DIETARY CALCIUM REDUCING EFFECTS OF WATERBORNE LEAD UPTAKE IN NILE TILAPIA (*OREOCHROMIS NILOTICUS*)

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ABSTRACT

This study investigated the influence of dietary calcium supplementation (Ca^{2+}) to reduce the toxicity of sublethal lead concentration in Nile tilapia with emphasis on hematological, biochemical, histopathological, and nuclear morphology analysis. The values of 24, 48, 72, and 96 h LC_{50} of lead in tilapia were 247.51, 197.47, 193.36, and 182.38 mg/L, respectively. Therefore, lead (Pb) concentration tested in the sublethal experiment was 45 mg/L, which corresponds to 25% of the 96 h LC_{50}. Fish were fed with 0, 20, and 60 mg Ca^{2+}/g food for 30 days. Regarding the hematological investigation erythrocytes count, MCHC showed significant elevation ($p \leq 0.05$). The Hct, Hb, and MCV were decreased significantly with Pb exposure when compared with the control group ($p \leq 0.05$). In the gills of tilapia exposed to Pb treatment, edema, lamellar cell hyperplasia, epithelial lifting, lamellar fusion, and aneurysm were observed. In the liver, there were blood congestion in sinusoids, vacuolation of hepatocytes, and necrosis. In the kidney, glomerulus's atrophy, tubular swelling, and also necrosis were seen. In the spleen, there were cell swelling, blood congestion, and necrosis. In the testes, vacuolation, blood congestion, and necrosis were found. No recognizable changes were observed in ovaries and muscles. The structural damage could be correlated to the significant increase ($p \leq 0.05$) in aminotransferase activities and the total amount of protein. The micronuclei values in erythrocytes, gills, livers, kidneys, and fin cells showed significant increase. The nuclear abnormality (NA) shapes in erythrocytes, gills, livers, kidneys, and fin cells were sorted into blebbed nuclei (BL), lobed nuclei (LB), notched nuclei (NT), and binuclei (BN). The frequencies of each NA shape in the tissues of all treatments observed were as follows: NT > LB > BN > BL. Fish fed with Ca^{2+} supplemented diets showed slight alteration when compared to the Pb only treatment groups. These results indicate that dietary Ca^{2+} will be protective in reducing Pb burdens in fish exposed to environments contaminated with waterborne Pb.

KEY WORDS: NILE TILAPIA / *OREOCHROMIS NILOTICUS* / CALCIUM / HISTOPATHOLOGY / HEMATOLOGY

116 pages

ผลของอาหารเสริมแคลเซี่ยมต่อการลดความเป็นพิษของตะกั่วในปลานิล

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บทคัดย่อ

การศึกษาประสิทธิภาพของอาหารปลาผสมแคลเซี่ยมต่อการลดความเป็นพิษของตะกั่วในปลานิล โดยศึกษาการเปลี่ยนแปลงทางโลหิตวิทยาและชีวเคมี พยาธิสภาพที่เกิดในอวัยวะต่างๆ ได้แก่ เหงือก ตับ ไต ม้าม อัณฑะ รังไข่ และกล้ามเนื้อ และลักษณะนิวเคลียสของเม็ดเลือดแดง เหงือก ตับ ไต และครีบ โดยการผสมแคลเซียมในอาหารปลาที่เลี้ยงในน้ำผสมตะกั่ว รวมทั้งการตรวจวัดอัตราการเจริญเติบโต หาค่าดัชนีความสัมพันธ์ระหว่างน้ำหนักตัวและน้ำหนักของตับและไต ผลการศึกษาได้ค่าเฉลี่ยความเป็นพิษเฉียบพลันที่เวลา 24, 48, 72 และ 96 ชั่วโมง เท่ากับ 247.51, 197.47, 193.36 และ 182.38 มิลลิกรัมต่อลิตร ตามลำดับ คำนวณหาค่าความเข้มข้นเพื่อใช้ศึกษาความเป็นพิษแบบกึ่งเฉียบพลันได้เท่ากับ 45 มิลลิกรัมต่อลิตร ให้อาหารปลาผสมแคล เซี่ยมเท่ากับ 0, 20 และ 60 มิลลิกรัมต่อกรัม เป็นระยะเวลา 30 วัน พบการเปลี่ยนแปลงทางโลหิตวิทยา คือ จำนวนเม็ดเลือดแดง และ MCHC สูงขึ้นอย่างมีนัยสำคัญ ส่วน Hct, Hb และ MCV ลดลงอย่างมีนัยสำคัญในกลุ่มที่เลี้ยงด้วยตะกั่วเมื่อเปรียบเทียบกับกลุ่มควบคุม ($p \leq 0.05$) เมื่อศึกษาด้วยกล้องจุลทรรศน์ พบพยาธิสภาพที่เกิดขึ้นในอวัยวะต่างๆ ได้แก่ เหงือกบวม หนาตัวขึ้น มีการเชื่อมรวมกันของซี่เหงือก พบมีเลือดคั่ง ช่องว่างภายในเซลล์และเนื้อตายในตับ พบการฝ่อของโกลมูโรลัส การบวมของท่อไตและเนื้อตายในไต พบเซลล์มีการบวม เลือดคั่งและเนื้อตายในม้าม พบช่องว่างภายในเซลล์ เลือดคั่งและเนื้อตายในอัณฑะ ส่วนรังไข่และกล้ามเนื้อปลาไม่พบการเปลี่ยนแปลง ซึ่งการเปลี่ยนแปลงทางพยาธิสภาพนี้มีความสัมพันธ์อย่างมีนัยสำคัญ ($p \leq 0.05$) กับการเปลี่ยนแปลงของระดับเอนไซม์ aminotransferase และ total protein การศึกษาลักษณะรูปร่างของนิวเคลียส พบค่า MN ในเม็ดเลือดแดง เหงือก ตับ และไต เพิ่มขึ้นอย่างมีนัยสำคัญ ($p \leq 0.05$) ส่วน NA ของเม็ดเลือดแดง เหงือก ตับ ไตและครีบ พบในลักษณะต่างๆ ได้แก่ blebbed nuclei (BL), lobed nuclei (LB), notched nuclei (NT) และ binuclei (BN) โดยมีอัตราความถี่ดังนี้ NT> LB> BN> BL ปลาที่ให้อาหารเสริมแคลเซี่ยมพบการเปลี่ยนแปลงทางโลหิตวิทยาและชีวเคมี พยาธิสภาพของอวัยวะต่างๆ และลักษณะของนิวเคลียส ลดลง เมื่อเทียบกับการเลี้ยงในตะกั่วเพียงอย่างเดียว จากผลการศึกษสรุปได้ว่าอาหารปลาผสมแคลเซี่ยมมีประสิทธิภาพต่อการลดความเป็นพิษของตะกั่วในปลานิลได้

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LIST OF ABBREVIATIONS

%	= Percentage
ALT	= Alanine aminotransferase
AST	= Aspartate aminotransferase
BL	= Body length
◦C	= Degree celcius
cm	= Centimeter
G	= Gauge
g	= Gram
h	= Hour
HPF	= High power field
HNE	= 4-hydroxy-2-nonenal
HSI	= Hepato-somatic index
kg	= Kilogram
KI	= Kidney-somatic index
L	= Liter
m	= Meter
MCH	= Mean corpuscular hemoglobin
MCHC	= Mean corpuscular hemoglobin concentration
MCV	= Mean corpuscular volume
mg	= Milligram
min	= Minute
mL	= Milliliter
MS-222	= Tricaine methan sulphonate
mt	= Metric tons

LIST OF ABBREVIATIONS (cont.)

nm	= Nanometer
PBS	= Phosphate buffer saline
PVCs	= Pavement cells
RBC	= Red blood cell
rpm	= Round per minute
TL	= Total length
WBC	= White blood cell

CHAPTER I
INTRODUCTION

1.1 Lead

Lead (Pb) is a highly toxic metal that is used for many years in products found in and around our homes. Lead is a bluish-white lustrous metal. It is very soft, highly malleable, ductile, and a relatively poor conductor of electricity. It is very resistant to corrosion but tarnishes upon exposure to air and non-biodegradable. The usual valence state in inorganic lead compounds is +2. Natural lead is a mixture of four stable isotopes, ^{208}Pb (53%), ^{206}Pb (26%), ^{207}Pb (20%), and ^{204}Pb (1.5%). Lead isotopes are the stable decay product of three naturally radioactive elements: ^{206}Pb from uranium, ^{207}Pb from actinium, and ^{208}Pb from thorium (WHO, 1995).

1.1.1 Applications

Lead pipes bearing the insignia of Roman emperors, used as drains from the baths, are still in service. Alloys include pewter and solder. Tetraethyl lead ($PbEt_4$) is still used in some grades of gasoline but is being phased out on environmental grounds. Lead is a major constituent of the lead-acid battery used extensively in car batteries. It is used as a coloring element in ceramic glazes, as projectiles, in some candles to threat the wick. It is the traditional base metal for organ pipes, and it is used as electrodes in the process of electrolysis. One if its major cusses are in the glass of computer and television screens, where it shields the viewer from radiation. Other uses are in sheeting, cables, solders, lead crystal glassware, ammunitions, and bearing as weight in sport equipment. The major used of lead found in battery productions, and some in paints, such as pigments and colored inks (Martinez et al., 2004).

1.1.2 Lead in the environment

Native lead is rare in nature. The level of lead in the earth's crust is about 20 mg/kg. Lead in the environment may derive from either natural or anthropogenic sources. Natural sources of atmospheric lead include geological weathering and volcanic emissions and have been estimated at 19,000 tons/year (WHO, 1995).

Currently lead is usually found in ore with zinc, silver and copper and it is extracted together with these metals. The main lead mineral in Galena (PbS) and there are also deposits of cerrusite ($PbCO_3$) and anglesite ($PbSO_4$) which are mined. Galena is mined in Australia, which produces 19% of the world's new lead, followed by the USA, China, Peru' and Canada. Some is also mined in Mexico and West Germany. World production of new lead is 6 million tons a year, and workable reserves total are estimated 85 million tons, which is less than 15 year's supply. Lead occurs naturally in environment.

However, most lead concentrations that are found in the environment are a result of human activities. Due to the application of lead in gasoline an unnatural lead-cycle has consisted. In car engines lead is burned, so that lead salts (chlorines, bromines, and oxides) will originate. These lead salts enter the environment through the exhausts of cars. The larger particles will drop to the ground immediately and pollute soils or surface waters, the smaller particles will travel long distances through air and remain in the atmosphere. Part of this lead will fall back on earth when it is raining. This lead-cycle caused by human production is much more extended than the natural lead-cycle. It has caused lead pollution to be a worldwide issue.

As a result, lead becomes one of the most everywhere environmental poisons encountered in everyday life. Levels of lead found in air, food, water and soil vary widely throughout the world and depend upon the degree of industrial development, urbanization and lifestyle factors (ATSDR, 2005).

1.1.3 Lead problem

Waste containing lead can lead to many serious health hazard implications. Exposure to excessive levels of lead is harmful to the health and intellectual development of millions of children and adults in all most region of the world. Lead has highly hazardous properties and is always included in the high-risk category of toxicants. It is known to be a potent neurotoxin, carcinogen, developmental toxicant and also a reproductive toxicant causing fetal deformities and mortality (Environmental Defense, 2005). Lead poisoning can cause damages to nervous system, kidney, liver and sterility, as well as inducing growth inhibition, developmental retardation and detrimental effects in blood (Amdur et al., 1991). In 1978, there were nearly three to four million children with elevated blood lead levels in the United States (EPA, 2006).

The problem of lead contaminated surface water and sediment in Klity Creek, Klity Village, Kanchanaburi province, Thailand was reported in April 1998. The Ministry of Public Health has studied the level of lead in blood of villagers and the water of the Klity Creek. The result showed that lead concentration in blood was about four to five times higher than the usually found 4.9 μg/dL in an average Thai adult. The contamination level of water was the highest ever recorded in Thailand, about ten times higher than the level deemed safe. Aquatic animals had high concentration of lead. Department of Pollution Control (DPC) reported the estimate of lead in the sediment of the Creek was about 15,000 tons in the distance 3 kilometers along the creek (Tonmanee, 2001). Moving onto the toxic substance disposal trends in Thailand, it has been recorded that about 2,000 tons of electronic devices and appliances are disposed off annually in Thailand, however, only 100 tons are processed properly by Thailand's only licensed operator of disposal systems, General Environmental Conservation PLC (GENCO) (Sanongphan, 2003). This would mean that the remaining waste is dumped and disposed off improperly causing high risk of contamination in the environment. The process of waste disposal is extremely costly and it needs to be monitored for up to about 30 years therefore a better approach would be to limit the amount of low quality imports of electronic devices from developed nations pouring into Thailand. A lot of illegal dumping of hazardous waste

also takes place at Bangkok's Klong Toey port. No action was taken to eliminate the waste thus an explosion took place in 1991 killing 10 people and causing chronic and acute health problems to thousands of people living in the area and claiming even more lives later (Eamsakulrat et al., 1994). The study by United States Agency for International Development (USAID) and EPA in 1990, it was found that lead was in the high-risk category of Bangkok toxicants. It caused thousands cases of hypertension, up to 400 male deaths and loss of I.Q points for 700,000 children up to the age of seven (Eamsakulrat et al., 1994). Like most rivers in Thailand, the Chao Phraya has been progressively contaminated by heavy metals, especially lead. A study in 1989 showed that 18 major rivers in Thailand were contaminated with heavy metals. A subsequent study in 1991 found that 25 of the total 43 rivers in Thailand were contaminated (Mahaphol, 1993). The contamination of lead in aquatic area does not only cause problems to humans but also affects animal and other organism, terrestrial organisms, aquatic organism, and plants. In fact, lead contamination has a far wider impact beyond its specific area and boundaries extending well into the entire ecosystem.

Lead has been the focus of investigation in this study because it thrives in the marine environment and is also accumulated into aquatic organisms such as fish, which are consumed by the masses of population (WHO, 1995).

1.2 *Oreochromis niloticus*

Tilapia have become one of the most commercially important groups of cultured freshwater fish with in excess of 850,000 tons produced annually in range of countries e.g. Thailand, Taiwan, China, Philippines, Belgium and USA, and tilapia are second only to carps as the most widely farmed freshwater fish in the world (Coward and Bromage, 2000).

The Nile tilapia (*O. niloticus*) was one of the first fish species cultured. Originally, tilapia came from Africa where it is found from Egypt to Cape Horn, but at present 90 percent of *Oreochromis* are farmed commercially and aquaculture projects in 85 countries around the world included India, Indonesia, Taiwan, and Thailand (Brown et al., 1996). Tilapia has been called Saint Peters fish in reference to biblical

passages about the fish fed to the multitudes. The Nile tilapia is still the most widely cultured species of tilapia in Africa.

Positive aquaculture characteristics of tilapia are their tolerance to poor water quality (high salinity, high water temperature, low dissolved oxygen, and high ammonia concentrations) and the fact that they eat a wide range of natural food organisms. Biological constraints to the development of commercial tilapia farming are their inability to withstand sustained water temperatures below 50 to 52 ^{o}F and early sexual maturity that results in spawning before fish reach market size. Following is a discussion of the characteristics and culture of non- hybrid tilapia (Popma and Masser, 1999).

World production of framed tilapia exceeded 2,002,087 metric tons (mt) in 2004 (Fig. 1), with China the major producer and consumer. The United Stated is the world's major importer of tilapia. Its 2005 imports were 126,000 mt, with a value of $374 million. Tilapia has already become one of the most important farm raised fish and has an increasing role in the international food trade (Lim and Webster, 2006).

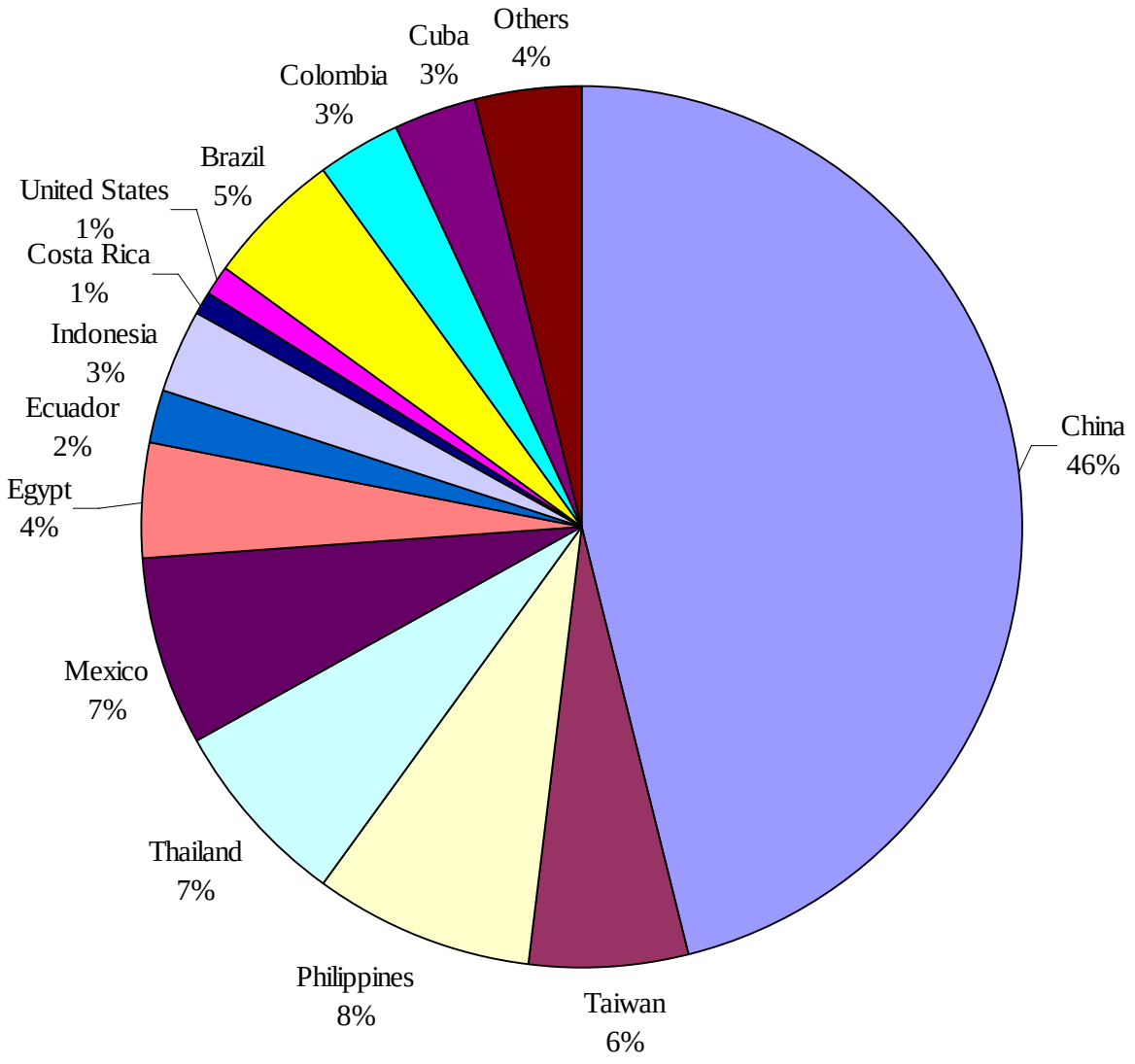


Figure1. Origin of tilapia aquaculture production in 2004 (Lim and Webster, 2006).

In Thailand, The Ministry of Agriculture and Co-operatives reported Nile Tilapia is the first one of Production by species for whole Inland fishery (including freshwater culture), 2000 – 2004 (Department of Fisheries, 2006).

Table 1. Production by species for whole inland fishery including freshwater culture, in 2000 – 2004.

Species	2000	2001	2002	2003	2004
Nile tilapia	122.4	127.6	120.9	123.6	203.1
Walking catfish	95.6	92.0	94.5	115.4	166.1
Common silver barb	87.3	85.6	88.5	88.0	106.8
Striped snake-head fish	24.9	24.9	23.8	28.2	29.8
Snake skin gourami	22.3	23.0	25.3	36.1	37.8
Catfish	14.5	15.7	16.5	26.0	33.7
Common carp	12.5	11.6	15.0	9.4	13.8
Other fish	74.0	78.4	80.7	85.6	86.7

1.2.1 Taxonomy (Nelson, 1984)

Taxonomy of Nile tilapia (*Oreochromis niloticus*)

Phylum	Chordata
Subphylum	Vertebrata
Superclass	Osteichthyes
Class	Actinopterygii
Subclass	Neopterygii
Order	Perciformes
Suborder	Labroidei
Family	Cichlidae
Genus	Oreochromis
Species	niloticus

1.2.2 Characteristic

Nile tilapia has one nostril on each side of the snout. The body is oblong, moderately deep, compressed. The dorsal and ventral profiles are about equally convex. The caudal peduncle is broadly short. The mouth is slightly oblique and protractile. The dorsal fin is with a long base; spinout dorsal fin with 16-17 spines, followed by 11-15 soft fin rays (Fig. 2). The anal fin base is relatively short, consisting of 3 spines and 8-11 soft fin rays. The posterior part of both fins, especially in adult male fishes, is usually and considerably extended. The pectoral fins are moderately large and pointed, with 15 soft fin rays. The caudal fin is broadly round in adult, but almost truncate in young. The scales are fairly large and cycloid. There are two lateral lines. The upper one extends from the upper corner of operculum vertically to below the origin of soft dorsal fin, with 19-25 scales. The lower portion begins perpendicular and transposes to the middle of caudal peduncle, with 11-18 scales, totally consisting (Nelson, 1984).

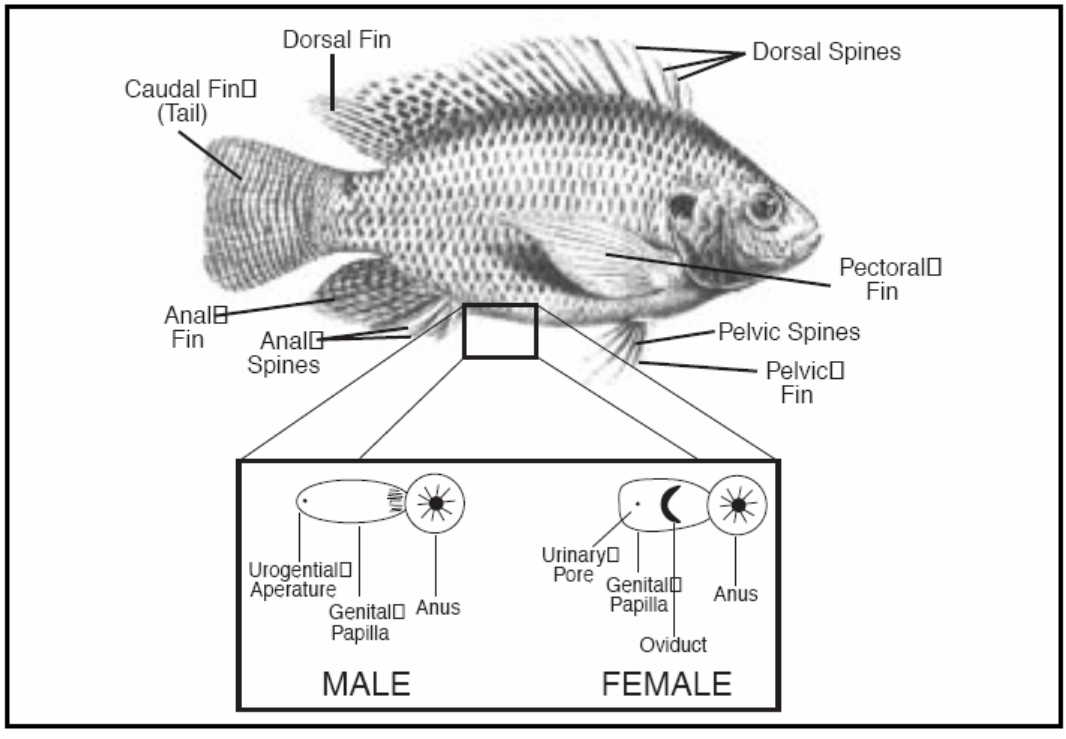


Figure 2. Fins and genital papilla of Nile tilapia (*Oreochromis niloticus*) (Popma and Masser, 1999).

1.2.3 Reproduction

In all *Oreochromis* species the male excavates a nest in the pond bottom and mates with several females. After a short mating ritual the female spawns in the nest, the male fertilizes the eggs, and holds and incubates the eggs in mouth or buccal cavity until they hatch. Fry remain in the female mouth through yolk sac absorption and often seek refuge in her mouth for several days after they begin to feed.

Sexual maturity in tilapia is a function of age, size and environmental conditions. When growth is slow, sexual maturity in Nile tilapia is delayed a month or two but stunted fish may spawn at a weight of less than 20 grams. Under good growing conditions in ponds, the Nile tilapia may reach sexual maturity in as little as 3 months of age, when they seldom weigh more than 60 to 100 grams.

The sex of a 25-gram tilapia fingerling can be determined by examining the genital papilla located immediately behind the anus (Fig. 2). In males the genital papilla has only one opening, the urinary pore of the ureter, through which both milt and urine pass. In females the eggs exit through a separate oviduct and only urine passes through the urinary pore. Placing a drop of dye, methylene blue or food coloring, on the genital region helps to highlight the papilla and its openings (Popma and Masser, 1999).

1.3 Calcium

Calcium was prepared by the Romans in the first century under the name calx, the metal was not discovered until 1808. After learning that Berzelius and Pontin for prepared calcium amalgam by electrolyzing lime in mercury, Davy was able to isolate the impure metal.

Calcium, a metallic element, is fifth in abundance in the earth's crust, of which it forms more than 3%. It is an essential constituent of leaves, bones, teeth, and shells. Never found in nature uncombined, it occurs abundantly as limestone, gypsum, and fluorite. Apatite is the fluorophosphate or chlorophosphate of calcium.

Calcium is a soft grey alkaline earth metal, and is the fifth most abundant element in the Earth's crust. It is essential for living organisms, particularly in cell physiology, and is the most common metal in many animals. Calcium is not naturally

found in its elemental state. Calcium is found mostly in soil systems as limestone, gypsum and fluorite.

Calcium is used as a reducing agent in preparing other metals such as thorium, uranium, zirconium, etc., and is used as a deoxidizer, desulfurizer, or decarburizer for various ferrous and nonferrous alloys. It is also used as an alloying agent for aluminum, beryllium, copper, lead, and magnesium alloys, and serves as a "getter" for residual gases in vacuum tubes, etc.

As mentioned above, the threat of lead contamination in the environment is alarmingly high and usually people do not even realize it until they are affected and start exhibiting adverse effects. Here, will study the effect of calcium on lead treated fish. Recently, a different approach to reduce metal toxicity in fish has been investigated; the protective role of the diet against metal uptake has been studied (Baldisserotto et al., 2004a; 2004b).

Waterborne Ca^{2+} has marked protective effect against waterborne Cd toxicity to brook trout (Carrol et al., 1979), tilapia (Pratap and Wendelaar Bonga, 1993), rainbow trout (Hansen et al., 2002), and zebra fish embryos (Meinelt et al., 2001). The mortality due to the waterborne Cd is reduced with the increase of waterborne calcium ion (Meinelt et al., 2001; Hansen et al., 2002). Waterborne calcium ion and Cd compete for the same transport pathway (Playle et al., 1993), reducing Cd uptake in the gills (Hollis et al., 2000). This explains why elevation of dietary Ca^{2+} protected against Cd accumulation in several fish tissues. Due to Cd and Pb have similar properties, it is claimed that elevated dietary Ca^{2+} levels will be protective in reducing Pb burdens in freshwater fish exposed to environments contaminated with waterborne Pb (Alves and Wood, 2006).

The lack of a method providing a rapid and accurate estimation of sublethal toxicity of heavy metals in aquatic organisms has led many researchers to investigate the physiological and biochemical responses of fish to these toxicants. In fish, the accumulation and effects of heavy metals in organs and tissues depends on the administration route, the concentration, and the length of time of exposure to contaminants. Moreover, the evaluation of toxic effects and pathological responses is related to physiological and functional aspects of the tissues as liver, kidney, and blood. Knowledge of these responses can provide valuable information on sublethal

inhibition of reproduction, growth, and behavior that could lead to the disappearance of a population without evidence of direct mortality (Hodson et al., 1977).

In this experiment will emphasize on dietary calcium supplemented using the inorganic calcium carbonate inhibits lead uptake in Nile tilapia. The result of this experiment will prove whether or not calcium will take the action in fish treated with lead. If calcium carbonate helps reducing lead toxic in fish, we will be able to find further solutions to improve aquatic life and reducing toxic that can be passed from food to man.

CHAPTER II
LITERATURE REVIEW

Heavy metals are serious pollutants of the aquatic environment because of their environment persistence and ability to be concentrated by aquatic organism. Several studies have been conducted to determine the effect of varying levels of heavy metals toxicity in water on fish tissues (Dugo et al., 2006; Pyle et al., 2005; Agusa et al., 2004). The testing environment could be either artificially controlled or simulated from the natural environment.

The concentrations of Pb in gill, liver, kidney, stomach and intestine of Arctic char, *Salvelinus alpinus* from two oligotropic high mountain lakes in Northern Tyrol, Austria during winter and summer were investigated to elucidate pathway for Pb uptake. The experiment showed in fish from both lake Pb concentrations of stomach and gut tissues correlated positively with Pb burden of their contents indicating dietary Pb uptake as a significant source of Pb load. However, in both lakes Pb concentrations of stomach and intestine were significantly lower in the ice-free period than during the ice-covered period cause by in this period diet becomes an increasingly important additional source of Pb contamination (Köck et al., 1998).

Cadmium and lead were determined in different tissues such as muscle, gill, stomach, intestine, liver, vertebral column and scales, of *Tilapia nilotica* from the High Dam Lake, Aswan in Egypt. The results showed that cadmium and lead concentrations were higher in fish scales and vertebral column than in the other parts of the fish. The fish muscles in this study were in the safety baseline levels for man consumption (Rashed, 2001).

Gale and co-workers considered lead concentration in fish and river sediments in the Old Lead Belt of Missouri, they found the sediment had significantly elevated Pb concentrations compared with control sites upstream from the mining activity and whole body Pb concentrations in small sunfish were also determined and correlated strongly with sediment concentrations (Gale et al., 2002).

Mahmood studied the effects of lead and nickel on the fish, *Catla catla, Labeo rohita* and *Cirrhina mrigala* in the Ravi River in Punjab province, Pakistan. According to the study, lead accumulation was higher in fish gills and liver than in kidney and muscle. Metal concentrations in water were significantly correlated with the temperature. However, lead in water showed positively non-significant regression on water pH (Mahmood, 2003).

Concentrations of cadmium, mercury, and lead were determined in sediment and fish tissue from several locations in Alaska and California. Tissue i.e., liver, muscle, gill, and stomach contents from white croaker (*Genyonemus lineatus*), English sole (*Pleronectes vetulus*) and flathead sole (*Hippoglossoides elassodon*) were analyzed. The result showed Cd in fish liver exhibited a negative correlation with sediment concentrations. No such correlations were found for Hg and Pb when fish liver and sediment were compared; however, these metals did exhibit a positive relationship between liver and organic carbon normalized sediment concentration. These results suggested that the enhanced bioavilability of the metal at some uncontaminated sites was the main determinate for the inverse correlation between metal and organic contamination in tissue (Meador et al., 2005).

Black scabbard fish, *Aphanopus carbo* caught off Madeira and the Azores in Portugal were determined the concentration of Hg, Ca and Pb in liver, muscle and skin. The results showed mean metal levels from both area was higher in liver than muscle and skin. In general, the results obtained did not allow the establishment of any relationship between metal accumulation and fish weight and length (Afonso et al., 2007).

Alves and co-workers conducted an experiment concerning lead accumulation in juvenile freshwater rainbow trout (*Oncorhynchus mykiss*). Lead accumulation was measured in the gills, intestine, liver and kidney. The results showed that the highest Pb burdens in intestine, liver, and kidney on day 21, unlike gills which had the highest lead accumulation on the first 7 days. Their experiment showed that gills are the primary site of lead toxicity, and toxicity may also occur through gastrointestinal tract (Alves et al., 2006).

Disturbance of living processes at the molecular and subcellular levels of biological organization by xenobiotics can lead to cell injury, resulting in degenerative

and neoplastic diseases in target organs (Pacheco and Santos, 2002). Therefore, histopathological effects of heavy metals are biomarkers of toxicity in fish organs are useful indicator of environmental pollution. Handy and colleagues demonstrated the utility and sensitivity of histopathological biomarkers by recording lesions to the gill, liver, and spleen in three spined sticklebacks (*Gasterosteus aculeatus*) (Handy et al., 2002). Schwaiger and his partners were quantified using semiquantitive as well as stereological or morphometric procedures to compare the severity of lesions in fish exposed to more polluted stream (Schwaiger et al., 1997).

There are several reports on the impact of environmental toxicants on fish revealed by histopathological studies of vital organs. *Carassius auratus* were exposed to mixtures of constant free lead and particulate lead (absorbed on gibbsite particles) at varied concentrations, 0, 0.4, 0.8, 1.2, 2.0, and 3.0 mg/L. The concentrations of lead in the gill and intestines of fish were determined after exposure. The examination results revealed that lead accumulation on the gill increased with increased particulate lead concentration in ambient water indicating the bio-availability of lead via gill. Gibbsite particles were also observed on gill surface after exposure. The results suggest that there was a two step process involved in the uptake of particulate lead by fish gill: (1) adherence of the particles on the gill surface where mucus was attached; and (2) adsorption of lead from the particles under condition of the gill microenvironment (Tao et al, 1999).

Trahira, *Hoplias malabaricus*, used to investigate the effects of successive Pb(II) or tributyltin doses, individuals were exposed to 21 µg/g Pb or 0.3 µg/g TBT for 5 day intervals. A number of morphological effects were observed in liver, including cytoskeleton disturbance, microautophagy of mitochondria, nuclear damage, and cell death. In kidney, necrosis area, increasing of the neutrophils cell number, changes in melanomacrophage centers, and free macrophages were frequently registered after both Pb(II) and tributyltin exposures (Rabitto et al, 2005).

The African catfish, *Clarias gariepinus*, has also been subject to study in lead toxicity studies. The gills and liver of fish showed the degree of distortion proportional to exposure periods and concentration of the metals was found to be dose and time dependant. The demonstrating of histopathological results epithelial uplifting in gills and distortion of lamellar, while in liver there was hepatic cirrhosis and fibrosis

as well as congestion of blood circulation (Olojo et al., 2005). The histopathological results were similar as in other study (Martinez et al, 2004).

Peebua and co-workers studied on Nile tilapia, *Oreochromis niloticus* exposed for one month to sediments from the Mae Klong River, Samutsongkram province, South West of Thailand, which contained elevated levels of heavy metals, lead and chromium, developed abnormalities of the gills, liver and kidney. In the gill filaments, cell proliferation, lamellar cell hyperplasia, and lamellar fusion were observed. In the liver, there was vacuolation of hepatocytes and nuclear pyknosis, Kidney lesions consisted of dilation of Bowman's space and accumulation of hyaline droplets in the tubular epithelial cell. No recognizable changes were observed in muscle tissue. Despite these histopathological changes, no firm correlation between levels of heavy metals in sediments and those in fish tissues could be established (Peebua et al., 2005).

Most of the metal is accumulated in the blood cells and many of the toxic effects correlate with its circulating concentrations. The main target organs of lead are the nervous, renal and hematopoietic systems (WHO, 1995). Therefore, hematological indices are gaining general acceptance as valuable tools in monitoring various aspects the health of fish exposed to contaminants. These adverse effects have been described not only in human but also in a number of other vertebrates such as adult South toad, *Bufo arenarum* exposed to sublethal Pb for 6 weeks. The number of red blood cells showed a tendency to decrease, whereas the number of white blood cells increased significantly (Chiesa et al., 2006).

In fish, the study about acute morphological and physiological effects of lead on *Prochilodus lineatus* or ray finned fish at 96 hrs period. The results showed there was no significant alteration in hematocrit (Martinez et al, 2004). In contrast, the effect of lead in *Barbus conchonius* after 30 and 60 days exposure, they were observed decrease in erythrocytes counts, hematocrit and hemoglobin concentration (Tewari et al., 1987; Gill et al., 1991). Similarly in *Cirrhinus mrigala* have shown hemoglobin content, red blood cell count, hematocrit, red blood cell indices mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) were significantly reduced. The adverse effect of lead was higher after long term exposure when compared to the short term (Kumar et al., 2005).

Some effects of methyl mercury, inorganic lead, and tributyltin in a tropical fish species *Hoplias malabaricus* over 70 days, evaluate hematological effects of metals on erythrocytes, total leukocytes and differential leukocytes counts, hematocrit, hemoglobin concentration, MCV, MCH, and mean corpuscular hemoglobin concentration (MCHC). Transmission electron microscopy and image analysis of erythrocytes were also used to investigate some morphometric parameters. Results showed no significant effects in MCH and MCHC for all tested metals, but differences were found in erythrocytes, hemoglobin, hematocrit, MCV, and white blood cells counts, image analysis revealed differences in area, elongation, and roundness of erythrocytes from individuals exposed to Pb^{2+} and tributyltin (Oliveira et al., 2006).

Santos and Hall found differential white blood cells count increased in number of lymphocytes in yellow eel (*Anguilla anguilla*) when exposed to an inorganic lead concentration of 300 µg/L for 30 days (Santos and Hall, 1990).

Tinca tinca were exposed to lethal and sublethal treatments with mercury, cadmium and lead for acute and chronic periods to study alterations in immunological parameters. Higher acute sublethal exposures caused a significant increase in total WBC count in all 3 treatments, and after 3 weeks exposure resulted in significant increase in total WBC count in the Hg and Pb treatments and a significant decrease in the Cd treatment (Shah, 2004).

Genotoxicity studies on toxic metals and their organic compounds are very important, especially so in the investigation of the effects of these compounds on the aquatic environments where they tend to accumulate. The detection of micronucleus (MN) and nuclear abnormalities (NA) in fish help to know the presence or absence of genotoxin in water. The study of Edwards et al. (2001) showed a non-specific biomarker of genotoxicity, micronuclei in erythrocytes, is potentially useful as monitoring technique in fish species to evaluate their exposure and genotoxic responses to pollutants in South Australian water (Edwards et al., 2001). Micronucleus analysis was performed in peripheral blood erythrocytes and gill cells of grey mullet (*Mugil cephalus*) to monitoring of genotoxic pollutant in the marine environment (Çavas and Gözükara, 2004). Not only that Arkhichuk and Garanko used fin cells to

determine cytotoxic and genotoxic effects of organic and inorganic substances. (Arkhichuk and Garanko, 2005).

Talapatra and Banerjee investigated the frequencies of nuclear abnormalities such as necrotic cells, apoptotic cells, notch nucleated cells and binucleated cells were also counted separately for gill and kidney erythrocytes of *Labeo bata* in the sewage fed fish farms, in which significantly increased values comparison to control. There genotoxicity results the sewage fed ponds contain genotoxic metal such as Cr, Zn, Cu, Pb, Mn, and Fe (Talapatra and Banerjee, 2007).

The study of micronucleus and abnormal nuclear morphology in *Hoplias malabaricus* by exposed to 21 mg/g of body weight of inorganic lead for two months the micronucleus assay showing morphological alterations of the nucleus (Ferraro et al, 2004). Generally, MN is formed by the condensation of whole chromosomes or fragmented chromosomes that are not incorporated into the main nucleus during mitosis due to aneugenic or clastogenic effects (Al-Sabti and Metcalfe, 1995). In case of abnormal nucleus, apoptotic cells are formed by actively dying cells that include cell shrinkage, membrane blebbing and chromatin condensation (Murakawa et al, 2001).

Polluted water habitants exert extensive stress impacts upon aquatic animals. Changes brought about by a stressor could be metabolic in nature, affecting molecular and cellular components such as enzymes (Barton and Iwama, 1991). Blood chemistry has long been a helpful diagnostic tool to prove after functional damage to tissues or organs of fish. The study of Hamnn et al., (1999) and Adham (2002) all showed in response, functional damage of the heart and liver in polluted fish was pointed out in view of the elevated serum enzymes such as alanine and aspartate aminotransferase (Hamnn et al, 1999; Adham, 2002).

Adham and colleague investigated blood chemical of Nile tilapia, *O. niloticus* under the impact of water pollution. The study found serum enzyme increased in alanine aminotransferase, lactate dehydrogenase, creatine kinase, alkaline phosphatate and cholinesterase, whereas, aspartate aminotransferase was suppressed in fish caught from water with the highest metal concentration (Adham et al, 2002).

Anabas scandens was exposed to subletal concentration of 10 ppm of lead nitrate for 15 days. The result showed total protein, free amino acid decreased,

whereas aspartate and alanine aminotransferase and glutamate dehydrogenate in gill and brain tissue were elevated during the expose period (Chandravathy and Reddy, 1994).

Cyprinus carpio were exposed to two non-essential, Hg and Pb, and two essential, Cu and Ni, heavy metal salts at lethal and sublethal concentrations. Blood serum total protein, serum globulin and serum albumin was analyzed every 2 hr for 24 hrs and again at 48 and 72 hrs. Serum protein and globulin levels showed an initial sharp increase from 2 to 20 hrs, followed by declines that extend over a period of 72 hrs. Serum albumin decline from 2 to 20 hrs and extend over a period of 72 hrs (Gopal et al, 1995).

Waterborne Pb causes the disruption of Na^+, Cl^- and Ca^{2+} regulation during acute exposure, the induction of spinal deformities and black tails during chronic exposure and disruption in hemoglobin synthesis during both types of exposure (Rogers and Wood, 2004).

The study of Alves et al., (2006), Mount et al., (1994), and Crespo et al., (1986) all found that dietary Pb accumulates in the whole body and in a number of internal tissues when present in the diet. In the study of Alves and Wood (2006), the impact of elevated dietary Ca^{2+} on the response to chronic dietary of Pb exposure in juvenile rainbow trout have revealed many interesting results. They claimed that simultaneous addition of Ca^{2+} to the diet had an over all protective effect in all the tissue analyzed in reducing Pb accumulation. They have also concluded that elevated dietary Ca^{2+} levels will be protective in reducing Pb burdens in freshwater juvenile rainbow trout exposed to environments contaminated with waterborne Pb. Many other studies have also found similar result. Generally, divalent metals such as Pb, Cd, and Zn are considered Ca^{2+} antagonists. Waterborne Ca^{2+} has marked protective effect against waterborne Cd toxicity to brook trout (Carrol et al., 1979), tilapia (Pratap and Wendelaar Bonga, 1993), rainbow trout (Hansen et al., 2002), and zebra fish embryos (Meinelt et al., 2001). The mortality due to the waterborne Cd is reduced with the increase of waterborne calcium ion (Meinelt et al., 2001; Hansen et al., 2002). Waterborne calcium ion and Cd compete for the same transport pathway (Playle et al., 1993), reducing Cd uptake in the gills (Hollis et al., 2000). In an early study, coho salmon (*Oncorhynchus kisutch*) force-fed gelatin capsules containing 8.4 mg of

calcium chloride ($CaCl_2$) and the exposed to 1,300 µg/L of waterborne Pb for 168 hour, had reduced Pb tissue burdens (Varanasi and Gmur, 1978). Similarly several more recent studies (Zohouri et al., 2001; Baldisserotto et al., 2004a, 2004b, 2005; Flankin et al., 2005) have shown that dietary Ca^{2+} (as $CaCl_2$ or $CaCO_3$) is protective against the uptake of waterborne Zn (Niyogi and Wood, 2006).

CHAPTER II
OBJECTIVES

The objectives of this study were:

1 To investigate the acute toxicity of lead to Nile tilapia (*Oreochromis niloticus*) in term of the median lethal concentration.

2 To evaluate the toxicity of lead and the efficiency of calcium supplementary dietary reduces the toxicity of sublethal exposure of lead to Nile tilapia via

- 2.1 Hematological analysis i.e., hematocrit, hemoglobin, red blood cell count, white blood cell count and red cell indicies.
- 2.2 Biochemical analysis i.e., the activities of aspartate and alanine aminotransferase, and level of total protein.
- 2.3 Nuclear morphology analysis i.e., micronuclei (MN) and nuclear abnormality (NA) in erythrocyte, gills, liver, kidney and fin cells.
- 2.4 Histopathological analysis of gills, liver, kidney, spleen, reproductive organ, and muscle using light microscopy.

CHAPTER IV
MATERIALS AND MATHODS

This study was performed at the Department of Pathobiology, Faculty of Science, Mahidol University, Bangkok, Thailand (Fig. 3).

4.1 Fish

Oreochromis niloticus (n=430) with mean body weight of 10-15 g. and total length 8-10 cm. They were bought from the commercial fish farm in Chonburi Province, Thailand.

4.2 Maintenance of fish

All fish were acclimatized under laboratory conditions for a minimum period of 7 days prior to experiments. Before performing the experiment, the fish were observed for signs of disease, stress, physical damage, and mortality. The dead and abnormal individuals were removed for accuracy of the experiment. If mortality rate exceeded 10%, the entire stock was discarded. A 12 hours light and 12 hours dark photoperiod was given. Fish were fed twice a day with 28%-protein, 3%-fat, and 4%-fibre commercial fish food (Charoen Pokphand Group, Bangkok, Thailand). The quantity of food was 2% of the initial body weight per day.

Figure 3. Flow-through fish aquarium at the Department of Pathobiology, Faculty of Science, Mahidol University**.**

4.3 Water conditions

The physiochemical characteristics of water were measured daily, according to the experimental procedures described in Standard Methods for the Examination of Water and Wastewater (APHA, 2005). The pH was measured with a Hanna microprocessor pH/mV/^{o}C meter model 8417 and the temperature was measured with a glass mercury thermometer. Dissolved oxygen was supplied by a diffused air system. A 12:12 hour light-dark cycle was maintained throughout.

4.4Chemicals

$Pb(NO_3)_2$ (CAS No. 10099-74-8), $CaCO_3$ (CAS No. 1317-65-3) were manufactured by Sigma, Germany. $Pb(NO_3)_2$ was directly diluted in water to obtain the desire exposure concentrations and $CaCO_3$ mixing with commercial fish food.

4.5 Diet preparation

Calcium supplemented diets were prepared by mixing 20 and 60 mg/g calcium carbonate with commercial fish food. The fish food was ground in a blender, followed by hydration with approximately 75% v/w deionized water and added to the food paste. The resulting paste was mixed well and put it into a pasta maker then breaks the food paste into small pallets by hands. It was air dried in 60^{o}C 3 hour hot air oven (Zohouri et al., 2001). The control diet was prepared by the same method but with the addition of deionized water only.

4.6 Experimental design

4.6.1 Acute toxicity tests

The acute toxicity test was made to determine LC_{50} and to estimate toxicant concentrations for chronic toxicity test. The acute toxicity tests were static non-renewal technique. Fish of similar size were selected. The length of the longest fish should not be more than 1.5 times the length of the shortest fish. Fish were randomly put into each tank (60x30x40 cm). The experimental chambers were filled with 54 L dechlorinated tap water with or without the testing solutions.

The fish were not fed 48 hours prior to the experiment and 96 hours during the experiment. Observations at 3 hours, 6 hours, and every 24 hours after the beginning of the test were desirable. The conditions and visible abnormalities should be recorded such as, loss of equilibrium, swimming behavior, erratic swimming, loss of reflex, increased excitability, and changes in appearance or physiology such as discoloration, excessive mucous production, hyperventilation, opaque eyes, curved spine, or area of hemorrhages. Fish were considered dead if there was no visible movement (e.g. gill movements) and if touching of the caudal peduncle produced no reaction. Dead fish were removed when discovered to prevent fouling of the water. Abnormalities and mortalities were recorded every 24 h. The LC_{50} and 95% confidence limits were computed using the probit analysis computer program (Finney, 1971).

4.6.1.1 Range-finding test (OECD, 1992)

The range-finding test was the preliminary test that was designed to establish the approximate concentration range of a chemical that should be used in the definitive test. The tests were consisted of a control and a down-scaled series of widely spaced sample dilution. Three replicates were used per group, with ten fish in each replicate. The test concentrations were composed of five concentration levels based on a logarithmic ratio having concentrations a factor of ten from each other. The levels include concentrations that would kill all organisms and others that would kill very few or no organisms. The numbers of mortal fish of range-finding tests were recorded.

4.6.1.2 Definitive test (OECD, 1992)

The goal of the definitive test was to determine the concentration-response curves for fish mortality, the LC_{50} and the 95 percent confidence intervals for each species tested at 24 h, 48 h, 72 h, and 96 h in a static system. The tests were consisted of a control and at least five concentration levels based on a geometric series, three replicates per group, with ten fish in each replicate. The numbers of dead fish of definitive tests were recorded.

4.6.2. Efficacy of dietary calcium in chronic treatment

Fish (n=60) were randomly assigned to six equally sized groups as follows: (1) Control: control group and provided with mormal food; (1) normal diet and 25% LC_{50} waterborne lead ($Pb(NO_3)_2$); (2) low Ca-supplemented diet (20 mg Ca/g) and normal water; (3) high Ca-supplemented diet (60 mg Ca/g) and normal water; (4) low Ca-supplemented diet and 25% LC_{50} waterborne lead; and (5) high Ca-supplemented diet and 25% LC_{50} waterborne lead.

The fish were kept separately in the glass flow-through aquaria (50x50x120 cm) with continuous aeration were filled with 200L of dechlorinated tap water whose physicochemical characteristics were the same as those described previously.

4.6.3. The process of tissue and blood collection

After 30 days of treatment, 7 fish of each group were anesthetized with 0.2 g/L MS-222 (tricaine methan sulphonate, Sigma, Germany, CAS No.886-86-2), weighed and measured (Fig. 4).

A B

Figure 4. Fish are measured the length (A) and weight (B)

Peripheral blood samples were obtained by caudal vein puncture for hematological and biochemical studies (Fig.5).

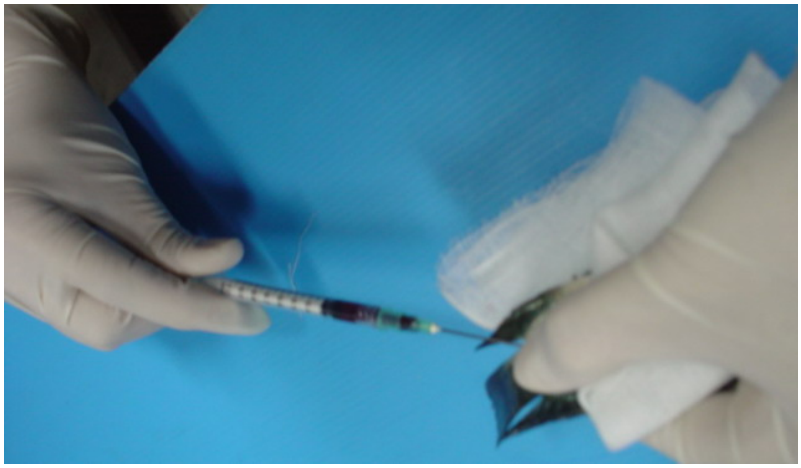


Figure 5. Blood collection

The organs (gills, liver, kidney, spleen, intestine, ovary, testis, muscle and fin cells) (Fig.6) were removed and prepared for histopathological studies and micronuclei (MN) and nuclear abnormality (NA) tests. Each organ was weighed for the determination of the organ index [the percentage of organ weight (mg) per body weight (mg)]

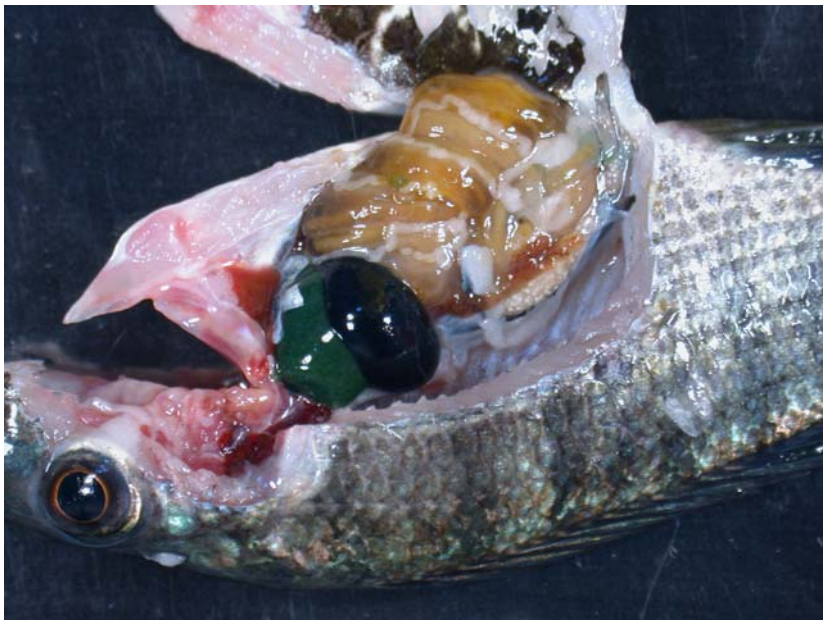


Figure 6. Internal organs including reproductive organ and spleen

4.6.4. Histopathological studies

4.6.4.1 Organ handling

Internal organs (gills, liver, kidney, spleen, intestine, ovary, testis, and muscle) were removed. Slide off a ventral surface in order to collect the internal organs, such as liver, intestine, reproductive organ and kidney. All organs were removed and washed with normal saline solution (0.85% NaCl) (Merck k15123600, Germany). Normal saline was an isotonic solution, which would not disturb an original ion concentration of organs, unlike hypotonic and hypertonic. Organs must measure by using weighing machine (Sartorius BP 3100 S, Minneapolis, U.S.A) to determine the organs index. After that, the procedure was followed by fixing individual organs in a 10% buffered formalin solution, prepared for histopathological study.

4.6.4.2 Specimen preparation for light microscopic study

The organs were fixed in 10% buffered formaldehyde for 24h, washed with 70% ethanol, dehydrated with a series of ethanol and cleared with xylene solutions.

The processes of light microscopy were followed by the Department of Pathobiology, Faculty of Science, Mahidol University (Appendix). The organs were embedded in a block using paraffin at the embedding machine (Axel Johnson Lab System, U.S.A) (Fig. 7).

Figure 7. Embedding machine (Axel Johnson Lab System, U.S.A)

After embedded in paraffin the organs would be sectioned at 4 µm using rotary microtome (HistoSTAT, Reichert, U.S.A) (Fig.8).

Figure 8. Rotary microtome (HistoSTAT, Reichert, U.S.A)

The slides were stained with hematoxylin and eosin. They were examined for abnomalities using a Nikon E600 light microscope (Fig. 9) and photographed by a Nikon DXM 1200digital camera (Tokyo, Japan) (Humason, 1972).

Figure 9. Nikon E600 light microscope

4.6.4.3 Semiquantitative scoring

Histopathological alterations were evaluated semi-quantitatively by ranking tissue lesion severity. Ranking from – to + + + depending on the degree and extent of the alteration as follows: (-) no pathological alteration; (+) mild; (+ +) moderate and (+ + +) severe pathological alterations. These ranking were used by Schwaiger et al. (1997) to establish an overall duassessment value of the histopathological lesion for each indiviual fish gill. Ten slides were observed for each treatment.

4.6.5 Hematological studies

Hematological parameters such as hematocrit, hemoglobin, white blood cell count, and white blood cell differential were determined as follows (Dacie and Lewis, 1991).

4.6.5.1 Hematocrit

Hct was the volume of erythrocytes expressed as a percentage of the volume of whole blood in a sample. The microhematocrit method was as following:

1. Filled two-thirds of heparinized hematocrit tube (Vitrex 0705427, Herlev, Denmark) with EDTA-blood and sealed one end of the tube with clay.
2. Placed the filled tube in the hematocrit centrifuge machine (BOECO, Germany), centrifuged at 10,000 to 12,000rpm for 5 minutes.

3. Placed the tube in the microhematocrit reader. Read the hematocrit by following the manufacturer's instruction on the microhematocrit reading device.

4.6.5.2 Hemoglobin

Hemoglobin was estimated by Drabkin's cyanmethemoglobin method. Drabkin's solution containing potassium ferricyanide, potassium cyanide and sodium bicarbonate was prepared. Twenty microliters of blood was added to 5mL of Drabkin's solution. Readings were taken at 530nm in a spectrophotometer. Hemoglobin values were calculated from a hemoglobin curve prepared using hemoglobin standard.

4.6.5.3 White blood cell count

The total white cell count determined the number of white cells per cubic millimeter of blood. The procedures were as following:

1. Draw anticoagulated venous blood to the 0.5mark on the white cell pipette.
2. Draw Natt and Herrick's solution to the 11.0mark on the white cell pipette. Shaked the pipette for 3minutes.
3. Load in the hemocytometer counting chamber (Precicolor, HBG, Germany) (Fig. 10), counted the white cells within each of the four large corner fields.
4. Multiply the count by 50 for the total white cell count.

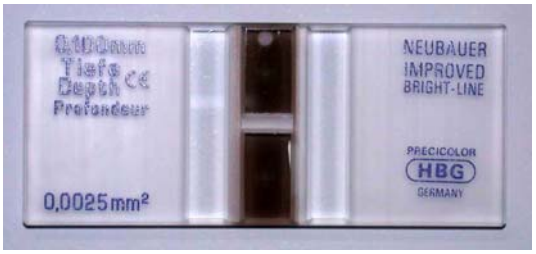


Figure 10. Hemocytometer counting chamber

4.6.5.4 Technique for making smears

Touched the drop of blood lightly with a clean, grease-free slide. Placed the slide on a flat surface with the drop of blood up. Hold a second slide between thumb and forefinger and placed the edge at a 45-degree angle against the top of the slide, holding the drop of blood. Back the second slide down until it touched the drop of

blood. The blood would distribute itself along the edge of the slide in a formed angle. Push the second slide along the surface of the other slide, drawing the blood across the surface in a thin, even smear. If this was done with uniform rapidity and without wobbling the slide, a good smear would result. Tried to keep the blood from reaching the extreme edges of the slides. Large cells had a tendency to stack up on the perimeter of the smear, and let the smear reach the edges of the slide will aggravate this tendency. The smear should show no wavy lines or blank spots. Let the smear air-dry.

4.6.5.5 Technique for staining smears

Placed the smear on a staining rack. Flood it with about 1mL of Wright's stain and allowed it to stand for 2 minutes. Added an equal quantity of buffer. There should be no run-off of fluid from the slide. Mixed the buffer and stained by blowing air through the rubber pipette tube and directing the current of air about the surface of the slide. Mixed until a metallic (copper looking) film appears. Let stood for 3 minutes. Washed with tap water, provided pH testing had shown the water to be neutral. If the tap water was not neutral, washed the slide with distilled water. The stain and the metallic film must be floated off to prevent streaking, so kept the slide flat and horizontal in the stream of water. If the slide was tilted, the metallic film would settle to the surface of the smear and remained there. Wiped the stain from the bottom of the slide. Let it air-dry. A good smear should be thin and evenly distributed, and it must be dry before staining.

4.6.6 Biochemical studies

Serum was determined for the enzyme activity as follows:

4.6.6.1 Aspartate aminotransferase (AST)

The enzymatic activity of AST was measured by the oxaloacetate formed in the reaction (Reitman and Frankel, 1957):

$$\text{alpha-ketoglutarate} + \text{L-aspartate} \xrightarrow{\text{AST}} \text{glutamate} + \text{oxaloacetate}$$

Oxaloacetate was measured colorimetrically as its hydrazone after the reaction with 2,4 – dinitrophenylhydrazine at 505nm. The procedures for the analysis of glutamic oxaloacetic transaminase activity

1. Aspartate transaminase substrate 0.25mL were pipetted into a 12 x 75mm cuvette and warmed for 5min in a water bath at 37°C.

2. Serum 0.05mL was added, mixed and covered with parafilm.

3. The mixture was incubated at 37°C for 60min.

4. Color reagent 0.25mL was added and shaken gently.

5. The tube was allowed to stand at room temperature for 20min.

6. The 2.5mL of 0.4mol/L NaOH was added and mixed.

7. Exactly 5min later, the optical density of the unknown was read with water as a blank at 505nm.

8. The optical density for the value of oxaloacetic transaminase was referred to the calibration curve.

4.6.6.2 Alanine aminotransferase (ALT)

The enzymatic activity of ALT was measured by the pyruvate produced in the reaction (Reitman and Frankel, 1957):

$$\text{alpha-ketoglutarate} + \text{L-alanine} \xrightarrow{\text{ALT}} \text{glutamate} + \text{pyruvate}$$

Pyruvate formed was measured colorimetrically as its hydrazone after the reaction with 2, 4-dinitrophenylhydrazine at 505nm. The procedures for the analysis of glutamic pyruvic transaminase activity were performed similar to those of oxaloacetic transaminase, substituting with the alanine transaminase substrate, and incubated at 37°C for 30min.

4.6.6.3 Total protein (Biuret method)

Proteins form a purple colored complex with cupric ions in alkaline solution. The intensity of the purple color was measured at 540 nm (Reinhold, 1953). Plasma 0.01 mL was added to 4.0 mL total protein color reagent. The solution was

mixed and the optical density values were record within 30 min at the wavelength of 540 nm by using 4.0 mL color reagent blank to set zero.

The optical density was read at 540nm, and the value is calculated by

Total protein (g/dL) = (Abs. Sample / Abs. Stand.) x conc. Stand. (10g/dL).

4.6.7 Micronuclei (MN) and nuclear abnormality (NA) studies

Peripheral blood samples was smeared immediately on clean grease free microscope slides, air dried for 12 h, and then fixed in absolute ethanol for 20 min. Each slide was stained with 5% Giemsa solution for 30 min.

Gill arches were removed and placed into vials containing Carnoy's fixative (3:1 methanol:acetic acid). Gills were transferred into 20% acetic acid solution for 15 min for tissue maceration. After this chemical maceration, epithelial cells were then scraped off the gills and placed on clean slides. After tissue clumps on slides were removed, slides were air-dried and stained with 5% Giemsa solution for 30 min.

A portion of liver and kidney tissues were removed and placed into vials containing Carnoy's fixative. Small pieces of tissues were transferred into vials containing 45% acetic acid solution for 30 min for tissue maceration. After maceration, tissues were gently minced and filtered to obtain a cell suspension. The obtained cells were smeared on a clean slide, air-dried and stained with 5% Giemsa solution for 30 min.

The edge of caudal fins was cut at a depth of 2-3 mm and placed into vials containing Carnoy's fixative. Small pieces of tissue were transferred into vials containing 45% acetic acid solution for 30 min for tissue maceration. Then tissues were gently minced and filtered to obtain a cell suspension. The obtained cells were smeared on a clean slide, air-dried and stained with 5% Giemsa solution for 30 min.

From each slide 1000 cells were scored under 1000× magnification using a Nikon E600 light microscope and photographed using a Nikon DXM 1200 digital camera. The slides were scored by a single observer using blind review. Frequencies of micronucleated (MN) and nuclear abnormality (NA) cells were expressed per 1000 cells (%).

4.6.7.1 Micronuclei and nuclear abnormality cells scoring

Only the cells clearly isolated from the surrounding cells were scored. The criteria for the identification of MN were earlier described: (a) MN must be smaller than one-third of the main nuclei, (b) MN must be clearly separated from the main nuclei, (c) MN must be on the same plane of focus, and have the same color. Cells having two nuclei with approximately equal sizes were considered as binucleates (Fenech et al., 2003). Nuclear abnormality shapes were scored into one of the following categories: blebbed nuclei (BL), lobed nuclei (LB), notched nuclei (NT), and binuclei (BN) (Carrasco et al., 1990). The result was expressed as the mean value (%) of the sum for all the individual abnormality observed

.

4.7 Statistical analysis

Data were analyzed using SPSS 12.0 for Windows software (SPSS, Chicago, IL). All data were expressed as mean values $\pm$ S.D. A two-way analysis of variance (ANOVA) was performed separately for each time, and separately tested in each group. Scheffe post hoc test for multiple comparisons was used for determination of significant differences between the control and treated groups. The level of statistical significance was set at the probability level of 0.05.

CHAPTER V
RESULTS

5.1 Acute toxicity test

5.1.1 Fish characteristic

No mortality was detected during the acclimatization. The characteristic of fish in the experiment are showed in Table 2.

Table 2. The characteristic of fish in the experiment.

Fish	Total Number	Weight (g) (mean ± S.D.)	Length (cm) (mean ± S.D.)
Nile tilapia *(O. niloticus)*	430	16.71 ± 2.78	9.81 ± 0.42

5.1.2 Water quality

Parameters of water quality among the experiment were in the same range throughout this test. They were presented in Table 3.

Table 3. The physical water quality in the experiment.

Parameters	Range
pH	6.5-7.8
Temperature	25-28 ºC

5.1.3 Acute toxicity tests of $Pb(NO_3)_2$ on Nile tilapia (*O. niloticus*)

5.1.3.1 Range-finding test

The tests consisted of 0, 80, 160, 240, and 320 mg/L concentration of $Pb(NO_3)_2$. The mortal fish was not found in control and 80 mg/L concentration of $Pb(NO_3)_2$. The 240 and 320 mg/L concentration of $Pb(NO_3)_2$ be killed all of fish wish in 24h. The mortality percentages are showed in the Table 4.

Table 4. Mortality percentage of Nile Tilapia (*O. niloticus*) at the concentration range of 0-320 mg/L of $Pb(NO_3)_2$ over 96 hours.

Conc.	Number of fish	Number of replica-tion	Percent mortality											
			24-h			48-h			72-h			96-h		
			No of dead	Death (%)	Mean death (%)	No of dead	Death (%)	Mean death (%)	No of dead	Death (%)	Mean death (%)	No of dead	Death (%)	Mean death (%)
Control	10	1	0	0	0	0	0	0	0	0	0	0	0	0
	10	2	0	0		0	0		0	0		0	0	
	10	3	0	0		0	0		0	0		0	0	
80 mg/L	10	1	0	0	0	0	0	0	0	0	0	0	0	0
	10	2	0	0		0	0		0	0		0	0	
	10	3	0	0		0	0		0	0		0	0	
160 mg/L	10	1	1	10	10	0	0	0	0	0	0	0	0	0
	10	2	0	0		0	0		0	0		0	0	
	10	3	0	0		0	0		0	0		0	0	
240 mg/L	10	1	10	100	100	10	100	100	10	100	100	10	100	100
	10	2	10	100		10	100		10	100		10	100	
	10	3	10	100		10	100		10	100		10	100	
320 mg/L	10	1	10	100	100	10	100	100	10	100	100	10	100	100
	10	2	10	100		10	100		10	100		10	100	
	10	3	10	100		10	100		10	100		10	100	

5.1.3.2 Definitive test

The tests included 0, 172, 174, 176, 178, and 190 mg/L concentration of $Pb(NO_3)_2$ from this tests was not found the death of fish occurred in the control group. The mortality percentages are showed in the Table 5.

Table 5. Mortality percentage of Nile Tilapia (*O. niloticus*) at the concentration range of 0-190 mg/L of $Pb(NO_3)_2$ over 96 hours.

Conc.	Number of fish	Number of replication	Percent mortality											
			24-h			48-h			72-h			96-h		
			No of dead	Death (%)	Mean death (%)	No of dead	Death (%)	Mean death (%)	No of dead	Death (%)	Mean death (%)	No of dead	Death (%)	Mean death (%)
Control	10	1	0	0	0	0	0	0	0	0	0	0	0	0
	10	2	0	0		0	0		0	0		0	0	
	10	3	0	0		0	0		0	0		0	0	
172 mg/L	10	1	1	10	10.0	1	10	13.3	2	20	20.0	3	30	23.3
	10	2	1	10		1	10		1	10		1	10	
	10	3	1	10		2	20		3	30		3	30	
174 mg/L	10	1	1	10	16.7	1	10	26.7	1	10	30.0	1	10	30.0
	10	2	2	20		3	30		3	30		3	30	
	10	3	2	20		4	40		5	50		5	50	
176 mg/L	10	1	1	10	13.3	1	10	16.7	2	20	30.0	2	20	30.0
	10	2	2	20		3	30		4	40		4	40	
	10	3	1	10		1	10		3	30		3	30	
178 mg/L	10	1	2	20	10.0	2	20	20.0	3	30	26.7	3	30	30.0
	10	2	0	0		2	20		2	20		2	20	
	10	3	1	10		2	20		3	30		4	40	
190 mg/L	10	1	2	20	20.0	4	40	40.0	7	70	70.0	8	80	73.3
	10	2	2	20		4	40		7	70		7	70	
	10	2	2	20		4	40		7	70		7	70	

The results of LC_{50} at 24, 48, 72, and 96 h. were calculated by probit analysis method.

From the probit transformed responses curve of Nile Tilapia (*O. niloticus*) exposed to 24, 48, 72, and 96 h of $Pb(NO_3)$ the value of LC_{50} are presented in Table 6.

Table 6. The LC_{50} value of $Pb(NO_3)$ exposure to Nile Tilapia (*O. niloticus*).

	24 h	48 h	72 h	96 h
LC_{50} (mg/L)	247.51	197.47	183.74	182.38

Therefore, the concentration of $Pb(NO_3)$ used in the sublethal experiment was 45 mg/L which corresponded to 25% of the 96 h. LC_{50}

5.2 The efficiency of dietary calcium on the toxicity of sublethal exposure of $Pb(NO_3)$ to Nile Tilapia (*O. niloticus*).

5.2.1 Growth and morphological measurements

The parameters of growth and morphological measurements are represented the mean ± S.D. in the Table 7. The data were tested at α=0.05 for growth and morphological measurements among experimental groups (Fig. 11).

Table 7. Growth and morphological measurements (mean ± S.D.) in the experimental groups.

Parameters	Experimental Groups					
	Control	1	2	3	4	5
Total length (cm)	11.38 ± 0.47	10.82 ± 0.31[a]	11.70 ± 0.36[b]	10.90 ± 0.45[c]	11.62 ± 0.48[b,d]	11.66 ± 0.74[b,d]
Body weight (g)	23.77 ± 3.74	21.26 ± 2.75	24.96 ± 2.18[b]	22.08 ± 2.39	26.62 ± 3.94[b,d]	26.03 ± 3.63[b,d]
Liver weight(g)	0.65 ± 0.25	0.59 ± 0.15	0.57 ± 0.20	0.62 ± 0.27	0.75 ± 0.18	0.57 ± 0.31
Kidney weight(g)	0.07 ± 0.03	0.06 ± 0.03	0.07 ± 0.03	0.07 ± 0.04	0.07 ± 0.03	0.05 ± 0.02
HSI[1] (%)	2.69 ± 0.79	2.31 ± 0.75	2.29 ± 0.73	2.77 ± 1.11	2.82 ± .51	2.15 ± 1.00
KI[2] (%)	0.32 ± 0.14	0.29 ± 0.16	0.34 ± 0.08	0.32 ± 0.15	0.28 ± 0.09	0.21 ± 0.06

[1] HSI : Hepatosomatic index = 100 [liver weight/body weight]

[2] KI : Kidney index = 100 [kidney weight/body weight]

[a] The mean difference was significant when compared to the control group at 0.05 level

[b] The mean difference was significant when compared to the group 1 at 0.05 level

[c] The mean difference was significant when compared to the group 2 at 0.05 level

[d] The mean difference was significant when compared to the group 3 at 0.05 level

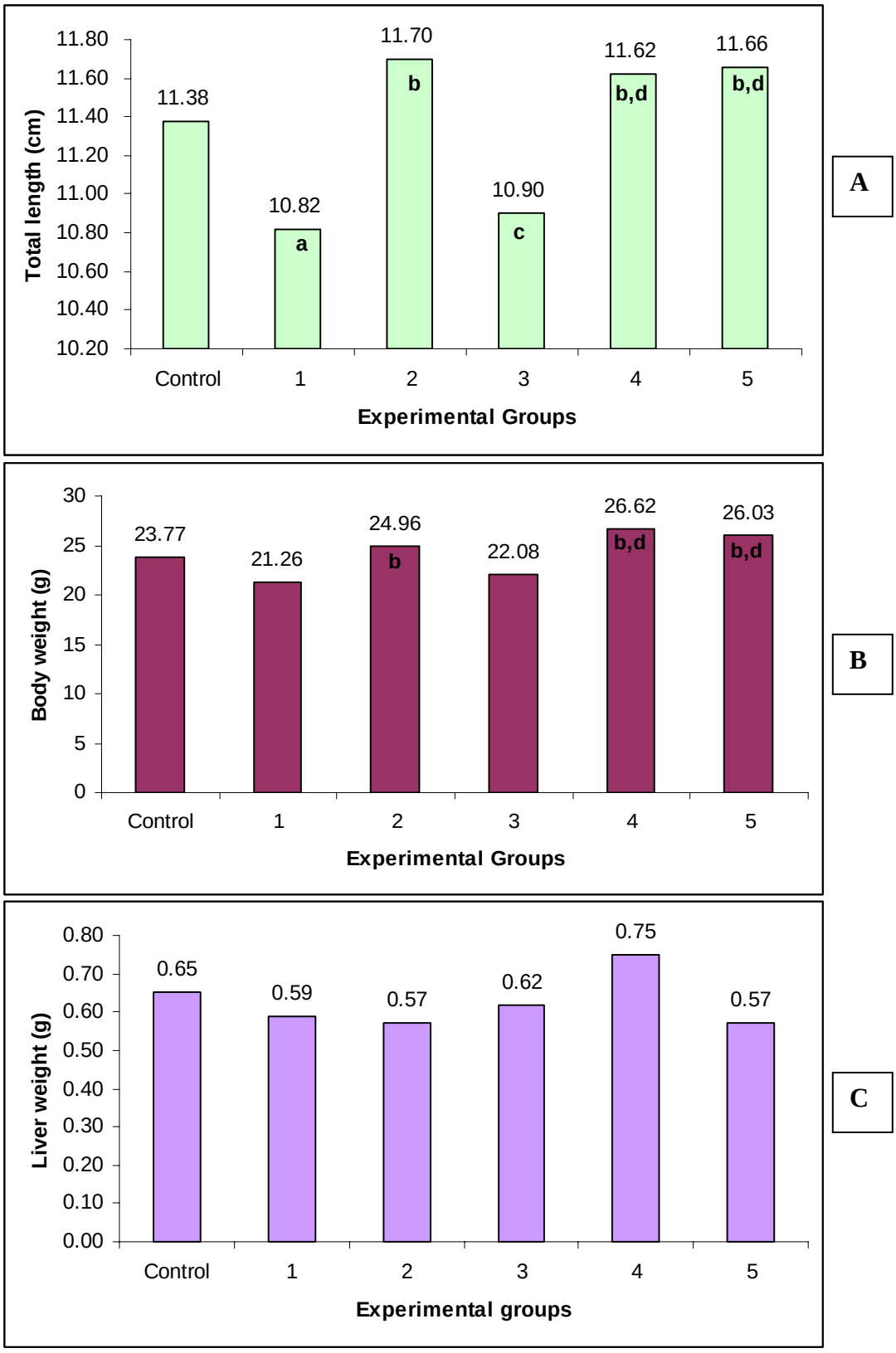


Figure 11. Growth and morphological measurements in the experimental groups.

A. Total length B. Body weight C. Liver weight

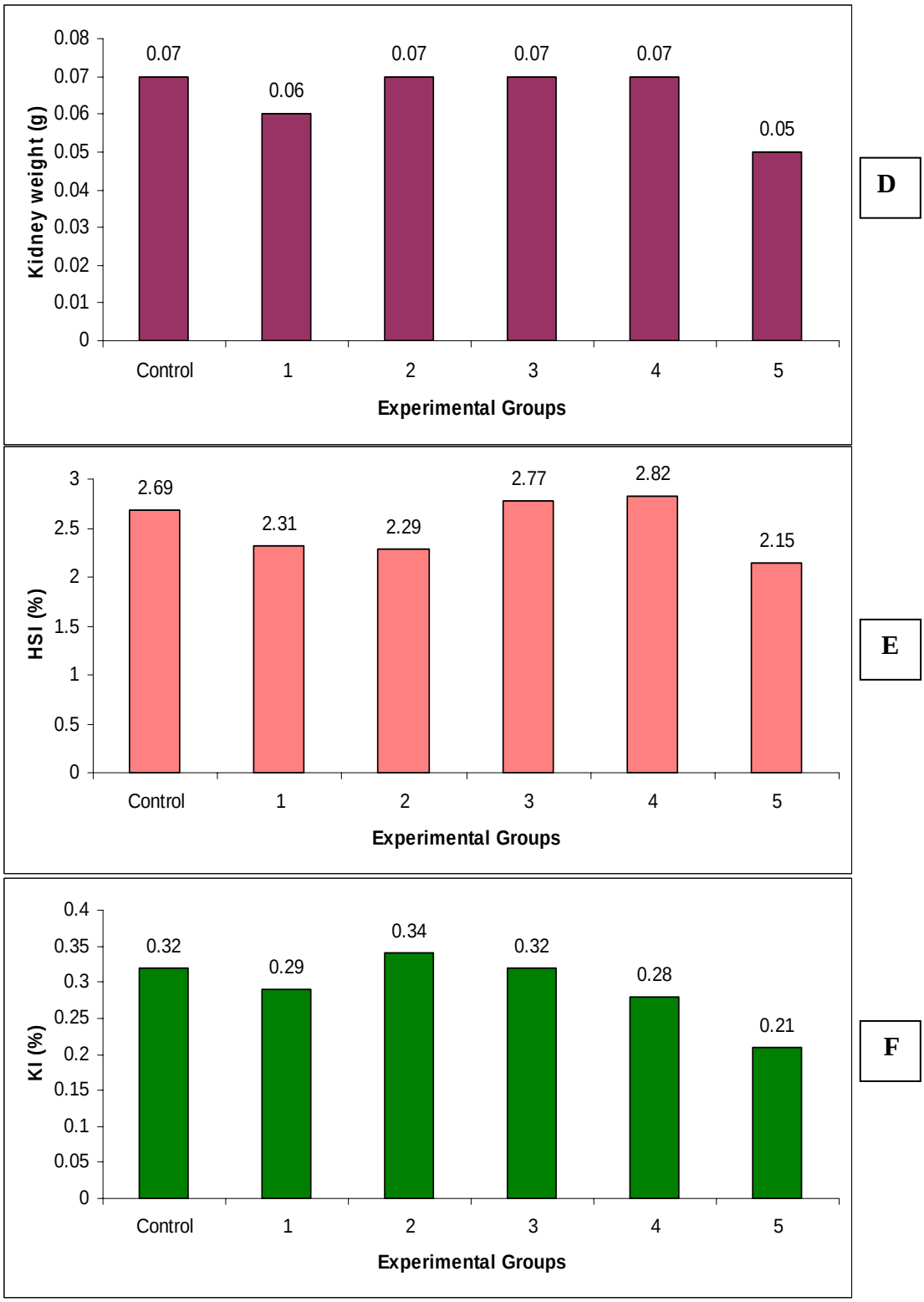


Figure 11. (cont.) Growth and morphological measurements in the experimental groups.

D. Kidney weight E. HSI F. KI

5.2.1.1 Total length

Group 1: The total length of group 1 was significantly reduced from 11.38 cm to 10.82 cm (4.92%) when compared with the control group ($p \leq 0.05$) but there was no significant difference among groups.

Group 2: The total length of group 2 was significantly increased from 10.82 cm to 11.70 cm (8.13%) when compared with the group 1 ($p \leq 0.05$) but there was no significant difference among the other groups including the control group.

Group 3: The total length of group 3 was significantly reduced from 11.70 cm to 10.90 cm (6.84%) when compared with the group 2 ($p \leq 0.05$) but there was no significant difference among the other groups including the control group.

Group 4: The total length of group 4 was significantly reduced from 11.62 cm to 10.82 cm (6.88%) and from 11.62 cm to 10.90 cm (6.19%) when compared with the group 1 and 3, respectively ($p \leq 0.05$) but there was no significant difference among the rest groups including the control group.

Group 5: The total length of group 5 was significantly reduced from 11.66 cm to 10.82 cm (7.20%) and from 11.66 cm to 10.90 cm (6.51%) when compared with the group 1 and 3, respectively ($p \leq 0.05$) but there was no significant difference among the rest groups including the control group.

5.2.1.2 Body weight

Group 1: The body weight of group 1 was no significant difference among groups.

Group 2: The body weight of group 2 was significantly increased from 21.26 g to 24.96 (17.40%) when compared with the group 1 ($p \leq 0.05$) but there was no significant difference among the other groups including the control group.

Group 3: The body weight of group 3 was no significant difference among groups.

Group 4: The body weight of group 4 was significantly increased from 21.26 g to 26.62 g (25.21%) and from 22.08 g to 26.62 g (20.56%) when compared with the group 1 and 3, respectively ($p \leq 0.05$) but there was no significant difference among the rest groups including the control group.

Group 5: The body weight of group 5 was significantly increased from 21.26 g to 26.03 g (22.43%) and from 22.08 g to 26.03 g (17.88%) when compared with the group 1 and 3, respectively ($p \leq 0.05$) but there was no significant difference among the rest groups including the control group.

5.2.1.3 Liver weight

There was no significant difference in liver weight among groups in each experimental group.

5.2.1.4 Kidney weight

There was no significant difference in kidney weight among groups in each experimental group.

5.2.1.5 HSI

There was no significant difference in HSI among groups in each experimental group.

5.2.1.6 KI

There was no significant difference in KI among groups in each experimental group.

5.2.2 Hematological analysis

The hematological parameters in each group were measured and show the mean ± S.D. in Table 8. An analysis of variance was performed separately for each experimental group. LSD was used for mean separation. The significant level was determined at the probability level of 0.05 (Fig. 12).

Table 8. Hematological analysis (mean ± S.D.) in the experimental groups.

Parameters	Experimental Groups					
	Control	1	2	3	4	5
Hct (%)	27.00 ± 1.73	17.28 ± 3.09[a]	32.00. ± 2.16[a,b]	26.14 ± 2.04[b,c]	33.00 ±3.91[a,b,d]	31.29 ± 3.04[a,b,d]
Hb (g/dL)	8.93 ± 0.83	7.08 ± 1.48[a]	10.76 ± 1.94[a,b]	8.30 ± 1.21[c]	10.88 ± 3.94[a,b,d]	10.63 ± 0.99[a,b,d]
Leukocyte count ($10^3/\mu L$)	26.87 ± 1.95	25.36 ± 2.42	31.63 ± 1.63[a,b]	27.42 ± 1.15[b,c]	28.83 ± 1.06[a,b,c]	28.12 ± 1.09[b,c]
Erythrocyte count ($10^5/\mu L$)	1.13 ± 0.14	1.56 ± 1.3[a]	1.56 ± 0.09[a]	1.11 ± 0.21[b,c]	1.71 ± 0.12[a,d]	1.53 ± 0.12[a,d,e]
MCV[1] (fL)	241.59 ± 36.15	112.58 ± 27.82[a]	205.76 ± 23.12[b]	243.24 ± 52.28[b,c]	194.04 ± 25.51[a,b,d]	205.31 ± 27.90[b,d]
MCH[2] (pg)	61.41 ± 5.35	61.20 ± 23.91	65.36 ± 8.87	69.19 ± 23.06	64.00 ± 8.45	69.57 ± 0.06
MCHC[3] (g/dL)	26.05 ± 5.43	56.87 ± 29.19[a]	31.96 ± 4.69[b]	28.88 ± 8.96[b]	33.40 ± 5.76[b]	35.43 ± 5.87[b]

[1]MCV : Mean corpuscular volume = 10 (Hct/RBC)

[2]MCH : Mean Corpuscular Haemoglobin = 10 (Hb/RBC)

[3]MCHC: Mean Corpuscular Haemoglobin Concentration =100 (Hb/Hct)

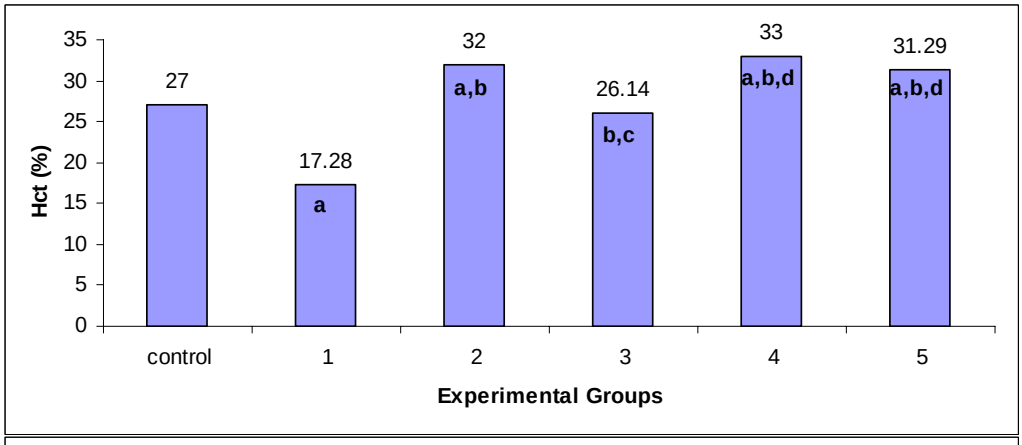
[a] The mean difference was significant when compared to the control group at 0.05 level

[b] The mean difference was significant when compared to the group 1 at 0.05 level

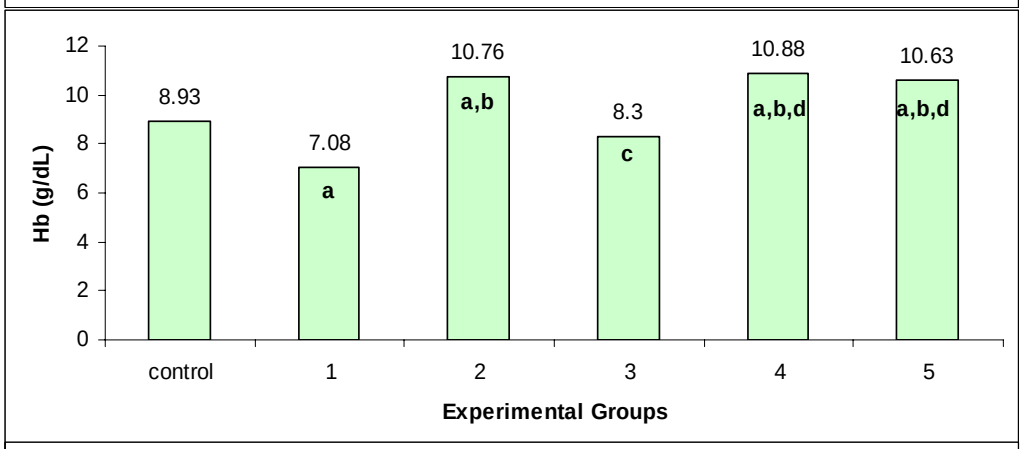
[c] The mean difference was significant when compared to the group 2 at 0.05 level

[d] The mean difference was significant when compared to the group 3 at 0.05 level

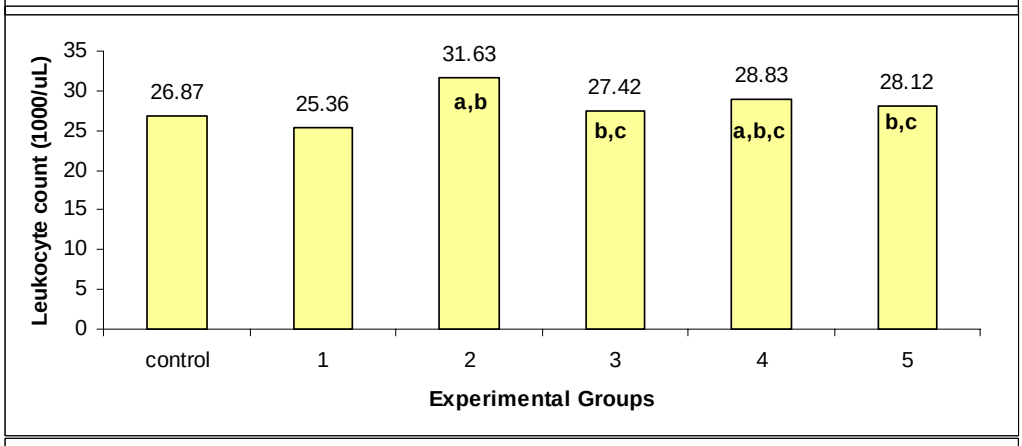
[e] The mean difference was significant when compared to the group 4 at 0.05 level


Hct (%)
35
30
25
20
15
10
5
0
27
17.28
32
26.14
33
31.29
a
a,b
b,c
a,b,d
a,b,d
control
1
2
3
4
5
Experimental Groups

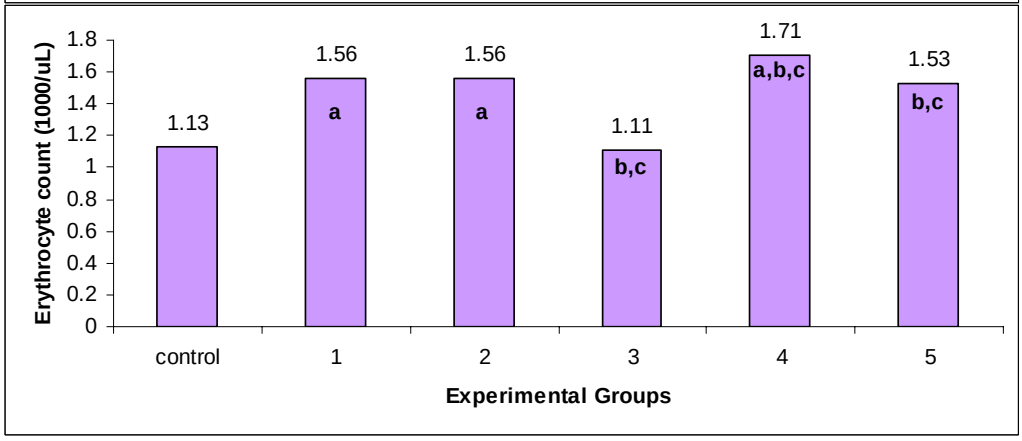
A


Hb (g/dL)
12
10
8
6
4
2
0
8.93
7.08
10.76
8.3
10.88
10.63
a
a,b
c
a,b,d
a,b,d
control
1
2
3
4
5
Experimental Groups

B


Leukocyte count (1000/uL)
35
30
25
20
15
10
5
0
26.87
25.36
31.63
27.42
28.83
28.12
a,b
b,c
a,b,c
b,c
control
1
2
3
4
5
Experimental Groups

C


Erythrocyte count (1000/uL)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1.13
1.56
1.56
1.11
1.71
1.53
a
a
b,c
a,b,c
b,c
control
1
2
3
4
5
Experimental Groups

D

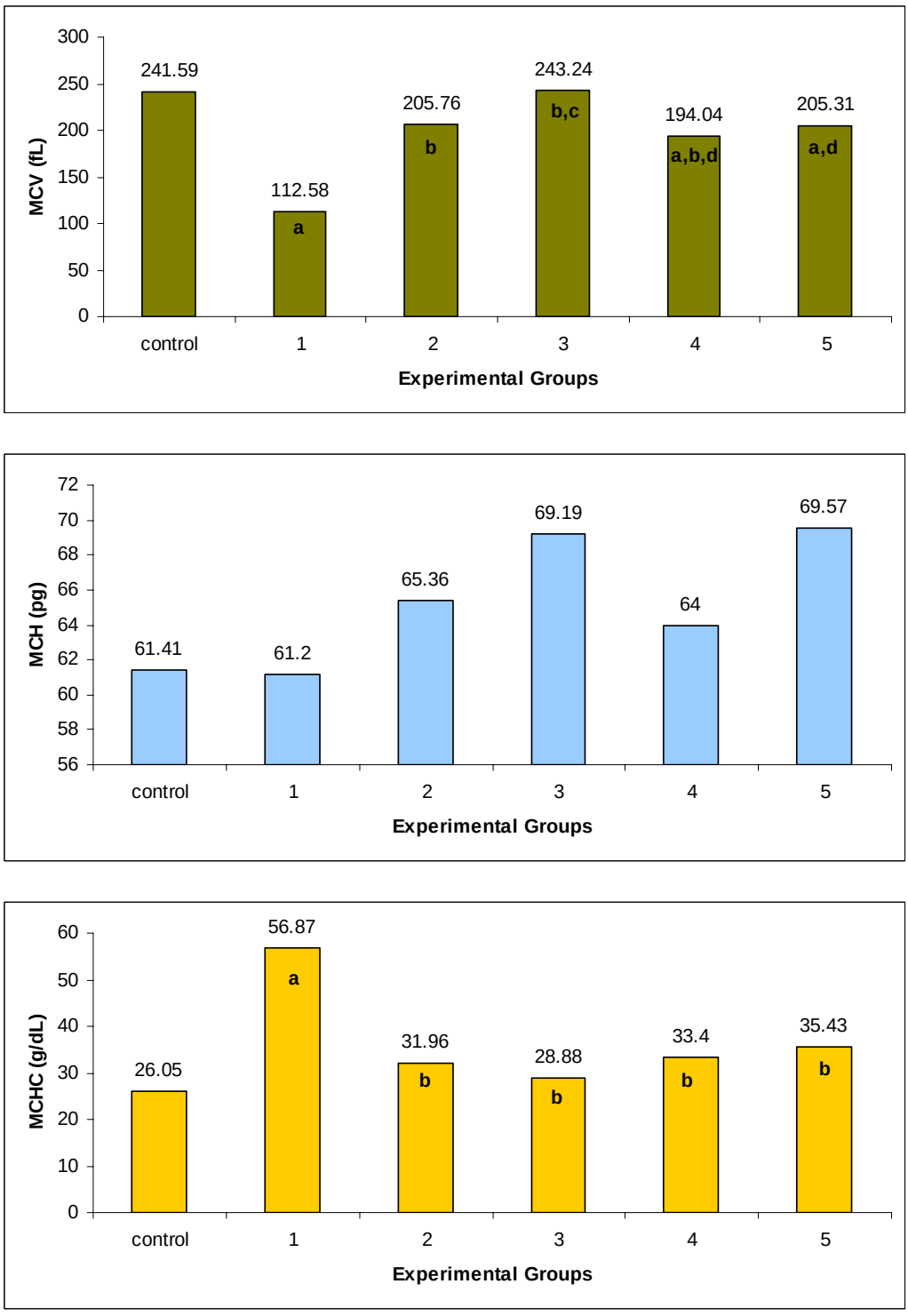


Figure 12. The hematological parameters measurements in the experimental groups.

A. Hct B. Hb C. Leukocyte count D. Erythocyte count

E. MCV F. MCH G. MCHC

5.2.2.1 Hematocrit (Hct)

Group 1: The hematocrit of group 1 was significantly reduced from 27.00% to 17.28% when compared with the control group ($p \leq 0.05$) but there was no significant difference among groups.

Group 2: The hematocrit of group 2 was significantly increased from 27.00% to 32.00% and from 17.28% to 32.00% when compared with the control group and group 1, respectively ($p \leq 0.05$) but there was no significant difference among the other groups.

Group 3: The hematocrit of group 3 was significantly increased from 11.28% to 26.14% when compared with the group 1 ($p \leq 0.05$), whereas there was significantly reduced from 32.00% to 26.14% when compared with the group 2.

Group 4: The hematocrit of group 4 was significantly increased from 27.00% to 33.00%, from 17.28% to 33.00% and from 26.14% to 33.00% when compared with the control group, group 1 and 3, respectively ($p \leq 0.05$).

Group 5: The hematocrit of group 5 was significantly increased from 27.00% to 31.29%, from 17.28% to 31.29% and from 26.14% to 31.29% when compared with the control group, group 1 and group 3, respectively ($p \leq 0.05$) but there was no significant difference among the other groups.

5.2.2.2 Hemoglobin (Hb)

Group 1: The hemoglobin of group 1 was significantly reduced from 8.93 g/dL to 7.08 g/dL (20.00%) when compared with the control group ($p \leq 0.05$) but there was no significant difference among the other groups.

Group 2: The hemoglobin of group 2 was significantly increased from 8.93 g/dL to 10.76 g/dL (20.49%) and from 7.08 g/dL to 10.76 g/dL (51.97%) when compared with the control group and group 1, respectively ($p \leq 0.05$).

Group 3: The hemoglobin of group 3 was significantly lower than the group 2 from 10.76 g/dL to 8.30 g/dL (29.63%) but there was no significant difference among the rest groups including the control group.

Group 4: The hemoglobin of group 4 was significantly increased from 8.97 g/dL to 10.88 (21.29%), from 7.08 g/dL to 10.88 g/dL (53.67%) and from 8.30 g/dL

to 10.88 g/dL (31.08%) when compared with the control group, group 1 and group 3, respectively ($p \leq 0.05$) but there was no significant difference among the other groups

Group 5: The hemoglobin of group 5 was significantly increased from 8.97 g/dL to 10.63 (18.50%), from 7.08 g/dL to 10.63 g/dL (50.14%) and from 8.30 g/dL to 10.63 g/dL (28.07%) when compared with the control group, group 1 and group 3 , respectively ($p \leq 0.05$) but there was no significant difference among the other groups

5.2.2.3 Leukocyte count

Group 1: The leukocyte count of group 1 was no significant difference among the other groups.

Group 2: The leukocyte count of group 2 was significantly increased from $26.87 \times 10^3/\mu L$ to $31.63 \times 10^3/\mu L$ (17.71%) and from $25.36 \times 10^3/\mu L$ to $31.63 \times 10^3/\mu L$ (24.72%) when compared with the control group and group 1, respectively ($p \leq 0.05$).

Group 3: The leukocyte count of group 3 was significantly increased from $25.36 \times 10^3/\mu L$ to $27.42 \times 10^3/\mu L$ (8.12%) when compared with the group 1 ($p \leq 0.05$), whereas there was significantly reduced from $31.63 \times 10^3/\mu L$ to $27.42 \times 10^3/\mu L$ (13.31%) when compared with the group 2 but there was no significant difference among the rest groups.

Group 4: The leukocyte count of group 4 was significantly increased from $26.87 \times 10^3/\mu L$ to $28.83 \times 10^3/\mu L$ (7.29%) and from $25.36 \times 10^3/\mu L$ to $28.83 \times 10^3/\mu L$ (13.68%) when compared with the control group and group 1, respectively ($p \leq 0.05$) whereas there was significantly reduced from $31.63 \times 10^3/\mu L$ to $28.83 \times 10^3/\mu L$ (8.85%) when compared with the group 2.

Group 5: The leukocyte count of group 5 was significantly increased from $25.36 \times 10^3/\mu L$ to $28.12 \times 10^3/\mu L$ (10.88%) when compared with the group 1 ($p \leq 0.05$), whereas there was significantly reduced from $31.63 \times 10^3/\mu L$ to $28.12 \times 10^3/\mu L$ (11.09%) when compared with the group 2 but there was no significant difference among the rest groups.

5.2.2.4 Erythrocyte count

Group 1: The erythrocyte count of group 1 was significantly increased from $1.13x10^5/\mu L$ to $1.5x10^5/\mu L$ (38.06%) when compared with the control group. There was no significant difference among the other groups.

Group 2: The erythrocyte count of group 2 was significantly increased from $1.13x10^5/\mu L$ to $1.56x10^5/\mu L$ (38.06%) when compared with the control group, there was no significant difference among the other groups.

Group 3: The erythrocyte count of group 3 was significantly reduced from $1.56x10^5/\mu L$ to $1.11x10^5/\mu L$ (28.85%) when compared with the group 1 and group2, respectively ($p \leq 0.05$).

Group 4: The erythrocyte count of group 4 was significantly increased from $1.13x10^5/\mu L$ to$1.71x10^5/\mu L$ (51.33%) and from $1.11x10^5/\mu L$ to $1.71x10^5/\mu L$ (54.06%) when compared with the control group and group 3.

Group 5: The erythrocyte count of group 5 was significantly increased from $1.13x10^5/\mu L$ to $1.53x10^5/\mu L$ (35.40%) and from $1.11x10^5/\mu L$ to $1.53x10^5/\mu L$ (37.84%) when compared with control group and the group 3, respectively ($p \leq 0.05$), whereas there was significantly reduced from $1.71x10^5/\mu L$ to $1.53x10^5/\mu L$ (10.53%) when compared with the group 4.

5.2.2.5 MCV : Mean corpuscular volume

Group 1: The MCV of group 1 was no significant difference among the other groups.

Group 2: The MCV of group 2 was significantly reduced from 241.59 fL to 112.58 fL (53.40%) when compared with the control group; there was no significant difference among the other groups.

Group 3: The MCV of group 3 was significantly increased from 112.58 fL to 243.24 fL (116.06%) and from 205.76 fL to 243.24 (18.22%) when compared with the group 1 and group 2 ($p \leq 0.05$).

Group 4: The MCV of group 4 was significantly reduced from 241.59 fL to 194.04 fL (19.69%) and from 205.76 fL to 194.04 fL (5.70%) when compared with the control group and group 3, respectively ($p \leq 0.05$), whereas there was

significantly increased from 112.58 fL to 194.04 fL (72.36%) when compared with the group 2 ($p \leq 0.05$).

Group 5: The MCV of group 5 was significantly increased from 112.58 to 205.31 (82.37%) when compared with the group 2 ($p \leq 0.05$), whereas there was significantly reduced from 243.24 fL to 205.31 (15.60%) when compared with the group 3 ($p \leq 0.05$).

5.2.2.6 MCH : Mean Corpuscular Haemoglobin

There was no significant difference in the MCH among groups in each experimental group.

5.2.2.7 MCHC: Mean Corpuscular Haemoglobin Concentration

Group 1: The MCHC of group 1 was significantly increased from 26.05 g/dL to 56.87 g/dL (118.31%) when compared with the control group ($p \leq 0.05$), there was no significant difference among the other groups.

Group 2: The MCHC of group 2 was significantly reduced from 56.87 g/dL to 31.96 g/dL (43.81%) when compared with the group 2 ($p \leq 0.05$), there was no significant difference among the other groups.

Group 3: The MCHC of group 3 was significantly reduced from 56.87 g/dL to 28.88 g/dL (49.22%) when compared with the group 2 ($p \leq 0.05$), there was no significant difference among the other groups.

Group 4: The MCHC of group 4 was significantly reduced from 56.87 g/dL to 33.40 g/dL (41.27%) when compared with the group 2 ($p \leq 0.05$), there was no significant difference among the other groups.

Group 5: The MCHC of group 5 was significantly reduced from 56.87 g/dL to 35.43 g/dL (37.70%) when compared with the group 2 ($p \leq 0.05$), there was no significant difference among the other groups.

5.2.3 Biochemical analysis

Changes in enzyme activity measurements in each group (mean ± S.D.) are presented in Table 9. An analysis of variance was performed separately for each experimental group. LSD was used for mean separation. The significant level was determined at the probability level of 0.05 (Fig. 13).

Table 9. Changes in enzyme activity measurements in each group (mean ± S.D.).

Parameters	Experimental Groups					
	Control	1	2	3	4	5
Aspartate aminotrasferase (U/L)	85 ± 1.45	140 ± 2.32 [a]	84 ± 2.10 [b]	61 ± 1.54 [b]	120 ± 3.31[a,d]	125 ± 3.18[a,d]
Alanine aminotrasferase (U/L)	77 ± 0.83	90 ± 1.48[a]	80 ± 1.94	49 ± 1.21[b]	37 ± 3.94[b]	74 ± 0.99
Total protein (mg/L)	4.34 ± 0.65	5.59 ± 0.49 [a]	2.23 ± 0.36 [a,b]	3.06 ± 0.53 [b]	5.32 ± 0.46[a]	4.37 ± 0.45 [b]

[a] The mean difference was significant when compared to the control group at 0.05 level

[b] The mean difference was significant when compared to the group 1 at 0.05 level

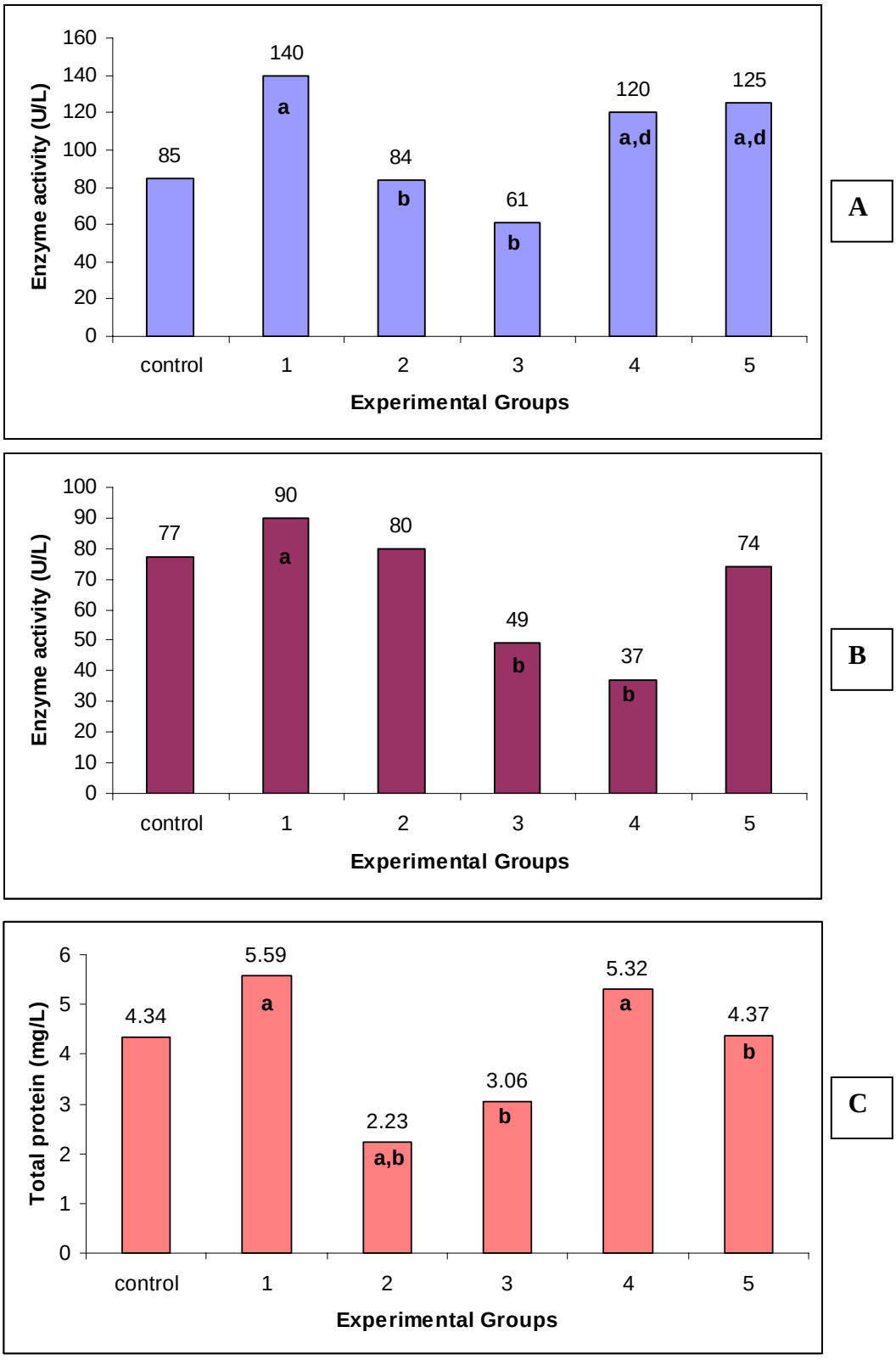


Figure 13. Change in enzyme activity measurements in the experimental groups.
A. Aspartate aminotrasferase B. Alanine aminotrasferase C. Total protein

5.2.3.1 Aspartate aminotrasferase

Group 1: The enzyme activity of aspartate aminotrasferase of group 1 was significantly increased from 85 U/L to 140 U/L (64.71%) when compared with the control group ($p \leq 0.05$), there was no significant difference among the other groups.

Group 2: The enzyme activity of aspartate aminotrasferase of group 2 was significantly reduced from 140 U/L to 84 U/L (40.00%) when compared with the group 1 ($p \leq 0.05$), there was no significant difference among the other groups.

Group 3: The enzyme activity of aspartate aminotrasferase of group 3 was significantly reduced from 140 U/L to 61 U/L (56.43%) when compared with the group 1 ($p \leq 0.05$), there was no significant difference among the other groups.

Group 4: The enzyme activity of aspartate aminotrasferase of group 4 was significantly increased from 85 U/L to 120 U/L (41.18%) and from 61 U/L to 120 U/L (96.73%) when compared with the control group and group 3, respectively ($p \leq 0.05$).

Group 5: The enzyme activity of aspartate aminotrasferase of group 5 was significantly increased from 85 U/L to 125 U/L (47.06%) and from 61 U/L to 125 U/L (104.92%) when compared with the control group and group 3, respectively ($p \leq 0.05$).

5.2.3.2 Alanine aminotrasferase

Group 1: The enzyme activity of alanine aminotrasferase of group 1 was significantly increased from 77 U/L to 90 U/L (16.89%) when compared with the control group ($p \leq 0.05$), there was no significant difference among the other groups.

Group 2: The enzyme activity of alanine aminotrasferase of group 2 was no significant difference among the other groups.

Group 3: The enzyme activity of alanine aminotrasferase of group 3 was significantly reduced from 90 U/L to 49 U/L (45.56%) when compared with the group 1 ($p \leq 0.05$), there was no significant difference among the other groups.

Group 4: The enzyme activity of alanine aminotrasferase of group 4 was significantly reduced from 90 U/L to 37 U/L (58.89%) when compared with the group 1 ($p \leq 0.05$), there was no significant difference among the other groups.

Group 5: The enzyme activity of alanine aminotrasferase of group 5 was no significant difference among the other groups.

5.2.3.3 Total protein

Group 1: The total of group 1 was significantly increased from 4.34 mg/L to 5.59 mg/L (28.81%) when compared with the control group ($p \leq 0.05$), there was no significant difference among the other groups.

Group 2: The total of group 1 was significantly reduced from 4.34 mg/L to 2.23 mg/L (48.62%) and from 5.59 mg/L to 2.23 U/L (60.11%) when compared with the control group and group 1, respectively ($p \leq 0.05$).

Group 3: The total of group 3 was significantly reduced from 5.59 mg/L to 3.06 mg/L (45.26%) when compared with the group 1 ($p \leq 0.05$), there was no significant difference among the other groups.

Group 4: The total of group 4 was significantly increased from 4.34 mg/L to 5.32 mg/L (22.58%) when compared with the control group ($p \leq 0.05$), there was no significant difference among the other groups.

Group 5: The total of group 3 was significantly reduced from 5.59 mg/L to 4.37 mg/L (21.83%) when compared with the group 1 ($p \leq 0.05$), there was no significant difference among the other groups.

5.2.4 Nuclear morphology analysis

5.2.4.1 Blood erythrocytes

Frequencies (%) of micronuclei and nuclear abnormalities in blood erythrocytes in each group (mean ± S.D.) are presented in Table 10. An analysis of variance was performed separately for each experimental group. LSD was used for mean separation. The significant level was determined at the probability level of 0.05

Table 10. Frequencies (%) of micronuclei and nuclear abnormalities in blood erythrocytes in each group (mean ± S.D.)

Parameters	Experimental Groups					
	Control	1	2	3	4	5
Micronuclei (%)	0.18 ± 0.83	1.10 ± 1.12[a]	0.24 ± 0.99[b]	0.28 ± 1.38[b]	0.65 ± 2.20[a]	0.70 ± 1.98[a]
Blebbed nuclei (%)	0.04 ± 5.79	0.06 ± 1.34	0.04 ± 2.59	0.04 ± 3.26	0.06 ± 3.69	0.06 ± 2.75
Lobed nuclei (%)	0.16 ± 12.44	0.28 ± 12.98	0.16 ± 11.35	0.18 ± 12.33	0.08 ± 11.89	0.08 ±12.57
Notched nuclei (%)	0.24 ± 8.23	0.40 ± 9.77[a]	0.16 ± 9.34[b]	0.18 ± 8.47[b]	0.28 ± 7.66	0.30 ± 8.89[a]
Binuclei (%)	0.16 ± 0.77	0.16 ± 0.45	0.16 ± 0.63	0.16 ± 0.57	0.16 ± 0.23	0.16 ± 0.39

[a] The mean difference was significant when compared to the control group at 0.05 level

[b] The mean difference was significant when compared to the group 1 at 0.05 level

5.2.4.1 Micronuclei

Group 1: The frequencies of micronuclei of group 1 was significantly increased from 0.18 % to 1.10%when compared with the control group ($p \leq 0.05$), there was no significant difference among the other groups.

Group 2: The frequencies of micronuclei of group 2 was significantly reduced from 1.10 % to 0.24% when compared with the group 1 ($p \leq 0.05$), there was no significant difference among the other groups.

Group 3: The frequencies of micronuclei of group 3 was significantly reduced from 1.10 % to 0.28% when compared with the group 1 ($p \leq 0.05$), there was no significant difference among the other groups.

Group 4: The frequencies of micronuclei of group 4 was significantly increased from 0.18% to 0.65%when compared with the control group ($p \leq 0.05$), there was no significant difference among the other groups.

Group 5: The frequencies of micronuclei of group 5 was significantly increased from 0.18% to 0.70% when compared with the control group ($p \leq 0.05$), there was no significant difference among the other groups.

5.2.4.1.2 Blebbed nuclei

There was no significant difference in liver weight among groups in each experimental group.

5.2.4.1.3 lobed nuclei

There was no significant difference in liver weight among groups in each experimental group.

5.2.4.1.4 Notched nuclei

Group 1: The frequencies of notched nuclei of group 1 was significantly increased from 0.24% to 0.40% when compared with the control group ($p \leq 0.05$), there was no significant difference among the other groups.

Group 2: The frequencies of notched nuclei of group 2 was significantly reduced from 0.40% to 0.16% when compared with the group 1 ($p \leq 0.05$), there was no significant difference among the other groups.

Group 3: The frequencies of notched nuclei of group 3 was significantly reduced from 0.40% to 0.18% when compared with the group 1 ($p \leq 0.05$), there was no significant difference among the other groups.

Group 4: The frequency of notched nuclei of group 4 was no significant difference among the other groups.

Group 5: The frequencies of notched nuclei of group 5 was significantly increased from 0.24% to 0.30% when compared with the control group ($p \leq 0.05$), there was no significant difference among the other groups.

5.2.4.1.5 Binuclei

There was no significant difference in liver weight among groups in each experimental group.

5.2.4.2 Gill cell

Frequencies (%) of micronuclei and nuclear abnormalities in gill cell in each group (mean ± S.D.) are presented in Table 11. An analysis of variance follow by LSD was performed separately for each experimental group. The significant level was determined at the probability level of 0.05

Table 11. Frequencies (%) of micronuclei and nuclear abnormalities in gill cell in each group (mean ± S.D.)

Parameters	Experimental Groups					
	Control	1	2	3	4	5
Micronuclei (%)	0.12 ± 2.73	0.60 ± 3.35[a]	0.17 ± 3.98	0.09 ± 3.42[b]	0.42 ± 2.20[a,b]	0.29 ± 1.98[b]
Blebbed nuclei (%)	0.03 ± 5.61	0.05 ± 4.89	0.03 ± 5.60	0.03 ± 5.34	0.04 ± 4.53	0.05 ± 4.98
Lobed nuclei (%)	0.12 ± 7.13	0.21 ± 8.53	0.15 ± 7.66	0.16 ± 7.34	0.16 ± 7.45	0.14 ± 8.92
Notched nuclei (%)	0.20 ± 2.35	0.38 ± 3.90[a]	0.31± 1.60	0.28 ± 2.77	0.28 ± 1.75	0.30 ± 2.75
Binuclei (%)	0.12 ± 6.98	0.10 ± 5.52	0.12 ± 5.72	0.11 ± 5.33	0.12 ± 4.96	0.12 ± 5.45

[a] The mean difference was significant when compared to the control group at 0.05 level

[b] The mean difference was significant when compared to the group 1 at 0.05 level

5.4.2.2.1 Micronuclei

Group 1: The frequencies of micronuclei of group 1 was significantly increased from 0.12% to 0.60% when compared with the control group ($p \leq 0.05$), there was no significant difference among the other groups.

Group 2: The frequency of micronuclei of group 2 was no significant difference among the other groups.

Group 3: The frequencies of micronuclei of group 3 was significantly reduced from 0.60% to 0.09% when compared with the group 1 ($p \leq 0.05$), there was no significant difference among the other groups.

Group 4: The frequencies of micronuclei of group 4 was significantly increased from 0.12% to 0.42% when compared with the control group ($p \leq 0.05$), whereas there was significantly reduced from 0.60% to 0.42% when compared with the group 1 ($p \leq 0.05$).

Group 5: The frequencies of micronuclei of group 5 was significantly reduced from 0.60% to 0.29% when compared with the group 1 ($p \leq 0.0$), there was no significant difference among the other groups.

5.2.4.2.2 Blebbed nuclei

There was no significant difference in blebbed nuclei among groups in each experimental group.

5.2.4.2.3 Lobed nuclei

There was no significant difference in lobed nuclei among groups in each experimental group.

5.2.4.2.4 Notched nuclei

Group 1: The frequencies of notched nuclei of group 1 was significantly increased from 0.20% to 0.38% when compared with the control group ($p \leq 0.05$), there was no significant difference among the other groups.

Group 2, 3, 4 and 5: There were no significant differences in notched nuclei among the other groups.

5.2.4.2.5 Binuclei

There was no significant difference in binuclei among groups in each experimental group.

5.2.4.3 Liver cell

Frequencies (%) of micronuclei and nuclear abnormalities in liver cell in each group (mean ± S.D.) are presented in Table 12. An analysis of variance follow by LSD was performed separately for each experimental group. The significant level was determined at the probability level of 0.05

Table 12. Frequencies (%) of micronuclei and nuclear abnormalities in liver cell in each group (mean ± S.D.)

Parameters	Experimental Groups					
	Control	1	2	3	4	5
Micronuclei (%)	0.16 ± 7.34	1.20 ± 7.90[a]	0.29 ± 6.15[b]	0.28 ± 8.55[b]	0.67 ± 7.11[a]	0.69 ± 1.98[a]
Blebbed nuclei (%)	0.03 ± 4.58	0.04 ± 3.22	0.03 ± 3.76	0.03 ± 3.72	0.03 ± 3.90	0.05 ± 4.12
Lobed nuclei (%)	0.15 ± 14.74	0.20 ± 13.48	0.16 ± 13.48	0.17 ± 12.59	0.16 ± 11.99	0.16 ± 14.78
Notched nuclei (%)	0.22 ± 1.12	0.34 ± 2.01[a]	0.26 ± 1.32	0.27 ± 2.35	0.28 ± 1.18	0.28 ± 2.45
Binuclei (%)	0.14 ± 0.45	0.15 ± 0.59	0.15 ± 1.14	0.15 ± 1.67	0.15 ± 2.45	0.15 ± 3.39

[a] The mean difference was significant when compared to the control group at 0.05 level

[b] The mean difference was significant when compared to the group 1 at 0.05 level

5.4.2.3.1 Micronuclei

Group 1: The frequencies of micronuclei of group 1 was significantly increased from 0.16% to 1.20% when compared with the control group ($p \leq 0.05$), there was no significant difference among the other groups.

Group 2: The frequencies of micronuclei of group 2 was significantly reduced from 1.20% to 0.29% when compared with the group 1 ($p \leq 0.05$), there was no significant difference among the other groups.

Group 3: The frequencies of micronuclei of group 3 was significantly reduced from 1.20% to 0.28% when compared with the group 1 ($p \leq 0.05$), there was no significant difference among the other groups.

Group 4: The frequency of micronuclei of group 4 was significantly increased from 0.16% to 0.67% when compared with the control group ($p \leq 0.05$).

Group 5: The frequency of micronuclei of group 5 was significantly increased from 0.16% to 0.69% when compared with the control group ($p \leq 0.05$).

5.2.4.3.2 Blebbed nuclei

There was no significant difference in blebbed nuclei among groups in each experimental group.

5.2.4.3.3 Lobed nuclei

There was no significant difference in lobed nuclei among groups in each experimental group.

5.2.4.3.4 Notched nuclei

Group 1: The frequencies of notched nuclei of group 1 was significantly increased from 0.22% to 0.3 % when compared with the control group ($p \leq 0.05$), there was no significant difference among the other groups.

Group 2, 3, 4 and 5: There were no significant differences in notched nuclei among the other groups.

5.2.4.3.5 Binuclei

There was no significant difference in binuclei among groups in each experimental group.

5.2.4.4 Kidney cell

Frequencies (%) of micronuclei and nuclear abnormalities in kidney cell in each group (mean ± S.D.) are presented in Table 13. An analysis of variance follow by LSD was performed separately for each experimental group. The significant level was determined at the probability level of 0.05.

Table 13. Frequencies (%) of micronuclei and nuclear abnormalities in kidney cell in each group (mean ± S.D.)

Parameters	Experimental Groups					
	Control	1	2	3	4	5
Micronuclei (%)	0.31 ± 7.23	0.92 ± 6.49[a]	0.44 ± 7.49	0.38 ± 7.52	0.60 ± 8.23	0.69 ± 5.43
Blebbed nuclei (%)	0.02 ± 9.81	0.02 ± 8.44	0.02 ± 8.87	0.02 ± 10.12	0.02 ± 9.52	0.01 ± 8.03
Lobed nuclei (%)	0.14 ± 9.05	0.18 ± 11.45	0.15 ± 12.78	0.15 ± 11.45	0.16 ± 11.59	0.15 ±12.23
Notched nuclei (%)	0.22 ± 14.13	0.24 ± 12.32	0.19± 13.45	0.18 ± 14.57	0.20 ± 14.37	0.22 ± 14.13
Binuclei (%)	0.12 ± 4.45	0.12 ± 1.67	0.12 ± 2.49	0.12 ± 3.57	0.12± 2.94	0.11 ± 4.66

[a] The mean difference was significant when compared to the control group at 0.05 level

5.2.4.4.1 Micronuclei

Group 1: The frequencies of micronuclei of group 1 was significantly increased from 0.31% to 0.92% when compared with the control group ($p \leq 0.05$), there was no significant difference among the other groups.

Group 2, 3, 4 and 5: There were no significant differences in micronuclei among the other groups.

5.2.4.4.2 Blebbed nuclei

There was no significant difference in blebbed nuclei among groups in each experimental group.

5.2.4.4.3 Lobed nuclei

There was no significant difference in lobed nuclei among groups in each experimental group.

5.2.4.4.4 Notched nuclei

There was no significant difference in lobed nuclei among groups in each experimental group.

5.2.4.4.5 Binuclei

There was no significant difference in binuclei among groups in each experimental group.

5.2.4.5 Fin cell

Frequencies (%) of micronuclei and nuclear abnormalities in fin cell in each group (mean ± S.D.) are presented in Table 14. An analysis of variance follow by LSD was performed separately for each experimental group. The significant level was determined at the probability level of 0.05.

Table 14. Frequencies (%) of micronuclei and nuclear abnormalities in fin cell in each group (mean ± S.D.)

Parameters	Experimental Groups					
	Control	1	2	3	4	5
Micronuclei (%)	0.19 ± 8.87	0.25 ± 7.26	0.22 ± 8.14	0.23 ± 8.13	0.20 ± 7.20	0.20 ± 5.79
Blebbed nuclei (%)	0.02 ± 6.67	0.01 ± 5.75	0.01 ± 4.90	0.02 ± 4.85	0.02 ± 3.12	0.01 ± 5.55
Lobed nuclei (%)	0.14 ± 13.67	0.18 ± 15.89	0.15 ± 14.87	0.15 ± 12.67	0.14 ± 13.76	0.13 ±12.45
Notched nuclei (%)	0.18 ± 9.31	0.20 ± 9.23	0.17 ± 8.25	0.18 ± 4.47	0.21 ± 7.75	0.19 ± 5.50
Binuclei (%)	0.08 ± 6.90	0.09 ± 9.78	0.08 ± 9.63	0.08 ± 12.98	0.07 ± 7.89	0.07 ± 8.64

5.2.4.5.1 Micronuclei

There was no significant difference in micronuclei among groups in each experimental group.

5.2.4.5.2 Blebbed nuclei

There was no significant difference in blebbed nuclei among groups in each experimental group.

5.2.4.5.3 Lobed nuclei

There was no significant difference in lobed nuclei among groups in each experimental group.

5.2.4.5.4 Notched nuclei

There was no significant difference in lobed nuclei among groups in each experimental group.

5.2.4.5.5 Binuclei

There was no significant difference in binuclei among groups in each experimental group.

5.2.5 Histopathological study

Histopathological alterations were observed in all fish in the experimental groups .Most tissue samples showed mind lesions and severe lesions were observed in some samples. Histopathological alterations were evaluated semi-quantitatively by ranking tissue lesion severity.

5.2.5.1 Gills

Control groups: No recognizable changes were observed in the gills of the control group and groups 2 and 3. There were four gill arches on each side of the buccal cavity. Each arch was composed of numerous gill filaments with two rows of secondary lamellae that run perpendicular to each filament (Fig. 14A). The secondary

gills lamellae were composed of a single layer of epithelial cells supported by pillar cells, which were contractile and separated the capillary channels (Fig. 14B). One of two erythrocytes was usually recognized within the lumen (Fig. 14B). Chloride cells were identified as large epithelial cells with light cytoplasm, usually present at the base of lamellae (Fig. 14B). Mucous cells were present in the in the epithelium of the lamellae, but they lacked the light cytoplasm and were smaller than chloride cells

Treatment groups: Light microscopic study of gills in each experimental group showed several pathological changes. In group 1 the gills showed hyperthrophy and hyperplasia of chloride cells and mucous cells at the base of gill filament and secondary lamellae (Fig.14C). Fusion of secondary lamellae was observed. Sever edema of epithelial cells and aneurysm was found (Figs. 14D-E). In groups 3 and 4, filament cell proliferation was quantified by the height of the filament epithelium. The thickening of the primary lamellar epithelium appeared regular. They showed similar alterations as those observed in group 1 but they were less severe (Fig. 14G). The semiquantitative scoring of gill lesion is shown in Table 15.

Table 15. Semiquntitative scoring of gill lesion in Nile tilapia (*O. niloticus*) in the experimental groups [a]

Lesion	Experimental Groups					
	control	1	2	3	4	5
Edema	-	+++	-	-	+	++
Aneurism	-	+++	-	-	-	-
Hyperplasia	-	+++	+	+	+	++

[a] (-) no histopathology; (+) mild histopathology (<25% of fields); (+ +) moderate histopathology (>75% of fields); (+ + +) severe histopathology (all fields).

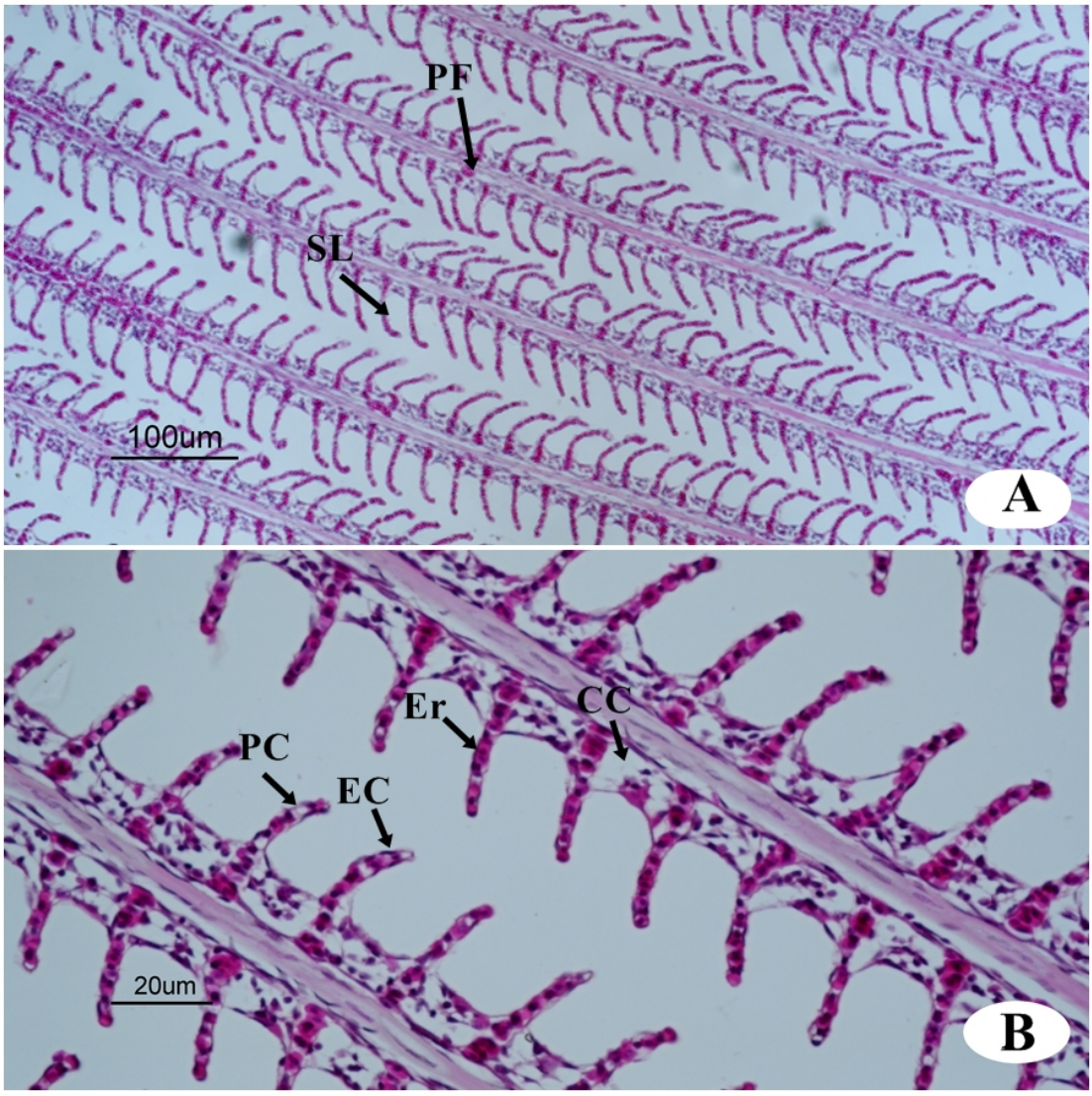


Figure 14. Light micrographs of a transverse section of *O. niloticus* gill tissues in each experimental group.

A. Control group showing normal appearance of primary filament (PF) and secondary lamellae (SL).
B. High magnification showing erythrocytes (Er) within capillary lumen delimited by pillar cells (PC) chloride cell (CC) and epithelial cell (EC).

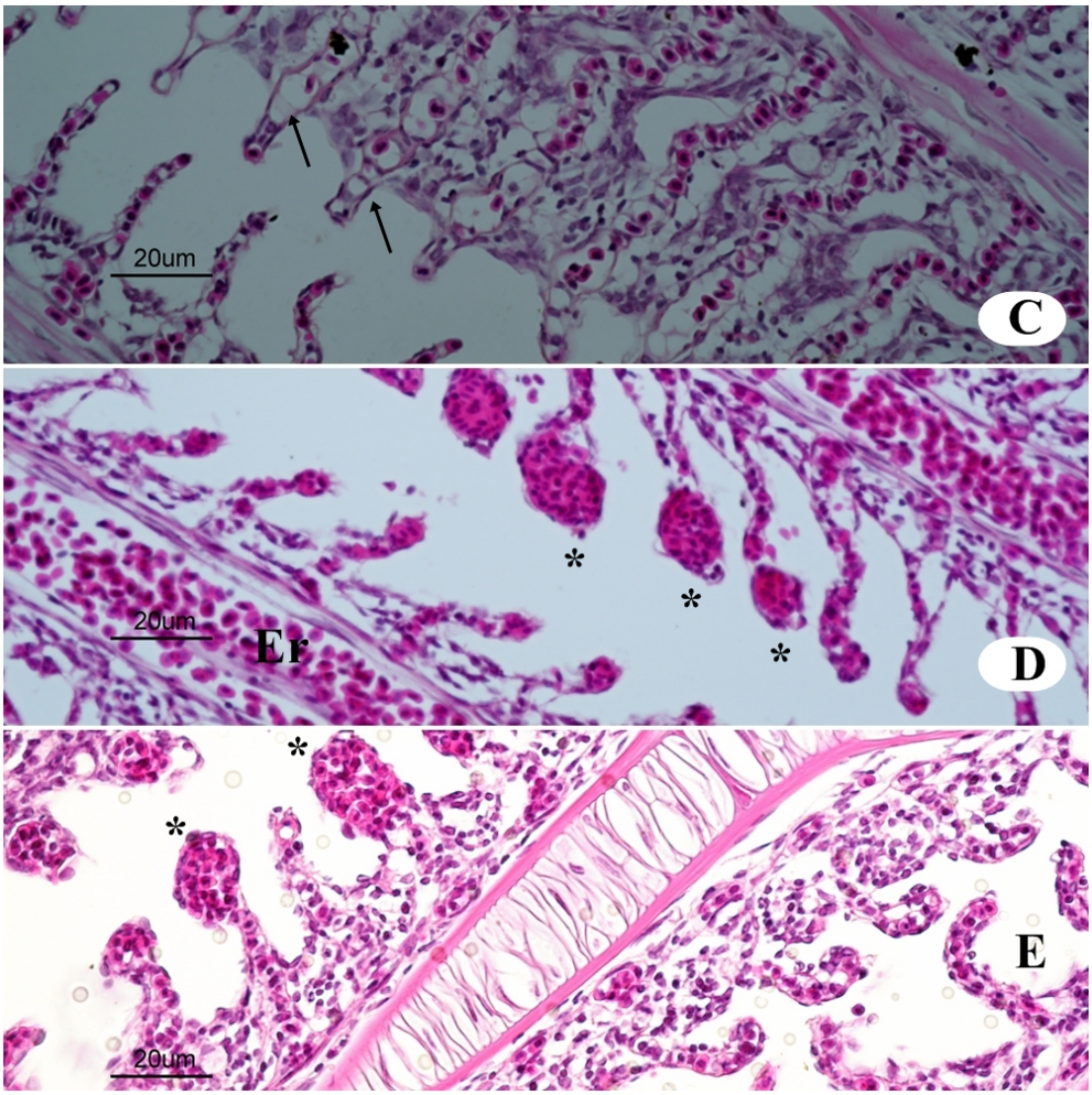


Figure 14 (cont).

C. Group 1, showing hyperthrophy and hyperplasia of chloride cells and mucous cells at the base of gill filament and secondary lamella and sever edema of epithelial cells (arrows).

D. and E. High magnification in group 1 showing aneurysm (arrows). Note the accumulation of erythrocytes (Er) in primary lamella.

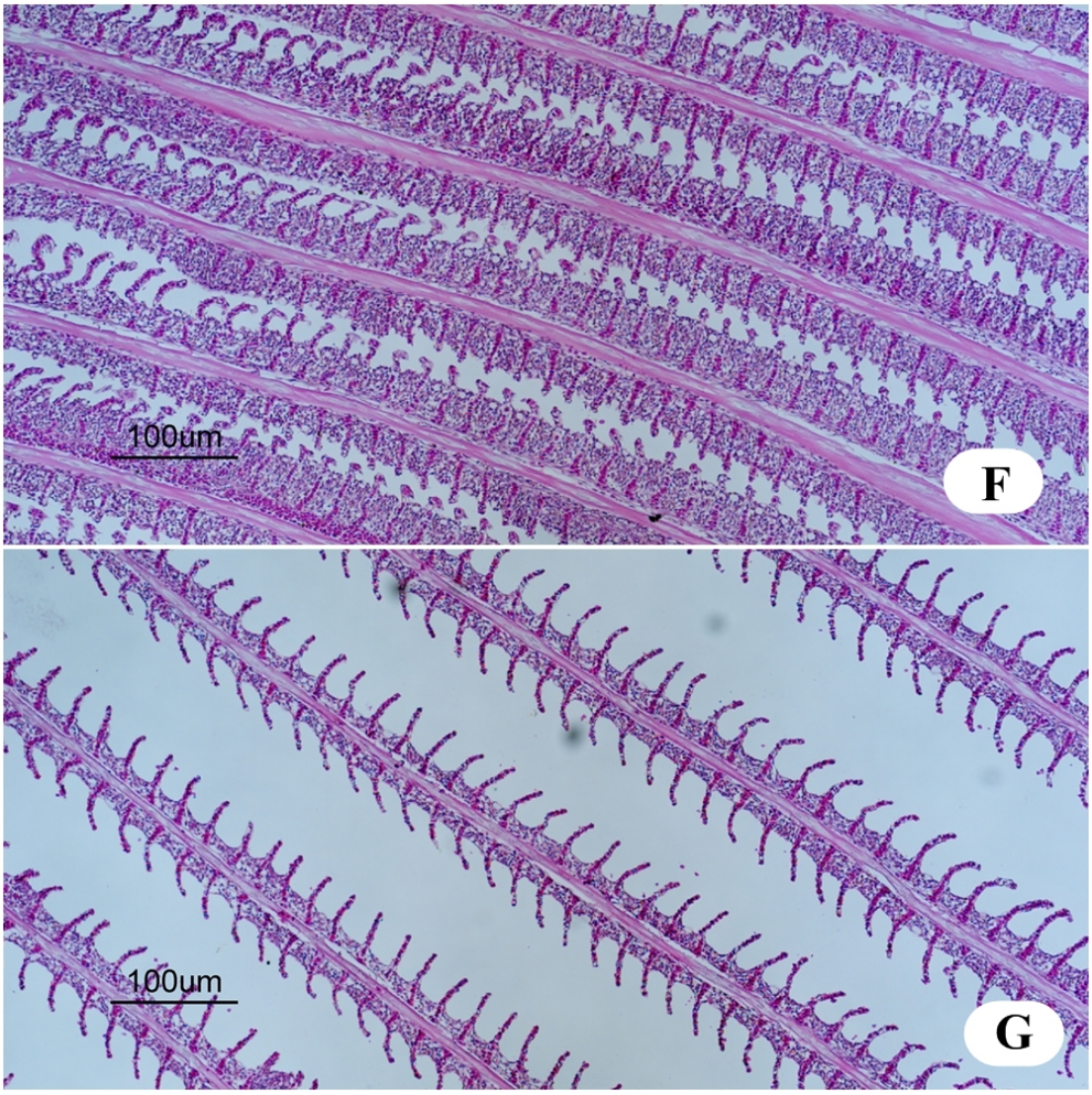


Figure 14 (cont).

F. Group 1, showing severe hyperplasia of epithelial cells.

G. Group 4 and 5 gills showed mild hyperplasia of epithelial cells.

5.2.5.2 Liver

Control group: The livers of the control group had a typical parenchymatous appearance. The parenchyma itself was primarily composed of polyhedral hepatocytes typically with central nuclei with densely stained chromatin (Fig. 15A). Venous blood entered the liver caudally from the intestine via the hepatic portal veins and branched into capillaries known as sinusoid (Fig 15A). Sinusoids were lined with reticuloendothelial cells which were in turn surrounded by hepatocytes (Fig. 15A).

Treated groups: The livers of fish in experimental groups showed several pathological changes. The general lesions were pyknotic nuclei and large lipid vacuoles in the cytoplasm of hepatocytes (Fig. 15B). Some liver areas showed focal necrosis and contained severe infiltration of leukocytes (Fig. 15C). In more severe cased, the liver showed slight blood congestion in sinusoids and hydropic swelling of hepatocytes (Fig 15C) In groups 2 and 3, mild congestion and vacuolization were observed in these types of treatment (Fig. 15D). In groups 4 and 5, the hepatocytes were still swelling and exhibiting necrotic nuclei, they showed similar alterations as those observed in the group 1 but they were less severe (Fig.15E). The semiquantitative scoring of liver lesion is shown in Table 16.

Table 16. Semiquntitative scoring of liver lesion in Nile tilapia (*O. niloticus*) in the experimental groups [a]

Lesion	Experimental Groups					
	control	1	2	3	4	5
Vacuolation	-	+++	+	+	+	++
Blood congestion	-	+++	+	+	+	++
Necrosis	-	+++	-	-	+	++
Hypertrophy	-	+++	-	-	+	+

[a] (-) no histopathology; (+) mild histopathology (<25% of fields);
(+ +) moderate histopathology (>75% of fields); (+ + +) severe histopathology (all fields).

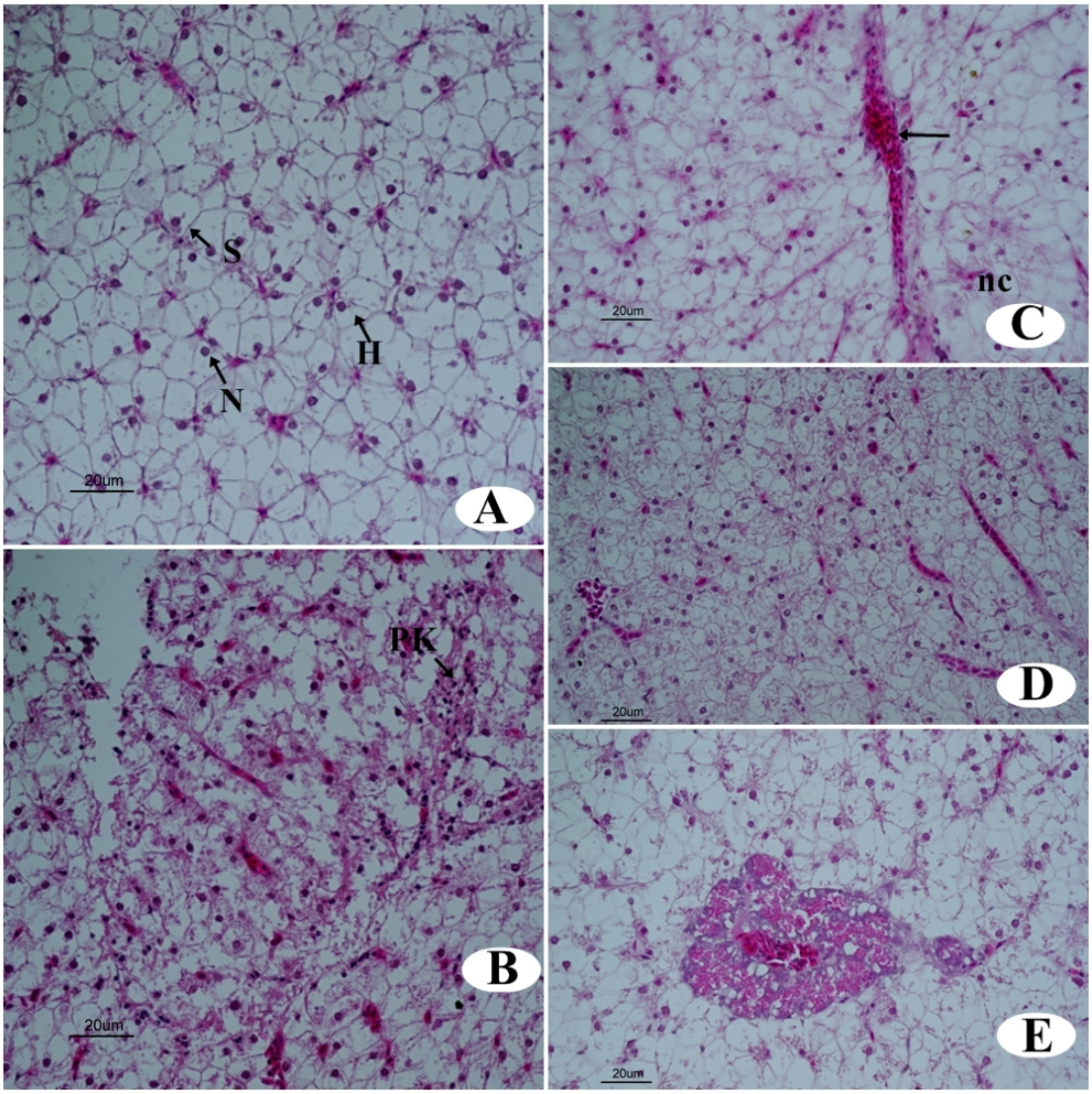


Figure 15. Light micrographs of a transverse section of *O. niloticus* liver tissues in each experimental group.

A. Control group showing normal appearance of central nuclei with densely stained chromatin (N), sinusoid (S) and hepatocyte (H).
B. Group 1, showing vaculolization of hepatocytes with pyknotic nuclei (PK).
C. Group 1, showing hepatocytes swelling, sinusoid dilation with blood congestion (arrow), vacuolation and cell necrosis (nc) were seen.
D. Groups 2 and 3, mild congestion and vacuolization were also observed.
E. Groups 4 and 5, hepatocytes were still swelling.

5.2.5.3 Kidney

Control group: No recognizable changes were observed in the kidney of the control group. The kidney was composed of numerous renal corpuscles with well developed glomeruli and a system of tubules. The proximal segment was covered by tall columnar epithelial cells with basal nuclei and brush border was reduced or absent. The collecting duct or glomerulus was larger in diameter than the distal segmenty, containing columnar epithelial cells with basal nuclei and brush border (Fig.16A).

Treated groups: In group 1 showed glomeruli were collapsed or atrophy and tubular necrosis (Fig. 16B). Hydropic swelling and hypertrophy of tubules with dilated nuclei and necrotic tissue were found (Fig. 16C). In more severe cases, the tubular cells showed hyaline droplet and dark granule accumulation (Fig. 16D.) In groups 2 and 3, the epithelial cells of many tubules had shown fewer damages, their morphology similar to those of the control (Fig.16E). In groups 4 and 5, their changes occurred similarly the group 2 but they were less severe (Fig.16F). The semiquantitative scoring of kidney lesion is shown in Table 17.

Table 17. Semiquntitative scoring of kidney lesion in Nile tilapia (*O. niloticus*) in the experimental groups [a]

Lesion	Experimental Groups					
	control	1	2	3	4	5
Glomerulus atrophy	-	++	-	-	+	+
Tubular cells-swelling	-	+++	-	-	+	++
Necrosis	-	+++	-	-	+	+

[a] (-) no histopathology; (+) mild histopathology (<25% of fields);
(+ +) moderate histopathology (>75% of fields); (+ + +) severe histopathology (all fields).

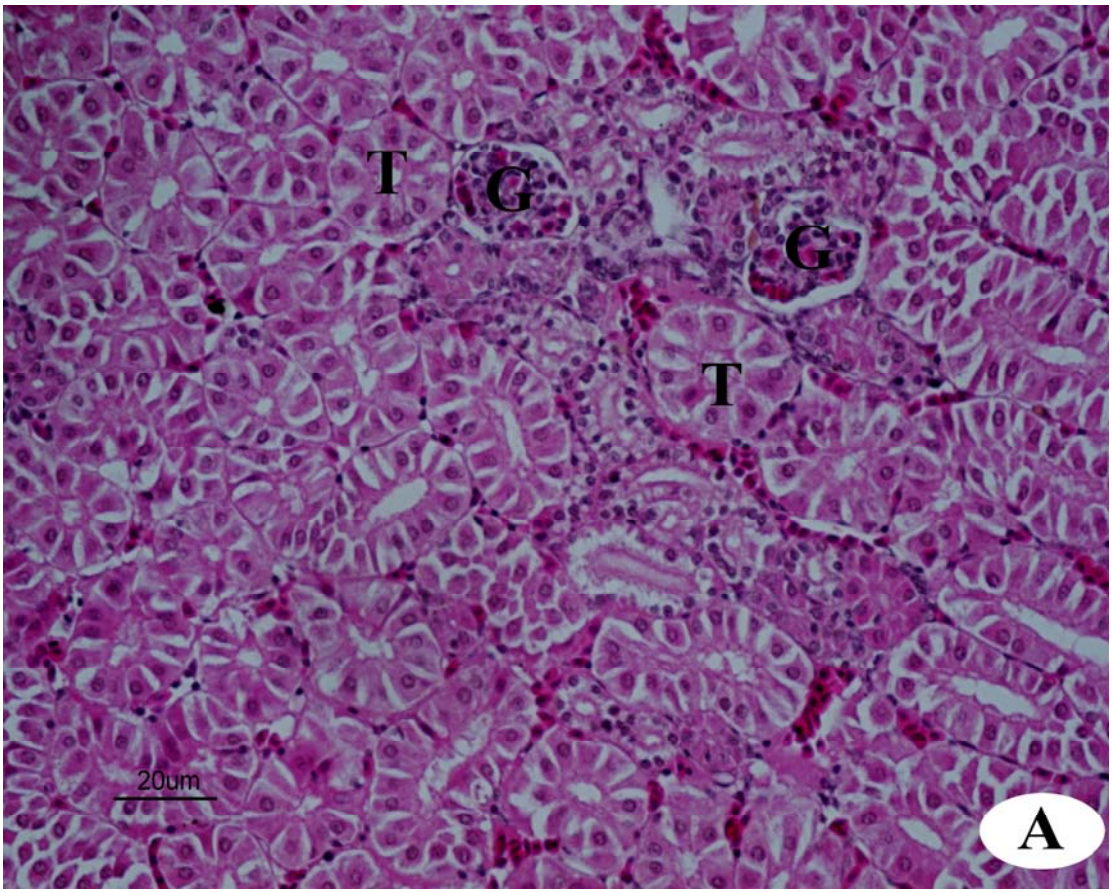


Figure 16. Light micrographs of a transverse section of *O. niloticus* kidney tissues in each experimental group.

A. Control group showing normal appearance of glomerulus (G) and proximal tubules (T).

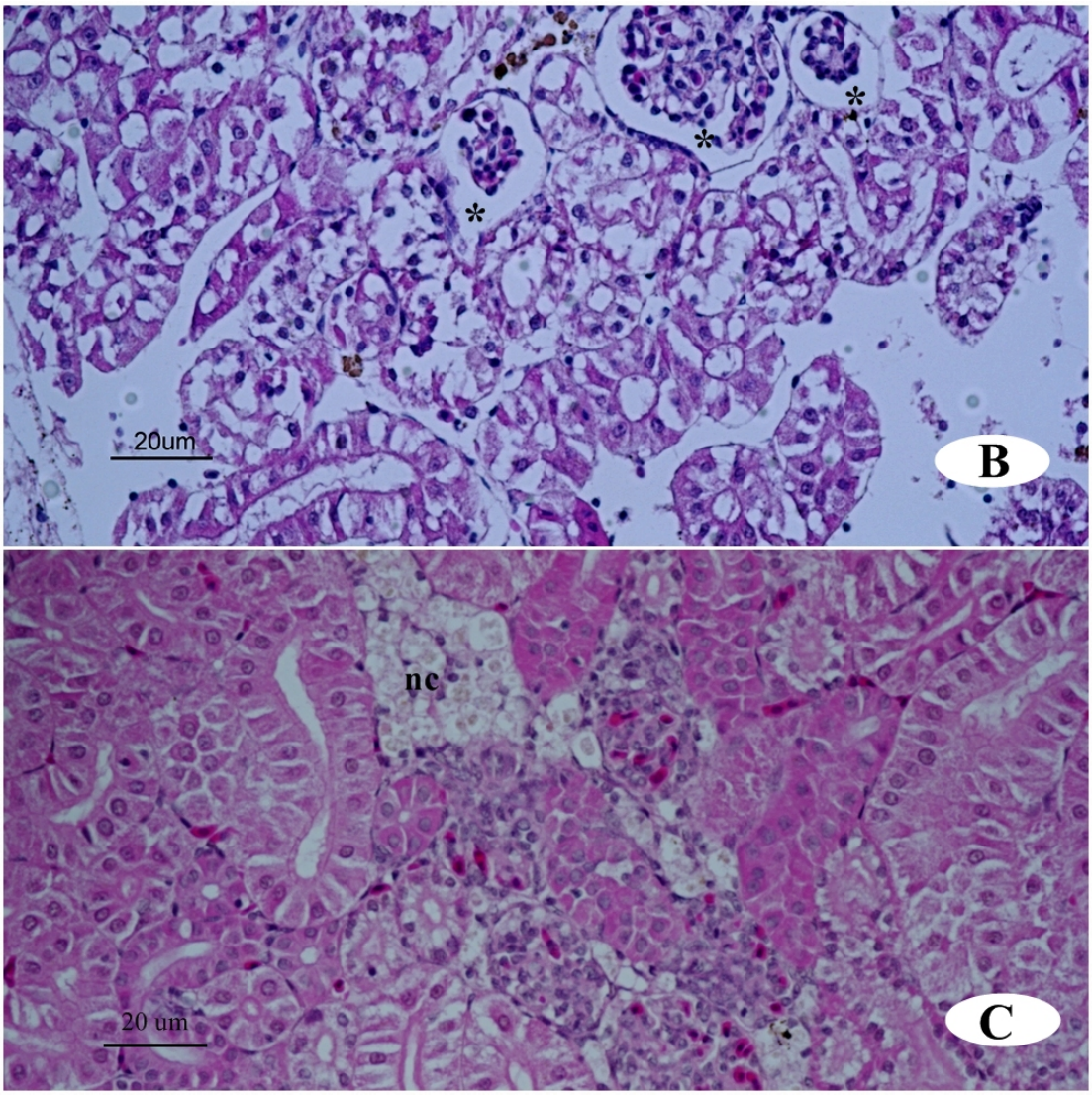


Figure 16 (cont.).

B. and C. Group1 showing tubular cells swelling, glomeruli atrophy (*) and tubular necrosis (nc) were observed in some areas.

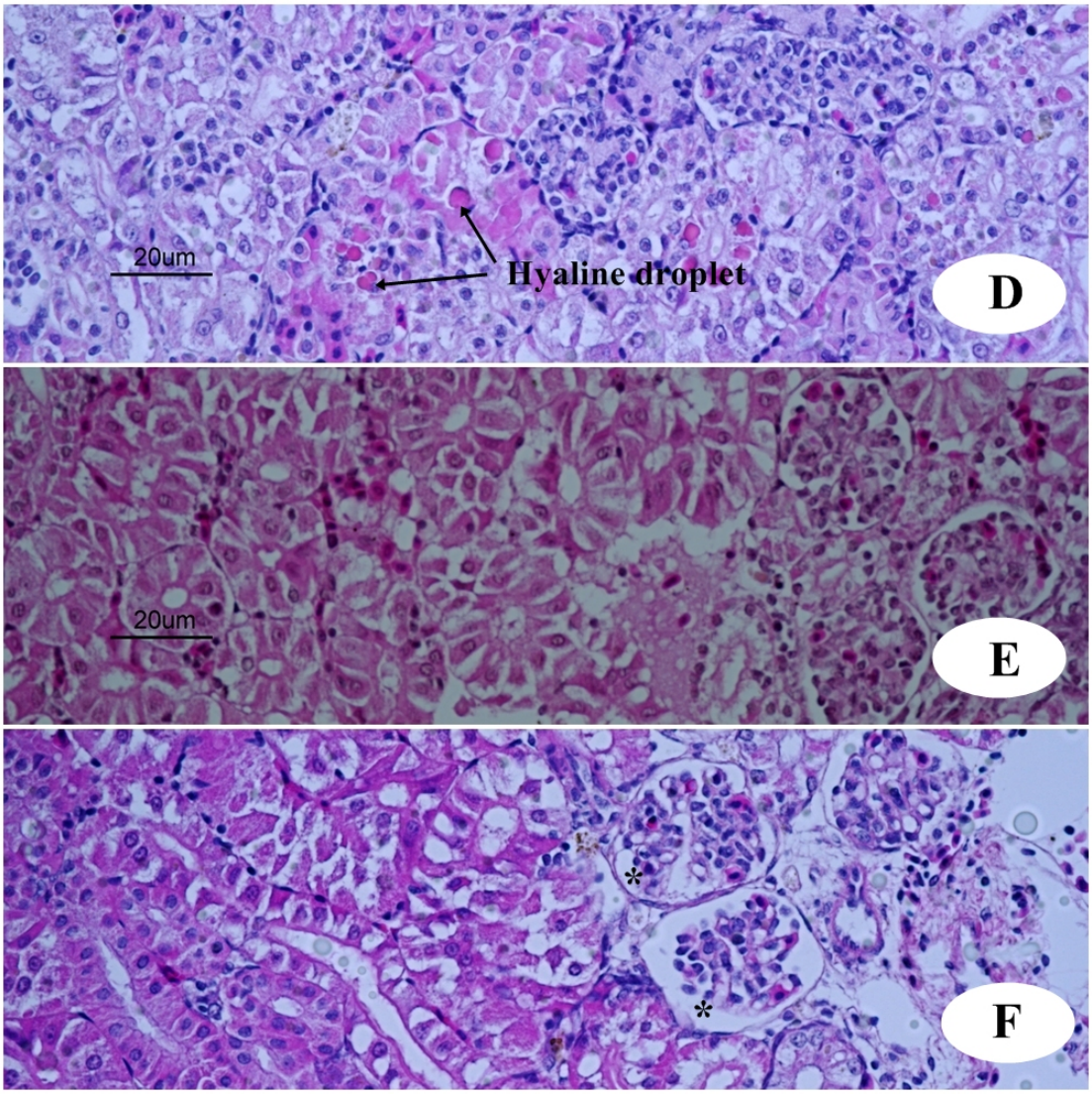


Figure 16 (cont.).

D. Group1 showing tubular hyaline droplet in severe case.

E. Groups 2 and 3, showing normal appearance similar to the control group.

F. Glomeruli atrophy (*) were observed in some area of groups 4 and 5.

5.2.5.4 Spleen

Control group: No recognizable changes were observed in the spleen tissue of the control group. The spleen was discrete organ in the mesentery of the fish. It was one of the major filters in the circulatory system. The spleen capsule was a thin squamous epithelium and connective tissue (Fig.17A). The red pulp is composed of sinusoids filled with erythrocytes, erythroblasts, macrophages and reticular cells and melanomacrophage centers (Figs. 17A-B.). The white pulp is basophilic and contains lymphocytes, some erythrocytes, mocrophages and macrophage centers (Figs. 17A-B.)

Treated group: Light microscopic study of the spleen in group 1 showed several pathological changes. There were large numbers of megakaryocytes due to the pollution. Thickening of membrane of the spleens (Fig. 18A), coarsening of trabeculae of the spleens, fibrosis round splenetic arteries, and stasis of blood and dilatation of the splenetic sinus and necrotic in some areas were main features of the spleens in group 1 (Fig. 18B). The fish in groups 2 and 3 showing mind blood congestion (Fig. 18D). The groups 4 and 5 (Fig. 18E.) showed similar the histopathological alteration in the group 1, but the alterations were not so extensive. The semiquantitative scoring of spleen lesion is shown in Table 18.

Table 18. Semiquntitative scoring of spleen lesion in Nile tilapia (*O. niloticus*) in the experimental groups [a]

Lesion	Experimental Groups					
	control	1	2	3	4	5
Cell - swelling	-	+++	+	+	+	++
Blood congestion	-	+++	+	+	+	++
Necrosis	-	++	-	-	-	+

[a] (-) no histopathology; (+) mild histopathology (<25% of fields);
(+ +) moderate histopathology (>75% of fields); (+ + +) severe histopathology (all fields).

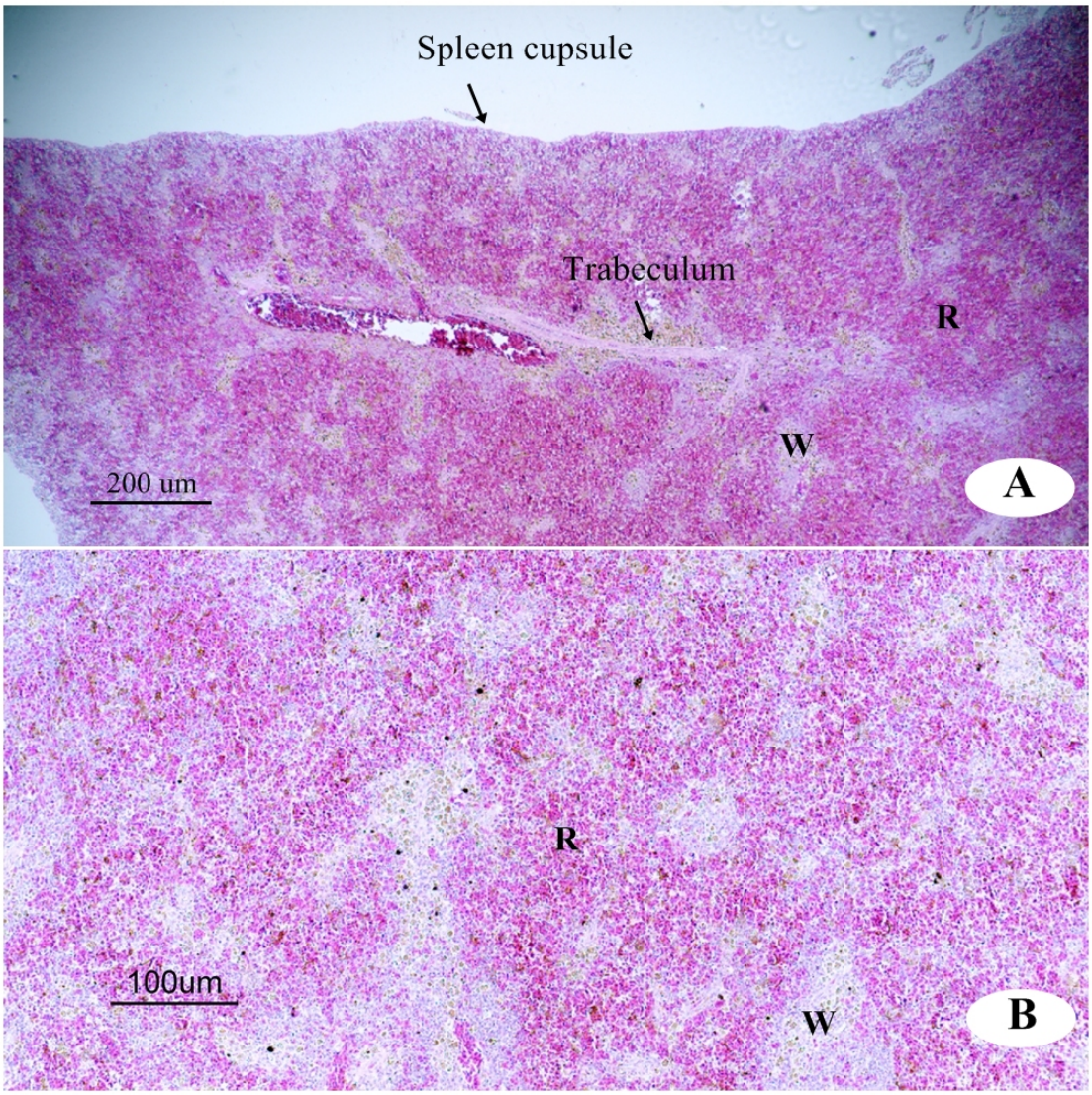


Figure 17. Light micrographs of a transverse section of *O. niloticus* spleen tissues in each experimental group.

A. and B. Control group showing normal appearance of spleen capsule, trabeculum, red pulp (R), and white pulp (W).

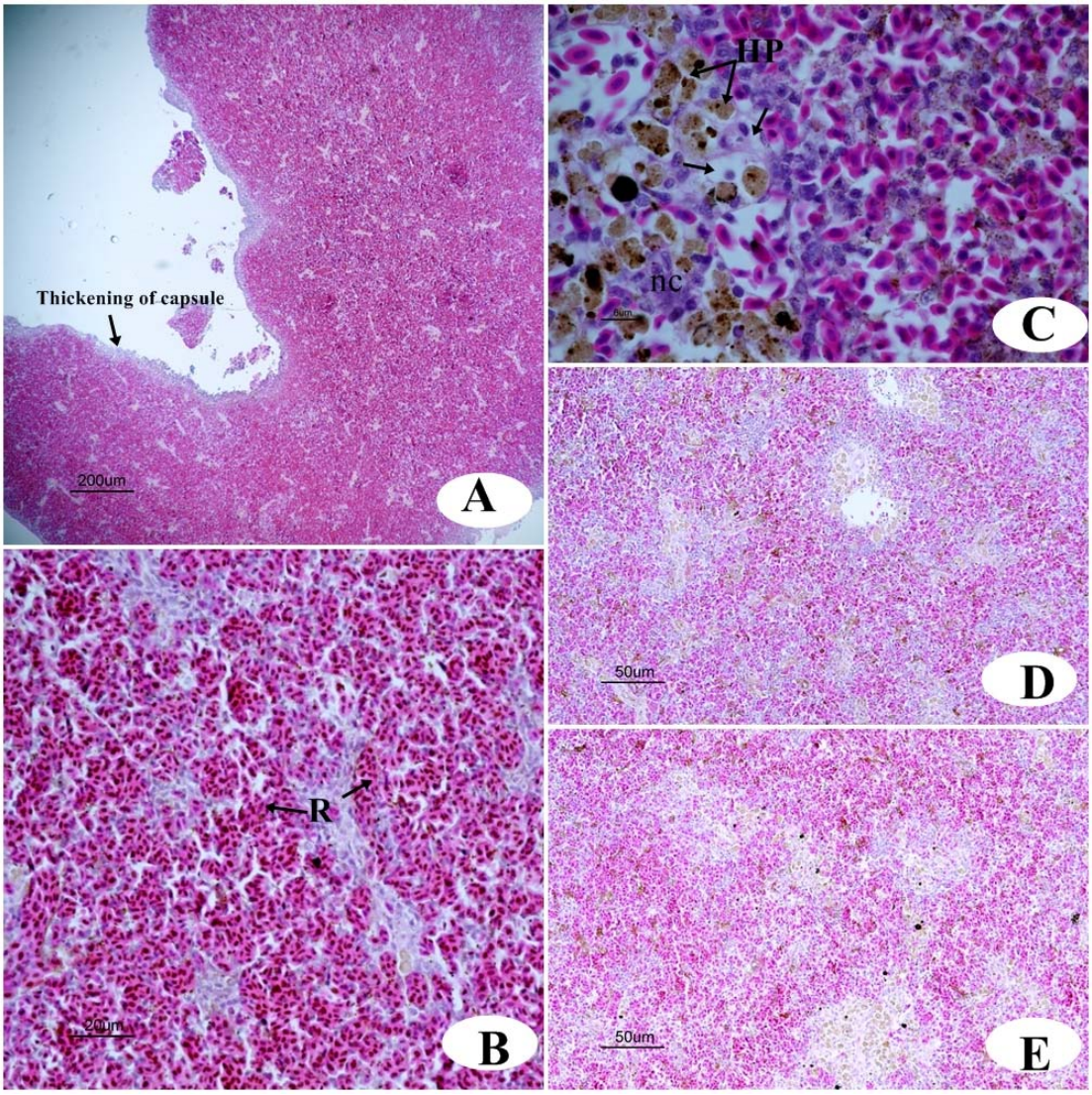


Figure 18. Light micrographs of a transverse section of *O. niloticus* spleen tissues in each experimental group.

A. The group 1 showing thickening of spleen capsule.

B. The group 1 showing vascular congestion and increase in red pulp (R).

C. The group 1 showing necrotic in some areas (nc), cell swelling (arrows) and heamosiderin pigments.

D. The groups 2 and 3 showing mind vascular congestion.

E. The groups 4 and 5 showed similar the histopathological alteration in the group 1, but the alterations were not so extensive

5.2.5.5 Testis

Control group: No recognizable changes were observed in the male reproductive system of the control group (Fig. 19A) groups 2 and 3 (Fig. 19C) throughout this experiment. The testes had normal appearance; containing cells at all spermatogenic stages, and was classified as maturing testes. Histological the testis of the fish consisted of lobules of various shapes, which were connected with each other by thin connective tissues. The external testis is covered by the tunica albuginea (Fig. 19A). The interstitial cells were observed in between the testis lobules. During the spermatogenic process, the sperm mother cell in the germinal epithelium multiplied and produced the primary and secondary spermatocytes, spermatozoa (Fig. 19C) and sperms. The lumina were filled with spermatozoa (Fig. 19A) and the lobules contained numerous spermatogenic cysts lines with sertoli cell (Fig. 19 C)

Treated group: The histopathological alteration of the testis of the group 1 fish showed disorganization of the testis lobules. Most of the lobules had either collapsed or ruptured; the germinal epithelium showed degeneration and desquamation and, in places, was reduced to a sheet of fibrous tissue. The spermatogenic cysts were less abundant than in the control group. Some blood vessel walls had collapsed and large numbers of erythrocytes were present surrounding the lobules (Fig. 19B). The nuclei of the lobuleboudary cells were pyknotic nuclei, indicating necrosis (Fig. 19B). Moreover, the presence of several vacuoles within testis tissue was also observed (Fig. 19E). The damaged testes of the fish in groups 4 and 5 (Figs. 19D-E.) showed similar injury, but the damages were not so extensive. The semiquantitative scoring of spleen lesion is shown in Table 19.

Table 19. Semiquntitative scoring of testis lesion in Nile tilapia (*O. niloticus*) in the experimental groups [a].

Lesion	Experimental Groups					
	control	1	2	3	4	5
Vacuolation	-	+++	-	-	+	++
Blood congestion	-	+++	-	-	-	++
Necrosis	-	++	-	-	-	+

[a] (-) no histopathology; (+) mild histopathology (<25% of fields);
(+ +) moderate histopathology (>75% of fields); (+ + +) severe histopathology (all fields).

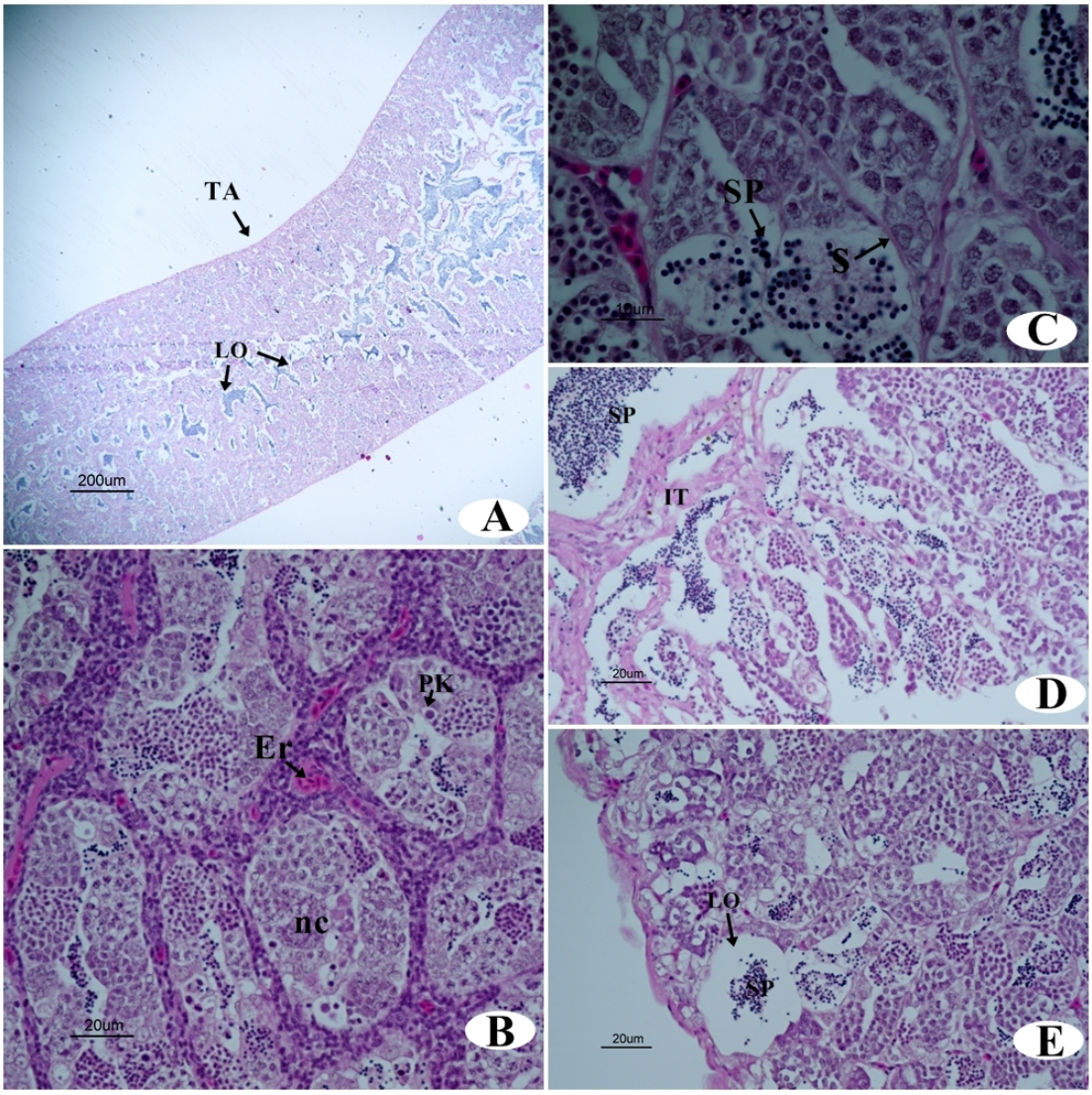


Figure 19. Light micrographs of a longitudinal section of *O. niloticus* testis tissues in each experimental group.

A. Control group showing normal appearance of the tunica albuginea (TA), seminiferus lobules (LO).

B. In group 1 showing pyknotic nuclei (PK), indicating necrosis (nc) in some areas and erythrocytes (ER) surrounding the lobules.

C. In groups 2 and 3 showing normal appearance of spermatozoa (SP) and sertoli cell (S).

D. In group 5 showing degenerative change in the interstitial tissue (IT).

E. In group 4 and 5 showed similar lesions with group 2, but the damages were not so extensive.

5.2.5.6 Ovary

Control group: No recognizable changes were observed in the ovary of the control group. The ovary was covered by membrane, the visceral peritoneum (Fig. 20A). The ovary was in the growth phase found yolk vesicle and yolk granules state. Yolk granules state oocyte has coarse granules that may occupy the whole cytoplasm (Fig. 20B).

Treated groups: No recognizable changes were observed in the ovary of exposed fish. Yolk vesicle and yolk granules had a normal appearance (Fig. 20C).

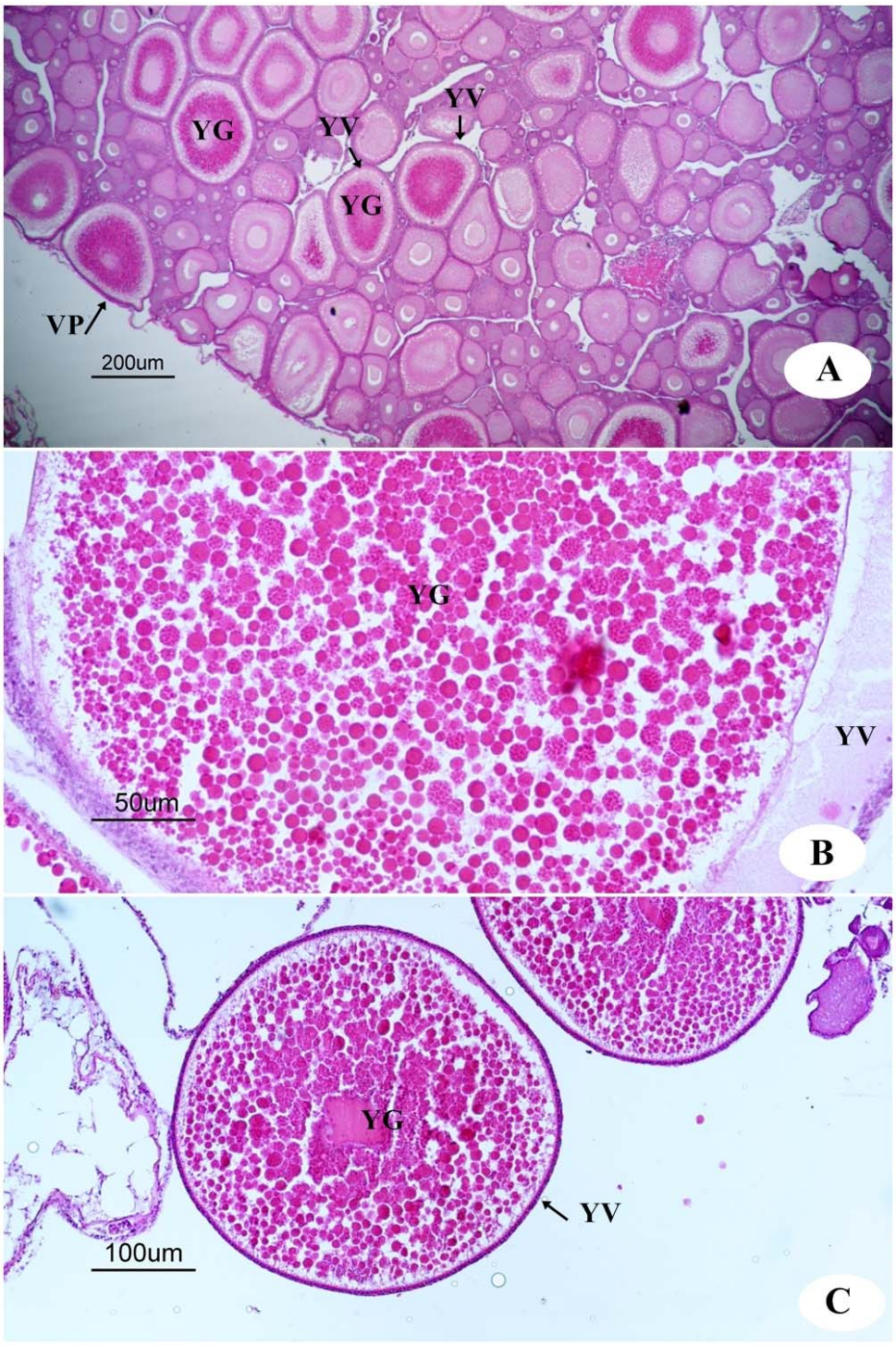


Figure 20. Light micrographs of *O. niloticus* ovary in each experimental groups.

A. Longitudinal section of ovary in control group showing visceral peritoneum (VP) yolk vesicle (YV) and yolk granules (YG).

B. Transverse section of ovary in control group showing yolk coarse granules.

C. Treated groups showing ovary without pathological alterations.

5.2.5.6 Muscle

Control group: No recognizable changes were observed in the muscle of the control fish. The multiple nuclei lay at the periphery of the muscle fibers. Groups of the fibers were surrounded again by larger pale area with loose connective tissues, the perimysium. The whole muscle or muscle bundle was surrounded by a denser connective tissue, the epimysium (Figs. 21A-B).

Treated groups: No recognizable changes were observed in the muscle of exposed fish. The muscle bundles and muscle fibers had a normal appearance (Fig. 21C).

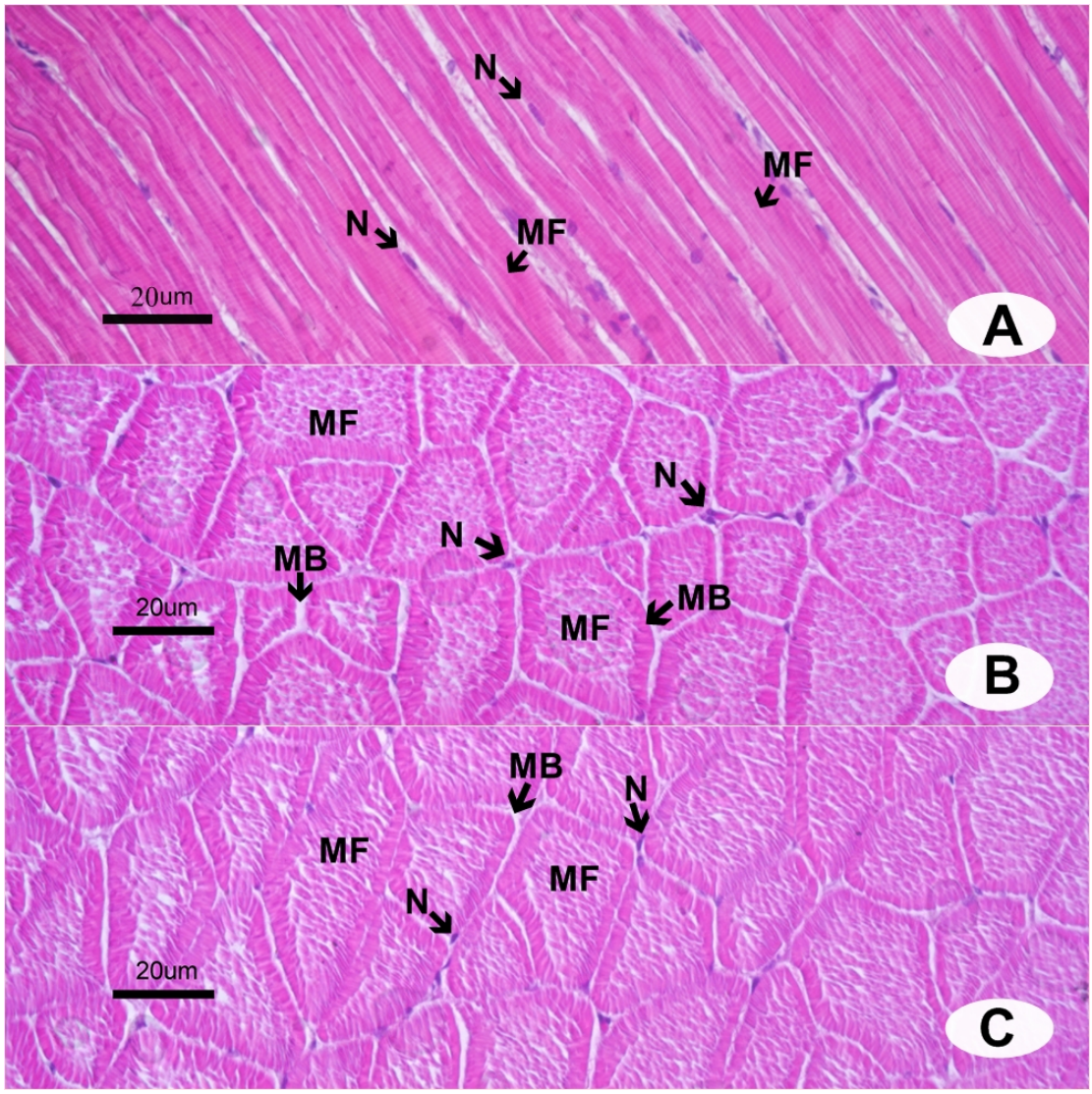


Figure 21. Light micrographs of *O. niloticus* muscle tissues in each experimental group.

A. Longitudinal section of muscle tissue in control group.

B. Transverse section of muscle tissue in control group showing normal muscle bundle (MB) and muscle fibers (MF) with nuclei (N).

C. Treated groups showing muscle tissue without pathological alterations.

CHAPTER VI
DISCUSSION

6.1 Hematological analysis

In the present study, the hematocrit (Hct) value in fish exposed to 25% LC_{50} (45mg/L) of waterborne lead was significantly decreased when comparing with control group. This result agreed with Maheswaran and co-workers who found the attributed such the decrease in hematocrit values due to increase in the size of the erythrocytes as being demonstrated for chromium and zinc treated in rainbow trout. Observed depression in hematocrit and hemoglobin values coupled with decreased and deformed erythrocytes are obvious signs of anemia (Maheswaran et al., 2008).

The hemoglobin (Hb) value in fish exposed to 45mg/L waterborne lead in our study was significantly decreased when comparing with control group. These results agree with that obtained by Sobecka, who studied the decreased of hemoglobin may be attributed to the destructive influence of nickel on the cell membranes of erythrocytes through binding of the toxicants with immunoglobulins or through disturbance of the activity of erythrocyte enzymes, especially those responsible for reduction of glutathione and thiol groups of proteins (Sobecka, 2001). According to Kleczkowski et al., (1998) the excessive loss of glutathione, increased release of iron to intracellular spaces, peroxidation, destruction of cell membranes, and release of metal ions to the surrounding tissues should be attributed to free oxygen radicals. The effect of the described processes is instability of hemoglobin, structural changes in erythrocytes and increased susceptibility to hemolysis. Consequently, the pool of the serum iorn from disintegrating erythrocytes increases, while the iron content in the spleen decreases. In contrast to this results, Ptashynski and Klaverkamp (2002) observed that the concentration of hemoglobin value was unaffected between control and treated lake white fish "*Coregonus clupeaformis*" and lake trout "*Salvelinus namaycush*" by exposure to nickel in diet 0, 10, 100 and 1000 µg for 10, 51, 104 days.

The Erythrocyte count was significantly increased in fish exposed to 45mg/L of waterborne lead. These finding are consistent with the mechanism of adenergically stimulated splenic contraction to release supplemental erythrocytes into the circulatory system to increase oxygen carrying capacity. This explanation agrees with Perry and Wood (1989). The increased erythrocytes count may be due to stimulation of erythropoietin by elevated demands for O_2 or CO_2 transport as a result of increased metabolic activity or destruction of gill membranes causing faulty gaseous exchange (Cyria et al., 1989).

Decrease or increase in certain blood parameters can be associated with the nature of species and the toxicants in different studies. Annune et al. (1994a**)** reported a significant increase in RBC count of *C. gariepinus* when subjected to Zn treatment. They attributed the red blood cell elevation to blood cell reserve combined with cell shrinkage as a result of osmotic alterations of blood by the action of the metal. In another study, a non-significant decrease in red cells for zinc treated *O. niloticus* was observed (Annune et al., 1994b).

The leukocyte count of fish exposed to 45mg/L of waterborne lead was decreased but not significantly along the experimental periods. Leucopenia may be attributed to the inhibition of white blood cell maturation, their release from tissue reservoir or occurrence of leucopoenia by an organism as a response to a stress caused by toxic compounds which associated with allergic reaction (Sobecka 2001). Moreover, the decreased number of white blood cells (leucopoenia) may be the result of bioconcentration of the tested metal in the kidney and liver. Other authors have associated the cause to hindering of granulopoiesis or lymphpoiesis, induced by primary or secondary changes in haematopoietic organs (Tomaszewski, 1997).

The results of this work are conflicting with the results of Maheswaran et al. (2008) who found the number of white blood cells significantly increased in mercuric chloride treated fish (*Clarias batrachus*) for 35 days. This contrast may be due to the different in heavy metal, dose, or even due to varied duration of treatment and time of administration.

In the values obtained in the hematological indices in fish exposed to 45mg/L of waterborne lead, no significant change was recorded in the mean corpuscular hemoglobin (MCH) and but there was significantly decreased in the mean

corpuscular volume (MCV). Cells released from the spleen, which is an erythropoietic organ would have the lower MCV values when compared with the control. As similar observation was made for *Cyprinus carpio* after cadmium exposure (Koyama and Ozaki, 1984). While mean corpuscular hemoglobin content (MCHC) was significantly increased. The increase of Hct value and MCHC may be attributed to swelling of RBCs due to increased CO_2 in blood, hypoxia or stressful procedures (Ellis, 1981; Nemesok and Boross, 1999**)**.

6.2 Biochemical analysis

Transaminases (AST, ALT) enzymes are frequently used to diagnose the sublethal damage to the different organs as well as liver (Rojik et al., 1983; Benedeczky et al., 1984). The aspartate aminotransferase belongs to the plasma non-functional enzymes which are normally localized within the cells of liver, heart, gills, kidneys, muscle and other organs. Monitoring of liver enzyme leakage into the blood has proved to be a very useful tool for toxicity studies in the liver. The present observation on the Nile tilapia in 45 mg/L of waterborne lead revealed a significantly decreased when comparing with control group. This is supported by a reported alteration in the enzyme activity due to the inhibitory effect of metals (Abou El-Naga et al., 2005). According to Oluah (1999), the plasma ALT activity was elevated in the cat fish *(Clarias albopunctatus)* when exposed to subletal zinc and mercury for 14 days.

Alanine aminotransferase is a key metabolic enzyme released on the damage of hepatocytes. This present result of ALT on the Nile tilapia in 45 mg/L of waterbone lead showed significantly increased when comparing with control group, indicating its adaptive response to its leakage into the blood stream due to the metal toxicity. Also, the AST has a part in transforming protein to glycogen, which is the major reserve fuel of the body during the stress-induced toxicity in the liver. This result is in accordance with the results of previous investigators on freshwater fish, the results indicate that under the influence of different heavy metals or in a state of stress,

the damage of tissues and organs may occur with concomitant elevation and liberation of transaminases into the circulation (Zikic et al., 1997).

According to Watson et al., (2008) nonspecific chronic hepatitis and increased activities of serum aminotransferases have been reported in dolphins, porpoises, and whales, that biochemical changes in these animals may provide clues as to potential causes of liver disease in cetaceans (Stephanie et al., 2008). Additional, Zaki and colleagues, who reported exposure of Nile tilapia to subletal concentration (1.5 mg/L) of lead acetate for 14 days resulted in marked increase in activities of serum AST and ALT (Zaki et al., 2008).

The present biochemical findings agree with the microscopic findings on histopathological analysis, which revealed a marked degeneration and necrosis of hepatocytes as the elevation in transaminases activities may be attributed to the liver injury.

The quantitative determination of the total protein in the blood serum gives an idea about the condition of the liver cells and consequently it is of vulnerable effect in the diagnosis of the toxicity of fish. In the present study, serum total protein of the 45 mg/L waterborne lead exposed tilapia fish was significantly increased during the exposure period. This results similar finding as Al-Attar, who found the total protein was significantly elevated at 4 and 7 days cadmium exposure on *O. niloticus* (Al-Attar, 2005) and Zaki et al., who reported the serum protein level significantly increased in 14 days post exposure to lead. The elevated protein concentration may be due to the induction of protein synthesis in liver (Zaki et al., 2008). The observed hyperproteinemia in *O. niloticus* following cadmium administration is possibly due either to water loss in the serum, the relative changes in the mobilization of blood protein, or elevated de novo synthesis. These findings and explanation are comparable to those considered by several studies on toad and fish exposed to cadmium, lead, mercury, copper and nickel. (Hilmy et al., 1968; Ruparelia at al., 1989; Gopal et al., 1997).

6.3 Histopathological analysis

In the fish gills, serve as a major organ for osmotic and ion regulation and respiration. Because of the highly vascular structure of the gill epithelium, it is a primary target for waterborne toxicants. Histopathological alteration on gill epithelial in several previous studies have been described similarly in this study i.e., gill cells degeneration, uplifting of epithelial, necrosis, lesions and inflammatory infiltration. The first sign of lesions in the present study included edema of epithelial cells. The secondary lamellae showed capillary congestion or aneurism, similar to those reported in *Puntius altus* exposed to cadmium (Jiraungkoorskul et al., 2006), *Poronotus triacanthus* exposed to copper (Jiraungkoorskul et al., 2007). The lamellar aneurism resulted from the collapse of the pillar cell system and the breakdown of vascular integrity with a release of large quantities of blood that push the lamellar epithelium outward. Otherwise, thickening of the primary lamellar epithelium, hypertrophy and hyperplasia of epithelial, chloride cells, lifting and fusion of secondary lamellae were also observed. The distal extremities of secondary lamellae were bent, with a reduction of the interlamellar space. Several studies pointed out that chloride cell hyperplasia occurred in response to the need to eject the toxicant absorbed by the gills (Gill et al., 1988; Marshall and Grosell, 2005).

In the fish liver not only acts as a storage organ, but is also the primary site for detoxification mechanisms (Olsson et al., 1996). The histopathological alterations in the liver observed in the present study were sinusoid dilation with blood congestion, vacuolation, hemosiderin accumulation and cell necrosis. The vacuolation of hepatocytes might indicate an imbalance between the rate of synthesis of substances in the parenchymal cells and the rate of their release into the systemic circulation (Gingerich, 1982). Toxic of Pb can result in the injured cells dying by necrosis. These results are in agreement with those obtained by El-Saeed and Ptashynski et al. (2001) and Sobecka (2001). The inactivation of the pancreatic acini and the vacuolar and hydropic degeneration of hepatocytes may be due to the irritation of toxic metabolites and impairment of potassium sodium pump that disturb the ion exchange through the cell wall. The periductal fibrosis and newly formed bile ductules may be due to the

persistent of the toxic effect for long time (sub chronic intoxication) which pointed out by fibrous tissue proliferation (Atallah et al., 1997).

The histopathological alterations occurred in the kidney in this study were dilation of Bowman's space, glomeruli atrophy and tubular swelling. The degeneration and necrosis of tubular epithelial cells of the first proximal tubule were also observed. Because the excretion of divalent ions is a major function of the renal tubular epithelium, pollution with heavy metals would be highly likely to affect these cells. On the same side, this pattern agrees with those obtained by Ptashynski et al., the congestion and hemorrhage among the renal tubules may be due to increase in the permeability and subsequently escape of the blood components especially RBCs out side by diapedesis leading to focal hemorrhage under the influence of toxic metabolites of nickel (Ptashynski et al., 2002). Some glomeruli appeared contracted as a result of the pressure of edematous fluid, which accumulated in the Bowman's capsule (Ferguson, 1989). Areas of activation of hematopoietic elements might be a general response due to the initiation of the toxicity of nickel. The depletion may be long persistence of the toxic effect. The hydropic degeneration may be due to the impairment of the electrolytes exchange between the intracellular and extracellular fluids (Roberts, 1978).

The spleen showed hyperplasia of megakartyocytes which increased in number also became darker in color (dark brown). Large areas of depletion of hematopoietic elements were encountered. The number, size and histological appearance of megakarytocytes changed with age, season, state of nutrition and outigenic exposure. The number and size of megakartocytes increase in the chronically sick fish, old fish or excessive catabolism (Ferguson, 1989). The catabolism highly increased due to persistence of stress.

Pollutants may have direct effects on the gonads, resulting in a disturbed development of germ cells. Indirect effects on reproduction, *via* interference with the regulating hormonal system, have also been suggested (Janssen et al., 1997). According to Ruby et al., the testis contains a fixed number of spermatogonia which are responsible, by mitosis, for the production of additional spermatogonia to maintain the processes of spermatogenesis and sperm production (Ruby et al., 1979). Therefore, the observed degenerative and necrotic changes in the cellular elements,

including spermatogonia, of the seminiferous tubules in the testis of both fish result in permanent testicular damage and may reduce the ability of fish to reproduce. High metal accumulation occurring in the testis, affects the process of spermatogenesis and suppressing sperm production. The present results are in agreement with those observed by many authors who have studied the effects of metals on fish testis (Hanna et al., 2005; Yamaguchi et al., 2007).

No recognizable changes were observed in the ovary. This result is conflicting with the results of Marwa et al., who found the tunica albuginea was loose and the oogonia were destroyed. The cytoplasm of early and late perinucleolus oocytes stained deep purple. The perinucleolus oocytes appeared as a solid mass, also late perinucleolus oocytes become atretic on the effect of pollution on the ovary of *O. niloticus* from Rosetta Branch, Nile River, Egypt (Marwa et al., 2009). This contrast may be due to the different dose, or even due to varied duration of treatment and time of administration.

In the muscle, there was no lesions occurred in fish. This agree with the results of Peebua et al., who studied on Nile tilapia exposed for one month to sediments from the Mae Klong River, Samutsongkram province, South West of Thailand, which contained elevated levels of heavy metals, lead and chromium. No recognizable changes were observed in muscle tissue (Peebua et al., 2005).

6.4 Nuclear morphology analysis

The results of this study similar to previous report in *P. altus* exposed to cadmium (Jiraungkoorskul et al., 2007). Fish exposed to Pb showed a statistically significant ($p \leq 0.05$) increased in the hematological parameters. MN was cytoplasmic chromatin masses with the appearance of small nuclei that arose from chromosome fragments or intact whole chromosomes lagging behind in the anaphase stage of cell division. Their presence in cells was a refection of structural and/or numerical chromosomal aberrations arose during mitosis. MN and NA tests in fish were generally performed in enucleated peripheral blood erythrocytes mainly due to its

technical feasibility. It was well known that heavy metals interfered the regular chromosome segregation during cell division mainly by inhibition of polymerization of actin tubules, an essential structure of the mitotic spindle (Miura and Imura, 1987). Furthermore, it was suggested that the mechanism of Pb genotoxicity was mainly conditioned by single strand broke in DNA through the direct lead-DNA interaction by single strand broke in DNA through the direct lead-DNA interactions as well as by the action of incision nucleases and/or DNA-glycosylase during DNA repair like those of cadmium (Privezentsev et al., 1996). Correspondingly, most of the toxic chemicals that produced genotoxic effects have been known to form reactive oxygen species as well as electrophilic free-radical metabolites that interacted with DNA to cause disruptive changes. It has been suggested that during the heavy metal exposure, electrophilic ions and radicals were produced, interacted with nucleophilic sites in DNA which lead to break and other related damage in the latter.

Arkhipchuk and Garanko reported the highest increased of cells having micronuclei were observed in blood after fish exposed to cadmium (Arkhipchuk and Garanko, 2005). In the present study, there was a general tendency of occurrence of notched and lobed types in higher frequencies. An analysis revealed spontaneous frequencies of nuclear abnormalities in erythrocytes, gill, liver, kidney and fin cells were found in the following order: NT >LB > BN > BL. Thus, in the present study seem to be in agreement with previous studies (Cavas and Ergene-Gozukara, 2003; Mallick and Khuda-Bukhasa, 2003).

Further, it would be revealed from the results of this study that toxicity of waterborne lead on hematology, histopathology, biochemical and nuclear morphology effects. Interesting the calcium supplementary dietary reduces the toxic of waterborne lead. Previous studies have found significant reductions in whole body Cd uptake from the water and diet when rainbow trout were fed a diet supplemented with 60 mg $CaCO_3$ g^{-1} (Baldisserotto et al., 2004b, 2005; Franklin et al., 2005), or 60 mg $CaCl_2$ (Zohouri et al., 2001; Baldisserotto et al., 2004a). Freshwater fish have two primary uptake pathways for essential ions i.e., Ca^{2+}, Na^{+}, the gills as waterborne ions and the gastrointestinal tract as dietary ions. They can regulate the total uptake by changing the proportion of each kind of uptake depending on the environmental situations. For example, tilapia (*O. mossambicus*) up-regulate intestinal Ca^{2+} uptake when living in

water with low Ca^{2+} concentration (Flik et al., 1995). Therefore, if the fish can acquire more ions via the gastrointestinal route, they may decrease branchial ion uptake rates and thereby subsequently reduce the uptake of metals sharing the common branchial pathway. For example, Cd^{2+}, Zn^{2+} shares the same transport pathway with Ca^{2+} (Verbost et al., 1989; Niyogi and Wood, 2004) and previous studies have shown that dietary Ca^{2+} supplementation decreased waterborne Ca^{2+}uptake and subsequently waterborne Cd^{2+}uptake (Zohouri et al., 2001; Baldisserotto et al., 2004ab, 2005). There is no available data of Ca^{2+} concentrations in the natural diet of feral fish, but Ca^{2+} is available in abundance in crustacean exoskeleton and mollusk shells. Interestingly though, Sherwood et al. (2000) reported that wild yellow perch (*Perca flavescens*) in heavy metal impacted lakes tend to eat relatively more invertebrates than fish.

CHAPTER VII
CONCLUSION

The present study investigated the influence of dietary calcium supplementation to reduce the toxicity of sublethal lead concentration in Nile tilapia with emphasis on hematological, biochemical, histopathological and nuclear morphology analysis.

The acute toxicity test to determine the LC_{50} of 24, 48, 72 and 96 h LC_{50} of lead nitrate ($Pb(NO_3)_2$ to tilapia were 247.51, 197.47, 193.36 and 182.38 mg/L respectively. Therefore, lead concentration tested in the sublethal experiment was 45 mg/L, which correspond to 25% of the 96 h LC_{50}. Fish were fed with 0, 20 and 60 mg Ca^{2+} g/g food for 30 days.

The hematological analysis investigation in Pb treatment, erythrocytes count, MCHC showed significant elevation, while the Hct, Hb and MCV were increased significantly in Pb expose when compare with the control group ($p \leq 0.05$). The lecucocyte count and MCH were slightly decreased but there were not significant.

The biochemical analyses were significant increased ($p \leq 0.05$) in aminotransferase activities and total protein. Change in biochemical analysis could be correlated to the structural damages.

The histopathological analysis in Pb treatment in the gills was showed hyperthrophy and hyperplasia of chloride cells and mucous cells at the base of gill filament and secondary lamellae. Fusion of secondary lamellae was observed. Sever edema of epithelial cells and aneurysm was found. In the livers of fish showed several pathological changes. The general lesions were pyknotic nuclei and large lipid vacuoles in the cytoplasm of hepatocytes. Some liver areas showed focal necrosis and contained severe infiltration of leukocytes. In more severe case, the liver showed mild to moderate blood congestion in sinusoids and hydropic swelling of hepatocytes. In kidney showed collapsed or atrophy glomeruli and tubular necrosis. Hydropic swelling

and hypertrophy of tubules with dilated nuclei and necrotic tissue were found. In more severe cases, the tubular cells showed hyaline droplet and dark granule. In the spleen showed several pathological changes. There were large numbers of megakaryocytes due to the pollution. Thickening of membrane of the spleens, coarsening of trabeculae of the spleens, fibrosis round splenetic arteries, and stasis of blood and dilatation of the splenetic sinus and necrotic in some areas were main features of the spleens. In the testis showed disorganization of the testis lobules. Most of the lobules had either collapsed or ruptured; the germinal epithelium showed degeneration and desquamation and, in places, was reduced to a sheet of fibrous tissue. The spermatogenic cysts were less abundant than in the control group. Some blood vessel walls had collapsed and large numbers of erythrocytes were present surrounding the lobules. The nuclei of the lobule boundary cells were pyknotic nuclei, indicating necrosis. Moreover, the presence of several vacuoles within testis tissue was also observed. No recognizable changes were observed in ovary and muscle tissue.

The MN values in erythrocytes, gills, liver, kidney and fin cells showed significantly increased. The NA shapes in erythrocytes, gills, liver, kidney and fin cells were scored into blebbed nuclei (BL), lobed nuclei (LB), notched nuclei (NT) and binuclei (BN). The frequencies of each NA shapes in tissue of all treatment observed in following NT > LB > BN > BL.

Fish fed with Ca^{2+} supplemented diets showed slightly alteration on hematological, biochemical, histopathological and nuclear morphology analysis when compare the only Pb treatment groups

The results presented in this study show that the efficacy of dietary calcium supplementation in reducing hematological, biochemical, histopathological and nuclear morphology alterations associated with sublethal exposed to waterborne lead uptake in fish.

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APPENDIX

LIGHT MICROSCOPE STUDY

Preservative solution (10% Formalin)

1. Formalin (40% Formaldehyde)	1000 ml
2. Distilled water	9000 ml
3. Di-sodium-hydrogen-phosphate, anhydrous	65 g
4. Sodium-di-hydrogen-phosphate, monohydrate	40 g

Haris's haematoxylin Stain

1. Aluminium alum	40 g
2. Distilled water	400 ml
3. Haematoxylin	2 g
4. Absolute alcohol	20 ml
5. Mercuric Oxide	1 g
6. Glacial Acetic Acid	5 ml

Eosin Stain

1. 1% Eosin in water	100 ml
2. 1% Phloxin-B in water	20 ml
3. 95% alcohol	780 ml
4. Glacial Acetic Acid	4 ml

Schedule for histopathological process

No	Method	Duration	
1	Dissection		**First day**: Removal and fixing tissues in 10% buffered formaldehyde 24 hours.
2	10% buffered formaldehyde	24 hours	
3	70% alcohol	24 hours	**Second day**: Washing out the fixative
4	80% alcohol	40 minutes	**Third day**: Dehydration
5	95% alcohol	40 minutes	
6	95% alcohol	40 minutes	
7	Absolute alcohol	40 minutes	
8	Absolute alcohol	40 minutes	
9	Xylene	1 hour	
10	Xylene	1 hour	
11	Paraffin	40 minutes	
12	Paraffin	40 minutes	
13	Embedding		
14	Sectioning	24 hours	**Fourth day**: Sectioning, spreading on glass slides, drying overnight
15	Staining		**Fifth day**: Staining, mounting, and examination
16	Examination		

Schedule for staining sections

No	Method	Duration
1	Xylene (I)	5 minutes
2	Xylene (II)	5 minutes
3	Xylene (III)	5 minutes
4	Absolute alcohol (I)	3 minutes
5	Absolute alcohol (II)	3 minutes
6	Absolute alcohol (III)	3 minutes
7	95% alcohol	3 minutes
8	80% alcohol	3 minutes
9	70% alcohol	3 minutes
10	Running water	7 minutes
11	Hematoxylin	7 minutes
12	Running water	7 minutes
13	Eosin	3 minutes
14	95% alcohol (I)	3 minutes
15	95% alcohol (II)	3 minutes
16	95% alcohol (III)	3 minutes
17	Absolute alcohol (I)	3 minutes
18	Absolute alcohol (II)	3 minutes
19	Absolute alcohol (III)	3 minutes
20	Xylene (I)	5 minutes
21	Xylene (II)	5 minutes
22	Xylene (III)	5 minutes
23	Mounting	
24	Examination	

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