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The Effect of Oil Exposure on the Tissues and Health Status of Gulf of Mexico Fishes

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By

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The Macondo 252 oil spill occurred on April 20, 2010 and persisted for 86 days. Oil spill exposed Gulf fish demonstrated reduced lymphocyte counts and increased ethoxyresorufin-O-deethylase (EROD) values. Alligator gar were exposed to 0, 0.5 and 4.0 g oil/L of tank water for 48 hours, then moved to oil-free water for a 7 day recovery period. After forty-eight hours exposure, lymphocyte and hematopoietic precursor populations were significantly decreased in dose responsive by exposure to oil. Following a 7 day recovery period, lymphocyte and precursor cell numbers increased. After 48 hours exposure, tissue changes included hepatocellular vacuolization and necrosis, necrotizing pancreatitis, splenic congestion and epicarditis. This demonstrates that oil exposure negatively impacts immune cells and tissues in fish and increases their disease susceptibility. Prolonged oil exposure has the potential to dramatically impact the health status of fish populations.

Keywords: *Atractosteus Spatula*, Gulf of Mexico, Effects of petroleum products

DEDICATION

This thesis is dedicated to my parents, who introduced me to the joy of reading from birth, enabling such a study to take place today. I would like to thank my wife, for her patience, encouragement, and support through this study. In addition, I would also like to thank my brothers and sisters for their love gave me forces to make this work.

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CHAPTER I

INTRODUCTION

Gulf of Mexico disaster

The Macondo 252 petroleum oil spill resulted from the blow out of the Deepwater Horizon drilling platform, and happened on April 20, 2010, 50 miles southeast of Mississippi canyon block 252 in the Gulf of Mexico (Fig 1.1). Approximately 4.9 million barrels (206 million US gallons) of crude oil were released into the water at that location [1]. This disaster was considered the largest environmental disaster that has occurred in the United States. In comparison, the EXXON Valdez oil spill that happened in 1989 in Prince William Sound, Alaska resulted in approximately 262 barrels (11 million US gallons) released onto the Alaskan coast [2]. Many attempts to plug the Macondo 252 leak failed, and oil spilled for 86 days. Approximately 28,958 square miles of Mississippi, Florida, Alabama and Louisiana were contaminated with oil. Heavy oiling threatened wildlife in coastal Louisiana marshes. The Fish and Wildlife Service estimated 32 species of wildlife were affected by contact, ingestion, inhalation, or absorption of the oil, and 36% of the federal water off the coast was banned for fishing during the spill. Oil can persist in the environment for a long time, continuing to affect wildlife and fish, and interacting with the environment, after a surface slick is no longer visible [1].

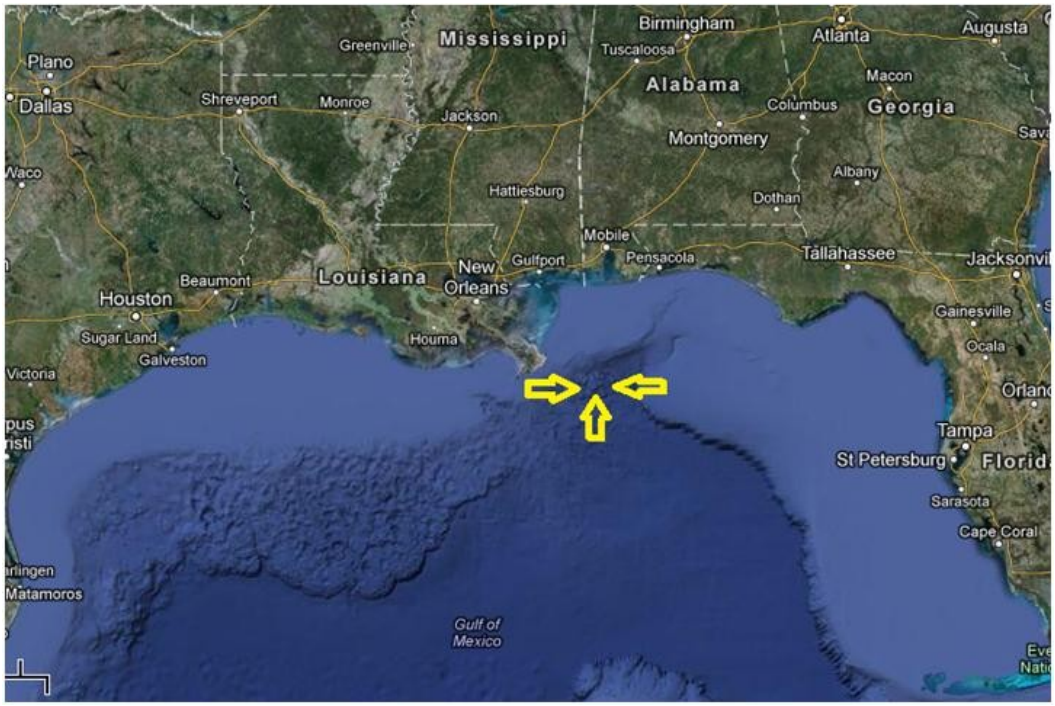

Figure 1.1 The oil spill originated 50 miles southeast of Mississippi Canyon Block 252, indicated by arrows.

After the Exxon Valdez oil spill, multiple sand, mud, water, bird and marine mammal studies were performed to determine the acute and chronic effects of oil and petroleum product contamination, reviewed in [3]. However, three studies examined the health status of affected fish [4-6]. Remarkable histological lesions were found in Dolly Varden (hepatic necrosis), Rockfish (hepatocellular megalocytosis) and Pacific herring (hepatocellular necrosis). Fish population declines began in the year following the EVOS [7] and increased occurrence of fish diseases occurred in the years following the EVOS [5] demonstrated that laboratory exposures of oil resulted in decreased inflammatory cells, leading to immunosuppression.

Many effects of the oil are not known, but absorption of chemicals in the oil has caused immunosuppression, skin lesions and ulceration in fish [8]. Two years after the oil

spill, shrimp, crab, and fish that were found have skin lesions, and lack eyes and gill opercles. A survey led by the University of South Florida after the spill revealed that 2 to 5% of the Gulf fish have skin lesions or having sores, compared to before the oil spill occurred [9].

There were two main objectives in this study. The first was to determine if oil exposure adversely affected the health status of fish in the Gulf of Mexico. The second was to conduct laboratory experiments using specific amounts of emulsified oil to determine if oil exposure resulted in predictable tissue damage and increased disease susceptibility. This study utilized hematology, toxicology, and histology to determine the effects of crude oil exposure on fish tissues and blood cells, and the resulting health status of these fish.

CHAPTER II

LITERATURE REVIEW

The effects of petroleum compounds on aquatic animals

Crude oil exposure affects different species of wildlife in different ways. Weather can increase the severity of affects in both fish and plants. In general, oil spreads into a thin layer on the surface on the water and interacting with the environment. Plants also respond differently to crude oil and may die or grow abundantly. Fish are uptake the oil directly by ingestion, by ingestion of contaminated fish, or by absorption through the gills or epidermis. Oil exposure can reduce the growth of fish, and cause systemic diseases or skin lesions, and enlarge the liver [10]. Following the 2010 Gulf Oil Spill, water sampling demonstrated significant increases in bioavailable polycyclic aromatic hydrocarbons [11]. The ultimate affects of oil exposure on fish biology are unknown. Genomic microarray assessment of Gulf killifish tissues found that oil exposure causes significant changes to the biology of fish [12].

What is crude oil?

Petroleum, or crude oil is a flammable liquid found very deep far below the earth's surface. Oil companies pump it to the surface and store it in barrels for future refinement. Crude oil consists of hydrocarbons and other organic liquids. Many of these components are toxic, and the main abundant components are Aliphatics, Alicyclics, and

Polycyclic Aromatic Hydrocarbons (PAHs). Most of the PAHs are toxic, and it is very difficult to remove them from water. These components are highly toxic to humans and wildlife, and are also carcinogenic. PAHs that sedimented in water can accumulate in the tissues of fish and can also effects on their food [10].

There are different types of crude oil. The first one known as a light or volatile oil that is volatile and can evaporate very quickly like gasoline and jet fuel. This type is the most toxic oil. The second one is non sticky oil; this one can potentially cause a long term contamination in the environment like diesel, light crude oil. The third type is sticky and also called heavy oil. It is the most of the crude oil, and it is brown to black in color. This one cannot be mixed to the water, and it is less toxicant to the environment [10].

Ethoxyresorufin-O-deethylase (EROD) is useful to use as a biomarker and an indicator of toxic compound uptake in fish. EROD activity performs by cytochrome P450 A1, an evolutionarily conserved enzyme involved in clearance of hydrocarbons [13]. This enzyme is produced following exposure to hydrocarbons that are abundant in crude oil. There are diverse group of cytochrome 450 superfamily, and the CYP 450 functions are to catalyze the oxidation of tissue substances such as steroidal hormones, lipids, and chemicals. CYP enzyme proposes to increase when fish exposed to chemical pollution such as crude oil. CYP enzyme is abundant mainly in the liver, and can be found in gastrointestinal tract, and in the kidney [14].

Alligator Gar life history

Alligator gar was first described in 1983 [15], and named *Lepisosteus spatula,* a Latin derivative of the Greek word meaning any tool with a board. In 1976 this name was changed by Wiley to *Atractosteus spatula* in order to distinguish two distinct extant

genus of gar. This fish is also called the alligator gar, gator, greater gar, or Mississippi Alligator gar [16]. This fish has a very broad head that is flattened, with two rows of sharp long teeth. The dorsal surface is mud green or olive, and there are black spots on the fins and rounded wide tail. Young alligator gar have stripes on their sides. The scales fit together and are covered with a durable substance called ganoine [17]. The air bladder of this fish is spongy and highly vascular, so it acts like a lung to aerate the fish's blood. There is a connection between the air bladder and esophagus, the pneumatic duct, which allows the fish to gulp air. This fish can live in turbid and stagnant water, and even stay alive out of the water for 2 hours. This fish eats anything that they can get in their mouths. Alligator gar is attracted to light, and can be seen near the surface of the water. This fish can grow to 10 feet in length, and 300 pounds in weight. The average lifespan in males is 26 years and 50 years in females [15].

Sea trout life history

The scientific name of this fish is *Cynoscion arenarius* from Sciaenidae family. The common names of this fish are white trout and sand seatrout. This fish can be confused with the silver seatrout, the sand seatrout is larger and abundantly found inshore than silver seatrout. Sand seatrout is a slight yellow hue, while the silver seatrout is more silver in general [18]. Seatrout distribution is widely along the Atlantic and gulf coasts of Texas, Gulf of Campeche, and Mexico. They were found abundantly from the eastern seaboard of Virginia to Florida, and the highest numbers were found along the Gulf of Mexico from Texas to west coast of Florida.

Gulf killifish life history

The scientific name of this fish is *Fundulus grandis* from Cyprinodontidae family. The other common name for this fish is Gulf killifish, and it is one of the largest killifish species. Killfish are distributed along the coast of Florida to the Gulf of Texas. The caudal peduncle is relatively deep, and their head are blunted. They have dorsal fin in the front of the anal, and the gills are rakers and in lacking well defined vertical bars in males and black spots in females on each side [19]. The base color of this fish is dull greenish on top of the shading to the yellow below, and half of the caudal and the anal are yellow too. The dorsal side of the caudal is dark with white splotches at base. Females are larger than males, and their colors are live to olive yellow [20]. This fish eats the larvae and pupae of mosquito and grass shrimp. In 2009 National Oceanic and Atmospheric Administration (NOAA) listed this fish as a species of concern for coastal areas of Mississippi, Louisiana, Alabama, and Florida because it is rare and threatened by human petrochemical activities [21].

CHAPTER III

SAMPLING DURING THE OIL SPILL: ALLIGATOR GAR AND GULF KILLIFISH IN TERREBONNE BAY AND SEA TROUT FROM THE NORTHERN GULF

Introduction

The Deepwater Horizon disaster was unprecedented. In 1989, the Exxon Valdez Oil Spill occurred in Prince William Sound, Alaska. Fish population declines and disease outbreaks began the following year. Increased occurrence of fish diseases suggested an oil exposure associated perturbation of fishes' immune systems [22].

In healthy fish, leukocytes are present in specific proportions and locations in body tissues. These cells orchestrate the initial line of defense against pathogens. Environmental conditions that negatively impact these cells result in disease outbreaks. Ethoxyresorufin-O-deethylase or EROD activity is performed by cytochrome P450 A1, an evolutionarily conserved enzyme involved in clearance of hydrocarbons. This enzyme is induced following exposure to hydrocarbons, such as those found in crude oil, and is an indicator that hydrocarbon exposure has occurred [14]. The first aim of this investigation was to examine leukocyte populations in fish exposed to the oil *in situ*. The second aim was to determine EROD levels in non-oil exposed and oil-spill exposed alligator gar, Gulf killifish and sea trout.

Materials and methods

Oil-exposed Alligator gar were captured in marshes near Terrebonne Bay via jug-lines that were set overnight. Fish were pulled out of the water in the morning, immediately stunned by a blow to the head, and blood collected via cardiac puncture in lithium-heparinized vacutainers using 22 gauge needles. Blood samples were mixed by inversion, placed on ice and transported to the College of Veterinary Medicine (CVM). Blood from control juvenile alligator gar held in flow-through tanks at the Mississippi Agricultural and Forestry Experiment Station (MAFES) was non-lethally collected by caudal venipuncture in lithium-heparinized vacutainers using 22 gauge needles. Blood samples were mixed by inversion, placed on ice and transported to CVM.

Gulf Killifish were captured by a small seine in the same location as described for the alligator gar, and transported to MAFES in Starkville, MS. Part of the killifish were euthanized with 340 ppm tricaine methane sulfonate. A dorsal incision was made and blood collected from the caudal vein. The remainder of the killifish were held in a recirculating system simulating estuarine conditions, and allowed to recover from the oil exposure. After two weeks, the killifish were bled.

Sea Trout were collected from oil-exposed waters in the north-eastern Gulf of Mexico, in a trawl haul. A dorsal incision was made, and blood collected from the caudal vein. Control sea trout were reared in an in-land facility in Louisiana. Ten fish were euthanized in 340 ppm tricaine methane sulfonate and blood collected from the caudal vein.

After the blood was collected for each type of fish, blood smears were prepared, fixed and stained using a Hema-3 Stat Pack according to the manufacturer's instructions.

Differential leukocyte counts were performed based on morphological appearance, and cells were identified based on previous descriptions of comparative teleosts [23-25]. Staining was performed using established procedures optimized for fish [25-26]. Viewing and interpretation followed the same methods. One hundred leukocytes were counted on each slide and thrombocyte numbers were determined by counting the number of thrombocytes seen while counting 100 leukocytes.

For the alligator gar flow cytometric analyses involved forward scatter (FSC) and side scatter (SSC) determinations on a FACS Calibur (Becton-Dickinson), and were performed as previously described [23]. $DiOC_6$ was used to enhance cell properties for flow cytometric analyses [27].

For EROD analyses, liver samples for all the fish were flash frozen in liquid nitrogen and transported to the Center for Environmental Health Sciences in the College of Veterinary Medicine at Mississippi State University, and stored at -70 C until processed. A microsome preparation was made from each fish liver, using standard procedure developed for fish [14]. Tissues were homogenized by grinding them in 8 mL of cold Tris-HCL buffer PH 8.5 in a glass Potter- Elvehjem apparatus. Microsome suspensions were then transferred to cold centrifuge tubes and centrifuged at 17,000 RFC for 15 minutes at 4C in high speed Beckman centrifuge. The supernated were transferred to cold ultra centrifuge tubes and centrifuged at 34,000 RFC for 1 hour at 4 C to generate microsome pellets. Pellets were rinsed and resuspended in glycerol.

The standards of resorufin and ethoxysesorufin were prepared daily by following the manufacture's instruction (SIGMA). The chemicals that were used for this experiment are showed below in (table 3.1), and were added to 96 well plate. Three wells

were used for each liver sample. Temperature of the Spectrophotometer was set on 23 C, and the Excitation was set on 530 nm and emission on 582 nm. The 96 well plate was preheated with all chemicals except substrate for 5 minutes. After 5 minutes, substrate was added and the plate returned into the Spectrophotometer. The plate was incubated again for 5 minutes, and then the absorbance documented (Fig 3.1).

Table 3.1 Chemicals and quantity used for EROD analyses.

	Sample µl	Blank µl	Standard µl
0.05 Tris-HCL Buffer	170	215	200
100% tissue	30	30	30
MgSo4	15	15	15
Generating System	30	30	30
G-6 Dase	10	10	10
Substrate	45	-	-
Standard	-	-	15

100% tissue is made by 1g/1 ml. Generating system is made from glucose-6-phosphate, NADP, glucose-6-phosphate dehydrogenase, and buffer. The substrate was 7-ethoxyresorufin and buffer.

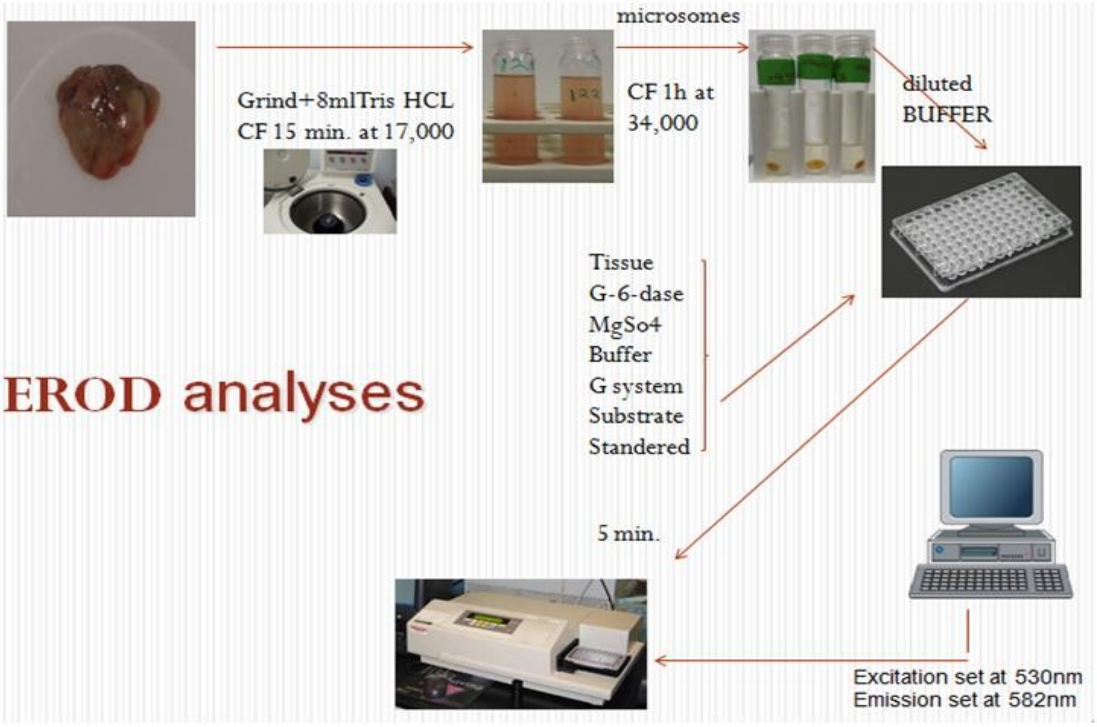

Figure 3.1 The process of EROD analyses.

Results

Oil exposed Gulf killifish demonstrated significantly reduced lymphocyte and eosinophil counts, and significantly increased monocyte counts compared to recovered killifish (Fig 3.3). In sea trout, peripheral blood lymphocytes were lowered in the Gulf fish compared to the land controls, while eosinophil numbers were significantly greater in the Gulf fish (Fig 3.4).

EROD values for the sea trout collected from the Gulf were significantly greater than EROD values from sea trout reared in an in-land facility (Fig. 3.5). EROD values for alligator gar and Gulf killifish sampled from Terrebonne Bay and control or recovered fish did not demonstrate statistically significant differences or any biological trends.

Interpretations

In Gulf killifish and sea trout, the response to oil exposure was a decreased number of circulating lymphocytes, which makes fish more susceptible to infectious diseases. Following the EVOS, oil exposure was associated with decreased peripheral blood lymphocyte counts. The 7 days recovery period, the peripheral blood lymphocyte counts returned to normal ranges. The wild alligator gar did not demonstrate significant differences in leukocyte cell counts from the hatchery reared control fish. However, this was the first time flow cytometry was compared to manual leukocyte differentials and the results indicated the methods corresponded well (Fig 3.2 A, B).

The sea trout EROD results suggest that fish caught in the Gulf of Mexico in November, 2010 had been exposed to hydrocarbons. EROD activity in all animal species is induced by hydrocarbons. The alternative explanation is there are naturally occurring hydrocarbons (unrelated to the oil spill) in the Gulf of Mexico. In alligator gar and Gulf killifish, EROD was not an indicator of hydrocarbon exposure. Possible explanations for this are the abnormal condition of the alligator gar liver (Chapter V), and the small sample size of the Gulf killifish livers, which necessitated pooling of samples that reduced the sample size to an unacceptable N number.

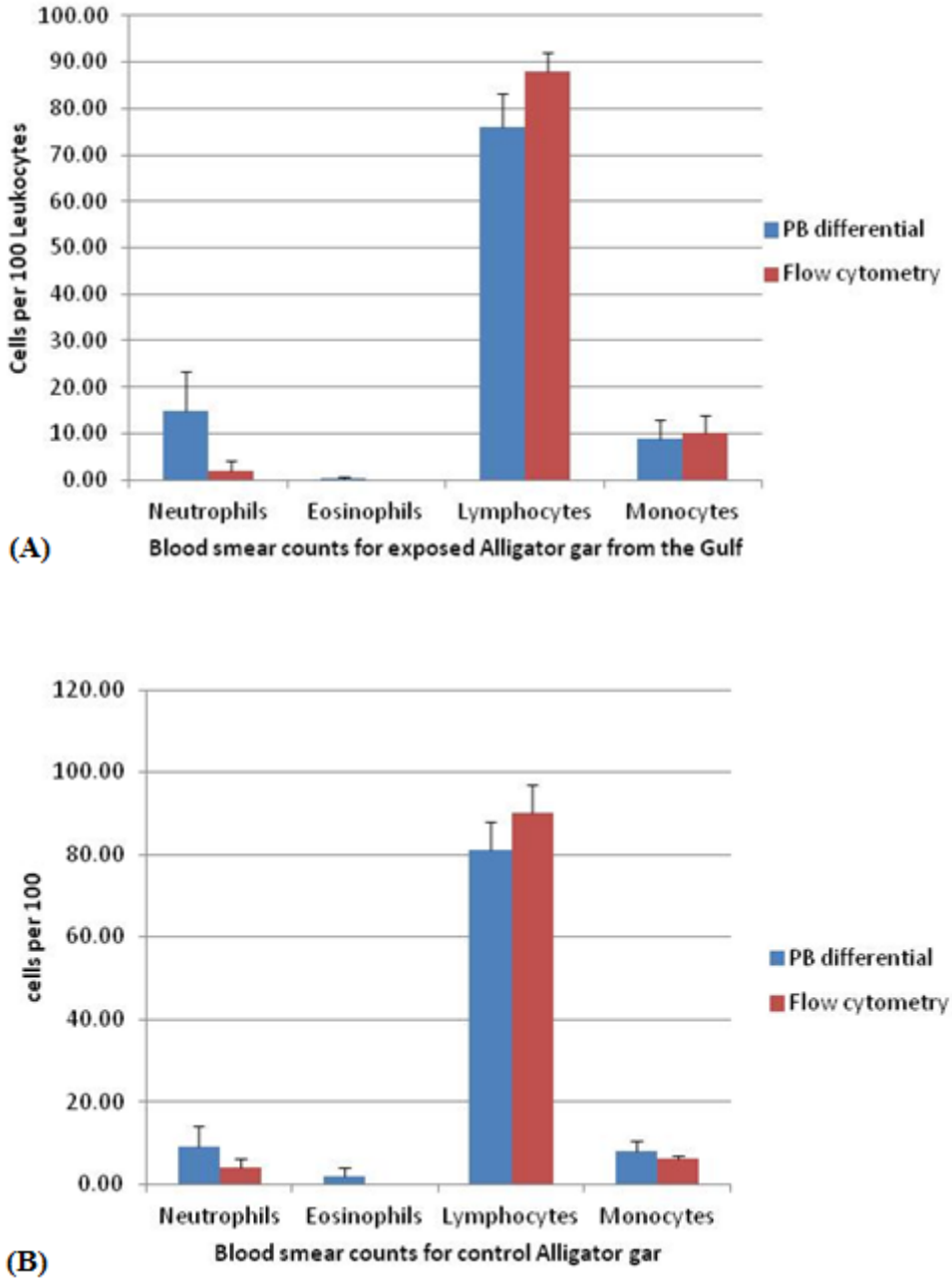

Figure 3.2 Oil-exposed (A) and control (B) *A. spatula* leukocyte peripheral blood differential counts and flow cytometry analyses.

Data was obtained manually or by flow cytometry and shows good correlation (n=17). Numbers in each of the bars is the percentage of each cell type that occurred.

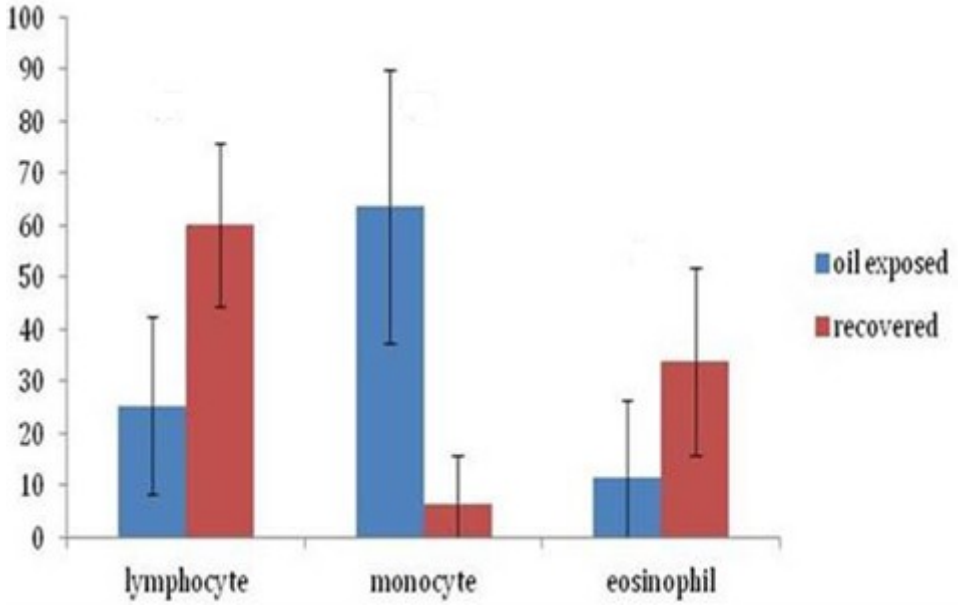

Figure 3.3 Killifish peripheral blood leukocyte differential counts.

Gulf killifish peripheral blood leukocyte differentials from wild fish caught in saltwater marshes near Terrebonne Bay, LA, during the oil spill. Recovered fish were held for two weeks in a recirculating system simulating estuarine conditions, and then sampled.

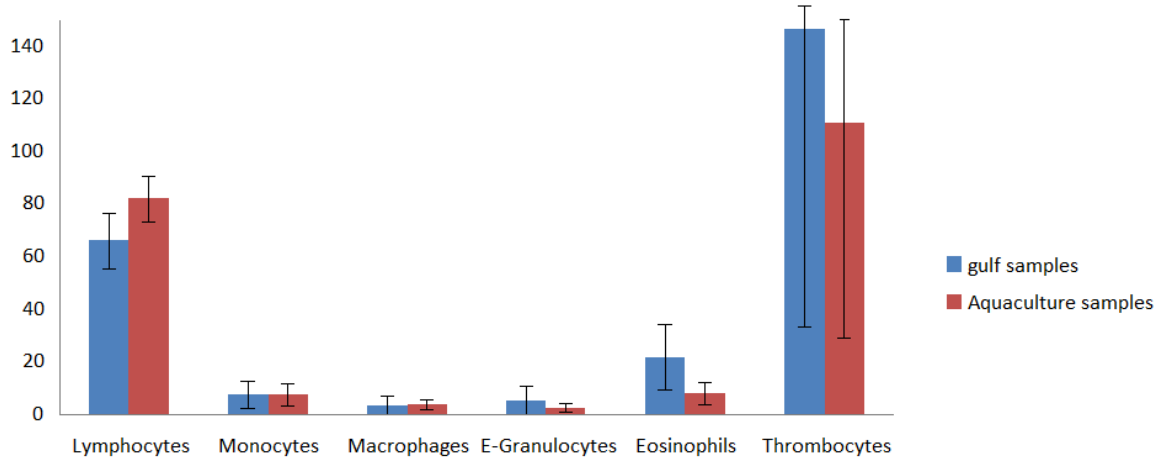

Figure 3.4 Peripheral blood leukocyte differentials from oil-exposed gulf sea trout and in-land reared sea trout were not significantly different.

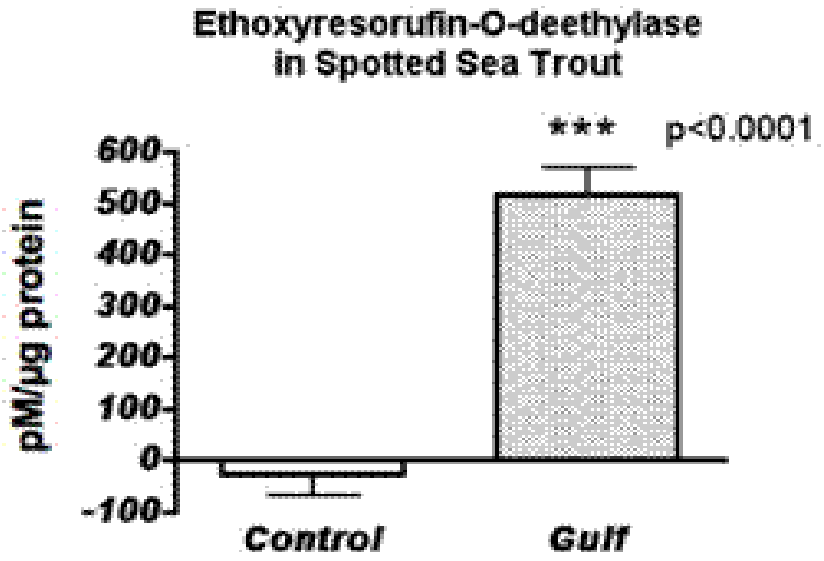

Figure 3.5 EROD in sea trout caught in the Gulf of Mexico, compared to the same those reared in an in-land facility.

Statistical significance by Student's t test is indicated by ***. N=5 for the control and N=8 for the Gulf sampled group.

CHAPTER IV

THE EFFECTS OF OIL EXPOSURE ON ALLIGATOR GAR, *ATRACTOSTEUS SPATULA*, BLOOD CELLS

Abstract

Oil exposure has been associated with devastating infectious disease outbreaks in wild populations of fish. The links between these outbreaks and oil exposure suggest an associated perturbation of fishes' immune systems. We determined that wild fish collected from waters that had been polluted with oil had decreased lymphocyte counts. To further define the basis of oil exposure and increased disease occurrence in fish, and to determine if there were dose-related exposure effects, controlled crude oil exposures were performed in a laboratory setting. Alligator gar were exposed to 3 different concentrations of crude oil, and their peripheral blood and kidney interstitial tissue (bone marrow equivalent) samples were analyzed after 48 hours of oil exposure, and again after 7 day oil-free recovery period. Blood cell analyses included flow cytometric and manual peripheral blood leukocyte differential counts. Blood smears were also analyzed by cytochemical staining methods used to differentiate leukocytes.

Introduction

For centuries, hematology has been a valuable tool to diagnose many human diseases. The blood cells of fish are produced in hematopoietic tissue which is located in

the spleen and kidney, and there are no lymph nodes in fish. In these tissues, hemocytoblast is the most important cell that gives rise to white blood cells, and red blood cells [28]. Leukocytopenia in fish is induced by many types of stress, and increases the susceptibility of fish to diseases. Leukocytes are divided into the granulocytes, lymphocytes, monocytes and thrombocytes. Fish do not have platelets in their blood. Lymphocytes are the highest number of WBCs in the peripheral blood, and they are round or ovoid cells with a large round centric nucleus that occupies most of the cell (Fig 4.4, A). Small lymphocytes have more compact chromatin than the medium and larger one. Neutrophils or heterophils are usually oval or rounded cells with granulated cytoplasm. The nucleus is multilobed from one to five lobes and eccentric in location. Eosinophils are round cells with rounded nucleus, and the cytoplasm is filled with a large rounded eosinophilic granular that takes pink color with Giemsa stain (Fig 4.4, B). Monocytes and macrophages are round cells with kidney shape nucleus, and the cytoplasm takes blue to pale blue gray color with Giemsa stain (Fig 4.5, B). The nucleus of macrophages is irregular in shape. The cytoplasm usually is vacuolated and basophilic when stained with Giemsa stain (Fig 4.5, A).M macrophages are resident in tissues, but it can be also found in blood such as monocytes [29]. The main object of this part was to determine whether oil exposure can change the normal counts of differential leukocytes in peripheral blood.

Special stains

Enzyme histochemical stains are one of the useful tools for identifying type's cells, and immunological cells types in disease processes [25]. Lymphocyte enzyme is use in detections of acid phosphatase (AP), α- naphthyl butyrate esterase (NBE), and β

glucoronidase(BG) in blood and tissues. α- Naphthyl acetate esterase (NAE) is used for cytologic demonstration of specific and non specific leukocyte esterase, and all of these stains are use in vitro for diagnosis (Fig 4.1). These stains were used in mammals and showed that all leukocytes were positive to AP stain, and all developmental stages of granulocyte were represented. NBE is present in T lymphocytes and monocytes, but B lymphocytes were negative to the stain. T lymphocytes and subpopulation B lymphocytes were positive for BG stain. NAE is one of the specific satins for monocytes, and it did show black granulation [26]. In channel catfish, tissue sections showed that AP stains were positive to all leukocytes cells. T lymphocytes demonstrated foci in the cytoplasm, and a blush cytoplasm appeared in B lymphocytes. NBE demonstrated small, medium, large T lymphocytes with cytoplasmic foci patterns. All leuckocytes were not positive to NAE stain. BG, NBE, and AP were used to identify the developmental stages of lymphocytes [25].

Methods

Controlled exposures to crude oil

The oil used was surrogate oil for MC252 oil, and was sample ID SO-20110212-HMPA10-009. Forty-three alligator gar (6 months old) were used in this study, and were exposed to either 0.5g of oil/L of water, 4g of oil/L of water, or no oil at all for the duration of 48 hours (Table 4.1). Exposure solutions were prepared as water accommodated fractions in which the oil was emulsified, or suspended in the water. This is similar to the form the oil would be in after being emulsified by a dispersant, as was done during the Deepwater Horizon Oil Spill.

The water-accommodated fractions (WAFs) of crude oil were prepared according to standardized methods. Briefly, a known mass of oil was added to a known volume of water. Oil was equilibrated with the water by mechanical stirring at a constant rate (100-200 rpm) for 48 hours. The salinity of the water was maintained at 9 g/L to represent an estuarine environment. Following mixing, oil was carefully transferred to experimental tanks. Concentrations of the WAFs were determined via gas chromatography with flame ionization detector at the Hand chemical laboratory, Mississippi State University, to establish total hydrocarbon content. Total organic carbon analysis was conducted to determine the toxicant decline rate, and liver ethoxyresorufin O- diethylase (EROD) was also determined.

After 48 hours, twenty-two fish were sampled (Table 4.1): whole blood was used for differential blood smears and flow cytometry, and kidney tissue was prepared for flow cytometric analyses. Tissues were surgically removed and placed in phosphate buffered 10% formalin and processed for microscopic viewing. Tissue samples were also placed in cryomolds with freezing medium, flash frozen in liquid nitrogen and stored at -70C until sectioned and stained. Muscle and liver samples were also flash frozen in liquid nitrogen and analyzed for EROD activity and polycyclic aromatic hydrocarbons (PAH) content.

Whole blood was collected from each fish by caudal venipuncture in lithium-heparinized vacutainers using 22 gauge needles at South Farm at Mississippi State University. Blood smears were made by the regular way which by using another slide to make the smear and air dried. Then slides were fixed by ethanol for 45 seconds, and stained by Giemsa stain (Sigma) following the manufacturer's instruction. Giemsa stain is useful stain to differentiate nuclear and /or cytoplasmic morphology of WBCs. Slides

were rinsed for at least 2 minutes in running tap water and air dried. All slides were covered by Permount and cover slip. Slides were viewed under light microscope at 100 X (oil lens), and 100 WBCs were counted in each slide by using laboratory counter. All cells that have been observed were documented. The remaining fish were transferred to oil-free water and maintained at 9 g/L seawater to stimulate estuarine condition. After a 7 day recovery period, fish were sampled again as described above. All experiments were approved by the MSU institutional animal care and use committee (IACUC).

Peripheral whole blood and kidney tissue samples were analyzed by flow cytometry on a FACS Aria High Through-put Flow cytometer. Whole blood erythrocytes were lysed and the blood was prepared according to standard procedures for our fish in our laboratory. The kidney was processed to a single cell suspension according to standard procedures in our laboratory.

Table 4.1 Number of alligator gar sampled for each oil exposure level and time.

Exposure time	48 hours	48 hours	48 hours	7 day recovery	7 day recovery	7 day recovery
Oil level	0	0.5	4.0	0	0.5	4.0
Fish #	8	7	7	6	8	7

0, control fish, 0.5 g/L oil, 4.0 g/L oil.

Enzyme cytochemical stains

Four blood smears were made from each Alligator gar, and were stained by using commercially available kits following the manufacturer's instruction (Sigma). Slides were air dried for 15 minutes, and blood smears were fixed in citrate acetone formaldehyde (CAF) for 30 seconds for AP, NAE, and BG stains except the slides that were stained by NBE for just 10 seconds. Slides were rinsed in distilled water for 45-60

seconds. Cytochemical stains were prepared following the procedures of commercially available kits (Sigma). AP solution will be amber, and NAE solution should turn to greenish color (fig 4.1). AP, NBE blood smears were incubated for 1 hour at 37 C. NAE blood smears incubated for 30 minutes at 37 C, and BG blood smears were incubated for 90 minutes at 37 C. All slides were rinsed in running tap water for 2-3 minutes, and air dried for 15 minutes. NBE slides concentrated for 5 minutes in methylene blue counter stain and BG slides for 3 minutes. NAE slides were concentrated for 2 minutes in hematoxylin solution. All slides were rinsed in tap water and dried in air for 15 minutes.

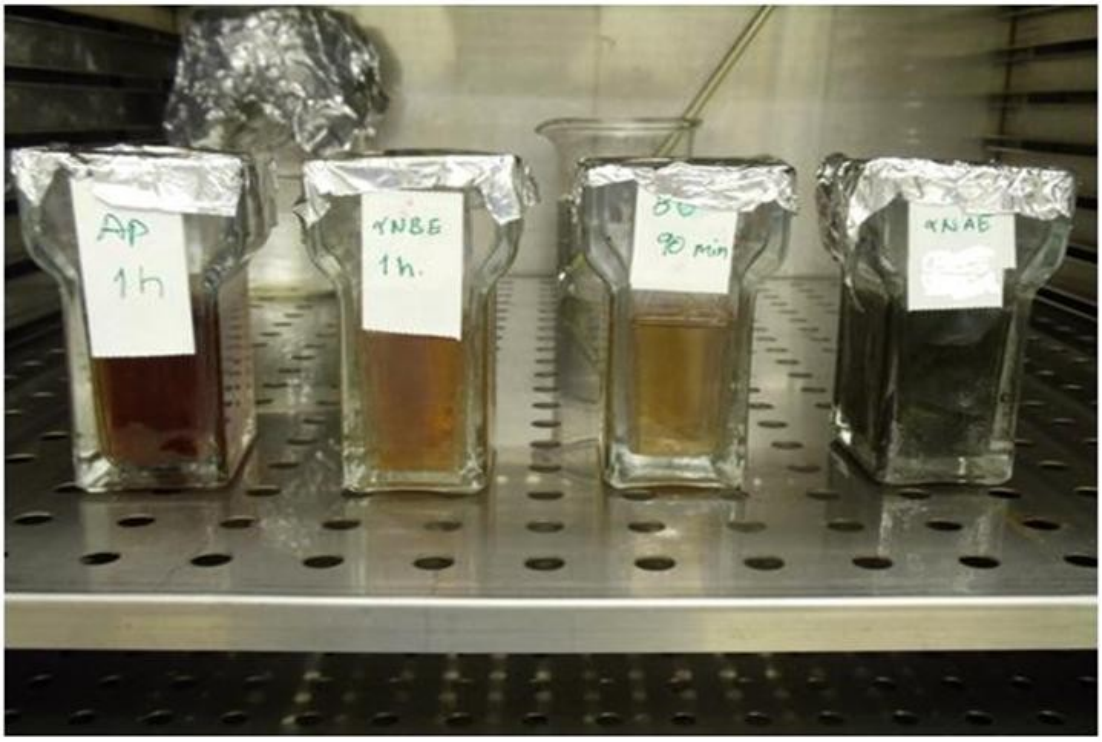

Figure 4.1 This picture shows the histochemical stains in incubator during the staining processes.

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Results

Giemsa stain

The diameter of Alligator gar leukocyte cells was slightly bigger than described in other fish species (Table 4.2). There are no previous studies describing gar blood cells. The blood peripheral counts from 48 hours exposure showed significant decrease in lymphocytes count in all different treatments, and increase in granulocyte cells compared to the differential counts of control fish (Fig 4.2). The most significant increase was in treatment 4 gm oil/L. Macrophage counts did not show any significant changes. The results from 7 days recovery showed that the lymphocytes started to increase again to the normal distribution, and granulocytes increased and recovered (Fig 4.3). Macrophage counts did not change.

Table 4.2 This table shows the cell diameter ranges of alligator gar leukocyte cells.

Cell Type	Cell size
Lymphocytes	5 to 10 µ
Neutrophils	9 to 13 µ
Eosinophils	11 to 15 µ
Monocytes	11 to 13 µ
Macrophages	13 to 14 µ

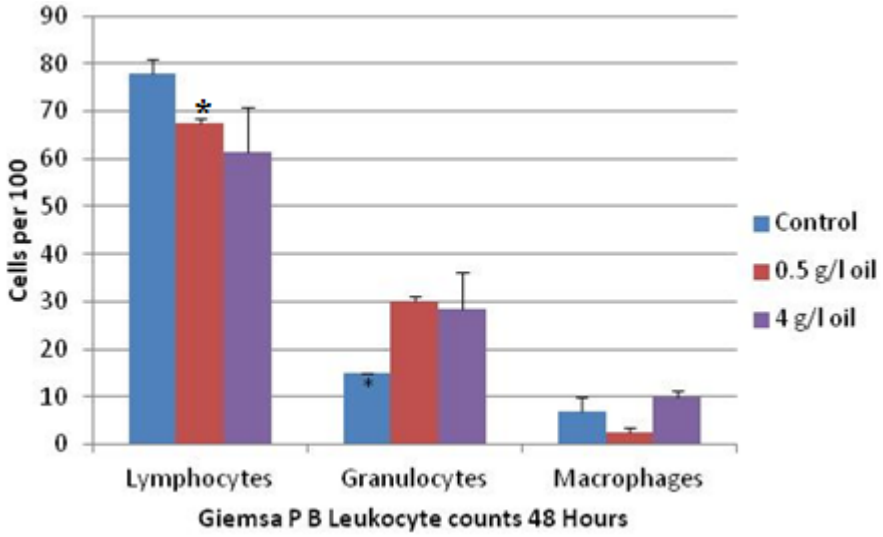

Figure 4.2 Differential blood smear counts after 48 hours oil exposure (Giemsa 100 X).

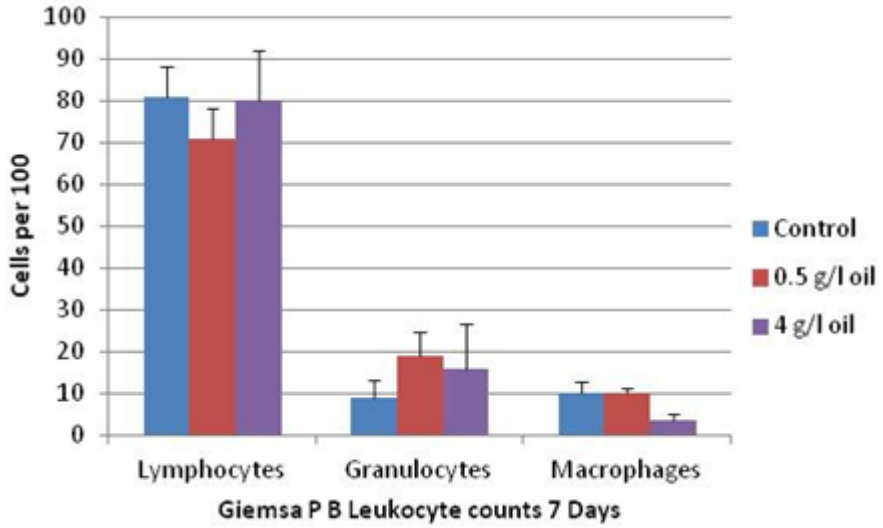

Figure 4.3 Differential blood smear counts after 7day recovery period (Giemsa 100 X).

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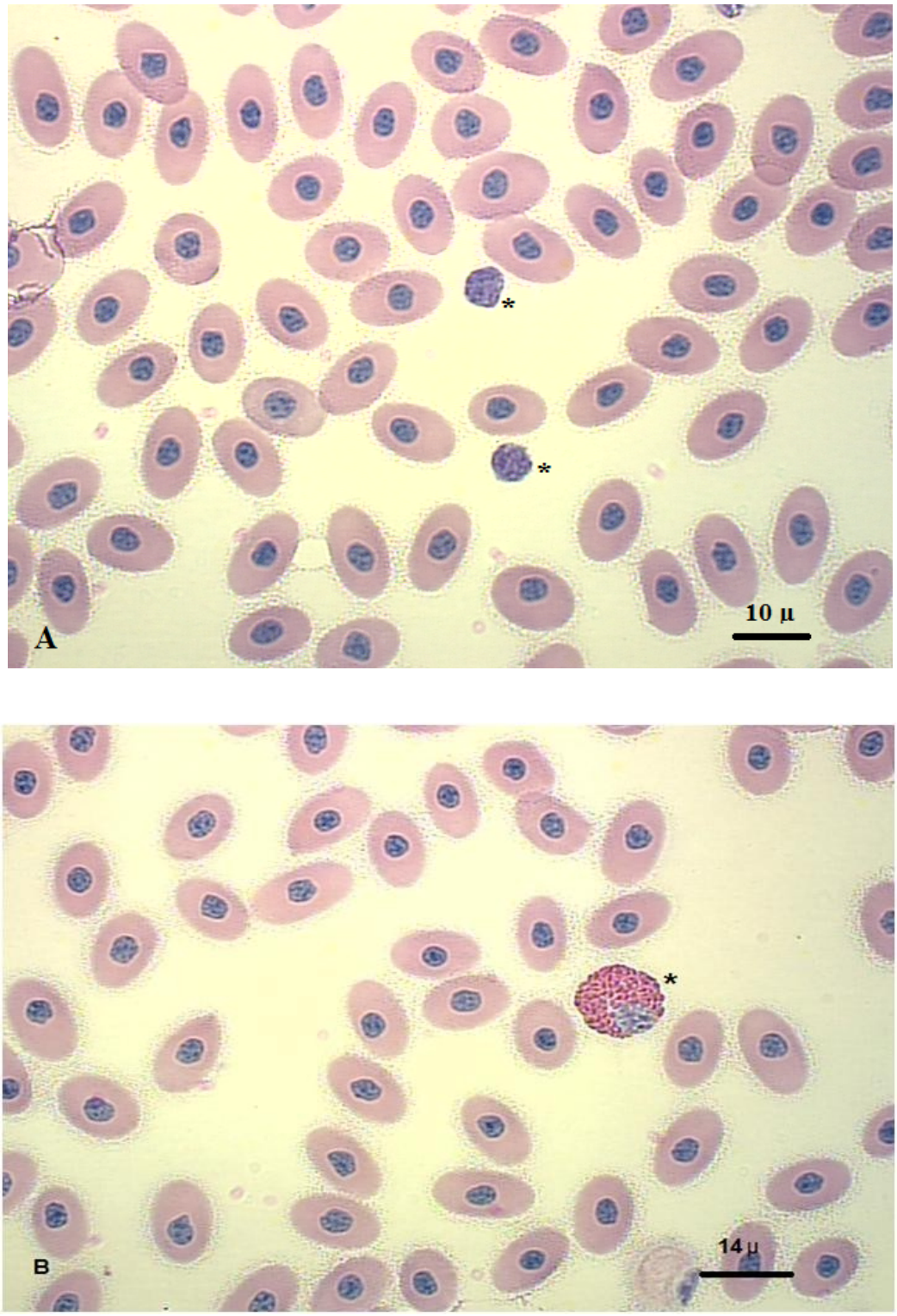

Figure 4.4 Peripheral blood cells from Alligator gar (Giemsa stain, 100 X). (A) Lymphocyte, (B) Eosinophil.

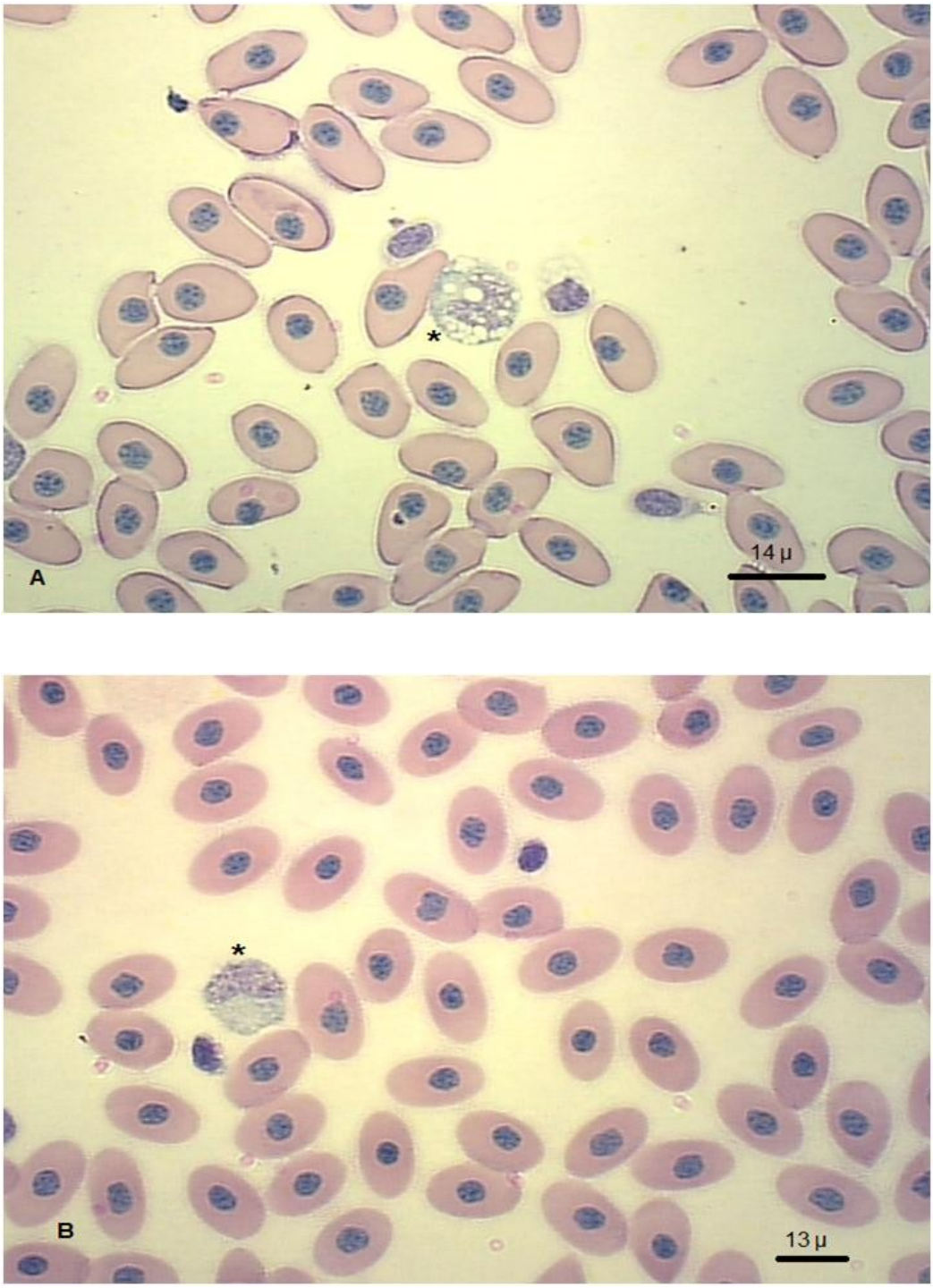

Figure 4.5 Peripheral blood cells from Alligator gar (Giemsa stain 100 X) (A) Macrophage. (B) Monocyte.

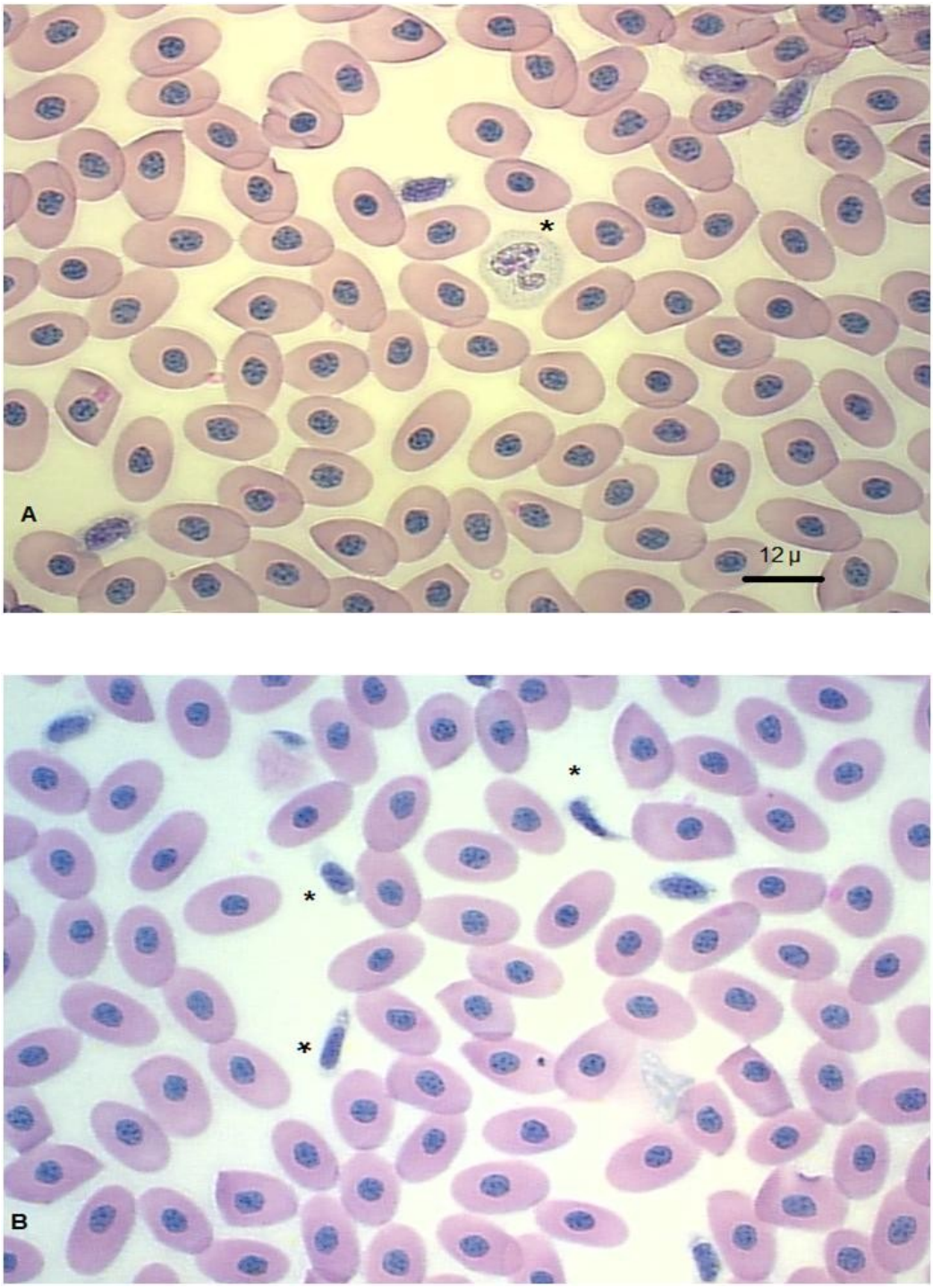

Figure 4.6 Peripheral blood smears from Alligator gar (Giemsa stain 100 X) (A) Neutrophil. (B) Thrombocyte.

The peripheral blood differential counts from 48 hours exposure showed significantly decreased lymphocyte counts in all treatments, and increased granulocyte counts (Fig 4.2). The most significant increase was observed in the 4 gm oil/L treatment. Macrophage counts did not significantly change. The results from 7 days recovery showed that the lymphocytes started to increase again to the normal distribution, and granulocytes increased and recovered (Fig 4.3).

Enzyme cytochemical stains

When slides were stained with AP, all leukocytes cells were positive in all treatments (Table 4.3). All developmental stages of lymphocytes were presented and demonstrated red or bluish cytoplasm. Monocytes and macrophages demonstrated red vacuolated cytoplasm with AP and NBE. When sections stained with NAE monocytes and macrophages presented as black granulation. Granulocyes were negative to BG, NAE and NBE stains as well. Granulocytes counts showed that the neutrophils were presented in low percentage compared to eosinophils.

Table 4.3 Alligator gar peripheral blood leukocytes stained with histochemical stains.

Cell type	Stain type			
	AP	BG	NBE	NAE
lymphocyte	+	+	+	+
Monocyte	+	+	+	+
Granulocyte	+	-	-	-

-; non reactive. +, reactive. AP; acid phosphates. BG; β glucuronidase. NBE; α-naphthyl butyrate esterase. NAE; α- Naphthyle acetate esterase.

Overall, trends were observed and correlated with other study findings. However, due to the variability that can occur in fish, many of the cytochemical findings were not significantly different. The AP peripheral blood smears results from 48 hours revealed

that there were decreases in granulocytes and macrophages counts compared to the counts from unexposed fish. Observing the lymphocytes counts showed that there was increase in the numbers (Fig 4.7). The AP peripheral blood smears results from 7 days recovery showed that there was a decrease in the number of lymphocytes cells compared to the counts from the unexposed fish. Granulocytes and macrophages counts were decreased in the numbers compared to the unexposed fish counts (Fig 4.8).The macrophages showed that there were increases in the counts when compared to the control counts from control fish.

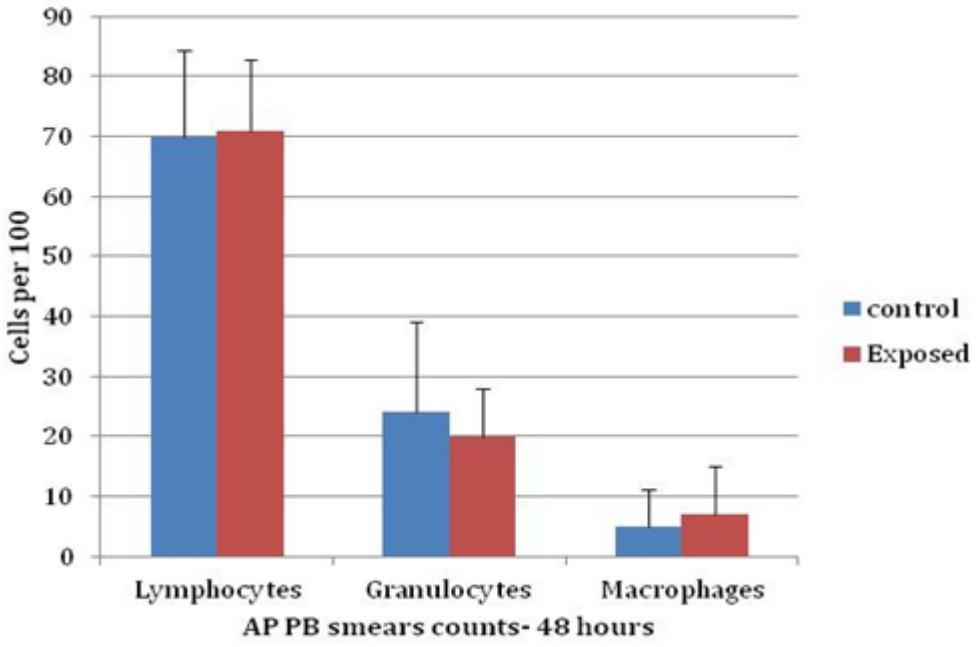

Figure 4.7 AP differential blood smears counts from 48 hours exposure.

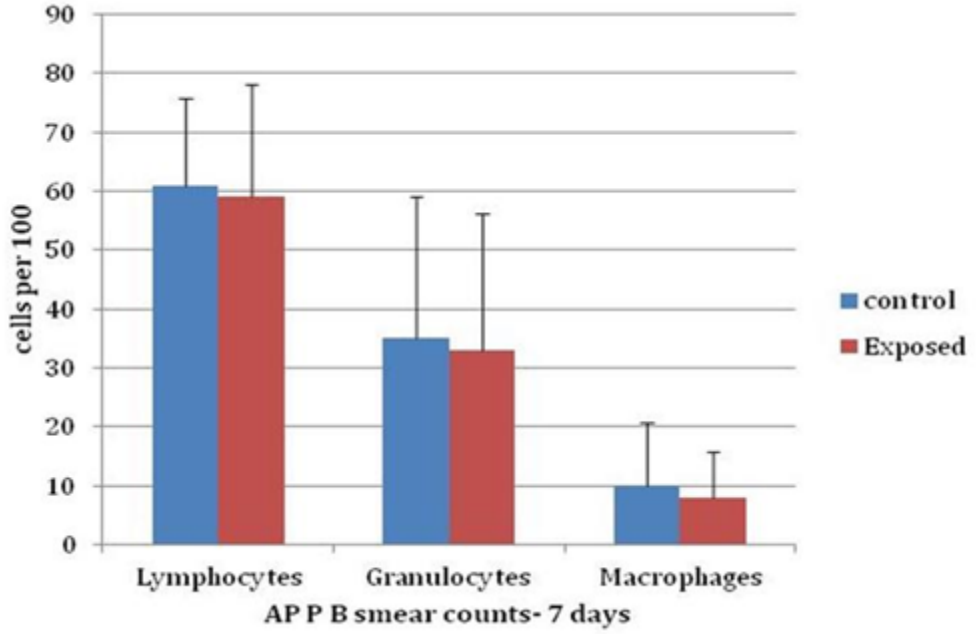

Figure 4.8 AP differential blood smears counts from 7 days recovery.

Flow cytometry results

Alligator gar peripheral blood showed increases in granulocytes including eosinophils due to oil exposure consequently reduced numbers of granulocytes were observed in the kidney of the same fish. Lymphocyte counts in the kidney were also lower in oil exposed fish compared to control fish. Peripheral blood smears demonstrated significant decreases in lymphocytes after 48 hours exposure, and increases in macrophages and granulocytes (Fig 4.11). Flow cytometric analyses demonstrated that the initial response is a decrease of all leukocytes in the kidney marrow (Fig 4.9). After the 7 day recovery period, there are no differences between the leukocytes in the kidney marrow of any of the fish; new cells have been produced (Fig 4.10).

Interpretations

Oil exposure results in decreased leukocyte counts. This condition predisposes fish to disease. If the oil exposure stops, hematopoiesis increases and leukocyte counts recover. Prolonged oil exposure would lead to greater disease susceptibility.

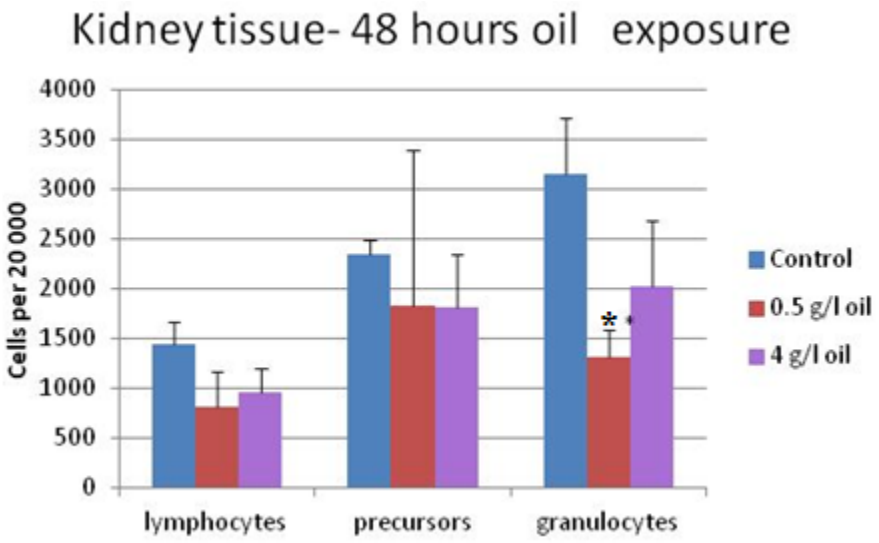

Figure 4.9 Flow cytometry leukocyte counts from the kidney after 48 hours exposure.

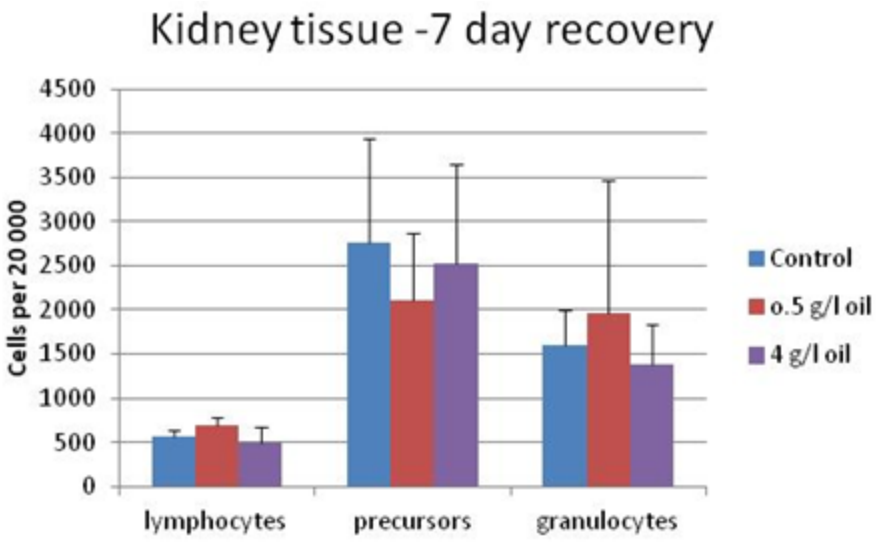

Figure 4.10 Flow cytometry leukocyte counts from the kidney after 7 days from the exposure.

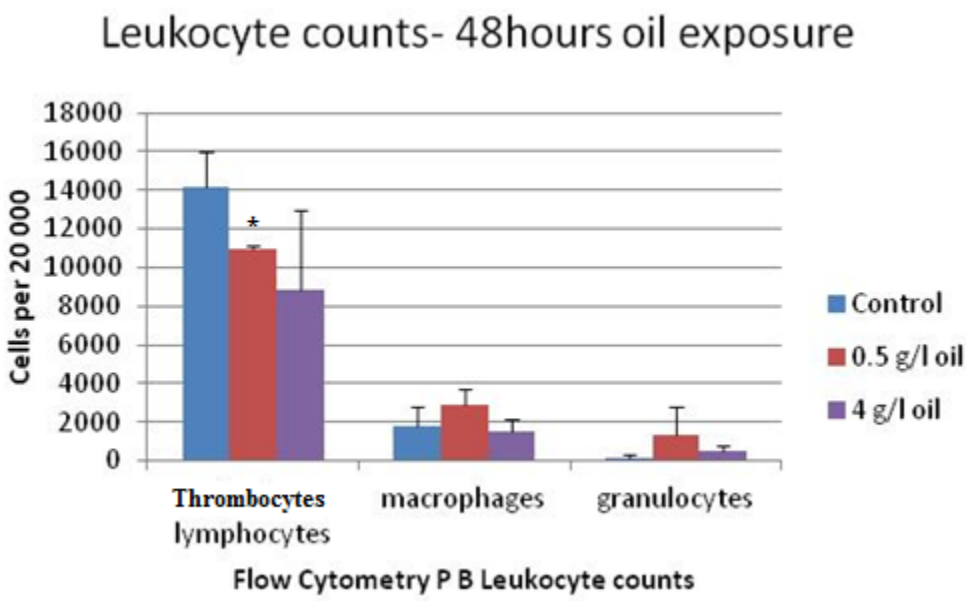

Figure 4.11 Flow cytometry peripheral blood leukocyte counts after 48 hours exposure.

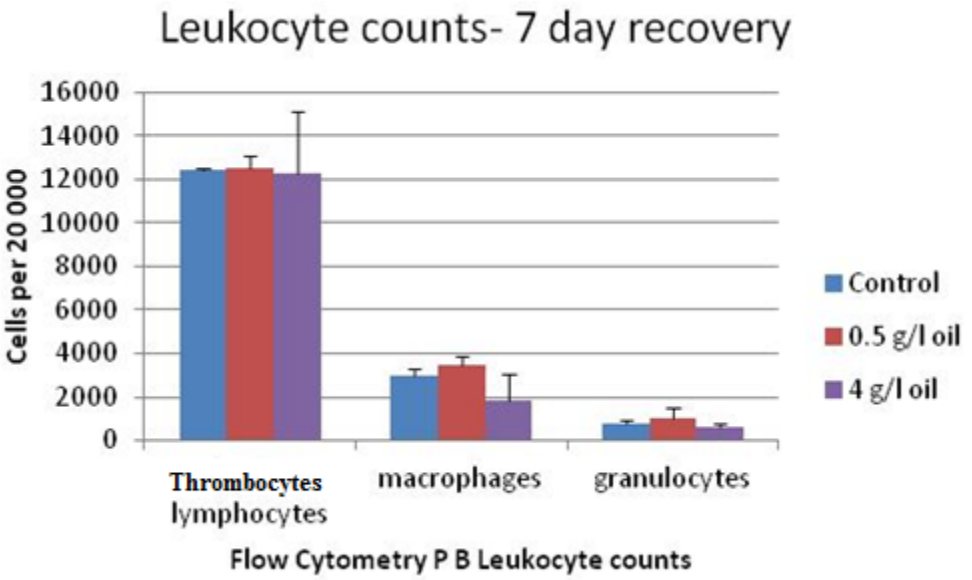

Figure 4.12 Flow cytometry peripheral blood leukocyte counts after 7 days for recovery.

CHAPTER V

THE EFFECTS OF OIL EXPOSURE ON ALLIGATOR GAR, *ATRACTOSTEUS SPATULA*, TISSUES

Introduction

Fish living in an aquatic environment are highly susceptible to many types of pollution. Fish are good indicators of environmental pollution in the water because many of the chemicals can concentrate in fish tissues, resulting in gross and microscopic lesions[30]. Organs that can be used as indicators of environmental pollution or toxicity are liver, kidney, gills, and spleen [31]. Liver is one of the important organs in fish for carbohydrate metabolism, lipid storage, and metabolizing toxins [32]. Studies have observed changes in the liver after exposure to chemicals or petroleum products that include increased vacuoles in cytoplasm, enlarged lysosomes, changes in nuclear chaps, focal necrosis, hepatocellular shrinkage, fatty degeneration, and loss of glycogen [28]. In general, hepatovacuolation is associated with toxicity. Since the kidney receives a large amount of blood, renal lesions are a good indicator of environmental pollution [32]. In one study, spotted sea trout were collected from an area that had been exposed to mercury. Liver lesions included necrosis, inflammation, bile duct hyperplasia, and glycogen cytoplasmic deposits [33]. Another study investigated the effects of pesticide run-off. Liver tissue from exposed fish showed degeneration, dilation of sinusoids, hypertrophy of hepatocytes, and focal necrosis. In the same study the kidney showed

cloudy swelling of renal tubules and necrosis in the tubular epithelium. The pancreatic tissues of the exposed fish also showed that there was infiltration of eosinophils [34]. A histological study of *Oreochromis niloticus* from a polluted wetland area in Saudi Arabia revealed vacuolization in hepatocytes. Vacuolization was a result of lipid dystrophies. In gills, a curved tips were noticed at the end of the secondary lamellae, hyperplasia, vasodilatation and necrosis was observed in epithelium of gills [35].

The Shatt Al-Arab River has been polluted by different sources of pollutants. One hundred twenty *Cyprinus carpio* were captured from the river, and histopathology revealed gill changes that included hyperplasia, clavate structures, loss of regular shape, separation of lamellar epithelium, atrophy of secondary lamellae of gill, and necrosis [30].

Frozen tissue sections are one of the rapid histological diagnostic methods, and is usually used when known antigens need to be visualized. In catfish, positive cells with small and large foci were visualized in the parenchyma around the arterioles by using AP and NBE stains. Cells that were positive to BG stain demonstrated bluish cytoplasm, and were adjacent to the arterioles [25].

Enzyme histochemical stains are important for identifying cell types in tissues and are critical for identifying immunological cell types involved in developmental and pathological processes. Leukocyte staining characteristics determined by enzyme cytochemistry and used in conjunction with standard histological stains allow more accurate assessments of the tissue distribution of leukocytes [25]. Acid phosphatase (AP) is a phosphoric monoester hydrolase that acts on ester bonds. In mammals, AP is present in lymphocytes, plasma cells, monocytes, histiocytes and all stages of granulocyte

development. A-naphthyl butyrate esterase (NBE) occurs in lymphocytes; T lymphocytes stain positive, while B lymphocytes are negative. NBE also demonstrates non-specific esterases in monocytes and macrophages. β-glucuronidase (BG) is a glycoside hydrolase that occurs in mature thymocytes, circulating T lymphocytes and a subpopulation of B lymphocytes. Acid phosphatase, NBE and BG also identify lymphocyte developmental stages. Histochemical staining characteristics of teleost leukocytes have been described in Atlantic salmon *Salmo salar* L. [36], carp *Cyprinus carpio* L. [37], rainbow trout *Oncorhynchus mykiss* (Walbaum) [38];[39, 40], European flounder *Platichthys flesus* (L.) [41], channel catfish (Rafinesque) [25], and *Polyodon spathula* [26].

Methods

Fixed formalin tissues

Liver, spleen, kidney, gut, and heart were removed from each fish, rinsed in physiological saline and fixed in phosphate buffered 10% formalin to preserve tissues in their natural state and fix all components for 24 hours. The formalin was then replaced with 70% ethanol. Tissues were embedded in paraffin, and sectioned at 4-6 μ, stained with haematoxylin and eosin staining (H & E). Slides were covered by per mount and cover clips. All slides were viewed under light microscope.

Frozen tissues

Spleen and liver were removed from each fish. Tissues were placed in disposable plastic cryomolds, and covered with optimal cutting temperature medium (OCT), snap frozan in liquid nitrogen and stored at - 70 °C until sectioned. Tissues were sectioned at 10 microns by using Cryostat at -16 °C. Sections were manipulated with a cold fine brush

to open it, and were transferred carefully to the slides. Sections on slides were dried in air, and fixed in citrate acetone formaldehyde (CAF). Enzyme substrate stains AP, NBE, NAE, and BG were used following the manufacturer's instructions (Sigma), with modifications to optimize the findings in fish tissues, as previously described in Chapter IV. All slides were viewed under light microscope.

Results

Fixed formalin tissues

Grossly, livers from fish that were exposed to 4 g/L oil for 48 hours were very dark in color (Fig.5.1). After the 7-day recovery period, they appeared creamy in color, very similar to the color of the control liver (Fig 5.2).

After 48 hours exposure to 4 g oil/L, livers demonstrated severe hepatocellular vacuolization, hepatocellular necrosis, necrotizing hepatopancreatitis and reduced numbers of melano-macrophage aggregations (Fig 5.9). Many of the spleens were congested and had neutrophilic infiltration (Fig 5.11). Posterior kidney tissue revealed vacuolization, increased Bowman's space and eosinophilic infiltration in the epithelium. In addition, melanin granule accumulations occurred close to the veins. The heart demonstrated neutrophilic infiltration of the epicardium (Fig 5.14). After the 7-day recovery period, the anterior kidney, or the hematopoietic tissue demonstrated increased numbers of stem cells. The anterior region of the alligator gar kidney consists of hematopoietic tissue and endocrine elements: interrenal cells and the adrenal homolog, chromaffin cells and the Corpuscle of Stannius. In the gar sections viewed, the interrenal and chromaffin cells occurred separately within the kidney marrow and were not intermingled. As the kidney proceeds posteriorly, the portion of hematopoietic tissue

diminishes. The posterior region of the alligator gar kidney is predominately renal corpuscle elements, the glomerulus, Bowman's capsule and proximal and distal tubules. The spleen was structurally similar to other described fish spleen [25]. No remarkable pathological changes were observed in the gut of 48 hour exposed or 7-day recovery fish.

Table 5.1 Tissues histopathological changes were observed during the oil exposure for unexposed fish.

TRT.	FFISH#	TISSUES	FINDINGS
48C	105	Liver	Normal
		Spleen	Normal
		Heart	Normal
		AK	Normal
		Gut	Normal
48C	107	Liver	abundant diffuse lipid-type hepatocellular vacuolization, MMA throughout parenchyma, Eosinophilic Granular Cells assoc w/intrahepatic pancreas, zymogen granules smaller and less intense than in teleosts
		Spleen	Normal
		Heart	Normal
		AK	Normal
		Gut	Normal
48C	112	Liver	Normal
		Spleen	Normal
		Heart	Normal
		AK	Normal
		Gut	Normal
48C	120	Liver	Normal
		Spleen	Normal
		Heart	Normal
		AK	Normal
		Gut	Normal
48C	143	Liver	Hepatocellular vacuolization
		Spleen	Normal
		Heart	Normal
		AK	Normal
		Gut	Normal
48C	149	Liver	Hepatocellular vacuolization
		Spleen	Normal
		Heart	Normal
		AK/PK	Normal
		Gut	Normal

Table 5.2 Tissues histopathological changes were observed during 48 hours exposure.

TRT.	FFISH#	TISSUES	FINDINGS
48 h-4	110	Liver	Diffuse hepatic necrosis, marked hepatocellular lipid vacuolization, hepatopancreatic necrosis
		Spleen	ECG infiltration, neutrophilic infiltration
		Heart	Mild hemorrhagic pericarditis
		AK/PK	Normal
		Gut	Serosal inflammation
48h-4	125	Liver	Diffuse hepatic necrosis, marked hepatocellular lipid vacuolization, hepatopancreatic necrosis
		Spleen	Congested; neutrophilic infiltration
		Heart	Normal
		AK/PK	Increased stem cells
		Gut	Normal
48h-4	128	Liver	Diffuse hepatic necrosis, hepatopancreatic necrosis
		Spleen	Neutrophilic infiltration, congestion
		Heart	Normal
		AK/PK	Normal
		Gut	Normal
48h-4	144	Liver	Marked hepatocellular vacuolization, marked EGC infiltration
		Spleen	Congested
		Heart	Normal
		AK/PK	Normal
		Gut	Normal
48h-4	148	Liver	Diffuse hepatocellular necrosis; congested hepatopancreas
		Spleen	Normal
		Heart	Normal
		AK/PK	High numbers of EGCs
		Gut	Normal
48h-4	150	Liver	Diffuse hepatic necrosis
		Spleen	Normal
		AK/PK	Increased stem cells
		Heart	Normal
		Gut	Normal

Table 5.3 Tissues histopathological changes were observed during 7 days recovery.

TRT.	FFISH#	TISSUES	FINDINGS
7D-R	116	Liver	Diffuse necrosis; MMC #s increasing from 48 h- trt4
		Spleen	Neutrophilic infiltration
		AK/PK	Increased stem cells
		Heart	Normal
		Gut	Normal
7D-R	119	Liver	Diffuse hepatocellular necrosis; hepatopancreatic necrosis
		Spleen	Mild congestion
		AK/PK	Normal
		Heart	Normal
		Gut	Normal
7D-R	136	Liver	Mild hepatocellular vacuolization
		Spleen	Mild congestion
		AK/PK	Increased stem cells
		Heart	Mild epicarditis
		Gut	Normal
7D-R	139	Liver	Mild hepatocellular vacuolization
		Spleen	Mild congestion
		AK/PK	Increased stem cells
		Heart	Normal
		Gut	Normal
7D-R	151	Liver	Focal hepatic necrosis; focal pancreatic necrosis
		Spleen	Congested
		AK/PK	Increased stem cells
		Heart	Normal
		Gut	Focal necrotizing enteritis
7D-R	158	Liver	Necrotizing hepatitis; necrotizing hepatopancreatitis
		Spleen	Mildly congested, neutrophilia
		AK/PK	Neutrophilic infiltration
		Heart	Epicarditis
		Gut	Not present
7D-R	159	Liver	Mild hepatocellular vacuolization
		Spleen	Normal
		AK/PK	Increased stem cells
		Heart	Mild epicarditis
		Gut	Normal

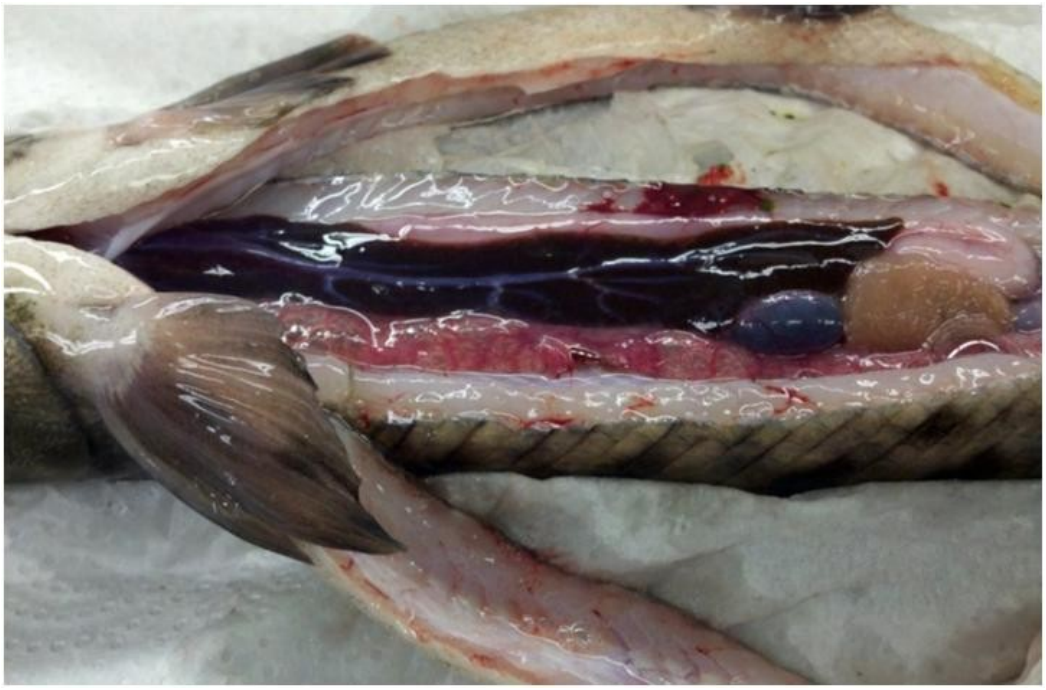

Figure 5.1 Liver of Alligator gar 48 hours fish showed how it became very dark.

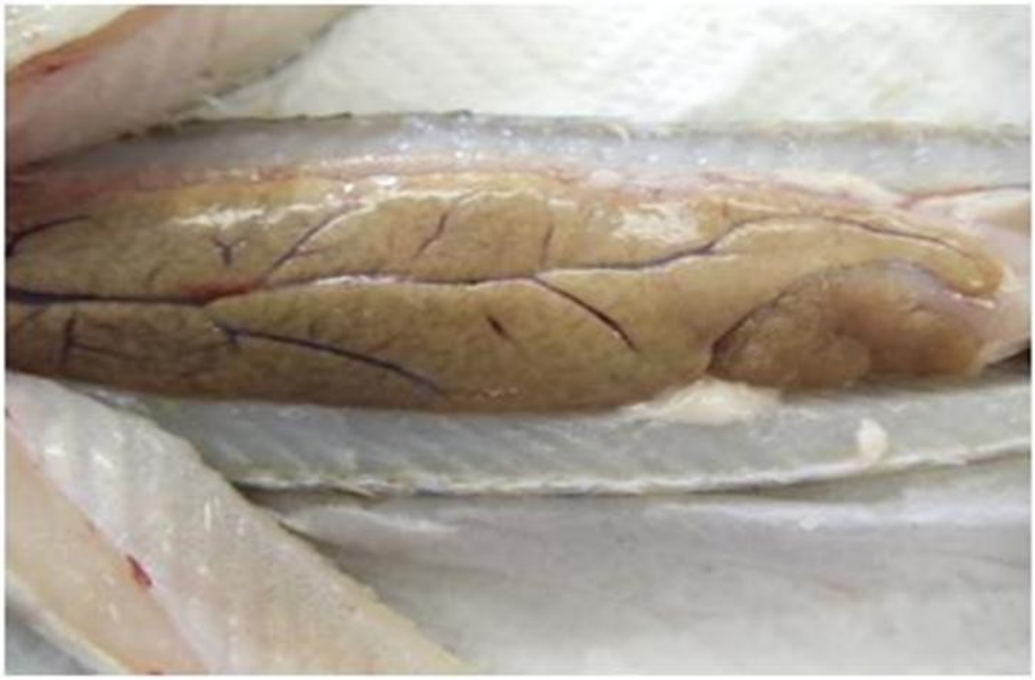

Figure 5.2 Liver of Alligator gar Liver from 7 days recovery group.

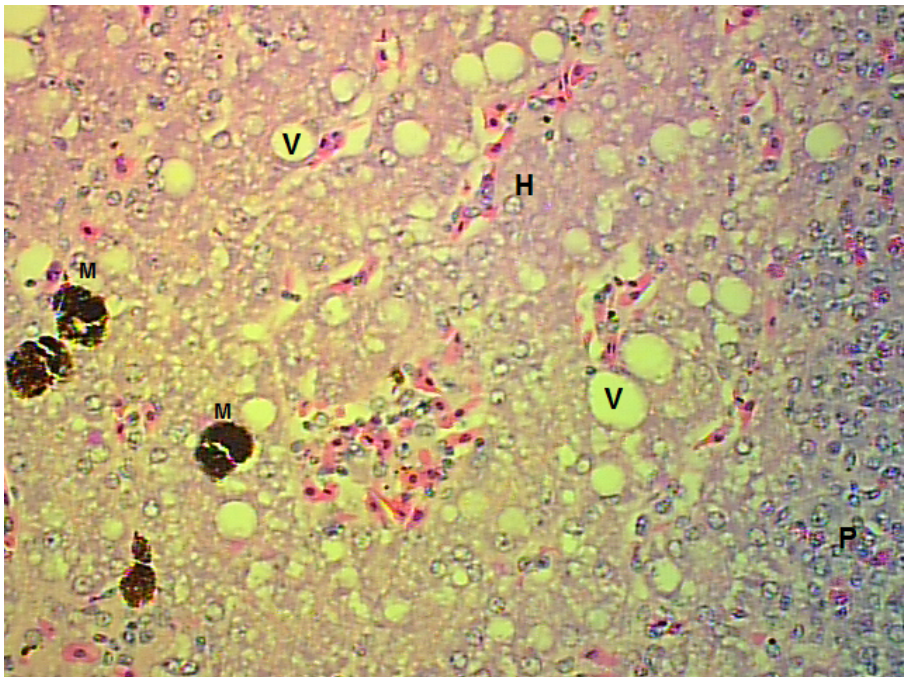

Figure 5.3 Liver tissue from unexposed Alligator gar, vacuolization (V), Hepatocyte (H), melano-macrophage centers (M).

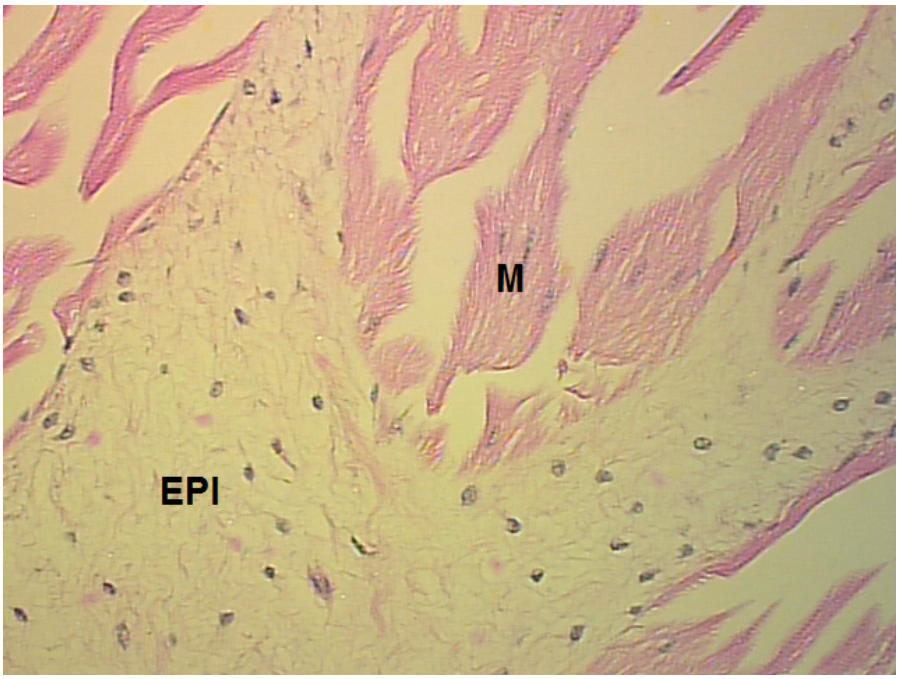

Figure 5.4 Heart tissue from unexposed Alligator gar, epicardium (EPI), myocardium (EPI).

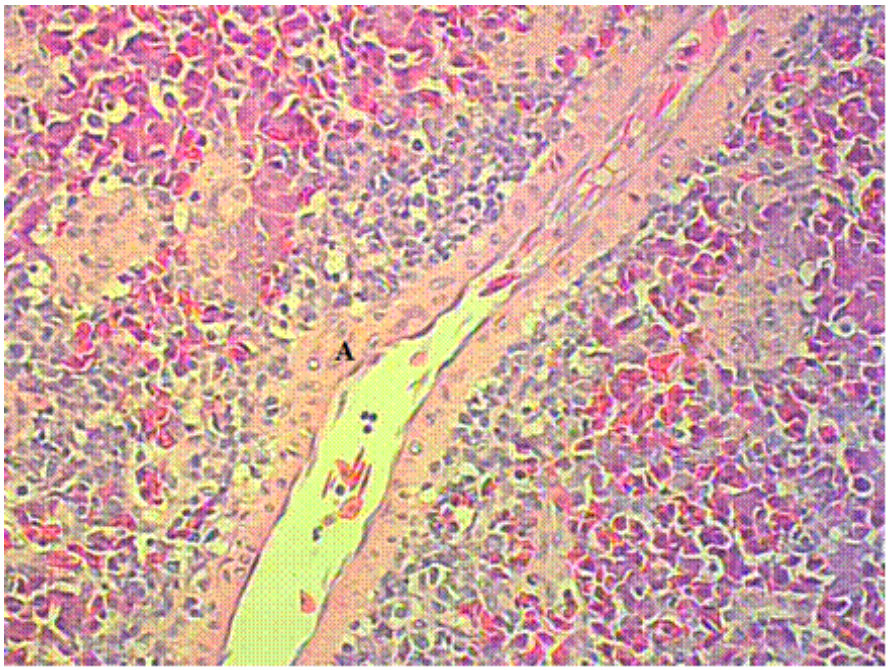

Figure 5.5 Spleen tissue from unexposed Alligator gar, artery (A).

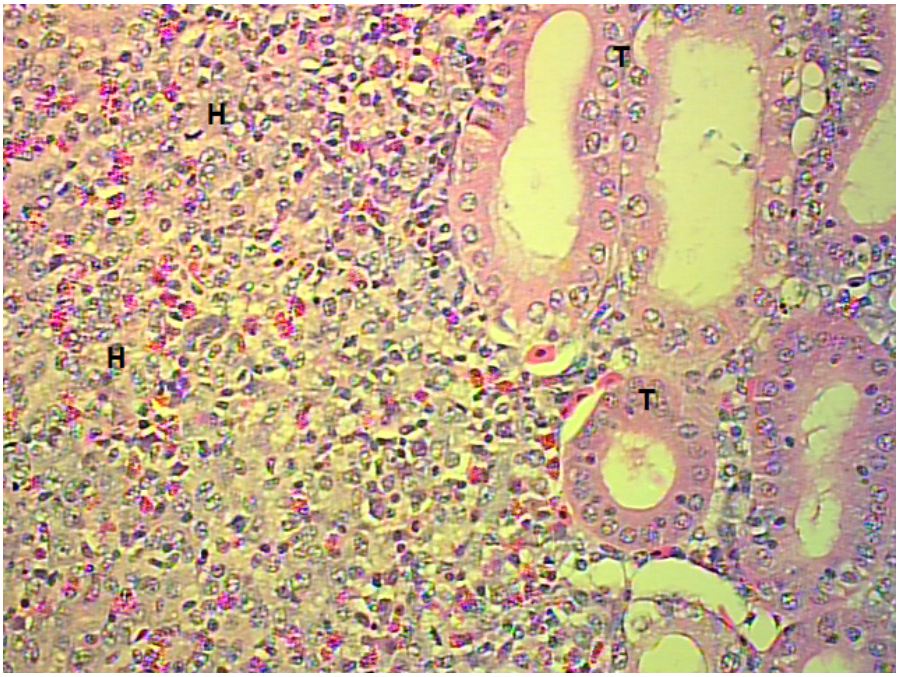

Figure 5.6 Kidney tissue from unexposed Alligator gar, hematopoietic tissue (H), renal tubules (T).

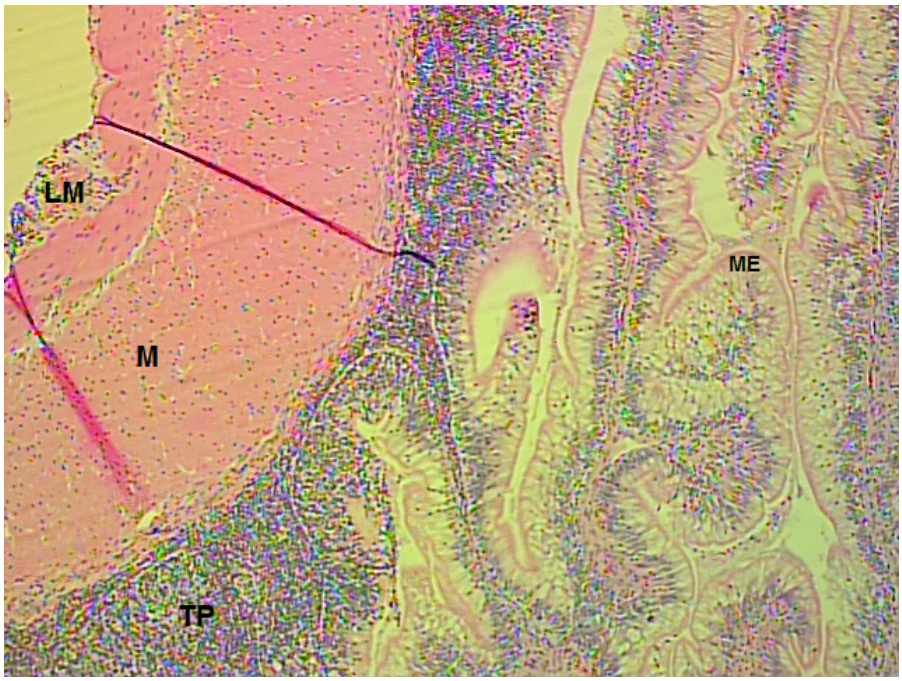

Figure 5.7 Intestine from unexposed Alligator gar, longitudinal muscle (LM), circular muscle coat (M), tunica propria (TP), mucosal epithelium (TN), mucosal epithelium (ME).

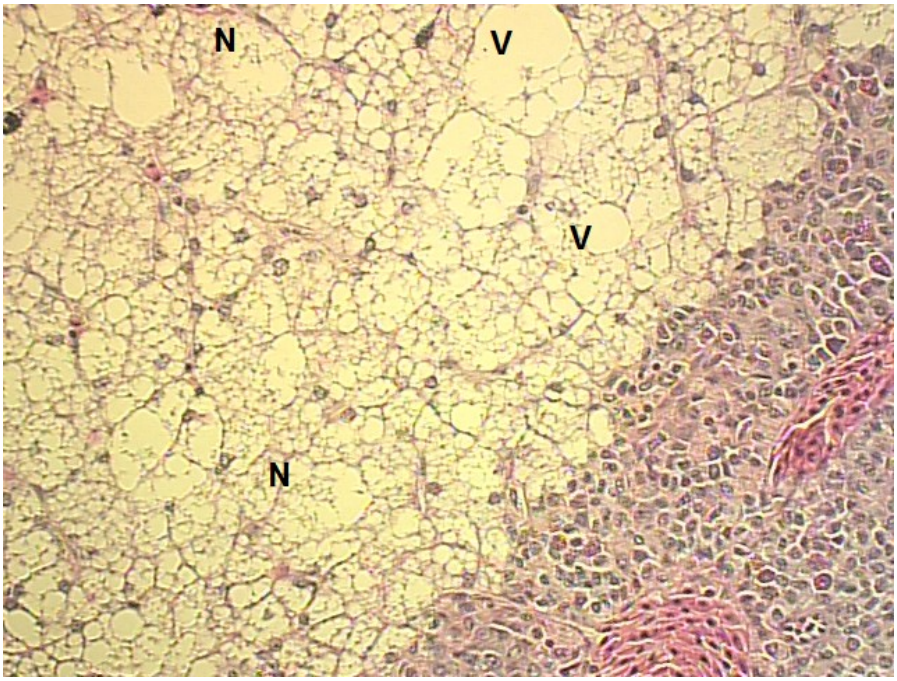

Figure 5.8 Liver tissue from exposed Alligator gar 4g/L after 48hours, necrosis (N), vacuolization (V).

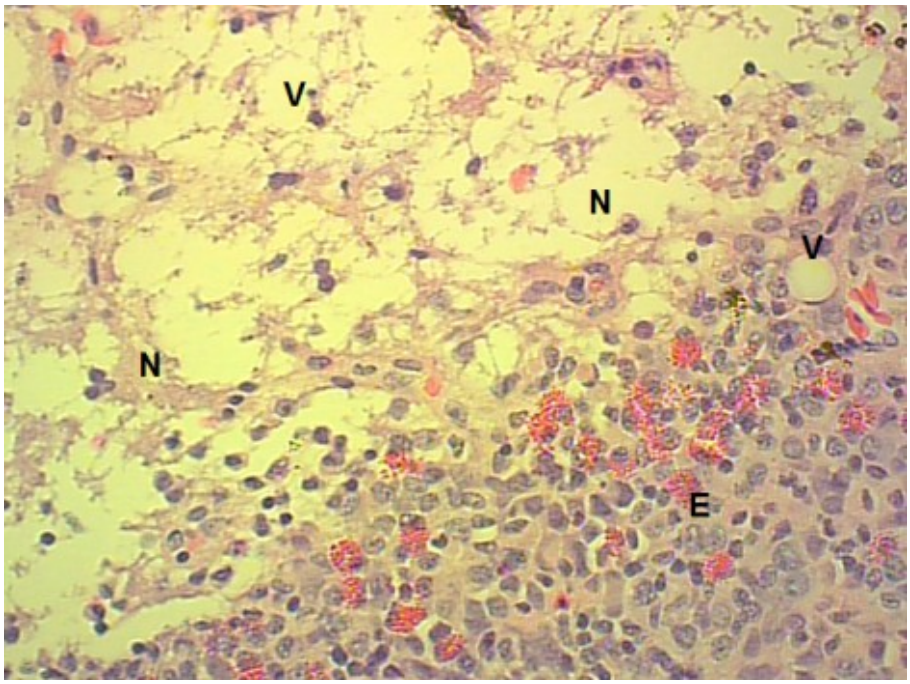

Figure 5.9 Liver tissue from exposed Alligator gar 4g/L after 48hours, necrosis (N), vacuolization (V), eosinophilic infiltration in pancreatic tissue (E).

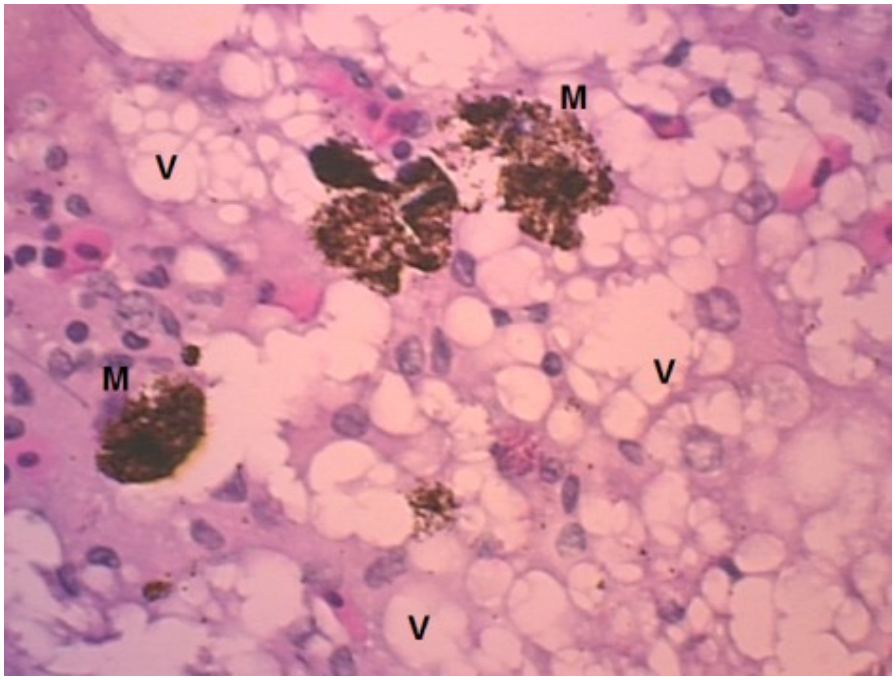

Figure 5.10 Hepatopancearatic tissue from exposed Alligator gar 4g/L after 48hours, vacuolization (V), melano-macrophage centers (M).

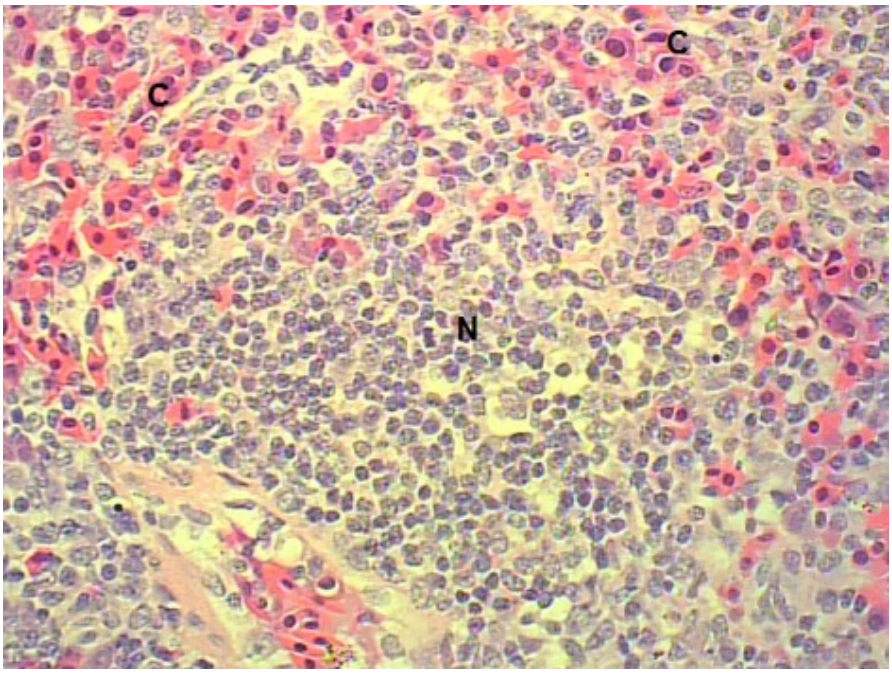

Figure 5.11 Spleen tissue from tissue from exposed Alligator gar 4g/L after 48hours, congested(C), neutrophilic infiltration (N).

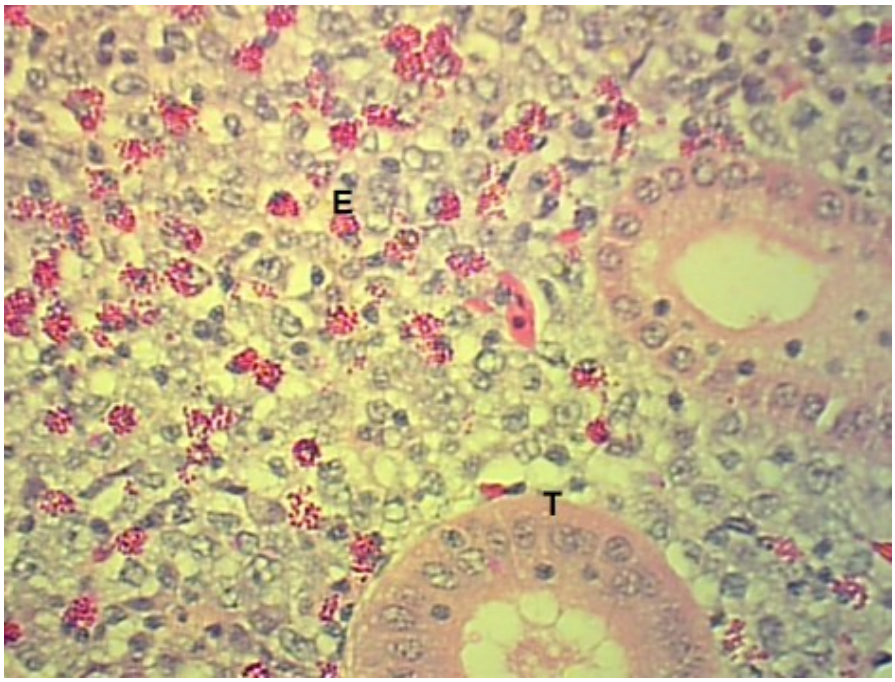

Figure 5.12 Kidney tissue from exposed Alligator gar 4g/L after 48hours, eosinophilic infiltration (N) hematopoietic tissue, tubules (T).

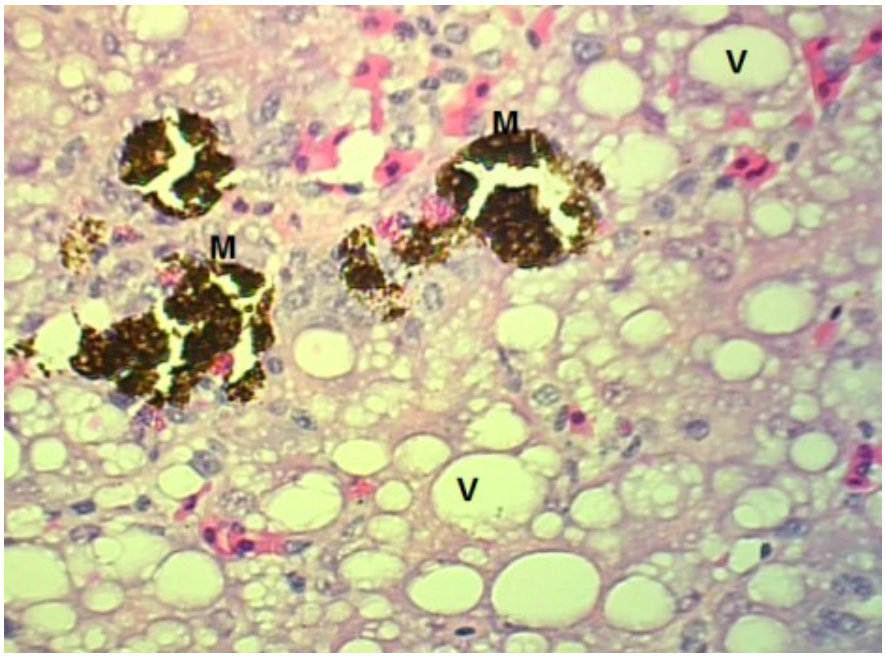

Figure 5.13 Kidney tissue from exposed Alligator gar 4g/L after 48hours, melanin aggregation (M), vacuolization (V) in hematopoietic tissue.

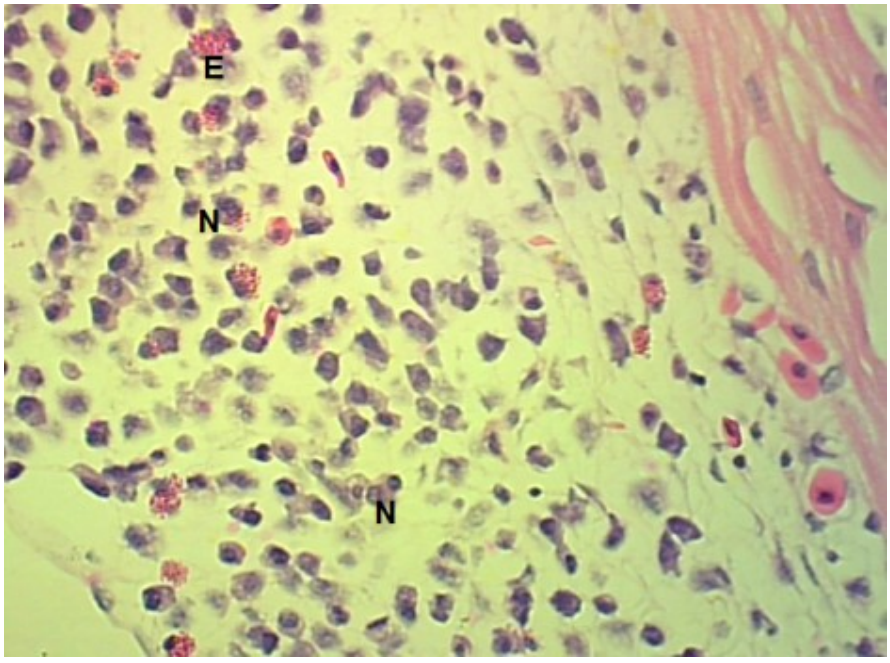

Figure 5.14 Heart tissue from exposed Alligator gar 4g/L after 48hours, inflammation in heart epicardium with eosinophilic (E) and neutrophilic infiltration (N).

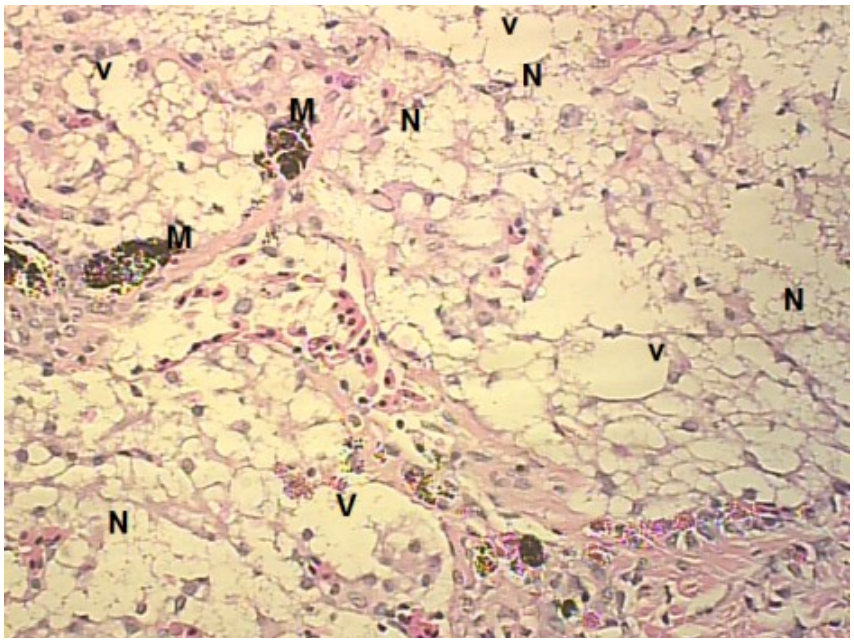

Figure 5.15 Liver tissue from exposed Alligator gar after 7 days, necrosis (N), Vacuolization (V), Melano-macrophage centers (M).

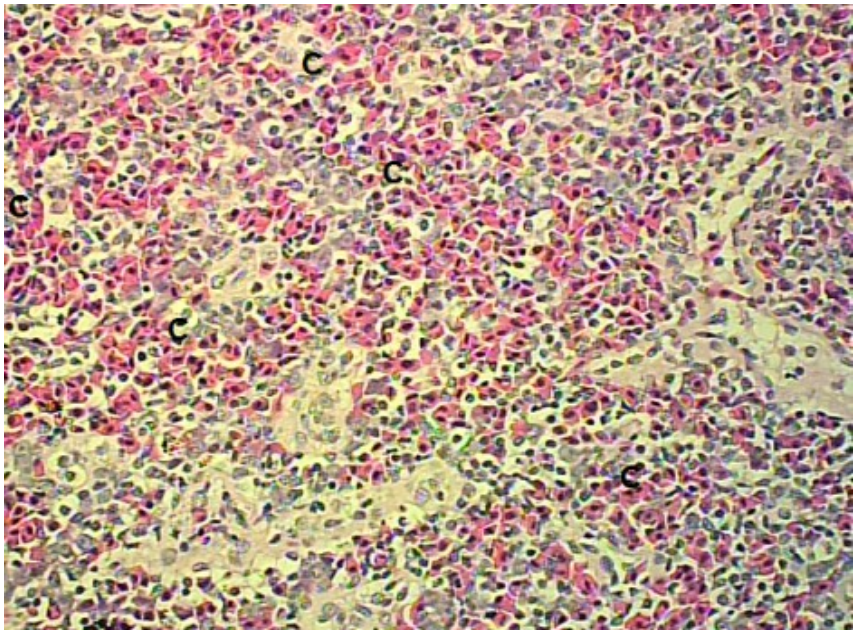

Figure 5.16 Spleen tissue from Alligator gar after 7 days, congestion(C).

Frozen sections

No positive cells were observed in any of the liver sections from unexposed fish tissues, 48 hours and, 7 days recovery treatments. Acid phosphatase positive cells from unexposed fish were seen in spleen sections. These cells were located close to arterioles, and also distributed throughout the parenchyma (Fig 5.21). Alpha-naphthyl-butyrate esterase from treatment 4g/L after 48 hours demonstrated positive cells, and melanin granule aggregations adjacent to the blood vessels (Fig 5.23). None of the cells stained positive for NAE and BG stain. The counting of the positive cells from the AP sections after 48 hours with treatment 4 g/L revealed that there were increased in the numbers when compared to the positive cells from unexposed fish, but there no significant differences between the positive cells counts from treatment 4 g/L and unexposed fish (Fig 5.17). While the counting of the positive cells from the AP sections after 7 days recovery with treatment 4 g/L showed that there were decreased in the numbers when compared to the positive cells from unexposed fish. Also, there were no significant difference between the positive cells counts from treatment 4 g/L and unexposed fish (Fig 5.18). The counting of the positive cells from the AP sections after 48 hours with treatment 0.5 g/L showed increases in the numbers of the positives cells when compared to the counting numbers of positive cells from unexposed (Fig 5.19). The counting numbers of positive cells from treatment 0.5 g/L after 7 days recovery decreased compared to the positive counting numbers from unexposed fish (Fig 5.20). Finally, there were no significant difference between the positive cells counts from treatment 0.5 g/L and unexposed fish in 48 hours and 7 days recovery.

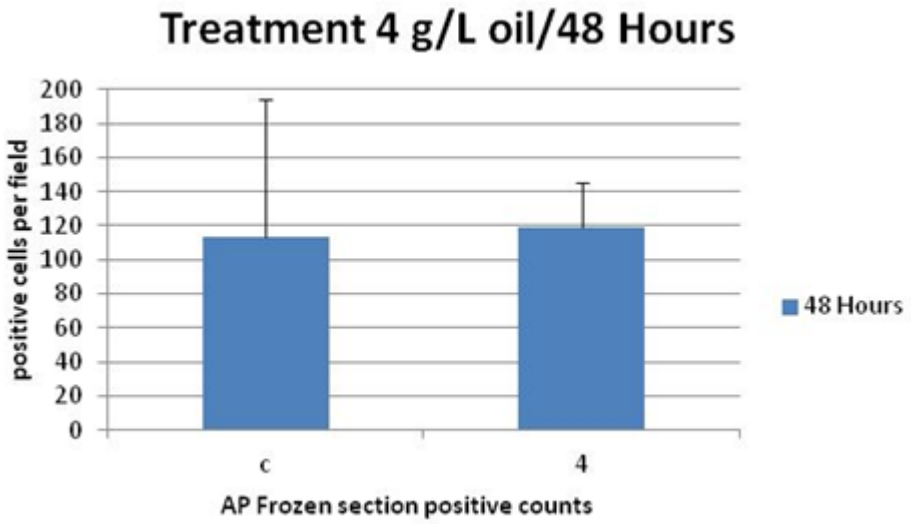

Figure 5.17 AP frozen section; positive cells counts with treatment 4g/L oil, 48hours.

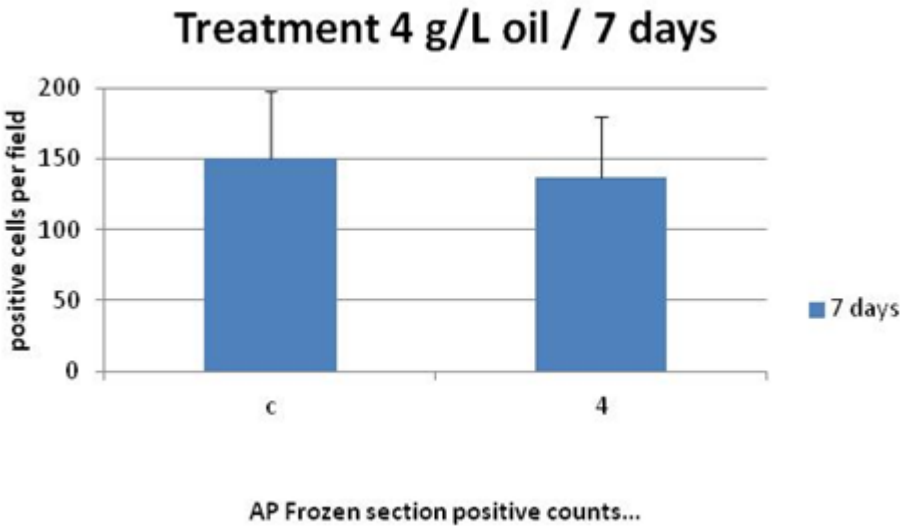

Figure 5.18 AP frozen section; positive cells counts with treatment 4g/L oil, 7days recovery.

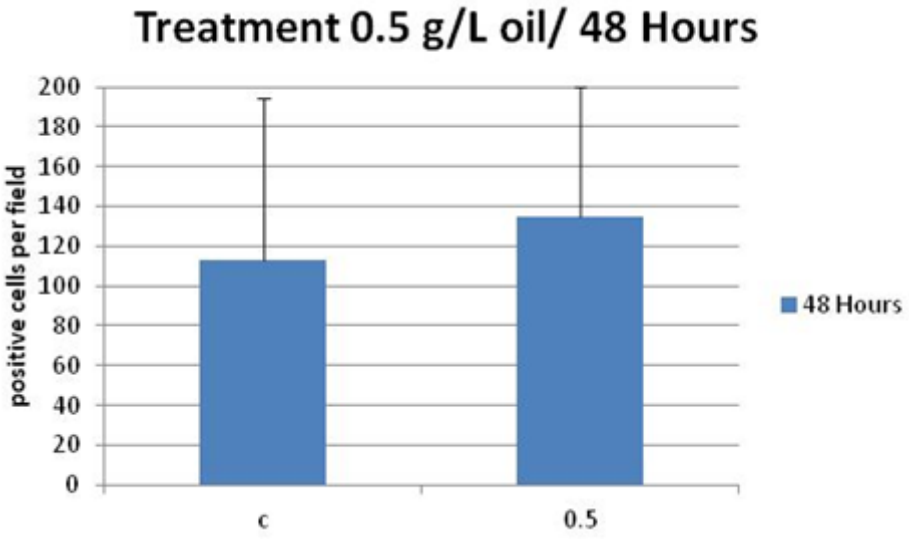

Figure 5.19 AP section; positive cells counts with treatment 0.5g/L oil, 48 hours.

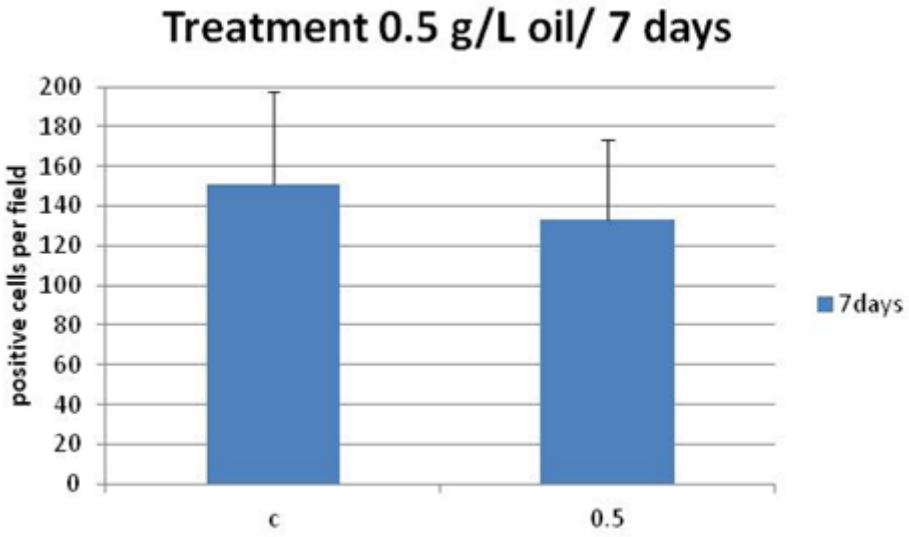

Figure 5.20 AP frozen section; positive cells counts with treatment 0.5g/L oil, 7 days recovery.

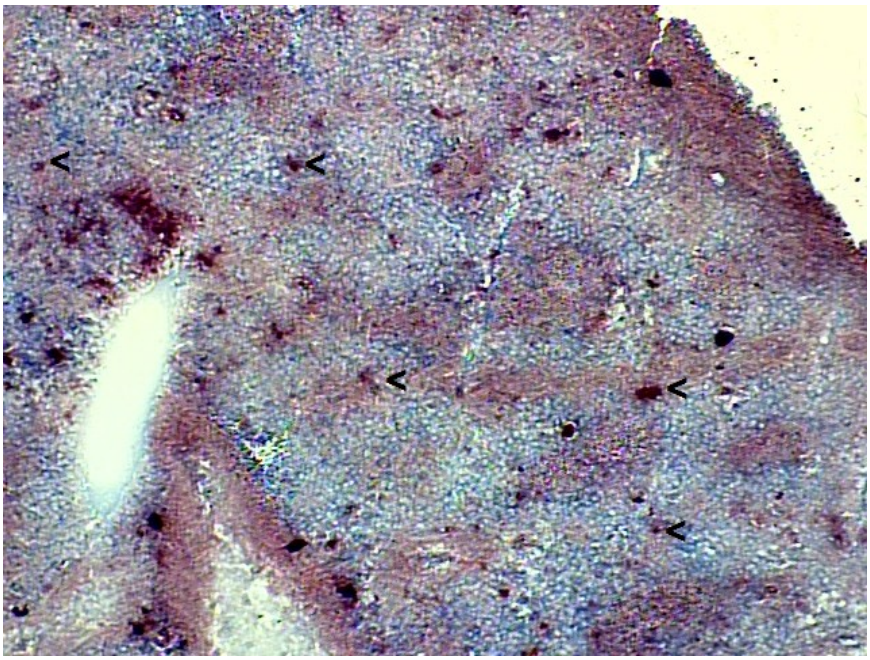

Figure 5.21 Alligator gar spleen from control fish; positive cells with AP stain (<).

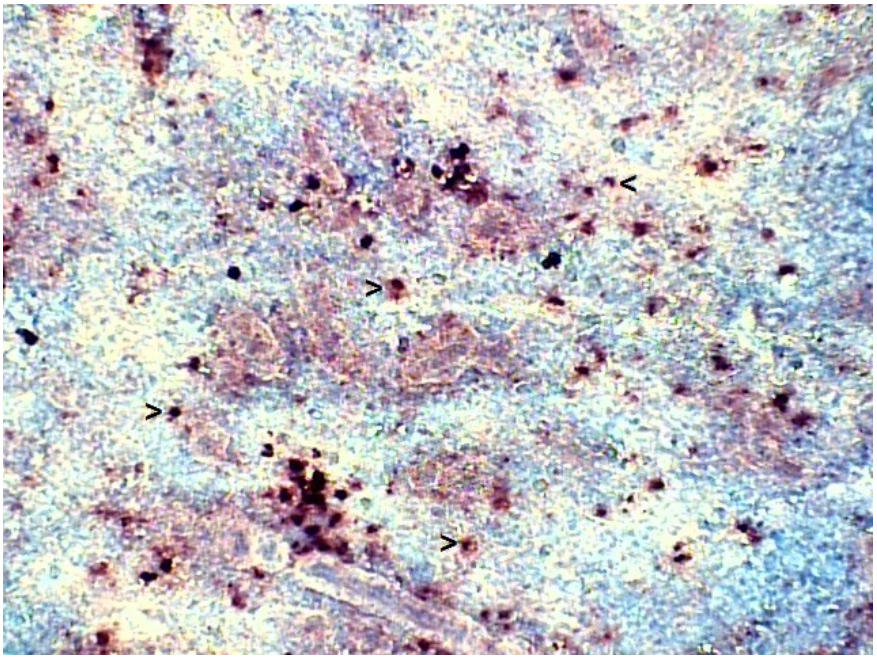

Figure 5.22 Alligator gar spleen from treatment 4g/L; positive cells with AP stain (<).

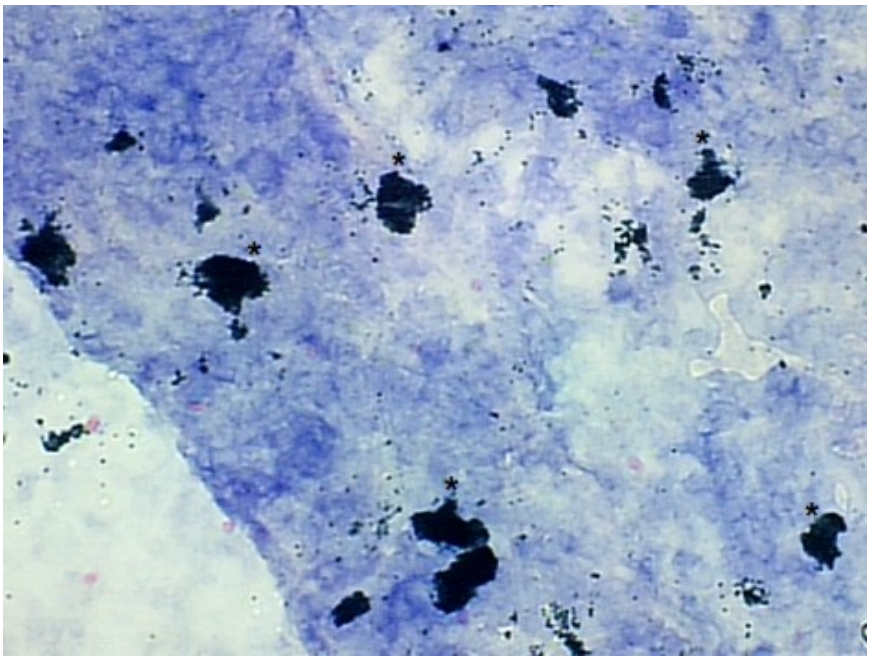

Figure 5.23 Aggregation of melanin with NBE stain (*).

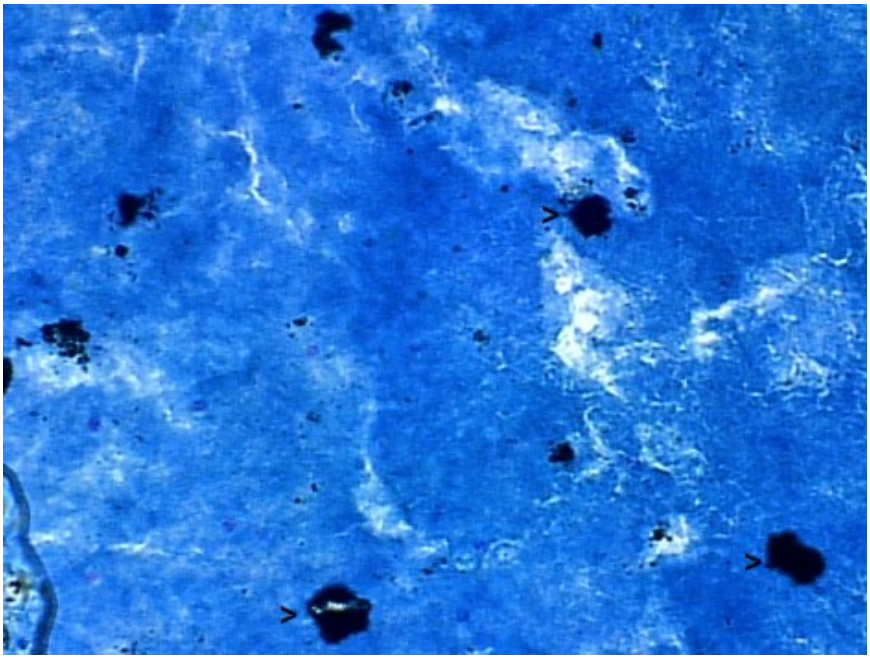

Figure 5.24 Aggregation of melanin with BG stain (<).

CHAPTER VI

DISCUSSIONS AND CONCLUSIONS

The current study examined the hematological, toxicological, and the histological responses to crude oil in fish. Alligator gars obtained from a private hatchery in Tupelo, MS, were exposed to different treatments of oil (4g/L- 0.5g/L-control fish). Fish were sampled after 48 hours of oil exposure and after 7 day oil free recovery period.

EROD activity is performed by cytochrome P 450 A1 that is used as a biomarker of exposure for PAHs. The EROD demonstrates the effect of the uptake of toxic planar compounds in fish, whether the presence of these agents have been analytically detected or not. In our study, EROD values for the sea trout from the Gulf were significantly greater than the values from the land sea trout facility, and it means that CY P 450 A1 was produced abundantly, most probably as a result of exposure to hydrocarbon. While EROD values from Alligator gar and killifish did not reveal any pointer of hydrocarbon as a result of oil exposure. An explanation for this is the livers from all treatments, including many of the control livers, did not show normal construction of the liver, and we think that affected the EROD levels. The liver samples from killifish were very small to process, and that can be the reason for the negative results.

In 1999 a study was performed by exposing channel catfish to Aroclor, and liver samples were removed from the fish for EROD analysis. The EROD was induced in the fish that had been exposed to Aroclor, and the EROD values were significantly higher in

Aroclor treated fish compared to the unexposed fish. Also, they did another experiment using the same chemical, and it revealed that the EROD values were significantly greater in the treated fish [14].

Another study explained that chemicals like oil, gas, and pesticide had been affecting the fish along the Rio Grande River. Liver samples were collected from the fish from ten different sites along the river for EROD analysis. EROD values were significantly higher in the fish from the sites close the Gulf of Mexico than from the fish that were sampled in the area close to New Mexico [42].

Leukocytes counts are one of the valuable tools to diagnose many diseases in fish, and blood cells of fish are produced from hematopoietic tissues which are located in the spleen and kidney. Anything that affects these organs will affect the normal distribution of leukocytes by increasing or decreasing leukocytes counts.

An experiment was performed by exposing humans to petroleum by inhalation, and there was a significant decrease in leukocyte counts compared to the control counts. In addition, the red blood cells counts were significantly reduced as well as the leukocyte counts [43].

Leukocytes counts from the Gulf of Mexico fish revealed that crude oil affected the normal distribution of WBCs compared to the counts from unexposed fish by decreasing the counts of lymphocyte in peripheral blood. The counts of leukocytes cells for Alligator gar after 48 hours indicates there were decreases in the lymphocytes counts as well as fish from the Gulf with increases in granulocyte counts especially eosinophils compared to the unexposed fish. After 7 days, leukocyte counts recovered close to the

normal counts. This suggests that the crude oil affected the immune system, and increased the fish's susceptibility to infectious diseases.

In this study, liver from 48 hours exposure and 7 days recovery demonstrated lipid vacuolization was observed in the cytoplasm of hepatocytes and severe necrosis in the parenchyma. Eosinophilic infiltration was observed in hepatopancreatic tissue and hematopoietic tissue of the kidney. In exposed fish the liver was the most dramatically effected tissue, and after 7 days recovery, severe lesions were still observed. Structural damage suggests these fish may not ultimately survive the 48 hour oil exposure.

A study exposing fish to petroleum products demonstrated vacuoles in the cytoplasm of the hepatocytes with focal necrosis, and fatty degeneration. Also, there was eosinophilic infiltration of pancreatic tissue [28]. Another study investigated the pathological changes in tissues of adult Herring from the site of the Exxon Valdez oil spill. Observing the tissues of the livers from each fish revealed that there was severe hepatic lipidosis, and marked hepatocellular necrosis in the fish from the exposed area. In addition, hepatocellular lipidosis was the major pathological change that was observed in rockfish from the site of that oil spill [6].

The present study showed that crude oil has affected fish that have been exposed in the Gulf, or in the laboratory (48 hours and caused white blood cells changes leading to immunosuppression in these fish, which make them more susceptible to infectious diseases. The EROD values from the Gulf fish indicated that the oil was still affecting the fish after several months from the oil spill. This may explain why skin lesions in the carp and fish in the Gulf of Mexico are still occurring. The pathological changes in the spleen, heart, and kidney after 48 hours exposure indicates that crude oil caused effects in many

fish tissues other than the liver. These changes caused inflammation with severe functional problems that may lead to the death of the fish. After 7 days oil free recovery, histopathological changes were less in severity in these fish tissues than 48 hours fish tissues. In addition, liver organ is responsible for the detoxification for the fish body, and if it exposed to high levels of chemicals that will cause a serious damage in the structure of the hepatocytes. As long as the oil exposure persists, fish will have increased susceptibility to infectious disease.

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