between PER genes and cellular components that control fundamental cellular processes, such as cell division and DNA damage. Data shed light on mechanisms by which circadian oscillators operate in peripheral organs to influence tissue-dependent metabolic and hormonal pathways. Circadian cycles are linked to basic cellular expression and protein interactions. By controlling global networks, such as chromatin remodeling and protein families, which themselves regulate a broad range of cellular functions, circadian regulation impinges upon almost all major physiological functions.

An investigation examined effects of exogenous melatonin and circadian synchronization on tumor progression in mice [60]. The circadian organization is responsible for predictable changes in the tolerability and efficacy of anticancer agents, and also controls tumor promotion and growth [61]. The clinical relevance of the chronotherapy principle, *i.e.*, treatment administration as a function of rhythms, has been demonstrated in randomized multicenter trials, using programmable-in-time drug delivery systems. Chronotherapeutic schedules first documented the safety and the activity of oxaliplatin-based combination chemotherapy in patients with metastatic colorectal cancer. The chronotherapy concept offers further promise for improving current cancer treatment options, as well as for optimizing the development of new anticancer agents.

A meeting reported the role of environmental lighting and circadian disruption in cancer and other diseases [62]. Overexpression of either PER1 or PER2 in cancer cells inhibits their neoplastic growth and increases their apoptotic rate [63]. *In vivo* studies showed that mice deficient in PER2 showed significantly higher incidences of tumor development after genotoxic stress. Loss and dysregulation of PER1 and PER2 gene expression have been found in many types of human cancers. Studies demonstrate that both PER1 and PER2 are involved in damage response pathways and implicate normal circadian function as a factor in tumor suppression. The molecular pathways that may couple the circadian machinery to breast cancer are elaborated [64].

4. Aging

The causes of aging and their treatment and prevention have attracted much scientific attention. One theory of aging which has achieved increasing acceptance is the free radical theory of aging [1]. Our review presents representative examples from the extensive literature dealing with the involvement of circadian rhythm with aging. It is well established that increasing age is associated with increased cancer risk.

Aging affects all organic structures and processes, including the circadian system and its principal sign, the biological rhythms [65]. Aging produces losses in function of three components: receptors (the eye), the central pacemaker and the outputs. This leads to the alteration of overt rhythms, with losses in the phase relationship between them.

Circadian rhythms are generally determined biological rhythms that are considered an important adaptive mechanism to the cyclical light/dark alterations in the earth environment [66]. Age-related changes in the circadian time-keeping mechanism are well known, and seemingly contribute to various pathologies of aging. The circadian system and circadian proteins play direct roles in many physiological processes, including those associated with aging. Core circadian proteins, in addition to their known functions in the circadian oscillator, play an essential non-redundant role in the control of tissue homeostasis and aging. The involvement of circadian proteins in the regulation of metabolism, genotoxic stress response and ROS homeostasis can be responsible for the premature aging observed in some circadian mutants.

Aging alters numerous aspects of circadian biology, including the amplitude generated by the suprachiasmatic nuclei (SCN) of the hypothalamus, the site of the central circadian pacemaker in mammals, and the response of the pacemaker to environmental stimuli, such as light [67]. Age alters the 24 h expression profile of the clock and its binding partner Bmal1 genes in the hamster SCN.

Effects of aging on the circadian rhythm of locomotor activity in males of *Dorsophila nasuta* were investigated [68]. Aging is associated with weakened coupling between the activity-rest cycle and the circadian clock [69].

The relationship between aging and daily circadian behavior in humans is bidirectional: on the one hand, dysfunction of circadian clocks promotes age-related maladies; on the other, aging per se leads to changes and disruption in circadian behavior and physiology [70]. For the latter case, recent research suggests that changes to both homeostatic and circadian sleep regulatory mechanisms may play a role.

A review discusses the ways in which the circadian rhythms of older people are different from those of younger adults [71]. Older people undoubtedly have earlier circadian phases than younger adults, and have more trouble coping with shift work and jet lag.

Chronic disruptions in circadian function are associated with an increase in a variety of disease states, including heart disease, ulcers and diabetes [72]. With advanced age, the genes regulating circadian function at the cellular level become disorganized. The review explores aging-associated changes in circadian function and examines evidence linking such alterations to adverse health consequences in late life and promotion of the aging process.

Aging in zebrafish is associated with major but selective circadian alterations [73]. By 3-5 years of age, zebrafish have reduced amplitude and increased fragmentation of entrained circadian rhythms of activity, with fast desynchronization of the rhythms. Aging is also associated with a reduction in the overall duration of nighttime sleep, followed by a higher arousal threshold during day. All 24-hour endocrine rhythms partially reflect the interaction of circadian rhythmicity with sleep-wake homeostasis [74]. The decrease in sleep quality which characterizes aging may contribute to age-related alterations in hormonal function and their metabolic consequences. Literature is reviewed indicating that aging is characterized by changes in circadian rhythms and sleep quality [75]. The most marked change is an attenuation of amplitude. An advance of phase, a shortening of period, and a desynchronization of rhythms are also evident. Circadian disruption induced by light-at-night accelerates aging and promotes tumorigenesis in rats [76] [77]. Shift-work that involves circadian disruption is probably carcinogenic to humans.

Circadian clock gene expression is changed in human pathologies, and transgenic mice with mutations in clock genes develop cancer and premature aging [78]. There is circadian clock-dependent control of cell proliferation, together with control of metabolism, oxidative and genotoxic stress response, and DNA repair.

A review is dedicated to the age-related changes in circadian rhythms as they occur in humans [79]. Alterations in the regulation of circadian rhythms are thought to contribute to the symptoms of a number of conditions for which the risk is increased in old age (e.g., sleep disturbances, dementia, and depression). During aging, and in particular in Alzheimer's disease, circadian rhythms are disturbed [80]. These disturbances include phase advance, reduced period and amplitude, increased intradaily variability and a decreased interdaily stability of the rhythm. Among the factors underlying these changes, the loss of SCN neurons seems to play a central role.

In human beings and animal models, cognitive performance is often impaired in natural and experimental situations where circadian rhythms are disrupted [81]. This includes a general decline in cognitive ability and fragmentation of behavioral rhythms in the aging population of numerous species. There is evidence that rhythm disruption may lead directly to cognitive impairment. These results support the notion that age-related rhythm fragmentation contributes to the age-related memory decline.

Resetting the circadian clock leads to well being and increased life span, whereas clock disrupting is associated with aging and morbidity [82]. Increased longevity and improved health can be achieved by different feeding regimens that reset circadian rhythms and may lead to better synchrony in metabolism and physiology. A review focuses on the relationship between circadian rhythms, aging attenuation, and life-span extension in mammals. An article deals with circadian sleep, illumination, and activity patterns involving influences of aging and time reference [83].

Sleep/wake and circadian rest-activity rhythms become irregular with age [84]. Typical outcomes include fragmented sleep during the night, advanced sleep phase syndrome and increased daytime sleepiness. These changes lead to a reduction in the quality of life due to cognitive impairments and emotional stress. More importantly, severely disrupted sleep and circadian rhythms have been associated with an increase in disease susceptibility. Additionally, many of the same brain areas affected by neurodegenerative diseases include the sleep and wake promoting systems. An article discusses the relationship between circadian clock gene, circadian rhythmicity, aging-related phenotypes, and cancer [85] [86].

Circadian rhythm disturbances, such as sleep disorders, are frequently seen in aging and are even more pronounced in Alzheimer's disease (AD) [87]. Alterations in the biological clock, the suprachiasmatic nucleus (SCN), and the pineal gland during aging and AD are considered to be the biological basis for these circadian

rhythm disturbances. Pineal melatonin secretion and pineal clock gene oscillation were disrupted in AD patients. Circadian rhythms have are disturbed as a consequence of both normal aging and age-associated pathologies, like Alzheimer's disease (AD) [88]. Because sleep loss in young subjects results in endocrine disturbances which mimic those observed in aging, it is conceivable that the decrease in sleep quality which characterizes aging may contribute to age-related alterations in hormonal function and their metabolic consequences [89].

Chronic, night administration of melatonin to aging mice and transplantation of a young pineal gland into the rhythmic rudiment of older mice and rats have been studied with the aim of evaluating their effects on aging of gonadal, sexual, and reproductive functions. Both melatonin administration and young-to-old pineal grafting positively affected size and function of testes and maintenance of juvenile hippocampal and testicular LHRH-receptors and beta-adrenergic receptors in the testes of old rats and mice. Results demonstrate that a pineal-directed circadian function and cyclicity is fundamental for the regulation of sexual reproductive physiology, and that proper intervention with melatonin may potentially postpone aging effects of both neural and gonadal sexual function [90].

Data indicate that there are dramatic changes in light-activated molecular responses in the suprachiasmatic nuclei of old hamsters, and suggest that these molecular changes may underlie age-related changes in the effects of light on the circadian clock system [91].

5. Conclusion

Melatonin, a pineal hormone, plays an important role in operations of the circadian clock. The focus is on influences surrounding cancer and aging. The mechanism based on ET-ROS-OS appears to occupy a fundamental role. The multifaceted approach puts focus on melatonin and circadian rhythm in relation to various physiological processes.

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Abbreviations

ET, electron transfer; ROS, reactive oxygen species; RNS, reactive nitrogen species; OS, oxidative stress; NADPH, nicotinamide adenine dinucleotide phosphate-oxidase; SCN, suprachiasmatic nucleus; AT1, angiotensin II type 1; PER2, period circadian protein homolog 2; SNP, single nucleotide polymorphism; 5-HT, serotonoin receptor; NAT, N-acetyl transferase; Mel 1b, melatonin 1b; PKA, protein kinase A; ER alpha, estrogen receptor alpha; BP, bipolar disorder; RF-amide peptides, peptides with C-terminal arginine-phenylalanine amid; AD, Alzheimer's disease; REV-ERB alpha or NR1D1, nuclear receptor subfamily 1 proteins; AVP, arginine vasopressin.

10

Application of Hartree-Fock Method for Modeling of Bioactive Molecules Using SAR and QSPR

Cleydson B. R. Santos[1,2,3*], Cleison C. Lobato[1], Francinaldo S. Braga[1], Sílvia S. S. Morais[4], Cesar F. Santos[1], Caio P. Fernandes[1,3], Davi S. B. Brasil[1,5], Lorane I. S. Hage-Melim[1], Williams J. C. Macêdo[1,2], José C. T. Carvalho[1,2,3]

[1]Laboratory of Modeling and Computational Chemistry, Federal University of Amapá, Macapá, Brazil

[2]Postgraduate Program in Biotechnology and Biodiversity-Network BIONORTE, Campus Universitário Marco Zero, Macapá, Brazil

[3]Laboratory of Drug Research, School of Pharmaceutical Sciences, Federal University of Amapá, Macapá, Brazil

[4]Laboratory of Statistical and Computational Modeling, University of the State of Amapá, Macapá, Brazil

[5]Institute of Technology, Federal University of Pará, Belém, Brazil

Abstract

The central importance of quantum chemistry is to obtain solutions of the Schrödinger equation for the accurate determination of the properties of atomic and molecular systems that occurred from the calculation of wave functions accurate for many diatomic and polyatomic molecules, using Self Consistent Field method (SCF). The application of quantum chemical methods in the study and planning of bioactive compounds has become a common practice nowadays. From the point of view of planning it is important to note, when it comes to the use of molecular modeling, a collective term that refers to methods and theoretical modeling and computational techniques to mimic the behavior of molecules, not intend to reach a bioactive molecule simply through the use of computer programs. The choice of method for energy minimization depends on factors related to the size of the molecule, parameters of availability, stored data and computational resources. Molecular models generated by the computer are the result of mathematical equations that estimate the positions and properties of the electrons and nuclei, the calculations exploit experimentally, the characteristics of a structure, providing a new perspective on the molecule. In this work we show that studies of Highest Occupied Molecular Orbital Energy (HOMO), Low Unoccupied Molecular Orbital Energy (LUMO) and Map of molecular electrostatic potential (MEP) using Hatree-Fock method with different basis sets (HF/3-21G*, HF/3-21G, HF/6-31G, HF/6-31G*, HF/**

*Corresponding author.

6-31G and HF/6-311G), that are of great importance in modern chemistry, biochemistry, molecular biology, and other fields of knowledge of health sciences. In order to obtain a significant correlation, it is essential that the descriptors are used appropriately. Thus, the quantum chemical calculations are an attractive source of new molecular descriptors that can, in principle, express all the geometrical and electronic properties of molecules and their interactions with biological receptor.**

Keywords

Molecular Modeling; Meps Maps; Orbital Frontier; Quantum Chemical Methods

1. Introduction

Brazil has the largest genetic diversity in plant species in the world, however it is estimated that less than 10% were evaluated as their biological characteristics and less than 5% were submitted the detailed phytochemical studies. Despite a recent increase in research in this area, plants still constitute a source relatively underused and potentially very valuable for discovery of new biologically active substances [1] [2]. The bioactive compounds present in the vegetable kingdom have important functions and biological actions and may be considered as promoters of human health. We have already recognized the association between intake of fruits and vegetables and decreased risk of development various disorder chronic degenerative, such as cancer, inflammation, cardiovascular disease, cataracts, macular degeneration and other being carotenoids and phenolic compounds, some of the groups of bioactive compounds to which are attributed such actions. The seeds and extracts of annatto (*Bixa orellana* L.) are used as colorants in food, pharmaceutical and cosmetic industries due to the predominant presence of the carotenoid bixin [3]. The efficiency of biological activity of bioactive compounds of vegetable origin depends on their structure and concentration. In turn, the amount of these substances in vegetables is largely influenced by genetic factors and environmental conditions, in addition of the degree of maturation and plant variety, among other things. It is known also that the biological capacity is influenced by the substrate used in the assay, by the solvent and by extraction technique used, as well as the binomial time-temperature. Regarding the organic solvents, methanol, by managing to extract high amount of bioactive compounds, it has been touted as the most effective [4]-[7].

The *Montrichardia linifera* (Arruda) Schott, Araceae family, popularly known as “aninga”, is an aquatic macrophyte that forms large clonal populations along the rivers and streams of the Amazon. Those bordering consider this poisonous plant because its sap causes skin burns and eye contact can cause blindness [8]. However, paradoxically, it is widely used in traditional Amazonian medicine, mainly due to the healing properties of sap and of the juice of this plant, which has already been mentioned in the literature since the nineteenth century for the treatment of wounds and ulcers [9]-[12], leading to the hypothesis that this species may contain biologically active substances. However, very little is known about their chemical composition and biological activities that eventually may be useful also against human infections caused by parasites, which are serious problems in tropical and subtropical in developing countries, despite the discovery of new anti-protozoal drugs [13]. Between antimalarial compounds isolated from plants, artemisinin is one of the most important discoveries nowadays [14]-[16]. Artemisinin (or Qinghaosu, QHS, **Figure 1**) represents the most relevant advance in the treatment of malarial disease for the last 20 years [17]. Artemisinin is a sesquiterpene lactone with an endoperoxide group, which has been used in traditional Chinese medicine for many centuries as a natural product for fever and malarial treatment. This drug was isolated by Chinese chemists in the early 1970s from the ancient *Artemisia annua* L. Nowadays, artemisinin and derivatives are widely used around the world because of their potent antimalarial activity, fast action, and low toxicity. As a result, artemisinin and its derivatives have become recognized as a new generation of antimalarial drugs [18].

Pinheiro, Ferreira and Romero (2001) techniques combined quantum chemical (Hartree-Fock 3-21G) and multivariate analyzes methods (PCA, HCA, KNN and SIMCA) to study and propose diidroartemisinin derivatives. Through the technique PCA and HCA, seven (7) descriptors that were responsible for the classification of compounds into two distinct classes were selected, and with construction of qualitative models KNN and SIMCA

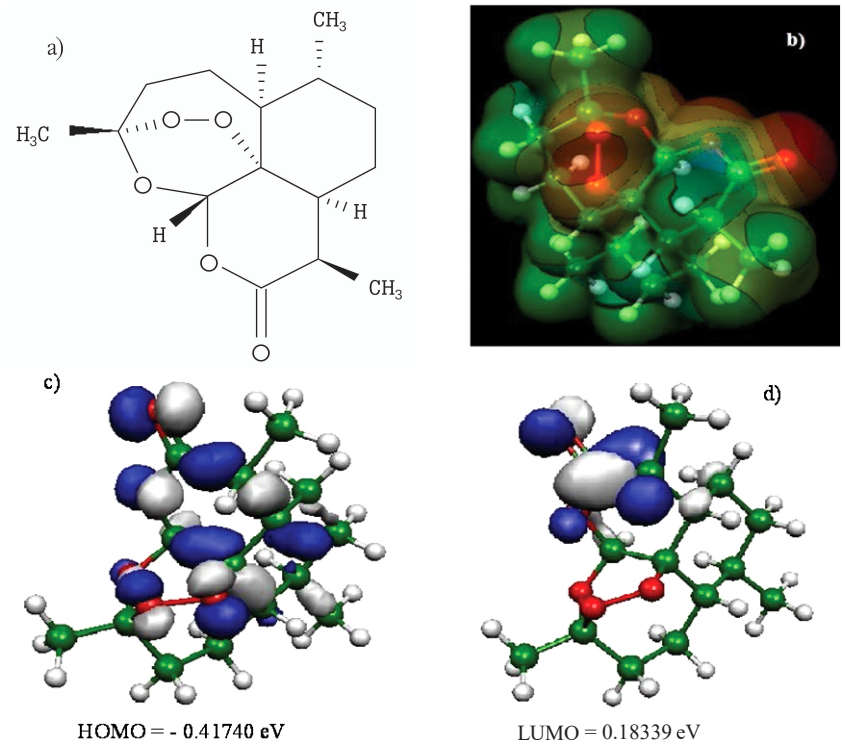


Figure 1. (a) The artemisinin structure and the region essential for expression of the biological activity (pharmacophore) was visualized using ChemSketch 12.00 program [23]. (b) Map of molecular electrostatic potential (MEP). ((c) and (d)) Orbital's energy Homo and Lumo. The MEP, Homo and Lumo were visualized and calculated using Hartree-Fock (HF) method and HF/6-31G** basis set by the Molekel program [24].

two (2) compounds of a set of twelve (12) test predicted as of high activity were proposed [19]. Artemisinin derivatives with antimalarial activity against *Plasmodium falciparum*, which is resistant to mefloquine, were studied using quantum chemical methods (HF/6-31G*) and the partial least-squares (PLS) method. Three main components explained 89.55% of the total variance, with $Q^2 = 0.83$ and $R^2 = 0.92$. From a set of 10 proposed artemisinin derivatives (artemisinin derivatives with unknown antimalarial activity against *Plasmodium falciparum*), a novel compound was produced with superior antimalarial activity compared with the compounds previously described in the literature [20]. Cardoso *et al.* (2008) studied artemisinin and some of its derivatives with activity against D-6 strains of *Plasmodium falciparum* using the HF/3-21G method. To verify the reliability of the geometry obtained, Cardoso *et al.* compared the structural parameters of the artemisinin trioxane ring with theoretical and experimental values from the literature. MEP was used in an attempt to identify key features of the compounds that are necessary for their activities, and they use those to propose new artemisinin derivatives [21]. There is however, the need for discovery of new antimalarial drugs using techniques of quantum chemistry which has been extensively used in the pharmaceutical industry, where predicting the probable activity of a drug from molecular orbital calculations is much less expensive than manufacturing it in order to perform expensive tests [22].

2. The Importance and Application of the Methods of Quantum Chemistry to Molecular Modeling

The central importance of quantum chemistry is to obtain solutions of the Schrödinger equation for the accurate determination of the properties of atomic and molecular systems. Within the area of physical chemistry, quantum chemistry is used to calculate various thermodynamic properties such as entropy, heat capacity of gases, for the interpretation of molecular spectra, calculations of bond length, bond angles, dipole moment and the understanding of intermolecular forces. In organic quantum chemistry can estimate the stability of molecules, calculate the properties of intermediate reactions, reproduce the aromaticity of organic compound and simulate spec-

tra of nuclear magnetic resonance (NMR). In analytical chemistry is used for the interpretation of intensities of the spectral lines. In the area of inorganic chemistry is used for the ligand field theory, where predictions are made, and properties justify complex ions of transition metals. In Biochemistry of quantum chemistry calculations are used to study the conformation of biological molecules such as enzyme-substrate binding and solvation of organic molecules [25]-[28].

The application of quantum chemical methods in the study and planning of bioactive compounds has become a common practice nowadays. From the point of view of planning, it is important to note that, when speaking of the use of molecular modeling (a collective term that refers to theoretical methods and computational techniques to model or mimic the behavior of molecules), it is not intended to reach a bioactive molecule simply through the use of computer programs. The development process of these molecules due to their complexity, necessarily involves multidisciplinary approach, which employs a large set of computational methods of systematic way form facilitate and optimize the development process of bioactive compounds, on a constant exchange of information with groups of chemical synthesis and evaluation of the activity of these compounds. These computational methods can be used as tools of rational design of bioactive compounds, so called because it is guided by a rational hypothesis about the mechanism of action of these compounds. The action of bioactive molecules is a very complex phenomenon, but one of the paradigms of medicinal chemistry is that these molecules are related to their effects or interactions to chemical reactions with macromolecular structures present in living systems, proteins, in its large majority. Cases these proteins are cell receptors, bioactive molecules are classified as receptor agonists or antagonists, in the case of enzymes, these molecules act as enzyme inhibitors [29] [30].

The Origin of Quantum Chemistry Methods

The history and debates related to the so called “semiempirical method” of London-Eyring-Polanyi are reported on a fascinating work of Nye [31]. The approach of Eyring and Polanyi, aiming to merge theory with experimental results to construct potential energy surfaces, showed that it is possible to gain insights into the mechanisms of adiabatic reactions, leading to important concepts related to the dynamics of chemical reactions, such as transition state and activated complex. Already at that time, there were debates comparing the approaches based on first principles with the semiempirical. In 1933 James and Coolidge [32] published an ab initio calculation when obtained the binding energy of H_2 molecule with an accuracy of 98%. This calculation took a year to be done, but its success increased confidence of authors that then criticized the semiempirical method as a “happy cancellation of errors” that occurs for not taking into account “terms of considerable importance” [32]. This expression “cancellation of errors” remains to this day in the imagination of researchers, although it is not possible to identify precisely that are these errors, and mainly, how and why they if would cancel. Polanyi in 1937 counter-argued: “Personally, I do not attach importance to an exact agreement between theory and experiment at this stage, but I believe that the theory [referring to the semiempirical method] can claim to provide a reasonable description of the mechanism of reactions chemical that would otherwise remain obscured” [33].

In 1941 Hirschfelder presented the calculation of at least one hundred activation energies for several reactions using the method of London-Eyring-Polanyi, showing that only in some cases the values did not agree with the experimental, with an error of 10 kcal/mol [34]. Hirschfelder defended the semiempirical method as “a method sufficiently flexible so that it could be made consistent with any set of chemical facts, maintaining consistent with the basic principles of quantum mechanics”. According to Nye [31], in this statement Hirschfelder has correctly identified that an ad hoc character is inherent to semi-empirical approaches. This ad hoc character involves risk, since it may well be possible to force an agreement between the semi-empirical and experimental model, even if the model is based on an erroneous theory. Such risk is even greater if the model is used for extrapolation. The final triumph of the first principles approach to the reaction $H + H_2 \rightarrow H_2 + H$, occur only came in 2003 when Mielke and colleagues [35] presented a new constants of experimental and theoretical speeds. The theoretical calculations were obtained using quantum dynamics in an exact adiabatic potential surface including Born-Oppenheimer corrections. The experiment and theory now agree in the range of 167 K to 2112 K, within experimental error, causing the problem can be solved now.

The “semiempirical” expression was first used in theoretical chemistry in 1931 by Michael Polanyi (1891-1976) and Henry Eyring (1901-1981) [36] [37] in their attempt to combine thermodynamics, chemical kinetics, quantum mechanics and the theory of binding electrons valence. In these 82 years, semiempirical method of London-Eyring-Polanyi to construct potential energy surfaces, provided a work tool useful to get insights about

how the physical and chemical processes occur, and to motivate the development of new techniques and experiments to study the temporary combination of atoms called transition state as in the works of Ahmed Zewail. The novelty of the visual maps of potential energy surfaces and the language of potential wells and barriers of activation has also become an important pedagogical tool for chemistry. In contrast, first-principles calculations of quantum dynamics, with equivalent accuracy to that of Mielke and colleagues [35], remain prohibitive, almost impossible to be performed for the vast majority of chemical reactions of practical interest. Undoubtedly: the ab initio methods, based entirely on first principles eventually prevail. However, if the reaction of the hydrogen atom with a hydrogen molecule could be regarded as definitely settled 10 years ago, what about all the other chemical and biochemical reactions? [38]

3. Hartree-Fock Equations

A development of great importance in quantum chemistry occurred from calculation functions accurate wave diatomic and for many polyatomic molecules, by the method of self-consistent field developed by Douglas Hartree [39]. In his theory Hartree considered the wave function as being formed by an antisymmetric linear combination of products of spin-orbitals. Then the wave function Hartree-Fock for atoms or molecules obeying the Pauli exclusion principle, should be written as a product antisimetrizado spin-orbitals, called Slater determinant, that in the formula restricted and normalized to a system of closed layers containing $2m$ electrons, is given by:

$$\Psi(1,2,2m)=\frac{1}{\sqrt{2m!}}\begin{vmatrix}\Phi_1(1) & \overline{\Phi}_1(1) & \cdots & \overline{\Phi}_m(1)\\ \Phi_1(2) & \overline{\Phi}_1(2) & \cdots & \overline{\Phi}_m(2)\\ \vdots & \vdots & \ddots & \vdots\\ \Phi_1(2n) & \overline{\Phi}_1(2m) & \cdots & \overline{\Phi}_m(2m)\end{vmatrix} \tag{1}$$

in which each spin-orbital $\Phi_i(x,\alpha)$ is a product of a spatial function $\varphi_i(x)$ and a spin function α or β

$$\Phi_i(x,\alpha)=\varphi_i(x)\alpha(i),\overline{\Phi}_i(x,\beta)=\varphi_i(x)\beta(i) \tag{2}$$

The functions of an electron $\varphi_i(x)$ are known as “orbitals”, a term proposed by Mulliken [40], which is the quantum mechanical analogue of the classical orbit [41]. It is considered that the orbitals are orthonormal

$$\langle\varphi_i(1)|\varphi_j(1)\rangle=\delta_{ij},\delta_{ij}=\begin{cases}0 & \text{se } i\neq j\\ 1 & \text{se } i=j\end{cases} \tag{3}$$

All elements forming a given column of Slater determinant involve the same spin-orbital already all elements in the same row involve the same electrons. When the configuration of an atom or molecule layer is closed this is represented by a single determinant, unlike a configuration layer to open, since in this case it is used a sum of Slater determinant. The analysis made the following refers the systems configurations closed layer. Open layer settings will not be discussed here, viewed the complexity of the formulas. Aiming to show a simple format for the expressions, it is used the system of atomic units in the equations.

The expression refers to the total electronic energy Hartree-Fock E for a system of electrons n nuclei in fundamental state is given by the theorem of the variational method.

$$E=\frac{\langle\Psi|\hat{H}|\Psi\rangle}{\langle\Psi|\Psi\rangle} \tag{4}$$

where Ψ comes to be wave function in the form of Slater determinant and $\hat{H}$ is the Hamiltonian operator without considering the coordinates spin system and non-relativistic. In the case of molecules that in our case the operator $\hat{H}$ is the Hamiltonian purely electronic which is obtained after the separation of Born-Oppenheimer [42] that written in atomic units has the form:

$$\hat{H}=-\frac{1}{2}\sum_i\nabla_i^2-\sum_\alpha\sum_i\frac{Z_A}{r_{\alpha i}}+\sum_i\sum_{<j}\frac{1}{r_{ij}} \tag{5}$$

The first term in Equation (5) is the operator for the kinetic energy of the electrons, the second term comes to be potential energy of attraction between electrons and nuclei, and the last term refers to the potential energy of repulsion between the electrons, Z_A is the charge of the nucleus A, $r_{\alpha i}$ is the distance of the electron i the nucleus α and r_{ij} is the distance of the electron i to the electron j.

The operator $\hat{H}$ can be separated into two other operators

$$\hat{H} = \hat{H}_1 + \hat{H}_2 \tag{6}$$

The operator $\hat{H}_1$ is relative to the kinetic energy of the electrons and the potential energy of interaction of the electrons with the nuclei:

$$\hat{H}_1 = \sum_{i}^{2m} \hat{h}(i) \tag{7}$$

The operator $\hat{h}(i)$ is the corresponding Hamiltonian to the motion of an electron in the field generated by the nuclei only

$$\hat{h}(i) = -\frac{1}{2}\nabla_i^2 - \sum_{\alpha} \frac{Z_A}{r_{\alpha j}} \tag{8}$$

The operator $\hat{H}_2$ is associated with the interelectronic repulsion

$$\hat{H}_2 = \sum_{i} \sum_{<j} \frac{1}{r_{ij}} \tag{9}$$

Utilizing the wave function (1) and the Hamiltonian (5) in the expression for the energy functional (4), has been as a result the expression for the total electronic energy of a system represented by a wave function in the form of Slater determinant.

$$E = 2\sum_{i=1} h_{ii} + \sum_{i}\sum_{j}\left(2J_{ij} - K_{ij}\right) \tag{10}$$

This relationship has reduced the integration of many electrons the set of three dimensional integrals (h_{ij}) and six (J_{ij}) and (K_{ij}), written of following form:

$$h_{ii} = \left\langle \varphi_i(1) \middle| \hat{h}(1) \middle| \varphi_i(1) \right\rangle \tag{11}$$

$$J_{ij} = \left\langle \varphi_i(1)\varphi_j(2) \middle| \frac{1}{r_{12}} \middle| \varphi_i(1)\varphi_j(2) \right\rangle \tag{12}$$

$$K_{ij} = \left\langle \varphi_i(1)\varphi_j(2) \middle| \frac{1}{r_{12}} \middle| \varphi_j(1)\varphi_i(2) \right\rangle \tag{13}$$

The integral of an electron h_{ii} represents the sum of the kinetic energy of an electron in the orbital $\varphi_i(1)$ and its potential integral due to the action of the nuclei. The Coulomb integrals J_{ij} and of exchange K_{ij} are associated to interaction between an electron in the orbital $\varphi_i(x)$ and another in the orbital $\varphi_j(x)$. In classical mechanics the integral Coulomb is the interaction energy between two distributions of charges, already integral exchange does not have analogue in classical mechanics, their presence arises due to anti-symmetry of the wave function [41]. Varying E in Equation (4) for each orbital and making $\delta E = 0$ while keeping orthonormality of the wave function arrives at the 1-electron equations that define the Hartree-Fock method

$$\hat{F}(1)\varphi_i(1) = \varepsilon_i\varphi_i(1), i = 1, 2, \cdots, m \tag{14}$$

where Fock operator $\hat{F}(1)$ is given by

$$\hat{F}(1) = \hat{h}(1) + \sum_{j}^{m}\left[2\hat{J}_i(1) - \hat{K}_j(1)\right] \tag{15}$$

where $\hat{J}_i$ and $\hat{K}_j$ are respectively operators of Coulomb and exchange

$$\hat{J}_i(1)\varphi_i(1) = \left[\int \varphi_j^*(2)\frac{1}{r_{12}}\varphi_j(2)\,\mathrm{d}\tau_2\right]\varphi_i(1) \tag{16}$$

$$\hat{K}_j(1)\varphi_i(1) = \left[\int \varphi_j^*(2)\frac{1}{r_{12}}\varphi_i(2)\,\mathrm{d}\tau_2\right]\varphi_j(1) \tag{17}$$

The Hartree-Fock equations are solved in general by an interactive procedure (SCF). At the end of the procedure, ε_i are the eigenvalues monoeletrônicos HF system. Each ε_i is often called orbital energy and is interpreted as being the energy of an electron in the orbital φ_i, resulting from its kinetic energy, energy of attraction with the nuclei and their energy exchange and repulsion due to all other electrons in their charge density $|\varphi_i|^2$.

The Equation (14) provides

$$\varepsilon_i = \langle \varphi_i(1)|F(1)|\varphi_i(1)\rangle \tag{18}$$

Substituting Equation (15) in (18) we can relate the orbital energies with the integrals (11), (12) and (13).

$$\varepsilon_i = h_{ii} + \sum_{j=1}^{m}\left(2J_{ij} - K_{ij}\right) \tag{19}$$

It may be also find the total electron energy E from the orbital energies. But this total energy is not simply equal the sum of the energies of an electron. This fact is due to the sum of the energies of an electron including twice each electron-electron interaction, *i.e.*, repulsion between electrons 1 and 2 contributes to the energy of an electron associated with both electrons. Thus it follows that the second term in the equation below corrects this problem

$$E = 2\sum_{i}^{m}\varepsilon_i + \sum_{i}^{m}\sum_{j}^{m}\left(2J_{ij} - J_{ij}\right) \tag{20}$$

Thus substituting (19) in (20) have

$$E = \sum_{i=1}^{m}\left[2h_{ii} + \sum_{j=1}^{m}\left(2J_{ij} - K_{ij}\right)\right] \tag{21}$$

which equals

$$E = \sum_{i=1}^{m}\left(\varepsilon_i - h_{ii}\right) \tag{22}$$

with h_{ii} being the same Equation (11).

3.1. Hartree-Fock-Roothaan Method

The problem of solving the Hartree-Fock equations for atoms and molecules is due to the fact of absence of central symmetry. Therefore it is necessary to use approximations for the best orbital. Thus for systems containing many electrons, an approximated way of revolver the Hartree-Fock equations consists of expand the orbital Hartree-Fock $\varphi_i(x)$ on a linear combination of K basis functions χ_μ, in accordance proposed by Roothaan [43], and this method denominated of Hartree-Fock-Roothaan (HFR) or also Molecular-Orbital Linear Combination Atomic Orbitals (LCAO-MO). Thus the orbital can be expanded in a linear combination as follows.

$$\varphi_i = \sum_{\mu}^{K} c_{\mu i}\chi_\mu \tag{23}$$

where the $c_{\mu i}$ are the coefficient of expansion, which will be treated as variational parameters and basis functions χ_μ are Slater type atomic orbitals or Gaussian type. To accurately represent the orbitals, functions should form a complete set. However, this requires an infinite number of such functions. What you should use is actually a finite number of basis functions. Orbitals should obey the orthonormality condition. Otherwise you can make a linear transformation thereby making them orthonormal

$$\sum_{\mu\nu} c_{\mu i}^{*} c_{\mu j} S_{\mu\nu} = \delta_{ij} \tag{24}$$

where $S_{\mu\nu}$ is the overlap integral

$$S_{\mu\nu} = \left\langle \chi_{\mu}(i) \middle| \chi_{\nu}(i) \right\rangle \tag{25}$$

For the molecules $\varphi_i(x)$ are molecular orbitals and χ_{μ} are the atomic orbitals. Thus there is a significant improvement in computational calculations when the orbital functions are expanded in terms of a finite set of basis functions. The integro-differential equations are then transformed into algebraic equations for the expansion coefficients [41]. The total electronic energy is obtained when Equation (23) is replaced in (21).

$$E = 2\sum_{i}^{ocup}\sum_{\mu\nu} c_{\mu i}^{*} c_{\nu i} h_{\mu\nu} + \sum_{ij}^{ocup}\sum_{\mu\nu\lambda\sigma} c_{\mu i}^{*} c_{\lambda j}^{*} c_{\nu i} c_{\sigma j} \left[2\left\langle \mu\lambda \middle| \nu\sigma \right\rangle - \left\langle \mu\nu \middle| \lambda\sigma \right\rangle \right] \tag{26}$$

where

$$h_{\mu\nu} = \left\langle \chi_{\mu}(1) \middle| \hat{h}(1) \middle| \chi_{\nu}(1) \right\rangle \tag{27}$$

with $\hat{h}(1)$ given by Equation (8) by replacing *i* by 1.

The integrals $\left\langle \mu\lambda \middle| \nu\sigma \right\rangle$ and $\left\langle \mu\nu \middle| \lambda\sigma \right\rangle$ are integral interaction two electrons are represented by

$$\left\langle \mu\lambda \middle| \nu\sigma \right\rangle = \int\int \chi_{\mu}^{*}(1)\, \chi_{\lambda}^{*}(2) \left| \frac{1}{r_{12}} \right| \chi_{\nu}(1)\, \chi_{\sigma}(2)\, \mathrm{d}\tau_1 \mathrm{d}\tau_2 \tag{28}$$

$$\left\langle \mu\nu \middle| \lambda\sigma \right\rangle = \int\int \chi_{\mu}^{*}(1)\, \chi_{\nu}^{*}(2) \left| \frac{1}{r_{12}} \right| \chi_{\lambda}(1)\, \chi_{\sigma}(2)\, \mathrm{d}\tau_1 \mathrm{d}\tau_2 \tag{29}$$

The best coefficients $c_{\mu i}$ are determined by varying the electron energy given by Equation (26) with respect to them, obeying the condition of orthonormality. Thus we arrive at the equations of Hartree-Fock-Roothaan

$$\sum_{\nu} c_{\nu i} \left\{ h_{\mu\nu} + \sum_{j}^{ocup}\sum_{\nu\sigma} c_{\nu j}^{*} c_{\sigma j} \left[2\left\langle \mu\lambda \middle| \nu\sigma \right\rangle - \left\langle \mu\sigma \middle| \lambda\sigma \right\rangle \right] \right\} = \sum_{j}\sum_{\nu} \varepsilon_{ij} c_{\nu j} S_{\mu\nu} \tag{30}$$

where the operator Fock defined as

$$F_{\mu\nu} = h_{\mu\nu} + \sum_{j}^{ocup}\sum_{\nu\sigma} c_{\nu j} c_{\sigma j} \left[2\left\langle \mu\lambda \middle| \nu\sigma \right\rangle - \left\langle \mu\nu \middle| \lambda\sigma \right\rangle \right] \tag{31}$$

We can replace the Equation (31) in (30) and thus obtain the equations of Hartree-Fock-Roothaan the following notation

$$\sum_{\nu} c_{\nu i} F_{\mu\nu} - \sum_{j}\sum_{\nu} \varepsilon_{ij} c_{\nu j} S_{\mu\nu} = 0, \mu = 1, 2, \cdots, k \tag{32}$$

Applying a unitary transform on Equation (32) to diagonalize the matrix ε, has been

$$\sum_{\nu} \left(F_{\mu\nu} - \varepsilon_i S_{\mu\nu} \right) c_{\nu i} = 0, \mu = 1, 2, \cdots, k \tag{33}$$

Equation (33) can be written in matrix form in this way has been

$$FC = SCE \tag{34}$$

The equations of Hartree-Fock-Roothaan are resolved in the same way that the equations of Hartree-Fock, *i.e.*, by an interactive process (SCF-MO-LCAO). Those obtained at the end of the procedure are the eigenvalues of HFR system.

3.2. Hartree-Fock Limit

In Hartree-Fock method, is used a electronic wave function composed only by a Slater determinant, thereby providing only approximate description of exact wave function because this can not be described by a single

Slater determinant. The exact solution of the Schrödinger equation would not be obtained in case the spatial orbitals $\varphi_i(x)$ be expanded into a linear combination of basis functions χ_μ, although the larger and more complete is the set of those functions greater the degree of flexibility in expansion for spin-orbital and lowest expected value for energy. Larger bases sets decrease the HF energy up to a certain limit. This limit is the lowest that can be obtained from a wave function of a determinant and it is called of Hartree-Fock limit. Even so, as a variational method, this limit energy E_{HF} will still be above the exact nonrelativistic energy $E_{ex}(NR)$ due to the energy of electron correlation E_{corr}

$$E_{ex}(NR) = E_{HF} + E_{corr} \tag{35}$$

The SCF method is a valid approach, but generates errors in energy because it describes of approximate mode the interactions between the electrons. Should be also consider the instantaneous interactions between the electrons. The moves of the electrons are correlated with each other, *i.e.*, there is a correlation in the different positions of the electron and must be taken into account.

The exact value of the energy E including electron correlation and relativistic effects E_{rel} is given by:

$$E = E_{HF} + E_{corr} + E_{rel} \tag{36}$$

4. Application of Basis Sets Separate Valence Used in the Molecular Modeling

To perform atomic and molecular calculations, determines a basis set formed by mathematical functions. These bases consist of a linear combination of the wave functions of an electron in terms of a finite number of base functions, which contains a set of parameters to be optimized. The denomination of the base sets depends on various types of basis functions, of the number of those employed functions in the expansion of monoeletrônicas functions (orbital) and of characteristics of the parameters to be optimized. Thus it is of fundamental importance the careful choice these functions when you want to get accurate results. The main basis sets separate valence available in computational chemistry programs for performing calculations of molecular properties are presented below:

4.1. Slater Type Functions

The atomic systems containing only one electron, hydrogenic atoms, have as a solution of the Schrödinger nonrelativistic equation, a function of type

$$\Psi_{nlm}(r,\theta,\varphi) = R_{nl}(r)Y_{lm}^{(\theta,\varphi)} \tag{37}$$

where n, l and m are the principal quantum numbers, orbital angular momentum and magnetic, $R_{nl}(r)$ is the radial part and $Y_{lm}^{(\theta,\varphi)}$ is the angular part denominated spherical harmonics. The radial part has the form

$$R_{nl}(r) = N_{nl}\left(\frac{2Zr}{na}\right)^{l}\exp\left(-\frac{Zr}{na}\right)L_{n+1}^{2l+1}\left(\frac{2Zr}{na}\right) \tag{38}$$

where N_{nl} comes to be a normalization factor, Z is nuclear charge, r is radius, a is a constant and $L_{n+1}^{2l+1}\left(\frac{2Zr}{na}\right)$ represents in a generic form a polynomial called associated Laguerre polynomial. The angular part is equal to

$$Y_{lm}^{(\theta,\varphi)} = N_{lm}P_l^{(m)}(\cos\theta)e^{im\phi} \tag{39}$$

being N_{lm} a normalization factor and $P_l^{(m)}$ the associated Legendre polynomial.

For more that hydrogenic orbitals are orthogonal they do not form a complete set of continuous functions. They have limited application because many of the required integrals in molecular orbital calculations are somewhat hard to calculated, especially when you have high values of the principal quantum number because of the complexity of the polynomial in r. Then Slater (1930) proposed a form analytical simpler for the radial function, then introducing functions such as Slater (Slater Type Functions-STFs) [42].

$$R_{nl}(r) = \frac{\left[2\zeta/a_0\right]^{n+1/2}}{\left[(2n)!\right]^{1/2}}r^{n-1}\exp\left(-\zeta r/a_0\right) \tag{40}$$

Since the orbital exponent is written as

$$\zeta = \frac{Z - s}{n^*} \tag{41}$$

where ***s*** is a constant related to the shielding effect of the electrons of the internal layers of atoms and n^* is an effective principal quantum number. Therefore the general shape of Slater type functions can be written as follows, which is similar to the equation.

$$\Psi_{nlm}(\zeta, r, \theta, \varphi) = \left[(2n)!\right]^{1/2} (2\zeta)^{n+1/2} r^{n-1} e^{-\zeta P} Y_{lm}^{(\theta,\varphi)} \tag{42}$$

Slater evaluated through empirical rules to select of parameters s and n^*, thereby aiming at a good approximation for the atomic orbitals best of this type. The values of ζ were determined by the variational method by Clementi (1963 and 1967) for neutral atoms to the ruthenium in their fundamental states, using for this the SCF method [44] [45]. The exponents ζ are positive numbers and adjustable in the methods of calculation. These exponents determine the size of the orbital, thereby large exponents characterize dense orbital and small exponents characterize diffuse orbitals.

The so-called Slater type orbitals (STOs) are formed by the product of the angular part of Equation (39), by the product of the radial part of Equation (40). The STOs generate reasonable representations of atomic orbitals. However, as they replace the polynomial in r in the hydrogenic orbitals by a simple power r, do not have the proper number of radial nodes, just not well represent the internal part of an orbital.

One of the limitations is that they are not of mode some orthogonal, as much as it can be corrected using a set of STOs orthogonalized. In addition the use of these orbitals in molecular SCF calculations makes the integrals multicenter, that involve interactions between electrons, becomes somewhat difficult to be solved numerically, thereby increasing the computational time.

4.2. Minimum Base Sets

Minimum base sets or base "single-zeta" is set containing a single function to represent each occupied atomic orbital of different quantum numbers ***n*** and ***l*** of electron configuration. Thus, this set presents a reduced computational time to make these calculations, so can be used in calculations involving large molecules which are our case. In the calculations where a minimum base set is used are used in general Slater type orbitals. Due to the small size of the base, minimum base sets generate results only form qualitative of the properties. Nevertheless the calculations of the electronic structure were until around 1960 all performed in terms of minimum base. The first semiempirical methods more used were based in a set of minimum base STOs [46] [47].

4.3. Gaussian Type Functions

4.3.1. Contracted Gaussian Functions

The use of STOs in electronic structure calculations generates computational problems due to the emergence of multicenter integrals, although these orbitals well describe the functional behavior of molecular orbitals. In order to simplify the calculation of multicenter integrals, Boys (1950) proposed for calculations involving molecules using Gaussian type functions (GTFs) [48]

$$R_{nl}(r) = \left[\frac{2^{2n+3/2}}{(2n-1)!\sqrt{\pi}}\right]^{1/2} \alpha^{2n+1/4} r^{n-1} \exp\left(-\alpha r^2\right) \tag{43}$$

where n and l are the main quantum numbers and orbital angular momentum and α is the orbital exponent which is a variational parameter. The use of GTFs in calculations of electronic interaction integrals presents an enormous advantage, because the product two of these functions entries in different centers is equivalent to a single function centered on a new center [49], thereby there is a reduction of multicenter integrals to integrals calculated in terms of functions centered on the same point.

The functions of the type Gaussian have a poor behavior of atomic orbitals in relation to Slater type functions, the reason is that the GTFs do not have the "cusp" in the region near the nucleus. Thus the GTFs have a behavior different functional that seen for molecular orbitals, so it is necessary to use two (2) to five (5) GTFs to represent adequately each STO. As the number of electronic interaction integrals originated using of a basis set dimension m increases in the order of m^4, fastest speed and simplify the calculations of the electronic interaction

integrals in terms of GTFs compensates the large number of integrals to be calculated when comparing using STFs.

The solution of the equations of SCF method in the calculation HFR is also a process using an enormous computational time, it is also proportional to the fourth power of the number of base functions. However, the number of cycles of the interactive process SCF method increases with the number of coefficients to be optimized. Thus the use of GTFs contracted originated from linear combinations of Gaussian primitive is generally more adequate [47] [50] [51].

The contracted basis functions can be chosen in order to resemble STOs, atomic orbitals HF or any other set of functions. A type of GTFs contracted is set STO-NG. This nomenclature comes being a set of GTFs contracted that describes a STF through N GTFs primitives used in contraction. Each STO is then approximated as a linear combination of N GTFs, in which the coefficients and exponents contracted are chosen so that the basis function approach is an STF. Thus the computational time decreases, but the results generated by the set of contracted GTFs are not good.

The STOs *ns* and *np* are approximated by their respective functions

$$\varphi_{ns}(r) = \sum_{k=1}^{m} d_{ns,k} g_{1s}(\alpha_{nk}, r), n = 1,2,3 \tag{44}$$

$$\varphi_{np}(r) = \sum_{k=1}^{m} d_{np,k} g_{2p}(\alpha_{nk}, r), n = 1,2,3 \tag{45}$$

where g_{1s} and g_{2p} are GTFs, α_{nk} are gaussian exponents and $d_{ns,k}$ and $d_{np,k}$ are coefficients of the expansion, where the last three parameters are determined by the least squares method [51].

4.3.2. Basis Sets Separate Valence

In order to obtain better results, many studies have been made aimed at finding basis sets with this capability. In recent years, besides the basis sets previously described, other sets are being used in electronic structure calculations. Among these, there are basis sets separate valence [42].

Basis sets separate valence are extended sets GTFs contracted, where the most usual are listed below: 4-31G, 3-21G, 6-31G e 6-311G. In such sets are used two functions for the valence orbitals and only one for the orbitals of the internal layer, may each of these functions is a linear combination or not primitive gaussian. The occurrence of this is due the internal layer contribute little to the chemical properties of interest. As the functions of the internal layers not duplicate it generates effects on total energy, however represents little it comes of dipole moments, ionization potentials of the valence, charge density, dissociation energy and other chemical properties. For example, the base 4-31G each atomic orbital of the internal layers is described by only one GTF contracted which is formed by the combination of four linear (4) GTFs primitive. For each atomic orbital of the valence shell there are two basic functions, being that of them is a GTF contracted from the linear combination of three (3) GTFs primitive, describing the internal part of valence orbital, since the other is a single GTF primitive, which describes the outer part of the valence orbital. Considering the first row atoms (Li to F) we have:

$$\varphi_{1s}(r) = \sum_{k=1}^{4} d_{k,1s} g_{1s}(\alpha_{k,1s}, r) \tag{46}$$

$$\varphi'_{2s}(r) = \sum_{k=1}^{3} d'_{k,2s} g_{1s}(\alpha'_{k,2sp}, r) \tag{47}$$

$$\varphi''_{2s}(r) = g_{1s}(\alpha''_{2sp}, r) \tag{48}$$

$$\varphi'_{2p}(r) = \sum_{k=1}^{3} d'_{k,2p} g_{2p}(\alpha'_{k,2sp}, r) \tag{49}$$

$$\varphi''_{2p}(r) = g_{2sp}(\alpha''_{2sp}, p) \tag{50}$$

For the hydrogen atom, which has no internal layer, it has been:

$$\varphi'_{1s}(r) = \sum_{k=1}^{3} d'_{k,1s} g_{1s}\left(\alpha'_{k,1s}, r\right) \quad (51)$$

where the functions which appear with (') are internal functions while appearing with (“) are external functions. Similarly to the base structure previously described is the basis 6-31G. Only now each orbital of internal layers is represented by a GTF contracted originated from linear combination of six (6) GTFs primitive. In the case of the base 6-311G, includes a GTF primitive the set 6-31G to represent a new layer of the outer valence.

4.4. Polarization Function

All basis sets discussed so far have a peculiar characteristic, *i.e.*, they comprise functions restricted to be centered in the nuclei. However, there is evidence showing that the atomic orbitals distort or polarize when they form a molecule. For this reason one should take into consideration the possibility of non-uniform displacement of electric charges outside the atomic nucleus, *i.e.* the polarization. Thus it is possible to obtain a better description of the changes as well as also deformations in the atomic orbitals within the molecule.

One way to consider such a polarization is introduced on the basis in question functions wherein the values of *l* (quantum number of the orbital angular momentum) larger than those of fundamental state of a given atom. In this type of functions gives the name of polarization functions. For the hydrogen atom of the description fundamental state uses only functions *s*. The functions *p*, *d*, ... centered on *H*, for molecular calculate, are considered as functions of polarization. Generally, with the inclusion of polarization functions in the molecular basis there is a higher possibility of obtaining better results for many of the properties of chemical interest, such as dissociation energy and dipole moments. In real terms, it is noted that it is not satisfactory to include polarization functions of symmetry *d* and *f* together with the basis sets *s* and *p* small, that is, the polarization functions should only be added when working with basis sets said saturated [52].

Of the bases valence separate with polarization functions most commonly used in molecular calculations are the STO-3G*, 3-1G*, 6-31G*, 6-31G**, 6-311G* and 6-311G** [53]. The base 6-31G* and 6-31G** are formed by the inclusion of polarization functions to the base 6-31G, being that the base 6-31G* is constructed by adding a set of five (5) polarization functions of type GTFs of symmetry d to the base 6-31G, for each different atom of hydrogen and helium, already the basis 6-31G** is constructed by adding a set of three (3) polarization functions of type GTFs of symmetry p to the set 6-31G*, for each hydrogen atom.

4.5. Diffuse Functions

The use of valence basis set with polarization function in calculations involving anions does not generate good results, because the electron cloud of the anionic systems possess a tendency to expansion. Thus it is necessary to also add appropriate diffuse functions because they allow a greater occupation of space by the orbital region. The importance of diffuse functions in calculations of transition metals is due to the metal atoms present orbital type d and these have diffuse characteristics. So it becomes necessary adding diffuse functions to basis function associated to configuration of the neutral metal atom in order to obtain a better description of the metal complex. The great importance of diffuse functions is due to the fact that they better describe the farthest molecular orbital of the nuclei [54].

5. Computational Chemistry Software for Calculating Molecular Properties

Several computational chemistry softwares make available calculations of molecular properties through semi-empirical and Hatree-Fock methods. We can cite: MOPAC2002 [55], VAMP [56], MNDO97 [57], Gaussian98 [58], Spartan [59], GAMESS [60], Chem3D [61], CAche [55], HyperChem [62], ZINDO [63], GEOMOS [64]. Most of the great advances obtained in the research of semi-empirical methods were essentially incorporated on a series of programs entitled MOPAC [62]. MOPAC should be understood as an abbreviation of Molecular Orbital PACkage. Initiated in 1983, on group of Prof. M. Dewar, with the participation of Prof. J. J., P. Stewart, the MOPAC project currently has 30 years, and the main landmarks of this story are:

1) 1983: MOPAC → Geometry optimization, vibrational analysis and transition states.
2) 1984: MOPAC 2 → Solution of errors.
3) 1985: MOPAC 3 → AM1 method, calculation of polymers and CI (*Configuration interaction*).

4) 1987: MOPAC 4 → Solution of errors.
5) 1988: MOPAC 5 → PM3 method and way of reaction.
6) 1989: MOPAC 6 → Repair errors, calculation of electrostatic potential and extent of PM3 for 28 elements.
7) 1993: MOPAC 7/93 → Model solvent, nonlinear optical properties and analysis of symmetry.
8) 1997: MOPAC 97 → MNDO-d method
9) 1999: MOPAC2000 → Dynamic memory allocation, large molecules, solids, transition metals, improving the search algorithm of the transition state.
10) 2001: MOPAC2002 → Improvement of the package solvate, all atoms of the main group, PM5 method and parameterization for more transition metals.
11) 2004: MOPAC2005 → Calculation of complex Eu(III) [65] and Th(IV) [66] by Sparkle Model.
12) 2006: MOPAC 7.1 → A translation of MOPAC 6 for Fortran 90 is available on the Internet, having incorporated the Sparkle/AM1 model for calculating all trivalent lanthanide ions [67];
13) 2007: MOPAC2007 → An application of MOPAC7.1 incorporating new methods of RM1, PM6, Sparkle/AM1 and Sparkle/PM3. This program is available free of charge to the academy and for applications nonprofit [67].

Several programs two-dimensional of design of molecules are available and easy to use, as ChemWindow, Isis Draw, ChemDraw [68] and Chem3D [69]. They allow the preparation of figures and diagrams with desired quality and accuracy and facilitate the documentation and scientific communication. The software ChemSketch 12.00 [23] is an advanced design that provides chemical molecular properties, optimization and 3D visualization, ability to name the molecules, as IUPAC, and still has a large database of chemical structures and laboratory materials. The software automatically calculates the valence of each atom and restricts the construction of the molecule based on the octet rule, unless instructed to do this restriction. Then is possible to request the construction of 3D spatial form of the species studied, which triggers another window where the academic can rotate tridimensionally the species studied, in addition to observing these species in different visualizations with possibility to visualize bonds and spatial arrangement of species prominently in each of these representations. The design and visualization of 3D drugs, with steric factors relevant to biological activity, are important for analysis of the size, volume and shape of the molecules [70]. The Molekel is a free software multiplatform molecular visualization. It was originally developed at the University of Geneva by Flükiger in the 1990s for Silicon Graphics computers. In 1998, Stefan Portmann took responsibility and released version 3.0. The version 4.0 was almost one version of the platform independent. Other developments lead version 4.3, before Stefan Portman moved and stopped developing the codes. In 2006, the Swiss National Supercomputing Centre (CSCS) restarted the project and version 5.0 was released on December 21 of the same year [24].

To obtain the conformational analysis and energy minimization, we can cite: the program Chem3D [69] widely used in studies with this objective [70]. However, other programs like Molecular Modeling Pro [71], ChemSite (ChemSW) [72], Alchemy, Sybyl, ChemX, cache and WebLab Viewer are also available.

In the area of molecular modeling, graphics construction and projects of drugs, the program Hyperchem [73] for being a tool specializing in 3D structures of interest to the medical, pharmaceutical and organic chemistry. The program lets you design complicated molecules. This software is also an alternative in the field of spectroscopy, which besides the ability to simulate a priori by the NMR spectra quantum methods, contains a database of approximately 10^4 molecules applicable to macromolecules as well as small molecules. The software also includes animations, and quantum chemical calculations and molecular mechanics.

The choice of method for energy minimization depends on factors related to the size of the molecule, parameters of availability and stored data and computational resources. Molecular models generated by the computer are the result of mathematical equations that estimate the positions and properties of the electrons and nuclei, the calculations exploit experimentally, the characteristics of a structure, providing a new perspective on the molecule [74].

In quantum chemistry the softwares more used are Gaussian and GaussView that uses the laws of quantum mechanics to predict the energies, structures and properties and vibrational frequency of molecular systems [75]. The GaussView 5.0 is a program that can work on Windows and responsible for building the structures under study, by viewing these as well as for generating the input of the species under study for the program calculations–Gaussian 03W. This includes an advanced molecular modeler, which can be used for construction and molecular dimensions of the three test [75].

The Gaussian 03W is a program that can work on Windows and Linux that performs computations used in the

study of reaction mechanisms, equilibrium geometries of neutral molecules, radicals and ions, and the determination of physicochemical parameters. Appreciates structure, reactivity, thermodynamic properties, energy barriers (transition states), conformational analysis, employing the optimization of molecules and theoretical calculations of vibrational spectra. From the optimization is obtained the most appropriate structure to the molecule, whereas the lengths and bond angles and power stabilization calculated by $E(RB = HF - LYP)$ ua [75].

6. Application of Hartree-Fock Method Using Computational Tools

Obtaining molecular properties depends of method and basis sets, and represents a means of chemical information contained in the molecular structure of the compound studied. The structure-activity relationships (SAR) represent a core aspect of medicinal chemistry. The fact that a small change in structure leads to a small change in biological activity and allows chemists to rationalize substitutions at specifics positions, giving them the freedom to modify a molecule to improve various properties such as lipophilicity, bioavailability, and so on without sacrificing potency (to a large extent). From the modelers' perspective, the principle of similar structures having similar activities [76] is a cornerstone of quantitative structure-activity relationship (QSAR) modeling [77] [78]. This information is transformed and encoded for lots of problems chemical, pharmacological and toxicological studies on the relationship between structure-activity, quantitative structure-activity and structure-property (SAR, QSAR and QSPR) [79]-[81].

Ferreira *et al*. [82] studied artemisinin and 18 derivatives with antimalarial activity against W-2 strains of *Plasmodium falciparum* through quantum chemistry and multivariate analysis. The geometry optimization of structures was realized using the Hartree-Fock method and the 3-21G** basis set. Maps of molecular electrostatic potencial and docking molecular were used to investigate the interaction between the ligands and the receptor (Heme).

Santos *et al*. [83] performed studies using Hartree-Fock method and the 6-31G** basis set were employed to calculate the molecular properties of artemisinin and 20 derivatives with antimalarial activity. Maps of molecular electrostatic potential (MEPs) and molecular docking were used to investigate the interaction between ligands and the receptor (heme). Principal component analysis and hierarchical cluster analysis were employed to select the most important descriptors related to activity. The correlation between biological activity and molecular properties was obtained using the partial least squares and principal component regression methods. The regression PLS and PCR models built in this study were also used to predict the antimalarial activity of 30 new artemisinin compounds with unknown activity. The models obtained showed not only statistical significance but also predictive ability. The significant molecular descriptors related to the compounds with antimalarial activity were the hydration energy (HE), the charge on the O11 oxygen atom (QO11), the torsion angle O1-O2-Fe-N2 (D2) and the maximum rate of R/Sanderson Electronegativity (RTe+). These variables led to a physical and structural explanation of the molecular properties that should be selected for when designing new ligands to be used as antimalarial agents.

Case Study on Aspirin

Aspirin introduced in 1899 was one of the first drugs developed and is still one of the most widely used. Estimated 20 billion aspirin tablets are consumed each year in the United States. Originally planned to ease the pain and relieve sore muscles, proved to be a highly complex drug with the power and unexpected limitations. It turned out that it reduces the incidence of heart attacks and is effective in reducing the incidence of Alzheimer's disease and cancer of the digestive tract. At the same time, however, aspirin attacking the stomach lining, causing bleeding or even ulcers, and usually cause intestinal problems [84]. One of the forms of action of aspirin is blocking an enzyme (a type of protein) called COX-2, which promotes inflammation, pain and fever. Unfortunately, it also interferes with COX-1 a correlate enzyme that produces essential hormones to the health of stomach and kidney. An analgesic and anti-inflammatory agent is efficient in COX-1. **Figure 2** is shown the structure of aspirin which acts transferring part of its molecule known as acetyl group, for COX-2, disabling it. This drug-receptor interaction is irreversible nature due to the formation of a covalent bond resulting from the nucleophilic attack of the hydroxyl group of the amino acid serina$_{530}$ to the electrophilic acetyl grouping present in aspirin [85].

A substitute for aspirin (new drug) has to keep this aspect of the molecule, and the replacement should the general format and size of the molecule in such a way it clicks into place in molecular target the same manner that aspirin.

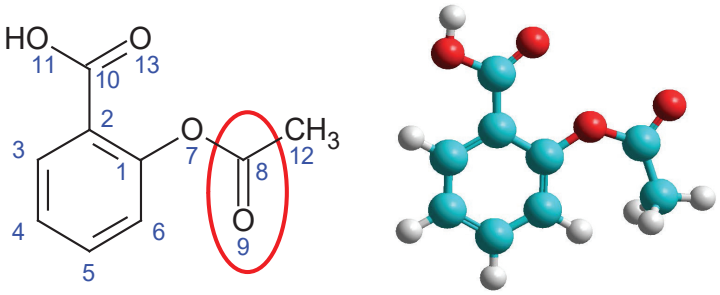


Figure 2. Aspirin (structure) and region essential for the expression of biological (pharmacophore) was visualized using ChemSketch 12.00 program [23] and HyperChem Release 6.02 [73].

The use of maps of electrostatic potential (MEP) is a computational tool that aids in the process of recognition of one molecule by another, as interactions of types receptor drugs and enzyme substrates, because being by their potential that chemical species interact with other in biological recognition process. The electronic parameters are one of the main factors governing drug-receptor interaction, in this sense; the MEP can be considered an alternative approach in order to understand the electrostatic contribution of this drug and its new derivatives for biological activity [86]. In the construction of the MEP are necessary three steps: construction of the surface density of the molecule, construction of the electrostatic surface potential and applying colors the surface obtained to designate potential values. One of the frequent topics of theoretical chemistry is research to improve methods to elucidate the behavior of molecules and other reactive chemical species. Among the numerous existing reactivity indices the molecular electrostatic potential $V(r)$ that is generated around a molecule by its nuclei and electrons, is known for being a real physical property can be determined experimentally by diffraction methods, as well as computationally [87].

In **Figure 3** are shown the MEPs of aspirin with Hatree-Fock method in different basis sets (HF/3-21G*, HF/3-21G**, HF/6-31G, HF/6-31G*, HF/6-31G** and HF/6-311G). In this figure we can observe that in the HF/6-31G** method, showed the lowest positive electrostatic potential (region blue color) of 0.06715 au (atomic unit). However, HF/6-311G method, showed higher positive electrostatic potential 0.07607 au. The variation between them was ±0.00892au (HF/6-311G and HF/6-31G**); Also in this figure we observed that the HF/6-311G method, showed the lowest negative electrostatic potential (region red color) equal to −0.10235 au. However, HF/3-21G** method showed higher negative electrostatic potential of −0.09071 au. The variation between them was ±0.01164 au (HF/6-311G and HF/3-21G**). Thus, the presence of a negative potential surface in carbonyl oxygen atom of the acetyl group is nucleophilic (affinity for positive nuclei), while the carbonyl carbon is electrophilic (electron affinity) in accordance with the literature [85]. Thus, we have shown that the use of different basis sets present values of different electrostatic potentials for the same case study. Therefore, obtaining molecular properties will depend on the method and basis set to reproduce experimental data with greater accuracy. In this case, the MEP of aspirin in different basis set was used to evaluate the key features of aspirin from qualitative comparisons in the region of the acetyl group, which according to Bernardinelli *et al.* [88] the geometric form of the electrostatic potential is similar for all active compounds. However, new derivatives of aspirin must have some structural similarity in terms of their electrostatic potentials that allow one to be recognized by the other, with similar biological activities [87] [89] [90].

Structure-activity relationship (SAR) indicates molecular structure modifications that increase the drug effectiveness. In general, reports show that these modifications are made throughout small changes in the leading compound structure, followed by trials in laboratory to quantify the variations in the biological activity due to changes in the molecular structure [91].

The quantum-chemical descriptors widely used in SAR, QSAR and QSPR studies are related to the energy of the frontier orbitals (HOMO and LUMO). The reason for this is related to the fact that these properties provide information about the character electron donor and/or electron-acceptor and a compound thus forming a charge transfer complex (CTC) [92]. The energy of Highest Occupied Molecular Orbital Energy (HOMO) and Lowest Unoccupied Molecular Orbital Energy (LUMO) are quantum-chemical descriptors, which play an important role in chemical reactions and the formation of many complex charge transfer [93]. In **Figure 4** is shown the fron-

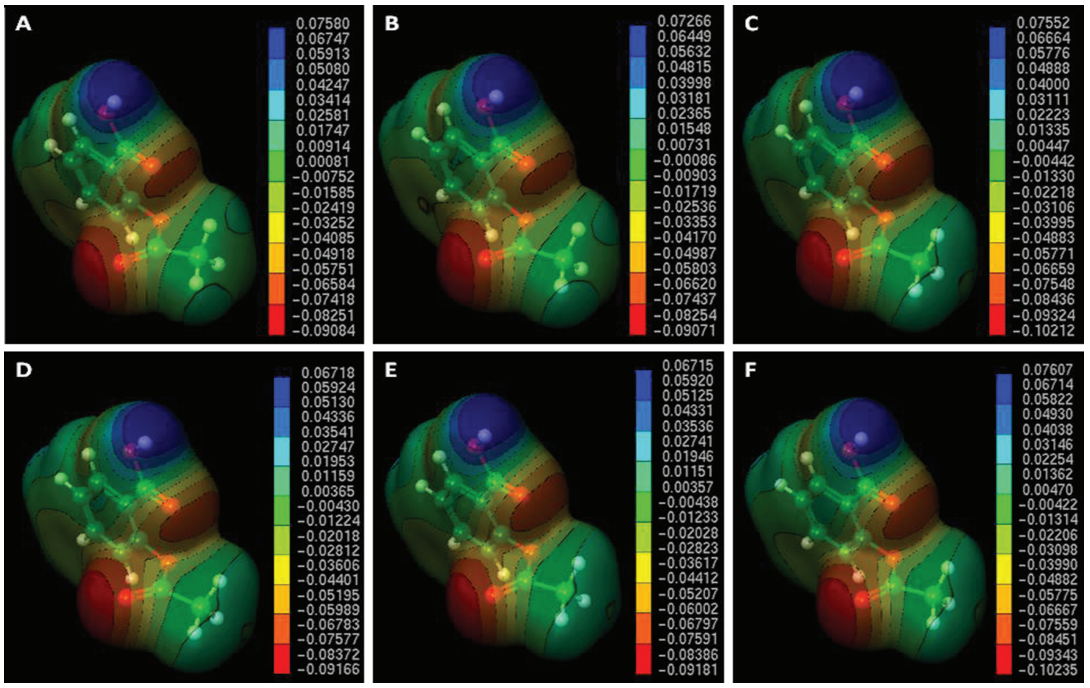


Figure 3. Molecular Electrostatic Potential Map of aspirin in different basis sets (A – HF/3-21 G*, B – HF/3-21G**, C – HF/6-31G, D – HF 6-31G*, E – HF 6-31G**, F – HF 6-311G). The MEPs were visualized and calculated by the Molekel program [24].

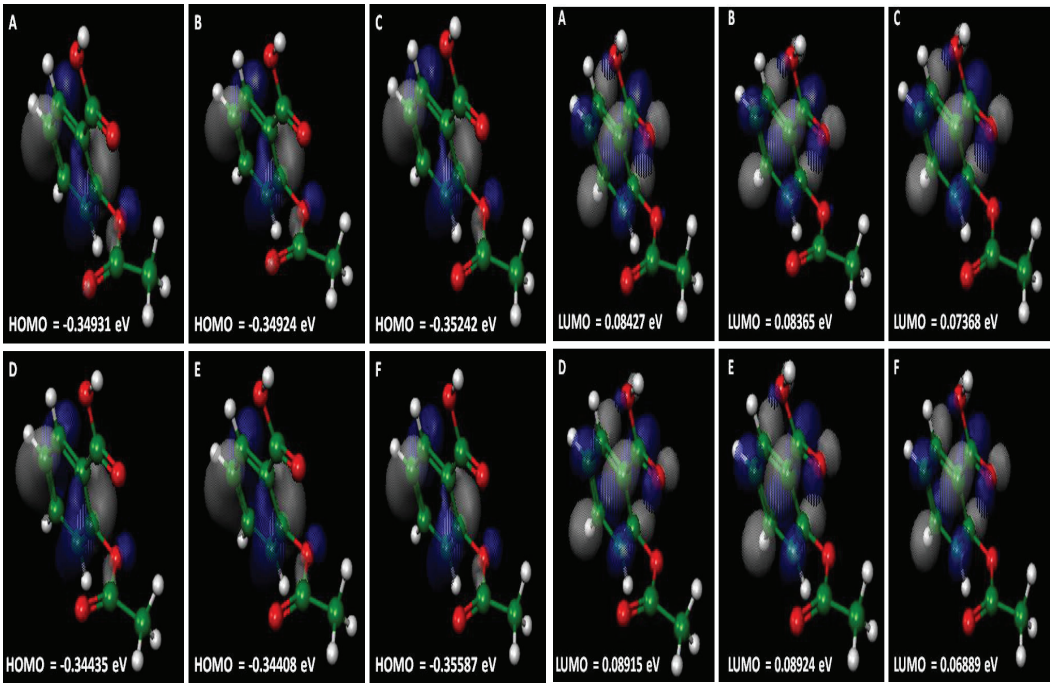


Figure 4. Orbital's energy Homo and Lumo of aspirin in different basis sets (A – HF/3-21 G*, B – HF/3-21G**, C – HF/6-31G, D – HF 6-31G*, E – HF 6-31G**, F – HF 6-311G). The Homo and Lumo were visualized and calculated by the Molekel program [24].

tiers orbital (HOMO and LUMO) with the respective values of energy (eV), using Hatree-Fock method at different basis sets (HF/3-21G*, HF/3-21G**, HF/6-31G, HF/6-31G*, HF/6-31G** and HF/6-311G) for aspirin,

and this figure we note the delimited region for the HOMO orbital which measures the electron-donor character of aspirin, and the LUMO which measures the electron-acceptor character. From these definitions, two important features can be observed: the higher the energy of the HOMO greater electron-donating ability, which can be observed in HF/6-31G** with a value of HOMO = −0.34408 eV, and the lower the energy of the LUMO will be lowest resistance to accept electrons that can be noticed in HF/6-311G with a value of LUMO = 0.06889 eV. In this figure the HOMO is located in the region around the benzene ring and the acetyl group, when the substituents are made in the aromatic ring or acetyl group, depending on the substituent will have a high electron density, such as the carbonyls, amines or amides, the more pronounced HOMO region is strongly influenced to perform electronic stereo secondary effects, which may compromise the pharmacological activity of the compound. The energy of HOMO is directly related to the ionization potential of the compound and characterizes the ability of the molecule to perform nucleophilic attacks. Also in **Figure 4**, the LUMO is located in close region to the benzene ring and of the carboxylic group, so the energy of LUMO is directly related to the electron affinity, characterized by the susceptibility of the compound in relation to attacks by nucleophiles [94].

The energies of HOMO and LUMO have been used for decades as indices of chemical reactivity and are commonly correlated with other indices, such as electron affinity and ionization potential [95]-[99]. The difference between the energies of the HOMO-LUMO orbitals (*gap*) is an important indicator of molecular stability. Molecules with low *gap* value are generally reactive, while molecules with higher gap value indicate high stability of the molecule, in the sense of low reactivity in chemical reactions [100]. In **Table 1** are shown some molecular properties obtained with Hartree-Fock method in different basis sets (HF/3-21G*, HF/3-21G**, HF/6-31G, HF/6-31G*, HF/6-31G** e HF/6-311G) and the correlation matrix of Pearson [101]-[103], considering how an independent variant the total energy (Etotal) of aspirin, and other properties as the dependent variable, this treatment was performed using Stastica 6.2 program [104]. In this table it is observed that the correlation between the molecular properties of aspirin is less than or equal to 0.96890 (LUMO), while the correlation between the molecular properties and the total energy is less than or equal to 0.85448 (QC8), which represents the charge on the carbon atom 8, see **Figure 2**. Among these properties obtained those that had greater relevance to building a model QSPR in function of the total energy were the volume (−0.78677), hydration energy (−0.45775), GAP (−0.43419) and QC_8 (0.85448). Therefore, we can represent the QSPR model according to the values of the statistical parameters in Equation (52) below. The statistical quality [105] of the regression equations was gauged by parameters like correlation coefficient (r) or squared correlation coefficient (r^2), explained variance (R_A^2, *i.e.*, adjusted R^2), standard error of estimate (SEE), and variance ratio (F) [106]-[108]. The better regression models were selected on the basis of the higher r, F value (a statistic of assessing the overall significance) and the lower SEE.

$$E_{\text{total}} = 286.1621 - 1.1850(V) + 8.7851(HE) + 615.3683(GAP) + 39.8875(QC_8) \quad (52)$$

This type of treatment shows that QSPR studies are important to obtain of molecular properties that take into account different aspects of chemical information, this information can be through experiments or theoretical calculations simple counting, consider the entire molecule, fragments or functional groups, knowledge of the 3D structure of the molecule or molecular graphics his or her simply formula, information defined by scalar values, vectors or scalar fields [109].

In the discovery of a superaspirin Jacob *et al*. (2012) [110] synthesized by conjugation aspirin-glucose (**Figure 5**) in order to study the solubility in water and anticancer activity as compared to aspirin and evaluated that aspirin-glucose was seven times more soluble in water than aspirin, and about 8 to 9 times more active in inhibiting cell growth than aspirin in its anticancer activity in cell culture breast, pancreatic, and cell lines prostate, while the activity was similar in a line benign non-cancer cells. According to the computer calculations performed to aspirin and aspirin-glucose in HF/6-31G** verified that the anticancer activity of aspirin-glucose was increased by the fact of presenting high value GAP = −0.43232 eV, having greater molecular stability and low chemical reactivity, being that the value of GAP to aspirin in HF/6-31G** was −0.43332 eV, having a variation of GAP = $\pm 10^{-3}$ eV (between aspirin and aspirin-glucose).

In **Figure 5** is shown the map of electrostatic potential for aspirin-glucose, and observed the region of negative electrostatic potential, characterized by red color, which is the region essential for the expression of biological activity (pharmacophore). Therefore, these results are directly related with the conclusion that Jacob *et al*. indicated that the hydrolysis of the aspirin-glucose in human serum is at a relatively slower rate compared to aspirin where there has been significant anticancer activity at the doses studied under the experimental conditions.

Table 1. Molecular properties obtained at different basis sets of aspirin and Pearson correlation matrix.

Method and basis set	Total energy (u.a)	Surface Area (Grid) (Å^2)	Volume (Å^3)	Hydration Energy (kcal/mol)	HOMO (eV)	LUMO (eV)	GAP (eV)	QC_8 (C)	QO_9 (C)	C8-O9 (Å)
HF/3-21G*	−641.3663	345.70	527.07	−8.00	−0.34931	0.08427	−0.43358	0.856	−0.584	1.19329
HF/3-21G**	−641.4183	345.02	526.10	−8.04	−0.34924	0.08365	−0.43289	0.825	−0.585	1.19312
HF/6-31G	−644.6781	347.46	530.81	−8.04	−0.35242	0.07368	−0.42610	0.778	−0.541	1.20473
HF/6-31 G*	−644.9649	345.45	528.40	−7.89	−0.34435	0.08915	−0.43350	0.783	−0.544	1.18114
HF/6-31G**	−644.9837	345.33	528.31	−7.89	−0.34408	0.08924	−0.43332	0.772	−0.545	1.18127
HF/6-311	−644.8264	345.99	529.85	−8.03	−0.35587	0.06889	−0.42476	0.723	−0.490	1.20201
Total energy	1.00000									
Surface Area (Grid)	−0.36056	1.00000								
Volume	−0.78677	0.83299	1.00000							
Hydration Energy	−0.45775	−0.42636	−0.09622	1.00000						
HOMO	−0.05826	−0.53886	−0.44601	0.86149	1.00000					
LUMO	0.18092	−0.65181	−0.64085	0.75742	0.96890	1.00000				
GAP	−0.43419	0.72982	0.81092	−0.58584	−0.86310	−0.96123	1.00000			
QC_8	0.85448	−0.27889	−0.73507	−0.09622	0.33329	0.52087	−0.69165	1.00000		
QO_9	0.34835	0.28008	0.03220	−0.61938	−0.54970	−0.45390	0.31382	0.18617	1.00000	
C_8-O_9	0.10478	0.71376	0.48478	−0.91231	−0.94599	−0.92477	0.83323	−0.18682	0.55693	1.00000

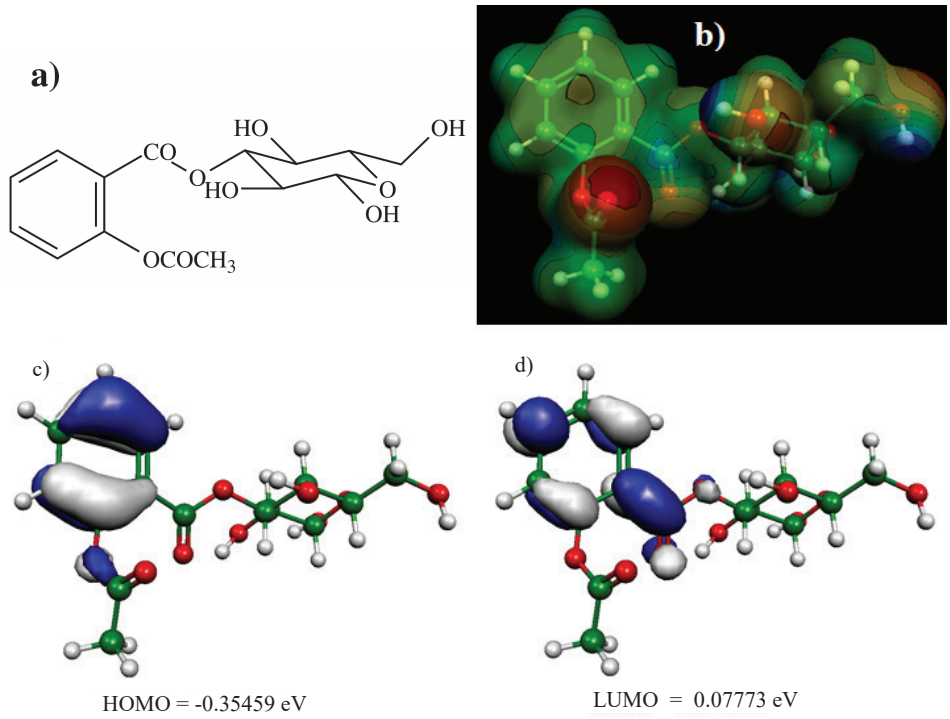


Figure 5. (a) The glucose-aspirin structure was visualized using ChemSketch 12.00 program [23]. (b) Map of molecular electrostatic potential (MEP). ((c) and (d)) Orbital's energy Homo and Lumo. The MEP, Homo and Lumo were visualized and calculated using Hartree-Fock (HF) method and HF/6-31G** basis set by the Molekel program [24].

The anticancer activity in vitro was much stronger for aspirin-glucose compared to aspirin in cancer cell lines. Therefore, further studies are needed among them we can mention the use of maps of electrostatic potential as an indicator of site of chemical reactivity to confirm this finding in a system *in vivo*. The high solubility of the conjugated glucose aids the development of a form of injection of aspirin.

This new derivative can be considered in the future a "superaspirin" that must pass safety tests for long periods before being placed on pharmacy shelves, but the time to replace aspirin and other non-steroidal anti-inflammatory drugs.

7. Final Considerations

The computational chemistry is considered as one of the greatest intellectual realizations of the twentieth century, and has been the conceptual basis that allows the understanding of chemistry of a way much deeper than that existing before age 20, epoch wherein were launched the bases of quantum theory. The impact of this theory in chemistry can be verified for its practical implications in various fields such as spectroscopy, electron microscopy, molecular modeling, among others.

The molecular modeling provides important information for the process of drug discovery, because it facilitates obtaining of specific molecular properties of a molecule that can influence the interaction with the receptor and biological activity.

The Hartree-Fock method provides a quantitative prediction of high quality for a wide variety of chemical and biological systems, but the calculations are time consuming and high computational cost. A resource commonly employed is optimizing geometry with a basis set simpler, and then perform calculations "Single Point" with a basis set most complete allowing the determination of energy and other molecular properties of a system, using a base of more sophisticated calculation.

The application of the Hartree-Fock method depends on the basis set and of system to be studied to obtain different molecular properties related to the biological activity of bioactive molecules. The computer calculations should describe the necessary characteristics related to the experimental data of the molecule or set of molecules under study, for that molecular modeling can be represented of effective and/or efficient form. However, these descriptors are not completely universal, because they are dependent on the structures and systems studied. Even though it is based on a minimum of energy, the calculated descriptors have values very close to their respective empirical values and indicate trends electronic systems under study. The aspects highlighted in this work are evident, therefore, the quantum-chemical descriptors have a wide range of applications in SAR, QSAR and QSPR studies, as well as in many areas of integration of fundamental knowledge of Organic Chemistry, Biochemistry, Molecular Biology, Pharmacology and Pharmaceutical Chemistry.

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11

Effect of Petroleum Products on Soil Catalase and Dehydrogenase Activities

Fidelis Ifeakachuku Achuba*, Patrick Nwanze Okoh

Department of Biochemistry, Delta State University, Abraka, Nigeria

Abstract

The effect of refined petroleum products on the activities of selected enzymes (catalase and dehydrogenase) was studied. There was a significant decrease ($p < 0.01$) in catalase activity. Catalase activity was higher in diesel and engine-oil treated soil after twelve days relative to petrol and kerosene. These observations indicate that the enzyme activity is the order of petrol > kerosene > diesel > engine oil. However, a significant increase ($p < 0.01$) was observed in dehydrogenase activity after twelve days relative to control values. Although, the refined petroleum products caused a similar pattern in the alteration of soil dehydrogenase activity, as they affected catalase activities, the general results indicate that the toxic effect is in the order of kerosene > diesel > petrol > engine oil. On the whole the results reveal that refined petroleum products alter soil biochemistry.

Keywords

Catalase Activity, Dehydrogenase Activity, Kerosene, Diesel, Engine Oil, Petrol

1. Introduction

Petroleum compounds have multifarious industrial and domestic uses. They are extensively employed in the making of solvents, dry cleaning fluids quick-products, automobile fuel, household solvents, and lubricants, cosmetics, water proofing agents, cleaning agents, and specialty chemicals [1]. These activities have led to the widespread contamination of the environment. Oil spill is the major cause for the high influx of petroleum to the biosphere [2] [3]. However, other points of soil pollution with refinery products are petrol stations, car and tractor servicing parks and, seaport areas [4]. Other areas of concern are mining and distribution of petroleum-based products [5] [6]. Besides, heavier use of machinery in agriculture together with unsatisfactory care while disposing of old or used petroleum products leads to considerable pollution of the natural environment [7].

*Corresponding author.

The soil is a key component of natural ecosystem because environmental sustainability depends largely on sustainable soil ecosystem [8]. When soil is polluted, the physiochemical properties are affected which may decrease its productive potentials [9]-[12]. In Nigeria, most of the terrestrial ecosystem and shoreline in oil-producing areas are important agricultural land under cultivation [13]. Any contact with petroleum and/or refined petroleum products causes damage to soil conditions of these agricultural lands, which culminates in loss of soil fertility [11] [12] [14]-[16]. Soil enzymes are important biotic components which are responsible for soil biochemical reactions [17]. The effects of petroleum hydrocarbon on soil enzyme activities are well documented [18]-[21].

Catalase activity, alongside with the dehydrogenase activities, gives information on the microbial activities in soil. Both catalase and dehydrogenase activity are very sensitive to heavy metal pollution [22]-[24]. Their values can therefore be used as a simple toxicity testing tool [25]. The aim of this study was to determine the effect of petroleum products (kerosene, diesel, engine oil and petrol in soil on soil catalase and dehydogenase activities.

2. Materials and Methods

Refined petroleum products of known physical properties were obtained from Warri Refining and Petrochemical Company, Warri, Nigeria. The soil (sand 84%, silt 5.0%, clay 0.4% and organic matter 0.6%, pH 6.1) was obtained from a fallow land in Delta State University, Abraka. Some of the chemical properties of the soil are listed in **Table 1**.

Soil (1600 g) was taken in each of small size planting bags (1178.3 cm^3, 15 cm deep) and divided into six groups of five replicates. Groups 1 to 5 contained 0.1%, 0.25%, 0.5%, 1.0% and 2.0% (v/w) respectively of each of the petroleum products while group six served as control (0.0%). To the first bag, 1.0 ml of kerosene, corresponding to 0.1%, was added. The petroleum products treated soil samples were mixed vigorously with the hand to obtain homogeneity of the mixture. The procedure was repeated for 0.25%, 0.5%, 1.0%, 1.5% and 2.0%. Each treatment, including control, was replicated five times.

2.1. Preparation of Extract and Assay of Catalase Activity

One hundred ml of phosphate buffer, pH 7.4, was added to 10 g of soil and stirred vigorously. The soil suspension was filtered using cheesecloth. The filtrate was centrifuged at maximum speed of 7000 g for 10 min to obtain supernatant (S1). Catalase activity was determined as described by Rani *et al.* [26]. Catalase breaks down hydrogen peroxide to give oxygen that oxidises potassium dichromate. The oxidation of chromate gives a chromophore that absorbs maximally at 610 nm. The enzyme extract (0.5 ml) was added to the reaction mixture containing 1 ml of 0.05 M phosphate buffer (pH 7.5), 0.5 ml of 0.2 MH_2O_2, 0.4 ml H_2O and incubated for different time period t_1, t_2 and t_3 for 1 minute, 2 minutes and 3 minutes respectively. The reaction was terminated after each time interval by the addition of 2 ml of acid reagent (dichromate/acetic acid mixture) which was prepared by mixing 5% potassium dichromate with glacial acetic acid (1:3 by volume). To the control, the enzyme was added after the addition of acid reagent. All the tubes were heated for 10minutes in boiling water and the absorbance was read at 610 nm. Catalase activity was expressed in terms of moles of H_2O_2 consumed/min.

Table 1. Physicochemical properties of test soil.

Parameters	Value
pH	6.09
Total organic carbon, %	2.90
Phosphorus, mg/kg	<0.01
Nitrogen, mg/kg	8.47
Nitrate, mg/kg	9.86
Cation exchange capacity, meq/100g	0.74
Sodium, mg/kg	9.06
Potassium, mg/kg	6.72
Calcium, mg/kg	2.98
Magnesium, mg/kg	0.31

2.2. Assay of Soil Dehydrogenase Activity

Dehydrogenase activity was determined using the method described by Tabatabai [27]. Dehydrogenases convert 2, 3, 5-triphenyl tetrazolium chloride to formazan. The asbsorbance of formazan was read spectrophotometrically at 485 nm. Sieved soil (1 gm) was placed in test tubes (15 × 100 mm), mixed with 1 ml of 3% aqueous (w/v) 2, 3, 5-triphenyl tetrazolium chloride and stirred with a glass rod. After 96 h of incubation (27˚C) 10 ml of ethanol was added to each test tube and the suspension was vortexed for 30 s. The tubes were then incubated for 1 h to allow suspended soil to settle. The resulting supernatant (5 ml) was carefully transferred to clean test tubes using Pasteur pipettes. Absorbance was read spectrophotometrically at 485 nm. Extinction coefficient of 15433 Mol/cm [28] was used for evaluating the concentration of formazan formed.

2.3. Statistical Analysis

The results were expressed as mean + SEM. All results were compared with respect to the control. Comparisons between the test and control were made by using analysis of variance (ANOVA). Differences at $p < 0.01$ were considered as significant.

3. Results

The results of the effect of refined petroleum products on soil catalase activites after four , eight and twelve days soil treatment are presented in **Figure 1** The activities of catalase in soil samples were decreased significantly ($p < 0.01$) after four days of the treatment of soil samples with kerosene, diesel, engine oil and petrol. At the highest concentrations petrol decreased catalase activity significantly ($p < 0.01$) more than did engine oil, and engine oil decreased catalase significantly ($p < 0.01$) activity more than did either kerosene or diesel. After eight days of treatment of soil with peroleum produts, kerosene treatment of soil resulted in significant ($p < 0.01$) decrease of catalase activity at 2% concentration compared with control. Diesel treatment of soil gave rise to a significant ($p < 0.01$) decrease of catalase activity at 1% and 2% concentrations. However, engine oil treatment of soil resulted in significant ($p < 0.01$) decrease in catalase activity in all the concentrations tested (0.25% - 2%). Finally, petrol treatment of soil brought about a significant increase of soil catalase activity at 0.25%, 0.5%, 1.5% and 2% compared with control. Comparing the activities of soil catalase in kerosene, diesel, engine oil and petrol treated soil; it was found to be significantly lower in all the tested concentrations in engine oil treatment of soil except at 0.25% concentration than the other petroleum products. But petrol treatment resulted in significantly higher catalase activity at 0.25% and 0.5% concentrations, than in other petroleum products treated soils

The activities of catalase after twelve days following the treatment of soil with kerosene significantly ($p < 0.01$) decreased catalase activity at a concentration of 0.25% but not at higher concentrations. Diesel treatment of soil resulted in decrease in catalase activity that was significant ($p < 0.01$) only at 0.25% level of contamination. Engine oil treatment of soil resulted in significant ($p < 0.01$) increase of catalase activity from 0.5% up to 1.5%, thereafter, an additional increase in concentration brought about a significant ($p < 0.01$) decrease of catalase activity relative to the control. Petrol treatment of soil resulted in a significant ($p < 0.01$) decrease of catalase activity only at 0.25%, 1% and 1.5% concentrations compared with control. When the activities of soil catalase in petrol, kerosene, and diesel and engine oil treatment of soil are compared; it was found to be significantly higher in engine oil treatment of soil at 1% and 1.5% concentrations than the other petroleum products. However, catalase activity was significantly lower in petrol treatment of soil at 1% concentration than the other petroleum products.

The activities of soil dehydrogenase after four days following the treatment of soil samples with kerosene, diesel, engine oil and petrol are presented in **Figure 2**. Kerosene treatment of soil resulted in significant increase of soil dehydrogenase activity from 0.5% up to 2%. Similarly, diesel treatment of soil led to a significant increase of soil dehydrogenase activity at 0.5% up to 2%. Engine oil and petrol treated soils had an all round significant ($p < 0.01$) increase of soil dehydrogenase activity in all the concentrations tested compared with control. When the activities of soil dehydrogenase in engine oil treatment of soil were compared with other petroleum products treated soil samples, they were found to be significantly ($p < 0.01$) higher at 0.25%, 0.5%, 1.5% and 2% concentrations over other petroleum products. Petrol treatment of soil recorded a significantly higher activity of soil dehydrogenase only at 1% than other petroleum products. The activities of soil dehydrogenase after eight days following treatment of soil samples with kerosene, diesel, engine oil and petrol are shown in **Figure 2**. In

all the concentrations tested, all petroleum products, treated soil samples resulted in significant increase ($p < 0.01$) of soil dehydrogenase activity compared to control. When the activities of soil dehydrogenase in soils treated with kerosene, diesel, engine oil and petrol are compared, it was found to be significantly higher in engine oil at all levels of concentrations than the other petroleum products. The activities of soil dehydrogenase after twelve days of following treatment of soil with kerosene, diesel, engine oil and petrol are shown in **Figure 6**. After twelve days, kerosene treated soil resulted in significant increase ($p < 0.01$) of dehydrogenase activity at 1%, 1.5% and 2% levels of soil contamination. Diesel treatment of soil in all the concentrations tested, gave rise to significant increase ($p < 0.01$) of dehydrogenase activity from 1% to 2%, while, engine oil and petrol treatment of soil samples resulted in significant ($p < 0.01$) increase of dehydrogenase activity at 0.5% to 2% concentrations compared to the control. when the dehydrogenase activities of soils treated with kerosene, diesel, engine oil and petrol were compared, the activities were found to be to almost the same at each level of soil contamination except that of kerosene, at 2%, which was significantly lower than the other petroleum products. On the whole, the activities of soil dehydrogenase, in all the concentrations were significantly ($p < 0.01$) higher compared to control.

4. Discussion

The refined petroleum products altered soil catalase activity. The activity of the enzyme decreased after four days of treatment of soil with petroleum products (**Figure 1**), increased by the eight day (**Figure 2**) at lower

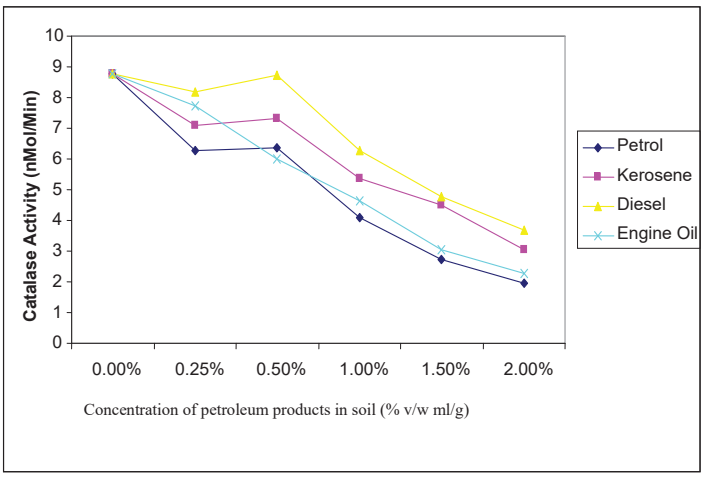


Figure 1. Dependence of catalase activity on concentration of four petroleum products in soil after four days.

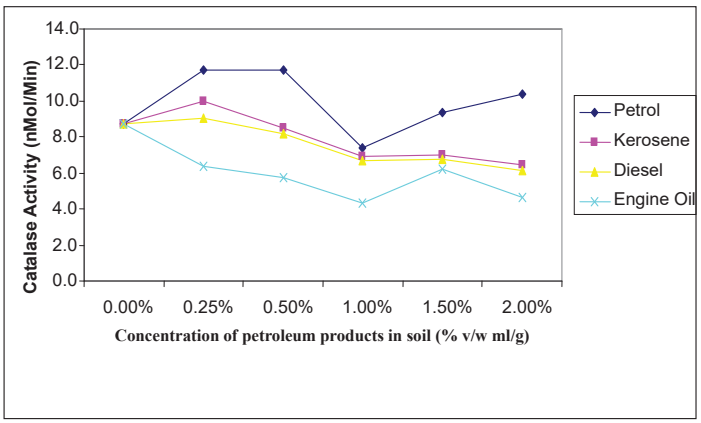


Figure 2. Dependence of catalase activity on concentration of four petroleum products in soil after eight days.

concentrations of kerosene, diesel and petrol in soil and then started to decrease after twelve days (**Figure 3**) of treatment of soil. Petroleum products, when present in soil, creates an unsatisfactory condition for soil organisms, mainly due to poor aeration, immobilization of soil nutrients and lowering of soil pH which culminates in petroleum mediated reduction in the number of hydrocarbon degrading microorganisms [11] [15] [17] [19] [29]. This may be the basis for decrease in catalase activity after four days of treatment. Decrease in catalase activity when soil is exposed to petroleum was previously reported [19]. However, the increase in the activity of the enzyme after eight days (**Figure 2**) could be predicated on increased microbial activity towards biodegradation of available petroleum hydrocarbon. This might explain why the activity of the enzyme started to decrease after twelve days of incubation with petrol and kerosene (**Figure 3**). Earlier reports indicated that there is a decrease in catalase activity after biodegradation has decreased [30]-[32]. However, the enzyme activity was higher in diesel and engine oil treated soil samples after twelve days relative to petrol and kerosene. These observations indicate that biodegradation was in the order of petrol > kerosene > diesel > engine oil.

Earlier reports indicated that soil polluted with petroleum hydrocarbon experienced increased dehydrogenase activity [18] [19]. The result of the current investigation agrees with this fact. The activity of the enzyme increased after four (**Figure 4**) and eight days of post treatment (**Figure 5**) with the four refined petroleum products (petrol, kerosene, and diesel and engine oil). However, the enzyme activity decreased after twelve days (**Figure 6**) in all the soil treated with the four refined petroleum products. The increase in enzyme activity has been attributed to the involvement of certain microorganisms in the metabolism of hydrocarbons whereas; the

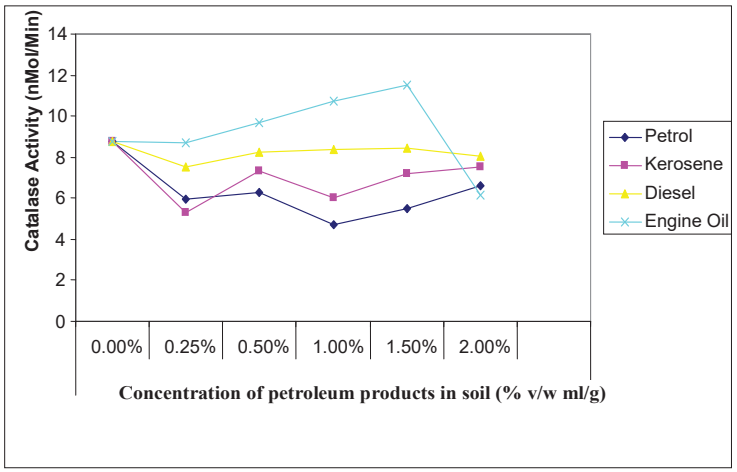


Figure 3. Dependence of catalase activity on concentration of four petroleum products in soil after twelve days.

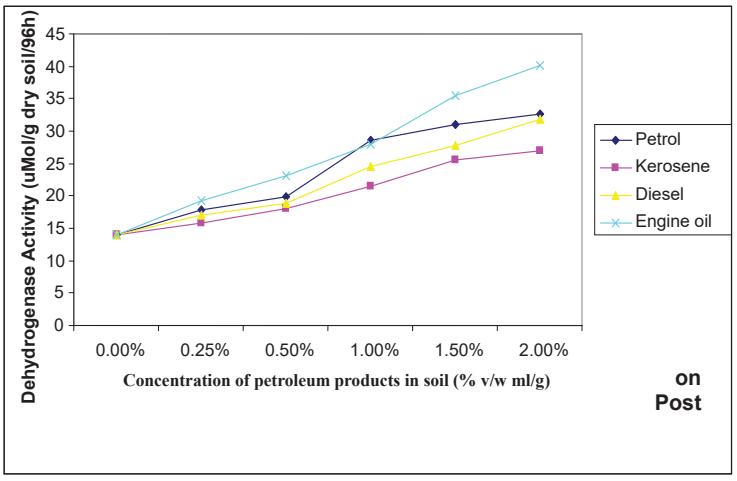


Figure 4. Dependence of dehydrogenase activity on concentration of four petroleum products in soil after four days.

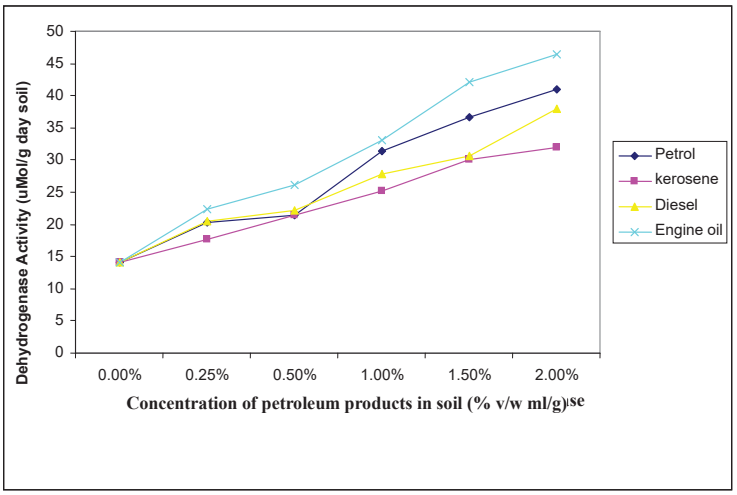


Figure 5. Dependence of dehydrogenase activity on concentration of four petroleum products in soil after eight days.

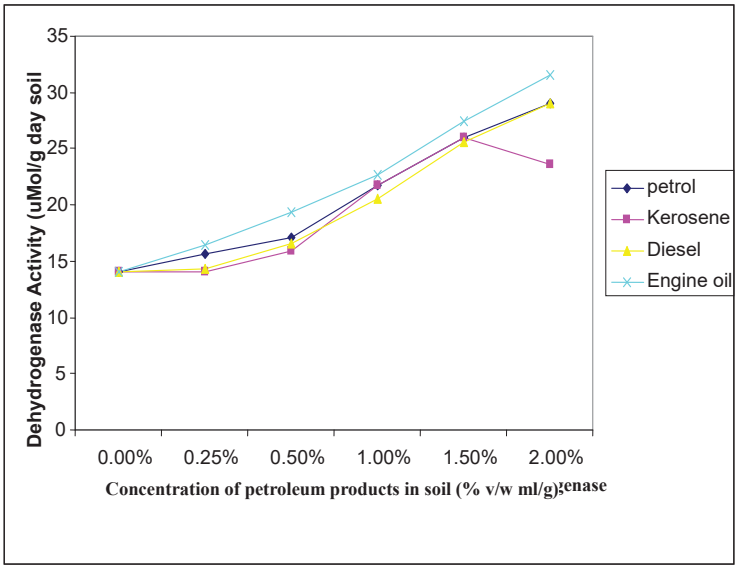


Figure 6. Dependence of dehydrogenase activity on concentration of four petroleum products in soil after twelve days.

decrease in the enzyme activity after twelve days of incubation could be due to decease in petroleum content of the soil. Decrease in soil dehydrogenase activity after biodegradation of petroleum has been reported [33] [34]. Although, the refined petroleum products caused a similar pattern, as they affect catalase activity, in the alteration of soil dehydrogenase activity, the general results seem to reveal that the toxicity effect is in the order of kerosene > diesel > petrol > engine oil. The toxicity of kerosene and diesel was reported earlier [20] [35].

5. Conclusion

In conclusion, it is pertinent to say that kerosene is more toxic to soil microorganisms, by decreasing the soil enzyme activities, in short term. However, engine oil stayed longer in the soil compared to the other three refined petroleum products, as reflected by sustained dehydrogenase after four days of post treatment.

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12

Toxicological Evaluation of Dietary Garlic (*Allium sativum*) Powder in European Sea Bass *Dicentrarchuslabrax* Juveniles*

Latife Ceyda Irkin[1], Murat Yigit[1], Sevdan Yilmaz[1], Masashi Maita[2#]

[1]Department of Aquaculture, Faculty of Marine Sciences and Technology, Canakkale Onsekiz Mart University, Canakkale, Turkey

[2]Graduate School of Marine Science and Technology, Tokyo University of Marine Sciences and Technology, Course of Applied Biosciences, Konan 4-5-7, Minato-ku, Tokyo, Japan

Abstract

The effects of dietary garlic (*Allium sativum*) powder as a feed additive on hematological and biochemical health characteristics of European Sea bass *Dicentrarchus labrax* juveniles were studied. Experimental fish were fed diets supplemented with garlic powder at 0 (control), 2%, 4%, or 6% levels for a period of 60 days. Results showed that the red blood cell count (RBC), hemoglobin (Hb) concentration, hematocrit (%), and mean corpuscular Hb in fish fed garlic powder diets at dietary inclusion levels of 4% and 6%, were significantly lower than the control values. Serum glucose was significantly lower in Sea bass that were fed garlic powder diets (4% and 6%) compared to the control group. Serum triglyceride and globulin levels in fish fed a 4% garlic powder diet were significantly higher than the control values, whereas these two variables in the 2% and 6% garlic treatments were similar to the control values. The cholesterol levels in the 2% and 6% treatment groups were lower than the value recorded for the control group. As a result, it is suggested that garlic powder supplementation in diets for Sea bass juveniles should not exceed 2%. The present study is the first attempt to examine the effects of dietary garlic powder on the hematological and biochemical status in Sea bass juveniles.

Keywords

Dietary Garlic, *Allium sativum*, European Sea Bass, *Dicentrarchus labrax*, Blood Biochemistry, Toxicological Evaluation

1. Introduction

There is an increasing need to understand the roles of phyto-additives in aquaculture. To date, a variety of herbs and spices have been successfully used in fish culture as growth promoters and immune-stimulants.

Garlic (*A. sativum*), an herb found virtually throughout the world, has been used in humans to treat rheumatoid arthritis, the common cold, diabetes, malaria, and tuberculosis for over 4000 years [1]. Furthermore, garlic has been reported to have antioxidant, antimicrobial, antifungal, antiviral and antiparasitic effects, and to improve the immune system [2].

Earlier studies have reported that garlic, as a feed additive in fish feed, may stimulate growth, improve antioxidant status, and enhance immunological, hematological and serum biochemical parameters [3]-[8]. The findings of previous studies to determine the optimum dietary inclusion levels varied depending on the dose used (0.05% to 4%), but the efficacy of different doses, particularly at high doses, appeared to be worse based on investigations of toxicology. Overall, the results suggested that high concentrations of phyto-additives may result in negative effects on fish health [9].

Hematological and biochemical variables are known as the most significant physiological indicators of fish health, stress, and welfare [9]-[11]. Previous studies reported that garlic was associated with a reduction in serum GLU in fish [3] [8] [12]. Garlic contains an odorless sulfur-containing compound known as S-allyl-cysteine sulfoxide, which stimulates hypoglycemic activity [13]. Several studies have concentrated on the use of phyto-additives, such as *Thymus vulgaris*, *Rosmarinusofficinalis* and *Trigonellafoenumgraecum* powders [14], garlic and ginger oils [9], and carvacrol [15] to improve immune function, hematological and serum biochemical status, growth and feed utilization or to control bacterial infection in European Sea bass. However, few studies have evaluated the dietary supplementation of garlic powder in terms of its effects on hematological and biochemical blood variables of European Sea bass. Thus, the objective of the present study was to evaluate the toxicological effects of garlic powder in European Sea bass in order to effectively and safely use garlic powder in aqua-diets.

2. Methods

2.1. Fish and Experimental Conditions

A total of 144 European Sea bass juveniles (mean weight ± SD. 10.60 ± 0.18 g) were obtained from a local marine fish farm (Ida Gıda) in Canakkale, Turkey. Fish were kept at the Marine Fish Research and Development Center of the Faculty of Marine Sciences and Technology at Canakkale Onsekiz Mart University (Dardanos-Canakkale, Turkey).

Water quality characteristics (mean ± SD) during the experiment were as follows: temperature was 24˚C ± 1˚C, dissolved oxygen concentration was 7.2 ± 0.2 mg/L, pH was 8.3 ± 0.2, salinity was 27.3 ± 0.1‰, and conductivity was 48.1 ± 0.5 mS/cm. The experiments were performed in accordance with the fish research guidelines from the animal ethics committees at Canakkale Onsekiz Mart University, Canakkale-Turkey.

2.2. Experimental Herbs and Diets

Garlic powder (Kotanyi, GmbH, Wolkersdorf-Austria) was added to the feed at increasing levels of 2%, 4% and 6% (g garlic powder/100g diet). Additionally, a control group was fed a diet without garlic powder supplementation. Four isoenergetic (gross energy, 18.8 ± 0.38 kJ/g diet) and isonitrogenous (protein content, 43.2% ± 0.8%) diets were formulated using commercial ingredients (**Table 1**). All ingredients were mixed in a mixer and pressed through a 2-mm die in a pelleting machine. The pellets were dried in a drying cabinet (40˚C) until moisture dropped to approximately 10%, and stored in bags in a freezer at −20˚C until use.

2.3. Experimental Design and Feeding Trials

A total of 144 fish were randomly distributed into recirculating aquariums each with a volume of 84-L (12 fish/aquarium). Experimental fish were acclimatized to the new conditions for 15 days. The fish were fed a commercial diet twice a day during the adaptation period. The experiment was designed in triplicate for each diet. During the feeding trial, fish were fed each experimental diet twice a day to satiation. The total amount of feed consumption was recorded daily. The recirculating system used in the experiment consisted of a sump, filters, a protein skimmer, and a biofilter containing bio-balls. During the experiment, water was exchanged daily at a rate

Table 1. Percentage ingredients (g/100g) of the experimental control and garlic powder supplementation diets fed to sea bass.

Ingredients	Control	GP2	GP4	GP6
Fish Meal	57.5	57.3	57.1	56.9
SBM	20.0	20.0	20.0	20.0
Fish Oil	10.6	10.6	10.6	10.6
b-Corn Starch	8.9	7.1	5.3	3.5
Vit-Min Premix	3.0	3.0	3.0	3.0
Garlic powder	0.0	2.0	4.0	6.0

C = controldiet, GP2 = 2% garlicpowderdiet, GP4 = 4% garlicpowderdiet, GP6 = 6% garlicpowderdiet; Fish Meal: Anchovy, Black Sea origin; SBM: Defatted soybean meal; Fish Oil: Anchovy, Black Sea origin; b-Corn starch: KENTON Corn Starch, Istanbul-Turkey; Garlic powder: KOTANYI GmbH, Wolkersdorf-Austria (Granulated garlic; Energy: 1.498 kJ·100g^{-1}, Protein 15.9 g·100g^{-1}; Carbohydrate 71.1 g·100g^{-1}, Lipid 0.3 g·100g^{-1}).

of approximately 10% of the total volume.

2.4. Blood Collection

At the end of the experiment (60 days), 12 fish per treatment group (4 fish/aquarium) were randomly selected and used for the hematological and serum biochemical analysis. Fish were anesthetized with clove oil at 20 mg/L [16]. Blood samples from fish were collected from the caudal vein by using a syringe, and added to tubes containing EDTA (BD Microtainer, UK). Blood samples were then subjected to hematological analysis. Blood serum was separated by centrifugation (2142 × g for 10 min) in plastic biochemistry tubes (Kima-vacutest, Italy) and stored at −20˚C until use in serum biochemical analysis [17].

2.5. Hematological Analysis

Red blood cell (RBC) count ($\times 10^6$ per mm^3), hematocrit (Hct, %), and hemoglobin (Hb) concentrations (g/dL) were determined by using the method of Blaxhall and Daisley [10]. The RBC count was obtained with a Thoma hemocytometer using Dacie's diluting fluid. The Hct value was determined using a capillary Hct tube. The Hb concentration was determined by spectrophotometry (540 nm) via the cyanmethemoglobin method. Mean corpuscular volume (MCV), mean corpuscular Hb (MCH), and mean corpuscular Hb concentration (MCHC) were calculated with the following formulae [18]:

$$\mathrm{MCV}\left(\mu\mathrm{m}^3\right)=\left[\left(\mathrm{Hct},\%\right)\times 10\right]\Big/\left(\mathrm{RBC},\times 10^6 \text{ per } \mathrm{mm}^3\right),$$

$$\mathrm{MCH}\left(\mathrm{pg}\right)=\left[\left(\mathrm{Hb},\mathrm{g/dL}\right)\times 10\right]\Big/\left(\mathrm{RBC},\times 10^6 \text{ per } \mathrm{mm}^3\right),$$

and

$$\mathrm{MCHC}\left(\%\right)=\left[\left(\mathrm{Hb},\mathrm{g/dL}\right)\times 100\right]\Big/\left(\mathrm{Hct},\%\right).$$

2.6. Biochemical Analysis

Biochemical indices in serum, including glucose (GLU), triglyceride (TG), cholesterol (CHOL), total protein (TP), and albumin (ALB) were determined using bioanalytic test kits (Bioanalytic Diagnostic Industry Co.) and were measured with an Optizen POP QX spectrophotometer (Mecasys Co. Ltd., Daejeon, Korea). The serum albumin/globulin ratio (A/G) was also calculated from the biochemical results as the ratio of albumin/(total protein-albumin).

2.7. Statistics

Each value was expressed as mean ± SE for each of the measured variables. The statistical significance ($P < 0.05$) of growth parameters, hematological and serum biochemical variables were tested using one-way ANOVA followed by a Tukey multi-comparison test with SPSS 17.0 packaged software.

3. Results

3.1. Feed Intake and Garlic Powder Consumption

During the experiment, survival rate was 100% in all treatment groups, showing that all fish were acclimated to experimental conditions and test diets. Overall, the highest feed intake was recorded for fish in the control group fed diets without garlic powder and for fish in the GP4 treatment group which were fed on diets with 4% garlic powder, while the lowest feed consumption was observed for fish in the GP6 treatment group, which received a diet containing 6% garlic powder. Feed intake of fish in the GP4 treatment group was similar to those in the control group, and no significant difference ($p > 0.05$) was recorded between the GP4 and control groups. However, when dietary garlic powder was increased from 4% to 6%, feed consumption of fish in the GP6 treatment group significantly decreased ($p < 0.05$), compared to those in the control, GP2 and the GP4 treatment groups. Garlic powder consumption in the experimental groups significantly increased ($p < 0.05$) with the increase in dietary garlic powder levels from 2% to 6%. The highest consumption of garlic powder was recorded for fish in the GP6 diet group, while the lowest consumption was found in the GP2 treatment group (**Table 2**).

3.2. Hematological Variables

The effects of dietary garlic powder on Sea bass hematological variables are presented in **Table 3**. The RBC count, Hb concentration, Hct, MCH, and MCHC in the GP2 treatment group did not vary significantly from the values observed for the control group ($P > 0.05$). The MCV and MCHC levels in the GP6 treatment groups were lower than the levels recorded in the control group ($P < 0.05$). The RBC count, Hb concentration, Hct, and MCH of fish in the GP4 and GP6 treatments groups were significantly lower than the control values ($P < 0.05$).

3.3. Blood Variables

The effects of dietary garlic powder on Sea bass biochemical variables are given in **Table 4**. Relative to the value

Table 2. Total feed intake (g/fish) and garlic powder consumption (g/fish) of sea bass juveniles during a 60-day feeding period.

	Control	GP2	GP4	GP6
TFI	18.504 ± 1.107^{c}	16.656 ± 0.172^{b}	18.289 ± 0.837^{c}	14.867 ± 0.446^{a}
GPC	0.00 ± 0.00^{a}	0.333 ± 0.003^{b}	0.732 ± 0.033^{c}	0.892 ± 0.027^{d}

Values with different superscripts in the same row are significantly different at $p < 0.05$ (mean ± SD of triplicate groups); (One way ANOVA and Duncan's multiple range test, $P < 0.05$); TFI (total feed intake, g/fish) = (total feed intake (g)/number of fish); GPC (garlic powder consumption, g/fish) = (total feed intake (g) × percent of garlic powder)/number of fish.

Table 3. Hematological variables (mean ± SD; $n = 12$ fish/group) in sea bass fed diets containing different conentrations of garlic powder.

Variable	Control	GP2	GP4	GP6
RBC ($\times 10^6$ per mm^3)	3.20 ± 0.03^{a}	3.15 ± 0.08^{a}	2.84 ± 0.07^{b}	2.78 ± 0.04^{b}
Hb (g 100 ml^{-1})	6.08 ± 0.18^{a}	5.63 ± 0.29^{a}	4.73 ± 0.11^{b}	4.42 ± 0.12^{b}
Hct (%)	32.38 ± 0.38^{a}	32.00 ± 0.73^{a}	28.63 ± 0.78^{b}	27.63 ± 0.46^{b}
MCV (μm^3)	101.25 ± 0.92^{a}	101.65 ± 0.39^{ab}	100.77 ± 0.37^{ab}	99.41 ± 0.31^{b}
MCH (pg)	19.07 ± 0.70^{a}	17.84 ± 0.72^{ab}	16.70 ± 0.52^{b}	15.90 ± 0.42^{b}
MCHC (%)	18.84 ± 0.71^{a}	17.56 ± 0.73^{a}	16.58 ± 0.55^{ab}	16.00 ± 0.43^{b}

Variables are red blood cell count (RBC), hemoglobin (Hb) concentration, hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular Hb (MCH), and mean corpuscular Hb concentration (MCHC). Superscript letters indicate inter group statistical differences. Means ± standard deviation of data for triplicate groups with different superscripts in the same row differ significantly (One way ANOVA and Duncan's multiple range test, $P < 0.05$).

Table 4. Serum biochemical variables (mean ± SD; n = 12 fish/group) in sea bass fed diets containing different concentrations of garlic powder.

Variable	Control	GP2	GP4	GP6
GLU (mg 100 ml^{-1})	176.72 ± 3.83^{a}	153.67 ± 6.45^{a}	105.02 ± 8.43^{b}	88.10 ± 5.68^{b}
TG (mg 100 ml^{-1})	94.08 ± 5.85^{a}	112.50 ± 7.45^{a}	64.23 ± 6.43^{b}	95.41 ± 2.16^{a}
CHOL (mg 100 ml^{-1})	138.20 ± 6.05^{a}	72.30 ± 6.43^{b}	121.70 ± 10.78^{a}	81.00 ± 4.70^{b}
TP (g 100 ml^{-1})	9.84 ± 0.30^{a}	9.72 ± 0.41^{a}	9.53 ± 0.40^{a}	11.27 ± 0.74^{a}
ALB (g 100 ml^{-1})	3.13 ± 0.18^{c}	4.15 ± 0.09^{b}	5.18 ± 0.31^{a}	5.84 ± 0.12^{a}
GLO (g 100 ml^{-1})	6.72 ± 0.35^{a}	5.57 ± 0.45^{ab}	4.35 ± 0.26^{b}	5.43 ± 0.72^{ab}
A/G Ratio	0.48 ± 0.05^{b}	0.79 ± 0.08^{a}	1.22 ± 0.10^{a}	1.24 ± 0.19^{a}

Variables are glucose (GLU), triglyceride (TRI), cholesterol (COL), total protein (TP), albumin (ALB), globulin (GLO), and albumin/globulin ratio (A/G). Super script letters indicate inter group statistical differences. Means ± standard deviation of data for triplicate groups with different superscripts in the same row differ significantly (One way ANOVA and Duncan's multiple range test, $P < 0.05$).

for the control group, serum GLU was significantly lower ($P < 0.05$) in Sea bass in treatment groups GP4 and GP6 (**Table 4**). Serum TG and GLO levels in fish fed the GP4 diet were significantly lower than the control values ($P < 0.05$), whereas these two variables for the GP2 and GP6 treatment groups were the same as the control values ($P > 0.05$). The CHOL levels in the GP2 and GP6 treatment groups were lower than the level recorded in the control group ($P < 0.05$). The TP levels in Sea bass were not significantly affected by feeding with garlic powder diets ($P > 0.05$). Serum ALB and A/G ratios in fish fed the GP2, GP4 and GP6 diets were significantly higher than those of the control group ($P < 0.05$).

3.4. Biochemical Variables

The effects of dietary garlic powder on Sea bass biochemical variables are given in **Table 4**. Relative to the value for the control group, serum GLU was significantly lower ($P < 0.05$) in Sea bass in treatment groups GP4 and GP6 (**Table 4**). Serum TG and GLO levels in fish fed the GP4 diet were significantly lower than the control values ($P < 0.05$), whereas these two variables for the GP2 and GP6 treatment groups were the same as the control values ($P > 0.05$). The CHOL levels in the GP2 and GP6 treatment groups were lower than the level recorded in the control group ($P < 0.05$). The TP levels in Sea bass were not significantly affected by feeding with garlic powder diets ($P > 0.05$). Serum ALB and A/G ratios in fish fed the GP2, GP4 and GP6 diets were significantly higher than those of the control group ($P < 0.05$).

4. Discussion

Physiological stress indicators such as hematological and serum biochemical variables could be useful to evaluate the effects of phyto-additives in fish [9] [14] [19], but for the application of these findings in fish feed preparation, more detailed studies of the dietary effects are necessary to produce safe and effective diets.

Changes in hematological variables are important for the evaluation of the health status of organs [20]. In particular, anemia is an important trigger of increased sensitivity to infectious diseases [21]. The present study demonstrated that hematological variables (RBC, Hct, Hb and MCH) decreased in a dose dependent manner (GP4 and GP6). Earlier studies in which garlic was used as a supplement in fish feed reported that diets containing 0.05% to 3% garlic resulted in hematological improvements [3]-[5] [8]. However, there is no information about the effects of higher levels of dietary garlic (over 4%) on hematological variables in fish. In our study, dietary garlic powder at inclusion levels of 4% and 6% resulted in significant decreases in RBC, Hb, and Hct, leading to anemia in Sea bass. These parameters were also used for the assessment of anemia in fish [22] [23]. The anemia may be due to the inhibition of RBCs (erythropoiesis) and hemesynthesis and an increased rate of erythrocyte destruction in hematopoietic organs [24].

The present study demonstrated that the GLU level decreased with increasing doses of garlic powder. These results suggest that garlic powder will cause hypoglycemia in Sea bass juvenile. Reference [25] reported that

persistent hyperglycemia causes hepatomegaly and other health problems, which could negatively affect any growth promoting effects of hyperinsulinemia in fish. This hypothesis about hyperglycemia is also supported by the increased activity of serum AST and ALT enzymes, which are known as indicators of liver injury, in *Oncorhynchus mykiss* fed 40 mg/kg (4%) and 50 mg/kg (5%) garlic diets over a period of 8 weeks [7]. Although the influence of chronic hyperglycemia or hypoglycemia in fish is still unclear, hypoglycemia induced by garlic powder supplementation may be considered as a beneficial effect of garlic powder. Further investigations are needed to address this issue.

Major blood lipid components, such as triglycerides, phospholipids, and cholesterol are affected by diet and stress levels in fish [26]. In our study, the levels of serum TG in Sea bass decreased in the GP4 group. On the other hand, the levels of CHOL in theGP2 and GP6 groups were increased. Reference [27] also demonstrated that garlic supplementation resulted in an inhibition of the plasma cholesteryl ester transfer protein (CETP) activity, lowering the concentration of total cholesterol and triglycerides in blood. On the other hand, in this study, serum TG or CHOL levels showed fluctuations in response to garlic diets. The present findings seem to be consistent with other research, which found a similar and regular trend [7]. Serum cholesterol levels are affected by dietary cholesterol (exogenous cholesterol) and synthesized cholesterol in the liver (endogenous cholesterol) [21]. The feed consumption in theGP2 and GP6groups was significantly lower than that in the control and GP4 groups. These findings suggest that lowered serum CHOL would be affected by a decrease in exogenous cholesterol. If the decrease in serum TG observed in GP4 is due to the influence of garlic powder, it is expected that the levels of TG in the GP2 and GP6 groups would show significant decreases compared to the control. However, the levels of TG in GP2 and GP6 were similar to that of the control. In yellowtail, slight starvation caused an elevation of serum TG levels (Maita, unpublished data). It is therefore likely that the levels of TG observed in GP2 and GP6 resulted from both the garlic powder itself (lowered TG) and insufficient feed intake.

The serum TP, ALB and GLO contents are considered to reflect the health status of fish during the herb treatments [9]. In this study, the serum TP and GLO levels were not significantly affected by feeding garlic powder diets. However, serum ALB and A/G ratios in fish fed with all garlic diets were higher than the control and this result was dose-dependent. Therefore, it appears that the increase in serum ALB and A/G ratios were caused by garlic power supplementation. Similarly, [3] conducted an experiment with *Labeo rohita* fingerlings fed a basal diet containing 0%, 0.5% or 1.0% garlic powder for 60 days, and found that 0.5% garlic powder increased serum ALB and A/G ratio values compared to that of the fingerlings fed a control diet. In general, the serum ALB level indicates the status of dietary protein intake. When we consider the significant decrease of feed intake in GP2 and GP6, the dose-dependent increases in ALB observed in the present study seem to be contradictory. Based on the increased levels of ALB and A/G ratios of the fish reported here, the garlic powder seemed to exert a stronger effect than that of the decreased feed intake in this study.

It is well known that GLO concentrations are indisputably essential for a healthy immune system and for maintaining immune functions in fish blood [8]. An increase in serum GLO in fish with the addition of dietary garlic has also been reported in previous studies [4] [8]. In contrast, our study indicated that the blood GLO concentration was decreased in fish fed the experimental diet containing 4% garlic. Similar results have been reported by an earlier study [3], indicating that garlic powder supplementation at 0.5% decreased the serum GLO values in *L. rohita* fingerlings after 20 days of feeding. However, they found that the use of 0.5% garlic powder significantly increased serum GLO values compared to the fingerlings fed the control diet after 60 days of feeding. These fluctuations in GLO values are in agreement with the findings of an earlier study [4], who also reported a decrease and an increase in serum GLO contents in *O. mykiss* fingerlings after 14 days of feeding of oven-dried garlic bulbs at 0.05% and 0.5%, and 0.1% and 1.0%, respectively. Based on the findings in different fish species, it may be concluded that the dose and time of exposure to garlic may alter the effects of garlic on GLO levels in blood.

5. Conclusion

Various studies of dietary herbal supplements in different fish species have demonstrated hematological, serum biochemical or immunological enhancements. In the present study, results showed that the choice of herbs, their dose and time of application is very important for obtaining higher efficiency. High levels of garlic supplementation in diets of Sea bass may have negative hematological and biochemical consequences. Hence, it is suggested not to use more than 2% dietary garlic powder in juvenile European Sea bass diets. The present study is the

first attempt to examine the effects of dietary garlic powder on the hematological and biochemical status of Sea bass. Further studies are needed to determine the optimal application frequency for favorable garlic performance and to assess its impacts on infection.

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The Effects of Low Intensity Endurance Activity on Various Physiological Parameters and Exercise Induced Oxidative Stress in Dogs

Janice L. Huntingford[1], Corri B. Levine[2], Debbie J. Mustacich[3], Devin Corrigan[3], Robert L. Downey[4], Joseph J. Wakshlag[2*]

[1]Essex Animal Hospital, Essex, Canada

[2]Department of Clinical Sciences, Cornell University, Ithaca, NY, USA

[3]Linus Pauling Institute, Oregon State University, Corvallis, OR, USA

[4]Annamaet Petfood Company, Sellersville, PA, USA

Abstract

The study of canine athlete physiology has focused on endurance sled dog racing and high intensity short duration Greyhound racing, yet the number of dogs competing in low intensity endurance activities is rising due to the increased numbers of hunting and companion dog activities. There is little information on the physiological effect of longer duration low intensity endurance activities. We set out to evaluate the serum biochemistry, oxidative stress, and cortisol response before and after two consecutive days of exercise in ten healthy unconditioned male dogs. Exercise sessions consisted of 120 minutes on an exercise wheel at 11 km/hour on 2 consecutive days. Blood was collected at four time points: 24 hours pre-exercise (sample 1, Day 0, resting); 2 min post-exercise on days 1 and 2, (samples 2 and 3, respectively); and 20 hours post-exercise, collected on day 3(sample 4). Hematocrit, blood gases, serum chemistry, uric acid, cortisol, and F_2-isoprostanes were determined. Serum biochemistry and hematocrit suggested hemoconcentration, mild muscle damage and respiratory alkalosis during exercise, which was expected in the unconditioned canine athlete. In addition, plasma indices of oxidative damage (F_2-isoprostanes) increased, as did plasma uric acid (an endogenous antioxidant). Importantly, similar to human studies, plasma F_2-isoprostanes decreased 24 hours after exercise suggesting a protective effect of exercise. Serum cortisol concentrations were also markedly elevated at the end of exercise on both days suggesting that timing of sampling may play a role in interpreting cortisol results when looking at previous field studies.

*Corresponding author.

Keywords

Exercise, Cortisol, Isoprostane, Dehydration, Uric Acid

1. Introduction

The study of exercise physiology in canine athletes has focused primarily on the endurance sled dog and the sprinting Greyhound [1]-[4]. However, in the last 10 years, the number of dogs competing in other canine sports has increased significantly. These include the low intensity endurance activities (30% - 40% VO_2 max) typical amongst law enforcement, military, detection, search and rescue, recreational hunting dogs and companion dog "athletes". In addition, pet owners are also engaging in more physical activities with their canine companions including running, swimming, biking and skijoring (http://www.akc.org/about/annual_report.cfm). In many cases, unlike racing greyhounds and sled dogs, dogs participating in these low intensity endurance activities are not well conditioned and perform activities only on weekends or sporadically due to seasonality of the activities [5].

Presently, the only information on canine endurance exercise comes from the examination of the physiological extreme of the high intensity endurance activity of sled dog racing in highly conditioned athletes [2] [3] [6]. Depending on population and/or duration of activity, studies of physiological changes in sled dogs have suggested both hemoconcentration and hemodilution [3] [7] [8]. In addition, hydration status and kennel management have been shown to significantly affect hemodynamics in dogs within the same race [7].Considering all of these possible confounding variables present in many sled dog studies, it is not surprising that these studies have demonstrated significant inter-individual, as well as inter-study, variation in measures of exercise-induced muscle damage, *i.e.*, plasma creatine kinase (CK) and aspartate amino-transferase (AST) [3] [6].

Limited field studies have been conducted on agility dogs, hunting foxhounds, Labrador retrievers, search and rescue dogs, and scenting dogs [9]-[12], and the current literature contains little information on longer duration low intensity exercise in the unconditioned canine. In addition, similar to sled dog studies, field studies of low intensity endurance activities are difficult to standardize and the inherent variability in exercise intensity makes interpretation difficult and may lead to contradictory inter-study results. Thus, we were interested to utilize additional measures of exercise-induced stress during low intensity endurance exercise using a controlled field setting for the exercise sessions.

Exhaustive exercise, *i.e.*, human ultramarathon or endurance sled dog racing, increased production of free radicals (particularly oxygen-and nitrogen-centered radicals)leads to exercise-induced oxidative stress, including increased formation of F_2-isoprostanes [13] [14]. Four groups of F_2-isoprostane regioisomers (5-, 8-, 12-, and 15-series F_2-isoprostanes) can be formed during free-radical mediated oxidation of arachidonic acid. The 15-series F_2-isoprostanes (15R- and 15S-8-isoprostanes (8-isoPs))are the most extensively studied and are considered a biomarker of choice to assess *in vivo* oxidative stress, particularly when measured by either GC/MS or LC/MS[15] [16]. In studies using a commercial ELISA method for measurement of 8-isoPs, endurance sled dogs show 3- to 5-fold increase in plasma F_2-isoprostanes after running 100 km, while agility dogs during competition showed no increase in F_2-isoprostanes [12] [14]. These discrepancies may be genuine or may be the result of cross-reactivity to other compounds by the polyclonal antibody used in these commercial ELISA assays [15]. Importantly, 8-isoP levels have not been determined during exercise activities falling between these two extremes, *i.e.*, during the more common low intensity longer duration activities of the unconditioned canine companion athlete. Interestingly, in contrast to exhaustive exercise, moderate intensity exercise in humans results in an adaptive response whereby markers of oxidative stress are significantly increased after the initial bout of moderate exercise, but become progressively lower after repeated bouts of moderate exercise[17]-[19]. Similar studies in canines are unavailable.

Uric acid, a potent antioxidant, is suggested to prevent elevations in oxidative damage [14] [20]. Elevated serum uric acid during both short and long duration strenuous activities is associated with decreased exercise-induced oxidative damage [20]-[22]. In addition, supplementation of humans with uric acid prevented exercise-induced elevation of 8-isoPs [23]. To date, similar studies have not been undertaken during low intensity endurance activities in either conditioned or unconditioned dogs.

Similar to exercise-induced oxidative stress, measures of the exercise-induced physiological stress have not

been reported in dogs immediately following low intensity endurance activity. Physiologic stress is commonly assessed through serum cortisol as an indication of the activation of the adreno-hypothalamus axis. Cortisol has been shown to be elevated during an array of athletic and non-athletic canine activities [24]-[26], but the data varies with the activity. Sled dogs running between 10 and 120 minutes exhibited a marked rise in serum cortisol, while cortisol concentrations did not increase in agility dogs during short duration exercise [7] [11] [24]. These differences may be a reflection of the timing of sampling as well as variation in exercise, excitement and breed. To date, F_2-isoprostane and uric acid levels, as well as markers of muscle damage, hemodynamics and cortisol, have not been determined during low intensity endurance activities in unconditioned dogs.

The purpose of this study was to assess biochemical and physiological parameters in unconditioned dogs during repeated low intensity endurance activity (30% - 40% VO_2 max)in a controlled field environment by measuring clinical biomarkers of exercise-induced stress (serum chemistry and cortisol), as well as uric acid and plasma F_2-isoprostanes.

2. Materials and Methods

2.1. Dog Exercise Sessions

All procedures in this study were approved by the Cornell University institutional care and use committee and owner consent was acquired for the study.

Ten healthy, unconditioned, male mixed breed dogs from a single kennel between the ages of 6 - 9 years of age weighing between 24 and 27 kilograms were used in this study. All of the dogs in the study were fed a commercial kibble containing 26% crude protein and 16% crude fat (Annamaet Extra, Annamaet Pet food, Sellersville, PA).Blood was collected at four different time points: a pre-exercise resting sample 24 hours before the first exercise session (day 0); a post-run sample on day 1 within 2 minutes of cessation of exercise (day 1); a post-run sample on day 2 within 2 minutes of cessation of exercise (day 2); and a sample 24 hours after the cessation of the day 2 exercise session (day 4). Exercise consisted of all dogs running simultaneously on an exercise wheel as is typical for conditioning canine athletes (sled dogs and hunting dogs) at 11 km/hour for 120 minutes on two consecutive days between 7 and 9 am. This exercise regimen simulates a low intensity endurance exercise equivalent to approximately 30% - 40% of their maximal oxygen consumption [20]. At each half hour interval of exercise, the direction in which the dogs ran was changed. Immediately after exercise (within 60 seconds) venipuncture of the jugular vein was performed using a 20-gauge needle on each dog and 10 milliliters of blood was collected.1-ml of blood was transferred to a 1 ml lithium heparin tube, 5-ml to a coagulation tube and 4 ml to another lithium heparin tube. Serum and plasma were separated within 20 minutes of collection by centrifugation of the respective tubes for 6 minutes at 4000 × g, transferred to separate tubes, immediately frozen on dry ice, and placed in liquid nitrogen for transportation to the laboratory. After transportation to the respective laboratories the samples were stored at −80˚C until analysis.

2.2. Hematocrit

Heparinized hematocrit capillary tubes were filled for each dog at each time point and spun on a hematocrit centrifuge.

2.3. Serum Chemistry

Serum chemistry was determined using an Olympus AU5400 automated analyzer (Olympus America, Center Valley, PA, USA).

2.4. Blood Gas, Lactate, Uric Acid and Cortisol

Blood gas and serum lactate was determined using an iSTAT CG8+ cartridge per the manufacturer (Abbott Laboratories, Deerfield IL). The cartridge was filled with heparinized blood within 5 minutes of blood draw. Cortisol was determined utilizing a validated radioimmunoassay for dogs (Cornell University Diagnostic Laboratory, Ithaca, NY). Uric acid was determined utilizing a commercial ELISA kit per manufacturer's instructions (Cayman Chemical, Anna Arbor, MI).

2.5. Plasma F2-Isoprostanes

Plasma samples were shipped to the Linus Pauling Institute at Oregon State University for F_2-isoprostane analysis. Plasma 15-series F2-isoprostanes (15R- and 15S-8-isoprostanes, 8-isoPs) and 5-series F_2-isoprostane metabolites were extracted and measured by LC/MS as described by Taylor *et al.* [27]. Briefly, plasma was extracted by SPE as described [28] using a Strata-X SPE cartridge (500 mg/3 mL, Phenomenex, Torrance, CA). Eluted samples were dried under nitrogen and reconstituted with 200 µL of methanol containing 0.1% formic acid and stored at −80˚C until analysis. Samples were analyzed using an HPLC coupled to a triple-quadrupole mass spectrometer operated in negative mode. 5- and 15-series F_2-isoprostanes were quantified based on an internal standard [27].

2.6. Statistical Analysis

Data was analyzed by one-way ANOVA using Prism (Graphpad, La Jolla, CA). Any data that was not normally distributed based on Shapiro Wilks testing was transformed prior to analysis. Tukey's Post hoc tests were performed when overall time point effects were found to be significant. Comparisons between groups with p values < 0.05 were considered significant. All data for **Figures 1-3** (cortisol, uric acid and isoprostanes) are expressed at mean ± standard error of the mean.

3. Results

Unconditioned dogs participated in 2 low intensity endurance exercise sessions on 2 consecutive days (days 1 and 2). Measures of physiologic and oxidative stress were determined 24 h pre-exercise (day 0), 2 min post-exercise (days 1 and 2), and 20 h post-exercise (day 3).

3.1. Hematocrit and Serum Chemistry

Compared to pre-exercise levels serum total protein, albumin, sodium, and chloride, as well as hematocrit and total iron binding capacity, increased immediately following each low intensity endurance exercise session and returned to pre-exercise levels on day 3 ($p < 0.05$; **Table 1**). In contrast, immediately following the day 1 and day 2 low intensity endurance exercise sessions, phosphorus levels decreased to 65% and 60%, respectively, compared to pre-exercise levels ($p < 0.05$; **Table 1**). Phosphorus levels remained decreased 20 h post-exercise. Blood urea nitrogen (BUN) levels increased 6% following day 2 exercise and returned to pre-exercise resting levels on day 3 ($p < 0.05$). Glucose and potassium levels decreased to approximately 90% of pre-exercise levels following day 2 exercise, but returned to pre-exercise levels by day 3 ($p < 0.05$; **Table 1**). Blood calcium levels decreased 3% following the 2nd day of low intensity endurance exercise, compared to pre-exercise levels, and recovered by 20 h post-exercise ($p < 0.05$). Alkaline phosphatase levels was unaltered following the day 1 low intensity endurance exercise, but increased 25% following low intensity endurance exercise on day 2 and remained elevated 20 h post-exercise, compared to pre-exercise levels ($p < 0.05$; **Table 1**). Importantly, following low intensity endurance exercise on day 1, AST and CK levels increased 1.8- and 2.5-fold, respectively, compared to pre-exercise levels (28 ± 10 U/L and 96 ± 17 U/L, respectively). Following the 2nd low intensity endurance exercise session (day 2), AST and CK levels further increased to 2.4- and 4.6-fold pre-exercise levels ($p < 0.05$; **Table 1**). AST, but not CK, levels returned to pre-exercise levels 20 h post-exercise ($p < 0.05$). Finally, iron and iron saturation increased approximately 2-fold following each low intensity endurance exercise session, compared to pre-exercise levels ($p < 0.05$) and both indices remained elevated 20 h post-exercise. Cholesterol, creatinine, magnesium, and ALT levels were unchanged during this study.

3.2 Blood Gas and Lactate

Blood gas parameters were determined pre-exercise and immediately following low intensity endurance exercise on days 1 and 2. Base Excess (1.7- and 1.7-fold), pH (0.5% and 1.4%), and lactate (2.3- and 2.0-fold) levels increased following each exercise session compared to pre-exercise levels (**Table 2**, $p < 0.05$). Total CO_2, pCO_2,

Table 1. Hematological and serum chemistry parameters in dogs at rest on day 0, after exercise on day 1 and day 2, and on day 3 20 hrs post-exercise. Data are expressed as mean ± standard deviations. * = $p < 0.05$ compared to pre-exercise levels (resting).

Serum Chemistry	Day 0 (Rest)	Day 1 Ex.	Day 2 Ex.	Day 3 20 hr Post
Total protein (5.0 - 7.4 g/dL)	6.1 ± 0.3	6.5 ± 0.2*	6.4 ± 0.3*	6.1 ± 0.3
Albumin (2.7 - 4.4 g/dL)	3.7 ± 0.1	3.9 ± 0.1*	3.9 ± 0.1*	3.7 ± 0.2
Globulin (1.6 - 3.6 g/dL)	2.4 ± 0.3	2.6 ± 0.1	2.5 ± 0.2	2.4 ± 0.2
AST (SGOT) (15 - 66 U/L)	28 ± 10	51 ± 12*	68 ± 17*	42 ± 16
ALT (SGPT) (12 - 118 U/L)	90 ± 47	99 ± 48	107 ± 58	99 ± 50
Alkaline phos. (5 - 131 U/L)	52 ± 17	53 ± 18	65 ± 26*	70 ± 22*
Urea Nitrogen (6 - 31mg/dL)	15 ± 3	15 ± 2	16 ± 3*	12 ± 3
Creatinine (0.5 - 1.6 mg/dL)	0.9 ± 0.1	0.9 ± 0.1	1.0 ± 0.2	0.8 ± 0.1
Phosphorus (2.5 - 6.0 mg/dL)	4.4 ± 0.5	2.9 ± 0.8*	2.7 ± 0.8*	3.5 ± 0.5*
Glucose (70 - 138 mg/dL)	96 ± 6	93 ± 9	86 ± 9*	98 ± 10
Calcium (8.9 - 11.4 mg/dL)	10.2 ± 0.2	10.2 ± 0.2	9.9 ± 0.3*	10.1 ± 0.2
Magnesium (1.5 - 2.5 mEq/L)	1.7 ± 0.1	1.7 ± 0.1	1.7 ± 0.1	1.7 ± 0.1
Sodium (139 - 154 mEq/L)	148 ± 1	153 ± 1*	151 ± 3*	146 ± 2
Potassium (3.6 - 5.5 mEq/L)	4.9 ± 0.2	4.7 ± 0.1	4.5 ± 0.3*	4.9 ± 0.2
Chloride (102 - 120 mEq/L)	115 ± 2	118 ± 2*	117 ± 2*	114 ± 2
Cholesterol (92 - 324 mg/dL)	179 ± 37	186 ± 36	187 ± 37	186 ± 40
Creatine Kinase (59 - 895 U/L)	96 ± 17	216 ± 60*	440 ± 147*	196 ± 108*
Hematocrit % (40 - 56)	51 ± 3	55 ± 2*	55 ± 4*	52 ± 2
Iron (ug/dL)	98 ± 15	210 ± 39*	200 ± 41*	126 ± 20*
Iron Saturation%	31.7 ± 5.4	64.4 ± 13.9*	61.8 ± 11.3*	40.9 ± 8.5*
TIBC (ug/dL)	310 ± 36	328 ± 34*	327 ± 43*	311 ± 38

Table 2. Blood gas parameters in dogs at rest on day 0, after exercise on day 1 and day 2. Data are expressed as mean ± standard deviations. * = $p < 0.05$ compared to pre-exercise levels (resting).

	Day 1 Rest	Day 1 Exercise	Day 2 Exercise
Base Excess (−2 - +3 mmol/L)	−3 ± 1	−5 ± 2*	−5 ± 2*
pH (7.31 - 7.41)	7.40 ± 0.21	7.44 ± 0.15*	7.50 ± 0.10*
HCO_3 (23 - 28 mmol/L)	22 ± 2	19 ± 2*	19 ± 2*
TCO_2 (24 - 29 mmol/L)	23 ± 2	20 ± 2*	19 ± 2*
PCO (41 - 51 mmHg)	36 ± 4	29 ± 5*	26 ± 4*
Lactate (0.9 - 1.7 mmol/L)	0.7 ± 0.3	1.6 ± 0.9*	1.4 ± 1.8*

and bicarbonate levels decreased following each exercise session (**Table 2**, $p < 0.05$).

3.3. Cortisol and Uric Acid

Serum cortisol concentrations increased 5.6- and 5.1-fold following low intensity endurance exercise on days 1

and 2, compared to pre-exercise levels (1.9 ± 0.3 ug/mL, $p < 0.01$), but returned to pre-exercise levels by day 3 (**Figure 1**). Following the low intensity endurance exercise session on day 1 serum uric acid levels were increased more than 2-fold compared to pre-exercise levels (59.6 ± 5.2 µM vs. 26.9 ± 1.8 µM, $p < 0.01$) (**Figure 2**). Serum uric acid levels were also increased (47.7 ± 4.2 µM) following day 2 exercise, compared to pre-exercise levels ($p < 0.05$), but recovered by 20 h post-exercise (32.6 ± 2.8 µM) (**Figure 2**).

3.4. F_2-Isoprostanes

Due to the inter-individual variation (range 384 - 744 ng/mL 15-series F_2-isoprostane; 954 - 1893 ng/mL 15 series-F_2-isoprostanes) for pre-exercise F_2-isoprostane levels, day 1, 2 and 3 plasma F_2-isoprostane levels are expressed as percent of pre-exercise levels, *i.e.*, %day 0. Immediately following low intensity endurance exercise on day 1, 15-series F_2-isoprostane (8-IsoPs) levels increased 20%, compared to resting levels ($p < 0.01$). However, following the low intensity endurance exercise session on day 2, 15-series F_2-isoprostanelevels did not differ from resting levels. Interestingly, plasma 8-IsoPs levels were 77% of resting levels by 20 h post-exercise ($p < 0.05$; **Figure 3(a)**). Although the 5-series F_2-isoprostane levels show an increasing trend following low intensity endurance exercise on day 1, this increase did not reach significance. Similarly to the 8-IsoPs, 5-series F_2-isoprostane levels were decreased 20 h post-exercise (day 3) compared to pre-exercise levels ($p < 0.05$; **Figure 3(b)**).

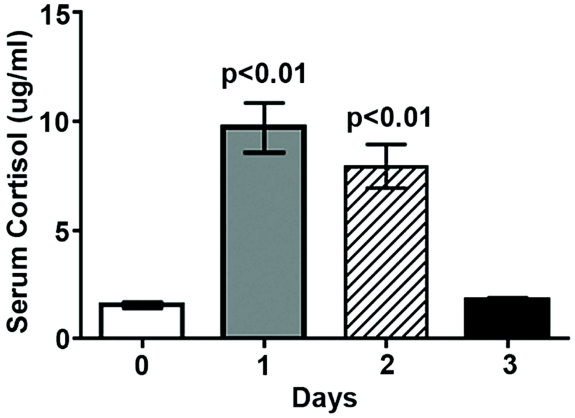


Figure 1. Low intensity endurance exercise increases serum cortisol. Unconditioned dogs participated in 2 low intensity endurance exercise sessions on 2 consecutive days (days 1 and 2) followed by a day of rest. Serum cortisol was determined 24 h pre-exercise (day 0, resting), 2 min post-exercise (days 1 and 2), and 20 h post-exercise (day 3). Data are expressed as mean ± SEM, n = 10; $p < 0.01$ compared to pre-exercise (day 0).

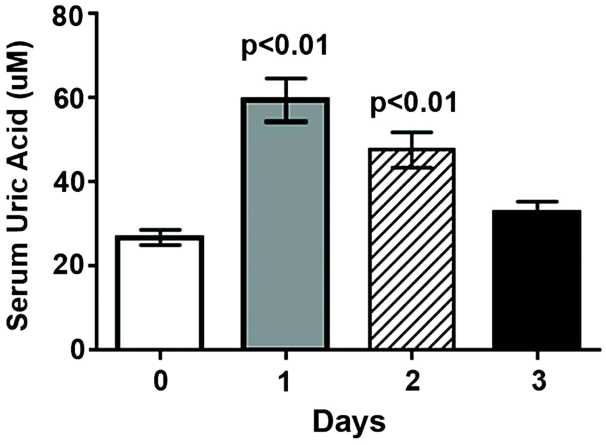
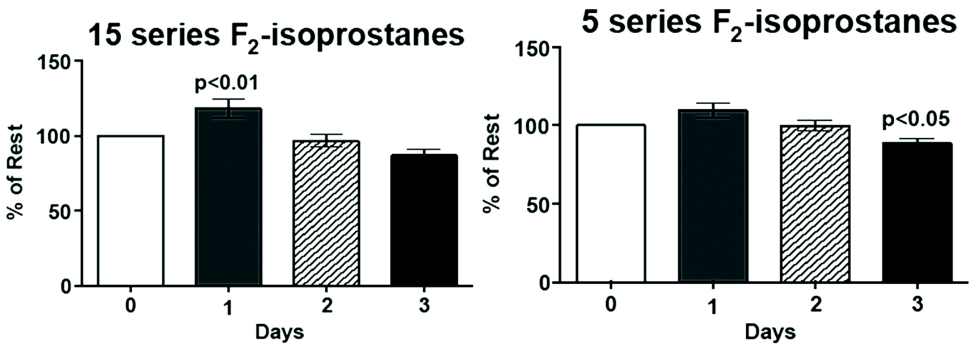


Figure 2. Low intensity endurance exercise increases serum uric acid levels. Unconditioned dogs participated in 2 low intensity endurance exercise sessions and serum was collected as described in **Figure 1** and Methods. Data are expressed as mean ± SEM, n = 10; $p < 0.01$ compared to pre-exercise (day 0).



(a) (b)

Figure 3. Low intensity endurance exercise alters plasma 15- and 5-series F_2-isoprostane levels. Unconditioned dogs participated in 2 low intensity endurance exercise sessions on 2 consecutive days (days 1 and 2) followed by a day of rest. F_2-isoprostanes were determined 24 h pre-exercise (day 0), 2 min post-exercise (days 1 and 2), and 20 h post-exercise (day 3) as described in Methods. a) 15-series F_2-isoprostane levels and b) 5-series F_2-isoprostane levels. Data are expressed as mean ± SEM, n = 10. Comparisons with p values < 0.05 are considered significant as compared to pre exercise (day 0).

4. Discussion

To date, canine athlete physiology studies have focused primarily on endurance sled dog racing and high intensity short duration Greyhound racing. However, the number of dogs competing in other canine activities has risen significantly over the past 10 years, particularly low intensity endurance activities. Presently there is a lack of information on the physiological effect of low intensity endurance activities in canines, particularly in the unconditioned dog. In the current study, we determined serum biochemistry, oxidative stress, and cortisol response before and after two consecutive days of controlled low intensity exercise on an exercise wheel, as well as 20 h post-exercise, in ten healthy unconditioned male dogs. Interestingly, although F_2-ioprostanes increased modestly following the day 1 exercise session, 20 h following the day 2 exercise session these indicators of oxidative stress were decreased below day 0 pre-exercise levels. These data suggest an adaptive response to repeated moderate exercise that may be beneficial to the long-term health of canines.

Although more consistent with physiologic changes seen in hunting or agility dogs, low intensity endurance exercise produced a unique set of biochemical changes compared to either hunting and agility dogs [1] [4] [9] [29] or endurance sled dogs [2]-[4]. Similar to agility dogs following competition, dogs in our study showed increased HCT, total protein, sodium, alkaline phosphatase, AST, and CK, but not ALT [11]. In addition, in contrast to agility dogs, our dogs demonstrated decreased serum phosphorus and glucose levels, which may have been a reflection of diet and/or energetics and phosphorus utilization in unconditioned dogs. Modest drops in phosphorus are common, reflecting the need for negative ions to move intracellularly due to respiratory alkalosis and increased glucose utilization in skeletal muscle requiring more intracellular phosphorus [9] [30] [31].

Following an endurance sled dog race changes in serum biochemistry were opposite compared to those seen following low intensity endurance exercise, *i.e.*, decreased albumin, total protein and sodium, and increased phosphorus. The only similarity between the findings in sled dogs and our study dogs was increased chloride and decreased potassium. Elevations in HCT, total protein, albumin, sodium, chloride and blood urea nitrogen can be explained in part by dehydration and inter compartmental fluid shifts causing hemoconcentration [1] [3]. Sympathetic induced splenic contraction occurs in agility dogs and Greyhounds and is thought to partially explain elevations in hematocrit post exercise [1] [29]. However, our increases were mild compared to those observed in Greyhounds. Thus it would appear that both of these processes occurred in the study dogs with complete recovery 20 hours post-exercise.

Immediately following low intensity endurance exercise, the serum enzymes CK and AST increased significantly indicating that exercise induced damage to the muscle cell membrane had occurred [2] [7] [32]. CK did not recover within 20 hours, likely due to continual rise that occurs after the cessation of exercise for up to 12 hours [33]. Lactate has been shown to be an indicator of glycolytic activity in skeletal muscle and the magnitude of the rise is related to intensity of exercise and fitness of the individual. Lactate was elevated on day 1 and day

2 of the exercise, however the rises were still within the reference interval. When compared to Greyhounds and agility dogs who show a 3- to 10-fold higher serum lactate concentration after an exercise bout, our study dogs showed little muscle glycolytic activity after 2 hours of low intensity exercise [12] [34]. This suggests that the dogs were running primarily from energy generated from the beta-oxidation of fatty acids, which would be expected for dogs running between 30% - 50% of their maximal oxygen capacity [20] [35]. However, even at this low intensity of exercise, our study dogs appeared to be alkalotic, similar to field trial Labradors who showed a pronounced respiratory alkalosis following exercise [36]. This is expected since panting is the major thermoregulatory mechanism for the dog. Panting causes increased loss of CO_2 through respiration resulting in a mild alkalosis that recovers quickly in dogs [1] [36]. This drop in serum bicarbonate and modest decrease in phosphorus may have led to the mild hyperchloremia observed in our dogs. These changes may represent an effort to replace intravascular negative ions for acid base balance [8] [30].

Uric acid is a constantly formed metabolite of purine metabolism in all mammals, and also a potent endogenous antioxidant that acts in concert with ascorbic acid [37] [38]. Typically, uric acid concentrations do not rise dramatically unless purines are being metabolized from diet or from endogenous sources into carbon moieties for other metabolic purposes [37]. However, uric acid levels have been shown to increase following endurance exercise in both dogs and humans. Specifically, uric acid levels increased 2-fold in endurance sled dogs running 30 minutes at high intensity (70% VO_2 max) and were increased as much as 3-fold in sled dogs after running 100 miles [20]-[22]. Increased uric acid levels have been proposed as a protective mechanism against exercise-induced oxidative stress [20] [22]. The 2- to 3-fold increase following low intensity endurance activity suggests that this protective mechanism may be in place during most exercise of any intensity or duration and should be examined in sprint athletes.

Similar to racing sled dogs, plasma F_2-isoprostane levels increased immediately following the first bout of exercise, compared to resting levels. However, F_2-isoprostane levels in our study dogs increased only 20% while levels in the racing sled dogs increased more than 3-fold [14]. In addition, while F_2-isoprostane levels remained elevated following the third day of the sled dog race, day 2 F_2-isoprostane levels did not differ from resting levels in our study dogs. Furthermore, in our study, 20 h post-exercise F_2-isoprostane levels were decreased below resting levels. Our data are in contrast with data reported by Dunlap and colleagues [20] in which rises in uric acid were coupled with non-significant elevations in plasma F_2-isoprostane levels. Additionally, Baltzer and colleagues [12] showed no differences in urinary or serum F_2-isoprostane levels in agility dogs after exercise. These differences may be due to the higher specificity and sensitivity of the mass spectroscopy method used in our current study compared to the ELISA method used by the earlier studies [15] [39]. ELISA based assays utilize antibodies that have shown significant cross reactivity with other compounds in addition to the 8-F_2-isoprostane they are intended to measure [27]. Mass spectroscopy coupled with either gas chromatography or HPLC is the preferred analytical technique for *in vivo* measurement of F_2-isoprostanes [15] [39]. In addition, Dunlap indicates that the recommended sample extraction was not done in their study and this may further explain why increases did not reach significance in that study [20]. Importantly, our data shows that 20 hours after 2 consecutive days of low intensity exercise F_2-isoprostane levels decreased significantly suggesting a protective effect of exercise, as previously observed in human studies of moderate exercising [17] [19].

Finally, our data demonstrates a dramatic rise in serum cortisol in unconditioned dogs that was similar to, but considerably more robust than studies of endurance sled dogs, sprinting sled dogs, field trial or agility dogs [3] [6] [11] [12] [32]. In studies of endurance and sprinting huskies, blood draws occurred within 30 minutes of cessation of exercise, whereas the agility trial blood draw was within 2 minutes and again 4 hours after cessation of exercise [3] [7] [12]. Since the serum half-life of cortisol is approximately 30 minutes, it is entirely possible that the more elevated concentrations observed in our study were due to the timing of our blood draw, *i.e.*, within 60 seconds of the discontinuation of exercise. Together these data point to the importance of venipuncture timing when planning studies and interpreting results [40] [41].

Another possibility for the differences in cortisol results is that the dogs used in our study were unconditioned. Thus there may be greater stress associated with beginning a new exercise regimen. Further studies are needed to determine cortisol responses in the conditioned versus unconditioned dog. Finally, there could have been excitement by the unconditioned dogs when they were allowed to discontinue this exercise session. Such a response would prompt sympathetic stimulation resulting in a burst of cortisol. However, this is doubtful based on behaviors observed by researchers during and after the exercise sessions.

5. Conclusion

In conclusion, this study demonstrated that the unconditioned canine athlete undergoes physiological changes similar to those observed in other exercising canines. However, unlike the endurance sled dog, the unconditioned dog shows modest hemoconcentration and physiological changes that favor fat oxidation rather than glycolysis. The low intensity endurance exercise used in this study did not induce a level of oxidative stress indicative of significant muscle damage. Rather, levels of the endogenous antioxidant uric acid were increased. In addition, there was a significant decrease in resting levels of F_2-isoprostanes within 2 days of beginning training. These data suggest that, like humans, moderate exercise leads to conditioning and an improved ability to respond to oxidative stress in canines. Finally, the oxidative indices and serum cortisol measurements in this study highlight that sampling times and methodology are absolutely critical when interpreting findings in field studies.

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14

Effect of Sodium Metabisulphite on Blood Metabolic Status of Wistar Rats

Fatima Zohra El Kadi[1], Amina Imène Bénali[1], Mohammed Bénali[1], Slimane Belbraouet[2*]

[1]Laboratoire de Biotoxicologie, Département de Biologie, Faculté des Sciences de la Nature et de la vie, Université D Liabés, Sidi Bel Abbés, Algérie

[2]École de Nutrition, Université de Moncton, Moncton, Canada

Abstract

The objective of this work was to determine the sodium metabisulphite (NaMBS) subchronic toxicity used as a food additive in Algeria. Three groups of female Wistar rats were treated with 0.25%, 1% and 4% of NaMBS in their drinking water for 90 days. An immunization protocol was conducted during the experiment. Mortality, comportmental and weight modifications, and food and water consumption were recorded. At the end of the experiment, the control and experiment rats were killed, and their blood and organs were removed. Immunoglobulin levels were evaluated; biochemical and hematological parameters were investigated. Our results showed that the administration of NaMBS at 1% and 4% had significant effects on body weight, food and water consumed. There was an increase in biochemical parameters (calcium, urea, creatinine, uric acid, transaminases) and decrease immunoglobulin levels. The hematology revealed a decrease in red blood cells and hemoglobin, as well as leucocytosis. Physiological study showed enlarged spleen, kidney, liver and stomach. In light of our results, we can conclude that subchronic intake of NaMBS 1% and 4% seems to alter immune function, biochemical, hematological and physiological parameters in Wistar rats.

Keywords

Sodium Metabisulphite, Subchronic, Haematology, Biochemistry, Physiology, Wistar Rat

1. Introduction

Sulphites are substances naturally present in foods and in the body. They are also regulated food additives that

*Corresponding author.

are used as preservatives to maintain the color and prolong the shelf life of food. They protect the preparations against enzymatic or non-enzymatic browning [1]. The European Union has classified them among the food preservatives [2]. Endogenous sulphites are normally generated in the human body by degradation of amino acids such as methionine and cysteine-sulphur. They can also be generated by polymorphonuclear neutrophils [3]. Several mammalian cells produce sulphites from H_2S [4]. Exogenous sources of sulphites include food, drinks, ambient air and pharmaceutical products [5].

There are several amino acid preparations used for parenteral nutrition that contain high levels of sulphites [6]. Although sulphites are very efficient, they are subject to restrictions of use due to their toxic effects. They have the ability to react with several molecules of biological importance such as DNA and may also be neurotoxic molecules by generating free radicals such as superoxide [7]. These radicals can also react with proteins and lipids [8]. Similarly, sulphites are additives and allergens to which the majority of asthmatics are sensitive [9].

Consumption of 0.25% $Na_2S_2O_5$ supplemented food is equivalent 72 mg/kg of SO_3^{2-} intake daily. With this finding, Joint Expert Committee in the World Health Organization (WHO) has added 100 fold safety factor (by multiplying this number with 0.01) and has established acceptable daily intake (ADI) level of sulfites as 0.7 mg/kg/body weight [10].

Thus, the aim of this study was to evaluate, in a sub-chronic study, the effects of ingestion of sodium metabisulphite (NaMBS) at levels of 0.25%, 1% and 4% on the biochemical, hematological and physiological parameters of Wistar rats.

2. Material and Methods

2.1. Animals and Experimental Design

Twenty four females Wistar rats (weighing 130.41 ± 22.68 g, 8 weeks old), were used in this study. During the entire experimental period, the animals were housed under standard conditions of temperature (21˚C ± 2˚C) and humidity (55% ± 10%) with a 12-hour light/dark cycle and received a specific diet and water *ad libitum* according to the method of Lane-Peter and Pearson [11]. food and water were measured every day and body weight weekly. The animals were separated into four groups (n = 6) and received daily three different doses of NaMBS (0.25%, 1%, and 4%) in water for 12 weeks, the doses chosen are based on the value of acceptable daily intake ADI [12]. A 42-day immunization protocol was used [13].

At the end of the experiment (day 90), animals were fasted for approximately 16 h and were sacrificed by exsanguinations sunder urethane anesthesia. The sacrifice was done according to the guidelines of the regional Committee of Ethics of Animals, consistent with those agreed upon by the European Community on 24 November 1986 (86/609/EEC). Samples blood for hematological and clinical chemistry examinations were withdrawn from the abdominal aorta of rats, under conditions of food and water deprivation. Blood aliquots were put in different anticoagulants, according to the type of investigations. For the evaluation of hematological parameters, an aliquot of blood per animal was placed in ethylen-diamino-tetracetic-acid (K3-EDTA). For the evaluation of biochemical parameters, one aliquot of blood per animal was placed in a tube containing lithium-heparin and centrifuged at 4000 × g for 10 minutes at room temperature to obtain serum. The obtained serum is aliquoted in volumes of 50 µL in Eppendorf tubes and frozen at −20˚C. For the determination of immunochemical parameters blood samples were then collected to the nonanticoagulated tubes centrifuged and aliquoted for until analysis.

The organs (liver, spleen, thymus, kidney, and stomach) were carefully collected, rinsed with physiological saline (0.9% NaCl), weighed, snap-frozen in liquid nitrogen, and stored at −80˚C until further analysis.

2.2. Methods

The chosen test substance was NaMBS- $Na_2S_2O_5^-$ (Biochem International Chemopharma: 7681-57-4; Canada). The provider indicates a 95% degree of purity of the product. The substance presents itself as a very fine powder of white colour, pungent and very soluble in water.

The hematologic analyses were determined on the same day after the blood samples were taken from the rats,using automated Advia 60 CT (Bayer DC): white blood cells, red blood cells, hematocrit; haemoglobin was determined by the colorimetric method of Drabkin (cyanomethemoglobin) [14].

Biochemical assays were measured by spectrophotometer using commercial assay kits. Kidney functions, creatinine was measured by Jaffé. Colometric-kinetic method and urea was assayed by Berthelot. Enzymatic

colorimetric method [15] using Spinreact kits (Spain) and recorded at spectrophotometer with wavelength 492 nm and 580 nm, respectively. The level of uric acid was measured using enzymatic colorimetric test (PAP-aminophenarose method) with lipid clearing factor (LCF) and recorded at spectrophotometer with wavelength 520 nm, using Human kits (Germany) [16] [17].

Total Cholesterol (TC) was estimated by enzymatic colorimetric assay (CHOD-PAP method) [18], triglycerides (TG) was determined by Glycerol Phosphate Oxidase/Peroxidase(GPO-PAP) method [19] [20] and recorded at spectrophotometer with wavelength 500 nm, using Human kits (Germany).

Total protein (TP) was assayed by using the Biuret, colorimetric method and recorded at spectrophotometer with wavelength 540 nm [21], using Spinreact kits (Spain).

Liver functions, the activities of alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) were adapted to determine in blood serum according to the kinetic method without activation by the pyridoxal phosphate [22], using Human kits (Germany).

Calcium value was determined by colorimetric method according to Stern & Lewis [23], using Biomaghreb kits (France) and recorded at spectrophotometer with wavelength 570 nm.

The rate of immunoglobulins is assayed by the reverse radial immunodiffusion technique of Mancini. The principle consists in depositing the antibody solution to be assayed in dug wells in a gel containing antigen. Antibodies diffuse into the gel and form rings of precipitation when the relative concentrations of the two elements are close to the equivalence point. The disc surface is proportional to the concentration of antibody [24]. For the optimization of this technique, we based our procedures on the protocols described by Mancini [25] and Vearman [26].

2.3. Statistics

The data is expressed as mean ± standard deviation (X ± SD). Statistical test one way ANOVA was applied to find significant difference between values of various parameters recorded for control and treated animals. $p < 0.05$ was considered statistically significant.

3. Results

Regular monitoring of animals receiving water with 4% sulphites resulted in the following observations: diarrhea from the 2nd week; blood around the nose from the 4th week; and loss of hair from the 6th week. Anorexia and inhibition of growth were also observed.

The weight change of the control and experimental rats is illustrated in **Figure 1**. The values of weight gain in the control group and those receiving 0.25% and 1% of NaMBS were respectively 100.83 ± 6.49 g, 115.33 ± 7.55 g and 72.66 ± 13.75 gr Variations between these groups remain insignificant. In contrast, the rats treated with 4% of NaMBS showed very low weight gain and the inhibition of growth, and the difference was highly significant compared to the controls ($p < 0.0001$). At the highest doses of NaMBS food and water intakes were strongly diminished in the treated rats with 4% of NaMBS compared with controls during the 12 weeks of observation (**Figure 2**).

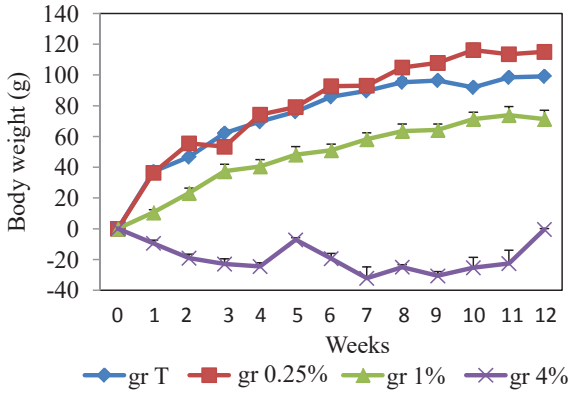


Figure 1. Body weight changes of control rats and rats treated with 0.25%, 1% and 4% of sodium metabisulfite daily for 12 weeks. Each value represents the mean ± SD.

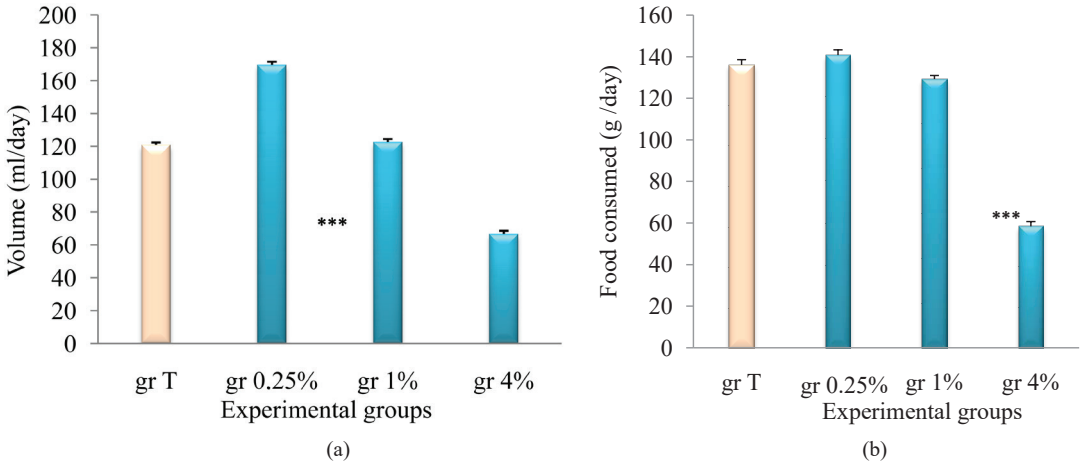


Figure 2. (a) Water intake (ml/day) of control rats and rats treated with 0.25%, 1% and 4% of sodium metabisulfite daily for 12 weeks. Each value represents the mean ± SD; (b) Food consumption (g/day) of control rats andrats treated with 0.25%, 1% and 4% of sodium metabisulfite daily for 12 weeks. Each value represents the mean ± SD. ***Statistically significant compared to control group; $p < 0.001$.

According to the immunochemical test results, there was a decrease in the diameter of precipitates (immune complex antibody-antigen Ab-Ag) of the experimental groups receiving 1% and 4% of NaMBS compared with the control (**Figure 3**).

At the end of the experiment, various hematological parameters of treatment rats were measured and were compared with those of controls (**Table 1**). The results of the hematological study revealed a decrease in the number of red blood cells and the content of haemoglobin associated with leucocytosis in the group treated with 4% of NaMBS compared with the control group. The results of the different biochemical assays of the serum in the treatment groups compared to the control group are presented in **Table 2**. Rats treated with high doses of NaMBS (4%) expressed a hypercalcemia and high rates of urea combined with hypercreatinemia and hyperuricemia. Increased serum transaminases were also found.

The assessment of the relative weights of taken organs of control and experimental rats treated with 1% showed an enlargement in spleen and liver. The group receiving 4% expressed a hypertrophy in spleen, liver, stomach, kidneys. However, no significant change was found in the weights of the thymus in the different experimental groups compared with the control group (**Table 3**).

4. Discussion

In this work, the effects *in vivo* of subchronic NaMBS consumption at doses of 0.25%, 1% and 4% were studied. The doses of sulfite administered in this project was determined in reference to previous studies designed to investigate the effects of high level of sulfite exposure via consuming certain foods and beverages containing sulfite [12] [27] [28]. In particular, there are several amino acid preparations utilized in total parenteral nutrition (TPN) solutions that contain large amounts of sulfites [6]. Sulfite toxicity is also considered possible with peritoneal dialysis fluids, some of which contain $Na_2S_2O_5$ in concentrations of 0.005% - 0.012% [29]. Considering that many foods such as sausage, dried fruit, beer and wine contain SO_2^{3-}, the daily level of normal SO_2^{3-} intake can easily be exceeded [30].

The results show that the effect of subchronic high-dose (4%) of NaMBS administration in rats translates to clinical and behavioural changes (diarrhea, anorexia, hair loss). These neurobehavioural changes can be explained by the neurotoxic effect of sulphites, the toxicity of which is attributed to the release of sulphur and oxygen free radicals that can damage the central nervous system [8] [31] [32]. Reist *et al*. [33] showed that sulphite exerts toxic effects on neuronal cells grown directly or in combination with peroxynitrite. In addition, individuals suffering from a congenital disease caused by a deficiency of sulphite oxidase, a key enzyme in the metabolism of sulphites in the body that causes their oxidation to sulphate, develop severe neurological abnormalities with mental retardation, attenuated brain growth and early death [34].

The results obtained showed a significant decrease in food and water consumption associated with body

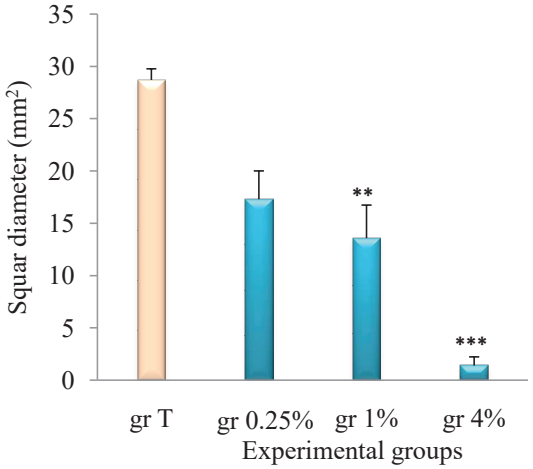


Figure 3. Square diameter (D^2) of the precipitate Ab-Ag of controls rats andrats treated with 0.25%, 1% and 4% of sodium metabisulfite daily for 12-weeks. Each value represents the mean ± SD. **Statistically significant compared to control group; $p < 0.01$; ***Statistically significant compared to control group; $p < 0.001$.

Table 1. Blood levels of some hematological parameters in controls rats and daily treated with 0.25%, 1%, and 4% of sodium metabisulphite for 12 weeks.

Parameters	Controls	Experimental		
		0.25%	1%	4%
Red blood cell (10^6/mm^3)	7.17 ± 0.14	7.72 ± 0.24	6.56 ± 0.44	6.13 ± 0.34*
White blood cell (10^3/mm^3)	3.13 ± 0.42	3.54 ± 0.22	2.5 ± 0.11	5.8 ± 0.17**
Haemoglobin) (g/dl)	13.98 ± 0.25	14.9 ± 0.4	12.7 ± 0.43	11.55 ± 0.43*
Hematocrit (%)	40.11 ± 1.17	40.14 ± 1.67	40.85 ± 1.83	36.97 ± 1.76

*Statistically significant compared to control group; $p < 0.05$; **Statistically significant compared to control group; $p < 0.01$; Results are expressed as Mean + SD standard deviation.

Table 2. Serum levels of biochemical parameters in controls rats and treated daily with 0.25%, 1%, and 4% of sodium metabisulphite for 12 weeks.

Parameters	Controls	Experimental		
		0.25%	1%	4%
T. protein (gr/L)	67.7 ± 2.13	65.6 ± 2.16	69.75 ± 1.46	67 ± 1.73
Calcium(mmol/L)	2.26 ± 0.12	2.37 ± 0.21	2.21 ± 0.08	2.78 ± 0.02*
Urea (mmol/L)	11.77 ± 0.02	11.72 ± 0.02	10.92 ± 0.01	11.99 ± 0.01*
Creatinine(µmol/L)	42.47 ± 2.76	42.92 ± 6.9	52.92 ± 3.37	57.7 ± 2.93*
Uric acid (µmol/L)	160.05 ± 28.07	159.46 ± 20.95	61.89 ± 23.8	232.05 ± 13.75*
T. cholesterol (mmol/L)	2.21 ± 0.15	2.16 ± 0.17	1.91 ± 0.17	1.83 ± 0.04
Triglycerides (mmol/L)	0.98 ± 0.29	0.54 ± 0.05	0.76 ± 0.08	1.08 ± 0.03
ASAT (IU/L)	68.66 ± 4.27	69.5 ± 4.46	131 ± 22.69*	151 ± 44.3*
ALAT (IU/L)	35 ± 2.78	41.6 ± 10.63	39.8 ± 6.64	30.8 ± 0.38

T: total; ASAT: aspartate aminotransferase; ALAT: alanine aminotransferase; Results are expressed as mean + SD standard deviation; *Statistically significant compared to control group; $p < 0.05$.

Table 3. Relative organ weights (g) in controls rats and treated daily with 0.25%, 1%, and 4% of sodium metabisulphite for 12 weeks.

Organ	Controls	Experimental		
		0.25%	1%	4%
Spleen	0.37 ± 0.01	0.38 ± 0.01	0.45 ± 0.02*	0.50 ± 0.03**
Thymus	0.12 ± 0.01	0.17 ± 0.007	0.13 ± 0.01	0.2 ± 0.02
Stomach	0.69 ± 0.01	0.76 ± 0.09	0.76 ± 0.05	1.48 ± 0.09**
Liver	3.13 ± 0.15	2.93 ± 0.11	3.85 ± 0.12*	4.65 ± 0.40**
Kidneys	0.66 ± 0.02	0.7 ± 0.02	0.68 ± 0.03	0.98 ± 0.0**

Results are expressed as mean + SD standard deviation. *Statistically significant compared to control group; $p < 0.05$. **Statistically significant compared to control group; $p < 0.01$.

weight loss in the group receiving the highest dose (4% of NaMBS). Sulphites have an unpleasant taste and smell so that their administration in drinking water might cause a decrease in the water consumption. In addition, the destruction of thiamine by sulphite is an important factor in the toxicological evaluation of this food additive. Thiamine destruction not only occurs when sulphite is added to the food, but also when it is present in the organism It thus appears as an antivitamin factor B1 [35]. This may explain the decrease in weight gain of animals treated with the highest dose.

These results are consistent with those of other studies, including those Till *et al*. [36], which showed that male and female rats fed high doses of NaMBS Joglur (0% - 8%) have reduced food consumption and weight growth. In a follow-up study [37], it was shown that a dose of 1.72% of NaMBS Joglur added to the diet causes a decrease in growth and feeding in male and female pigs during an experimental study of 12 months. Similarly, a study by inhalation was conducted by Miyata *et al*. [38]. Six rabbits exposed to dioxide of sulphur (186.2 mg/m^3) for 6 weeks. The main symptom was a decrease in growth.

The significant decrease in immunoglobulin in a manner inversely proportional to levels of administered of NaMBS Joglur explains the immunomodulatory effects of NaMBS. Several studies have been conducted to measure the impact of exposure to known concentrations of sulphites on the normal immunological mechanisms. The results show that sulphites in particular can influence the production of antibodies and agglutinins [39].

In order to assess the actual toxicity of NaMBS Joglur, different hematological and biochemical parameters were measured at the end of the experiment in treated rats and were compared to those of control rats. Our results show that a 4% dose causes a significant decrease in red blood cells and haemoglobin levels and a significant increase in white blood cells in the experimental rats compared to the controls. These results are in agreement with the work of Til [36], which showed the appearance of anaemia at 2% and a leucocytosis to 6% of NaMBS in Wistar rats. According to Gunnison [40], rats fed a diet containing 6% of NaMBS Joglur for 21 days became severely anaemic. The results showed a significant increase in the concentration of serum calcium in the experimental group receiving 4% of NaMBS Joglur. Sulphites act on the excretion of the calcium through sulphate, to which they give rise *in vivo* [41].

Uric acid is the final product of the catabolism of purine [42]. The levels of uric acid in most mammals are lower than in humans due to the presence of uricase, a liver enzyme that degrades uric acid into allantoin [43]. Our results illustrate hyperuricemia exclusively in the group treated with the highest dose (4%). The observed increase in serum levels of uric acid may be the result of reduced urinary excretion of the metabolite. Creatinine and urea are excellent markers of renal function, and their increase or decrease reflects a dysfunction of the renal function [44]. Transaminases are enzymes with important metabolic activity inside cells. Their increase in serum reflects cell damage, in particular at the hepatic level [45].

The results indicate that doses of 4% cause an increase in serum urea, creatinine and transaminases marking abnormal renal and hepatic function. Degradation of hepatic protein compounds may explain the increase in urea and serum creatinine in the treated rats, where proteins can be degraded into amino acids and then into urea and creatinine. Thus, these amino acids can be transformed by the action of the serum transaminase in carboxylic compounds such as pyruvic acid [46] which requires the high enzymatic activity of transaminases in rats treated with 4% of NaMBS. In this case, we may suggest that the high enzyme activity of the aspartate ami-

notransferase (ASAT) and alanine aminotransferase(ALAT) is linked to the hepatotoxic effect of the xenobiotic, given that the liver is the principal organ of detoxification because it contains most of the enzymes of metabolism.

The assessment of the relative weight of the various bodies reveals that the doses of 4% cause an enlarged liver, spleen, kidney, stomach. The increase in the hepato-body and reno-body report has been highlighted indirectly by a disruption of the renal and hepatic enzyme system (increase serum in urea, creatinine, uric acid and transaminases in the animals treated with the highest dose). The increase of thespleen weight is the result ofan increased extra-medullary splenic hematopoietic activity and increased leucocyte counts. This result is fully consistent with the work of Til *et al*. [36], which showed the presence of splenomegaly in rats receiving doses of 4% or more of NaMBS. In a follow-up study, they showed an enlarged liver, kidney and heart of pigs treated with 0.83% and 1.72% of NaMBS [36].

5. Conclusions

Females Wistar rats oral exposed to NaMBS, at levels up to 4% in the water, during 12 weeks, shown alterations in immunochemical, biochemical (calcium, uric acid, urea, creatinine, transaminases), hematological (hemoglobin, red blood cell, and white blood cells) parameters in relation to the control group. Similarly, the oral administration of 4% of NaMBS for 12 weeks causes hypertrophy of kidneys, liver, spleen and stomach. These results lead to considerations of the development of other research focused on the determination of biomarkers of oxidative stress induced by sulphite (glutathione, superoxide dismutase, cytochrome P450, catalase, substances reacting with thiobarbituric acid, malondialdehyde, vitamins, etc.) for a better analysis of potential toxic effects of this synthetic preservative. Our recommendations relate to the control of their unauthorized presence in foods or their presence at unauthorized rates:

- remove or replace their use in foodstuffs for which their use is not required;
- reduce the use of these additives when technologically possible;
- increase the legibility and visibility of labelling (this is particularly useful for asthmatics).

In addition, sulphites do not meet the current demand for consumer which is turning more and more towards a natural diet free from synthetic additives. Natural additives such as vitamin C, citric acid and tocopherols can also be used as alternative preservatives to be able to propose products to consumers without hazards, natural, good for health and practices.

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15

Serum Biochemistry and Hematology in Blue Fox (*Vulpes lagopus*)

Hannu T. Korhonen, Hanna Huuki

MTT Agrifood Research Finland, Animal Production Research, Kannus, Finland

Abstract

The blue fox (*Vulpes lagopus*) is a colour mutation of wild arctic fox. It is commonly farmed for its fur. The aim of the present study was to analyse a baseline biochemical profile of serum from adult farmed blue foxes during pelting season. Serum biochemical profile can be used later on as a reference in chemical analysis of blood serum. Biochemical profile here consists of liver enzymes alanine aminotransferase (ALAT), aspartatate aminotranasferase (ASAT), alkaline phospahatase (ALP), and total bilirubin (T-BIL); kidney function substances creatinine, urea and inorganic phosphate (Pi); muscle enzyme creatinine kinase (CK); glucose (GLUK) from pancreas; serum total proteins (PROT) and albumin (ALB); electrolytes potassium (K), natrium (Na), chloride (CL), calcium (S-Ca); and lipids such as total cholesterol (T-Cho). For some of the measured parameters reference values were also available from previous studies. Certain values were close to those previously published but some differed markedly. These differences can be explained by differences e.g. in techniques, conditions, unit reports, and breed and age of the animals. The present study additionally reviewed previously published haematological data concerning small blood count, *i.e.* hemoglobin, hematocrit, white blood cells and red blood cells. Summarized material revealed that these variables are very similar despite of study and animal material. Thus, small blood count can be reliably used to screen general welfare status of farmed blue foxes. The reference values presented in this paper will be valuable as a guide for clinical diagnosis and research.

Keywords

Farm-Raised Fox, Blood Analysis, Haematology, Animal Welfare, Small Blood Picture

1. Introduction

Serum is the liquid portion of blood. It is extracted from blood sample by spinning down or centrifuging the sample to separate cellular components and coagulation factors of blood from the liquid part. Serum biochemis-

try refers to the chemical analysis of blood serum. The analysis can include many different tests, each of which provides targeted information about one or more organs [1]. Assessment of the test results may indicate which organ system is affected, and may provide valuable information about the nature and severity of the problem or disease [2]. Biochemical profile of serum is typically species-specific [3]. However, for example in foxes, the concentrations of serum biochemical indicators seem to be fairly similar to the reference values previously reported for the dog [4] [5].

The most common fox species raised for its fur are the blue fox, a colour mutation of wild arctic fox (*Vulpes lagopus*), and the silver fox, a colour mutation of red fox (*Vulpes vulpes*). Farming history of both fox species is about 100 years old. Earliest evaluations on haematology and blood biochemistry in farmed foxes were published over 70 - 80 years ago [6]-[8]. These data are from red and silver foxes. As concerns blue foxes, earliest haematology screenings derive from 1980's [2] [3] [9] [10]. Some haematology and serum biochemistry evaluations have also been made for wild arctic foxes [11] [12]. The latest haematological measurements regarding farm-raised foxes were published by [13] and [14]. A total serum biochemical profile, however, is lacking from farmed blue foxes. The small blood count is best illustrated by [15] and [16].

The purpose of the current study was to screen baseline serum biochemical profile for adult farm-raised blue foxes during pelting season. A selected group of tests for serum biochemistry applied for dog-like canids were employed. Previously published haematological data in farmed blue foxes mainly includes small blood count [15] [16]. The second aim was to summarize the existing data for background screening of general health status.

2. Material and Methods

2.1. Animals and Health Evaluation

The present study was carried out at the Fur Farming Research Station of MTT, in Kannus, western Finland (63.54˚N, 23.54˚E). The use of experimental animals was evaluated and approved by the Animal Care Committee of MTT Agrifood Research Finland. Health of animals was visually checked daily. Health evaluation was based on general appearance of animals, including consistence of faeces.

The animals were born at the Fur Farming Research Station in Kannus. Blood samples were taken from fifteen 21 months old female blue foxes in November. Because of artificial insemination, adult females are more common than adult males on farms. Therefore, only females were used here. Body weights of animals were measured with a Mettler SM 15 balance, accuracy ±1 g. Before sampling, experimental animals were kept in wire-mesh cages measuring 105 cm long × 115 cm wide × 70 cm high. Each cage had a wire-mesh platform (105 cm long × 25 cm wide) and a wooden block for chewing (diameter 7 cm, length 35 cm). Daily routine treatments were conducted according to standard farming procedures [16]-[18]. Feed composition was based on the recommendations of the Finnish Fur Breeders' Association [13] [18]. Watering was automatic *ad libitum*.

2.2. Blood Collection and Analysis

Animals were clinically healthy at the time of blood collection. They were fasted overnight prior to blood sampling. Each fox was manually restrained and blood was collected from the jugular vein through a 20-gauge needle into a syringe. Serum and plasma were separated by double centrifugation. For the serum biochemical evaluation, 3 mL blood samples were centrifuged at 3000 rpm for 10 min within 1 h of collection. Sera were stored at −80˚C in a freezer prior to analysis.

Serum biochemical assays (**Table 1**) were carried out by using an automatic analyzer (Shimadzu CL-7200, Kyoto, Japan), and automatic electrolyte analyzer (Ciba-Corning 644, USA) [5] [12] [19]. Haematological analyses here include the small blood count, *i.e.* hemoglobin, hematocrit, white blood cell count and red blood cell count. This data is based on previous publications listed in **Table 2**.

3. Results

Body weights of the foxes averaged 8.0 ± 0.9 kg (mean ± SD). The main biochemical results are presented in **Table 1**. Serum biochemical assays here consisted of liver enzymes alanine aminotransferase (ALAT), aspartate aminotranasferase (ASAT), alkaline phospahatase (ALP), and total bilirubin (T-BIL); substances describing kidney function creatinine, urea and inorganic phosphate (Pi); muscle enzyme creatinine kinase (CK); glucose

Table 1. Serum biochemistry analyses in blue foxes (N = 15). Results are presented as means with standard deviation (SD). Reference values (mean or range) from previous publications (a - e) are presented on the right. See References in more details. For variables in details see Material and methods.

Parameter	Mean	SD	Reference values
ALAT (U/l)	171.5	56	132[a], 121[b], 260[c], 49 - 65[d], 12 - 14[e]
ASAT (U/l)	49.2	25	48[a], 53.8[b], 99 - 168[c], 87 - 104[d], 23 - 28[e]
CK (U/l)	84.0	27	227[a], 184 - 363[c]
ALP (U/l)	101.2	32	34.6[b]
UREA (mmol/l)	6.4	3.3	6.4[b]
KREA (µmol/l)	52.6	3.9	62[b]
ALB (G/l)	35.9	2.0	
S-Ca (mmol/l)	2.9	0.1	
GLUK (mmol/l)	7.6	1.2	
T-Cho (mmol/l)	5.1	0.6	
T-BIL (µmol/l)	1.7	0.4	
Pi (mmol/l)	1.5	0.4	
K (mmol/l)	4.6	0.3	
Na (mmol/l)	145.7	1.9	
CL (mmol/l)	108.2	2.7	

[a][15], [b][14], [c][13], [d][9], [e][2].

Table 2. Small blood count for farmed blue foxes. Hb = hemoglobin, HC = hematocrit, WBC = white blood cells, RBW = red blood cells. Results are with or without mean and standard deviation (SD), except in [20] as standard error (SE). N = number of animals.

Hb	HC	WBC	RBC	N	Reference
(g/l)	(%)	(10^9 cells^{-1})	(10^9 cells^{-1})		
155 ± 10	49.3 ± 50	8.4 ± 7.2	8.4 ± 8.7	20	[16]
160 ± 2	51.7 ± 51	8.8 ± 8.7	8.9 ± 8.0	20	[19]
159 ± 0.2	50.2 ± 0.8	7.7 ± 0.8	9.0 ± 0.2	12	[20]
171	57.0	8.9	9.5	20	[15]
164	52.0	7.4	8.8	60	[17]
178	57.1	7.8	9.2	50	[18]
168	58.4	7.9	9.7	16	[21]

(GLUK) from pancreas; serum total proteins (PROT) and albumin (ALB); electrolytes potassium (K), natrium (Na), chloride (CL), calcium (S-Ca) , and lipid substance total cholesterol (T-Cho). **Table 1** also provides some reference values from previous publications in farm-raised blue foxes.

Table 2 summarizes previously published haematological data concerning small blood count, *i.e.* hemoglobin, hematocrit, white blood cells and red blood cells. These variables are very similar despite of study and animal material.

4. Discussion

It is known that individual animal variability can affect study results. To minimize animal variability, research-

ers should be provided with normal, healthy, well-adapted animals having a narrow range of physical, behavioural, and clinical parameters. The establishment of standard basic data is therefore important. The present biochemical study was carried out at pelting season in November. At that time of the year there is the highest number of animals on farms. By then, juvenile animals have already reached maturity, too. Comparison of serum biochemistry profile can be expected to be then most needed and justified. Animals in this study were clinically healthy at the time of blood collection and maintained under similar husbandry practices.

Values of certain blood parameters determined in the present study were close to previously documented reference values, but there were also variables that differed markedly from each other. These differences quite likely can be explained by differences in techniques, conditions, unit reports, and breed and age of the animals [12].

Serum biochemical profile contains a series of blood tests that provide an overview of many of the body's functions. The term "liver function tests" is often used to describe a panel of laboratory measures of a variety of hepatic enzymes, including serum aspartate aminotransferase or AST and alanine aminotransferase or ALT and alkaline phosphatase (ALP). With a disturbance of the structure and function of hepatocytes the cytoplasmic enzyme ALAT is liberated and supplied to the blood earlier than the mitochondrial enzyme ASAT [2]. ASAT is most abundant in the heart, the liver and the skeletal muscle, while the liver and kidneys are good sources of ALAT. Cirrhosis of the liver, where hepatic cells are irreversibly damaged and part of the tissue becomes fibrous, increases serum ASAT too, as a result of a deep organ disturbance of hepatic cells [2]. Normal ASAT values in blood serum for blue foxes at pelting time have been described to be between 23 - 28 U/l, and ALAT values between 12 - 14 U/l [2]. It has, however, found higher values, 87 - 104 U/l for ASAT and 49 - 65 U/l for ALAT in Finnish blue foxes [9]. In dogs, ALAT and ASAT value in the serum range between 21 - 102 U/l and 23 - 66 U/l [22]. High ALAT and ASAT values can be related to the seriousness of liver lipodosis [13]. In the present study, ALAT values found were higher than previously measured. ASAT values, on the other hand, were very similar than those documented by [14] and [15]. In dogs, ASAT value has been described to be 35 U/l [5]. ALP values found were higher than those found in blue fox study [14]. In silver foxes, ALP value of 57 - 122 U/l has been measured [4]. These are rather close to values found in the present study. In human beings, normal ALK range typically is from 44 to 147 U/l.

The two substances most commonly measured to assess kidney function are urea and creatinine. Urea is a by-product of protein breakdown. It is produced in the liver and secreted from the body in urine. Creatinine is a by-product of muscle metabolism. Increased levels of creatinine indicate decreased kidney function. Both urea and creatinine values measured here were comparable to those values previously found [14].

The enzyme most frequently evaluated to assess muscle health is creatinine kinase (CK). Elevations in muscle enzymes are often found with muscular activity, trauma, and muscle inflammation. CK values in the present study were lower compared to previously values by [2] and [14]. In silver foxes, on the other hand, it was found similar values (84.9 U/l) than in the present study for blue foxes [4].

For the rest of measured parameters there are no actual reference values available in the literature. Because of lack of specific reference it is difficult to estimate their true relevance. Typically values outside of normal range are suggestive of a lack of good health. Further studies will be needed to evaluate normal range of certain serum biochemistry variables in farmed foxes.

Summarized haematological data on small blood count revealed that these four blood parameters are fairly similar despite of experiment or animal material. All haematological analyses were done by the same laboratory which probably declined variability of results. It looks that small blood count can be reliably used to screen general welfare status of farmed blue foxes [22]. More data on serum biochemistry is needed. However, it can be expected that small blood count together with serum biochemistry profile is a potential test combination for analysis of animal welfare and health.

5. Conclusion

The present study evaluated a wide scale serum biochemical assays for adult farm-raised blue foxes. For some of the measured blood parameters, also reference values were available from previous studies. Some of the measured variables were close to values previously published, but some differed markedly. These differences can be most likely explained by the differences in techniques, conditions, unit reports, and breed and age of the animals. It is essential to reduce errors by determining the normal ranges as accurately as possible. Small blood

count can be reliably used to estimate general welfare status of blue foxes. The reference values presented in this paper will be valuable as a guide for clinical diagnosis and research.

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Influence of Carvacrol on the Growth Performance, Hematological, Non-Specific Immune and Serum Biochemistry Parameters in Rainbow Trout (*Oncorhynchus mykiss*)

Ebru Yilmaz[1], Sebahattin Ergün[2], Sevdan Yilmaz[2]

[1]Aquaculture and Fisheries Engineering Department, Faculty of Agriculture, Adnan Menderes University, Aydın, Turkey

[2]Department of Aquaculture, Faculty of Marine Sciences and Technology, Çanakkale Onsekiz Mart University, Çanakkale, Turkey

Abstract

The present study investigated the effects of phytoadditive carvacrol on growth performance, feed utilization, hematological, non-specific immune and serum biochemical parameters in rainbow trout (*Oncorhynchus mykiss*). In trial, 240 rainbow trout which had average weight of 10.79 ± 0.57 was used. Fish were divided into four groups before being fed for 60 days with 0, 1 (C1), 3 (C3) or 5 (C5) g/kg of carvacrol. There were no particular differences in the growth performance, feed utilization, whole body composition, hematological parameters between the experimental group and the control group. However, as compared to control group, a significant increase of serum lysozyme activity was seen in groups receiving feed containing C3 and C5 groups after 30 days. The myeloperoxidase activity in groups (C1 and C3) was significantly higher on the 30th day of sampling. Myeloperoxidase activity of C5 treated group was significantly higher on the 60th day in comparison with the control group. Serum glucose levels significantly decreased on 60th day for C1, C3 and C5 treated groups. The serum total protein, globulin and triglyceride levels of C3 treated groups was significantly higher on the 60th day in comparison with the control and other carvacrol treated groups. The level of serum cholesterol in C5 treated groups was significantly lower on the 30th day in comparison with the control and other carvacrol treated groups. The results of the present study indicated that dietary supplementation of 3 g/kg of carvacrol in commercial diets could improve some non-specific and biochemical status in rainbow trout.

Keywords

Carvacrol, Growth Performance, *Oncorhynchus mykiss*, Blood Parameters

1. Introduction

Aquaculture industry has shown a rapid growth in recent years. The main aims of industry area is both increasing growth rates of fish and protecting their health. Antibiotics have been used for promoting growth and struggling with illness for a long time. However, use of antibiotics in fish aquaculture have been limited and prohibited in many countries [1]. That's why; researchers have been looking for alternatives to antibiotics and the other synthetic chemicals.

Especially herbs or essential oils of herbs can be used for struggling against pathogen bacterias with its antimicrobial and antioxidant features. The features of herbals can be related to terpenoid and phenolic compounds [2]. It was seen that herbal sources can promote growth in fish, develop fish health and rise the resistance to illness as a result of their addition to fish-feed [3] [4].

Carvacrol is a major component of oregano and thyme essential oils. It is recognized as a safe component by the U.S. Food and Drug Administration (2010), by the Council of Europe (2000), and FAO/WHO Committee on Food Additives (2001). Some studies have reported that oral administration of combination of carvacrol and thymol in *Ictalurus punctatus* [5] and *O. mykiss* [6] improved growth performance, disease resistance and/or immunity. Channel catfish fed with carvacrol-based diet, significantly enhanced growth, dismutase and catalase antioxidant activity in plasma, as well as fish resistance to a challenge with *Aeromonas hydrophila* [5]. When a 0.025% carvacrol-based diet was fed to *Dicentrarchus labrax* for 9 weeks, it decreased the mortality of fish challenged with *Listonella anguillarum* [7]. Dietary carvacrol supplementation at doses of 200 ppm decreased the mortality of *Oreochromis niloticus* challenged with *Edwardsiella tarda* [8]. Since, only one study demonstrated that carvacrol (12 g/kg) had a positive effect on trout growth performance with apparent effects towards antioxidant defence and innate immunity status [9]. However, only one dosage was used by Giannenas *et al*. [9]. Positive effects of carvacol treatment may be obtained provided that a different carvacrol doses can be determined and applied. Therefore, the aim of the present study was to determine dietary phytoadditive carvacrol (1, 3, or 5 g/kg) can influence growth performance, proximate composition, and some hematological, non-specific immune or serum biochemical parameters in rainbow trout.

2. Material and Methods

2.1. Fish and Experimental Protocol

Trial was applied in commercial trout farm (Çobanlar) in Fethiye. In trial, 12 polyester tanks (3 × 0.8 × 0.4 m) consisted of 480 liter were used. Trial was carried out in continuous flow system and water was exchanged daily 6 - 8 times. In trial, 240 rainbow trout (*O. mykiss)* which has average weight of ±SD = 10.79 ± 0.57 was used. Fish were fed by hand 2 times a day in the ratio of % 2 of their body weight during 60 days trial. Water temperature, dissolved oxygen, pH and EC (electrical conductivity) were measured at 7˚C, 5.72 $mg{\cdot}L^{-1}$, 8.04, 1408 µmhos/cm.

2.2. Diet Preparation

Carvacrol (W224502, Sigma-Aldrich, Munich, Germany) was added to a commercial trout extruder feed (Çamlı Feed Company, Turkey, **Table 1**) at a dose of 0 (Control), 1 (C1), 3 (C3), 5 (C5) g/kg by mixer. Briefly, after heating (40˚C) for 3 h the diet was top-dressed with fish oil containing the carvacrol by slowly mixing in a food mixer. No herbal oil supplementation was made to control feed. Feeds were offered to fish two times a day as ad libitum for 60 days.

2.3. Growth Performance and Proximate Analyses

Growth performance of rainbow trout with different diets was considered by calculating weight gain (WG), specific growth rate (SGR), feed conversion rate (FCR) and death rate.

Table 1. Fish diet (pellet size: 2 mm).

Chemical Analyses	
Crude Protein (%)	49
Crude Lipid (%)	19
Crude Cellulose (%)	3
Crude Ash (%)	13
Energy (kcal/kg)	4329
***Amino Acids* (%)**	
Lysine	4.7
Methionine + Cystine	2.4
***Vitamins* (*per kg Feed*)**	
A (IU)	2500
D3 (IU)	3050
E (mg)	240
K (mg)	10
C (mg)	250
***Macro Elements* (%)**	
Calcium	1-2
Total Phosphor	1.5
Sodium	0.2/1

Ingredients: fish meal, fish oil, soybean and by products, wheat and by products, yeast and by products, amino acids, vitamins and minerals.

$$\mathrm{WG}(\mathrm{g}) = \text{final weight } (\mathrm{FW})\ (\mathrm{g}) - \text{initial weight } (\mathrm{IW})\ (\mathrm{g})$$

$$SGR(\%/d) = \left[\left(\ln \text{final weight } (\mathrm{g}) - \ln \text{initial weight } (\mathrm{g})\right)/\text{days}\right] \times 100$$

$$FCR = \text{feed intake}(\mathrm{g})/\text{weight gain}(\mathrm{g})$$

Proximate analyses of the diets were performed using standard methods [10]. Moisture was detected after drying at 105˚C until a constant weight was achieved. Crude protein was analyzed by the Kjeldahl method, and crude ash by incineration at 525˚C in a muffle furnace for 12 h. Crude fat was analyzed by methanol/chloroform extraction [11].

2.4. Blood Collection

Blood samples of six fish/groups were collected randomly from the caudal vein using a vacutainer fitted 5 mL on days 30 and 60. For blood sampling, fish were anaesthetized with MS222 (Sigma Aldrich, Steinheim, Germany) [12]. They were well wiped and cleaned in order to avoid mucus mixing into the blood, and blood was taken from the fish through the caudal vein by a 2.5-mL plastic syringe without harming the fish [13] Then, 200 µL of blood was transferred to ethylenediaminetetraacetic acid (EDTA) tubes (BD, Oxford, UK) for hematological analysis. The other 600 µL of blood was harvested in plastic biochemistry tubes (Vacutest Kima s.r.l., Piove di Sacco, Italy). After the blood was coagulated, the tubes were centrifuged at 4000 × g for 10 min for serum separation, which was stored below −20˚C [14].

2.5. Hematological Analysis

Red blood cells (RBC, 10^6 mm^3), hematocrit (Hct, %) and hemoglobin (Hb, g/dL) were determined by using the

method by Blaxhall and Daisley [15]. RBC was counted with a Thoma hemocytometer using Dacie's diluting fluid. Hct was determined using a capillary hematocrit tube. Hb concentration was determined by spectrophotometry (540 nm) using the cyanomethahaemoglobin method. The hematological indices of mean cell haemoglobin concentration (MCHC: $g \cdot dL^{-1}$), meancell haemoglobin (MCH: pg) and meancell volume (MCV: fL) were calculated using the total RBC count, Hb concentration and Ht [16]. Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were calculated using the following formula [17].

$$\mathrm{MCV}\left(\mu m^3\right) = \left[(Hct,\%)\times 10\right] \Big/ \left(RBC,\times 10^6 \text{ per } \mathrm{mm}^3\right),$$

$$MCH\,(\mathrm{pg}) = \left[(Hb, g/dL)\times 10\right] \Big/ \left(RBC,\times 10^6 \text{ per } \mathrm{mm}^3\right),$$

and

$$MCHC\,(\%) = \left[(Hb, g/dL)\times 100\right] \Big/ (Hct,\%)$$

2.6. Immunological Analysis

2.6.1. Myeloperoxidase Activity

Total myeloperoxidase (MPO) content was measured according to Quade and Roth [18] with slight modification. A volume of 10 µl serum was diluted with 90 µl of Hank's balanced salt solution without Ca^2 or Mg^2 in 96 well plate. Then, 35 µl of 0.1 mg/mL (w/v) 3,3′,5,5′-tetramethylbenzidine dihydrochloride (TMB, Sigma-Aldrich, Munich, Germany) and 0.006% fresh hydrogen peroxide were added. The reaction was followed kinetically by measuring the increase of absorbance. Reaction velocities were determined as International Units, defined as the amount of enzyme required to produce an increase in absorbance of 0.001/min in 0.5 mL reaction mixture (ΔA 450/min/ml).

2.6.2. Lysozyme Activity

Serum lysozyme was assessed using the turbidometric assay [19]. The same amount of PBS was added onto the 100 µl serum sample. Then, 800 µl of *Micrococcus lysodeikticus* (Sigma, ATCC 4698) suspension at 0.2 mg/mL in PBS was added, and at the minutes 0.5 and 4.5, readings were taken from the spectrophotometer at 530 nm. The mixture was incubated at RT, and its OD was measured after 0.5 and 4.5 min at 530 nm using a spectrophotometer. One unit of lysozyme activity was defined as the amount of enzyme producing a decrease in absorbance of 0.001/min.

2.6.3. Serum Biochemical Analyses

Biochemical indices in serum including glucose (GLU), total protein (TPROT), albumin (ALB), triglyceride (TRI), cholesterol (CHOL) globulin (GLO) were determined using bioanalytic test kits (Bioanalytic Diagnostic Industry, Co) and measured by a spectrophotometer (PG Instruments, UK). Serum globulin was determined by the following formula; globulin = total protein − albumin.

2.6.4. Statistic

In this study, the variation analyses were carried out with Duncan multiple comparison tests, and the differences between groups were carried out via the use of SPSS 17 statistics program in order to evaluate the relationships between the data of blood, proximate composition and growth parameters obtained from the test group.

3. Results and Discussion

Growth results are presented in **Table 2**. No particular difference was found in the final weight gain, FCR and SGR ($P > 0.05$). In parallel, no significant difference was found between rainbow trout (IW: 111.8 g) fed with the carvacrol supplement at 12 g/kg and the control diet in terms of weight gain, although the carvacrol group had lower FCR than the control group [9]. Zheng *et al*. [5] reported that carvacrol (0.05%) significantly improved weight gain and FCR in rainbow trout (IW: 50 g). These results indicate that the action modes of different levels of carvacrol in small fish and big fish are different and this may result from physiological differences in different stages of the fish life cycle.

Table 2. Growth performance, feed utilization, and survival in rainbow trout that were fed diets containing different levels of carvacrol (0, 1, 3, or 5 g/kg of feed; diets Control, C1, C3, and C5, respectively) for 60 day.

	Control	C1	C3	C5
IW (g)	11.32 ± 0.27[a]	11.07 ± 0.54[a]	10.67 ± 0.16[a]	10.75 ± 0.29[a]
FW (g)	27.40 ± 0.57[a]	26.49 ± 0.21[a]	26.71 ± 0.67[a]	28.27 ± 0.35[a]
WG (g)	16.09 ± 0.78[a]	15.41 ± 0.64[a]	16.04 ± 0.63[a]	17.52 ± 0.49[a]
FCR	0.97 ± 0.05[a]	1.01 ± 0.13[a]	1.06 ± 0.05[a]	0.95 ± 0.05[a]
SGR (%/d)	1.47 ± 0.07[a]	1.45 ± 0.09[a]	1.53 ± 0.04[a]	1.61 ± 0.05[a]

Values are mean ± SE (n = 6). Within a row, means with differing letters are significantly different ($P < 0.05$).

The whole-body proximate compositions of fish at the end of the experiment are presented in **Table 3**. There was no significant difference related to protein, lipid, ash and moisture between the experimental group and the control group. Similarly, there was no significant difference in proximate composition of rainbow trout fed with the carvacrol supplement at 0.05 % in comparison with the control group [5].

It is often suggested that hematological parameters are useful stress or disease indicators for fish; and the changes in RBC count, Hct value, Hb value and erythrocyte indexes are important to detect organ health status [4] [20] [21]. The effects of carvacrol on rainbow trout hematological variables are presented in **Table 4**. The RBC count, Hb concentration, Hct, MCV, MCH, and MCHC in the treatment groups did not vary significantly from the values observed for the control group. In the published literature, no previous report written on dietary carvacrol's effects on the hematological parameters of fish was found. However, one study has reported that the combination of carvacrol and thymol additives (1, 2, or 3 g/kg) did not change hematological parameters (Hct, Hb concentration, RBC, MCV, MCH or MCHC) in rainbow trout [6].

Myeloperoxidase is contained in the polymorphonuclear neutrophils, monocytes, and macrophages [22]. It is known to participate to microbicidal activity and its activity gives clues about fish neutrophil ability to kill microorganisms [23]. An exceptionally widespread defence molecule lysozyme is important for protection against fish pathogen as it directly activates the polymorphonuclear leukocytes and macrophages or it promotes phagocytosis as an opsonin of freshwater and marine fish [24]. The myeloperoxidase activity in groups (C1 and C3) was significantly higher ($P < 0.05$) on the 30th day of sampling (**Figure 1**). Myeloperoxidase activity of C5 treated group was significantly ($P < 0.05$) higher on the 60th day in comparison with the control group. As compared to control group, a significant increase ($P < 0.05$) of serum lysozyme activity was observed in groups with the feed containing C3 and C5 groups after 30 days (**Figure 2**). However, lysozyme levels of carvacrol treated groups were not significantly ($P > 0.05$) different on the 60th day. Similarly, there was no significant difference in lysozyme levels of rainbow trout fed with the carvacrol supplement at 0.05% when compared to control group after 8 weeks [5]. Volpatti *et al*. [7] reported that when fish fed with 0.025% carvacrol for 1 to 4 week exhibited a similar trend in the level of lysozyme, but this parameter was significantly lower when compared with the control after 8 weeks. Feeding the diet which contains 0.05% carvacrol compared with the control diet resulted in a significant decrease of the lysozyme after 4 weeks, although the lysozyme didn't significantly change after 8 weeks.

In the present study and other similar studies showed that non-specific immune responses exhibited significant enhancement in their activity in different time of feeding with the carvacrol diet and didn't show any modulation of the immune parameters on all sampling days. It is known that the fish innate immune system lacks memory, the duration of its response is always shorter than the specific system [25]. So, each activity shows peaks that disappear in time and that may not coincide with magnitude or time together with the peaks of other activities [26] [27].

Serum glucose has often been suggested as a useful nonspecific stress indicator [28]. The present study showed that the GLU levels (**Table 5**) significantly decreased on 60th day for C1, C3 and C5 treated groups when compared to control groups ($P < 0.05$). The decreased glucose levels might be associated with hypoglycemic effects of carvacrol [29].

The increase of serum protein, albumin and globulin levels indicates the increase in innate immune response of fish [4] [30]. The level of TPROT and GLO in treatment groups was insignificant on 30th day of sampling. However, TPROT and GLO levels of C3 treated groups were significantly ($P < 0.05$) higher on the 60th day in

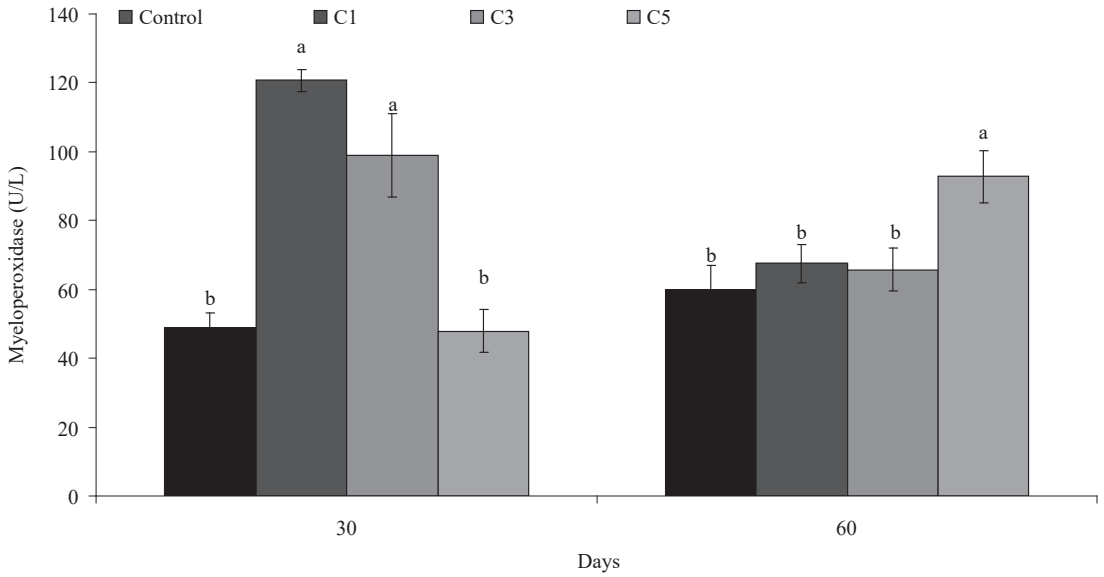


Figure 1. Changes of myeloperoxidase activity in rainbow trout fed with carvacrol diets as compared to control diet on days 30 and 60. Data represent the mean ±SE. Data in the same row with different superscript are significantly different ($P < 0.05$). (See **Table 2** for definition of treatment codes).

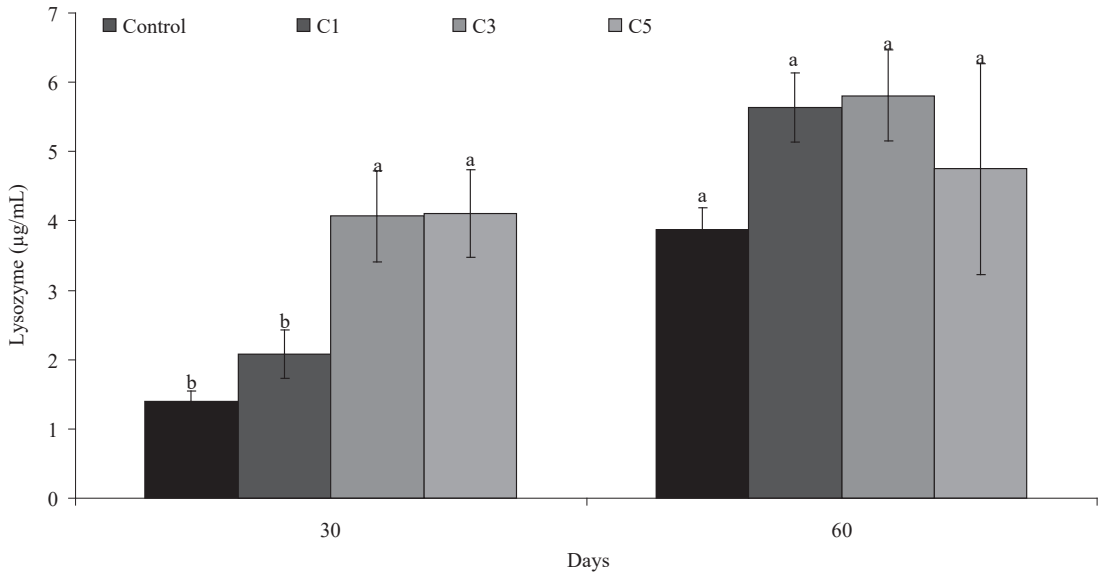


Figure 2. Changes of lysozyme activity in rainbow trout fed with carvacrol diets as compared to control diet on days 30 and 60. Data represent the mean ± SE. Data in the same row with different superscript are significantly different ($P < 0.05$). (See **Table 2** for definition of treatment codes).

Table 3. Whole-body proximate composition (%) of rainbow trout fed diets with different levels of carvacrol for 60 day.

Composition (%)	Control	C1	C3	C5
Protein	17.81 ± 0.58^a	18.02 ± 0.47^a	16.51 ± 0.58^a	16.78 ± 0.62^a
Lipid	6.28 ± 0.80^a	5.56 ± 0.29^a	6.66 ± 0.61^a	6.07 ± 0.18^a
Ash	2.72 ± 0.10^a	2.58 ± 0.08^a	2.60 ± 0.02^a	2.51 ± 0.04^a
Moisture	72.94 ± 1.30^a	73.10 ± 0.03^a	73.22 ± 0.41^a	73.22 ± 0.15^a

Values are mean ± SE (n = 6). Within a row, means with differing letters are significantly different ($P < 0.05$). (See **Table 2** for definition of treatment codes).

Table 4. Hematological parameters in rainbow trout that were fed diets containing different levels of carvacrol for 60 day.

Blood Parameter	Control	C1	C3	C5
Hb ($g \cdot dL^{-1}$)	5.07 ± 0.15^{a}	5.26 ± 0.18^{a}	4.87 ± 0.16^{a}	5.00 ± 0.37^{a}
Hct (%)	35.60 ± 0.42^{a}	37.80 ± 0.30^{a}	37.00 ± 0.58^{a}	36.60 ± 0.67^{a}
RBC ($10^{6}mm^{3}$)	3.55 ± 0.06^{a}	3.78 ± 0.06^{a}	3.70 ± 0.07^{a}	3.65 ± 0.09^{a}
MCV (fL)	100.42 ± 0.43^{a}	100.14 ± 0.56^{a}	100.03 ± 0.49^{a}	100.21 ± 1.00^{a}
MCH (pg)	14.31 ± 0.50^{a}	13.93 ± 0.44^{a}	13.19 ± 0.49^{a}	13.66 ± 0.96^{a}
MCHC ($g \cdot dL^{-1}$)	14.25 ± 0.48^{a}	13.92 ± 0.45^{a}	13.18 ± 0.46^{a}	13.67 ± 1.02^{a}

Hb, haemoglobin; Hct, hematocrit; RBC, red blood cells; MCV, mean cell volume; MCH, mean cell haemoglobin; MCHC, mean cell haemoglobin concentration. Data represent as mean ± SE. Within a row, means with differing letters are significantly different ($P < 0.05$). (See **Table 2** for definition of treatment codes).

Table 5. Changes of serum biochemical parameters in rainbow trout fed withcarvacroldiets as comparedtocontroldiet on days 30 and 60.

	Days	Control	C1	C3	C5
GLU (mg/dL)	30	119.26 ± 5.28^{a}	141.12 ± 8.67^{a}	139.42 ± 17.52^{a}	136.93 ± 11.06^{a}
	60	116.83 ± 6.63^{a}	63.18 ± 4.16^{b}	59.90 ± 5.28^{b}	54.88 ± 8.63^{b}
TPROT (g/dL)	30	8.72 ± 0.87^{a}	10.63 ± 1.07^{a}	10.21 ± 0.39^{a}	10.02 ± 0.87^{a}
	60	7.89 ± 0.34^{bc}	9.27 ± 0.52^{b}	11.25 ± 0.61^{a}	7.40 ± 0.53^{c}
ALB (g/dL)	30	3.06 ± 0.31^{a}	2.93 ± 0.29^{a}	3.07 ± 0.34^{a}	3.58 ± 0.54^{a}
	60	2.43 ± 0.06^{a}	2.57 ± 0.11^{a}	2.22 ± 0.04^{a}	2.39 ± 0.16^{a}
GLO (g/dL)	30	5.65 ± 0.91^{a}	7.71 ± 1.01^{a}	7.14 ± 0.38^{a}	6.44 ± 0.87^{a}
	60	5.47 ± 0.36^{bc}	6.70 ± 0.47^{b}	9.03 ± 0.59^{a}	5.01 ± 0.56^{c}
TRI (mg/dL)	30	124.11 ± 12.64^{a}	130.85 ± 12.29^{a}	121.81 ± 13.40^{a}	112.32 ± 17.01^{a}
	60	161.91 ± 12.76^{b}	151.28 ± 12.21^{b}	215.96 ± 17.76^{a}	154.89 ± 8.35^{b}
CHOL (mg/dL)	30	253.07 ± 12.31^{a}	260.33 ± 10.21^{a}	253.33 ± 11.02^{a}	153.53 ± 15.76^{b}
	60	178.08 ± 12.16^{a}	187.20 ± 8.84^{a}	178.56 ± 9.23^{a}	192.24 ± 10.23^{a}

Values are mean ± SE (n = 6). Within a row, means with differing letters are significantly different ($P < 0.05$). (See **Table 2** for definition of treatment codes).

comparison with the control, C1 and C5 treated groups. In addition, serum ALB levels were similar in all treatment groups.

Major blood lipid components, such as triglycerides, phospholipids, and cholesterol are affected by diet and stress levels in fish [31]. The level of TRI in treatment groups was insignificant on 30th day of sampling ($P > 0.05$). However, TRI levels of C3 treated groups were significantly ($P < 0.05$) higher on the 60th day in comparison with the control, C1 and C5 treated groups. Carvacrol can enhance dietary lipid emulsification and transportation of absorbed lipids into the serum. Although more detailed studies are required for a more clear understanding. Because, the level of CHOL in C5 treated groups was significantly ($P < 0.05$) lower on the 30th day in comparison with the control and other carvacrol treated groups. Furthermore, the level of CHOL in treatment groups was insignificant on 60th day of sampling ($P > 0.05$).

4. Conclusion

In conclusion, the results of the present study indicate that dietary supplementation of 3 g/kg of carvacrol in commercial diets could improve some non-specific immune and biochemical status in rainbow trout, without

adversely affecting hematological parameters, growth performance or feed utilization. Thus, carvacrol can be utilized as health promoter in fish culture. Further investigation on the potential effects of carvacrol on control of trout diseases is encouraged.

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The Effects of Exercise on Serum Chemistry, Non-Esterified Fatty Acid, Insulin and Glucagon Dynamics during a 400 Meter Sprint in Racing Greyhounds

Michael J. Bell[1], Emily M. Donaldson[2], Sabine Mann[3], Robert L. Gillette[4], Corri B. Levine[2], Joseph J. Wakshlag[2*]

[1]Craigieburn Animal Hospital, 9 Craigieburn Rd., Craigieburn, Australia

[2]Cornell University College of Veterinary Medicine, Department of Clinical Sciences, Ithaca, NY, USA

[3]Cornell University College of Veterinary Medicine, Department of Population Medicine, Ithaca, NY, USA

[4]Red Bank Referral Center, Sports Medicine and Rehabilitation Service, Red Bank, NJ, USA

Abstract

The effects of Greyhound racing on plasma insulin, glucagon and non-esterified fatty acid have not been extensively studied. We sought to examine the effects of exercise in 19 greyhounds trialed over 400 meters on serum biochemistry, osmolality, nonessential fatty acids, and the major gluco-regulatory hormones insulin and glucagon, before and after racing. Due to the non-traditional dietary patterns of many racing Greyhounds, the diet was analyzed for macro and micro-nutrient assessment to determine adequacy of the diet. Many significant biochemical changes were observed and agreed with previous studies, except for low resting glucose, low post-exercise globulin and phosphorus concentrations, and a high CK both before and after exercise. Insulin concentrations increased 10 minutes post-exercise which might be a result of the rise in serum glucose observed. Non-esterified fatty acids decreased 10 minutes post-exercise, as a likely result of the insulin facilitated cellular uptake of fatty acids early in recovery. Glucagon concentrations did not change after exercise despite the rise in insulin. The diet analysis found some mineral deficiencies in sodium, selenium, iodine and copper; and contained a far higher protein and fat content, being atypical of diets that have been shown to improve the performance in racing Greyhounds.

Keywords

Greyhound, Insulin, Glucagon, Non-Esterified Fatty Acid, Glucose

1. Introduction

Greyhound racing continues to be a popular sport in Australia, and portions of Europe, and the United States. The racing Greyhound has been a common sprinting dog prototype for evaluation of hemodynamic and intramuscular changes in relationship to exercise [1]-[8]. Commonly observed changes include significant shifts in acid-base balance and electrolyte concentrations [1]-[8]. Elevated osmolality and hematocrit are seen after racing in nearly every study [1]-[7]; however, these changes are transient and return to resting (or below resting) within 1 hour post-racing [5] [7]. The predominant theory is that intravascular fluid moves into the extravascular space during this strenuous exercise and leads to hemoconcentration [2]-[7]. However, it is possible that the increase in hematocrit is due to dehydration [5] [7] or splenic contraction [1] [2] [5] [8] [9].

To date, few studies have focused on glucose homeostasis and its relationship to sprinting dogs [10]. In general, dogs are considered as highly aerobic animals which has major implications for energy utilization during exercise [11]-[13]. In dogs, regulation of glucose production shifts from glucagon to catecholamines when performing high intensity exercise [10]. This change in glucose regulation is well documented in humans where a catecholamine spike occurs with a concomitant rise in insulin during intense exercise that dramatically declines during recovery [14].

The resting blood glucose observed in racing Greyhounds has been highly variable, between 60 - 100 mg/dL at rest, and the diet composition is often not reported [1] [5] [7] [8]. When examining the literature many studies performed by Hill and colleagues [4] utilized retired Greyhounds being fed commercial dog food, while the prior studies in the field do not report the dietary constituents or the nutrient composition of the diet fed during the trials, which may be influencing the resting glucose homeostasis [1]-[5] [7] [8]. This makes it difficult to fully understand the glucose dynamics observed across these studies. In humans as well as in dogs, hyperglycemia can occur after short intense bouts of exercise and has been observed in Greyhounds. Decreases in serum insulin have been observed in sled dogs and foxhounds immediately after exercise, yet whether or not insulin or glucagon changes occur in racing Greyhounds after sprinting has not been reported [2]-[5] [7] [14] [15].

Conversely, fat metabolism has not been thought to be a major component of energy regulation in racing Greyhounds. However, McClelland and colleagues studied the capability of dogs (breed unspecified) to utilize fatty acids during exercise of varying intensity [13]. They observed that blood fatty acid concentrations increase during periods of exercise in dogs, which was presumed to be due to increased binding of fatty acids to albumin during lipolysis. A single Greyhound study has shown a rise in free fatty acids which can be partially attributed to hemoconcentration as well as increased lipolysis [8]. Changes in non-esterified fatty acid (NEFA) concentrations have been reported to increase or decrease depending on duration of running, with sprinting activities showing some discrepancy regarding increases and decreases due to exercise [8] [13] [16].

The aim of our study was to examine the serum chemistry, NEFA, osmolality and the major glucoregulatory hormones (plasma insulin and glucagon) in competitive racing Greyhounds that are fed a typical non-commercial ration, similar to what is fed in many kennels (chicken, horse meat, bread and supplements).

2. Materials and Methods

2.1. Greyhound Trial and Collections

The exercise and sampling was approved by the Cornell University Institutional Animal Care and Use Committee. Nineteen Greyhounds from a single competitive kennel managed similarly to other regional kennels, were enrolled in the study with informed owner consent. All dogs were between the age of 16 and 20 months old with 11 being intact females and 8 being intact males undergoing conditioning for competitive Greyhound racing. The trial started at 8 am (12˚C) and all exercise was finished by 9:30 am (12˚C) with the last blood draws occurring by 11:30 am (14˚C). Relative humidity during the trial was 95%. All dogs were fasted before the trial with their past meal being 4 pm the evening before the trial, and no dogs had been trained within 72 hours of the trial. Ten milliliters of whole blood was collected from each Greyhound at three time points: prior to the exercise bout in the kennel environment which was 200 meters away from the track with no visual contact with any activities on the track, 10 minutes post exercise at the track, and 120 minutes post exercise at the kennel. In all cases, the distance trialed was 400 meters on an oval dirt track chasing a lure individually. Body condition score on a 1 - 9 scale [17] was also assessed and recorded. All samples were collected from the cephalic vein and immediately placed in 10 ml lithium heparin vacutainer blood tubes. They were then transported back to the clinic within 10

minutes and centrifuged at 3800 g for 5 minutes and minimally 0.5 milliliters of separated plasma from each sample was placed into each of 3 plastic polypropylene collecting vials. Samples were then frozen at −4˚C until transportation approximately 1 week later on dry ice to the principle investigators laboratory where the samples were stored at −80˚C for approximately 1 week before analyses.

2.2. Plasma Analyses

Serum biochemical analytes (sodium, chloride, potassium, bicarbonate, calcium, phosphorus, magnesium, glucose, total protein, albumin, globulin, creatinine, urea, cholesterol, alanine aminotransferase [ALT], aspartate aminotransferase [AST], and creatine kinase [CK]) were analyzed at baseline, 10 minutes after exercise and 120 minutes after exercise using a Olympus AU5400 automated analyzer (Olympus America, Quakertown, PA). Plasma osmolality was assessed at baseline and 10 minutes after exercise using the Advanced 3250 Osmometer (Advanced Inc., Norwood, MA). Concentration of NEFA was analyzed for all samples in duplicate by colorimetric measurement of an enzymatic reaction according to the manufacturer's directions (HR Series NEFA-HR [2]; Wako Life Sciences, Mountain View, CA) with a microplate spectrophotometer (Epoch, Biotek, Winooski, VT). Intra- and inter-assay CV were 4.9 and 7.4%, respectively; and all samples were run on three plates. Insulin and glucagon concentrations were measured using competitive radioimmunoassays at the certified New York State Diagnostic Laboratory at Cornell University (Porcine Insulin RIA, Millipore, Concord MA). All samples for insulin were run in two assays. Intra- and interassay coefficients of variation were 4.5% and 5.0%, respectively. Glucagon was determined for all samples in duplicate using a radioimmunoassay kit according to manufacturer instruction (Glucagon RIA kit, Millipore, St. Charles, MO). In-house canine control samples were run every 28 samples. All samples were run in two assays. Intra- and interassay (CV) was determined as 3.9 and 4.9%, respectively.

2.3. Statistical Analyses

Data was assessed for normalcy using a Shapiro Wilkes test, which revealed that a majority of the data was normally distributed except for NEFA, insulin and glucagon analyses. For the serum chemistry parameters, a one-way analysis of variance was performed with a Tukey's post hoc analysis to determine differences between resting (time 0), 10 minutes after exercise and 120 minutes after exercise. Lack of normality in NEFA, glucagon and insulin led to Friedman's analysis of variance with Tukey's post hoc analysis. Statistical significance was set at $p < 0.05$ for all testing.

2.4. Diet Analyses

A typical meal for one Greyhound was made and a sample of the diet was sent in for a basic macronutrient and mineral analysis through the Australian Government National Measures Institute. Substrates assessed were crude protein, crude fiber, crude fat, nitrogen free extract and total kilojoules. Minerals assessed were calcium, phosphorus, sodium, potassium, magnesium, iron, zinc, manganese, and copper. Further vitamin, selenium and iodine content of the data were assessed utilizing the United States Department of Agriculture Nutrient Database to complete the dietary analysis.

3. Results

3.1. Plasma Electrolytes and Osmolality

Plasma electrolytes exhibited a range of changes (**Table 1**). Plasma sodium showed a mild yet significant increase after exercise ($p < 0.01$) and by 120 minutes after exercise the plasma sodium concentrations fell below the resting concentrations ($p < 0.01$). Potassium concentrations in plasma were no different between resting and 10 minutes after exercise, but were decreased from baseline by 120 minutes after exercise ($p < 0.01$). Chloride showed a decrease 10 minutes after exercise when compared to baseline, but not 120 minutes after exercise ($p < 0.01$). Plasma bicarbonate concentrations were markedly decreased 10 minutes after exercise when compared to baseline and 120 minutes after exercise ($p < 0.01$). Calcium in the plasma was significantly higher at 10 minutes after exercise ($p < 0.01$), while it was even higher at 120 minutes post exercise ($p < 0.01$). Plasma phosphorus was significantly lower when comparing baseline to 10 minutes post exercise ($p < 0.01$) and recovered to

Table 1. Serum chemistry mean and standard deviations before, 10 minutes after a 400 meter run and 120 minutes after exercise in 19 Greyhounds. [a]indicates a significant difference between baseline racing samples ($p < 0.05$). [b]indicates a significant difference between 10 minutes after racing and 120 minutes post racing ($p < 0.05$).

	0 minutes	10 minutes	120 minutes
Na (142 - 150 mEq/L)	150.2 ± 2.7	152.7 ± 2.9[a]	146.3 ± 3.5[ab]
K (3.8 - 5.4 mEq/L)	5.4 ± 0.3	5.3 ± 0.2	4.5 ± 0.4[ab]
Cl (105 - 116 mEq/L)	104.2 ± 3.2	99.6 ± 2.4[a]	104.2 ± 2.8[b]
Bicarbonate (mEq)	20.1 ± 2.3	9.1 ± 4.7[a]	19.4 ± 2.0[b]
Ca (9.3 - 11.4 mg/dL)	7.7 ± 0.5	9.0 ± 1.4[a]	10.1 ± 0.6[ab]
P (2.9 - 5.2 mg/dL)	3.8 ± 0.7	2.6 ± 0.4[a]	3.6 ± 0.5[b]
Mg (1.4 - 2.2 mEq/L)	1.8 ± 0.3	1.8 ± 0.2	1.7 ± 0.1
Total Protein (5.3 - 7 g/dL)	6.1 ± 0.8	6.4 ± 0.6	5.5 ± 0.3[ab]
Globulin (1.9 - 3.6 g/dL)	2.5 ± 0.9	2.3 ± 0.4	1.9 ± 0.2[ab]
Albumin (3.1 - 4.2 g/dL)	3.6 ± 0.3	4.1 ± 0.3[a]	3.6 ± 0.2[b]
BUN (10 - 32 mg/dL)	21.6 ± 4.3	21.9 ± 4.2	19.9 ± 3.9[ab]
Creatinine (0.6 - 1.4 mg/dL)	1.6 ± 0.2	1.7 ± 0.2[a]	1.7 ± 0.2
ALT (20 - 98 U/L)	65.2 ± 10.4	80.5 ± 16.5[a]	68.2 ± 11.7[b]
AST (14 - 51 U/L)	85.6 ± 60.0	76.4 ± 35.7	65.2 ± 32.5[b]
ALP (17 - 111 U/L)	32.8 ± 17.4	43.6 ± 17.6[a]	37.7 ± 12.6[ab]
CK (48 - 261 U/L)	484.1 ± 394.6	469.1 ± 487.6	347.6 ± 432.6[b]

baseline concentrations by 120 minutes after exercise. Magnesium concentrations showed no significant difference across all three time points. Plasma osmolality was performed only before and 10 minutes after simulated racing and was significantly increased from 304.5 ± 5.8 to 317.5 ± 13.7 mOsm/L ($p < 0.01$).

3.2. Plasma Proteins, Urea and Creatinine

Plasma total protein concentrations were modestly, but significantly decreased 120 minutes after exercise compared to 10 minutes after exercise ($p < 0.01$) as well as baseline concentrations ($p < 0.01$). The plasma globulins showed a similar pattern to total protein with the only significance being a difference at 120 minutes after exercise from the prior two blood draws ($p = 0.02$). Plasma albumin had a significant rise 10 minutes after exercise ($p < 0.01$) which returned to baseline within 120 minutes. Renal parameters were examined; plasma urea nitrogen showed no significant differences before and 10 minutes after exercise, however there was a significant decrease ($p < 0.01$) between 120 minutes post exercise and prior time points examined. The plasma creatinine showed a mild significant increase 10 minutes after exercise ($p < 0.01$) when compared to before or 120 minutes after exercise (**Table 1**).

3.3. Plasma Enzymes

Plasma liver enzymes revealed changes due to exercise (**Table 1**). ALT concentrations significantly increased 10 minutes after exercise ($p < 0.01$) and returned to baseline within 120 minutes of exercise. Alkaline phosphatase activity was modestly increased from baseline ($p < 0.01$) 10 minutes after exercise and remained elevated at 120 minutes after exercise, but was mildly decreased from immediately after exercise ($p < 0.01$). Plasma AST and CK as markers of muscle cell permeability were not elevated from baseline 10 minutes after exercise and were significantly decreased from baseline by 120 minutes post exercise ($p < 0.01$).

3.4. Plasma Insulin, Glucagon, NEFA and Glucose

Plasma glucose concentrations were markedly increased 10 minutes after exercise (81.7 ± 27.3 mg/dL), when compared to before exercise (48.8 ± 9.5 mg/dL) and remained elevated at 120 minutes post exercise (74.5 ± 14.2; $p < 0.01$; **Figure 1**). The serum concentration of NEFA at baseline was 942.9 ± 242.7, while 10 minutes after exercise it was 244.0 ± 94.2 mEq/L ($p < 0.01$) and returned to baseline at 120 minutes after exercise at 922.4 ± 214.5 mEq/L (**Figure 2**). The serum insulin also increased from 7.1 ± 1.7 uIU/mL at baseline to 10.8 ± 7.2 uIU ($p < 0.03$) 10 minutes after racing, and returned below baseline at 4.0 ± 2.4 uIU by 120 minutes post exercise ($p < 0.01$; **Figure 3**). The mean serum glucagon concentration at baseline was 68.1 ± 24.0 pg/mL, 10 minutes after exercise it was 63.3 ± 20.3 pg/mL, and 120 minutes after exercise was 58.9 ± 20.8 pg/mL showing no significant difference between time points (**Figure 4**).

3.5. Diet Analysis

A daily portion of the diet for the average Greyhound was made containing 500 grams of ground whole chicken,

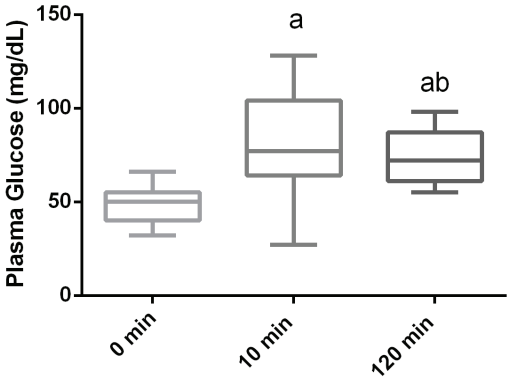


Figure 1. Box and whisker plots of plasma glucose concentrations immediately before 400 meter sprint and 10 minutes and 120 minutes after exercise in 19 Greyhounds. Boxes represent the median, 25th and 75th percentiles while the whiskers represent the minimums and maximums. a—represents a significant difference ($p < 0.05$) from 0 minutes (resting). b—represents a significant difference ($p < 0.05$) from 10 minutes after exercise.

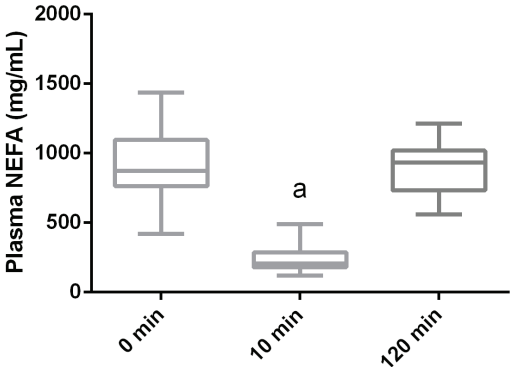


Figure 2. Box and whisker plots of plasma non-esterified fatty acids (NEFA) concentrations immediately before 400 meter sprint and 10 minutes and 120 minutes after exercise in 19 Greyhounds. Boxes represent the median, 25th and 75th percentiles while the whiskers represent the minimums and maximums. a—represents a significant difference ($p < 0.05$) from 0 minutes (resting).

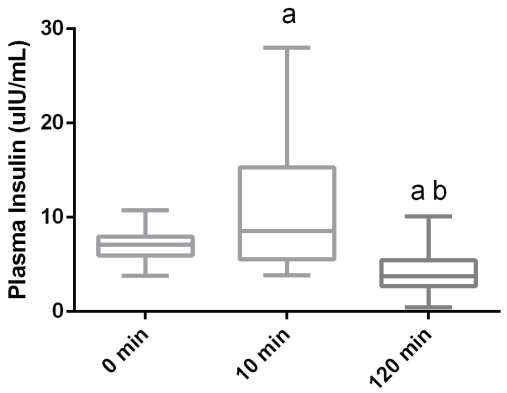


Figure 3. Box and whisker plots of plasma insulin concentrations immediately before 400 meter sprint and 10 minutes and 120 minutes after exercise in 19 Greyhounds. Boxes represent the median, 25th and 75th percentiles while the whiskers represent the minimums and maximums. a—represents a significant difference ($p < 0.05$) from 0 minutes (resting). b—represents a significant difference ($p < 0.05$) from 10 minutes after exercise.

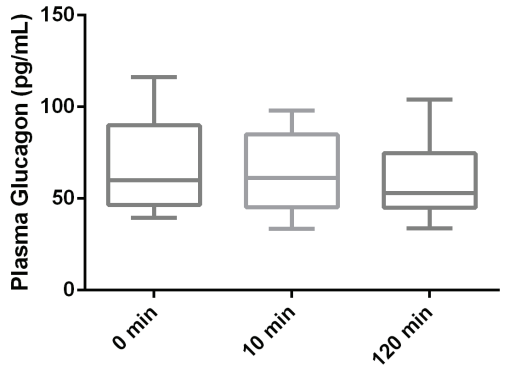


Figure 4. Box and whisker plots of plasma glucagon concentrations immediately before 400 meter sprint and 10 minutes and 120 minutes after exercise in 19 Greyhounds. Boxes represent the median, 25th and 75th percentiles while the whiskers represent the minimums and maximums.

500 grams of ground horse meat, 4 slices of whole wheat bread and 4 grams of Ranvet B Canine Multivitamin/Mineral supplement (Ranvet Corp, Banksmeadow NSW, Australia). The dietary constitution is reported in **Table 2** based on dry matter and is adjusted for a typical 4000 kcal diet. This adjustment was performed to allow for comparison to the National Research Council recommended daily intake based on dry matter content of a typical 4000 kilocalorie diet. Based on the analysis, this average daily ration contained approximately 2290 kilocalories and the metabolizable energy from protein, fat and carbohydrate were 42, 41 and 17%, respectively. When compared to the NRC recommended daily allowances of vitamins and minerals the diet was shown to be deficient in vitamin A, vitamin D, choline, sodium, iodine, selenium and copper (**Table 2**).

4. Discussion

Many of the serum biochemical alteration due to exercise were similar to those observed in other Greyhound studies [3] [5] [17] [19], showing rises in sodium and decreases in bicarbonate and chloride in the initial 10 minutes after exercise; while some studies found no alterations in chloride or potassium [4] [5]. The change in sodium is likely related to the increased osmolality post-exercise, while bicarbonate would be expected to fall as a compensatory mechanism for expected post-exercise lactic acidosis and rapid exchange of carbon dioxide due

Table 2. Dietary analysis and calculation for the diet fed to the Greyhounds in this study compared to the National Research Council's recommended daily intake of essential nutrients based on a 4000 kcal diet. Nutrient intake for the diet (4660 kcals/kg) was adjusted for the increased caloric content of the diet being fed. Vitamin K and biotin were not included due to gastrointestinal microbe contributions that cannot be accounted for.

	Diet Dry Matter	NRC Dry Matter
Protein (g)	360.0	100
Fat (g)	193.7	25
Carbohydrate (g)	180.8	NR
Crude Fiber (g)	4.1	NR
Calcium (g)	15.3	4
Phosphorus (g)	11.3	3
Magnesium (g)	0.9	0.6
Sodium (mg)	378.9	800
Potassium (g)	6.3	4
Iron (mg)	112.8	30
Zinc (mg)	166.9	60
Copper (mg)	4.2	6
Manganese (mg)	20.8	4.8
Selenium (ug)	64.8	350
Iodine (ug)	333.3	880
Vitamin A (IU)	3245.6	4545
Vitamin E (IU)	72.1	30
Vitamin D (IU)	433.0	552
Thaimine (mg)	352.1	2.3
Niacin (mg)	58.4	17
Riboflavin (mg)	9.1	5.3
Pantothenate (mg)	20.4	15
pyridoxine (mg)	4.7	1.5
Folic acid (ug)	436.8	270
Cobalamin (ug)	34.9	35
Choline (mg)	476.0	1,700

NR – no requirements.

to panting [2] [19] [20]. Lactate concentrations were not recorded in this study, but were expected to rise precipitously due to the 400 meter sprint, creating some acidosis and a reciprocal decrease in plasma chloride observed. This accumulation of unmeasured anions, such as lactate, leads to a fall in the measured anions, chloride and bicarbonate, in order to maintain electroneutrality.

Within 120 minutes post-exercise sodium concentrations returned to below baseline, probably as a result of rehydration from post-exercise water consumption and re-establishment of intracellular and extracellular fluid homeostasis. Plasma potassium was no different after exercise, but was lower than baseline at the post 120 minutes most exercise time point. After exercise the potassium is expected to be elevated mildly due to hemoconcentration, however, the short 10 minute delay in blood collection after exercise may have been enough recovery time to allow for potassium normalization which is the time frame for normalization observed in other studies [5] [8].

Serum urea and creatinine changed little post-exercise, while others have shown more dramatic rises within the first 5 minutes after exercise [3] [5] [8]. Our less dramatic increase might be associated with plasma osmolality rise being approximately half of that observed in prior studies [5] [8]. Again, this might be a reflection the blood draw being 10 minutes after exercise allowing for skeletal muscle recovery and re-establishing of intracellular and extracellular fluid dynamics.

Contrary to other reports, our study showed that globulin concentrations decreased after exercise, especially by the 120 minutes mark [2] [6]. Globulin concentrations would have been expected to rise with the plasma fluid shifts, but did not. Similarly there were no rises in serum CK after exercise, yet the baseline concentrations were elevated in this group of dogs. We could not attribute this high CK to recent exercise, since the dogs were last exercised 3 days or prior to this simulated event. Resting CK values in other more recent studies in Greyhounds show values approximately half of what we observed [3] [4], suggesting some differences in kennel management of this particular population of Greyhounds. Although hemolysis can affect serum CK, the amount of hemolysis in a majority of the samples was minimal and should have had little to no significant effect [21]. In addition, serum phosphorus was depressed after exercise which is thought to be due to intracellular increases needed during glycolysis; however many studies have shown differing results which could be dietary relationships as well [3] [6] [8].

Further discrepancies in the blood work may be the result of dietary patterns, particularly due to the numerous deficiencies we observed in this diet. Prior field studies made no dietary assessment in relationship to the results reported [1] [5] [7] [8]. In this study, mineral deficits in copper, selenium and iodine were evident when compared with the National Research Council's (NRC) recommended daily intake [18]. The copper deficits were mild and likely insignificant since the copper content was around 70% of NRC recommended daily intake which would be considered a minimum requirement; while the selenium deficit was more substantial leading to concerns related to antioxidant pathway sufficiency and might be related to the smoldering elevation in CK in these dogs [24]. Further examination of antioxidant status utilizing a diet of this nature is warranted on properly collected and stored serum or plasma samples. In addition, the inadequate dietary iodine intake has not been previously reported in the Greyhound literature; however low serum thyroid hormone concentrations in kennels of racing Greyhounds has been reported [22] [23]. In light of these findings, we speculate that some of the low serum thyroid concentration reported could be related to low iodine intake in populations of racing Greyhounds.

Insufficiency in dietary vitamin A and D were also noted, yet these concentrations in the in the diet were close to the presumed minimum adequate intake of these vitamins [25]. Choline intake was only around 25% of the adequate intake for dogs. Yet, choline hepatic synthesis can occur when dogs consume a high protein diet [25]. This diet supplied approximately 6 - 8 grams of protein per kilogram body weight which is 3 times the current NRC protein requirement, therefore hepatic choline synthesis might be adequate to make up for the low dietary intake. Equally concerning is the sodium deficiency in the diet. Although sodium is highly regulated through the renin-aldosterone angiotensin system, there is concern that the intake could result in clinical deficiency, yet plasma sodium was not perturbed in this group of Greyhounds. This is likely due to the fact that deficiencies noted when consuming 0.16 g/kg dry matter or less in feed include obscure clinical signs such as polyuria, polydipsia, and increase heart rate. These dogs were consuming about twice that amount [25]. In addition, the higher kilocalorie consumption for daily energy requirements in Greyhounds leads to a 20% increase in consumption of all the mineral and vitamins intakes recommended [4], therefore many of the vitamin and mineral intakes might have been adequate due to the slightly higher energy intake of these dogs when compared with normal house dwelling domestic pet dogs.

The most striking glucoregulation difference observed in these dogs was the low resting blood glucose concentrations, compared to Greyhounds on higher carbohydrate commercial diets [3]-[5]. This lower resting plasma glucose concentration suggests some fundamental dietary differences. Hill and colleagues have shown that a high carbohydrate moderate protein diet may be beneficial to racing times in Greyhounds [3]. Greyhound trainers in Australia have fed diets similar to the one utilized in this study for many years, and in most cases have been reluctant to change despite the reported literature [26]. To our knowledge this is the first study to detect such a significant insulin rise post-exercise. Previous studies have reported that catecholamines increase during exercise and are responsible for regulation of hepatic glucose production [8] [10] [14]. Catecholamines would initially be expected to inhibit insulin-mediated glycogenesis [27] [28] [29], hence an insulin surge would not be expected until there was a catecholamine induced rise in glucose. This is likely the case as we observe an increase in glucose with a concomitant rise in plasma insulin concentrations 10 minutes post-exercise, with insulin

being required to help transport the increased plasma glucose and free fatty acids intracellularly. This hyperglycemic-hyperinsulinemic response after glycogen-depleting exercise would create the appropriate milieu for restoration of muscle glycogen and energetics [1]. At 120 minutes after exercise there is a marked decrease in resting serum insulin concentrations to below baseline; most likely due to their not being fed at any time during that post-exercise interval.

Glucagon concentrations remained stable throughout the testing period. It is entirely possible that the bout of exercise was not long enough to stimulate the typical rise in glucagon for glycogenolysis or reciprocal decrease in the face of an insulin rise. Thirty minutes of modest exercise in sled dogs induces a rise in serum glucagon to promote glycogenolysis [30]. Our data suggest that the effects of glucagon in early regulation of hepatic glycogenolysis during short anaerobic activity are minimal to non-existent.

Non-esterified fatty acids have been reported to increase immediately after exercise [8], yet we showed a decrease in plasma NEFA at 10 minutes post-exercise. As previously mentioned, the catecholamine increase associated with strenuous exercise would have been expected to cause hepatic glycogenolysis and lipolysis leading to the release of glucose and free fatty acids into the circulation more rapidly than taken up by the working muscle. This could explain Snow *et al.*'s findings of elevated NEFA immediately post exercise [8]. The insulin surge that we detected 10 minutes post-exercise would then help to drive these fatty acids into the cells, hence explaining the subsequent fall in NEFA detected in this study.

Overall this work captures a distinct phase in elevated insulin concentrations which are responding to rises in serum glucose that are likely due to hepatic glycogenolysis which is expected after sprinting anaerobically. Surprisingly, glucagon is not responsive to short anaerobic activity. The plasma glucose at rest was lower than typical domestic dogs and increased significantly after exercise as a response to hepatic and muscle glycogenolysis associated with the acute bout of exercise and subsequent catecholamine surge. Ten minutes after exercise there is a pronounced decrease in non-esterified fatty acids, which may be due to insulin response in skeletal muscle promoting intracellular uptake for fuel. Serum chemistry parameters were typical for post exercise short anaerobic activity in Greyhounds except for a persistently elevated creatine kinase at all time points and decreases in plasma globulins well after recovery from exercise. The reasons for these differences observed are unknown; however, there were multiple subclinical deficiencies found in the raw meat-based diet formulation, including selenium which is involved in skeletal muscle integrity and antioxidant status.

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18

Effects of High Dietary Fluoride on Serum Biochemistry and Oxidative Stress Parameters in Broiler Chickens

Yubing Deng, Hengmin Cui*, Xi Peng, Jing Fang, Zhicai Zuo, Junliang Deng, Qin Luo

Key Laboratory of Animal Diseases and Environmental Hazards of Sichuan Province, College of Veterinary Medicine, Sichuan Agricultural University, Ya'an, China

Abstract

The aim of this study was to investigate the effects of high dietary fluoride (F) on serum biochemistry and oxidative damage in broiler chickens. 280 one-day-old healthy avian broiler chickens were randomly allotted into four equal groups and fed with a corn-soybean basal diet containing 22.6 mg·F/kg (control group) or same basal diets supplemented with 400, 800, and 1200 mg·F/kg (high F groups I, II, and III) in the form of sodium fluoride for 42 days. At 42 days of age, the serum F content was markedly higher in the three high F groups than that in the control group. From 28 to 42 days of age, the contents of serum total protein (TP) and albumin (ALB) were significantly lower in the three high F groups. From 14 to 42 days of age, the activities of alkaline phosphatase (ALP), alanine aminotransferase (ALT) and the creatinine (Crea) contents in the serum showed a marked increase in the three high F groups; aspartate aminotransferase (AST) activity and uric acid (Ua) content were significantly increased, and a significant increase in the content of malondialdehyde (MDA) along with marked decreases in the activities of total superoxide dismutase (T-SOD), glutathione peroxidase (GSH-Px), catalase (CAT), the glutathione (GSH) content and the ability to inhibit hydroxyl radical were observed in the high F groups II and III. In conclusion, F has accumulated in the blood circulatory system and dietary F in the range of 800 - 1200 mg/kg could significantly induce abnormalities of bone, liver and kidney, inhibit the synthesis of protein, enhance lipid peroxidation and disturb the antioxidative system of broiler chickens.

Keywords

Biochemical Indexes, Broiler Chickens, High Dietary Fluoride, Oxidative Stress, Serum

1. Introduction

Fluoride (F) is known to cause fluorosis in large doses and can even lead to death in excessive amounts [1]. In addition, F is a well-known inhibitor of numerous enzymes [2] [3]. Study has reported that changes on some biochemical parameters are possibly ascribed to increased oxidative stress in F toxication [4]. In recent decades, many investigations have focused on the relationship between F and free radical toxicity in humans, rats, mice, pigs, sheep or rabbits [5]-[10].

In our recent studies, we found that high dietary F induced oxidative stress in the cecal tonsil and the intestinal mucosa of broiler chickens [11] [12]. However, limited studies have focused on the effects of high dietary F on serum biochemistry and oxidative damage parameters in broiler chickens. Chen *et al*. [13] have reported that high dietary F decreases the superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) activities and increases the malondialdehyde (MDA) content in the serum of broiler chickens. In addition, Liu *et al*. [14] have reported that F decreases the activities of SOD, catalase (CAT), GSH-Px and increases the MDA content in the serum of chicks, but the serum biochemical indexes, and the ability to inhibit hydroxyl radical and glutathione (GSH) content in the serum have not been reported in their experiments. Abdelhamid and Dorra [15] have studied that feed-borne F intoxication decreases the content of total protein (TP), albumin (ALB) and hepatic enzymatic activity in broiler chicks.

Thus, the objective of this study was to investigate the effects of high dietary F on serum biochemical parameters and oxidative stress indicators by detecting the activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total superoxide dismutase (T-SOD), CAT, GSH-Px, the ability to inhibit hydroxyl radical and the contents of TP, ALB, creatinine (Crea), uric acid (Ua), GSH and MDA.

2. Materials and Methods

2.1. Broiler Chickens and Diets

280 one-day-old healthy avian broiler chickens (purchased from Wenjiang commercial poultry hatchery in Sichuan Province) were randomly divided into four equal groups with 70 broiler chickens in each and fed a corn-soybean diet as follows: a control group with 22.6 mg·F/kg, and three high F groups I, II, and III with 400, 800, and 1200 mg·F/kg diet, respectively. They were housed in corresponding cages with electrically heated units and had unrestricted access to water and the above-mentioned diets for 42 days.

All the dietary nutrition requirements were adequate according to the US National Research Council (NRC) (1994) [16].

Animal care and protocol of the experiment were approved by Sichuan Agricultural University Animal Care and Use Committee.

2.2. Serum Samples Collection

At 14, 28, and 42 days during the experiment, five broiler chickens in each group were phlebotomized from jugular vein to collect serum after a 12-h overnight fast. Non-anticoagulative blood samples were clotted for 15 min at room temperature and then centrifuged at 3000 rpm for 15 min. The serum samples were collected in eppendorf tubes and stored at −20˚C until analysis.

2.3. Determination of the Content of Serum F

Before determination, the serum samples were thawed. The content of serum F ions was determined with fluoride ion-selective electrode INESA PF-1-01 (China).

2.4. Determination of Serum Biochemical Parameters

The activities of T-SOD, CAT and GSH-Px and the contents of GSH, MDA, and the ability to inhibit hydroxy radicals in the serum were assayed by biochemical reagent kits (T-SOD, A001-1; CAT, A007-2; GSH-Px, A005; GSH, A006-1; MDA, A003-1; ability to inhibit hydroxyl radicals, A018). Meanwhile, the activities of AST, ALT and ALP, the contents of TP, ALB, Crea, Ua were measured using the corresponding reagent kits (AST, C0010-2; ALT, C009-2; ALP, A059-2; TP, A045-2; ALB, A028-1; Crea, C011-1; Ua, C012, respectively). All

the aforementioned commercial reagent kits were purchased from Nanjing Jiancheng Bioengineering Institute (China). All the experimental operating procedures followed the manufacturer's instructions. The absorbances of T-SOD, CAT, GSH-Px, GSH, MDA, ability to inhibit hydroxy radicals were measured at 550, 240, 412, 420, 532, 550 nm and the absorbances of AST, ALT, ALP, TP, ALB, Crea, Ua were detected at 510, 510, 520, 595, 628, 510 and 690 nm, respectively, with a microplate reader Bio-Rad 680 (USA).

2.5. Statistical Analysis

All the grouped data were statistically evaluated with SPSS software 17.0 for Windows and expressed as means ± standard deviation (SD). Comparison of means between four groups were conducted using one-way analysis of variance (ANOVA) followed by least significant difference (LSD) post hoc test and P-values of less than 0.05 ($p < 0.05$) were considered to indicate statistical significance.

3. Results

3.1. Changes in the Content of Serum F

As shown in **Table 1**, the serum F content was markedly higher in the three high F groups than that in the control group at the 42 days of age ($p < 0.01$).

3.2. Changes in the Serum Oxidative Stress Parameters

From 14 to 42 days of age, the activities of T-SOD, CAT, GSH-Px, the content of GSH and the ability to inhibit hydroxyl radicals were significantly lower ($p < 0.01$) in high F groups II and III than those in the control group, whereas the MDA content was significantly increased ($p < 0.01$) in the abovementioned high F groups in comparison with the control group (**Table 2**).

3.3. Changes in the Serum Biochemical Indexes

As shown in **Table 3**, the contents of serum TP and ALB were significantly lower in the three high F groups from 28 to 42 days of age ($p < 0.05$ or $p < 0.01$), the activities of ALP, ALT and the contents of Crea in the serum were markedly higher ($p < 0.05$ or $p < 0.01$) in the three high F groups; AST activity and Ua content were significantly higher in the high F groups II and III ($p < 0.05$ or $p < 0.01$) than those in the control group from 14 to 42 days of age.

4. Discussion

4.1. Effects of High Dietary F on the Content of Serum F in Broiler Chickens

In blood, about 75% of F remains free in plasma, therefore, determination of the content of serum F reflects the F content in blood to some extent. In our present study, the serum F content was markedly higher in the three high F groups than that in the control group at 42 days of age ($p < 0.01$). The increased content of serum F was reported by many anthors [17]-[19]. Likewise, the content of urine F significantly increases in rat [20], rabbit [18] and fluorotic human [19] has been reported. Kono *et al*. [21] reported that serum F concentrations in F-exposed workers with impaired renal function were strikingly higher than in other patients with chronic renal failure (CRF) and F-exposed workers. We may speculate that the increased content of serum F is due to renal dysfunction induced by F, which makes the ability to excrete F decreased and eventually leads to F accumulation in the blood circulatory system.

4.2. Effects of High Dietary F on Serum Oxidative Stress Parameters in Broiler Chickens

The generation and concentration of reactive oxygen species (ROS) in the body is controlled by the antioxidative system, which enables transformation of ROS into inactive and harmless compounds or molecules [22] [23]. Oxidative stress is a result of disturbed antioxidative/pro-oxidative balance and leads to biological damage, and is regarded as one of the causes of several pathologies that affect poultry growth [22] [24]. Lipid peroxidation is the fundamental parameter of oxidative stress and MDA is a terminal product of lipid peroxidation, and the content of MDA can be used to estimate the extent of lipid peroxidation [25] [26].

Table 1. Changes of the serum F content in broilers.

Days	Groups	F (μg/mL)
42 days	Control group	0.06 ± 0.01
	High F group I	0.22 ± 0.01**
	High F group II	0.75 ± 0.05**
	High F group III	1.79 ± 0.09**

Data are presented with the means ± standard deviation (n = 5). **p < 0.01, compared with the control group.

Table 2. Changes in the serum oxidative stress parameters of broilers.

Parameters	Groups	Days		
		14 days	28 days	42 days
T-SOD (U/ml)	Control group	127.74 ± 4.15	150.91 ± 2.10	162.34 ± 3.14
	High F group I	127.48 ± 2.52	150.65 ± 2.69	161.92 ± 2.37
	High F group II	102.25 ± 1.39**	122.65 ± 1.93**	145.77 ± 4.72**
	High F group III	101.56 ± 1.12**	119.20 ± 2.20**	140.91 ± 1.79**
GSH-Px (U/ml)	Control group	544.70 ± 6.94	497.70 ± 5.33	442.57 ± 14.17
	High F group I	527.63 ± 11.06*	483.51 ± 14.39	421.37 ± 11.12*
	High F group II	485.10 ± 4.95**	382.90 ± 16.02**	326.24 ± 10.19**
	High F group III	446.92 ± 13.87**	348.97 ± 14.16**	302.12 ± 6.55**
CAT (U/ml)	Control group	8.24 ± 0.58	12.99 ± 0.75	13.76 ± 0.55
	High F group I	7.80 ± 0.36	12.39 ± 0.76	13.12 ± 0.77
	High F group II	6.64 ± 0.52**	9.34 ± 0.33**	11.65 ± 0.51**
	High F group III	6.45 ± 0.50**	8.17 ± 0.38**	11.19 ± 0.57**
GSH (mgGSH/L)	Control group	13.88 ± 0.71	16.96 ± 1.06	17.41 ± 0.65
	High F group I	13.61 ± 0.86	16.05 ± 0.61	16.60 ± 1.04
	High F group II	11.57 ± 0.83**	12.92 ± 0.93**	13.78 ± 0.99**
	High F group III	11.02 ± 0.83**	10.61 ± 0.73**	11.83 ± 0.45**
MDA (nmol/ml)	Control group	3.44 ± 0.18	3.84 ± 0.76	5.96 ± 0.09
	High F group I	3.64 ± 0.05	4.66 ± 0.03	6.45 ± 0.33*
	High F group II	5.03 ± 0.96**	5.58 ± 0.85**	6.73 ± 0.17**
	High F group III	5.27 ± 0.33**	5.89 ± 0.46**	8.90 ± 0.40**
The ability to inhibit hydroxyl radicals (U/ml)	Control group	1357.86 ± 2.01	1361.47 ± 1.59	1362.05 ± 1.55
	High F group I	1356.67 ± 3.12	1357.80 ± 2.64	1360.33 ± 1.38
	High F group II	1348.80 ± 0.18**	1345.37 ± 3.59**	1355.48 ± 0.98**
	High F group III	1345.24 ± 1.56**	1344.64 ± 1.30**	1350.09 ± 3.30**

Data are presented with the means ± standard deviation (n = 5). *p < 0.05, compared with the control group. **p < 0.01, compared with the control group.

GSH-Px and CAT, as preventive antioxidants, and T-SOD, a chain-breaking antioxidant, play an important role in protecting against the deleterious effects of lipid peroxidation [27]. The limited activity of CAT causes accumulation of hydrogen peroxide, which in turn is the inhibitor of SOD [23]. GSH plays an important role in antioxidant defense against ROS through a nonenzymatic mechanism and regarded as an early biological marker of the oxidative stress [28] [29]. Meanwhile, GSH-Px metabolizes hydrogen peroxide to water by using GSH as a hydrogen donor [30]. The decreased GSH-Px activity may also be due to the decreased availability of GSH in the serum. Hydroxyl radical is one of the major oxygen radicals that can cause oxidative stress [12]. In our recent studies, we have found the abilities to inhibit hydroxyl radicals in the cecal tonsil [11] and intestinal mucosa

Table 3. Changes in the serum biochemical indexes of broilers.

Parameters	Groups	Days		
		14 days	28 days	42 days
TP (g/L)	Control group	27.47 ± 0.92	28.34 ± 1.13	30.85 ± 0.43
	High F group I	27.12 ± 1.00	26.77 ± 0.49*	25.73 ± 1.02**
	High F group II	24.33 ± 1.07**	23.57 ± 0.65**	25.94 ± 0.98**
	High F group III	18.09 ± 0.60**	18.72 ± 1.02**	22.49 ± 0.56**
ALB (g/L)	Control group	16.10 ± 0.77	19.90 ± 0.65	31.51 ± 1.13
	High F group I	17.60 ± 0.59**	17.30 ± 0.40**	28.93 ± 0.92**
	High F group II	19.80 ± 0.56**	17.10 ± 0.37**	27.27 ± 0.40**
	High F group III	18.70 ± 0.65**	15.19 ± 0.37**	23.70 ± 0.92**
ALP (U/100mL)	Control group	597.71 ± 8.82	781.70 ± 16.58	1260.39 ± 19.40
	High F group I	998.03 ± 12.11**	1884.29 ± 21.05**	2228.92 ± 21.15**
	High F group II	1133.82 ± 23.53**	2237.26 ± 14.23**	2407.08 ± 17.71**
	High F group III	1483.33 ± 15.56**	2566.02 ± 26.35**	2491.80 ± 22.19**
Crea (μmol/L)	Control group	204.28 ± 5.35	239.64 ± 2.48	269.11 ± 4.13
	High F group I	243.56 ± 4.36**	246.19 ± 2.96**	275.03 ± 5.27*
	High F group II	261.26 ± 4.59**	251.42 ± 2.71**	294.64 ± 1.39**
	High F group III	267.15 ± 3.34**	280.89 ± 1.61**	304.46 ± 3.08**
Ua (mg/L)	Control group	33.80 ± 0.75	36.62 ± 0.94	38.73 ± 0.92
	High F group I	34.68 ± 0.97	39.79 ± 0.84**	40.66 ± 0.67**
	High F group II	39.08 ± 1.30**	42.72 ± 0.80**	46.24 ± 0.92**
	High F group III	42.02 ± 0.84**	47.72 ± 1.31**	48.83 ± 0.97**
AST (U/L)	Control group	37.86 ± 2.85	40.05 ± 1.64	43.51 ± 1.96
	High F group I	40.94 ± 1.59	46.56 ± 1.90**	62.76 ± 1.29**
	High F group II	41.76 ± 1.37*	48.79 ± 1.70**	68.04 ± 1.91**
	High F group III	53.94 ± 2.49**	68.13 ± 2.87**	70.13 ± 2.09**
ALT (U/L)	Control group	2.50 ± 0.16	2.72 ± 0.09	6.21 ± 0.21
	High F group I	3.53 ± 0.14**	4.04 ± 0.11**	9.05 ± 0.74**
	High F group II	7.41 ± 0.42**	8.25 ± 0.66**	14.87 ± 0.56**
	High F group III	10.30 ± 0.34**	13.93 ± 0.99**	16.70 ± 0.92**

Data are presented with the means ± standard deviation (n = 5). *$p < 0.05$, compared with the control group. **$p < 0.01$, compared with the control group.

[12] of broiler chickens were decreased in the high F groups II and III. In the present study, a significant increase ($p < 0.01$) in the content of MDA along with the marked decreases ($p < 0.01$) in the activities of T-SOD, GSH-Px, CAT, the content of GSH and the ability to inhibit hydroxyl radical were observed in high F groups II and III from 14 to 42 days of age, suggesting that F inhibited the activities of serum antioxidants and increased the concentration of ROS in the blood circulatory system of broiler chickens. The results in this study are similar with the results from chicks [14], pigs [8]. Likewise, Li and Cao [31] have found a decrease in the activities of SOD and GSH-Px in people living in areas with endemic fluorosis. However, Chlubek *et al*. [32] found no changes in the activity of GSH-Px or concentrations of MDA in the pancreas of rats exposed to NaF in drinking water during four months. Reddy *et al*. [10] reported that there was no significant difference in the lipid peroxidation, GSH and vitamin C, as well as in the activities of SOD, GSH-Px, and CAT in the blood of fluorotic humans and rabbits. Shivarajashankara *et al*. [33] found the increased GSH level and GSH-Px activity in the erythrocytes, brain and liver of F-treated rats. Results obtained by different authors are contradictory, but most reports favor the oxidative stress theory of F.

4.3. Effects of High Dietary F on Serum Biochemical Parameters in Broiler Chickens

F toxicity in animals is multifarious [2]. F ions pass the intestinal barrier and are deposited in several organs and body through the blood system [34]. It has been reported that F toxication affects protein synthesis by primary causing destruction of polypeptide chains and weakness of amino-acid bindings in proteins [35] [36]. In our study, the serum TP and ALB contents were significantly decreased ($p < 0.05$ or $p < 0.01$) in the three high F groups I, II and III from 28 to 42 days of age, which demonstrated that excessive F caused a reduction of protein synthesis. Our results are agreed with other findings of the literature realized on mice [37], children [38], fish [39]. However, Cenesiz *et al*. [40] reported there was no significant change between the serum ALB values of control and fluorosed sheep.

As a very active site of metabolism, the liver is especially susceptible to F intoxication [37]. ALP, ALT and AST are important indicators of liver damage in clinic findings. These enzymes were secreted to blood in hepatocellular injury and their levels increase [41]. In the present study, a marked increase ($p < 0.05$ or $p < 0.01$) in the activities of serum AST, ALT and ALP was observed in the high F groups II and III or in the three high F groups from 14 to 42 days of age, which is similar with the observations of children [38], rats [42] and pigs [26]. These data indicated that the hepatic function of broiler was weakened by excessive ingestion of F. It remains controversial about whether or not excessive F intake induces damage to human liver functions. Xiong *et al*. [43] found no significant differences in the levels of serum AST and ALT in children with and without dental fluorosis. ALP also is the marker enzyme of F toxicosis and bone pathology [42]. The increased serum ALP activity following F exposure may reflect a toxicity of F for both osteoblasts and resorbing osteocytes [44]. In the present study, the increased serum ALP activity in the three high F groups may imply that dietary F induced bone disorder in broiler chickens. However, Pillai *et al*. [2] reported that a sublethal concentration of F induced a decline in the serum ALP activity.

The major route for the removal of F from the body is by the kidney [45]. High concentrations of F usually lead to kidney damage, including vacuolization and necrosis of tubules, hypertrophy and atrophy of glomeruli, interstitial edema, and interstitial nephritis [46]. Serum Crea levels are frequently used as a screening test for renal dysfunction [47]. Ua is the main end-product in nitrogen metabolism in birds [48]. Elevated serum Ua is a marker for decreased renal function [49]. The increased serum Ua and Crea in this study may be explained by the dysfunction of kidney due to the damage induced by F. Also Ua has a protective role in defence mechanisms of the body and is a lipid peroxidation inhibitor and a radical scavenger [50]. Decreased serum Ua was reported in children [51] and mice [7].

5. Conclusion

Based on the results observed in the present study and the aforementioned discussion, it is concluded that F has accumulated in the blood circulatory system and dietary F in the range of 800 - 1200 mg/kg could significantly induce abnormalities of bone, liver and kidney, inhibit the synthesis of protein, enhance lipid peroxidation and disturb the antioxidative system of broiler chickens. Our data provide some information for clinical diagnosis of fluorosis and for further studying the mechanism of excessive F accumulation on the damage of soft tissues in broilers.

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List of Abbreviations and Acronyms

GSH: Glutathione
T-SOD: Total superoxide dismutase
CAT: Catalase
GSH-Px: Glutathione peroxidase
MDA: Malondialdehyde
TP: Total protein
ALB: Albumin
ALT: Alanine aminotransferase
AST: Aspartate aminotransferase
Crea: Creatinine
Ua: Uric acid
ALP: Alkaline phosphatase

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A Review of Enrofloxacin for Veterinary Use

Tessa Trouchon, Sébastien Lefebvre

USC 1233 INRA-Vetagro Sup, Veterinary School of Lyon, Marcy l'Etoile, France

Abstract

This review outlines the current knowledge on the use of enrofloxacin in veterinary medicine from biochemical mechanisms to the use in the field conditions and even resistance and ecotoxicity. The basics of biochemistry, the mechanisms of action and resistance and pharmacokinetics are presented. Then an overview of available veterinary products, their efficacy and their toxicity against target species, human and environment is provided.

Keywords

Enrofloxacin, Antibiotic Resistances, Veterinary

1. Introduction

Enrofloxacin (**Figure 1(c)**), or 1-Cyclopropyl-6-fluoro-7-(4-ethyl-1-piperazinyl)-1,4-dihydro-4-oxo-3-quinolinecarboxylic acid, belongs to fluoroquinolone family which is a subfamily of quinolone. The first quinolone is the Nalidixic acid (**Figure 1(a)**) used in animal at the beginning of 1980s, enrofloxacin is the first fluoroquinolone patented in 1984 [1]. The huge evolution in the quinolone family is the addition of a fluor atom on the 6th position which improves quinolones' antibacterial spectrum [2] and creates the fluoroquinolone subfamily. Quinolones have an action on bacterial topoisomerase. The marketing authorization reports a large antimicrobial spectrum for enrofloxacin, which is efficient on most gram-negative and gram-positive bacteria but not efficient on anaerobic bacteria [3]. But with 3.6 tons sold per year in France for animal use [4], fluroroquinolones are an important family in veterinary medicine that increases the probability of selecting resisting bacteria.

2. Action Mechanism of Enrofloxacin

2.1. Important Physicochemical Properties of Enrofloxacin

Enrofloxacin is a zwitterionic molecule with a pKa_1 = [5.88 - 6.06] and a pKa_2 = [7.70 - 7.74] [5]. The lowest

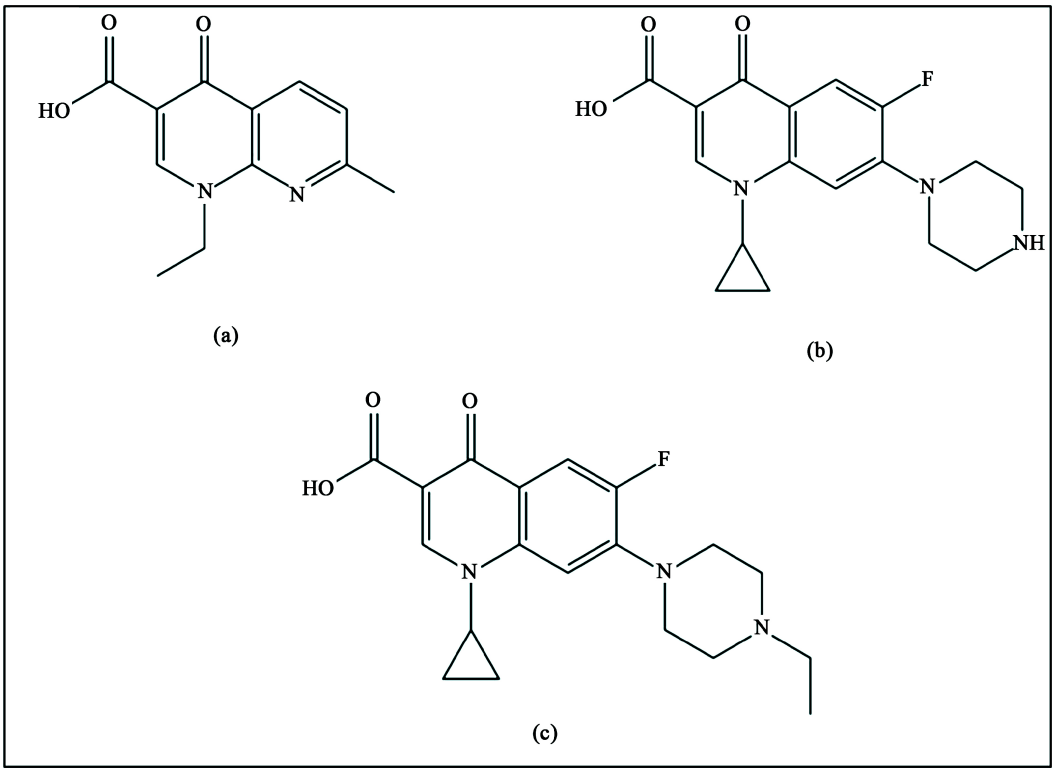


Figure 1. Structure of (a) Nalidixic Acid; (b) Ciprofloxacin and (c) Enrofloxacin.

pKa is due to the carboxyl acid group and the second to the basic tertiary amine. So enrofloxacin doesn't bear charge between this two pH. Moreover it is a lipophilic molecule with a logP of 4.70 at a pH of 7 [6], but 1.88 ± 1.43 with ACDLabs.

The Active metabolite of enrofloxacin, ciprofloxacin (**Figure 1(b)**) or 1-Cyclopropyl-6-fluoro-1, 4-dihydro-7-(1-piperazinyl)-4-oxo-3-quinoline carboxylic acid, which is available as a drug product on veterinary and human market, is a multiple acid with $pKa_1 = 5.15$ and $pKa_2 = 8.25$ [7]. Ciprofloxacin is less lipophilic than enrofloxacin with a logP of −1.11 at a pH of 7.4 [8].

2.2. Targets' Mechanism of Action

Enrofloxacin, like the other quinolones, has two mains targets of the topoisomerase family. Although these proteins exist in eukaryotes cells, quinolones have less affinity for eukaryotes' topoisomerases than for the DNA Topoisomerase II (Gyrase) and the DNA Topoisomerase IV (Topo IV) two major bacterial topoisomerase [9]. The Gyrase and the Topo IV are two tetramers (**Figure 2(a)**) formed respectively of two GyrA and two GyrB and of two ParC and two ParE. Moreover GyrA and GyrB are homologous respectively with ParC and ParE [10].

The Gyrase has an important role in bacteria's life by modifying the topology of the spiral DNA. Indeed, the positive supercoiling stabilizes the DNA and the strands' separation becomes more difficult [11]. Moreover transcription generates a positive supercoiling accumulation that can stop transcription. This positive supercoiling can be released by the Gyrase, which enhances transcription [12]. To do this, the Gyrase binds and wraps around itself a strand of DNA (**Figure 2(b)**) with the help of the C-terminal domain [13] and cleaves it (**Figure 2(c)**) with mediating of the catalytic tyrosine Tyr 122 [14] of each GyrA. Tyrosines form covalent phosphotyrosyls with each 5' phosphoryl terminus of both strands, which remain binding during the reaction [15]. This reaction forms a gap in sequence (called G-DNA). The GyrB part catches the other DNA sequence (called transported DNA or T-DNA). The T-DNA is passed through the opened G-DNA (**Figure 2(d)**) [16]. The G-DNA is closed in an ATP-dependent reaction (**Figure 2(e)**) [17]. Of this reaction results an adding of negative coiling.

The Gyrase is able to do an intermolecular strand passage at the end of the replication like the Topo IV but

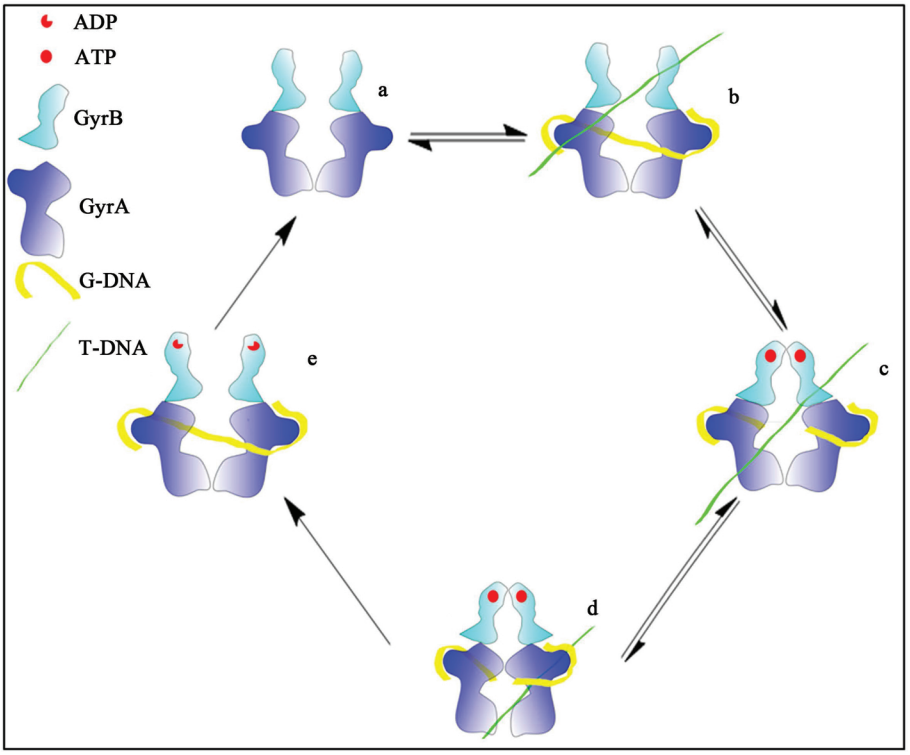


Figure 2. Mechanism of action of Gyrase, with intervention of Gyrase part A (GyrA) and B (GyrB), gap DNA sequence (G-DNA), transported DNA sequence (T-DNA), ATP and GDP. (a) Gyrase with GyrA dimer and GyrB dimer; (b) Interaction of Gyrase with two DNA sequences; (c) Fixation of an ATP molecule and cleavage of the G-DNA strand; (d) Passage of the T-DNA strand through G-DNA; (e) Closing of the G-DNA strand with ATP degradation.

without wrapping the DNA as the Topo IV, this is the major difference between reactions of both enzymes [18]. Topo IV catalyzes the segregation of the two daughter DNA molecules after the replication [19] more efficiently than Gyrase [20].

2.3. Interactions between Fluoroquinolones, DNA and Its Target

The penetration in the bacteria differs between Gram-positives and Gram-negatives bacteria. For Gram-negative bacteria, fluoroquinolones pass the outer membrane mainly through porins [21] [22]. The trimeric OmpF porin is mainly used by fluoroquinolone, this pathway can be modulated by Mg^{2+} [23]. But it seems that some quinolones can promote a pathway by interacting with the Lipopolysaccharide (LPS) and create a lipophilic passage [24]. For Gram-positive bacteria the diffusion process is the main uptake pathway [25].

In the case of mycobacteria the high lipid level of the membrane allows fluoroquinolones to diffuse through membrane [26].

After the entrance in the bacteria, quinolones have two effects on the bacteria: bacteriostatic at low concentration level or bactericide at high concentration level. Rapidly after the Gyrase has formed a complex with the DNA strand, two quinolones bind with this complex, before the DNA's cleavage [27]. This binding is reversible and induces a conformation modification in Gyrase [28]. This modification induces a cleavage of DNA in a particular location and forms a cleaved complex [29] [30]. At this step the action is still reversible, but reduces rapidly the activity of replication and has a bacteriostatic effect [31].

The next two pathways are possible one for bacteriostatic concentration and the other for bactericide concentration. For bacteriostatic concentrations, quinolones induce the SOS regulon controlled by the repressor lexA [32] [33]. Activation of the regulon induces the sfiA gene which codes for an inhibitor of cell division and bacteria form a long filamentous structure. This effect is still reversible [34]. This filamentous structure contributes to the death of bacteria [35].

For bactericide concentration quinolones induce a chromosome fragmentation by creation of a suicide factor or by destabilization of a GyrA dimer [35]. The fragmentation can induce a rapid death or, by reassociation of the GyrA dimer, genomic mutation in bacteria [36].

2.4. Resistance Mechanism

The resistance to fluoroquinolones in general, is today a main subject for the use of antibiotic substances in veterinary medicine and was the subject of an EMA report [4]. Many studies show an augmentation in the development of resistances to enrofloxacin [37] [38]. Moreover, another study shows the increase of resistance of *Escherichia coli* to quinolones and fluoroquinolones after using of enrofloxacin in calves [39].

The mechanism of resistance to fluoroquinolones is based on many pathways: the change of targets, the protection of target, the lower expression of target, the improvement of the efflux pumps, and inactivation of fluoroquinolones. These mechanisms can be chromosomal or plasmide-mediated (**Table 1**) [40].

The most common mechanism is the mutation of Gyrase. In the subpart GyrA the mutation is mainly between Ala67 and Gln 106 for *E.coli* and other bacteria [41] [42]. The mutation is near the GyrA active site which is called Quinolone Resistance-Determining Region (QRDR). For GyrB two mutation are reported at Asp426 and Lys447 [43]. Only one Gyrase mutation is able to increase the Minimum Inhibitory Concentration (MIC) up to 64 times [41], but the GyrB mutation seems to be less effective in resistance against nalidixic acid than the GyrA mutation [44].

The mutation of Topo IV is also described but, until today, always with at least one Gyrase mutation. Therefore, these mutations seem to be developed in a second time to increase the resistance more than only Gyrase's mutations. A QRDR can be also characterized in the parC of topoisomerase IV between Tyr 57 and Glu84 [45].

The protection of target can be attributed to a qnr gene often present on a Plasmide-Mediated Quinolone Resistance (PMQR) or in the chromosomal DNA. The Qnr protein is a dimere which binds with Gyrase and decreases the binding of quinolones with DNA-Gyrase-Qnr complex. But it seems that the Qnr protein reduces the interaction between DNA and Gyrase [46]. Another theory is that the Qnr protein destabilizes the DNA-Gyrase-quinolone complex and promotes the DNA repair after cleavage [47].

An hypothesis of resistance is the slow growth of cell, bacteria growing slowly seem to resist better, but this mechanism is not known today [40] [48].

Although resistances are often attributed to the Gyrase or Topo IV mutations, decrease of influx and increase of efflux mechanisms are important pathways of resistance common to a large number of unrelated antibiotics [49]. This defines a Multiple Antimicrobial Resistance (MAR) phenotype. *E. coli* bearing a MAR operon has higher resistance for the quick death by fluoroquinolones [50]. This operon can induce a decrease of OmpF pumps [51] and an increase of efflux with AcrAB protein [52]. The decrease of porins can also be attributed to PMQR [53].

2.5. Recommendations to Prevent Resistance

Given the rapidity of development of resistance, we have to consider with the MIC, the Mutant Prevention Concentration (MPC). MPC is a recent indicator which is not well standardized. Commonly MPC50 is the concentration where 50% of the colonies doesn't contain resistant mutant after 72 hours of incubation. To count the mutants, a Polymerase Chain Reaction (PCR) is used but only known resistant mutation is tested [54]. Between MIC and MPC there is a window where enrofloxacin is effective but also where mutants are selected.

The data recorded in **Table 2** show that the window between the MIC and the MPC can be very important with a MPC/MIC between 1.6 and 64. This high difference can explain the explosion of resistance against enrofloxacin and might be a base to define new guidelines and dosage for enrofloxacin.

Table 1. Possible origins of resistances to fluoroquinolones.

Chromosomal resistance	Plasmide-mediated resistance
Mutation of target	Lower expression of target
Lower expression of target	Protection of target Improvement of efflux pumps
Protection of target Improvement of efflux pumps	Inactivation of fluoroquinolones

Table 2. MIC and MPC of enrofloxacin for some bacteria.

Bacteria	MIC (µg/mL)	MPC (µg/mL)	(MPC/MIC)	References
Escherichia coli	[0.022 - 0.03]	[0.17 - 0.5]	[7.8 - 16]	[67] [107]
Staphylococcus pseudintermedius	0.13	0.27	2	[108]
Rhodococcus equi	[0.5 - 1]	[8 - 64]	[16 - 64]	[109]
Mannheimia haemolytica	0.16	0.25	1.6	[110]
Salmonella Typhimurium	[0.06 - 0.125]	[1 - 2]	16	[67]
Pseudomonas aeruginosa	2	[16 - 32]	[8 - 16]	[67]

3. Pharmacokinetic of Enrofloxacin

3.1. Absorption

There is high variation of bioavailability after oral administration of enrofloxacin between polygastric and monogastric animals from 10% to 80% [55] [56]. This has a direct consequences on the galenic, oral presentations are reserved for pigs, poultry, calves, and carnivores and only injectable solutions are available for cattle. Moreover, bioavailability depends on whether the animals are fed or fasted [55] [57], and of the presence of ion [58]. These interferences can be explained by the formation of a complex between cation and fluoroquinolone which cannot permeate through the digestive barrier [59]. Another consequence of this complex is the influence of the hardness of water notably in poultry farming where the dilution in water is used [60]. But lipophilic compounds in food can enhance oral bioavailability of fluoroquinolone [61].

In addition to passive diffusion allowed by enrofloxacin lipophilicity, active transporters have also an important role in intestinal absorption [62]. But these transporters are also important to eliminate fluoroquinolones [62].

The intramuscular bioavailability is 96% with a maximum concentration 3 hours after administration [3]. Intramuscular bioavailability can be enhanced by solid lipophilic nanoparticles, this technic enhances the duration of enrofloxacin in plasma [3].

3.2. Metabolism

After administration a high part of enrofloxacin is metabolized into ciprofloxacin in most of species (**Table 3**). Enrofloxacin has an active metabolite, ciprofloxacin [63], obtained by deethylation of the ethyl on the piperazin ring. Other metabolites are obtained but they don't have antimicrobial effects [64].

Only poultry does not have a huge part of enrofloxacin metabolized in ciprofloxacin. The first hepatic pass doesn't have a lot of effect, only 7% of enrofloxacin is metabolized [65]. Moreover ciprofloxacin seems a more potent drug than enrofloxacin [66] [67]. These elements are in favor to grant a more important place to ciprofloxacin in the effects of enrofloxacin-based drugs.

3.3. Distribution

Distribution of enrofloxacin and ciprofloxacin to tissues depends on drug's free concentration, which depends itself on the concentration of protein and the strength of this biding.

The difference between ciprofloxacin and enrofloxacin protein binding (**Table 4**) are in favor of the theory of enrofloxacin's role of prodrug. Indeed, in some species ciprofloxacin is less bound with proteins, so it is able to be more available to be effective. Moreover, enrofloxacin can interact with other protein-binding drugs for instance it increases the clearance of flunixin meglubine [68].

As described in **Table 5**, the volumes of distribution of enrofloxacin and ciprofloxacin in different species are all higher than 1. So enrofloxacin and its metabolite diffuse strongly in tissues, and molecules can be present in cells, so being inactive. Contrary to what we might expect considering its protein binding, ciprofloxacin doesn't diffuse in tissues more than enrofloxacin. Data of **Table 6** suggest a high affinity of drugs for lung and kidney, even if data confirm enrofloxacin's and ciprofloxacin's good diffusion.

Table 3. Percent of ciprofloxacin after enrofloxacin given.

Species	Ciprofloxacin percent of enrofloxacin plasma concentration	References
Dogs	40	[65]
Dairy cows	59	[75]
Steers	64	[75]
Chickens	<10	[111]
Pigs	51	[112]
Goats	34	[71]

Table 4. Protein binding of enrofloxacin and ciprofloxacin.

Species	Percent of bond enrofloxacin (%)	Percent of bond ciprofloxacin (%)	References
Dogs	34	18	[113]
Dairy cows	59.4	33.7	[75]
Steers	60.8	49.6	[75]
Chickens	23	ND	[114]
Pigs	[31.1 - 37.1]	35	[115]

Table 5. Pharmacokinetics parameters of enrofloxacin and its metabolite ciprofloxacin.

Species	$t_{1/2}$ (h) *		Cl (mL/min/kg) **		V_{ss} (L/kg) ***		References
	Enro	Cipro	Enro	Cipro	Enro	Cipro	
Dogs	2.3	2.8	12.16	7.8	2.45	1.92	[65]
Dairy cows	3.69	2.96	24.16	ND	1.56	ND	[75]
Steers	5.5	7.60	11.6	ND	1.59	ND	[75]
Chickens	6.99	3.11	3.30	15.45	1.98	4.04	[111]
Pigs	26.6	2.60	3.0	17.30	6.40	3.80	[69]
Goats	1.39	[1.82-2.72]	22.18	19.59	1.27	3.33	[71] [116]

*elimination half-live, **clearance, ***volume of distribution at the steady state. Enro: enrofloxacine, Cipro: ciprofloxacine.

Table 6. Maximal concentration (C) and area under the curve (AUC) of enrofloxacin (ENR) and ciprofloxacin (CIP) after an intravenous injection of 5 mg/kg of enrofloxacin [117].

	Plasma†		Muscle‡		Liver‡		Spleen‡		Lung‡		Kidney‡	
	ENR	CIP	ENR	CIP	ENR	CIP	ENR	CIP	ENR	CIP	ENR	CIP
C	4.44	0.18	3.48	0.19	3.67	2.95	11.04	0.25	3.82	0.86	8.98	2.78
AUC	3.52	0.61	5.61	0.62	5.76	2.02	10.44	2.08	9.44	4.79	9.03	4.91

†Unit of C is µg/mL and unit of AUC is µg.h/mL; ‡Unit of C is µg/g and unit of AUC is µg.h/g.

3.4. Elimination

Elimination parameters show great difference between species (**Table 5**), especially for pigs where the elimination half-life is high with 26 h [69]. Moreover, for chickens and pigs, clearance of ciprofloxacin is five times higher than enrofloxacin's clearance and their enrofloxacin clearances are lower than other species. This differences might be attributed to a difference of elimination way.

Enrofloxacin's elimination way is mainly renal. This has been proved in rat by nephrecotomizing and comparing with CCl_4 hepatic impairment [70] and in goat with probenecid [71]. Probenecid has been shown to reduce the renal clearance of fluoroquinolones [72]. On the other hand, ciprofloxacin's elimination is both hepatic and renal [73]. For both molecules there is an intestinal recirculation via the bile excretion, moreover, two hour after given, there is no significant difference in the concentration of enrofloxacin in the intestinal content between oral and intramuscular administration of enrofloxacin [74]. Lactation can influence significantly enrofloxacin's and ciprofloxacin's elimination by increasing two folds clearance in dairy cows comparative to steers [75]. It might be explained by an ionic trap.

4. Veterinary Medicinal Products

4.1. Available Veterinary Medicines

The first veterinary product based on enrofloxacin was launched by the laboratory Bayer in 1991 (marketing authorisation in 1991) under the trade name Baytril® and it was an oral form for poultry [3]. Now, many veterinary products on the basis of enrofloxacin are available on the market with at least thirty four veterinary medicines under different forms including oral and injectable forms, tablets or bolus [3]. There are many target species including domestic carnivores (dogs and cats), farm animals (cattle, pigs, poultry) and even exotic pets since 2010 [3].

4.2. Therapeutic Indications and Uses in the Field Conditions

Enrofloxacin is indicated in the treatment of local and systemic diseases caused by a wide range of Gram-negative and Gram-positive bacteria [4] [76]. The most important indication of enrofloxacin in all of the species is the treatment of respiratory infections but it is also indicated in the treatment of digestive, urinary, joint, genital, mammary and dermal infections [3] [77].

However, enrofloxacin is a third generation fluoroquinolone with a very large spectrum of activity so it has to be reserved to second intention. Indeed, in order to avoid fluoroquinolone resistances, it is important to reserve the use of enrofloxacin to infections resistant to over antibacterial agents and if possible under a susceptibility study [4].

In the field conditions, enrofloxacin is often off-label used empirically to prevent uterine infections in susceptible embryo-transfer mares. In fact, a conventional dose (5 mg/kg body weight) given pre-breeding followed by two further doses at 36 - 48 h post breeding are supposed to prevent bacterial adherence and provide effective bactericidal concentrations in utero [78].

Enrofloxacin is often used in aquaculture in Indonesia, Thailand and Vietnam. Indeed, because of the non-hygienic and stressful conditions in aquaculture facilities, the risk of bacterial infections is high and motivates the widely use of antibiotics in fish feed for prophylactic and therapeutic purpose [79].

5. Therapeutic Efficacy

Enrofloxacin is a powerful antimicrobial which have shown efficacy against a lot of bacterial diseases [4] [80]. The effectiveness of enrofloxacin against some bacterial infections in cattle, poultry, domestic carnivores (dogs and cats), rodents, lagomorphs and crustaceans has been assessed in many published studies, as well during natural infections as during experimental infections (**Table 7**). Among these studies, some relate to classical infections contained in the Summary of Product Characteristics (SPC) of veterinary products but some evaluate the efficacy of enrofloxacin in species for which there is no marketing authorisation or against specific bacteria such as *Anaplasma marginale* in cattle, *Ehrlichia canis* and *Brucella canis* in dogs, *Bartonella henselae* or *Bartonella clarridgeiae* and *Chlamydophila felis* in cats, *Toxoplasma gondii* in *Calomys callosus* or even *Vibrio harveyi* in Artemia franciscana (**Table 7**).

6. Adverse Effects

Overall, the fluoroquinolones are well tolerated with fewer adverse effects that are not very serious, especially when compared to their benefits [80] [81]. The most common side effects of enrofloxacin are digestive disorders including nausea, abdominal discomfort, vomiting and diarrhoea [81] [82] and inflammatory reaction at the site

Table 7. Twenty published studies examining the efficacy of enrofloxacin.

Target specie(s)	Disease(s) and/or bacterial agent(s)	Type of infection	Regimen of enrofloxacin	Purpose of the study	Number of animals	Result(s)	References
CATTLE							
Dairy cows	Acute clinical mastitis (*Escherichia coli*)	N	5 mg/kg IV then SC 24 h later	Comparative efficacy of a ENR + KET treatment and a KET treatment	132 (64 ENR + KET, no controls)	Inefficacy of ENR to treat acute clinical E. coli mastitis	[118]
Sahiwal cattle	Anaplasma marginale	N	5 mg/kg IV SID for 5 days	Comparative efficacy of ENR, OXY and IMI	60 (15 per group + 15 controls)	Inefficacy of ENR to clear persistent infection	[119]
Holstein calves	Anaplasma marginale	E	7.5 mg/kg in a single dose or twice every 3 days	Comparative efficacy of ENR (2 regimens) and LA-OXY	24 (6 per group + 6 controls)	Superiority of ENR	[120]
Calves	Pneumonic pasteurellosis (Pasteurella haemolytica A1)	E	2.5 mg/kg SC SID for 3 days	Efficacy of ENR	36 (12 ENR + 12 positive controls + 12 negative controls)	Inefficacy of ENR to treat experimentally induced pneumonic pasteurellosis	[121]
Feeder calves	Bovine respiratory disease (Mh, Pam, Hs, Mb)	N	12.5 mg/kg SC in a single injection	Comparative efficacy of ENR and TUL in two different states of USA	500 (125 per group per site)	Superiority of TUL	[122]
POULTRY							
Chickens	*Escherichia coli* (Ec) and Pasteurella multocida (Pam)	E	10 mg/kg BID in drinking water for 5 days	Efficacy of ENR (given immediately or 6 hours after infection)	Ec: 30 (10 per group + 10 controls) Pam: 20 (7 per group + 6 controls)	Efficacy of ENR against Ec and Pam	[123]
Broilers	Mycoplasma gallisepticum (Mg)	N	5 mg/kg in drinking water at 1 - 10 and 22 - 32 days of age	Efficacy of ENR in a treatment program against Mg in offspring of Mg-infected chicken-broiler breeders	45,000 (22,500 ENR + 22,500 controls)	Efficacy of ENR	[124]
Chicks (White Leghorn)	Salmonella enterica Serovar Typhimurium DT104	E	10 mg/kg for 5 days (1) or 25 mg/kg for 2 days (2) or 50 mg/kg for 1 day (3) (continuously or pulsed in water or gavage)	Efficacy of ENR (comparative efficacy of high dose short duration treatments and conventional treatment)	481 (151 in (1) + 141 in (2) + 89 in (3) + 100 controls)	Efficacy and best compromise for the 2-day 2.5 dosing treatment	[125]
Broilers (male chicks)	Colibacillosis (*Escherichia coli*)	E	50 ppm in drinking water for 7 days	Comparative efficacy of ENR and bacteriophage (individually and in combination)	320 (40 for each one of the 8 treatments)	Superiority of ENR but synergy between ENR and bacteriophage	[126]
Turkeys (poults)	Respiratory infections due to Ornithobacterium rhinotracheale (associated with avian metapneumovirus)	E	10 mg/kg in drinking water during 20 hours for 5 days or 50 mg/kg during 5, 10 or 20 hours in a single day	Efficacy of ENR (comparative efficacy of 4 regimens)	80 (16 per group + 16 controls)	Superiority of the 10 mg/kg 5-day ENR treatment	[127]

Continued

DOMESTIC CARNIVORES							
Dogs	Ehrlichiosis (*Ehrlichia canis*)	E	5 or 10 mg/kg PO BID for 21 days	Comparative efficacy of ENR (2 regimens) and DOX	13	Inefficacy of ENR to clear Ehrlichia canis infection	[128]
Dogs	Recurrent superficial (S) and deep (D) pyoderma	N	5 mg/kg PO SID continued up to 1 (S) or 2 (D) weeks after clinical recovery	Efficacy of ENR	9 (S) + 3 (D)	Efficacy, safety and convenience of ENR	[129]
Dogs	Uncomplicated urinary tract infections	N	18 - 20 mg/kg PO SID for 3 days	Comparative efficacy of ENR (high dose short duration treatment) and AMO-CLA	68 (35 ENR + 33 AMO-CLA)	Non-inferiority of a high dose short duration ENR treatment	[82]
Dogs	Brucellosis (*Brucella canis*)	N	5 mg/kg PO BID for 30 days	Efficacy of ENR for the eradication of Brucella canis in a kennel	12	Incomplete efficacy of ENR but safety use during gestation	[130]
Cats	Chronic bartonellosis (*B. henselae* and *B. clarridgeiae*)	N (25 cats) + E (18 cats)	22.7 mg PO BID for 14 or 28 days	Comparative efficacy of ENR and DOX (each one with 2 treatment durations)	43 (23 ENR + 17 DOX + 3 controls)	Efficacy of high dose long duration ENR treatment (4 to 6 weeks)	[131]
Cats	Conjunctivitis (*Chlamydophila felis*)	N	5 mg/kg SC SID for 3 days then PO SID for 11 days	Comparative efficacy of ENR and DOX	25 (14 ENR + 11 DOX)	Equal improvement of ENR and DOX in clinical signs and infection status	[132]
RODENTS							
Mice	Systemic infections (*Escherichia coli*)	E	5 mg/kg SC or PO	Efficacy of ENR (2 different routes of administration)	NP	Efficacy of ENR and superiority of the injectable form (SC)	[133]
Calomys callosus	Toxoplasmosis (*Toxoplasma gondii*)	E	3 mg/kg SC SID for 3 days	Comparative efficacy of ENR and SUL	15 (5 ENR + 5 SUL + 5 controls)	Efficacy of ENR as a potential alternative drug	[134]
LAGOMORPHS							
Rabbits	Pasteurella multocida (Pam)	N (11 rabbits) + E (12 rabbits)	5 mg/kg SC BID for 10 days	Efficacy of ENR in the elimination of Pam from asymptomatically infected rabbits	11 (N) + 12 (E)	Inefficacy of ENR to eliminate Pasteurella multocida	[135]
CRUSTACEANS							
Artemia franciscana (nauplii)	Vibrio harveyi (strain PN9801)	E	ENR 4h before infection (A) or 24 h after infection (B)	Comparative efficacy of ENR (2 regimens)	600	Efficacy of ENR to stop the course of a bacterial infection in Artemia franciscana	[136]

of injection for injectable forms, particularly in pigs [3]. However, some more serious adverse effects of enrofloxacin could appear targetting the juvenile joints, the reproductive system, the ocular system and the central nervous system.

The best known adverse effects of enrofloxacin concern the joints of young animals and result in arthropathy, articular cartilage degeneration, tendonitis and other forms of tendon injury [80] [81] [83] [84]. In order to try to understand quinolone related-arthropathy, a study was carried out in 1994 on juvenile New Zealand White Rabbits indicating that quinolones stimulate the cellular respiratory burst of immature articular chondrocytes which results in the production of oxygen-derived compounds highly toxic for the cartilage [83]. However the mechanisms underlying fluoroquinolones-induced tendinopathy and cartilage degeneration remained incompletely understood. Thus, further studies were necessary such as the study carried out in 2008 on canine Achilles tendon cells and chondrocytes suggesting that enrofloxacin-induced tendinopathy and cartilage damage could be explained by the inhibition of cell proliferation, induction of apoptosis and DNA fragmentation [84]. In young chickens, a study led in 2009 to determine the chondrotoxic effects of enrofloxacin on avian articular cartilage indicates that only very high dosage of enrofloxacin can induce toxic effects in articular cartilage of growing chickens and that the intensity of chondrotoxicity is dose- and time-dependent. Thus it is suggested that quinolone-induced arthropathy is far less expressed in birds than in mammals [85].

To assess the effects of enrofloxacin on adult joints, a study was carried out in 2000. The effects of long-term administration of an injectable enrofloxacin solution were evaluated by the monitoring of physical and musculoskeletal variables in adult horses. Adverse effects were only detected with high doses and consist of lameness, cellulitis, tendinitis, sheath effusion and even transient neurologic signs [86].

The adverse effects of enrofloxacin on the reproductive system were mainly investigated in males in order to assess the impact of this antibiotic on the fertility parameters. A study carried out in 2008 in male chickens suggested that enrofloxacin at therapeutical dose does not affect the sperm motility, the weight of testes, wattles and combs and the testicular concentration of testosterone, ascorbic acid, total protein and cholesterol [87]. On the opposite, a study led in the same year to evaluate toxic effects of enrofloxacin on sperm quality in male mice indicates that a fixed 150 mg/kg dose of enrofloxacin could lead to structural damages in the testicular tissue resulting in disruption of spermatogenesis in the testes with deterioration of motility, content and morphology of sperms [81]. Although the toxicity of enrofloxacin on the female reproductive tract was less investigated, a recent study on the use of intrauterine enrofloxacin infusion in healthy mares reveals acute effects including endometrial ulceration, necrosis and haemorrhage and chronic effects including fibrosis and inflammation [88].

The ocular toxicity of enrofloxacin has been suggested by the association of enrofloxacin, retinal degeneration and blindness in cats [89] [90]. A retrospective clinical study carried out to assess the possible connection between the administration of parenteral enrofloxacin and the onset of acute retinal degeneration in cats highlights the potential retinotoxicity of parenteral enrofloxacin which can result in acute and diffuse retinal degeneration, particularly with dosages exceeding 5 mg/kg once daily (which is the manufacturer's current dosage recommendation). Mydriasis and blindness were frequently observed but some cats may recover their sight [89].

At slightly higher doses, central nervous system signs of lethargy, anorexia and hypersalivation were observed in dogs as shown in a recent study carried out to assess a high dose short duration enrofloxacin protocol in dogs with uncomplicated urinary tract infections [82].

7. Residues and Toxicity for Consumers

In many animal species, the use of enrofloxacin lead to its de-ethylation to its primary metabolite ciprofloxacin and both enrofloxacin and ciprofloxacin would be found as drug residues in animal muscle and tissue [76] [91].

The consumption of meat containing these residues represent a significant threat against human health because it may result in disruption of the colonization barrier, development of drug-resistant bacterial strains or even allergies [91] [92]. In this regard, a recent study provides information on in vitro testing to determine if concentrations of veterinary antimicrobial agent residues entering the human colon remain microbiologically active [92]. Thus, the authorities have defined Acceptable Daily Intakes (ADI) of antimicrobial veterinary residues by human. The European Medicines Agency (EMA) have set the overall ADI value for enrofloxacin at 6.2 µg/kg body weight [92] [93] corresponding to the microbiological ADI because it is lower than the toxicological ADI of 12 µg/kg body weight which was calculated by applying a safety factor of 100 to the No Observed Effect Level (NOEL) of 1.2 mg/kg body weight per day [94].

In order to protect consumer's health, many countries have defined Maximum Residue Limits (MRL) of enrofloxacin and ciprofloxacin in animal-derived products [76]. In the European Union (EU), the MRLs of enrofloxacin and ciprofloxacin in muscle tissues and milk of all species are 100 µg/L [93] but as there is no MRL for enrofloxacin in eggs, enrofloxacin is forbidden in animals form which eggs are produced for human consumption [93].

To measure the enrofloxacin and ciprofloxacin residues in animal-derived foods including milk, eggs, honey and even mane and tail hair in horses, many analytical methods have been developed. Among these methods, we can mention High Performance Liquid Chromatography (HPLC); HPLC-Diode Array Detection method combined with liquid chromatography-mass spectrometry; HPLC-Ultraviolet Diode Array Detection method; molecularly imprinted solid-phase extraction procedure; liquid chromatography using a metal chelate affinity column; finally, the method of ChemiLuminescence Enzyme ImmunoAssay [76] [77] [91] [95]-[97].

8. Ecotoxicity

The release of enrofloxacin and ciprofloxacin in the environment is mainly due to the direct discharge of aquaculture products and the excretion in urine and feces of livestock animals [98]. It results in the contamination of soil, surface water, sediment, ground water and biota [99]. Once released into the environment the behavior of a chemical substance is determined by its tendency to partition from the aqueous phase to the atmosphere which is expressed by the Henry's Law Constant (HLC) and its affinity to adsorb on solid which is expressed by the Octanol-Water Partition Constant (Kow or log Kow) [99]. Enrofloxacin have a very low HLC at ambient temperature (<10 - 15 $atm \cdot m^3 \cdot mol^{-1}$) resulting in a negligible volatile loss and its Kow is low (0.83) while enrofloxacin have a high affinity for sludge, soils and sediments [99].

In the environment, enrofloxacin and ciprofloxacin can undergo degradations by different processes including photolysis, biodegradation and oxidation by mineral oxides but they are not sensitive to hydrolysis [99]. Despite these degradation mechanisms, environmental half-life time of enrofloxacin and ciprofloxacin are very long (half-life time estimated between 1155 and 3466 days for ciprofloxacin in a mesocosm soil study performed by Walters et al. in 2010, indicating an important persistence in soil matrices [100].

This long environmental persistence of enrofloxacin and ciprofloxacin can affect the growing or the activity of the soil microbial communities [101]. Indeed, although some edaphic organisms may utilize and decompose enrofloxacin as a nutrient, it is suggested that enrofloxacin or ciprofloxacin concentrations exceeding 0.2 mg/kg result in significant toxicity for the edaphon [101] [102]. Thus, edaphic ammonification and nitrification are affected and the edaphon community structure is modified, which can impact the soil fertility [102]. Furthermore, a quite recent study carried out in 2008 indicates that the exposure of whole earthworms and their different tissues to various concentrations of enrofloxacin can lead to changes in catalase activity and to a lesser extent, in growth rate [103].

The toxicity of enrofloxacin and ciprofloxacin for aquatic ecosystems has been assessed in many studies, as well on microorganisms as on algae or even aquatic vertebrates and invertebrates [104] [105]. Among these studies, three were carried out recently in zebrafish embryos [106], in tropical freshwater ecosystems with the monitoring of macro-invertebrates, zooplankton, phytoplankton, periphyton, bacteria, organic matter decomposition and nitrogen cycling [105] and in three tropical aquatic species (the green-algae Chlorella sp., the micro-invertebrate Moina macrocopa and the Nile tilapia Oreochromis niloticus) collected in a stream receiving effluents from a Pangasius catfish farm which uses enrofloxacin [104]. The results of these three studies are similar, suggesting that residual concentrations of enrofloxacin and ciprofloxacin in aquatic environment are not likely to result in direct or indirect severe toxic effects on aquatic ecosystems [104]-[106].

9. Conclusion

The fluoroquinolones are one of the most useful classes of antibiotics, as well in human medicine as in veterinary medicine. Thanks to their broad spectrum of activity against a wide range of bacteria and their physicochemical properties, their use is increasing. Enrofloxacin, which is a third generation fluoroquinolone only available in veterinary medicine, is thus used in many species with few adverse effects. However, there are recent concerns about the emergence of quinolone-resistant bacterial strains and the impact on the environment of the overuse of these drugs. Thus, there is now an important need to use fluoroquinolones with caution to preserve their effectiveness for many years. In veterinary medicine, it is essential to reserve these drugs for cases

requiring a powerful antibiotic and to prescribe and/or administer them only under a good clinical assessment and with appropriate regimens.

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Abbreviations

ADI: Acceptable Daily Intakes;
EMA: European Medicines Agency;
Gyrase: DNA Topoisomerase II;
G-DNA: Clived DNA Sequence;
HLC: Henry's Law Constant;
HPLC: High Performance Liquid Chromatography;
MAR: Multiple Antimicrobial Resistance;
MIC: Minimum Inhibitory Concentration;
MPC: Mutant Prevention Concentration;
MRL: Maximum Residue Limits;
PCR: Polymerase Chain Reaction;
NOEL: No Observed Effect Level;
PMQR: Plasmide-Mediated Quinolone Resistance;
QRDR: Quinolone Resistance-Determining Region;
SPC: Summary of Product Characteristics;
Topo IV: DNA Topoisomerase IV;
T-DNA: Transported DNA Sequence.

20

The Impact of Chronic Social Stress on Emotional Behavior in Mice and the Therapeutic Effect of Peripheral Mild-Heat Stimulation

Hang Liu[1], Takuji Yamaguchi[2], Kenji Ryotokuji[3], Satoru Otani[2,4], Hiroyuki Kobayashi[2,5], Masako Iseki[1], Eichi Inada[1]

[1]Department of Anesthesiology and Pain Management, School of Medicine, Juntendo University, Tokyo, Japan

[2]Center for Advanced Kampo Medicine and Clinical Research, Juntendo Graduate School of Medicine, Tokyo, Japan

[3]Institute of Transcultural Studies, Ryotokuji University, Chiba, Japan

[4]Center for Medical Education, Ryotokuji University, Chiba, Japan

[5]Department of Hospital Administration, Juntendo University Graduate School of Medicine, Tokyo, Japan

Abstract

In today's stressful society, mental diseases such as adjustment disorder, affective disorders including anxiety and depression, and chronic fatigue syndrome could be attributed to social stress and are considered as serious social problems. It has been long known in the acupuncture and moxibustion therapies in Japan and China that stimulation at Zhongwan and Taichong points have effects on stress-related anxiety and depression. In the present study, we therefore tested the effect of peripheral mild-heat stimulation at Zhongwan and Taichong on anxiety and brain biochemistry using a mouse model of chronic social stress. Male ddY mice (4 weeks old) were stressed by isolated rearing for 6 weeks. They received mild-heat stimulation (43˚C for 5 min) at Zhongwan and Taichong under isoflurane anesthesia after 4 weeks of the social isolation 3 times a week for 2 weeks, using the "Stress Free Apparatus" (Ryoken Co., Ltd). Control mice received only isoflurane anesthesia. Group-reared mice also underwent the same stimulus/anesthesia sessions. Two days after the last peripheral heat stimulation, anxiety was determined by the light/dark box test as the time spent in the light compartment and the number of transfer between the light and dark compartments. Before and after the last stimulation, blood was collected from an artery at the back of

the eye, and serum corticosterone, immunoglobulin E (IgE) which was an index of the inflammatory immune system, and interleukin 6 (IL-6) were measured using ELISA Kit. The frontal cortex was collected after the behavioral test, and the dopamine and serotonin contents were measured also by ELISA Kit. A significant decrease of the time spent in the light compartment was observed after the isolated rearing ($p < 0.01$). Mild-heat stimulation significantly increased the time spent in the light compartment, indicating an anti-anxiety effect. Serum corticosterone, IgE and IL-6 were increased after stress, and the amount of serotonin in the frontal cortex was found decreased. The increased corticosterone, IgE and IL-6 were cancelled by mild-heat stimulation. These results suggest that chronic social isolation has negative biological and emotional impacts in mice and that peripheral mild-heat stimulation alleviates at least part of these stress effects.

Keywords

Chronic Stress, Anxiety, Acupuncture, Frontal Cortex, Corticosterone

1. Introduction

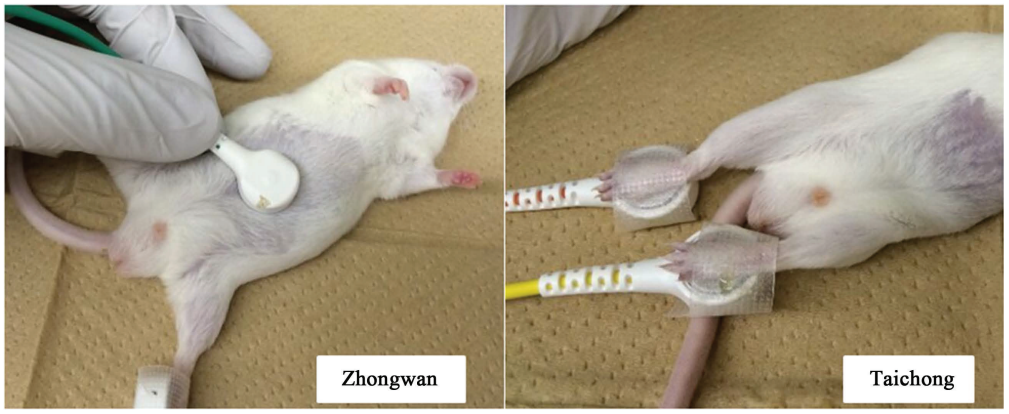
In our society, stress is known to be related to many clinical conditions. Especially, mental diseases such as adjustment disorder, depression, and chronic fatigue syndrome caused by chronic social stress are recognized as serious social problems [1]. Moreover, mood disorder has been linked to physical diseases for years. For example, endocrine diseases (e.g. hypothyroidism, hyperthyroidism, and Cushing's syndrome), organic brain diseases (e.g. Parkinson's disease, multiple sclerosis, and cerebrovascular disease), and other diseases including cancer, systemic erythematodes (SLE), cardiac infarct, and chronic pain, can be caused by anxiety and depression resulting from social stress [2]. Nevertheless, currently, there are no effective treatments available for these stress-related diseases.

It has been reported in the acupuncture and moxibustion therapies practiced in Japan and China that Zhongwan and Taichong stimulations have effects on stress-related anxiety and depression [3]-[5]. Zhongwan (CV-12) is the 12th acupuncture point that belongs to the conception vessel meridian. Zhongwan activates the autonomic nervous system [6], and has an anti-inflammatory effect [7] as well as a palliative effect on chronic anxiety disease [8] and severe depression [9] [10]. Taichong (LR-3) is the 3rd acupuncture point of the liver meridian of foot-jueyin, and is a transport point and a yuan-primary point of the foot. Taichong stimulation has been shown to have a palliative effect on epilepsy, insanity, bile, temper, and lowering of mood [3]-[5]. It is also thought that taichong stimulation has an antihypertensive property [11].

Recently, as a sophisticated version of the traditional acupuncture method, the pinpoint plantar low-wavelength infrared light irradiation (PP-LILI), known as stress-free therapy® was introduced (Trademark Registration No.5495960; patent Application No. PCT [Patent Cooperration Theaty]/JP [Japan] 2012/073972). Thus, application of a mild-heat stimulation at a specific plantar part with the PP-LILI was shown to cause a decrease of blood ACTH, an increase of deep body temperature, and an increase of bran blood flow [12]-[14], which are expected to have anti-anxiety effects. This novel method draws attention as a possible therapeutic option to treat stress-related disorders. To evaluate its effects objectively, the basic animal research is now required.

Along with the introduction of the concept of chronic stressor, a syndrome called functional somatic syndrome (FFS) is often mentioned [15] [16]. For example, the developmental environment in the juvenile stage is considered as one of the factors to cause pathogenesis of mental diseases, and rodents reared in isolation for a long time in the juvenile stage serve as important model animals [17] [18]. Rearing small laboratory animals such as mice and rats individually for a long time leads to behavioral changes not seen in group-reared normal animals, particularly called "isolation syndrome" [19] that includes anxiety, depression, increased aggression, and memory disorder [20]. The isolation syndrome is considered as a model for human mental diseases such as schizophrenia, anxiety disorder, mood disorder, and drug dependence [21] [22].

In the present study, behavioral effects of a 6-week isolated rearing in mice starting at the juvenile stage (28 days of age) were quantified. As a therapeutic option for stress-related disorders, we then tested the effect of mild-heat stimulation at Zhongwan and Taichong points (**Figure 1**), using the PP-LILI. Moreover, blood levels of corticosterone, immuno-globulin E (IgE), interleukin-6 (IL-6) as well as the amount of dopamine and seroto-



(a) (b)

Figure 1. The locations of the stimulation to Zhongwan and Taichong points in the mouse are shown. In our condition, heat stimulation was applied to these points as well as areas surrounding these points to some extent.

nin in the frontal cortex were measured to determine whether the PP-LILI also normalizes changes in these biological stress markers.

2. Material and Method

2.1. Animals and Stress Protocol

Three weeks old male ddY mice were purchased from Japan SLC Co, Ltd. (Shizuoka, Japan). The ddY strain is an outbred one, and has been maintained as a closed colony. Mice of the strain show good reproductive performance and superior growth. In Japan, this strain, along with ICR strain, has been widely used in trials for drug efficacy and various fields of researches, such as pharmacological, phamacokinetic, and toxicological studies [23]. After a week of habituation, animals of the experimental group (stressed group) were placed individually in a transparent cage (9 × 13 × 20 cm) for 6 weeks ("isolation" group). A wooden wall was inserted between the cages so that the isolated animals are deprived of visual contact with others. As the control group, 5 mice were reared together in a cage of the size 23 × 31 × 15.5 cm ("group" group). All animals were reared under the following conditions; 23˚C ± 2˚C, relative humidity 55% ± 10%, 12 hours of light (7:00-) and dark (19:00-) cycle, free drinking, and normal food consumption (MF, Oriental Yeast Co., Ltd., Tokyo, Japan). The experiments were conducted in accordance with the guideline of Juntendo University Medical School Animal Testing (JACUC) (Registration no. 1022).

2.2. Mild-Heat Stimulation Method

After 4 weeks of isolated rearing, mild-heat stimulation was applied at Zhongwan (CV-12) and Taichong (LR-3) by using a mild-heat stimulator (Stress Free Apparatus, Ryoken Co, Ltd. Tokyo) under isoflurane anesthesia. Briefly, Zhongwan is located at the midpoint between the bottom edge of the sternumand the navel, and Taichong is at the junction of the 1st and 2nd metatarsals of the foot. However, in our method, areas surrounding these points were also heat-stimulated to some extent (**Figure 1**). The temperature of stimulation was set at 43˚C, and the duration of stimulation was 5 min/day, which was repeated 3 times a week for 2 weeks. This stimulation parameter is based on the previous human studies [12]-[14], slightly modified to accelerate data acquisition. Control mice received only isoflurance anesthesia. The same operations were performed on the group-reared mice.

2.3. Light/Dark Box Test Method

Two days after the last mild-heat stimulation, behavioral changes were evaluated by using a light/dark box. After habituating the mice in the dark compartment of the light/dark box (20 × 16 × 20 cm) for 1 minute, the gate was opened, and the time spent in the light compartment and the number of transfer between two compartments were measured for 5 min as the indicator of anxiety state.

2.4. The Measurement of Blood Serum Component

After 6 weeks of isolated rearing (in the case of corticosterone measurement, also after 2 and 4 weeks), blood was collected from an artery at the back of the eye, and the blood serum was obtained after centrifugation (15,000 rmp × 20 minutes, 4˚C). The blood serum was frozen and preserved at −20˚C. Serum corticosterone, IgE, and IL-6 were measured by ELISA Kit: Mouse Corticosterone Kit (Assay Designs, Michigan, USA), Mouse IgE Kit (Morinaga Institute of Biological Science Inc. Yokohama, Japan), and Mouse IL-6 Kit (mouse IL-6 Immunoassay R & D Systems USA) were used for measuring, respectively, corticosterone, IgE, and IL-6 levels.

2.5. The Measurement of Brain Monoamines

Two days after the last stimulation, the frontal cortex was collected, frozen and preserved at −80˚C. The tissue was homogenized in the buffer solution (0.01 N hydrochloric acid (wako), 1 mM EDTA (sigma), 4 mM sodium disulfite (sigma)), and the supernatant fluid was obtained after centrifugation (15,000 rmp × 30 minutes, 4˚C). Dopamine (Dopamine Research RIA, Labor Diagnostika Nord, Nordhorn, Germany) and serotonin (Serotonin Research RIA, Labor Diagnostika Nord, Nordhorn, Germany) were measured by each ELIZA Kit. The total amount of protein was then measured (BCA Protein Assay kit Pierce, Rockford, IL, USA).

2.6. Statistical Analysis

All data were presented as mean ± S.E.M. The data were analyzed with Student's t-test to evaluate differences between mild-heat stimulation group and the control group. P values of <0.05 were considered as statistically significant.

3. Results

3.1. The Effect of Isolated Rearing on Behavior

The time spent in the light compartment and the number of transfer between the dark and light compartments in the isolation-stimulation group, the group-stimulation group and the control groups were quantified. The time spent in the light compartment was significantly decreased in the isolation-control group compared with the group-control group (the isolation-control group 144.33 ± 8.2 sec (n = 17), the group-control group 183.4 ± 8.4 sec (n = 19), $p < 0.01$; **Figure 2(a)**). This decrease of the time spent in the light compartment seen in the isolation-control group was significantly increased to the group-control level by peripheral mild-heat stimulation (5 min each, 3 times a week for 2 weeks; the isolation-stimulation group; **Figure 2(a)**). The time spent in the light compartment in the group-stimulation group was increased compared with the group-control group, but no significant difference was observed (**Figure 2(a)**).

No significant inter-group changes were observed in the number of transfer between two compartments (**Figure 2(b)**). The time spent in the light compartment per each transfer was decreased (the isolation-control group 9.4 ± 0.9 sec, n = 17; the isolated-stimulation group 15.1 ± 2.3 sec, n = 19, $p < 0.05$; **Figure 2(c)**) in the isolation-control group compared with the group-control group, and the peripheral mild-heat stimulation significantly increased this value (the isolation-control group 9.4 ± 0.9 sec, n = 17; the isolated-stimulation group 13.6 ± 1 sec, n = 20, $p < 0.01$). No significant difference was observed between the group-control group and the group-stimulation group.

3.2. The Change of Corticosterone by Isolated Rearing Stress

It was found that serum corticosterone was significantly increased at 2 weeks (1839.2 ± 115.6 pg/ml, n = 9, $p < 0.001$), 4 weeks (2903.1 ± 367.9 pg/ml, n = 9, $p < 0.001$) and 6 weeks (3647.2 ± 268.6 pg/ml, n = 4, $p < 0.01$) after isolation rearing compared with the group-rearing group (1024.3 ± 32.4 pg/ml at 2 weeks, n = 9; 1168.5 ± 57.2 pg/ml at 4 weeks, n = 9; 1284.3 ± 57.4 pg/ml at 6 weeks, n = 4; **Figure 3**). There were also significant differences between 2 weeks and 4 weeks of isolation ($p < 0.01$) and between 2 weeks and 6 weeks of isolation ($p < 0.001$; **Figure 3**).

3.3. The Effect of Mild-Heat Stimulation on Serum Corticosterone

The increase of serum corticosterone (see **Figure 3**) by isolated rearing in the isolation group was significantly

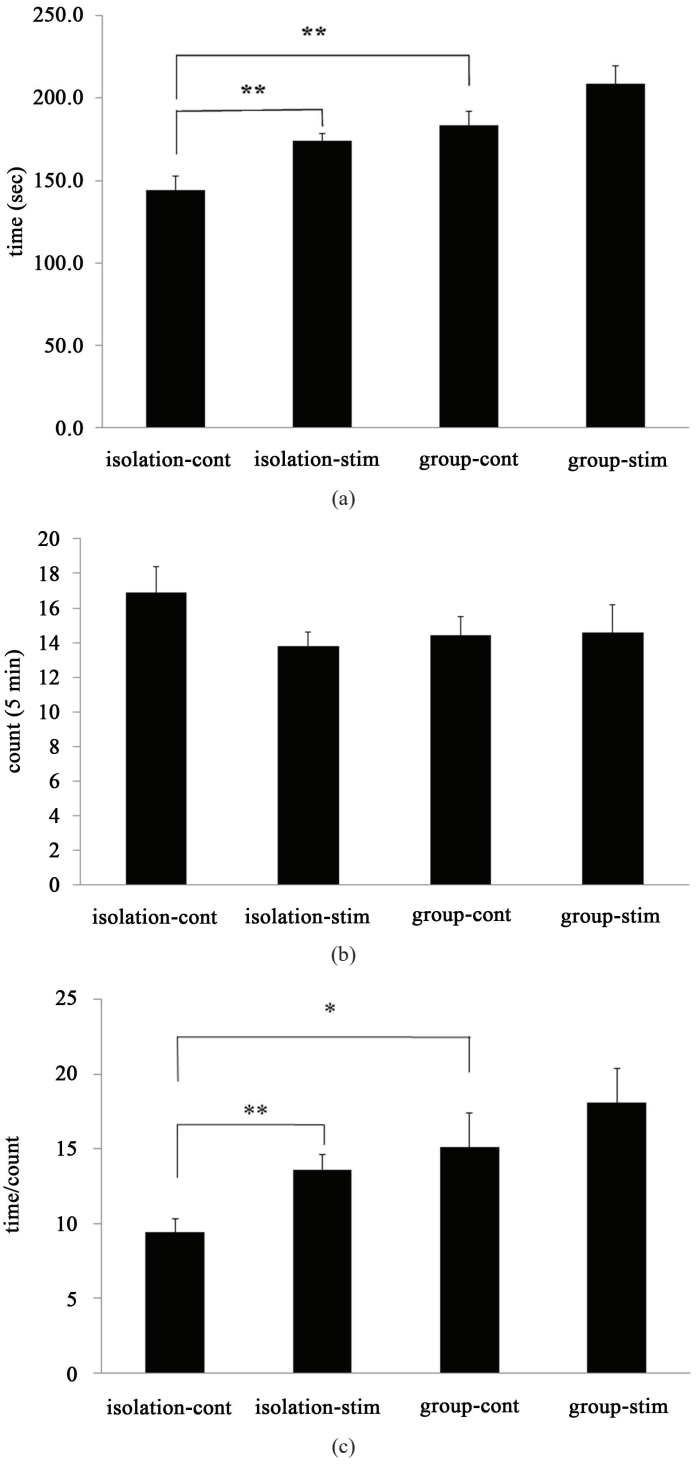


Figure 2. (a) The 6 weeks social isolation stress significantly decreased the time spent in the light compartment (isolation-cont) of light/dark box, compared with the group-reared control group (group-cont). This decrease was restored to the control level by brief (5 min) mild-heat stimulation applied during the last 2 weeks (3 times a week) of the 6 weeks stress (isolation-stim). Values are mean ± S.E.M. $^{**}p < 0.01$ vs. control; (b) The 6 weeks social isolation stress (isolation-cont) did not change the number of transfer between the light and dark compartments compared with the group-reared control group (group-cont). Mild-heat stimulation also did not change the number of transfer. Values are mean ± S.E.M; (c) The 6 weeks social isolation stress (isolation-cont) significantly decreased the time spent in the light compartment par each transfer, and this value was significantly increased by mild-heat stimulation. Values are mean ± S.E.M. $^{*}p < 0.05$, $^{**}p < 0.01$.

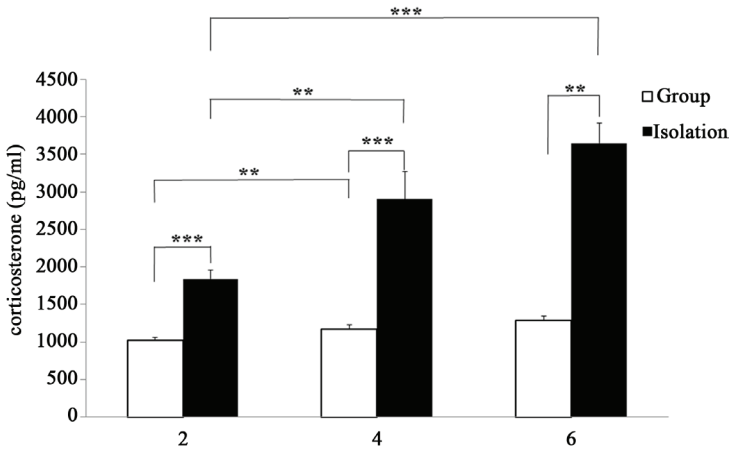


Figure 3. Serum corticosterone levels significantly increased at 2, 4, and 6 weeks after isolated rearing (black bars) compared with the group rearing group (white bars). There were also significant differences in corticosterone levels in the isolation rearing group between 2 and 4 weeks of isolation and between 2 and 6 weeks of isolation. Values are mean ± S.E.M. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

decreased by mild-heat stimulation applied during the last 2 weeks of isolation (the isolation-control group 3647.2 ± 68.6 pg/ml, n = 4; the isolation-stimulation group 1670.4 ± 126.4 pg/ml, n = 4, $p < 0.01$; **Figure 4**). No effects of mild-heat stimulation on serum corticosterone level were observed in the group-rearing group (control group, 1284.3 ± 57.4 pg/ml (n = 4), stimulation group, 1160.8 ± 97.8 pg/ml n = 5; **Figure 4**).

3.4. The Effect of Mild-Heat Stimulation on Serum Total IgE Level

The serum total IgE level was significantly increased in the isolation group after 6 weeks of isolated rearing compared with the group-rearing group (the isolation-control group 6.54 ± 0.52 pg/ml, n = 8; the group-control group 4.00 ± 0.47 pg/ml, n = 9 , p < 0.01; **Figure 5**). This increase was significantly lowered by mild-heat stimulation (the isolation-control group 6.54 ± 0.52 pg/ml, n = 8, the isolation-stimulation group 3.68 ± 0.34 pg/ml, n = 10, $p < 0.001$). In the case of the group-rearing group, the serum total IgE level in the stimulation group was also significantly decreased by mild-heat stimulation (the group-control group 4.00 ± 0.47 pg/ml, n = 9; the group-stimulation group 2.13 ± 0.29 pg/ml, n = 10, $p < 0.01$; **Figure 5**).

3.5. The Effect of Mild-Heat Stimulation on Serum Interleukin-6

Serum Interleukin-6 was significantly increased in the isolation group after 6 weeks of isolated rearing compared with the group-control group (the group-control group 106.7 ± 9.9 pg/ml, n = 7, the isolation-control group 177.1 ± 6.9 pg/ml, n = 6, $p < 0.001$; **Figure 6**). This increase was significantly lowered by mild-heat stimulation (the isolation-control group 177.1 ± 6.9 pg/ml, n = 6, the isolation-stimulation group 148.3 ± 7.7 pg/ml, n = 7, $p < 0.05$; **Figure 6**). A similar stimulation effect was observed also in the group-rearing groups (the group-control group 106.7 ± 9.9 pg/ml, n = 7, the group-stimulation group 71.5 ± 4.5 pg/ml, n = 8, $p < 0.01$; **Figure 6**).

3.6. The Effect of Mild-Heat Stimulation on Frontal Cortex Dopamine Level

There were no significant differences in the amount of dopamine in the frontal cortex between the group-control group and the isolation-control group after 6 weeks of isolated rerating (**Figure 7**). Mild-heat stimulation did not significantly increase the amount of dopamine in the isolation group, but a significant increase of dopamine by stimulation was observed in the group-rearing group (the group-control group 10 ± 2 pg/μg protein, n = 7, the group-stimulation group 17 ± 1 pg/μg protein, n = 7, $p < 0.05$; **Figure 7**).

3.7. The Effect of Mild-Heat Stimulation on Frontal Cortex Serotonin Level

The amount of serotonin in the frontal cortex was significantly decreased in the isolation group compared with

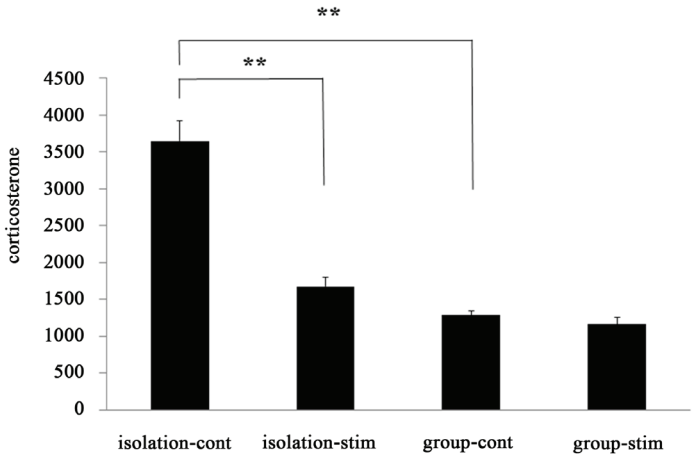


Figure 4. The increased serum corticosterone by 6 weeks social isolation stress (isolation-cont)was decreased to the control level by brief (5 min) peripheral mild-heat stimulation (3 times a week for 2 weeks; isolation-stim). Mild-heat stimulation did not change the corticosterone value in group-reared mice (group-stim). Values are mean ± S.E.M. $^{**}p < 0.01$.

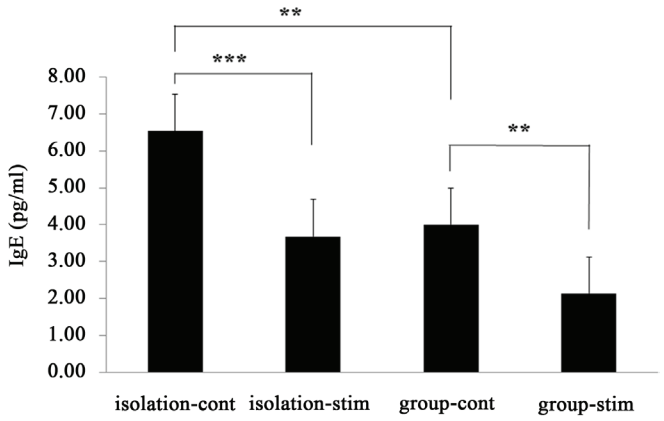


Figure 5. The 6 weeks social isolation stress (isolation-cont) significantly increased the serum IgE level compared with the group-reared control (group-cont), and this increase was lowered to the control level by brief peripheral mild-heat stimulation (isolation-stim). The heat stimulation also significantly decreased the IgE level in the group-reared control mice (group-stim). Values are mean ± S.E.M. $^{**}p < 0.01$; $^{***}p < 0.001$.

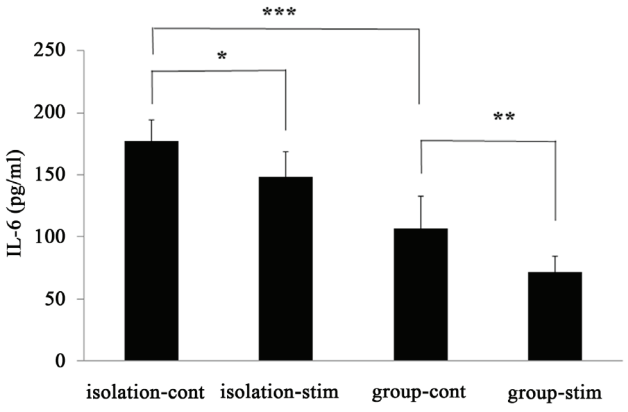


Figure 6. The 6 weeks social isolation stress (isolation-cont) significantly increased the serum IL-6 level compared with the group-reared control (group-cont), and this increase was significantly lowered by brief peripheral mild-heat stimulation (isolation-stim). The mild-heat stimulation also decreased the IL-6 level in the group-reared control mice (group-stim). Values are mean ± S.E.M. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

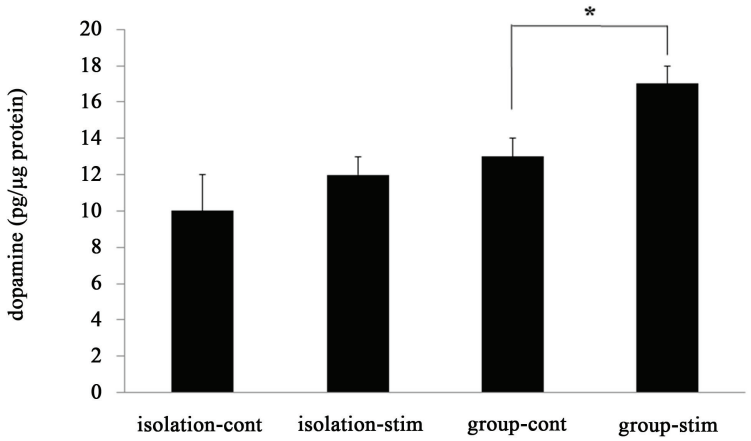


Figure 7. Dopamine content in the frontal cortex did not change after 6 weeks of social isolation stress (isolation-cont) compared with the group-reared control mice (group-cont). Brief peripheral mild-heat stimulation did not change the dopamine level in the stressed mice (isolation-stim), but it significantly increased the dopamine level in the group-reared mice (group-stim). Values are mean ± S.E.M. $^{*}p < 0.05$.

the group-control group after 6 weeks of isolation rearing (the group-control group 25 ± 2 pg/μg protein, the isolation-control group 17 ± 2 pg/μg protein, $p < 0.01$; **Figure 8**). This decrease in the isolation group was not significantly improved by mild-heat stimulation, but mild-heat stimulation caused a significant increase of the amount of serotonin in the group-rearing group (the group-control group 25 ± 2 pg/μg protein, n = 7, the group-stimulation group 30 ± 1 pg/μg protein, n = 8, $p < 0.01$; **Figure 8**).

4. Discussion

The present results showed that the 6 weeks social isolation stress in mice caused anxiety as indicated by a decreased time spent in the light compartment of the light-dark box and that this anxiety was ameliorated to the control group level by peripheral brief (5 min) mild-heat stimulation at abdomen and a plantar points, applied 3 times a week for 2 weeks. Moreover, the values of blood corticosterone, IgE and IL-6 were significantly increased by social isolation stress, and these increases were significantly inhibited by the mild-heat stimulation. Finally, while the amount of serotonin in the frontal cortex was significantly decreased by the social isolation stress, mild-heat stimulation had no effect on this serotonin change.

Chronic social stress is known to exert its impact on the body through the neuroendocrine system, autonomic nervous system, chronic inflammation, and oxidant stress [24]. Stimulation by stress is thought to be transmitted to the hypothalamic area through the cortex and the limbic system, and promotes the production of corticotrophin releasing hormone (CRH) in the paraventricular nucleus of hypothalamus; as a result, adrenocorticotropic hormone (ACTH) is released and promotes a production and release of glucocorticoid (the main component of which is cortisol in humans and corticosterone in rodents) from the adrenal cortex [25]. This hypothalamic-pituitary-adrenal (HPA) axis has been implicated for years as a pathological mechanism of mood disorder. For example, it has been indicated since 1960's that the level of cortisol in urine and blood and its metabolites are high in the patients of depression [26] [27]. In the present study, we found that serum corticosterone was indeed significantly increased by isolated rearing compared with the group-rearing control.

The physiological responses including the autonomic nervous system and the HPA axis and the behavioral reactions such as avoidance and escape, both caused by the exposure to stress, are essentially protective responses to the stress [21] [22]. Besides the peripheral effects such as increased blood sugar level, the release of glucocorticoid is known to release dopamine in the prefrontal cortex [28], another example for the adaptive response. However, it is also indicated that the brain system involved in stress reactions is formed dynamically depending on the developing process [29] [30], where stress given in the juvenile stage decreases hippocampal volume [31] and has impacts on emotions and cognitive functions after maturation [32]. Furthermore, a prolonged stress chronically decreases the release of dopamine in the prefrontal cortex and impairs working memory [33]. The results of the present study confirmed that a prolonged isolation stress from the adolescence stage, *i.e.* 28 days after birth, increased anxiety and declined the amount of serotonin in the frontal cortex. No statisti-

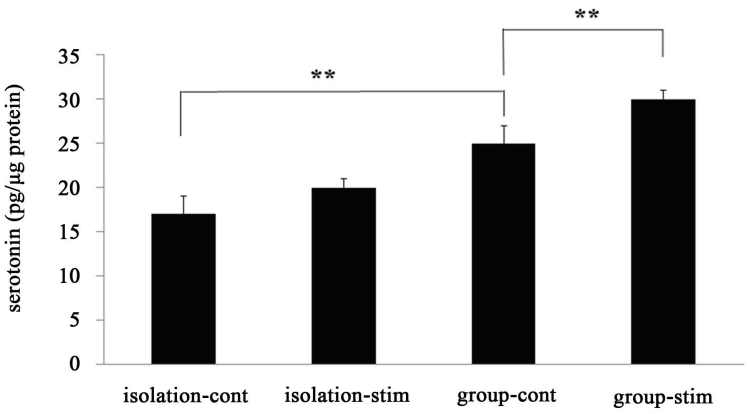


Figure 8. Serotonin content in the frontal cortex decreased significantly by the 6 weeks social isolation stress (isolation-cont) compared with the group-reared control group (group-cont). Brief peripheral mild-heat stimulation did not change the serotonin level in the stressed mice (isolation-stim), but it significantly increased the serotonin level in the group-reared mice (group-stim). Values are mean ± S.E.M. $^{**}p < 0.01$.

cally significant decrease of the amount of dopamine was observed. However, social isolation stress has been previously shown to decrease dopamine in the prefrontal cortex, as well as serotonin and glutamate [34]. The absence of the difference in dopamine content in the present study may be due to the fact that we dissected the whole frontal cortex rather than the confined, prefrontal area.

It is unknown at present how the prolonged social stress induces decreases of the amount of brain monoamine. But it is possible that the promotion of the production and release of glucocorticoid by stress stimulation is involved. As mentioned above, glucocorticoid receptor stimulation in the prefrontal cortex increases the release of stress-related neuromodulator dopamine [28] in the prefrontal cortex, through the positive-feedback system to midbrain dopamine neurons [35]. The present results demonstrated that serum glucocorticoid was significantly increased 2 weeks after the isolated rearing, and this increase was boosted further after 4 to 6 weeks of stress delivery (**Figure 3**). Such chronic increases of glucocorticoid may rather induce adapted decreases of dopamine release in the prefrontal cortex. It is also reported that the serotonin release in the prefrontal cortex is decreased by isolation stress [34], thus consistent with the present results, although the underlying mechanisms are still unclear.

Whatever the mechanisms, brain monoamines are involved in controlling emotions and cognitive behaviors, and a decrease of these neuromodulators would result in the abnormality of emotions and cognitive behaviors. As is well known, dopamine neurons project to the limbic and the cortical areas from the ventral tegmental area (VTA), and controls cognition, reward-related learning, and emotional behavior [36]-[38]. Especially, decreased dopamine in the prefrontal cortex results in the dysfunction of this cortex, and this may in turn lead to a decreased inhibition on the amygdala, resulting in the increased anxiety [39]. On the other hand, serotonin, synthesized in the regions including dorsal raphe nucleus, is projected to various areas in the brain including the cortex and the hippocampus [40] [41]. It is thought that a decrease of serotonin is the most important biological factor for causing depression [42], and serotonin is indeed involved in controlling of emotions [43]. It was shown in rodents that the decrease of serotonin synthesis in the brain caused declines of cognitive functions, depression, and anxiety [44]. In the present study, however, peripheral mild-heat stimulation did not restore the amount of serotonin in the frontal cortex, indicating that the anti-anxiety effect of mild-heat stimulation might involve other mechanisms. In the group-rearing group, on the other hand, the peripheral mild-heat stimulation did increase the amount of dopamine and serotonin: thus, the peripheral mild-heat stimulation can up-regulate frontal cortex monoamine levels in non-stressed animals.

We also demonstrated that chronic social stress increases serum IgE and IL-6, and that the increases of these factors are significantly inhibited by peripheral mild-heat stimulation. Enhanced release of glucocorticoid and catecholamine by stress changes the balance of Th1 cells and Th2 cells [45], where Th2 cells become predominant leading to an increase of serum IgE level [46]. It is known that the IgE level increases in the patients with allergic rhinitis, allergic asthma, and allergic dermatitis, and that the IgE value correlates with the severity of patients with allergic rhinitis and allergic dermatitis [46]. It is possible that peripheral mild-heat stimulation de-

creases corticosterone and inhibits its effect on Th1/Th2 balance; mild-heat stimulation thus may have a function to decrease the risk of developing allergic diseases by inhibiting the amount of serum IgE.

On the other hand, IL-6 is produced by astrocytes and microglia in the brain [47] and is known as an inflammatory marker cytokine for schizophrenia, depression, anxiety disorder, and mood disorders caused by chronic stress [48] [49]. According to the recent findings, IL-6 regulates the activity of ERK (extracellular signal-regulated kinases) which is an important second messenger regulating neuronal plasticity, and IL-6 indeed decreases hippocampal LTP and cortical synaptic transmission [47]. Therefore, the increase of IL-6 level in the prefrontal cortex may decrease the activity of this cortical area, which might result in an increased emotional behavior controlled by the amygdala.

It was previously shown in human subjects that the peripheral mild-heat stimulation decreases blood ACTH and cortisol, and increases the core body temperature and the cerebral blood flow [12]-[14]. The present study extended these results by using the laboratory animals for the first time. Thus, the increased corticosterone level by stress was significantly inhibited by peripheral mild-heat stimulation, and the increases of other stress markers IgE and IL-6 by stress were also significantly countered by peripheral mild-heat stimulation. This novel method is called "Stress Free" for its potential anti-stress effects, and the results of the present study using the mouse model suggest that this peripheral stimulation method indeed may normalize stress-related emotional changes. It remains to be demonstrated now how this peripheral mild-heat stimulation induces these endocrinological and behavioral changes. For example, moderate heat stimulation to specific peripheral sites might exert inhibitory effects on e.g. hypothalamus through afferent inputs.

5. Conclusion

In conclusion, chronic social stress is a major problem in our society. Nevertheless, there are no effective methods to date to treat cognitive and emotional abnormalities caused by social stress. The present study demonstrates the possibility that the brief, peripheral mild-heat stimulation alleviates stress-related anxiety. This result may provide a unique contribution to the field of neuropsychiatry.

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21

Magnesium: The Neglected Electrolyte? A Clinical Review

Pranev Sharma[1], Christine Chung[2], Marcela Vizcaychipi[1]

[1]Magill Department of Anaesthesia, Chelsea & Westminster Hospital, London, UK

[2]Imaging & Anaesthetics, Chelsea & Westminster Hospital, London, UK

Abstract

Magnesium, Mg^{2+}, is the second most abundant intracellular cation after potassium and the fourth most abundant in the body. It was first isolated in 1808 by the English chemist, Sir Humphrey Davy. Magnesium is essential to numerous biochemical reactions. It modulates key physiological processes such as metabolic biochemistry, nucleic acid synthesis, receptor-binding and ion flux. The western diet falls short of the recommended daily allowance of 4.5 mg/Kg/day and important dietary sources are seeds, grains, nuts and green vegetables. It is used as a therapeutic agent in a broad range of pathologies: neurological, cardiovascular, respiratory, gastrointestinal and obstetric. The pharmacokinetics and pharmacodynamics of magnesium, as a drug, are not well understood. Despite its fundamental importance to human physiology, it remains an electrolyte that is not routinely measured as part of the "urea & electrolytes" test and is the most overlooked electrolyte deficiency in hospital inpatients. This review will summarise the importance of magnesium homeostasis, its pharmacological effects and clinical applications.

Keywords

Magnesium, Pharmacology, Pharmacodynamics, Pharmacokinetics, Physiology, Arrhythmias, Eclampsia, Pre-Eclampsia, Phaeochromocytoma

1. Magnesium Intake and Regulation of Balance

A normal adult has approximately 1000 mmol of magnesium (22 - 26 g), the majority of which is stored within the bone (52.9%), followed by muscle (27%) and soft tissue (19%). Normal serum concentration ranges from 0.70 - 1.10 mmol/L [1] [2]. Serum magnesium levels are a poor reflection of total body stores as only less than 1% of total body magnesium is found in blood. This is an important consideration when supplementing in clinical practice [3].

The recommended daily allowance (RDA) for magnesium is 4.5 mg/Kg/day, or 13 mmol/day for a 70 kg individual, with requirements increasing in illness. Magnesium rich foods include seeds, grains, nuts and green vegetables, whilst processed foods tend to lack magnesium. Phytate, fibre and alcohol reduce the absorption of magnesium. Surveys have shown the average Western diet falls short of the RDA [1] [2].

Magnesium is mainly absorbed in the ileum and jejunum [4]. The mechanism of uptake is yet to be fully elucidated, with theories including passive paracellular uptake, passive leak and active extrusion [1] [5] [6].

Plasma magnesium levels are tightly controlled. Whilst the exact mechanism of regulation remains unknown, the renal system plays an essential role. In normal physiology, 80% of plasma magnesium is filtered, of which 95% is reabsorbed, leading to 3 - 5 mmols lost in the urine per day. The kidney is able to increase or decrease the urinary magnesium loss dependent on plasma magnesium concentration but the mechanism behind this is unclear [2].

Several hormones are also known to exert influence over magnesium homeostasis. For example, parathyroid hormone (PTH) promotes magnesium reabsorption in the loop of Henle and distal convoluted tubule [7] [8]. It stimulates gut uptake of magnesium and release of magnesium from bones [9].

The incidence of hypomagnesaemia is higher amongst diabetics, and this is correlated with the severity of hyperglycaemia, suggesting a link between insulin and magnesium [10]. *In-vitro* work has shown increased magnesium uptake in response to insulin in human platelet cells [11]. Studies have produced conflicting results on whether an oral-glucose loading test reduces [1] [12], or causes no change [13], in plasma magnesium in healthy controls.

Insulin's physiological opposite hormone, glucagon, increases magnesium reabsorption in the rat loop of Henle [14] but no change was found in plasma magnesium in humans, in response to intravenous glucagon [15].

These mechanisms are summarised in **Figure 1**. **Table 1** highlights the non-drug and drug causes of hypo- and hypermagnasaemia.

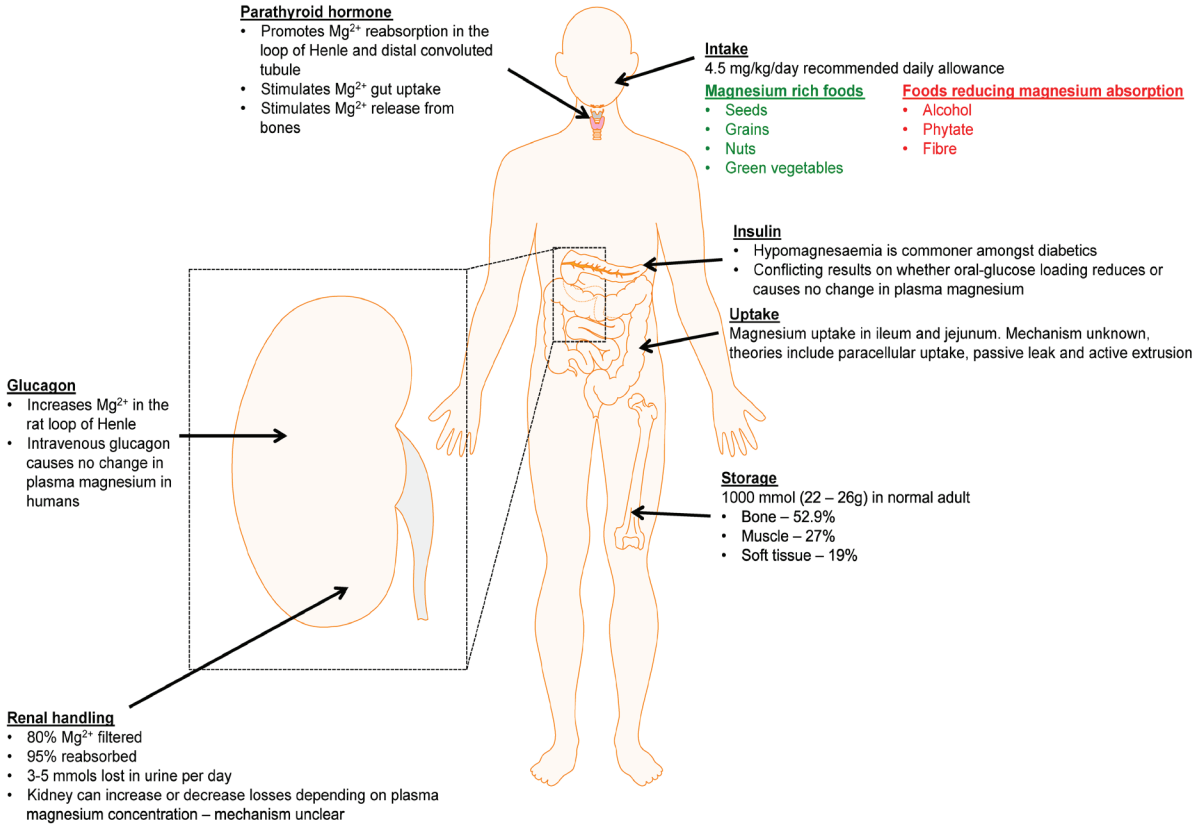


Figure 1. This cartoon illustrates the intake, absorption and regulation of balance of magnesium. Image modified from Motifolio Anatomy Drawing Toolkit available from http://www.motifolio.com/anatomy.html.

Table 1. This table shows the important non-drug and drug causes of hypo- and hypermagnesaemia. Reference: [70].

	Causes of hypomagnesaemia	Causes of hypermagnesaemia
Non-drug causes	Inadequate Mg^{2+} intake	Excessive intake
	High Ca^{2+} intake	Renal failure
	Malabsorption states	Hypothyroidism
	Gastrointestinal losses, e.g. diarrhoea, vomiting	Excessive tissue breakdown, e.g. sepsis, shock, burns
	Polyuria	
	Parathyroid dysregulation	
	Bowel resection	
Drug causes	Diuretics	Lithium
	Laxatives	Mg^{2+} containing antacids (excessive intake)
	Nephrotoxic drugs	Magnesium containing laxatives
	Insulin	

2. Physiological Role of Magnesium

Magnesium is of fundamental importance to hundreds of physiological processes [1] [16] [17], some of which are shown in **Figure 2**.

2.1. Magnesium in Enzymatic Reactions

Magnesium is a catalyst in enzymatic reactions; either Mg^{2+} induces a conformational change in the structure of an enzyme, or it can bind to a substrate, for which the enzyme will have a greater affinity [17].

Adenosine triphosphate (ATP) is a cellular "energy currency" used in several processes, such as fat, protein and nucleic acid synthesis. Enzymes responsible for transferring phosphate from adenosine diphosphate (ADP) or ATP are activated by Mg^{2+} [17].

Magnesium is also crucial in nucleic acid biochemistry. It is a co-factor to restriction endonucleases, exonucleases, polymerases and phosphatases, either as Mg^{2+} or bound to nucleotide triphosphate (NTP) as Mg-NTP [17].

2.2. Magnesium's Effect Platelet Activity and Coagulation

Magnesium inhibited the synthesis and release of pro-aggregation factors cyclooxygenase, lipooxygenase and thromboxane A2 during *in-vitro* work on human platelets [18]. Human *in-vivo* studies confirmed the theory, where magnesium infusions were shown to reduce platelet aggregation [19] and increase bleeding time [20]. It would, therefore, be prudent to monitor for signs of bleeding in hypermagnaesmic patients.

In vitro, magnesium enhanced the activity of factor IXa, increased the binding between factors IXa and VIII and together with Ca^{2+}, caused the activation of factor X [21]. However, the physiological relevance of this remains uncertain as intravenous magnesium did not alter the fibrin-degradation products or antithrombin III complex in human volunteers [22], the INR in human volunteers [23] or the coagulation index in a pre-eclamptic cohort [24].

2.3. Magnesium in Nucleic Acid Biochemistry

Magnesium serves as a co-factor to restrict endonucleases, exonucleases, polymerases and phosphatases, either as Mg^{2+} or bound to nucleotide triphosphate (NTP) as Mg-NTP [17].

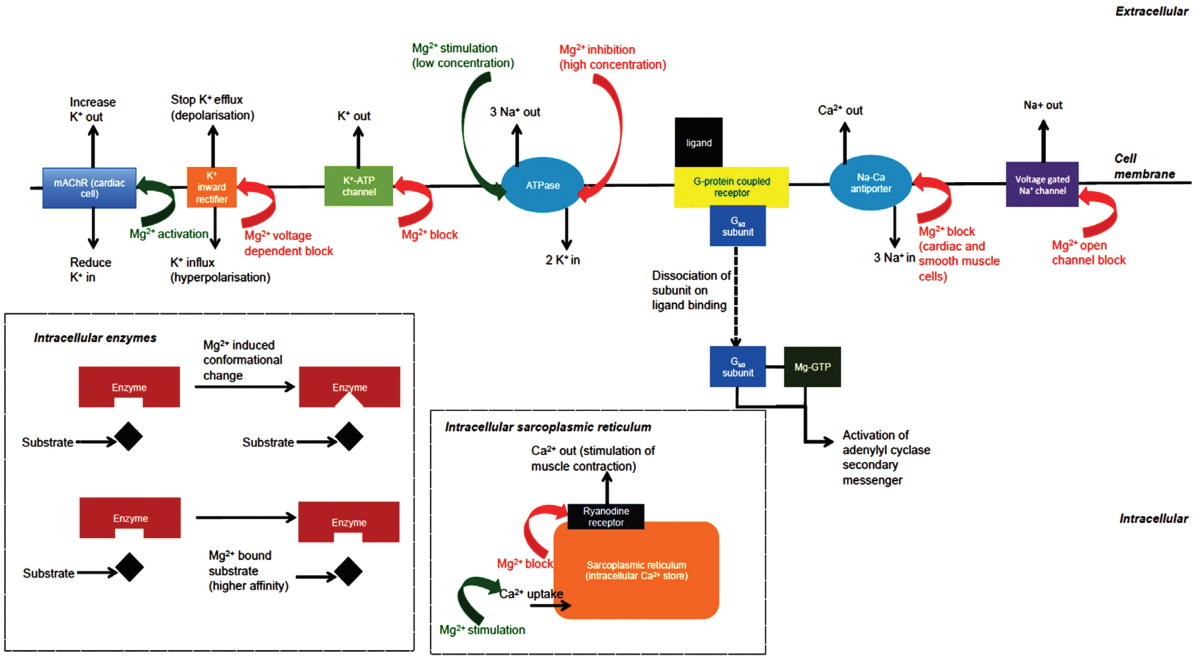


Figure 2. This cartoon illustrates schematically the key mechanisms of how Mg^{2+} modulates ionic transfer across cell membranes, how Mg^{2+} acts as a catalyst to intracellular enzymes and its interaction with the sarcoplasmic reticulum to modulate Ca^{2+} release. Image modified from Motifolio Anatomy Drawing Toolkit available from http://www.motifolio.com/anatomy.html.

2.4. Magnesium in Hormone-Receptor Binding

G-protein coupled receptors are ubiquitous and involved in many organ systems, serving cardiovascular, endocrine, neurological and reproductive functions [25] to name but a few. Upon activation of receptors with the $G_{s\alpha}$ subunit, the $G_{s\alpha}$ subunit dissociates and binds to magnesium-bound guanosine triphosphate (Mg-GTP), which is responsible for activation of adenylyl cyclase, the second messenger transduction system responsible for hormonal signal transmission [26].

2.5. Magnesium in Intracellular Calcium Regulation

Calcium influx into muscle cells triggers release of calcium from its intracellular store, the sarcoplasmic reticulum, via the activation of ryanodine receptors. This stimulates muscle contraction in both cardiac and skeletal muscle [27] [28]. Magnesium blocks this process via inhibition of the ryanodine receptors [27] [28] and it stimulates calcium uptake into the sarcoplasmic reticulum [29]. By competing with calcium binding sites on muscle filaments [30] [31], magnesium acts as a physiological antagonist to calcium [31].

The Na-Ca antiporter is essential in lowering intracellular calcium concentration after depolarisation [32], and this too is modulated by magnesium. Mg^{2+} inhibits this antiporter via competition with Ca^{2+} in cardiac cells [33] and smooth muscle cells [34], showing a role for magnesium in regulation of ion transfer across cell membranes.

2.6. Magnesium in Ionic Transfer across Cell Membranes

Magnesium is responsible for modulating ionic transfer of sodium or potassium via several pathways [35].

Intracellular Mg^{2+} causes an open-channel block of voltage-gated sodium channels, blocking the outward sodium current [36], which has been proposed as having a role in modulation of depolarisation [35].

Magnesium plays a role in potassium channels. Many cells contain a potassium inward rectifying channel that is responsible for influx of potassium under hyperpolarisation but stopping efflux under depolarisation. This behaviour is known as inward rectification and is responsible for prolonging depolarisation, which is of relevance in cardiac function and egg fertilisation. The inward rectification is caused by rapid closure of the potassium channel with a voltage-dependent block by intracellular Mg^{2+} [37].

Magnesium further affects inward rectification via modulation of the muscarinic acetylcholine receptor in cardiac cells. Activation of this receptor causes K^+ efflux and slows pacemaker activity. Depletion of intracellular Mg^{2+} not only increases the K^+ efflux but also reduces the K^+ influx. Mg^{2+} also participates in the initial activation of the muscarinic potassium channel via G-protein binding [38].

Cardiac muscle, skeletal muscle and insulin-secreting cells exhibit potassium-selective single membrane channels, inhibited by adenosine triphosphate (ATP), known as K^+-ATP channels [39] [40]. This K^+-ATP channel is blocked by intracellular Mg^{2+} [41] [42]. In cardiac muscle, this channel is activated under anoxia and causes a shortened plateau of the ventricular action potential and thus reduced contraction. This may be a protective mechanism preventing a dramatic fall in intracellular ATP, critical for cellular processes [40] [43]. In insulin-secreting cells, this channel has a role in initiating insulin release [40]. Its role in skeletal muscle remains unclear [40].

The symptoms of hypomagnesaemia are shown in **Table 2**.

3. Pharmacokinetics of Magnesium

Information on the pharmacology of magnesium is limited. Chuan *et al.* conducted a pharmacokinetic model, using a population approach, upon a cohort of 116 patients who received magnesium sulphate for pre-eclampsia with measurement of serum magnesium concentration. Their model estimated, along with interpatient variability (co-efficient of variation); a systemic clearance of 4.28 L/h (37.3%), a volume of distribution of 32.3 L (32.1%), a baseline concentration of 0.811 mmol/L (18.5%) and a half-life of 5.2 hours [44]. Similar pharmacokinetic modelling by Lu et al. on 51 pre-eclamptic patients given magnesium sulphate estimated a volume of distribution of 24.0 L (39%) [45].

4. Magnesium as a Drug

Magnesium's important therapeutic roles are summarised in **Table 3**.

4.1. Magnesium in the Central Nervous System

Magnesium has been used as a therapeutic agent in acute pain. A recent meta-analysis conducted on twenty randomised trials examined the effect of perioperative intravenous magnesium upon postoperative pain. Magnesium was superior to placebo in reduction of opiate consumption by a mean of 10.52 mg of morphine, as well as a reduction in pain scores at rest and with movement [46]. This may be due to magnesium's role as an NMDA-receptor blocker, similar to ketamine's analgesic properties [47].

There may be a role of magnesium in the treatment of chronic pain. A double-blinded randomised controlled trial found intravenous and oral magnesium therapy in patients with chronic low back pain lowered pain scores and increased range of movement compared to pre-treatment [48].

In long-term care patients with primary insomnia, magnesium, in conjunction with other oral supplements, improved sleep quality, sleep time, ease of getting to sleep, hangover on awakening and alertness the following day [49].

Table 2. This table shows the symptoms of hypomagneaemia. Reference: [71].

Range (mmol/L)	Range (mmol/L)	Symptoms
Mild	0.7 - 1.0	-
Moderate	0.5 - 0.7	-
Severe	<0.5	Weakness
		Convulsionscardiac arrhythmias
		ECG changes
		Nystagmus
		Positive Chvostek and Trousseau sign

Table 3. This table shows the potential therapeutic uses of magnesium.

System	Condition	Evidence base
Central nervous system	Acute pain	● Perioperative IV magnesium reduced opiate consumption postoperatively and led to reduced pain scores [46]
	Chronic pain	● IV and PO magnesium increased range of movement and reduced pain scores in chronic back pain with a neuropathic component [48]
	Memory dysfunction	● Increased brain magnesium enhanced learning and memory in rats [72] but no research in humans to date
	Neuromuscular blockade	● Potentiates the effects of non-depolarising neuromuscular blocking agents [73]-[76] ● Blunts the serum potassium rise induced by suxamethonium [77]
	Postoperative shivering and thermoregulation	● Reduction in hypothermic shivering threshold from 36.6˚C to 36.3˚C (not clinically significant) [78], some reports of reduced postoperative shivering [79]-[81] but not borne out in a systematic review [82]
	Insomnia	● PO supplementation gave improved sleep quality, sleep time and ease of getting to sleep [49]
Cardiovascular system	Arrhythmias	● Treatment for torsades de pointes, digoxin toxicity, most atrial and ventricular arrhythmias where hypokalaemia is present [50] ● Superior to amiodarone in cardioversion of atrial tachyarrhythmias in intensive care [51]
	Myocardial infarction	● Reduced infarct size in canine model [83] ● LIMIT-2 study showed reduced 28-day mortality in magnesium treated group [84] ● Larger and more recent ISIS-4 and MAGIC-2 trials showed no survival benefit [85] [86]
	Cardiothoracic surgery	● Prophylactic magnesium reduced incidence of postoperative atrial fibrillation from 28% to 18% [53] and ventricular arrhythmias by 48% [54] ● Magnesium in cardioplegic solution reduced postoperative ischaemia [55]
	Phaeochromocytoma anaesthesia	● Case series reported 15 out of 17 cases with good haemodynamic stability at induction and tracheal intubation, with 4 cases requiring additional pharmacological support for blood pressure control during tumour handling [59]
Respiratory system	Acute severe asthma	● Scottish Intercollegiate Guidelines Network (SIGN) and British Thoracic Society (BTS) 2012 recommend intravenous magnesium for patients with acute asthma, non-respondent to inhaled short-acting beta-2 agonists, inhaled anticholinergics, corticosteroids and oxygen [60] ● Reduced admission rates in acute severe asthma but not in non-severe [61]
	Respiratory muscle weakness	● Improved respiratory muscle power in hypomagnesaemic patients given supplementation [62]
Gastrointestinal system	Mendelson's syndrome	● Magnesium trisillicate is superior to cimetidine in achieving a higher gastric pH [66] in prophylaxis against Mendelson's syndrome (pulmonary aspiration of gastric contents in obstetric anaesthesia)
Obstetrics	Eclampsia	● Reduced risk of maternal death and seizure recurrence compared to diazepam [67]
	Pre-eclampsia	● Reduced risk of developing eclampsia compared to placebo, phenytoin and nimodipine [68]

4.2. Magnesium in the Cardiovascular System

Magnesium is established in the treatment of cardiac arrhythmias, particularly torsades de pointes, digoxin toxicity and most atrial and ventricular arrhythmias where hypokalaemia is present [50]. In fact, a randomised control trial showed magnesium sulphate to be superior to amiodarone in cardioversion of atrial tachyarrhythmias in intensive care patients [51].

Serum ionised magnesium is known to fall in patients undergoing cardio-pulmonary bypass [52]. Meta-analyses, have shown a reduction in postoperative atrial fibrillation from 28% to 18% with magnesium treatment [53] and a reduced risk of ventricular arrhythmias by 48% [54] compared to placebo in cardiac surgery. Magnesium in the cardioplegic solution is protective of the myocardium [55] and reduces the incidence of perioperative ischaemia [56] compared to controls.

Phaeochromocytoma is known to cause cardiovascular instability during induction of anaesthesia, tracheal intubation and tumour handling [50]. *Ex-vivo* work on cat adrenal glands showed the importance of calcium in co-ordinating catecholamine release from the adrenal gland [57], and it is probably via antagonism of calcium that Mg^{2+} is able to inhibit adrenal catecholamine release [58]. James discussed the clinical relevance of this in a case series, where magnesium sulphate was used as the primary anti-adrenergic agent in 17 phaeochromocytoma anaesthetics. Catecholamine levels were reduced from time of induction to tumour handling by magnesium sulphate in the 5 cases where measurements were taken. Fifteen of the seventeen cases had good haemodynamic stability at induction and tracheal intubation, with 4 cases requiring additional pharmacological support for blood pressure control during tumour handling [59].

4.3. Magnesium in the Respiratory System

The joint Scottish Intercollegiate Guidelines Network (SIGN) and British Thoracic Society (BTS) guidelines of 2012 on the management of asthma recommend intravenous magnesium should be considered for patients with acute asthma, non-respondent to inhaled short-acting beta-2 agonists, inhaled anticholinergics, corticosteroids and oxygen [60].

A Cochrane review found magnesium therapy was beneficial in reducing admission rates in acute severe asthma, with an improvement in the peak expiratory flow rate. These results were not seen in non-severe acute asthma [61].

Magnesium may have some direct effect upon respiratory muscle power as hypomagnesaemic patients display an improvement in respiratory muscle power when magnesium is replaced [62].

4.4. Magnesium in the Gastrointestinal System

Mendelson's syndrome is the pulmonary aspiration of acidic liquid gastric contents intra-operatively or early postoperatively in obstetric anaesthesia, leading to dyspnoea, cyanosis and shock [63] [64]. Antacid therapy has been an established treatment in the prophylaxis against Mendelson's syndrome [65] and magnesium trisillicate is superior to cimetidine in achieving a higher gastric pH [66].

4.5. Magnesium in Obstetrics

Magnesium is efficacious in the termination of seizures associated with eclampsia. A recent Cochrane review showed intravenous or intramuscular magnesium reduced the risk of maternal death and seizure recurrence (risk ratios 0.59 and 0.43 respectively) compared to diazepam. There was no impact on perinatal or neonatal mortality, with fewer babies needing admissions greater than 7 days in the magnesium group [67].

In pre-eclampsia, a Cochrane review showed magnesium reduced the risk of developing eclampsia compared to placebo or no drug therapy in six trials (risk ratio 0.41), and to a lesser extent when compared to phenytoin in three trials (risk ratio 0.08) and nimodipine in one trial (risk ratio 0.33) [68]. It should be noted that rates of progression from pre-eclampsia to eclampsia were as low as 1.9% in the Magpie trial [69], the largest trial on the matter.

5. Summary

Magnesium is fundamental to numerous physiological processes. Physical manifestations of hypomagnesaemia reflect the multitude of systems that depend on this unique electrolyte.

This article discussed magnesium homeostasis, important roles magnesium plays in enzyme interactions, nucleic acid synthesis and facilitating ionic transfer across cell membranes. Overall, the pharmacology of magnesium as a drug is not well known but the clearance, volume of distribution and half-life has been described by modelling data in humans.

Finally, we have discussed established indications for magnesium in the treatment of eclampsia, cardiac dysrhythmias, acute severe asthma and prevention of adrenergic surge in phaeochromocytoma surgery. We have explored more novel applications of magnesium in the prevention of perioperative complications, such as; aspiration in obstetric surgery, postoperative pain and arrhythmias following cardiac surgery.

Magnesium is often overlooked as an electrolyte, which deserves greater appreciation.

Statement of Conflict of Interest

None.

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22

Curricular Content Level of Complexity Is a Factor of Depression and Learning in Medical Students

Rosalinda Guevara Guzmán, María Esther Urrutia-Aguilar*

Facultad de Medicina, Universidad Nacional Autónoma de México, México D.F., México

Abstract

The aim of this paper was to analyze the impact of Human Physiology curriculum on depression and academic performance of medical students. Beck instrument was applied before exams' period to all students who voluntarily participated and the grades gotten in each of the three Human Physiology exams were correlated with all first year's averages. Descriptive and inferential statistics were carried out. We found a significant relationship between the complexity of Human Physiology curriculum and the number of cases of depression, resulting in low academic average; high correlations were among Human Physiology contents and those of Biochemistry and Anatomy. It is important to implement support programs to follow students' emotional welfare as well as to go over the subject's content.

Keywords

Depression, Medical Student, Human Physiology Curriculum, Academic Average

1. Introduction

1.1. Depression and Academic Performance

The academic field represents a set of stressing situations for students as they may experience a lack of control over the new environment and consequently turn into academic failure [1]. Several investigations have shown the existence of noticing indexes of stress in university populations, specially, during the first courses of the medical career and the time before exams [2]-[5].

Medical career, in particular, due to its curricular load, demands great commitment and discipline of study from students; some of them cannot cope with all the work or do not have a solid academic background, which consequently alters their mental and physical state. It has been reported the impact in mental health, stress, anxiety and depression experienced by medical students throughout their school year, which causes problems such as low academic performance [6]-[9].

Some authors have also reported that during the second year of medical career, the levels of anxiety and stress are much more elevated than during the first year; maybe because this second year is still within the adaptation period at the school for many students [10]-[14]. Others have determined that medical students' stress increases along with their studies in the clinical area, therefore representing a higher risk of academic failure [15].

The most significant causes of stress among medical students are the study material excess, lack of organizing time, pressure from professors, exams, fatigue as well as personal and familiar issues. Moreover, it has to be considered that academic stress affects three main areas: behavior, cognition and learning. In the behavior area, it has been studied how lifestyle changes during exams; tobacco, caffeine and alcohol consumption increase to keep awake.

1.2. Medical Curriculum

The 2010 Medical Curriculum of the Faculty of Medicine at UNAM is dedicated to teach according to new trends in medical education to fit the changing situation of the health system and the society's needs and expectations. During the first two years, the teaching of biomedical areas is emphasized: human physiology's objective in the formation of the future surgeon is to provide knowledge of the human organism's functions, to make students acquire the necessary scientific methodology for its study and to develop attitudes towards health maintenance and the treatment of diseases; in addition to this, inclusive macrostructure of the body, the function of the organs, the biochemistry and the pharmacological mechanisms, and consequently, the regular functioning as well as possible therapeutics [16] [17].

UNAM's medical students show a higher index of physiology failing in regards to other subjects. Given the importance in the formation of the future surgeon, the aim of this work is to learn whether the complexity of Human Physiology curriculum causes depression before exams affecting their grades.

2. Material and Methods

2.1. Ethical Aspects

The Ethics and Research Committees of the Faculty of Medicine at UNAM approved the research protocol. The participants, who volunteered, signed an acceptance letter. The results were used carefully guarding their confidentiality. The instruments were applied to second grade students taking Human Physiology and only those who completed their answers were considered.

2.2. Type of Curriculum and Course Setup

The UNAM's 2010 Medical Curriculum is a mixed program as areas and subjects are interacting to conform the curriculum to an orientation towards the development of competences. During the first two years, the teaching of the biomedical area is emphasized. First year: Anatomy, Biochemistry and Molecular Biology, Cellular Biology and Medical Histology as well as Human Embryology. Second year: Human Physiology is given with four hours of theory a week and four of practice during 32 weeks, with a total of 23 credits. The assessment is carried out through exams (50%) and the professor's criterion (50%).

The topics being dealt with are divided into thematic units. The first exam assesses cellular physiology, neurophysiology and the autonomic nervous system; the second, cardiovascular, respiratory and renal physiology; and the third one, endocrine system and the digestive apparatus.

2.3. Questionnaire

Beck Depression Inventory (BDI). This is one of the most used instruments for the assessment of depressive symptoms in adolescents and adults [18] [19]. It comprises 21 self-applicable questions; every question includes a series of four asseverations from which one has to be chosen in regards to the way the patient has felt

throughout the week. The total score varies from 0 to 63. A score higher or equal to 12 points was considered as a signaling cut to identify the cases with probable depression [20]-[22].

This instrument was applied to second grade medical students during the school year 2013-2014, a week before exams. The percentage of right answers in the three Human Physiology exams from August 2013 to May 2014 was considered as well as the final average of those from the first year August 2012 to May 2013 of only Anatomy, Biochemistry and Molecular Biology, Cellular Biology and Medical History as well as Human Embryology's. It is important to highlight that the exams were applied at the same time to all populations and each contained 50 to 70 items chosen by a group of experts from an items bank (Cronbach ≥ 0.87 Y 0.92; level of difficulty = 30 and 70; positive discrimination = 70 and 90).

In order to learn the levels of complexity of the themes covered in each of the three Human Physiology exams, we carried out correlations with grades obtained in the first year's biomedical area (Anatomy, Biochemistry and Molecular Biology, Cellular Biology and Medical Histology as well as Human Embryology).

2.4. Statistical Analysis

The data were analyzed with the SPSS version 20. The statistical analysis was descriptive and inferential; they were conducted under the assumption of a type I error rate of 0.05.

3. Results

The number of students that had answered the Beck questionnaire before each exam varied because, the day of its application, only those who had attended, out of 1035 students in second grade, answered it. In the first exam, 20% took it; in the second 27%; and, in the third, 46% of the total generation.

Table 1 shows the average of right answers in each exam, as well as the percentage of cases of depression. It was noted that during the second exam is where the highest percentage of depression cases were found (32%) and a lesser number of right answers in the exam (means 50) compared with that of the other two exams showing that it was during the cardiovascular course when students got the least checks.

The Kolmogorov-Smirnov statistical test showed no normality in the behavior of the percentages of assertiveness in the exams. We applied the Kruskal-Wallis test, which reported significant differences among them $p \leq 0.0001$.

Pearson correlations (**Table 2**) show that averages obtained in the second exam have the highest correlation with each one of the averages obtained in the curriculum first year biomedical area subjects.

Table 1. Results of score and depression during exams.

Exams	Media ± SD	% Cases	N
First	65 ± 2.8	23	207
Second	50 ± 3.8	**32**	279
Third	60 ± 3.9	13	280
Final	58 ± 3.5		

Table 2. Average correlation of the three human physiology exams and that of first year biomedical area subjects.

	Human physiology exams		
First year subjects	**First**	**Second**	**Third**
Anatomy	0.50	**0.58**	0.54
Cellular biology and medical histology	0.47	0.53	0.50
Biochemistry and molecular biology	0.49	**0.55**	**0.56**
Human embryology	0.45	**0.60**	0.44

In order to conduct a deeper correlation, we considered the averages per student in each Anatomy, Biochemistry, Histology and Human Embryology exam, which were correlated with the average of the second Human Physiology exam (**Table 3**). The results showed that the cardiovascular theme covered in the second Human Physiology exam has a higher correlation with that of Anatomy discussing Generalities of the spinal column, head and neck; with those of Cellular Biology and Medical Histology with themes on cells and tissues; with those seen in Biochemistry and Molecular Biology, carbohydrates metabolism, transport, glucogenesis, Krebs cycle and antioxidants; and lastly, with Human Embryology, where they learn about gastrulation, placenta and nexus.

4. Discussion

As it can be noted in the results from **Table 1**, we found that there is a relationship between the number of right answers and the level of depression that students show during the second exam, which coincides with literature in terms of a poor academic performance associated with levels of depression in the medical training [2]-[4] [6] [11]. Moreover, the results show that the cardiovascular topic addressed in the second set is the one that caused more depression to students and in which less percentage of right answers was obtained; the Kruskal-Wallis test reported significant differences among them $p \leq 0.0001$. It is important to highlight that the cardiovascular mechanisms are comprehensive components in the understanding of the physiopathological changes of diseases and in the integration of the basic and clinical sciences in a meaningful way [23]. In this research work, we showed the significant interaction of biomedical area's learning to integrate Human Physiology knowledge (Pearson correlations 0.44 to 0.60); some authors have reported the difficulty that students face to assimilate the physiological processes in these systems [24].

The latter shows the urgency to carry out a diagnosis of the complexity of the program's contents, as it shows that the objective to provide knowledge of the functions of the organism before the students' entry to clinical venues is not being accomplished and that this may lead to bad medical diagnosis that they give during their training in the clinical area. The medical student must handle knowledge, concepts and basic principles (core curricula) integrated from medical basic sciences [25], know when it is clinically important to focus in details, and use these data to back up the best medical decision for each individual patient. In addition to this, the General Medical Council "Tomorrow's Doctors", the Australian Medical Council, the Association of American Medical Colleges (AAMC), and the National Commission for Academic Accreditation & Assessment highlighted the importance of reducing the academic overload in the study curriculum [26]-[28].

With this study we show that the complexity of contents cause a high level of depression in students, which directly affects their academic performance. This is due mainly to the complexity of the cardiovascular course and its interrelation with the contents of first subjects taught the year before as shown by the Pearson correlations 0.49 to 0.50. We consider that previous knowledge of the biomedical area that students were supposed to have built as significant was not achieved thus becoming a depression factor that results in poor academic performance. It is probable that the implementation of a remedial course between the first and second years of medical school considering only the contents of those subjects that have a higher correlation with the understanding of Human Physiology may improve students' academic performance.

Table 3. Correlations of human physiology second exam averages in each of the biomedical area subjects.

Anatomy			
First	Second	Third	Fourth
0.49	0.36	0.36	0.41
Histology			
0.49	0.29	0.34	0.36
Biochemistry			
0.49	0.49	0.41	0.41
Embryology			
0.50	0.43	0.38	0.46

5. Conclusions

Biomedical area professors must promote interdisciplinary teaching for the student to integrate knowledge in the Human Physiology subject.

Finally, educational institutions must direct their efforts towards the analysis of Human Physiology contents and only teach those nuclear contents for the prevention and effective handling of depression in students.

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Chronic Toxicity Study in Rats Orally Exposed to Mulberry Sea-Buckthorn Beverage Concentrate

Qingping Sun*, Zhimin Xu

Guangdong Institute of Food and Drug Control, Guangzhou, China

Abstract

Objective: This study is designed to observe the chronic toxicity after the administration of mulberry sea-buckthorn beverage concentrate for 3 months on rats and to predict the possible adverse effect and the potential toxicity target organs. Method: The rats (SPF level) were randomly divided into high-dose (20 mL/kg BW), middle-dose (10 mL/kg BW), low-dose (5 mL/kg BW) groups and negative control group (20 mL/kg BW of purified water) with 30 rats in each group. Each group was orally given mulberry sea-buckthorn beverage concentrate for 3 months and recovered by stop feeding samples for 2 weeks for a recovery observation. The rats' general condition, the organ coefficient, the indexes of hematology and blood biochemistry and the histological changes of the main organs were determined. Result: The appearance and behavior of activity in rats showed no anomalies in all these groups and all the rats put on weight during this period. Comparing to the negative control group, no obvious differences were observed in the weekly weight and organ coefficient of each dose group. After 3 months of administration, HGB in both mulberry sea-buckthorn beverage concentrate low-dose group and high-dose group were increased. No significant differences were observed in the indexes of hematology after 2 weeks of recovery. CREA in low-dose, middle-dose and high-dose groups were significantly increased after 3 months of administration and it remained in the high level in middle-dose and high-dose group even after 2 weeks of recovery. No drug-related lesions were observed in the histological changes of major organs. Conclusion: The results show that long term use of mulberry concentrated sea-buckthorn beverage can lead to increased CREA, which suggested kidney toxicity. Although no obvious pathological change was found in kidney, we should pay attention to chronic kidney damage in the further research.

*Corresponding author.

Keywords

Chinese Traditional Medicine, Chronic Toxicity Test, Mulberry Sea-Buckthorn Beverage Concentrate, Rats

1. Introduction

In China, traditional Chinese medicines were used in almost all diseases for Chinese patients. Many Chinese people choose traditional Chinese medicines as their favorite because the efficacies of traditional Chinese medicines have been proved by Chinese people's experiences in the past 5000 years. However, the toxicities, especially the chronic toxicities, of traditional Chinese medicines were not comprehensively studied due to the technical limitation of toxicology. In fact, toxicities of some traditional Chinese medicines were reported recently [1] [2]. Safety re-evaluation of traditional Chinese medicines is needed.

Mulberry sea-buckthorn beverage concentrate is composed of the fruit of mulberry and sea-buckthorn. The fruit of mulberry was usually taken as food or medicine in China. Studies showed that the fruit of mulberry contained polyphenol, volatile oil, polysaccharide, amino acid, and so on. To Chinese experiences, mulberry has the efficacy of enhancing immunity, antioxidant, antibacterial, reducing blood sugar, protecting liver and cardiovascular system [3]. Literature [3] has summarized the clinical application of the fruit of mulberry. It can be used for chronic hepatitis, hypertension, hyperlipidemia, diabetes mellitus, aplastic anemia, gastrointestinal diseases (gastric ulcer, duodenal ulcer, chronic gastritis, colitis), senile constipation and sleep disorders, blood deficiency caused by dizziness and tinnitus, sequelae of cerebral concussion, eye disease, *et al*. [4].

Sea-buckthorn (*Hippophae rhamnoides* Linn) is one of common deciduous shrubs of Elaeagnaceae; its main chemical constituents are flavonoids, terpenoids, steroids, volatile oil, organic acids, phenols, carbohydrates, including 14 kinds of vitamins, 12 kinds of organic acids and sugar, 22 kinds of amino acid and amide class, 7 kinds of fatty acid, 32 kinds of fat, 30 kinds of phenolic and flavonoid, and 29 kinds of trace elements [5].

Sea-buckthorn has many pharmacological effects and clinical applications, including cardiovascular and liver protection, enhancing immunity, restraining cancer, preventing atherosclerosis and delaying aging [6]. Studies have shown that sea-buckthorn could delay aging in drosophila [6].

Chronic toxicity test, as the main content of pre-clinical toxicity evaluation, has been widely used for preclinical toxicity assessment in medicine and health food. In this study, a chronic toxicity test has been performed to evaluate the chronic toxicity of mulberry sea-buckthorn beverage concentrate.

2. Materials and Methods

2.1. Animals

120 healthy SPF SD rats, 5 - 6 weeks of age, provided by medical laboratory animal center of Guangdong, were randomly divided into 4 groups with half male and half female, which are control group, low-dose group, middle-dose group and high-dose group. During administration, external signs, behavioral, mental state, reaction, activity, stool, eyes and cavity secretion, hair color, the general condition were daily observed and recorded. And body weight and food intake were measured and recorded weekly. This study has been permitted by Ehics Committee of GDIFDC.

2.2. Sample and Experimental Design

Mulberry sea-buckthorn beverage concentrate was produced by Sichuan Chengdu Hongyi Food Co., Ltd., which clinical dose is 0.667 mL/kg BW. In this study, the clinical doses of 7.5, 15, 30 times respectively as low-, medium-, high-dose group experiment.

Rats were oral exposed to sample with dosage of 5 mL/kg BW, 10 mL/kg BW, 20 mL/kg BW by once a day, 6 days a week, 3 months (Weeks 0-13). Negative control group were given equal volume of purified water. 3 months after the last feeding sample, 2/3 rats (half male and half female) were sacrificed for hematological examination, blood biochemistry determination and histopathology analyzing. After additional 2 week (Weeks 14-15) recovery observation, the remaining 1/3 rats were sacrificed and analyzed in the same way.

2.3. Hematological Examination

The collected blood samples were analyzed by automatic blood cell analyzer (BECKMAN COULTER AC.T 5 Diff). Some items were selected for this study, they were red blood cell count (RBC), hemoglobin (HGB), hematocrit (HCT), white blood cell count (WBC), platelet count (PLT), mean red cell volume (MCV), hemoglobin concentration (MCH), mean corpuscular hemoglobin concentration (MCHC), red blood cell volume distribution (RDW), mean platelet volume (MPV) and platelet hematocrit (PCT). Prothrombintime (PT) was measured by Platelet aggregation coagulation analyzer (LG-PABER-I).

2.4. Blood Biochemistry Determination

Automatic biochemical analyzer (Hitachi 7020) was used for blood sample analyzing of serum total protein (TP), the serum albumin (ALB), total bilirubin (TBiL), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), blood glucose (GLU), blood urea nitrogen (BUN), creatinine (CREA), total cholesterol (CHO) and triglyceride (TG).

2.5. Organ Coefficient

Heart, liver, spleen, lung, kidney, brain, adrenal, thymus, bladder, the male rat testis, epididymis, female rats ovary, uterus were weighted, and the organ coefficient were calculated according to the following formula.

$$\text{Organ coefficient (\%)} = \text{organ weight (g)/body weight (g)} \times 100.$$

2.6. Histological Analysis

For histopathology, heart, liver, spleen, lung, kidney, stomach, brain, adrenal gland, thyroid gland, bladder, pancreas, trachea, male rats' prostate, testis, epididymis and female rats' ovary, uterus samples were harvested and fixed in 10% neutral buffered formalin. Subsequently, they were dehydrated in a series of ethanol and embedded in paraffin. Approximately 3.5 μm thick sections were cut, mounted on glass slides and stained with hematoxylin and eosin (H&E). The sections were observed under an optical microscope (ZEISS Axio Scope A1) at different magnifications.

2.7. Statistical Analysis

Results were statistically analyzed using SPSS17.0 software. Body and organ weights, food intake, biochemical analysis, Hematological examination and Organ coefficient results were analyzed with a one-way ANOVA with a Bonferroni post-test. For all statistical results, a P-value of ≤ 0.05 was considered significant.

3. Results

3.1. Influence on the General Condition and Body Weight of Rats

In this study, all rats showed no obvious abnormalities in animal appearance, behavior, eating, drinking, and all rats were weight increased weekly. Compared with the negative control group, the body-weight and food intake of each group rats showed no significant difference ($P > 0.05$) in each week through this study (**Tables 1-3**).

3.2. Influence on the Blood Cells of Rats

The influences of mulberry sea-buckthorn beverage concentrate on the blood cells were showed in **Table 4**. As shown in **Table 4**, compared with the negative control group, HGB was significantly increased in rats of low-dose group and high-dose group after 3 months oral exposed to mulberry sea-buckthorn beverage concentrate. However, it decreased to normal level after 2 weeks recovery by stopping feed mulberry sea-buckthorn beverage concentrate. The other indexes of Hematological examination showed no significant difference between groups of sample and group of negative control.

3.3. Influence on Blood Biochemistry of Rats

The influences of mulberry sea-buckthorn beverage concentrate on the blood biochemistry were showed in

Table 5. As shown in **Table 5**, compared with the negative control group, CREA was significantly increased in all sample groups' rats after 3 months oral exposed to mulberry sea-buckthorn beverage concentrate. Furthermore, it still showed significantly increased in rats of middle-dose group and high-dose group after 2 weeks recovery by stopping feed mulberry sea-buckthorn beverage concentrate. The other indexes of blood biochemistry examination showed no significant difference between groups of sample and group of negative control.

Table 1. Influence on the food intake of rats ($\bar{x} \pm s$).

	Groups	Negative control	Low-dose group	Middle-dose group	High-dose group
Food intake (g/cage)	Week 1	616.33 ± 34.78	595.72 ± 41.17	609.03 ± 20.47	600.82 ± 24.77
	Week 2	692.25 ± 81.17	700.02 ± 68.31	669.87 ± 57.42	694.53 ± 46.47
	Week 3	722.63 ± 107.20	749.17 ± 85.94	720.73 ± 71.07	723.07 ± 80.77
	Week 4	751.57 ± 112.02	763.18 ± 101.20	764.62 ± 102.72	754.87 ± 94.06
	Week 5	765.13 ± 97.83	769.35 ± 121.34	747.17 ± 99.32	735.28 ± 90.61
	Week 6	767.97 ± 108.34	798.05 ± 112.22	770.48 ± 104.44	765.85 ± 87.71
	Week 7	753.10 ± 108.41	741.13 ± 103.26	749.15 ± 100.48	709.58 ± 92.29
	Week 8	721.03 ± 105.25	715.47 ± 101.12	701.35 ± 98.57	671.77 ± 73.83
	Week 9	701.52 ± 91.93	719.85 ± 109.35	697.88 ± 96.60	682.92 ± 89.50
	Week 10	729.42 ± 114.22	748.77 ± 110.58	716.50 ± 98.92	712.23 ± 91.74
	Week 11	728.02 ± 123.92	756.53 ± 108.69	729.73 ± 84.15	719.18 ± 96.16
	Week 12	720.95 ± 91.67	755.43 ± 76.40	699.02 ± 95.82	711.18 ± 66.78
	Week 13	525.58 ± 60.49	524.58 ± 79.95	507.90 ± 70.40	499.98 ± 59.18
	Week 14	722.85 ± 150.83	748.05 ± 161.29	740.99 ± 133.48	732.20 ± 115.54
	Week 15	722.80 ± 150.61	778.30 ± 146.23	776.28 ± 134.31	773.75 ± 89.73

For Weeks 1-13, n = 6 cages, 5 rats/cage. For Weeks 14-15, n = 2 cages, 5 rats/cage.

Table 2. Influence on body-weight of female rats ($\bar{x} \pm s$).

	Groups	Negative control	Low-dose group	Middle-dose group	High-dose group
Body weight (g)	Week 0	91.06 ± 3.80	90.94 ± 3.77	89.42 ± 4.55	89.94 ± 3.08
	Week 1	124.66 ± 5.47	125.49 ± 6.97	126.12 ± 5.46	125.39 ± 4.30
	Week 2	150.23 ± 6.96	153.06 ± 8.46	151.85 ± 7.17	151.54 ± 4.17
	Week 3	173.31 ± 9.85	177.64 ± 11.58	180.70 ± 10.56	177.78 ± 6.95
	Week 4	188.19 ± 11.76	195.07 ± 13.92	195.60 ± 10.34	195.83 ± 7.73
	Week 5	202.90 ± 11.98	207.67 ± 14.67	208.45 ± 10.56	207.51 ± 7.11
	Week 6	216.22 ± 13.43	218.99 ± 14.92	219.35 ± 11.56	218.59 ± 8.04
	Week 7	224.07 ± 14.72	226.62 ± 16.16	227.39 ± 12.50	224.06 ± 8.21
	Week 8	230.61 ± 15.84	237.75 ± 16.08	234.26 ± 14.22	233.74 ± 10.87
	Week 9	239.44 ± 16.58	243.67 ± 17.35	243.12 ± 13.08	240.33 ± 10.01
	Week 10	246.29 ± 19.12	249.82 ± 17.77	249.01 ± 15.31	245.57 ± 10.15
	Week 11	251.25 ± 18.93	255.11 ± 18.45	254.36 ± 14.28	251.94 ± 13.14
	Week 12	256.09 ± 19.28	266.21 ± 20.56	258.29 ± 15.68	259.42 ± 15.12
	Week 13	262.86 ± 20.04	264.6 ± 20.70	260.41 ± 16.04	258.63 ± 15.04
	Week 14	263.43 ± 31.77	281.43 ± 21.80	274.85 ± 15.66	268.33 ± 8.38
	Week 15	266.73 ± 24.21	279.33 ± 24.00	278.15 ± 9.75	270.38 ± 8.58

For Weeks 0-13, n = 14. For Weeks 14-15, n = 4.

Table 3. Influence on body-weight of male rats ($\bar{x} \pm s$).

	Groups	Negative control	Low-dose group	Middle-dose group	High-dose group
Body weight (g)	Week 0	98.52 ± 3.64	98.50 ± 4.60	98.29 ± 4.97	96.56 ± 6.76
	Week 1	149.91 ± 9.53	147.01 ± 5.79	146.66 ± 6.72	146.54 ± 9.25
	Week 2	196.74 ± 10.90	196.84 ± 7.77	193.98 ± 10.26	192.09 ± 10.34
	Week 3	240.77 ± 11.23	241.71 ± 11.05	239.99 ± 10.93	237.49 ± 10.15
	Week 4	278.31 ± 13.98	279.09 ± 16.39	278.61 ± 16.12	276.55 ± 10.76
	Week 5	310.09 ± 14.71	313.66 ± 14.49	312.64 ± 15.99	306.60 ± 13.73
	Week 6	333.54 ± 15.96	337.06 ± 19.34	332.01 ± 20.41	328.91 ± 14.88
	Week 7	352.72 ± 17.71	358.74 ± 16.02	355.88 ± 21.34	340.98 ± 18.70
	Week 8	364.72 ± 17.78	369.91 ± 19.14	368.01 ± 27.26	359.38 ± 21.69
	Week 9	379.83 ± 21.00	385.04 ± 22.07	383.04 ± 29.74	372.39 ± 23.65
	Week 10	394.84 ± 25.37	400.12 ± 21.73	394.70 ± 33.86	386.28 ± 24.75
	Week 11	406.89 ± 27.73	415.04 ± 21.18	405.58 ± 34.11	399.01 ± 26.77
	Week 12	409.27 ± 32.89	423.30 ± 19.97	414.18 ± 32.36	408.64 ± 27.11
	Week 13	419.31 ± 28.47	431.71 ± 19.01	416.99 ± 38.48	410.37 ± 27.24
	Week 14	439.48 ± 20.25	462.15 ± 20.05	449.48 ± 37.77	435.28 ± 21.15
	Week 15	445.85 ± 24.77	468.40 ± 22.65	454.05 ± 41.99	435.53 ± 25.83

For Weeks 0-13, n = 14. For Weeks 14-15, n = 4.

Table 4. Influence on the blood cells of rats ($\bar{x} \pm s$).

	Groups	Negative control	Low-dose group	Middle-dose group	High-dose group
After feeding samples for 3 months (n = 16)	PT (s)	13.60 ± 0.43	13.30 ± 0.45	13.35 ± 0.54	13.34 ± 0.39
	WBC ($\times 10^9$/L)	6.14 ± 1.23	6.42 ± 1.30	6.30 ± 1.91	6.27 ± 1.48
	RBC ($\times 10^{12}$/L)	8.618 ± 0.439	8.909 ± 0.431	8.818 ± 0.578	8.887 ± 0.456
	HGB (g/dL)	16.48 ± 0.60	17.12 ± 0.69**	16.69 ± 0.87	17.28 ± 0.66**
	HCT (%)	47.69 ± 2.08	48.86 ± 1.97	47.71 ± 2.44	48.79 ± 2.28
	MCV (fL)	54.3 ± 1.1	54.6 ± 1.3	54.3 ± 1.4	54.8 ± 1.1
	MCH (pg)	18.92 ± 0.54	19.18 ± 0.60	18.86 ± 0.59	19.09 ± 0.55
	MCHC (g/dL)	34.87 ± 0.39	35.03 ± 0.49	34.95 ± 0.29	34.91 ± 0.38
	RDW (%)	11.44 ± 0.40	11.45 ± 0.32	11.44 ± 0.54	11.54 ± 0.32
	PLT ($\times 10^9$/L)	866.5 ± 88.5	882.6 ± 128.4	920.2 ± 61.4	910.1 ± 62.8
	MPV (fL)	7.44 ± 0.37	7.67 ± 0.51	7.44 ± 0.23	7.44 ± 0.37
	PCT (%)	0.6362 ± 0.0730	0.6543 ± 0.1015	0.6681 ± 0.0583	0.6652 ± 0.0550
After recovery for 2 weeks (n = 8)	PT (s)	13.81 ± 0.41	13.38 ± 0.53	13.79 ± 0.45	13.79 ± 0.37
	WBC ($\times 10^9$/L)	6.00 ± 2.11	5.50 ± 0.94	5.74 ± 1.91	6.35 ± 1.14
	RBC ($\times 10^{12}$/L)	8.754 ± 0.657	8.904 ± 0.309	8.975 ± 0.625	9.074 ± 0.304
	HGB (g/dL)	17.08 ± 1.14	17.05 ± 0.60	16.95 ± 0.75	17.21 ± 0.37
	HCT (%)	47.91 ± 2.98	47.63 ± 1.71	47.88 ± 2.49	48.41 ± 0.66
	MCV (fL)	54.4 ± 1.4	53.1 ± 1.1	53.8 ± 1.0	53.8 ± 1.0
	MCH (pg)	19.45 ± 0.55	18.94 ± 0.53	19.13 ± 0.49	19.04 ± 0.46
	MCHC (g/dL)	35.73 ± 0.21	35.61 ± 0.20	35.45 ± 0.43	35.58 ± 0.18
	RDW (%)	10.84 ± 0.37	11.15 ± 0.26	11.10 ± 0.54	11.05 ± 0.40
	PLT ($\times 10^9$/L)	874.1 ± 79.5	844.1 ± 42.2	904.3 ± 103.2	881.6 ± 94.9
	MPV (fL)	7.65 ± 0.29	7.43 ± 0.19	7.39 ± 0.36	7.61 ± 0.33
	PCT (%)	0.6655 ± 0.0554	0.6226 ± 0.0356	0.6683 ± 0.0988	0.6583 ± 0.0782

**Represent for $P < 0.01$.

Table 5. Influence on blood biochemistry of rats ($\bar{x} \pm s$).

	Groups	Negative control	Low-dose group	Middle-dose group	High-dose group
After feeding samples for 3 months (n = 16)	ALT (U/L)	41.9 ± 5.8	45.0 ± 8.0	41.6 ± 6.0	43.9 ± 7.7
	AST (U/L)	100.3 ± 8.1	106.3 ± 10.5	101.2 ± 11.3	103.1 ± 10.3
	ALP (U/L)	101.1 ± 18.0	105.8 ± 22.9	101.4 ± 16.9	95.4 ± 13.4
	TbiL (mmol/L)	1.781 ± 0.811	2.007 ± 0.844	2.036 ± 0.966	1.783 ± 1.252
	BUN (mmol/L)	5.786 ± 0.381	6.024 ± 0.409	5.854 ± 0.492	6.041 ± 0.437
	CREA (mmol/L)	26.08 ± 1.70	28.79 ± 3.14**	30.80 ± 3.21**	30.80 ± 3.79**
	GLU (mmol/L)	8.228 ± 0.614	8.087 ± 0.407	8.012 ± 0.610	7.775 ± 0.771
	TP (g/L)	62.02 ± 1.97	62.46 ± 2.02	61.00 ± 2.53	60.65 ± 2.46
	ALB (g/L)	33.10 ± 2.30	33.05 ± 2.16	32.85 ± 2.51	32.31 ± 1.80
	TG (mmol/L)	0.540 ± 0.100	0.567 ± 0.095	0.539 ± 0.125	0.555 ± 0.068
	CHOL (mmol/L)	1.805 ± 0.276	1.906 ± 0.220	1.977 ± 0.210	1.821 ± 0.166
After recovery for 2 weeks (n = 8)	ALT (U/L)	36.0 ± 5.7	36.1 ± 6.3	37.4 ± 5.9	35.0 ± 4.7
	AST (U/L)	106.6 ± 6.2	109.9 ± 7.2	108.3 ± 11.9	107.0 ± 3.6
	ALP (U/L)	86.0 ± 12.2	80.0 ± 18.7	77.4 ± 20.3	78.6 ± 14.6
	TbiL (mmol/L)	1.530 ± 0.989	2.058 ± 0.652	1.976 ± 0.833	1.515 ± 1.089
	BUN (mmol/L)	5.956 ± 0.563	6.015 ± 0.461	6.260 ± 0.548	6.334 ± 0.320
	CREA (mmol/L)	30.10 ± 4.54	31.68 ± 4.22	37.33 ± 4.57**	37.01 ± 3.50**
	GLU (mmol/L)	8.111 ± 0.332	7.968 ± 0.461	8.445 ± 0.801	8.166 ± 0.778
	TP (g/L)	59.90 ± 3.14	60.59 ± 4.46	60.03 ± 2.69	59.20 ± 3.37
	ALB (g/L)	32.91 ± 1.80	33.09 ± 2.30	33.00 ± 1.62	32.19 ± 1.61
	TG (mmol/L)	0.514 ± 0.072	0.535 ± 0.056	0.535 ± 0.092	0.599 ± 0.106
	CHOL (mmol/L)	1.879 ± 0.331	2.001 ± 0.290	1.973 ± 0.169	1.840 ± 0.130

**Represent for $P < 0.01$.

3.4. Influence on Organ Coefficient of Rats

In this study, all rats showed no obvious abnormalities in organ size and weight. Results were showed in **Table 6**. Compared with the negative control group, the organ coefficient of each group rats showed no significant difference ($P > 0.05$).

3.5. Histological Analysis

After administration for 3 months and after recovery for 2 weeks, major organs of rats were picked and carefully examined when anatomizing, which were heart, liver, spleen, lung, kidney, stomach, brain, adrenal gland, thyroid gland, pancreatic, bladder, trachea, thymus, male rats' prostate, testis, seminal vesicle and female rats' ovary, uterus. And no pathological changes were seen. Some major organs were selected for histological analysis. And histopathological assessment of the heart, liver, spleen, lung, kidney, stomach, brain, adrenal, thyroid, pancreas, trachea, bladder, prostate, male rats' testis, epididymis and female rats' ovary, uterus showed no differences between the treated animals and the controls (data no shown).

4. Discussion

Compared with the negative control group, HGB significantly increased in rats of low-dose group and high-dose group after 3 months oral exposed to mulberry sea-buckthorn beverage concentrate. However, it decreased to normal level after 2 weeks recovery by stopping feed mulberry sea-buckthorn beverage concentrate. Hemoglobin is a pigment containing cofactor binding protein, synthetized by the human nucleated red blood cells and reticulocytes, mainly in the organism responsible for carrying oxygen.

Table 6. Influence on organ coefficient of rats ($\bar{x} \pm s$).

	Groups	Negative control	Low-dose group	Middle-dose group	High-dose group
Organ coefficient of rats after feeding samples for 3 months (%) (n = 16)	Brain	0.5984 ± 0.1331	0.5988 ± 0.1286	0.6060 ± 0.1311	0.6209 ± 0.1234
	Liver	2.4979 ± 0.1527	2.5800 ± 0.1665	2.5275 ± 0.1432	2.5821 ± 0.1434
	Kidney	0.6577 ± 0.0329	0.6545 ± 0.0527	0.6671 ± 0.0340	0.6801 ± 0.0423
	Spleen	0.1598 ± 0.0137	0.1537 ± 0.0180	0.1597 ± 0.0207	0.1584 ± 0.0162
	Lung	0.3866 ± 0.0537	0.3899 ± 0.0511	0.3974 ± 0.0600	0.4079 ± 0.0541
	Heart	0.3460 ± 0.0346	0.3306 ± 0.0321	0.3400 ± 0.0438	0.3529 ± 0.0410
	Thymus	0.1027 ± 0.0311	0.1164 ± 0.0359	0.1057 ± 0.0259	0.0996 ± 0.0199
	Adrenal gland	0.0191 ± 0.0077	0.0198 ± 0.0068	0.0220 ± 0.0073	0.0202 ± 0.0065
	Testis	0.8150 ± 0.0357	0.8044 ± 0.0590	0.8513 ± 0.1002	0.8447 ± 0.0477
	Epididymis	0.3043 ± 0.0240	0.3027 ± 0.0445	0.3147 ± 0.0377	0.3091 ± 0.0114
	Ovary	0.0402 ± 0.0101	0.0409 ± 0.0076	0.0447 ± 0.0062	0.0406 ± 0.0108
	Uterus	0.2827 ± 0.0774	0.2451 ± 0.0502	0.2700 ± 0.0832	0.2386 ± 0.0456
	Bladder	0.0288 ± 0.0066	0.0302 ± 0.0050	0.0289 ± 0.0051	0.0310 ± 0.0040
Organ coefficient of rats after recovery for 2 weeks (%) (n = 8)	Brain	0.6003 ± 0.1025	0.5741 ± 0.1211	0.5819 ± 0.1330	0.6156 ± 0.1434
	Liver	2.5161 ± 0.1186	2.4622 ± 0.0764	2.4597 ± 0.0999	2.5195 ± 0.0689
	Kidney	0.6317 ± 0.0343	0.6469 ± 0.0343	0.6415 ± 0.0271	0.6586 ± 0.0401
	Spleen	0.1396 ± 0.0060	0.1459 ± 0.0118	0.1355 ± 0.0059	0.1480 ± 0.0074
	Lung	0.3815 ± 0.0258	0.3865 ± 0.0463	0.3680 ± 0.0517	0.4058 ± 0.0447
	Heart	0.3314 ± 0.0343	0.3540 ± 0.0460	0.3332 ± 0.0326	0.3464 ± 0.0570
	Thymus	0.0917 ± 0.0113	0.0936 ± 0.0171	0.0958 ± 0.0199	0.0998 ± 0.0279
	Adrenal gland	0.0182 ± 0.0036	0.0184 ± 0.0053	0.0180 ± 0.0058	0.0192 ± 0.0062
	Testis	0.7738 ± 0.0997	0.7580 ± 0.0255	0.7779 ± 0.0931	0.7575 ± 0.0101
	Epididymis	0.2724 ± 0.0328	0.2703 ± 0.0393	0.2698 ± 0.0216	0.2657 ± 0.0186
	Ovary	0.0280 ± 0.0039	0.0315 ± 0.0026	0.0312 ± 0.0008	0.0363 ± 0.0057
	Uterus	0.2324 ± 0.0715	0.2129 ± 0.0166	0.2498 ± 0.0801	0.2731 ± 0.0508
	Bladder	0.0284 ± 0.0058	0.0265 ± 0.0087	0.0263 ± 0.0054	0.0290 ± 0.0061

Note: For uterus, ovary, testis and epididymis, n = 8 for rats after feeding samples for 3 months; n = 4 for rats after recovery for 2 weeks.

In clinic, the HGB value increased probably due to water loss in the plasma, blood concentration, such as series fierce vomiting, severe diarrhea, chronic adrenocortical hypofunction, diabetes insipidus, hyperthyroidism and other diseases. The increased HGB value can also cause hypoxia. Hyposia leads to the increase of serum erythropoietin, and subsequently stimulates the release of red cells from bone marrow.

Research has shown that sea-buckthorn can obviously improve the athlete's vital capacity, hemoglobin, and cardiac function [7]. Another research shows that 30% mulberry liquid, once daily by gavage of 1 mL for 5 days can recover the HGB value of rats, which have been injected by acetyl phenylhydrazine to decrease the HGB value, to normal level [8].

In this study, the significant increase of HGB value appears in the low-dose group and high-dose group presumably due to the normal pharmacological effect of mulberry or sea-buckthorn.

Creatinine (CREA) is a small molecular substance. Almost all the daily produced creatinines *in vivo* are excreted in the urine. Because the value of CREA generally unaffected by urine volume, detection of CREA is one of the main methods used to understand the renal function in clinic. The concentration of CREA was determined mainly by the glomerular filtration rate. The decreased filtration capacity of glomerular leads to the higher concentration of CREA. So, CREA value can reflect the renal parenchyma damage.

In this study, CREA was significantly increased in all sample groups' rats after 3 months oral exposed to mulberry sea-buckthorn beverage concentrate. And the increase amplitude of CREA was positively related to

the dosage of mulberry sea-buckthorn beverage concentrate. Furthermore, it still showed the significant increase in rats of middle-dose group and high-dose group after 2 weeks recovery by stopping feed mulberry sea-buckthorn beverage concentrate. These results indicated that mulberry sea-buckthorn beverage concentrate could damage the kidney. However, related lesions were not found in the renal histopathological examination, and the organ coefficient of kidney showed no difference between sample groups and negative control group. This indicates that the damage on kidney is not serious.

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A Report on the Death of Mixed Infection of HEV and HBV

Yonggang Qu[1,2], Yujian Zheng[1], Chuangfu Chen[2], Guangze Zhu[3], Ningyi Jin[3]

[1]School of Public Health, Xinjiang Medical University, Urumqi, China

[2]College of Animal Science and Technology, Shihezi University, Shihezi, China

[3]Genetic Engineering Laboratory, Academy of Military Medical Sciences, Changchun, China

Abstract

Mixed infection with hepatitis E virus (HEV) in patients with chronic hepatitis B virus (HBV) infection is frequent. HEV mixed infection often leads to activation of hepatic pathological changes and worsens the inflammatory activity. However, it is not known clearly how these two types of virus influence each other in human body. Intensive investigation has revealed that HEV mixed infection inhibits HBV replication. We have just encountered a relative rare case. The patient who was a HBV carrier and was infected by HEV. Before he was infected by the HEV, the measurement of his HBV DNA fixed quantity examination on fluorescence was <10^3 copies/ml; his routine biochemistry was normal; and his anti HEV-IgM and anti-HEV-IgG appeared to be negative reaction. After he was infected by HEV, his routine biochemistry increased, and the measurement of his HBV DNA fixed quantity examination on fluorescence was 8.51 × 10^5 copies/ml. It indicated that the replication of HBV was activated after the patient infected HEV. Finally, he was dead. This case revealed that HEV mixed infection may activate the replication of HBV, not inhibit HBV replication, and demonstrated the needs for further studies about the mechanism of the interaction of the two viruses.

Keywords

Hepatitis E Virus, Hepatitis B Virus, Mixed Infection

1. Introduction

Mixed infection with hepatitis E virus (HEV) occurred in patients with chronic hepatitis B virus (HBV) infection is frequent [1]-[18]. It often leads to activation of hepatic pathological changes and worsens the inflammatory activity [1] [2], causes severe liver decompensation, which is frequently complicated with hepatic encephalopathy and renal failure. Acute hepatitis E in these patients has a protracted course with high morbidity and mortality [3]. However, it is not known clearly how these two types of virus influence each other in human body. Intensive investigation has revealed that HEV mixed infection inhibits HBV replication [1] [4]-[6].

Whereas, we encountered a relatively rare case. The patient had a acute HEV infection, who has been a HBV carrier for 20 years. The replication of HBV was activated remarkably after the patient infected HEV.

2. Case Report

The patient was 46 years old. 20 years ago, his HBsAg, HBeAb and HBcAb all appeared to be positive reaction without any relevant symptoms. He was diagnosed by the local physician as "hepatitis B" and was occasionally treated with the help of some liver-protecting medicine. He had no record of drinking, injury, operation, blood transfusion, or other infectious diseases, but had a 20-year-smoking history. Before he was infected by the HEV, the measurement of his HBV DNA fixed quantity examination on fluorescence was $<10^3$ copies/ml; his routine biochemistry was normal; and his anti HEV-IgM and anti-HEV-IgG appeared to be negative reaction.

On the 29th of December, the patient had the symptoms of sudden weakness, anorexia, fever and icterus. Physical examination on admission results are as following: body temperature 38.1°C; pulse 72/minute; blood pressure 120/80 mmHg; no abnormal evidence shown in neck artery and vein; soft abdomen; no pressed pain and no rebounding pain of the upper part of the side; touched appendix ensiformis to 3.0 cm with slippery surface, side parts and slight pressed pain; Murphy's sign appeared negative reaction; throbbing pain in liver area.

His laboratory tests on admission revealed: serum anti-HEV-IgM and anti-HEV-IgG were positive; alphafetoprotein (AFP) 5.0 ug/L; total bilirubin (TBiL) 95.8 μmol/L, alanine aminotransferase (ALT) 894 U/L, aspartate aminotransferase (AST) 1300 U/L, and gamma-glutamyl transpeptidase (GGT) 221 U/L, all were increased; total protein (TP) 75.3 g/L, albumin (ALB) 40 g/L, all were decreased; cholinesterase (CHE) 4653 U/L (**Table 1**).

He was diagnosed as acute HEV infection based on the symptom and the laboratory test results.

Immediately after he was hospitalized, his routine biochemistry increased (**Table 1**), and the measurement of his HBV DNA fixed quantity examination on fluorescence was 8.51×10^5 copies/ml. Blood clotting system test revealed: prothrombin time (PT), international normalized ratio(INR), activated partial thromboplastin time (APTT), thrombin time (TT), and fibrinogen (FIB) increased; prothrombin time ratio (PTR) decrease (**Table 2**).

It showed a large-area necrosis on the liver of the patient through transoesophageal echocardiography (**Figure 1**).

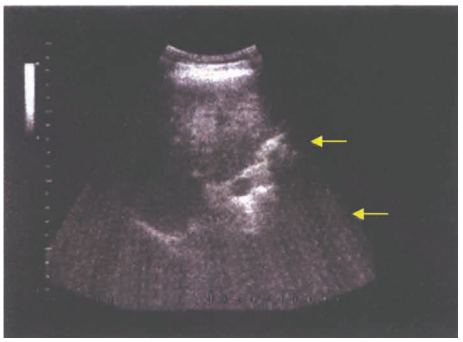


Figure 1. Transoesophageal echocardiography of the liver of the patient. It showed a large-area necrosis.

Table 1. Laboratory results of the 46-year-old man.

	Date							
	29 December 2005	31 December 2005	4 January 2006	9 January 2006	12 January 2006	23 January 2006	26 January 2006	2 February 2006
TBiL (μmol/L)	95.8	142.5	262.1	329.2	431.2	526.5	31.68	20.83
TP (g/L)	75.3	70.5	68.7	66.6	68.9	69.8	61	57.1
ALB (g/L)	40	37.9	35.6	33.6	33.5	33.4	30	34.79
ALT (U/L)	894	853	809	320	256.8	68	50.59	35.4
AST (U/L)	1300	893	864	245	196	131	129.7	88
GGT (U/L)	221	199	140	113	93	63	46	43
CHE (U/L)	4653	4487	2809	1792	1980	2400	2808	3611

Since the patient experienced the attack, we carried out the examination of HEV RNA in blood serum and excrement, and found that the HEV RNA appeared to be positive reaction. Subsequently, we sequenced the partial HEV gene, and analysised it by alignment and phylogenetic analysis. It showed that the HEV in blood serum and excrement of the patient belonged to the genotype IV. The homology with JYI-ChiSai01C, which is a HEV genotype IV strain, was 92.5%. The homology with the other genotype IV strains was above 85% (**Figure 2** and **Figure 3**).

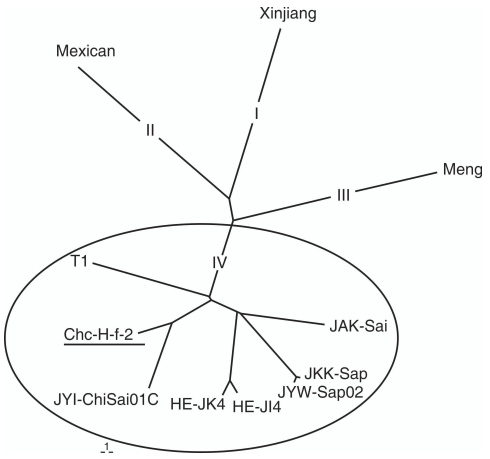


Figure 2. A phylogenetic tree of the HEV isolated from the patient based on nt 5997 to 6344 of the ORF2 gene region. The tree was constructed, and analysed by the Phylip software. The HEV isolated from the patient belong to the genotype IV. GenBank accession numbers with corresponding codes used in the text are as follows: Xinjiang (D11092), Mexican (M74506), Meng (AF082843), AB080575 (HE-JI4), swJ13-1 (AB097811), HE-JA1 (AB097812), JSN-Sap-FH (AB091395), HE-JK4 (AB099347), JAK-Sai (AB074915), JKK-Sap (AB074917), JYW-Sap02 (AB161719), JYI-ChiSai01C (AB197674), T1 (AJ272108), swCH25 (AY594119).

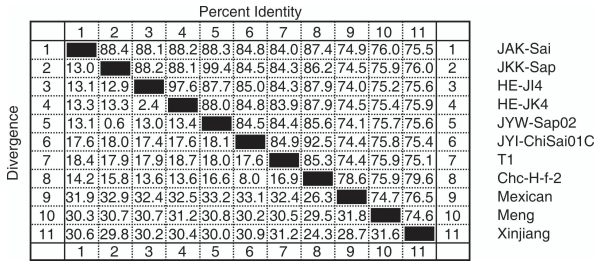


Figure 3. Nucleotide homology analysis between the HEV isolated from the patient and the other HEV. The nucleotide homology analysed by Clustal W. The homology with JYI-ChiSai01C, which is a HEV genotype IV strain, was 92.5%. The homology with the other genotype IV strains was above 85.0%. The homology with the other genotype was below 80.0%.

Table 2. Blood clotting system test.

	Date					
	31 December 2005	4 January 2006	9 January 2006	12 January 2006	23 January 2006	25 January 2006
PT (sec)	18.7	22.5	24.5	26.3	28.5	38.8
PTR (%)	52.6	37.7	32.3	30.8	24.6	17.8
INR	1.52	1.83	1.99	2.06	2.72	7.11
APTT (sec)	39.3	58.2	73.8	73.5	74.8	72.1
TT (sec)	21.3	25.3	29.8	28.4	27.5	29.4
FIB (g/L)	1.01	2.5	2.18	2.03	1.98	<1.4

After the patient was hospitalized, we took the anti-viral, decreasing ferment and jaundice measures. However, there was no significant improvement, so the patient was transferred to another hospital and received continuous treatment there with still no improvement. The patient died on the 11th day after he was transferred.

3. Discussion

Mixed infection with hepatitis E virus (HEV) occurred in patients with chronic hepatitis B virus (HBV) infection is frequent [1]-[18]. It often leads to patients has a protracted course with high morbidity and mortality [3].

Nowadays, Intensive investigation has revealed that HEV mixed infection inhibits HBV replication [1] [4]-[6]. At the same time, some researchers think that HEV infection may activate the replication of HBV [8]. However these finding mainly based on the epidemiology.

As we reported, the patient's HBV DNA was $<10^3$ copies/ml before he was infected by HEV, but after the infection, his HBV DNA reached 8.51×10^5 copies/ml along with other biochemical higher quota. It suggested that the patient died as a result of the HBV in his body being activated after he acquired HEV, which caused the large-area necrosis in his liver. This case revealed that HEV mixed infection may activate the replication of HBV, not inhibits HBV replication. However, the questions are required to be further studied, such as how HEV activates HBV, and what the vital mechanism is between the superinfection of HEV and HBV.

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Mood Psychopathologies: An Integrated Complexity-Based Interpretation

Massimo Cocchi[1,2,3], Lucio Tonello[1,3], Fabio Gabrielli[1,3]

[1]Paolo Sotgiu Institute for Quantitative & Quantum Psychiatry & Cardiology, L.U.de.S. University, Lugano, Switzerland

[2]Department of Veterinary Medical Sciences, University of Bologna, Bologna, Italy

[3]Faculty of Human Sciences, L.U.de.S. University, Lugano, Switzerland

Abstract

If the mechanistic-reductionist cognitive approaches have been characterised by the metaphor of the "edifice", of the solid Cartesian rock, all the forms of knowledge founded on complexity theory have been characterised by the metaphor of the "network", of thinking in relationships, in a dynamic, fluid, open manner. In the field of mental illness, this means setting aside both the organicist paradigm and the pseudo-phenomenological, "sentimentalistic", and therefore ideological, paradigm, in order to have an integrated view of biological objectiveness and *humanistic* psychotherapy. That is to say, an expression of diverse interrelated contributions from the various disciplines (psychiatry, psychology, biochemistry, anthropology, quantum physics, mathematics, philosophy). The observer thus becomes a builder of models, a manager of complexity, giving treatment the character of a truly empathic relationship. This is all the more so where distressing pathologies are involved, such as Major Depression (MD) and Bipolar Disorder (BD), *caput mortuum* of psychiatry, because the absence of cogent biological markers seriously compromises every form of therapy. Hence the identification of a biological platform (fatty acids of platelets) as a starting point for a correct classification of MD with respect to BD.

Keywords

Complexity; Major Depression; Bipolar Disorder; Fatty Acids; SOM; Biology; Phenomenology

1. Foreword

Drawing on the contributions of Culloch, Paget, Von Foerster, Bateson, Maturana, Varela, Atlan, the epistemology of the Irremovable Method and Foundation has been thrown into crisis in favour of a systemic view, not

characterised by linearity, where the observer is a builder of models, narrations to be shared in the *hic et nunc*, in the framework of the real world.

The observer is posed as an inter-worldly reality which operates through a plurality of cognitive strategies, within the epistemological-ecological, mind-world system, enabling an interpretation of reality that is neither ideological nor manipulatory.

Thus, from physical to biological systems, from mental processes to socio-economic structures, a *gestaltic reorientation* is needed in the name of complexity, beyond every interpretation that reduces the person to a mere expression of rules, practices, epistemic-Foucaultian discourses or to naïve spiritualistic emergences, the latter expressing the mere myth of the irreducibility of individual and collective behavioural dynamics to the properties of their elementary ingredients (a valid aid is provided by the general logical scheme of quantum state theory).

In extreme summary, complexity embodies a kind of self-organised, multifactoral knowledge, expressive of a plurality of models, open to unpredictability, to novelty, to the continuous intersection between observer and world in the construction of an open, mobile, non-dichotomic or foundationist weave.

So, as Bateson wrote prophetically, superseding the classic dichotomy between scientific culture and humanistic culture: "The beautiful and the ugly, the literal and the metaphoric, the sanity and madness, comical and serious… all these and even love and hate are matters that science presently avoids. But in a few years, when the split between problems of mind and problems of matter ceases to be a central determinant of what is impossible to think about, they will become accessible to formal thought" (G. Bateson & M.C. Bateson 1987).

Edgar Morin, perhaps more than anyone else, has grasped with absolute clarity the theoretical and applicational nodes of complexity, the perverse drifts of official thought, of "blind intelligence": "In many contexts, then, the parcelled, compartmentalised, mechanistic, disjointed reductionist intelligence breaks up the problems, separates what is connected, and makes one-dimensional what is multidimensional. It is an intelligence at one and the same time short-sighted, long-sighted, colour-blind and one-eyed; in most cases it ends up by being blind. It destroys at birth the possibilities of comprehension and reflection, eliminating all possibilities of a correct judgement or a long-term view" (Morin, 2008).

In particular, Morin has tried, on the one hand, to trace man back to the Universe from whence he emerged; on the other, to capture him in his entirety (the entire, multi-coloured man, between reason and imagination, emotion and reflection): "*The death knell sounds for an anthropology limited to a thin psycho-cultural band that hovers like a flying carpet over the natural universe*" (Morin, 1973).

The new subject, expressing a process of which it is at the same time creator and created, indicating relationship, interaction, is *homo sapiens-demens*: rational, but also uncertain, unstable, confused, an unpredictable mixture of objective, logical, technical, empirical thought and subjective, phantasmal, mythical, magical thought.

Alongside, or behind, the Apollonian *homo sapiens* there emerges, overbearingly, "a being that smiles, laughs, cries, an anxious, anguished being… inebriated, ecstatic, violent, furious, inclined to love…".

In this way, from a rigorous phenomenology (Jaspers, 1963), not reduced to mere poetics of interior resonances, a serious phenomenology of the person is preserved: the being located in a "here and now", expressing its own irreducible position in which to pour forth depth, creativity, ever-new initiality (Husserl, 1911; Baker, 2000, 2002). Preserving the free creativity of the subject/person demands complexity as a circular, dialogical opening, where the negative, the Hegelian contradictions are not overcome (*Aufhebung* as abolition-conservation) with a view to absolute concretisation, but remain to indicate complementarity, without a privileged meta-point, a particular "epistemological sovereignty", to use an expression of Morin's (Morin, 1984) and without remaining prisoners of the myth of the *punctum inconcussum* [irremovable foundation], with respect to which to establish a *good judgement* by homogenising them, on differences and contrapositions between various points of view. The important thing is to grasp how multiple points of view are produced reciprocally (against the hierarchisation of points of view, Magoroh Maruyama (1976) proposes the concepts of "demonopolisation" and "trans-spection"), that is to say the capacity to substitute and the asymmetry of the pluri-diversities of world views and entering into others' heads and, vice versa, without reducing the other's logic to one's own by an act of logical and dialectical domination.

In the treatment of psychopathologies, all this is translated into the reciprocal exchange and the mutual integration of phenomenological and biological approaches, in the intelligent dosing of biochemical and *logotherapeutic* dynamics, in the common sharing of the person as an autonomous, unique and irreducible expression.

2. Biological Roots and Phenomenological Emergence of Mental Illness

It is undeniable that the depth of the *ens sofferens*, to be approached with a curative word (Laín Entralgo, 1970), cannot be traced only to *disease*—pathology or biomedical classification—but also to *illness*—experience of the malaise as lived—and *sickness*—the social determination of the condition (Young, 1982; Augé, 1984; Good, 1994).

For all societies, illness is an event to be interpreted; it is not just a biological fact but also a cultural one. Basically, illness is representation, interpretation, of a portion or of all reality by individuals in a certain social context. The medical description of the human body and the illness always refer back to culturally peculiar meanings.

In strict terms, we could say that the pain articulates its meanings in suffering, which is a restless reflection on the ineluctable, and nevertheless unexpected, occurrence of the illness.

It can be understood, then, that only medical practice that does not limit itself to the biomedical dimension, which is in any case an indisputable point of departure, but is able to meet with the suffering person in his or her intimacy, can ensure, if not salvation against the perverse myth of recovery, at least dignity of the treatment as a profound ethical and existential relationship.

Hence the opening towards intimacy as a dual construction of meaning, the planning of significances contributed to both by the patient, in trusting abandon, and by the physician, as a warm, experienced responsibility. The intimacy is inhabited by the treatment (sphere of essential meanings of living) and not by anxiety (sphere of intra-worldly commerces), precisely because the experience of illness is experience of a relational wound which destructures the biological and existential narration of the subject:

- Wounded in relation to his own body, in *Der Zauberberg* Thomas Mann says that "illness makes men more corporeal, it makes them all body" (1996)—from which, on the one hand, we wish to distance ourselves as it is a sign of the precariousness of existence, while on the other hand we want to master it as we never did when in good health, because the anguish of feeling expropriated by it, not only by the almost fleshly possibility of death, but also by its medical visibility (the anguish—increasingly flaunted, for that matter, and reiterated like a mantra—of the reification of the medical approach), makes us feel the full weight of our vulnerability;
- a relational wound with respect to everyday life, whose narrative laceration provokes at first dismay, then a steady eclipse towards an indeterminate space-time, which for this reason is anguishing and inhospitable, and requires an approach that is not simply clinical but, precisely, one of intimacy, which, as a profound expression of empathy, configures itself as discretion, the word held back, the gesture experienced, total attention for the suffering countenance in a mutual exchange of meanings.

In the case of mental illnesses, then, the social, cultural weight takes on an almost transcendent value, due to the often blurred correspondences between classification and natural object, between nosology and effective reality of the illness.

When all this is recognised, what remains, excluding the interpretations of meaning, is the structural necessity, when coping with the illness—in our case the psychopathology—of starting from the biological fact, from objective biological markers, without which experience, biographical narrations, cultural rootings would be empty just as biology, on the other hand, would be blind.

The meaning is not only the prerogative of philosophy, which certainly remains a strong interlocutor, but also of biology and biochemistry: the corporeal meaning/significance of a pathological event.

Certainly, a medicine limited to the biological fact cannot be extensive and ostensive of the illness. At the same time, however, no one should doubt—as however happens unfailingly—that starting from a bio-medical platform can *ipso facto* reduce the physician to a pure functionary of the body and of the pathology connected with it.

Ultimately, we need to remind the alleged monolithic custodians of the thought of Husserl, Heidegger, Jaspers, Minkowski and Binswanger that rooting the pathology in biology does not mean expropriating the sick person of his illness and making the physician a mere functionary of the organism, an all-out pathologist who ignores biographies, experiences, corporeal dynamics and relational ontologies.

If anything, biographies, relationships, cultural expressions can be preserved in all their dignity, once their genuine biological matrix has been determined.

On the other hand, we risk only metaphysical hypostatisation and, therefore, a treatment rooted in an immo-

bile metempirical "elsewhere".

A fruitful heuristic synthesis between *Körper* and *Leib*, *erklären* and *verstehen* (Husserl, 1977), as a true commitment to healing the "living flesh" (*Fr. chair*) (Merleau-Ponty, 1964), is possible only if we start from the biological roots of our *being in the world* (*in der Welt sein*).

Heidegger's figures of omnipotence *(Allmacht)* and impotence *(Ohnmacht*) (Binswanger, 1963), can be taken up and re-elaborated in a synthesis between the naturalistic, classificatory power of science and the ever-open possibility of phenomenology, avoiding the same Heidegger's anti-technicist derailments and the exacerbated revivals of Binswanger's phenomenology as a mere therapeutic praxis, without theoretical rigour.

Like psychiatry and psychology—bio-reductionist and limited to the illness—so too "antipsychiatry", which reduces the illness only to a social construction, process of control, of exclusion/inclusion managed by bio-power (Goffman, 1961; Foucault, 1965, 1994, 2004, 2008), unable to recognise the productivity/creativity of schizophrenic processes (Deleuze-Guattari, 2004), ends up by prejudicing the genuine dynamics of the treatment.

Hence the need for an objective biological reference able to act as a cogent platform in the treatment relationship, with reference to two distressing psychopathologies: MD and BD (Bowden, 2001).

3. Biological Markers of Major Depression and Bipolar Disorder

In the first experimental phase we selected two mathematical instruments, one complex (Self-Organizing Map—SOM) and the other simple (the B2 index). These were to show their validity subsequently not only in defining the pathology of Major Depression and the Bipolar Disorder, but also for drawing plausible deductions as regards the biological value of the two molecular mood disturbances.

The SOM is an artificial neural network able to put together similar objects and separate them from very different objects according to their characteristics.

The B2 index [so called by the authors of the research (Cocchi and Tonello)] is derived from a mathematical operation that considers the characteristics of the molecular weight and melting point of the fatty acids isolated and recognised by the SOM, in order to distinguish between the two disorders.

Proceeding by stages we can say that the two groups of patients examined in the first phase of the research (apparently normal and depressed) were determined by the fatty acids of the platelets. This cell type was chosen for its morphological and functional peculiarities, i.e. the presence of receptors for neuro-transmitters, in particular serotonin, and for other characteristics that make them similar to neurons.

The results obtained experimentally, interpreted by a non-linear mathematical function, the SOM, and by a linear mathematical function, the B2 index, made it possible to distinguish "psychiatric" patients from "normal" patients.

The problem resided in the fact that the psychiatric diagnosis generally made was of Major Depression, while the arrangement of the subjects in the picture traced by the SOM and the B2 index made us feel that some aspects of the diagnosis were not entirely clear.

The arrangement of the cases on the SOM and the evaluation of the B2 index (negative or positive) led us to seek an opportunity to relaunch the experiment, which would be able to distinguish apparently normal subjects from those affected by Major Depression and Bipolar Disorder.

The opportunity arose with funding from the Marche Region in Italy and, in two years of intense work in collaboration with psychiatrists, biochemists, molecular biologists, mathematicians, quantum physicists and philosophers, the much sought-after result was obtained, confirming that our intuition was correct: the depressed subjects were isolated from the bipolar ones, beyond any interpretative, classificatory and psychiatric discussion.

With these results in our hands we began to reflect on the distribution and logic of the numbers that had been closely correlated with psychiatric illnesses, because they did not take into consideration the therapies conducted or anything which could be connected with the patients from the outside.

All this led us to suppose that there was something already written in the platelets able to simulate the condition of the neuron, at least as far as serotonin level was concerned. Consistently with this observation we wrote some papers concerning the molecular uniqueness of Major Depression, the role of membrane viscosity and the molecular reflections on the state of consciousness and the mechanical strength of the membrane.

This work will perhaps be reviewed when it is possible to carry out molecular mapping of the neuron, as agreed on the occasion of the Palermo Conference.

In the second stage of experimentation, research was then carried out using a combination of biology and non-linear mathematics to establish, in the psychiatric area of mood disorders, whether it was possible in the platelets, especially in their fatty acids, to identify molecular characteristics able to provide a clear, precise classification of subjects with Major Depression (MD) and Bipolar Disorder (BD). The results obtained once again confirmed the original data, using an artificial Neural Network, in particular a network known as a Self-Organizing Map (SOM) (Cocchi et al., 2008; Cocchi & Tonello, 2010). The SOM is a network algorithm of unsupervised competitive learning, devised by Teuvo Kohonen in 1981-1982 (Kohonen, 1982, 1998, 2001).

Thanks to the above combination, using platelet fatty acids (Palmitic, Linoleic and Arachidonic), the SOM and the mathematical index B2 (obtained by adding together the percentages of each fatty acid multiplied by their respective melting points and divided by their molecular weights) we were able to distinguish MD from BD for the first time ever. Thus we obtained the evaluation of an indirect expression of membrane viscosity, which led us to correlate with the membrane viscosity of the neuron thanks to the correlation shown between the passage of the arachidonic acid from the platelets to the brain (Cocchi, Tonello, & Gabrielli, 2012). The B2 index is negative in MD and positive in BD. This result leads to the observation that the membranes in MD are considerably less viscous than those of normal, BD and psychotic patients. Thus it can be said that subjects with MD have certain specific and exclusive molecular characteristics (Cocchi, Tonello, & Gabrielli, 2012b). On this basis we were able to explain the quantitative biomolecular approach used for Major Depression and hypothesise that in mood disorders there is a quantitative path, going from the viscosity of the cell membrane and passing through the Gsα protein and Tubulina (Cocchi et al., 2010b). **Figures 1-8** summarise the principal steps of the research.

Because it is not possible to manipulate the SOM, differently from the statistical evaluation, the SOM and the B2 index result (**Figures 4**, **5**) show that patients with Major Depression belong to a quite separate area from that of healthy and bipolar subjects. By observing the arrangement of the data on the SOM, we also see a region (high-left corner, **Figure 1**) which we attribute to psychotic subjects on a diagnostic basis.

We interpreted these events in terms of breakage of symmetry (Cocchi et al., 2012c), which confirms the fact that MD is an illness that stands quite apart from the normal, bipolar and psychotic conditions **(Figure 9)**.

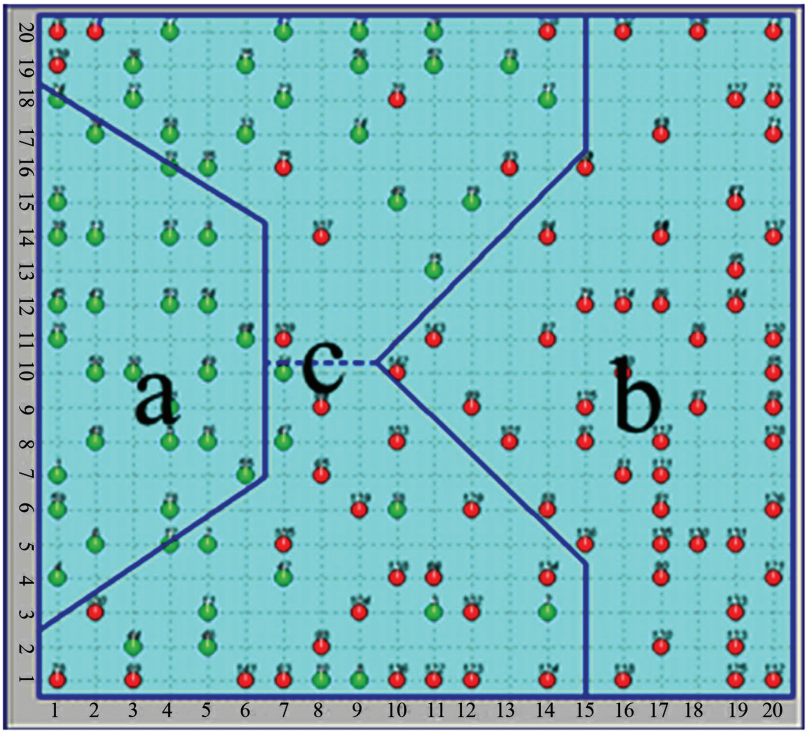


Figure 1. Distribution of all cases of the first experiment (first SOM).

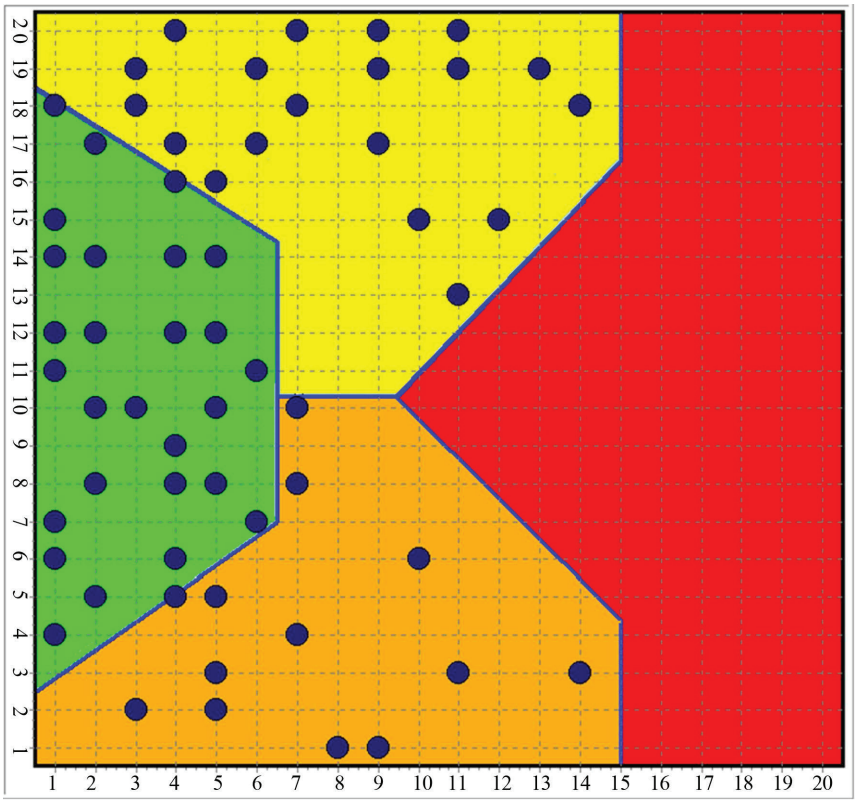


Figure 2. Distribution of normal subjects over the SOM.

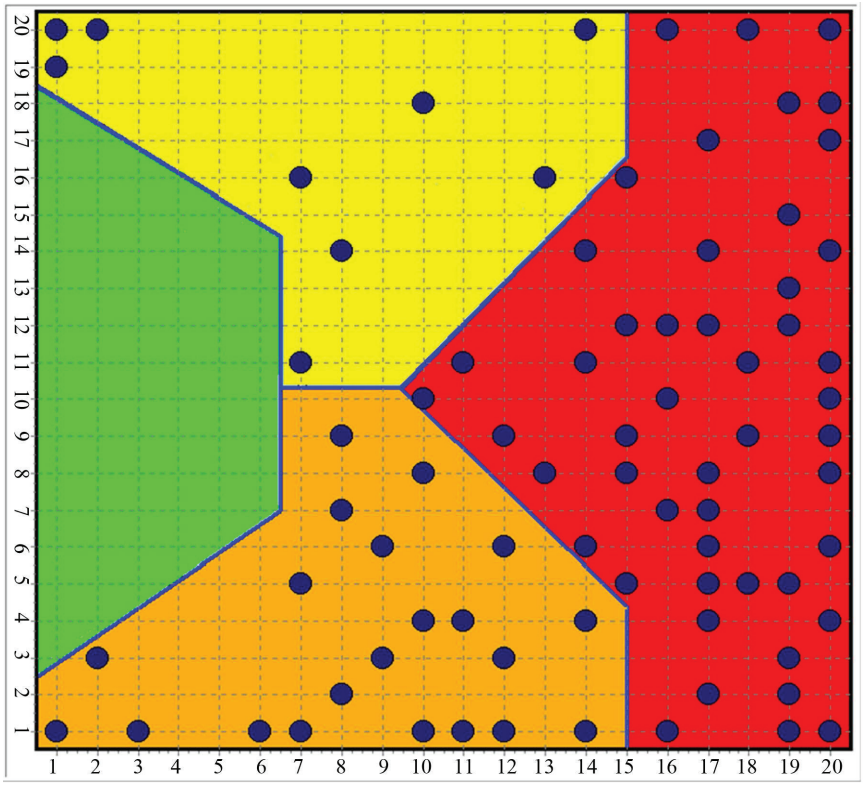


Figure 3. Distribution of subjects with mood disorders (MD and BD) over the SOM.

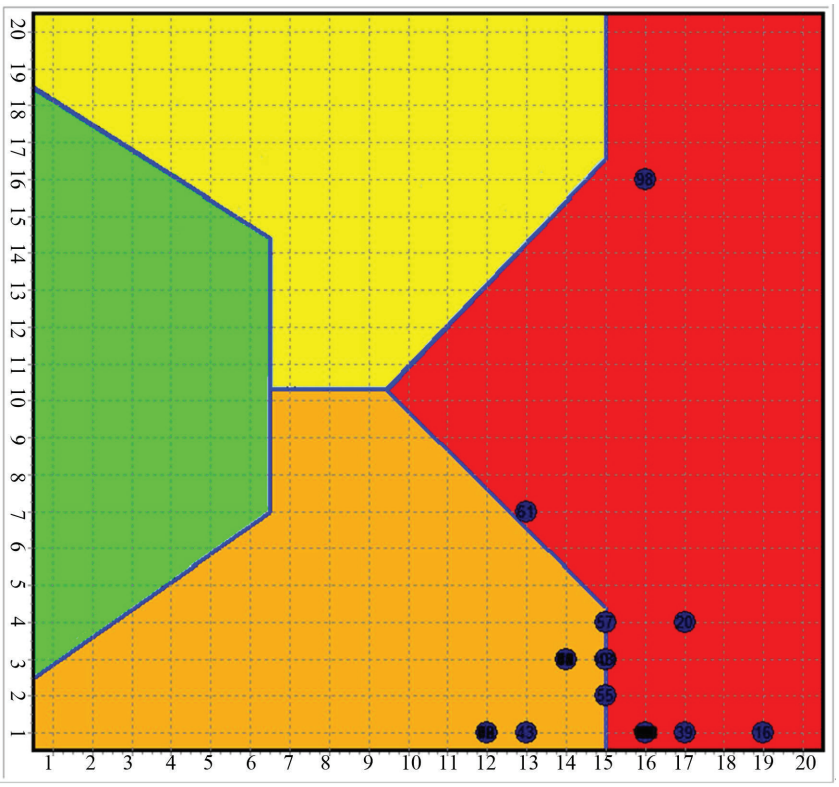


Figure 4. Distribution of the MD and BD subjects over the first SOM built.

20	2,24	1,17	1,92	1,89	1,92	2,04	2,11	1,85	1,68	1,59	1,58	1,56	1,64	1,60	1,30	0,99	0,50	0,02	-0,49	-1,02
19	1,92	1,94	2,25	2,08	1,98	1,98	2,00	1,71	1,53	1,44	1,37	1,53	1,70	1,54	1,32	0,96	0,48	0,10	0,12	-0,21
18	2,34	2,28	2,21	2,20	2,11	2,00	1,91	1,83	1,55	1,34	1,36	1,50	1,51	1,33	1,29	0,52	0,39	0,36	0,35	0,44
17	2,36	2,37	2,27	2,19	2,22	2,12	2,04	1,85	1,77	1,55	1,39	1,32	1,31	1,30	1,23	0,49	0,39	0,38	0,43	0,51
16	3,03	2,57	2,35	2,38	2,31	2,18	2,09	2,03	1,71	1,47	1,29	1,23	1,31	1,27	1,25	0,97	0,40	0,43	0,46	0,47
15	3,21	3,19	2,59	2,47	2,53	2,39	2,21	2,41	1,67	1,40	1,27	1,14	1,22	1,31	1,22	0,72	0,53	0,47	0,47	0,15
14	3,13	3,24	2,76	2,55	2,79	2,73	2,55	2,57	2,27	1,36	1,15	1,13	1,16	1,16	1,08	0,55	0,54	0,48	0,11	-0,49
13	2,99	3,05	2,53	2,44	2,53	2,47	2,42	2,53	2,17	1,17	1,09	1,11	1,02	1,01	0,67	0,49	0,51	0,18	-0,01	-0,24
12	2,91	2,83	2,35	2,28	2,34	2,18	2,01	2,06	1,97	1,63	1,55	1,48	0,79	0,67	0,60	0,43	0,49	0,12	-0,21	-0,27
11	2,39	2,54	2,44	2,31	2,22	2,12	1,93	1,97	2,22	2,20	2,01	1,91	0,79	0,73	0,63	0,48	0,31	0,07	-0,18	-0,38
10	2,49	2,55	2,45	2,41	2,28	2,13	2,01	1,97	2,27	2,40	2,15	1,68	1,07	0,69	0,55	0,50	0,35	0,05	-0,30	-0,42
9	2,83	2,78	2,54	2,52	2,46	2,26	2,05	1,91	2,02	2,21	1,81	1,57	1,37	0,69	0,56	0,51	0,22	0,04	-0,24	-0,40
8	3,28	3,10	2,78	2,53	2,57	2,43	2,23	2,03	2,01	2,05	1,91	1,71	1,23	0,87	0,58	0,51	0,32	0,19	-0,41	-0,57
7	3,45	3,33	2,88	2,69	2,57	2,58	2,22	1,93	1,97	1,82	1,66	1,38	1,19	0,85	0,63	0,64	0,38	0,32	-0,37	-0,51
6	3,78	3,74	3,07	2,86	2,88	2,59	2,43	2,06	2,07	1,54	1,50	1,40	1,11	0,83	0,76	0,44	0,26	0,08	-0,36	-0,46
5	4,05	3,80	3,40	3,13	2,93	2,76	2,54	2,49	1,99	1,81	1,83	1,57	1,12	0,87	0,75	0,33	-0,05	-0,05	-0,35	-0,54
4	4,60	4,20	3,75	3,18	3,18	2,91	2,85	2,71	2,20	1,99	1,89	1,85	1,23	1,01	0,91	0,10	-0,03	-0,14	-0,70	-0,80
3	4,45	4,20	4,19	3,68	3,45	3,36	2,90	2,69	2,42	2,09	1,93	1,77	1,51	1,34	1,18	-0,01	-0,16	-0,82	-1,07	-1,01
2	7,10	4,47	4,14	3,96	3,63	3,40	3,34	2,91	2,75	2,43	2,08	2,05	1,66	1,19	0,73	-0,14	-0,29	-1,00	-1,53	-1,95
1	8,23	6,58	4,26	3,98	3,36	3,06	3,89	3,07	2,90	2,50	2,16	2,34	1,72	1,03	0,50	-0,03	-0,19	-1,48	-1,79	-2,64
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20

Figure 5. Distribution of the B2 index and overlapped the SOM clusters (dark lines). The blue line delimits the negative indexes (MD).

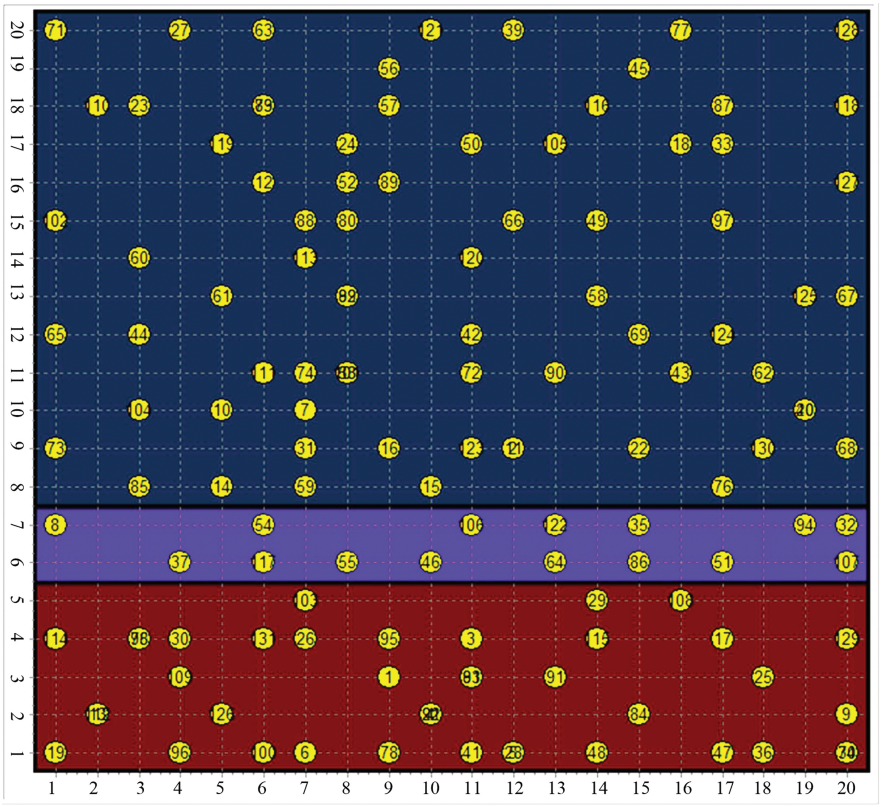


Figure 6. The new SOM recognize the MD (brown area) and the BD (blue area).

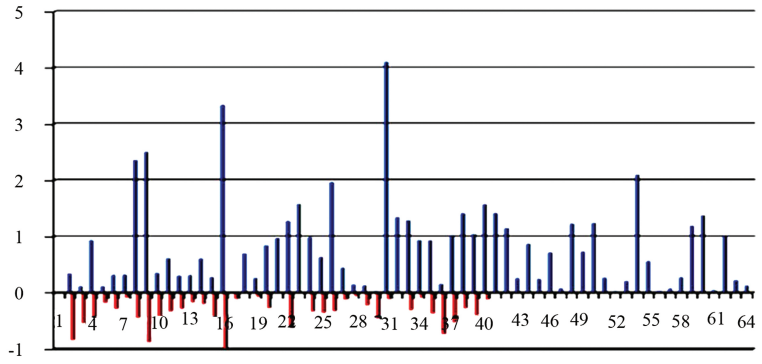


Figure 7. Histogram of the B2 index (blue = BD and red = MD).

4. Conclusive Anthropological Considerations

The identification of three platelet fatty acids (palmitic, linoleic and arachidonic), as well as enabling identification of subjects with Mood Disorders, has introduced some hypotheses which have been recognised over time in the stability of the experimental data obtained, also with regard to the concept of capture of serotonin, on the basis of the viscosity of the membrane, so that the platelet appears an element of great similarity with the neuron, given not only its embryogenetic origin but also some molecular aspects. This evidence, together with the possibility of classifying the two principal mood disorders (Major Depression-MD and Bipolar Disorder-BD), has led to some reflections as to the molecular specificity of major depression, understood, commonly, as a phenomenon concerning only human beings and, in particular, only some of them. In this situation we are able to trace the human condition between normality, bipolar disorder and major depression, the latter being considered as a biomolecular and existential niche.

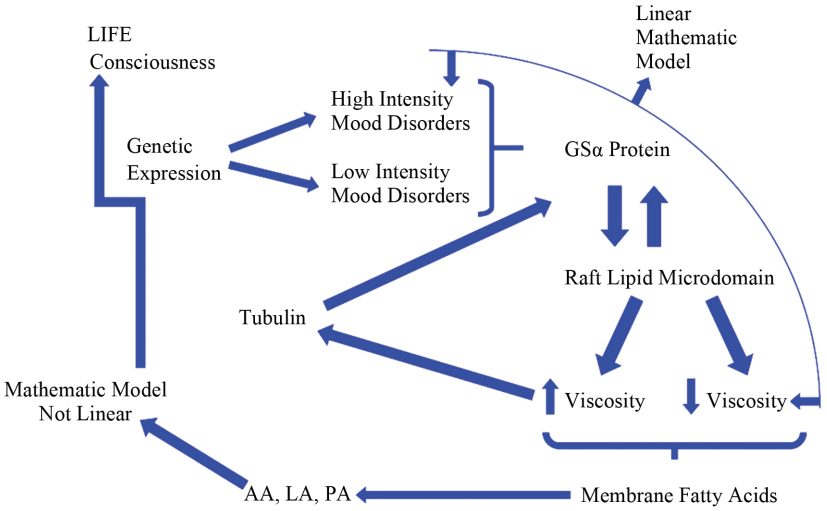


Figure 8. Molecular hypothesis (Cocchi, Hameroff, Mullis, Rasenik, Tonello, private meeting, Bologna, 2008) of consciousness that establishes a relationship between the membrane viscosity, Gs alpha protein and Tubulin (Cocchi &Tonello, 2010; Donati et al., 2008; Hameroff & Penrose, 1996; Hameroff, 2010).

U

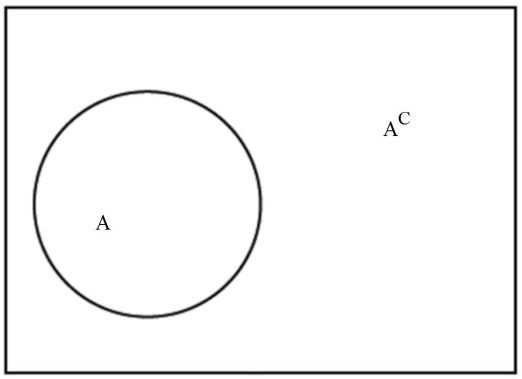


Figure 9. The U Bipartition. U is the universal representation of mankind. A is a cell whose elements are characterised by a positive B2 value. AC, as well as the completion of A in U, is the cell whose elements show a negative B2 value (B2 is the index that establishes a relationship between the three fatty acids isolated from the SOM and the molecular weight and melting point and enables us to discriminate bipolar patients (B2 positive) from those affected by major depression (B2 negative).

From this assumption we see the emergence in summary of some stringent theoretical and anthropological focuses:

- Firstly, the distinction between first-level ontological Depression and second-level ontological Depression.
- The first kind of depression, of an existential nature, expressed by the most varied cultural traditions, is rooted in our structural contingency, which in time and in becoming recognises the mark of its own finiteness (man as an anguished and depressed "natural" animal).
- Second-level ontological Depression, on the other hand, refers to MD, understood as a molecular,

bio-existential niche, marked cogently by serotonin and fatty acids, with its own specific "emotive tonality".

- The centrality of the concept of situation, that is of man as a being located "here and now", starting from biological markers able to offer the diagnosis of MD and BD an extremely real substrate, to be faced on an empirical, therefore public base, to construct a DSM that is not pregnant with pseudo-phenomenology or ideology, but which looks at the *dasein* of consciousness in its true biomolecular flesh (Cocchi et al, 2012, 2013a).
- The continuity between biology and culture, which finds full confirmation in the phenomenon of depression, whose original (ontological) biological nature (serotonin, fatty acids) thoroughly intercepts the structural (ontological) precariousness of living that the great cultural narrations have rooted in flesh and blood (ontic) existence.
- the view of man as a synthesis of *Körper* and *Leib*: life experience is always rooted in biology, the phenomenological "losability" and "impossibility" (Minkowski, 1970) are also, and firstly, biological "losability" and "impossibility".

To remain in the distinction, often denied only in the intentions, between *erklären* (causal explanation) and *verstehen* (psychological comprehension) means ignoring the prolific acquisitions of complexity theory; above all it means remaining prisoner to the "myth of the sense", on whose basis life experiences, the phenomenological approaches, the philosophical articulations, on which an authentic interpretation of the psychopathology should depend, are hypostatised. The reification of the metaphors, the empty sentimentalism of the interior resonances, the veneration of the illness *as a fruitful production of alternative worlds, the dilution of the tragedy of depression in the imaginative vis of melancholy*: this is the most injurious product of pseudo-phenomenology.

Hence the need for a "gestaltic reorientation": also biology produces sense, indeed it is the original meaning on which to graft other forms of meaning, of which philosophy is undoubtedly a strong interlocutor, but alongside other forms of knowledge (biochemistry, quantum physics, biomathematics, anthropology, sociology…), as an overall, heuristic synthesis, expressive of an autonomous, therefore "adult", approach to psychopathology (Cocchi et al., 2013a).

Lastly, it is necessary that the scientific community and the world of the clinical profession commit themselves increasingly so that psychiatry and psychology can constitute themselves as heuristic bio-analytical-existential knowledge, where the diagnosis is not placed under the Heideggerian "yoke of the idea" (classificatory ideology and diagnostic imperialism), but refers to convincing biological markers. In this context, comparison with the neurosciences appears inescapable, particularly in their quantistic standpoint (Pothos & Busemeyer, 2013; Woolf et al., 2010; Pylkkänen, 2010; Zizzi & Pregnolato, 2012; Mender, 2013; Cocchi et al., 2013b), with all the implications, also of an ethical nature, that this involves (Garland, 2004; Furman, Downey, & Jackson, 2004; Gazzaniga, 2005; Glannon, 2006; Illes, 2006; Levy, 2007; Illes & Sahakian, 2011; Benedikter & Giordano, 2012; Giordano, 2012; Kohls et al., 2013; Monti, 2013).

In other words, to start from biology to move towards increasingly complex systems, able to integrate biochemical expressions, living and irreducible existential experiences, social and cultural contexts. Depression therefore needs to be inscribed within a horizon of unmythicised, polyvocal meaning where the biological, physiological, clinical, existential, psycho-social and anthropological aspects are set as objective a hermeneutic framework as possible.

This is all the more so at a time like the present, when a person is often appraised only on the basis of successes achieved, of objects flaunted, of products voraciously consumed, in the instant, of his social visibility, of relentless efficacy, of perfect adaptation of the "thinkable to the possible": all contexts where the genetic and biological psychopathologies of mood are disproportionately amplified (Bauman, 2007; Bertman, 1998; Eriksen, 2001; Gabrielli, 2012).

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Clinical and Biochemical Evaluation of Facial Acanthosis Nigricans

Khalifa E. Sharquie[1,2*], Adil A. Noaimi[1,2], Halla G. Mahmood[3], Sameerah M. Al-Ogaily[4]

[1]Department of Dermatology, College of Medicine, University of Baghdad, Baghdad, Iraq
[2]Arab Board for Dermatology & Venereology, Baghdad Teaching Hospital, Medical City, Baghdad, Iraq
[3]Department of Clinical Biochemistry, College of Medicine, University of Baghdad, Baghdad, Iraq
[4]Department of Dermatology, Baghdad Teaching Hospital, Medical City, Baghdad, Iraq

Abstract

Background: Acanthosis nigricans is a well known cause of facial melanosis in Iraqi males and usually it is a part of ordinary acanthosis nigricans. It is commonly associated with many metabolic derangements. Objectives: To evaluate cases of acanthosis nigricans of the face for all metabolic disturbances including fasting blood sugar, fasting serum insulin, total cholesterol, triglyceride, growth hormone and serum leptin. Patients and Methods: Twenty seven cases of acanthosis nigricans of the face were included in this case descriptive, clinical and biochemical study. This was conducted in Department of Dermatology-Baghdad Teaching Hospital and Department of Clinical Biochemistry, College of Medicine, Baghdad, Iraq during the period from November 2012-August 2014. It consisted of 26 males and one female, their ages ranged from 16 - 58 (39 ± 4.9) years. The diagnosis was established by clinical and histopathological evaluation. Sharquie's ANSI scoring of acanthosis nigricans of face was carried out for all patients, also body mass index was assessed. Biochemical evaluation was carried out for all patients including total cholesterol, triglyceride, fasting blood sugar and insulin, insulin resistance, growth hormone and leptin enzyme immunoassay. Twenty seven healthy control non obese individuals with comparable ages and gender were assessed for all tests. Results: Biochemical results showed that fasting blood glucose, fasting serum insulin, insulin resistance, fasting serum triglyceride, total cholesterol, growth hormone and serum leptin were statistically significantly high in patients with acanthosis nigricans of the face in comparison with control individuals and all were positively correlated with the scoring of acanthosis nigricans of the face apart from high density lipoprotein was negatively correlated. Conclusion: Acanthosis nigricans of the face is a good marker for the associated metabolic diseases and these metabolic changes were statistically significantly correlated with the severity of acanthosis nigricans.

*Corresponding author.

Keywords

Acanthosis Nigricans, Face, Sharquie's Ansi Scoring, Facial Melanosis, Biochemical Changes

1. Introduction

Acanthosis nigricansis (AN) a skin disease characterized by hyperpigmented, thickened, verrucous plaques with a velvety texture typically symmetrical in distribution. It usually involves intertriginous areas including: neck, axillae, groin, antecupital and popliteal fossae, knuckles and umbilicus; occasionally it involves the mucosa such as oral, esophageal, pharyngeal, laryngeal, conjunctival, and anogenital mucosa [1]. The earliest changes in acanthosis nigricans of the face are usually dryness, pigmentation and roughness of the skin, which in affected area is gray-brown or black [1]. The hyperpigmentation is later accompanied by hypertrophy, increased skin marking, and velvety texture. The most commonly involved locations are the forehead, temples, nasolabial folds and cheeks [2]. Facial melanosis although is commonly caused by acanthosis nigricans of the face among adult males [3] but unfortunately was not reported and documented in the medical literatures until *Sharquie et al.* 2014, 2015 published their work. This study showed that acanthosis nigricans of the face has the same clinical and histopathological features of acanthosis of neck and axillae mainly pigmentation of the skin, thickening with or without velvety appearance and acanthosis with or without papillomatosis together with epidermal and dermal melanosis [2].

Acanthosis nigricans has a variety of causes but the likely common mechanism is stimulation of tyrosine kinase growth factor receptor signaling pathways in epidermis [4]. In insulin resistance syndromes, high levels of circulating insulin directly or indirectly activate the insulin-like growth factor 1 receptor (IGF1R) [5]. Insulin resistance due to obesity underlies the hyperinsulinaemia in obesity-associated acanthosis nigricans [6] [7]. An elevated fasting blood insulin level confirms the presence of hyperinsulinaemia underling the diagnosis of atypical acanthosis nigricans [8]. In malignant acanthosis nigricans, tumor deriver growth factors, in particular transforming growth factor-α, acting through the epidermal growth factor receptor (EGFR) is presumed, although anti-insulin receptor antibodies have also been implicated [9].

The metabolic disorder parameters like fasting blood sugar, fasting serum insulin, total cholesterol, triglyceride, serum growth hormone and serum leptin were not evaluated before. So the aim of present work is to assess these metabolic markers and to be correlated with the severity of acanthosis nigricans of the face.

2. Patients and Methods

This case descriptive, clinical and biochemical study was conducted in the Department of Dermatology-Baghdad Teaching Hospital and Department of Clinical Biochemistry, College of Medicine, Baghdad, Iraq during the period from November 2012 to August 2014. Twenty seven patients were enrolled in this study 26 males and 1 female. The nature and aim of this study were explained for each patient. Formal consent was taken from them before this study and photographs were taken. Also, the ethical approval was given by the Scientific Council of Dermatology & Venereology-Iraqi Board for Medical Specializations.

Clinical and histopathological study of acanthosis nigricans of the face of these patients were already published [2].

Body mass index for all patients were calculated in addition the atherogenic co-efficiency, atherogenic index plasma and cardiac risk ratio were assessed.

Color photographs for all patients were performed by 8 megapixels camera of mobile Samsung Galaxy Note II, in the same place and distance with fixed illumination.

Sharquie's ANSI scoring [2] was carried out for all patients regarding the followings:

Score 1 = Light brown;

Score 2 = Brown;

Score 3 = Dark brown;

Score 4 = Black.

1) Texture: Scoring from 0 - 3

Score 0 = No thickening;

Score 1 = Mild thickening;
Score 2 = Moderate thickening;
Score 3 = Severe thickening.

2) Percentage of the total area involved, this was measured by using transparent square paper. By this method the acanthosis nigricanc and the total surface areas were measured accurately by square centimeters, then the percentage of the acanthosis nigricansarea relative to the total area of the same region was measured and scoring was done as follows:

a) Forehead:
Score 1 = 1% - 25%;
Score 2 ≥ 25% - 50%;
Score 3 ≥ 50% - 75%;
Score 4 ≥ 75%.

b) Cheek:
Score 1 = 1% - 25%;
Score 2 ≥ 25% - 50%;
Score 3 ≥ 50% - 75%;
Score 4 ≥ 75%.

c) Nasolabial folds:
Score 1 = 1% - 25%;
Score 2 ≥ 25% - 50;
Score 3 ≥ 50% - 75%;
Score 4 ≥ 75%.

d) Temporal area:
Score 1 = 1% - 25%;
Score 2 ≥ 25% - 50%;
Score 3 ≥ 50% - 75%;
Score 4 ≥ 75%.

The scoring for each area were done separately including: forehead, right temporal, left temporal, right cheek, left cheek, right nasolabial fold and left nasolabial fold for all patients.

The **ANSI score** was calculated by the following equation:

$$\mathbf{ANSI} = (\mathbf{DF} + \mathbf{TF} + \mathbf{AF}) + (\mathbf{DTR} + \mathbf{TTR} + \mathbf{ATR}) + (\mathbf{DTL} + \mathbf{TTL} + \mathbf{ATL}) + (\mathbf{DCR} + \mathbf{TCR} + \mathbf{ACR}) + (\mathbf{DCL} + \mathbf{TCL} + \mathbf{ACL}) + (\mathbf{DNR} + \mathbf{TNR} + \mathbf{ANR}) + (\mathbf{DNL} + \mathbf{TNL} + \mathbf{ANL}).$$

where D is darkness, T is thickness, A is area, F is forehead, TR is right temporal, TL is left temporal, CR is right cheek, CL is left cheek, NR is right nsolabial, NL is left nasolabial [2].

The following biochemical tests were carried out: fasting blood sugar (FBS), fasting insulin, total cholesterol, triglyceride which determined by enzymatic methods, growth hormone and serum leptin enzyme immunoassay for the quantitative determination in human serum were used. DRG international Inc. USA is done by ELISA test and the results were correlated with the severity of acanthosis nigricans (Sharquie's ANSI score) using coefficient correlation.

Twenty seven healthy control individuals with age and gender matching with the patients of acanthosis nigricans of the face were studied for the same biochemical blood changes.

Data were statistically described in terms of range, mean, standard deviation (±SD), mode of frequencies (number of cases) and relative frequencies (percentages). Comparison between investigations of patients and controlled individuals was done using Chi square (x^2) test. A probability value (p value) less than 0.05 was considered significant. All statistical calculations were done using computer programs SPSS ver. 20 (Statistical Package for the Social Science; SPSS Inc. Chicago, IL, USA).

3. Results

Twenty seven patients with acanthosis nigricans of the face were included in this study, 26 (96.6%) males and 1 (3.3%) female, with a male to female ratio was 26:1. Their ages ranged from 16 - 58 years with a mean ± SD of 39 ± 4.9 years. The third and fourth decades of life were the commonest age groups affected. The duration of the

disease ranged from 1 - 8 years with a mean ± SD of 3.34 ± 5.67 years. All patients had bilateral symmetrical distribution of acanthosis nigricans on the face (**Figure 1**) in addition to acanthois nigricans of neck and axillae. No patients with underling malignancies, clinical diabetes or other important medical conditions were noted.

Twenty seven healthy control were assessed, 25 males and 2 females, their ages ranged from 18 - 56 years with a mean ± SD of 40 ± 2.93 years. the body mass index was ranged from 23 - 29.3 with a mean ± SD of 27 ± 0.93 (**Table 1**).

Normal weight was seen in 4 (13.3%) patients while overweight seen in 10 (33.3%) patients, and obese in 16 (53.3%) patients. The body mass index (BMI) ranged from 24.1 - 38 with a mean ± SD of 32.33 ± 0.72. So BMI was found to be highly significant elevated in patients with acanthosis nigricans of the face in comparison with healthy control individuals with P value = 0.00545.

Fasting blood sugar (P value = 0.00141), fasting serum insulin (P value = 0.00150), Homa-insulin resistance (P value = 0.00157), growth hormone (P value = 0.061446), Serum leptin (P value = 0.01550), total cholesterol (P value = 0.0014) and triglyceride (P value = 0.0032) were statistically significantly elevated in comparison with healthy control. Also, cardiac risk ratio (CRR) (P value = 0.001) and atherogenic coefficient (AC) (P value = 0.005) and atherogenic index plasma (AIP) (P value = 0.003) were highly significantly elevated (**Table 1**).

Sharquie's ANSI score was ranged from 4 - 62 and was positively correlated with the following parameter: serum insulin (r = 0.925), total cholesterol (r = 0.968), AIP (r = 0.95), *AC* (r = 0.976), serum leptin (r = 0.889) growth hormone (r = 0.945) and fasting blood sugar (r = 0.943), while there was negative correlation with HDL (r = 0.913) (**Figures 2-6**). (r = *coefficient correlation*).



Figure 1. Bilateral symmetrical distribution of acanthosis nigricans on the face.

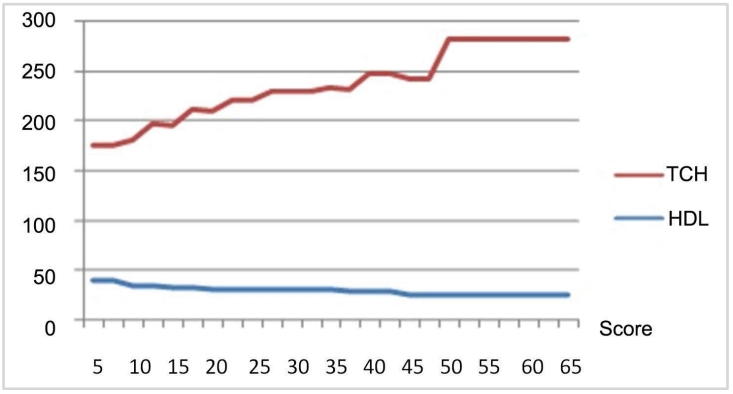


Figure 2. Showing the correlation between ANSI score & HDL & TCH levels.

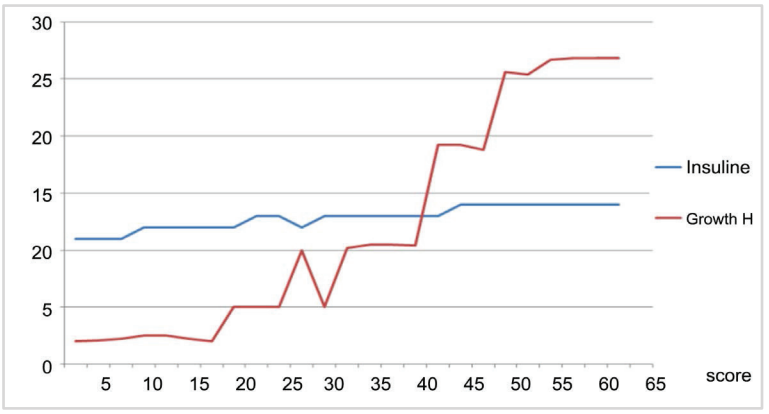


Figure 3. Showing the correlation between ANSI score & insulin & growth hormone levels.

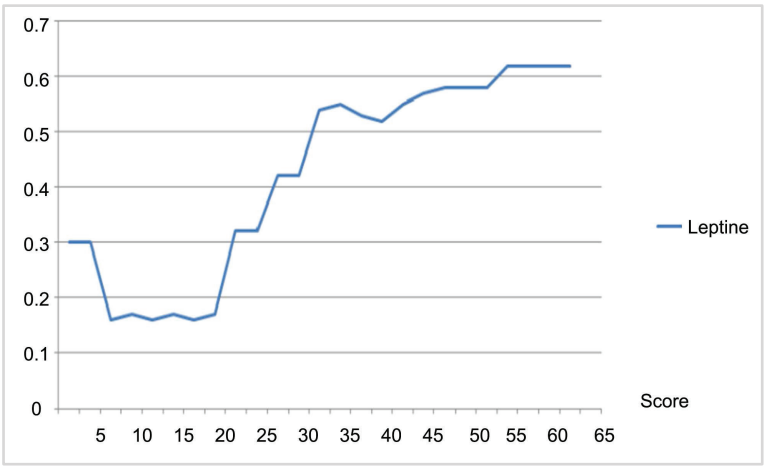


Figure 4. Showing the correlation between ANSI score & leptin level.

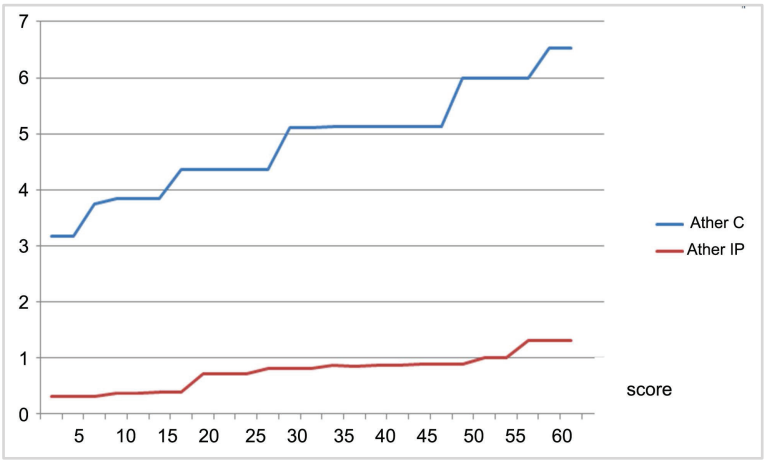


Figure 5. Showing the correlation between ANSI score & AC & AIP levels.

4. Discussion

Acanthosis nigricans is commonly associated with obesity and might be associated with many metabolic distur-bances like diabetes mellitus, elevated cholesterol and HBA1c [2]. No malignancies or drugs induced acanthosis nigricans were included in the present work.

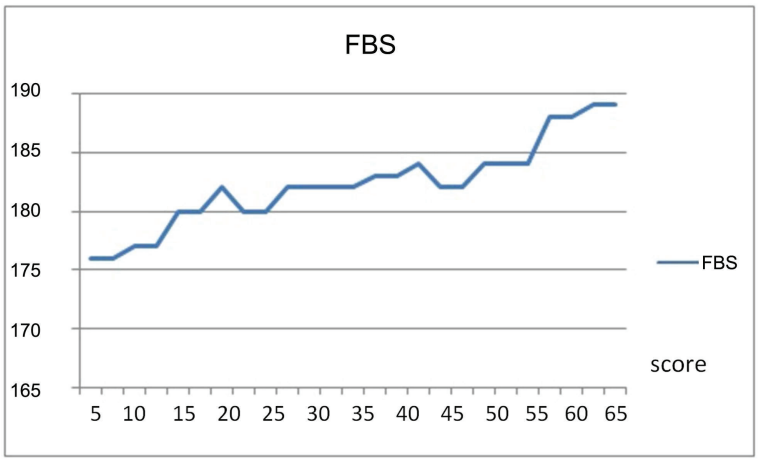


Figure 6. Showing the correlation between ANSI score & FBS levels.

Table 1. The result of fasting blood sampling from 27 patients with acanthosis nigricans of the face in comparison with 27 healthy controls.

	Control	Patients	P-value
Age	40 ± 2.93	39 ± 4.97	NS
BMI (Kg/m^2)	27 ± 0.93	32.33 ± 0.72	HS
FBG (mg/dl)	96.4 ± 3	180.3 ± 2.16	HS
Insulin (µlU/ml)	10.76 ± 0.4	12.3 ± 0.9	HS
Homa-IR	2.57 ± 0.1	5.46 ± 0.36	HS
Leptin (mg/dl)	0.38 ± 0.25	0.479 ± 0.07	S
CRP (mg/dl)	2.078 ± 0.432	1.68 ± o.79	S
GH (ng/ml)	5.66 ± 0.10	13.16 ± 9.43	S
TC (mg/dl)	187.3 ± 10.78	191.75 ± 34.34	HS
TG (mg/dl)	121 ± 9.55	223 ± 44	HS
HDL-C (mg/dl)	49.8 ± 6.78	33.12 ± 3.9	HS
CRR	3.80 ± 0.31	5.70 ± 1.21	HS
AC	2.80 ± 0.31	4.82 ± 1.03	HS
AIP	0.39 ± 0.03	0.85 ± 0.24	HS

The present study showed that Sharquie's ANSI score of acanthosis nigricans of the face was well correlated with the severity of acanthosis nigricans and body mass index. This score was also statistically significantly correlated with the following biochemical changes: FBS, fasting insulin, total cholesterol, triglyceride, growth hormone and serum leptin.

The present work also showed patients with acanthosis nigricans of the face had hyperinsulinemia and insulin resistance and this explain the pathogenesis of acanthosis nigricans like thickening of the epidermis and increased melanin deposition as was similarly reported about ordinary acanthosis nigricans [10], similarly this study also demonstrated elevated growth hormone in majority of cases of acanthosis nigricans of the face and this hormone also increase the activity of fibroblast and keratinocyte and play a role in pathogenesis of acanthosis nigricans. Serum leptin also elevated in majority of patients in the present study and this might explain the increased obesity among patients with acanthosis nigricans [11].

Total cholesterol and triglyceride were significantly elevated in majority of patients with decreased high density lipoprotein that lead to increase in cardiac risk ratio, atherogenic coefficient andatherogenic index plasma. These findings are very important as you can judge the changes in these parameter just by looking at the acanthosis nigricans of the face. To the best of our knowledge these findings are for the first time to be reported.

5. Conclusion

In conclusion, acanthosis nigricans of the face is an important cause of facial melanosis among Iraqi males where its clinical pictures and associated manifestations were similar to acanthosis nigricas of the body. Also, acanthosis nigricans of the face is a marker for many metabolic and biochemical changes in blood like elevated fasting blood sugar and lipid. Sharquie's ANSI scoring of acanthosis nigricans of the face was positively correlated with all biochemical changes.

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We would like to thank all the contributors and authors for lending their expertise to make the book truly unique. They have played a very crucial role in the development of this book. Without their invaluable contributions their work will not come in the form in the form of book wouldn't have been possible. They have made vital efforts to compile up to date information on the varied aspects of this subject to make this book a valuable addition to the collection of many professionals and students. This book was conceptualised with the vision of imparting current up-to-date information and advanced data in this field. To ensure the same, a matchless editorial board was set up for the review purpose. Every individual on the board went through rigorous rounds of assessment to prove their worth. After which they invested a large part of their time into researching and compiling the most relevant data for our readers. The editorial board has been involved for editing and reviewing in producing this book since its inception.

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List of Contributors

Joao Dias de Toledo Arruda-Neto
Physics Institute, University of São Paulo, São Paulo, Brazil
CEPESq/UniÍtalo—Italy-Brazilian University Center, São Paulo, Brazil

Guacyara Tenorio Cavalcante
Physics Institute, University of São Paulo, São Paulo, Brazil

Guilherme de Paula Nogueira
School of Veterinary Medicine, São Paulo State University/UNESP, Araçatuba, Brazil

Tulio Eduardo Rodrigues
Physics Institute, University of São Paulo, São Paulo, Brazil

Luiz Eduardo Correa Fonseca
School of Veterinary Medicine, São Paulo State University/UNESP, Araçatuba, Brazil

Mitiko Saiki
Institute for Energy and Nuclear Research, IPEN/CNEN-SP, São Paulo, Brazil

Godofredo Camara Genofre
CEPESq/UniÍtalo—Italy-Brazilian University Center, São Paulo, Brazil
Institute for Biomedical Sciences, University of São Paulo, São Paulo, Brazil

Gislei Maria Rigoni
Departamento de Ciências Biológicas, Universidade Estadual do Centro Oeste (UNICENTRO), Guarapuava, Brazil

Helio Conte
Departamento de Biotecnologia, Genética e Biologia Celular, Universidade Estadual de Maringá (UEM), Maringá, Brazil

Robert Louis Bertrand, Michael Okechukwu Eze
Department of Chemistry, University of Winnipeg, Winnipeg, Canada

Ting Liu, Lianqiang Hui, Chunyu Cao, Ran Hao, Shuangrong Gao, Rong He, Baoqiang Dai, Lifang Wang
Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences, Beijing, China

Cleydson B. R. Santos
Laboratory of Modeling and Computational Chemistry, Federal University of Amapá, Macapá, Brazil
Postgraduate Program in Biotechnology and Biodiversity-Network BIONORTE, Campus Universitário Marco Zero, Macapá, Brazil
Laboratory of Drug Research, School of Pharmaceutical Sciences, Federal University of Amapá, Macapá, Brazil

Cleison C. Lobato
Laboratory of Modeling and Computational Chemistry, Federal University of Amapá, Macapá, Brazil

Francinaldo S. Braga
Laboratory of Modeling and Computational Chemistry, Federal University of Amapá, Macapá, Brazil

Sílvia S. S. Morais
Laboratory of Statistical and Computational Modeling, University of the State of Amapá, Macapá, Brazil

Cesar F. Santos
Laboratory of Modeling and Computational Chemistry, Federal University of Amapá, Macapá, Brazil

Caio P. Fernandes
Laboratory of Modeling and Computational Chemistry, Federal University of Amapá, Macapá, Brazil
Laboratory of Drug Research, School of Pharmaceutical Sciences, Federal University of Amapá, Macapá, Brazil

Davi S. B. Brasil
Laboratory of Modeling and Computational Chemistry, Federal University of Amapá, Macapá, Brazil
Institute of Technology, Federal University of Pará, Belém, Brazil

Lorane I. S. Hage-Melim
Laboratory of Modeling and Computational Chemistry, Federal University of Amapá, Macapá, Brazil

Williams J. C. Macêdo
Laboratory of Modeling and Computational Chemistry, Federal University of Amapá, Macapá, Brazil
Postgraduate Program in Biotechnology and Biodiversity-Network BIONORTE, Campus Universitário Marco Zero, Macapá, Brazil

José C. T. Carvalho
Laboratory of Modeling and Computational Chemistry, Federal University of Amapá, Macapá, Brazil
Postgraduate Program in Biotechnology and Biodiversity-Network BIONORTE, Campus Universitário Marco Zero, Macapá, Brazil
Laboratory of Drug Research, School of Pharmaceutical Sciences, Federal University of Amapá, Macapá, Brazil

Latife Ceyda Irkin
Department of Aquaculture, Faculty of Marine Sciences and Technology, Canakkale Onsekiz Mart University, Canakkale, Turkey

Murat Yigit
Department of Aquaculture, Faculty of Marine Sciences and Technology, Canakkale Onsekiz Mart University, Canakkale, Turkey

Sevdan Yilmaz
Department of Aquaculture, Faculty of Marine Sciences and Technology, Canakkale Onsekiz Mart University, Canakkale, Turkey

Masashi Maita
Graduate School of Marine Science and Technology, Tokyo University of Marine Sciences and Technology, Course of Applied Biosciences, Konan 4-5-7, Minato-ku, Tokyo, Japan

Fatima Zohra El Kadi
Laboratoire de Biotoxicologie, Département de Biologie, Faculté des Sciences de la Nature et de la vie, Université D Liabés, Sidi Bel Abbés, Algérie

Amina Imène Bénali
Laboratoire de Biotoxicologie, Département de Biologie, Faculté des Sciences de la Nature et de la vie, Université D Liabés, Sidi Bel Abbés, Algérie

Mohammed Bénali
Laboratoire de Biotoxicologie, Département de Biologie, Faculté des Sciences de la Nature et de la vie, Université D Liabés, Sidi Bel Abbés, Algérie

Slimane Belbraouet
École de Nutrition, Université de Moncton, Moncton, Canada

Ebru Yilmaz
Aquaculture and Fisheries Engineering Department, Faculty of Agriculture, Adnan Menderes University, Aydýn, Turkey

Sebahattin Ergün
Department of Aquaculture, Faculty of Marine Sciences and Technology, Çanakkale Onsekiz Mart University, Çanakkale, Turkey

Sevdan Yilmaz
Department of Aquaculture, Faculty of Marine Sciences and Technology, Çanakkale Onsekiz Mart University, Çanakkale, Turkey

Yubing Deng, Hengmin Cui, Xi Peng, Jing Fang, Zhicai Zuo, Junliang Deng, Qin Luo
Key Laboratory of Animal Diseases and Environmental Hazards of Sichuan Province, College of Veterinary Medicine, Sichuan Agricultural University, Ya'an, China

Hang Liu
Department of Anesthesiology and Pain Management, School of Medicine, Juntendo University, Tokyo, Japan

Takuji Yamaguchi
Center for Advanced Kampo Medicine and Clinical Research, Juntendo Graduate School of Medicine, Tokyo, Japan

Kenji Ryotokuji
Institute of Transcultural Studies, Ryotokuji University, Chiba, Japan

Satoru Otani
Center for Advanced Kampo Medicine and Clinical Research, Juntendo Graduate School of Medicine, Tokyo, Japan
Center for Medical Education, Ryotokuji University, Chiba, Japan

Hiroyuki Kobayashi
Center for Advanced Kampo Medicine and Clinical Research, Juntendo Graduate School of Medicine, Tokyo, Japan
Department of Hospital Administration, Juntendo University Graduate School of Medicine, Tokyo, Japan

Masako Iseki
Department of Anesthesiology and Pain Management, School of Medicine, Juntendo University, Tokyo, Japan

Eichi Inada
Department of Anesthesiology and Pain Management, School of Medicine, Juntendo University, Tokyo, Japan

Rosalinda Guevara Guzmán, María Esther Urrutia-Aguilar
Facultad de Medicina, Universidad Nacional Autónoma de México, México D.F., México

Yonggang Qu
School of Public Health, Xinjiang Medical University, Urumqi, China
College of Animal Science and Technology, Shihezi University, Shihezi, China

Yujian Zheng
School of Public Health, Xinjiang Medical University, Urumqi, China

Chuangfu Chen
College of Animal Science and Technology, Shihezi University, Shihezi, China

Guangze Zhu
Genetic Engineering Laboratory, Academy of Military Medical Sciences, Changchun, China

Ningyi Jin
Genetic Engineering Laboratory, Academy of Military Medical Sciences, Changchun, China

Khalifa E. Sharquie
Department of Dermatology, College of Medicine, University of Baghdad, Baghdad, Iraq
Arab Board for Dermatology & Venereology, Baghdad Teaching Hospital, Medical City, Baghdad, Iraq

Adil A. Noaimi
Department of Dermatology, College of Medicine, University of Baghdad, Baghdad, Iraq
Arab Board for Dermatology & Venereology, Baghdad Teaching Hospital, Medical City, Baghdad, Iraq

Halla G. Mahmood
Department of Clinical Biochemistry, College of Medicine, University of Baghdad, Baghdad, Iraq

Sameerah M. Al-Ogaily
Department of Dermatology, Baghdad Teaching Hospital, Medical City, Baghdad, Iraq

Terry Golombick
Department of Endocrinology, St George Hospital, Sydney, Australia

Terrence H. Diamond
Department of Endocrinology, St George Hospital, Sydney, Australia

Arumugam Manoharan
Southern Sydney Haematology, University of Wollongong, Wollongong, Australia

Rajeev Ramakrishna
Southern Sydney Haematology, University of Wollongong, Wollongong, Australia

Jing Zhao, Yan Li, Bo Yan, Lintao Jia, Xia Li, Lifeng Wang, Xinping Liu, Libo Yao
Department of Biochemistry and Molecular Biology, Fourth Military Medical University, Xi'an, China

Husain A. El-Fadaly
Agricultural Microbiology Department, Faculty of Agriculture, Damietta University, Damietta, Egypt

Sherif M. El-Kadi
Agricultural Microbiology Department, Faculty of Agriculture, Damietta University, Damietta, Egypt

Mohamed N. Hamad
Dairy Department, Faculty of Agriculture, Damietta University, Damietta, Egypt

Abdelhady A. Habib
Agricultural Microbiology Department, Faculty of Agriculture, Damietta University, Damietta, Egypt

Heba S. Hamed
Department of Zoology, Faculty of Women for Arts, Science & Education, Ain Shams University, Cairo, Egypt

Peter Kovacic
Department of Chemistry and Biochemistry, San Diego State University, San Diego, USA

Ratnasamy Somanathan
Department of Chemistry and Biochemistry, San Diego State University, San Diego, USA
Centro de Graduados e Investigación del Instituto Tecnológico de Tijuana, Tijuana, Mexico

Fidelis Ifeakachuku Achuba, Patrick Nwanze Okoh
Department of Biochemistry, Delta State University, Abraka, Nigeria

Janice L. Huntingford
Essex Animal Hospital, Essex, Canada

Corri B. Levine
Department of Clinical Sciences, Cornell University, Ithaca, NY, USA

Debbie J. Mustacich
Linus Pauling Institute, Oregon State University, Corvallis, OR, USA

Devin Corrigan
Linus Pauling Institute, Oregon State University, Corvallis, OR, USA

Robert L. Downey
Annamaet Petfood Company, Sellersville, PA, USA

Joseph J. Wakshlag
Department of Clinical Sciences, Cornell University, Ithaca, NY, USA

Hannu T. Korhonen, Hanna Huuki
MTT Agrifood Research Finland, Animal Production Research, Kannus, Finland

Michael J. Bell
Craigieburn Animal Hospital, 9 Craigieburn Rd., Craigieburn, Australia

Emily M. Donaldson
Cornell University College of Veterinary Medicine, Department of Clinical Sciences, Ithaca, NY, USA

Sabine Mann
Cornell University College of Veterinary Medicine, Department of Population Medicine, Ithaca, NY, USA

Robert L. Gillette
Red Bank Referral Center, Sports Medicine and Rehabilitation Service, Red Bank, NJ, USA

Corri B. Levine
Cornell University College of Veterinary Medicine, Department of Clinical Sciences, Ithaca, NY, USA

Joseph J. Wakshlag
Cornell University College of Veterinary Medicine, Department of Clinical Sciences, Ithaca, NY, USA

Tessa Trouchon, Sébastien Lefebvre
USC 1233 INRA-Vetagro Sup, Veterinary School of Lyon, Marcy l'Etoile, France

Pranev Sharma
Magill Department of Anaesthesia, Chelsea & Westminster Hospital, London, UK

Christine Chung
Imaging & Anaesthetics, Chelsea & Westminster Hospital, London, UK

Marcela Vizcaychipi
Magill Department of Anaesthesia, Chelsea & Westminster Hospital, London, UK

Qingping Sun, Zhimin Xu
Guangdong Institute of Food and Drug Control, Guangzhou, China

Massimo Cocchi
Paolo Sotgiu Institute for Quantitative & Quantum Psychiatry & Cardiology, L.U.de.S. University, Lugano, Switzerland

Department of Veterinary Medical Sciences, University of Bologna, Bologna, Italy
Faculty of Human Sciences, L.U.de.S. University, Lugano, Switzerland

Lucio Tonello
Paolo Sotgiu Institute for Quantitative & Quantum Psychiatry & Cardiology, L.U.de.S. University, Lugano, Switzerland
Faculty of Human Sciences, L.U.de.S. University, Lugano, Switzerland

Fabio Gabrielli
Paolo Sotgiu Institute for Quantitative & Quantum Psychiatry & Cardiology, L.U.de.S. University, Lugano, Switzerland
Faculty of Human Sciences, L.U.de.S. University, Lugano, Switzerland