EFFECT OF *Vibrio* TOXIN ON HAEMATOLOGY AND HISTOPATHOLOGY OF *Clarias batrachus* (Linn)

M.F.Sc. DISSERTATION

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Dissertation submitted towards partial fulfilment for the degree of

MASTER OF FISHERIES SCIENCE
IN
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By

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1997

Dedicated to

Deota & Maa

DECLARATION

I hereby declare that the present dissertation entitled **Effect of *Vibrio* Toxin on Haematology and Histopathology of *Clarias batrachus* (Linn.)** is a record of authentic research work carried out by me under the guidance of Dr. K. Pani Prasad, Scientist, Division of Fish Pathology, Central Institute of Fisheries Education (ICAR), Mumbai.

The present experiments were carried out in the Pathology Unit of CIFE, Mumbai is submitted towards partial fulfilment of the requirement for the degree of Master of Science in Inland Aquaculture course during 1995-97 and the data not earlier formed the basis for any other publication or thesis.

CIFE, Mumbai
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This is to certify that the dissertation entitled "EFFECT OF *Vibrio* TOXIN ON HAEMATOLOGY AND HISTOPATHOLOGY OF *Clarias batrachus*" is a record of bonafide research work done by Mr. RUPAK NATH of the 1995 - 97 batch of M.F.Sc. (Inland Aquaculture) programme under our guidance and that it has not previously formed the basis for any publication or for the award of any other degree, diploma, or other similar titles.

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LIST OF ABBREVIATION

BHI	Brian Heart Infusion Agar
ECG	Econophilic Granule Cell
ECP	Extracellular Product
ESR	Erythrocyte Sedimatation Rate
Hb	Haematcrit Value
IU	International Unit
MCHC	Mean Cell Haemoglobin Concentration
MCH	Mean Cell Haemoglobin
MCV	Mean Cell Volume
mg	Milli gram
mm^3	Cubic milli meter
PBS	Phosphate Buffer Saline
pg	Picogram
RBC	Red Blood Cell
rpm	Rotation per minute
SGOT	Serum Glutamate Oxalacelate Transminase
SGPT	Serum Glutamate Pyruvate Transaminase
TG	Triglyceride
WBC	White Blood Cell
µl	Microlitre
µm	Micrometer
%	Percent

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RUPAK NATH

ABSTRACT

Disease diagnosis is a formidable task. Perfect diagnosis of disease is very much essential for effective treatement of the disease. Haematology and histopathology plays an important tool for the disease diagnosis. *Vibrios* are ubiquitous fish pathogen, producing endotoxin, which is haemolysin and cytoxin in nature. The toxin comprises of lipid polysaccharides and protein that was extracted from species *Vibrio alginolyticus* through centrifuging the eighteen hours broth culture. Significant chages were observed in haematological parameters of toxin injected fish *Clarias batrachus* which led to anaemic condition. The anaemic condition is one of the most important symptom of *vibriosis*. An increase in the number of WBC, ESR values and decrease in the haematocrit and haemoglobin concentration was observed in toxin injected *Clarias* when compared to the control group. A significant change in the concentration of uric acid, creatinine, Triglicerides, SGOT, SGPT in the serum of toxin injected fish was observed that indicated extensive liver and kidney damage. This results were confirmed by histopathological studies on different organs of the fish.

सारांश

रोग निदान एक कठिन कार्य है । रोग का उचित निदान रोग के प्रभावकारी उपचार के लिए अत्याधिक आवश्यक है । रक्त अध्ययन तथा औतिकी रोग अध्ययन रोग निदान में महत्वपूर्ण भूमिका निभाते हैं । वाहब्रियोस मुख्य सर्वव्यापी मत्स्य रोग कारक व अन्तर्विष उत्पादक हैं जो हीमोलाहटिक तथा साइटोटोक्सिन प्रकृति के हैं और वसा, सैकेराइडस, प्रोटीन के बने होते हैं जो कि वाहब्रियोस एलजिनोलाहटिकस जाति से 18 घंटे के अपकेन्द्र करण से निकाले जाते हैं । विष से उपचारित मछली क्लेरियस बैट्रेकस के हिमेटोलोजिकल मानकों मे चमत्कारिक परिवर्तन देखे गए । जो मछली को रक्ताल्पता की दशा की ओर ले जाते थे । ये वाहब्रियोसिस के महत्वपूर्ण लक्षणों में से एक है । विष अन्तर्वेशित मछली के यूरिक एसिड, क्रियेटिनीन ट्राईग्लिसरीन, S G O T, S G P T की सान्द्रता में महत्वपूर्ण परिवर्तन देखे गए । जो वाहब्रियो विष की रोग जनकता दर्शाते है । ऐसे औतिकीरोगजनक अध्ययन से किए गए जो ऊतक स्तर की संरचना में बहुत सी असामान्यताओं का प्रमाण है ।

INTRODUCTION

1 INTRODUCTION

Disease is a complex interaction between the host, pathogen and the environment. Although primary pathogens may cause infections when the environmental conditions are inadequate, facultative or opportunistic pathogens can cause disease only when both the physiological status of the host and the environmental conditions of the culture system are improper. In a culture facility, the environmental conditions such as unrealistically high stocking densities, incorrect feeding in both the quality and quantity, poor handling adversely affect the physiological status of the cultured organism. Once the organisms under culture are stressed, it allows both primary and facultative pathogens to casuse infections easily.

Diseases are among the greatest deterrents in achieving maximum production in aquaculture. They not only reduce production but also cause severe financial losses to the global aquaculture industry. According to recent estimates, fish disease accounted for lost revenue of at least US $ 1.36 billion in 1990 in Asia alone. Outbreaks of diseases and inadequate health management have been considered to be the most significant causes of these bitter incidents.

The foremost important step in aquaculture health management is to provide best quality environment within the culture unit (FAO Newsletter, 1995). It is difficult to completely eliminate infections in cultured animals. Diseases will occur in aquaculture regardless of the quality or intensity of management. It is essential to treat the fish with

appropriate chemicals, drugs, or antibiotics when disease occur. To make the treatment to be sucessful, it is essential to know the disease. A swift and correct diagnosis is a formidable task. Perfect diagnosis is very much important to know the causative agent of the disease as well as to enlighten about the abnormalities of the host.

Clinical haematology and histopathology are the most popular branch of medical physiology as they act as the most convenient and economically viable techniques for the diagnosis of physiological status of an animal. Haematological and chemical properties of blood are highly sensitive to even slight changes in the physiological condition of animal.

Histopathological studies also plays an important role in disease diagnosis. Comparative microscopic study of normal and abnormal tissues given a clear picture for any pathological changes and toxigenecity of the toxic compound.

The genus *vibrio* consists of gram negative straight or curved rods, 0.5 -0.8 in width and 1.4-2.6 µm in length. This genus contains the most significant fish bacterial pathogens. Among *vibrios*, *Vibrio algin olyticus, Vibrio ordali, Vibrio salmonicida* and *Vibrio vulnificus* are fish pathogens are associated with accute bacterial septicaemias or chronic focal lession in fish

Vibrios have emerged as the scourge of fish and shell fish. Many species have been described as fish pathogens. *Vibrio alginolyticus* (Miyamoto *et al.*, 1961) is very common in coastal waters of temperate and tropical regions (Goldern & Scheffers, 1975).

Vibrio alginolyticus infected fish was observed to become sluggish, the skin darkened, scales lossened and sloughed off and ulcers developed. Anaemia and gill rot were also seen. *Vibrio alginolyticus* abundant in the marine and estuarine environments and therefore present a constant threat for any susceptible host.

Microbial toxin cause a highly specific type of tissue damage in its host. The genus gram negative *vibrios* produced two types of toxin, haemolysin and cytotoxin (Venkateshwaran *et al.* 1989). The heat stable toxin associated with the cells of gram-negative bacteria known as endotoxin. The endotoxin are relatively nonspecific, all producing much the same clinical and pathological symptoms when injected into experimental animal. Endotoxins are lipopolysaccharide-protein complexes, derived from the outer layers of the cell walls of gram-negative bacteria (Stainier *et al.*, 1976).

Catfish are widely distributed in Asia. *Clarias batrachus* commonly known as walking catfish which is well established in India. *Clarias batrachus* (Linn) belongs to family Claridae can be cultured in stagnant or running water ponds. *Clarias batrachus* is considered as an esteem food fish in many parts of India. It has excellent culture potentialities in India.

Hence, a clinical toxicological approach was conducted in the study to evaluate the effect of *Vibrio* toxin on hematology and histopathology of *Clarias batrachus*.

REVIEW OF LITERATURE

2 REVIEW OF LITERATURE

2.1 History of *Vibrio* toxin

The genus *Vibrio* consists of gram negative straight curved rods 0.5-8 μm x 1.4-2.6 μm. They are non spore forming and motile by monotrichous or multitrichous sheathed polar flagella (Hjeltnes and Roberts, 1993). This genus contains the most significant marine fish bacterial pathogen and are associated with acute bacterial septicaemia or chronic focal lesion in fish. *Vibrios* are found in saline waters and freshwaters. They are more frequent where static and benthos occurs in combination with high organic load. These *Vibrios* release extracellular toxin but are not actively excreted into medium. The toxin is accumulated intracellularly and are liberated when cells die. An important toxic component of gram negative bacterial cell envelops, lipopolysaccharides, consists of lipid and polysaccharide. The lipopolysaccharides is commonly referred to as endotoxin because it is a structural component of bacterial cell (Arbuthnott, 1978).

Roberts (1975) studied on capacity of the *Vibrio* microorganisms, to produce haemolytic anemia, which results in high circulating, and melano-macrophage related to iron levels in affected fish, is related to its high requirement of iron. Pathogenic strains have a well developed iron sequestering mechanism based on secretion of a iodophore, which induces respiration of plasma and tissue iron from its transferin or ferritin binding proteins.

The purified endotoxin of virulent, as well as of avirulent enteric bacteria cause many of the symptoms of disease when injected

into animals. These toxins are inflammatory agent, which increase capillary permeability and produce cellular injury. Stainer *et al.*(1979) mentioned that toxin that filtrates of *Vibrio cholerae* when injected into ligated intestinal loops of rabbits, it produced the gross fluid loss and mucosal damage characteristic of the natural disease. The endotoxins of enteric bacteria cause transient changes in nonspecific resistance to infection when injected into animal in minute dose (Stanier *et al.*, 1979).

Karunasagar *et al.* (1987) studied about the human pathogenic vibrios *Vibrio parahaemolyticus*, *Vibrio vulnificus, Vibrio minicus* and non 01 *V.cholerae* isolated from seafoods of their ability to produce haemolysins and lethality to mice. He reported strains of *Vibrio parahaemolyticus* producing a heal stable haemolysin had a lower LD_{50} for mice compared to non haemolytic ones. Both haemolytic and non haemolytic strains of *Vibrio vulnificus* were isolated from seafoods and the former had greater lethality to mice. Even in non 01 *Vibrio cholerae*, haemolytic strains had greater mouse virulence than non-haemolytic strain.

Venkateshvaran *et al.* (1989) reported the occurrence and characterization of toxigenic *Vibrios* in surface water and sediment samples of the freshwater environment of the Ohta river. In the identification of 361 strains, 82% of the haemolytic *Vibrios* produced various toxins. About 71% elaborated cytotoxin and 55% produced haemolysin and 44% responded for both cytotoxin and haemolysin in the crude toxin extracts.

Powell (1992) studied on rainbow trout (*Oncorhynchus mykiss* Walbaum) intestinal eosinophilic granule cell (EGC) response to *Aeromon as* and *Vibrio anguillar.um* extracellular product. Bacterial extra cellular product was prepared from a broth culture of bacteria and injected intraperitoneally at a dose of 0.07 and 0.08 $\mu g\ g^{-1}$ body na-1 weight into O+ rainbow trout. A rapid decrease in the number of extra cellular product in control fish.

2.2 Haematology

Haematology concern mainly investigations on the cells present in the blood. Blood is defined as a fluid connective tissue has two components, fluid component and formed component. Fluid component is the watery component of blood called plasma whereas the formed component is cellular component viz. Red Blood Cells, White Blood Cells and thrombocytes or platelets. All fishes possess two main types of blood cells - Erythrocytes and Leucocytes which are derived from early fishlike ancestors (Nelson, 1984). He reported that the structures and function of the blood cells varies between different groups of fishes.

Larsson *et al.* (1976) determined the Hamatocrit Value and Haemoglobin concentrations in fish blood. He reported normal values of Hamatocrit Value and Haemoglobin for teleosts, slow swimmers (*Lophius piscatorius* and *Cyclopterus lumpus*) were 17.2%, 3.2g% and 19.3%, 3.3 gm%. For fast swimmers, *Scomber scombrus* and *Clupea barengus* the Hct value and Hb concentrations were 52.5%, 15.2 g% and 51.2 g%, 14.0 g% respectively.

Gutierrez (1967) reported the normal values of haematocrit, Erythrocyte, Haemoglobin concentration and Erythrocyte length for fish *Thunnus thunnus* were 52.4%, 2.15 x 10^3 µl, 15.4 g% and 13.1 µm respectively.

Glazova (1977) studied the normal blood parameters viz. Hct, RBC count, Hb, Erythrocyte length of elasmobranch fish (*Prionace glauca*) and reported the values as 25%, 0.76 x 10^3 µl, 10.1 g% and 15.7 µm respectively.

Haematological investigations are now employed for diagnostic purposes in fish pathology. Haemotological investigation on fish have confirmed that the variations in the blood gives indication of health condition (Schaperclaus, 1986).

Schumacher *et al.* (1956) reported increased ESR in brook trout infected by furunculosis and Murad and Mustafa (1988) also found a similar response in catfish (*Heteroneustes fossilis*) parasitized by matacercariae and also reported low haemotocit and increased white cells counts in the diseased fishes.

Among Indian workers Banerjee (1966), Srivastava (1968 a, b), Dube (1972); Pandey (1987); Ahmad (1982) studied on normal haematological parameters *viz.* Haemoglobin concentration , Erythrocyte count, Leucocyte count, Haematocrit value, erythrocyte sedimentation rate, Red Blood indices on freshwater fishes of India are given in table 1.

Tripathi *et al.* (1979) studied on blood parameters of *Clarias batrachus* under normal respiratory submerged water and exclusive air

Table 1: Normal blood values of the air-breathing fishes of India

Fish species	Erythrocyte number × 10^6/ $m.m^3$	Hb g/ 100 ml	Hct%	ESR mm/hr	MCV (fl)	MCH (pg)	MCHC %
Channa punctata	8.24 -3.30	14.8 -15.20	30.2 - 32.4	4.30 -4.50	94.12-98.99	46.44-46.53	46. 97 - 48. 55
Clarias batrachus	1.63 -21.02	7.52 -7.8	31.0-36.0	-	150.46 -214.07	41. 02	23. 09
Clarias batrachus	1.58 -2.59	10.22-18.8	20.4 -35.2	2.0 -3.4	87.81-150	30.73-53.87	33.55 -38.62
Heteropneustes fossilis	1.58-2.39	8.9-14.0	36.5-52.0	-	173.0-325.0	-	17.30-38.8
Anabus testudineus	3.37-7.06	19.0-19.8	-	-	-	-	-

(In Dutta Munshi, J.S. G.M. Hunghen (1992) Air breathing Fishes of India. Oxford & IBH Publishing Co. Pvt. Ltd.

Table 2: Blood parameters of *Clarias batrachus* during different respiratory conditions at 25.1^{0}C (Tripathi, *et. al.*, 1979)

Parameters	**Normal respiration**	**Submerged water condition**	**Exclusive air breathing**
Wt. of fish (g)	70.3 ± 2.08	72.1 ± 1.17	71.9 ± 2.11
RBC in million/ mm^3	2.26 ± 0.11	2.78 ± 0.10	3.14 ± 0.15
Hb g%	12.6 ± 0.28	14.2 ± 0.17	15.8 ± 0.31
PCV%	37.3 ± 1.35	43.7 ± 1.31	48.1 ± 1.48
MCV μm^3	165.04	157.18	153.19
MCH pg	55.75	51.08	50.32
MCHC %	33.79	32.49	32.85

breathing condition. It was reported that the RBC counts, Hb content and Hct were significantly higher in *Clarias batrachus* under submerged and exclusive air-breathing conditions compared to control groups. Absolute values of Mean cell volume (MCV), mean cell haemoglobin (MCH) and Mean cell Haemoglobin concentration (MCHC) were higher in control groups than experimental groups. All the blood parameters of *C.batrachus* during respiratory condition are given in Table 2.

2.3 Blood chemistry

Blood plasma carries the blood proteins, blood sugar, blood fat, varius salts and many other substances which are needed in the fish body. The level of all substances depends on physiological condition of the fish.

2.3.1 Uric Acid

Uric acid is formed by the degradation of pruine nucleotides and protein catabolism via purins, primarily in the liver and white muscle. Uric acid is generally converted to urea for excretion, so blood levels are typically low. In rainbow trout, plasma uric acid ranges from 40 μm to 100 μm (Hille, 1982).

2.3.2 Creatinine

Creatinine is formed by spontaneous cyclization of creatinine. Its level in plasma are typically low, 10-80 μm (Sandnes *et al.*, 1988), and appear to be unaffected by stress (Wells *et al.*, 1986) in African lungfish, estivation (Delaney *et al.*, 1977). Creatinine is not metabolized further and is excreted by the kidney. Creatinine level

increases due to acute and chronic renal failure, protein losing nephropathies urinary trout obstruction, congertive heart failure, decreased cardiac output and shock and dehydration.

2.3.3 Triglycerides

Triglycerides (TG) are the primary storage form of lipid in most fish species and are readily mobilized in response to physiological demand. The level of plasma TG varies throughout the life cycle of many fish and is affected by several factors like sexual maturation, spawning and nutritional status. Starvation results in a loss of TG from the plasma. In sea bass and starved for 40 days, plasma TG levels dropped from 560 to 280 mg dl^{-1} (Zammit and Newsholme, 1979). The level of Triglyceride concentration is raised due to biliary stasis, cirrhosis, extra-hepatic binary obstruction and concentration lowered because of acute liver disease.

2.3.4 Serum Glutamate-pyruvate transaminase (SGPT) and serum Glutamate-oxalacetic tramminase (SGOT)

Measurement of the activities of the plasma nonspecific enzymes has diagnostic potential in fish toxicology and pathology because enzyme activities is related to cell damage in specific organs. Mukhopadhyay (1987) reported the disturbances in the enzyme systems of cellular organelles of liver and gills of *Rita rita* when they were exposed to sublethal concentration of 0.5 ppm malathion for 40 days and 1.0 ppm for 30 days. Liver is rich in glutamic oxalacetic transaminase (GOT) and glutamic-pyruvate transaminase (GPT), and changes in plasma levels of these enzymes indication of liver dysfunction.

In trout treated with carbon tetrachloride (CCl_4), a hepatotoxicant, plasma GOT and GPT levels increased significantly (Racicot *et al.*, 1975). He reported that the GOT and GPT level concentration increased to 347 from 244 IU^{-l}.

Tandon and Chandra. (1978) studied on asphyxiation stress on serum transaminases level of fresh water cat fish, *Clarias batrachus*. He reported the levels of the two enzymes immediately fall after sudden asphyxiation stress of 15 minutes. SGOT level then rose to 22.16% when the stress was continued for longer periods. SGPT levels, rose when stress was continued upto 30 minutes, but fell to 24.54% when stress was continued to 60 minutes.

The SGOP level in serum raised due to muscle injury or necrosis. SGPT concentration in blood serum is raised due to cardio vascular diseases and hepatic diseases.

2.4 Histopathology

Histopathology is the study to structure of abnormal tissue. Histological structure of the body and organ of fishes are fundamentally the same as there of higher vertebrates.

M.F.X Bichatt (1715-1802) who provided the first general concept of animal histology. Virchow (1821-1902) proposed that disease could be characterised by changes at the cellular level (Hibiya, 1982).

Histopathology is of extreme importance in the diagnosis, etiology and prevention of disease (Hibiya, 1982). Microscopical examination of normal and diseased (pathological) tissues can be most

satisfactorily performed on suitably prepared material (Clayden, 1962).

Mayers and Hendricks (1984) reported that quantitatively or qualitatively measure changes prior to the death of an organism can often provide early indication of toxicity and valuable insights into the mechanism of toxicity. Examination of tissues from fish and other aquatic organism after death serve to identify the cause of death, possibly the causative agent. This information along with physiological and biochemical data will provide more complete and accurate description of the activity of a chemical agent.

Siegelman *et al.* (1983) studied about the effect of peptide toxin of the cyanobacterium, *Microcystistis aeruginuosa* which was injected to mouse with a medium lethal dose of about 0.06 µg/gm of body weight. The toxicity associated with marked reduction in the number of blood platelets, microscopic clot in the lung and engorement of the liver with blood. Frerichs and Roberts (1989) studied on histopathological changes on the atrium of Doversole with peracute vibriosis. He reported extensive vacuolation of the sarcoplasm with small foci of inflammatory cells and hyperaemia of the pericardium.

Inglis (1993) studied the mid kidney of salmon with acute vibriosis. He reported tubular necrosis as well as haemopoietic depletion and necrosis in the mid kidney.

MATERIALS AND METHODS

3 MATERIALS AND METHODS

3.1 PREPARATION OF GLASSWARE

All the glassware was soaked overnight in a detergent solution. Next day, they were washed thorouhgly in water and finally with triple distilled water. After air drying they were wrapped in paper and sterilized in hot air over at 180^0C for an hour.

3.2 LOCATION

Experiments were conducted under laboratory condition in fish Pathology Laboratory of CIFE.

3.3 EXPERIMENTAL FISH

Apparently healthy *Clarias batrachus* fish of 80 - 90 gm and 20 - 22 cm in size were procured from local Andheri Fish Market, Mumbai. They were acclimatized and kept in glass aquarium 1.5 x 1 x 1 untill further use. The fish were fed with balanced pelleted feed and provided with continuous aeration. The water was exchanged every three days.

3.4 BACTERIA

Pure culture of *Vibrio alginolyticus* was obtained from Division of Fish Pathology, CIFE, Mumbai.

3.5 EXTRACTION OF TOXIN

To 100 ml of BHI broth a loopful of organism was inoculated and incubated at 30 - 33 °C for 24 hours. Then the culture was centrifuged at 10,000 rpm for 20 minutes. Supernatant was discarded and the precipitate was washed by centrifugation at 5,000 rpm for 15

minutes with phosphate buffer saline. The supernatant was discarded and the precipitate was mixed in 100 ml PBS. The PBS with the cells were heated to 80° C for three hours. After three hours solution was immediately cooled to 0°C (Thermal shock treatment). Then the solution was centrifuged at 5,000 rpm for 15 minutes. The supernatant was crude toxin and was stored at -20 °C until further use.

3.6 COLLECTION OF BLOOD

Blood was collected from experimental fish to conduct the different studies. The haematological studies conducted for normal fish as soon as they were caught. For toxin injected fish haematological studies were done after 48 hours injecting *Vibrio* toxin to the fish. 20 numbers of normal and toxin injected fishes were used to perform all haematological tests. From each fish about 3 ml blood was drawn of which 1.5 ml was mixed with anticoagulant and 1.5 ml was allowed to clot for serum separation.

3.6.1 Preparation of Ethylenediamine Tetra Acetic Acid Solution

EDTA was used as an anticoagulant to perform all haematological tests. 2.7 gm of EDTA salt was dissolved in 100 ml distilled water. The solution was sterilised by autoclaving at 15 lbs. pressure at 121^0 C for 15 minutes. 0.2 ml of the sterilized EDTA solution was taken in glass vials of 3 ml capacity and kept them in hot air oven at 60^0C untiil the EDTA solution dried completely. After drying the vials were used for blood collection. In each tube approximately 2 ml of blood was collected and used for different tests immediately.

3.6.2 Procedure

The fishes were caught gently and stress was avoided during the time of handling. Fishes were held horizontally by hand and absolute alcohol swabbed where the needle was inserted. Hypodermic needle of 24 guage 1 inch length was inserted between the two pectoral fins on the ventral side in the heart and blood was drawn gently. The blood was mixed in a vial with anticoagulant.

3.7 HAEMATOLOGICAL STUDIES

3.7.1 Enumeration of Red Blood Cells

In a test tube 3980 µl of RBC diluting fluid was taken. 20 µl of whole blood was taken and mixed thoroughly with RBC diluting fluid to make the dilution 200 times. Immediately, after proper mixing, the Neubauere's counting chamber was charged with RBC suspension. Care was taken that there were no air bubbles trapped. The red blood cells were counted in five groups of squares. All the cells lying inside the five small squares are counted under high power (40 x) of light microscope.

Calculation

$$\text{Number of RBC/mm}^3 = \frac{\text{Number of Red Blood Cell couunted(X) x Dilution}}{\text{Area counted x Depth of the fluid}}$$

$$= \text{Number of Red Blood Cell counted x } 200 / 0.2 \text{ x } 0.1$$

$$= X \text{ x } 10{,}000$$

3.7.2 Enumeration of White Blood Cells

3980 µl of WBC diluting fluid was taken in a test tube. 20 µl whole blood was added to test tube to make dilution 200 times and mixed thoroughly. A small drop of this mixture was charged to Neubauer's counting chamber of haematocytometer.

The number of cells were counted in four big squares under high power (40x) magnification of light microscope.

Calculations

$$\text{Number fo white} = \frac{\text{Number of White Blood Cell counted x dilution}}{\text{Area counted x Depth of the fluid}}$$

$$= \frac{\text{Number of White Blood Cell counted x 200}}{4 \text{ x } 0.1}$$

$$= \text{Number of White Blood Cell counted x } 500$$

3.7.3 Determination of Haemoglobin Concentration
(Sahli's method)

The method followed for determination of haemoglobin was acid haematin method. The calibrated tube of the Sahli's haemoglobinometer was filled with 0.1 N Hcl upto 20 mark. 20µl of whole blood was drawn by Sahli's pipette and was blown into the acid solution inside the graduated tube. The contents of the pipette was washed by repeated drawing in and blowing out of the fluid. The mixture was allowed to stand at room temperature for 10 minutes. Continuously the mixture was diluted by 0.1N HCl till the colour of test mixture and colour of the reference tube was same. Reading was taken directly from graduated tube.

3.7.4 Determination of Haematocrit Value (Hct) and Erythrocyte Sedimentation Rate (ESR): (Macrohaematocrit Method)

The Macrohaematocrit method has been employed for determining Hct and ESR simultaneously. The Wintrobe tube used for these two tests was a 110 mm long narrow test tube with a 3 mm internal bore, graduated from 0 to 10 cm with the graduation both in ascending and descending order on the two sides of the tube. The scale with the marking ascending order was used in ESR determination, while the scale with descending order was used for haematocrit determination. The Wintrobe tube was filled with whole blood with a long fine capillary Pasteur pipette. During filling, the tube was kept in slanting position and blood was filled carefully upto 10 cm mark which represents 100% of calibrated tube. Then the tubes were kept for one hour in a vertical position to dtermine ESR of the blood sample. After one hour the ESR value was noted and the blood sample was used for determining haematocrit value.

The Wintrobes tube was centrifuged at 3000 rpm for 30 minutes. After centrifugation, the Wintrobe tube were taken out and haematocrit value read directly from the graduated Wintrobe tube, in descending order.

3.7.5 Differential Count

3.7.5.1 Preparation of smear

For preparation of smear a small drop of blood was placed on one end of clean glass slide which is free from grease. A second slide was held at an angle of 45^0 approximately in the center of first slide. The slide was brought back against the blood until the blood spread by

capillary action along the interface between slides. The second slide was then moved in the reverse direction, creating a thin uniform film of the blood on the first slide. The blood film was air dried.

3.7.5.2 Staining of blood film

The air dried blood film was flooded with Leishman's stain for 10 minutes. The stain on the slide was diluted with distilled water and again kept for 10 minutes. Then the stain was drained off, washed with tap water, air dried and observed under oil immersion for identification and enumeration of different cells.

3.7.6 Calculation of Red Blood Cell Indices

The quantitative measurement of the average sizes by volume (V), haemoglobin content by weight (W) and haemoglobin concentration of the Red Blood Cells were calculated against corresponding value of the Haematocrit, Haemoglobin and Red - Blood Cell.

3.7.6.1 Mean Cell Volume (MCV)

This is the average volume (V) of red cells. The volume is expressed in femto litre (fl)

$$MCV = \frac{\text{Haematocrit (\%) x 10}}{\text{RBC count in million}}$$

3.7.6.2 Mean Cell Haemoglobin (MCH)

The MCH is the average haemoglobin content of a red cell. The weight is expressed in picogram (Pg)

$$MCH = \frac{\text{Haemoglobin (g/100 ml x 10}}{\text{RBC count in million}}$$

3.7.6.3 Mean Cell Haemoglobin Concentration (MCHC)

The MCHC is an expression of the average haemoglobin concentration per unit volume of packed red cells (W/V).
It is expressed in g/dl.

$$MCHC = Hb\ (g/dl) / Hct\ (\%) \times 100.$$

3.8 BLOOD CHEMISTRY

The different parameters like triglycerides, uric acid, creatinine, glutamate pyruvate transaminase (SGPT) and glutamate oxaloacetate transaminase (SGOT) were performed employing. Diagnostic kits manufactured by Sigma Diagnostics kits, Mumbai and marketed by Qualigens Fine Chemicals, Mumbai.

3.8.1 Separation of Serum

Blood was obtained by puncturing the heart and was allowed to clot in a test tube in a slanting position at room temperature. Then the tubes were centrifuged 10 minutes at 3000 rpm. The serum was separated and utilized for enzyme studies.

3.8.2 Measurement of Triglyceride (neutral fat) Concentration in Serum

Triglycerides of normal and toxin injected fish were measured as per the procedure of Fossati (1982). Three test tubes were taken for this assay and marked as test, standard and blank tubes which were filled with 1 ml of working reagent. Working reagent was allowed to equilibrate at room temperature and was prepared by dissolving. One vial of the enzyme was dissolved with 3 ml of buffer and mixed

thoroughly and gently. 10 µl of serum sample and 10 µl of standard sample supplied with the kit was taken in the respective test tube and mixed thoroughly. They were incubated at 37^0C for 10 minutes. Absorbance was read against reagent blank on a spectrophotometer at 520 µm and triglyceride was calculated by the following formula.

$$\text{Tryglyceride concentration (mg/dL)} = \frac{\text{A of (T)}}{\text{A of (S)}} \times 200$$

3.8.3 Measurement of Creatinine in Serum

Creatinine of normal and toxin integrated fish were measured as per the procedure of Henry (1974).

Two test tubes were taken for this assay and marked as test and standard tubes. Each tube was filled with 1 ml working reagent. Equal volumes of reagent I (Picric acid) and Reagent II (Sodium Hydroxide) mixed to prepare working reagent the working reagent was allowed to equilibrate at room temperature. 50 µl of serum sample and standard available with the kit were taken in respective test tubes and mixed thoroughly. They were incubated for 20 seconds. Absorbance was read against blank on spectrophotometer at 505 nm. and creatinine concentration was calculated by the formula:

$$\text{Mg/dl creatinine in the sample} = \frac{\text{A of (T)}}{\text{A of (S)}} \times 2.0$$

3.8.4. Measurement of Uric Acid in Serum

Uric acid of normal and toxin injected fish were measured as per the procedure of Henry (1974).

Three test tubes were taken for this assay an marked as test, standard and blank that were filled with 1 ml of working reagent. Working reagent was prepared by dissolving the one vial with 10 ml of buffer. It was mixed thoroughly and gently and was allowed to equilibrate at room temperature. 25 µl serum sample a standard and distilled water mixed thoroughly with working reagent respective tubes. They were incubated for 5 minutes at 37^0C. Absorbance was read at 510 nm. Uric acid concentration was calculated by the following formula:

$$\text{Uric acid in sample in mg\%.} = \frac{\text{A of (T)}}{\text{A of (S)}} \times 3$$

3.8.5 Measurement of Serum Glutamate Pyruvate Transaminase

SGOT of normal and toxin injected fish were measured as per the procedure of Henry (1960).

Two test tubes were taken for this assay and marked as test and blank. The test tubes and blank were filled with 1 ml of working reagent and 1 ml distilled water respectively. Working reagent was prepared by mixing the contents of each enzymes vial with the 10 ml of buffer solution and mixed thoroughly and gently. Working reagent was allowed to equilibrate at room temperature. 100 µl of serum sample was mixed with working reagent. The tube were incubated for one minute and three absorbance readings were taken on a spectrophotometer at 340 nm at 60 seconds interval twice.

SGPT was calculated by the formula
Serum ALT activity (IU/L) = A / min X F
Where F = 1768

3.8.6 Measurement of Serum Glutamate Oxaloacetate Transaminase (SGOT)

SGPT of normal and toxin injected fish were measured as per the procedure of Henry (1960).

For this assay, two test tubes were taken and marked as test and Blank. Working reagents was prepared by mixing the contents of each enzymes vial with the 10 ml of buffer solution and mixed thoroughly and gently. Working reagent was allowed to equilibrate at room temperature. The test tube was filled with 1 ml working reagent whereas the blank was filled with 1 ml distilled water. 100 µl of serum sample was mixed with test tube thoroughly and gently. The tubes were incubated for one minute. Three absorbance readings were taken on spectrophotometer at 340 nm at 60 seconds intervals twice.

Sgot was calculated by following formula :
Serum AST activity (IU/L) = A / min X F
Where F = 1768

3.9 HISTOPATHOLOGICAL STUDIES

The organs liver, kidney, heart, stomach, intestine were taken out from normal and toxin injected fishes to observe gross anatomical changes such as haemorrhages, congestion, dropsy, necrosis, depigmentation and textural changes. These tissues were fixed in 10% formalin for 48 hrs. They were then dehydrated in 90% alcohol for an hour and three times in absolute alcohol for 45 minutes separately. The samples were then cleared two times in xylene for 30 minutes and embedded in paraffin thrice each time for 45 minutes.

The samples were then in blocked, allowed to cool, cut on a rotary microtome at 7 µm and mounted sections were dewaxed in xylene and dehydrated serially in alcohol and then stained section were then washed in tap water for 1 minutes, stained in haemotoxylene for 12 minutes washed in tap water, dipped in 2% acid alcohol and washed in tap water, followed by Scotts for water substitute. The section were dehydrated through 50%, 70%, 90% alcohol for 2 minutes each then stained in eosin for 4 minutes and dipped in absolute alcohol for 1 minute each. Finally stained section were cleaned in xylene for 5 minutes each and mounted a slide with DPX. Prepared section were examined and photographed under a light microscope.

RESULTS

4 RESULTS

4.1 EXTRACTION OF TOXIN

Bacteria centrifuged from 18 hours broth culture was suspended in 100 ml distilled water and toxin was released by heat and cold treatment. The solution was centrifuged at 5000 rpm for 15 minutes and the supernatant was used as toxin.

4.2 HAEMATOLOGICAL STUDIES

Results of haematological studies conducted on normal and toxin injected fish are given in Table 3. A marked difference in haematological values were observed in the toxin injected fishes.

4.2.1 Enumeration of Red Blood Cells

The total RBC count in the normal fish was 2.0 x $10^6/mm^3$. Fishes injected *vibrio* toxin showed reduction in RBC count to 1.18 X $10^6/mm^3$ (Fig. 1).

4.2.2 Enumeration of White Blood Cells

An increase in the number of WBC in toxin injected fish was observed. The total WBC count in the normal fish was 33 X $10^3/mm^3$ which was increased to 54.5 x $10^3/mm^3$ in toxin injected fish (Fig. 2).

Table 3: Blood parameters of normal and toxin injected *Clarias batrachus*

Parameter **Wt. of fish**	**Normal** **80 gm - 90 gm**	**Toxin injected** **80 - 90 gm**
RBC in million/mm^3	2	1.18
WBC in $\times 10^3$/ mm^3	33	54.5
Haemoglobin gm/100 ml	9.85	6.15
Haematocrit %	33.3	23.5
ESR mm/hour	0.2	3.625
MCV (fl)	168.6	204.0
MCH (pg)	55.40	50.805
MCHC %	29	25

FIG. 1: TOTAL RBC COUNT IN NORMAL AND *Vibrio* TOXIN INJECTED *C.batrachus*

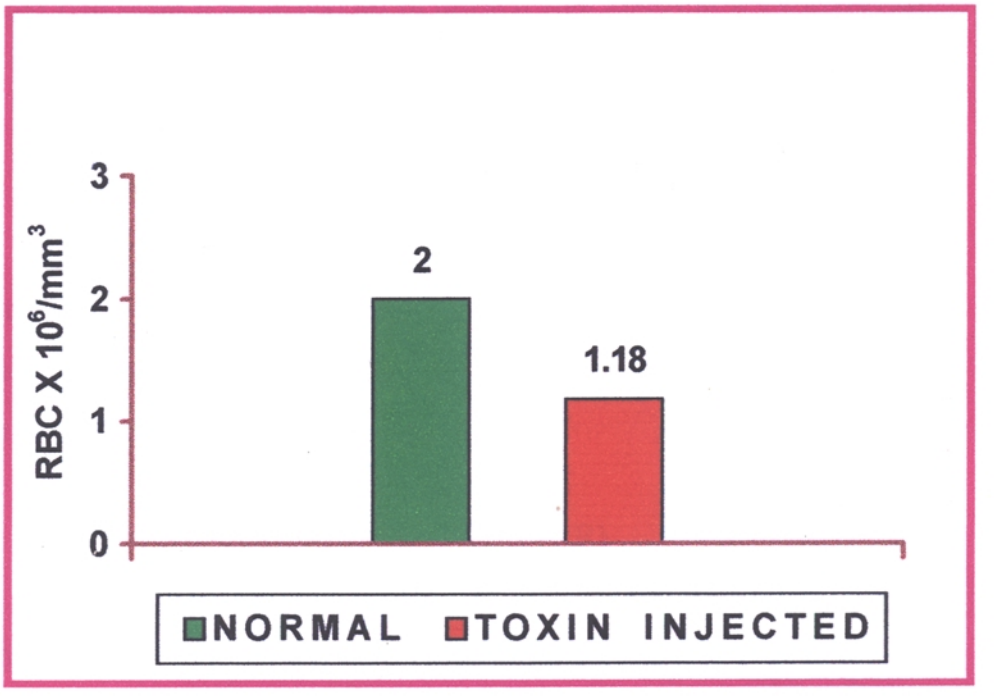
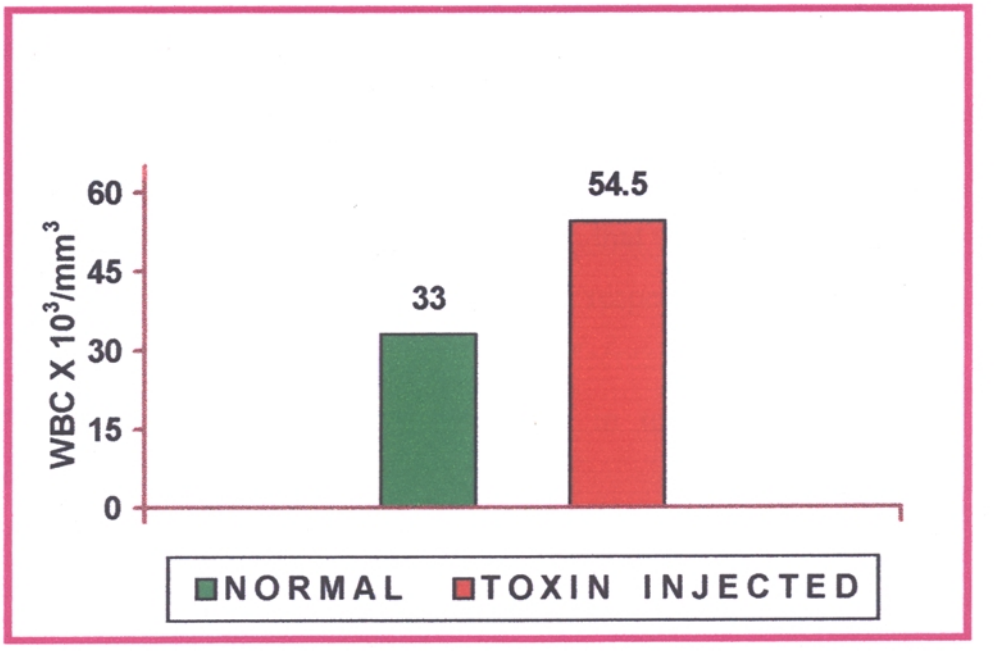


FIG. 2: TOTAL WBC COUNT IN NORMAL AND *Vibrio* TOXIN INJECTED *C.batrachus*



4.2.3 Haemoglobin concentration (Hb)

There has been a decrease in Haemoglobin content in toxin injected fish from 9.85 g/100 ml in normal fish blood to 6.15 g/100 ml (Fig. 3)

4.2.4 Haematocrit Value (Hct)

The results of the haematocrit value of normal and toxin injected fish are represented in Figure 4 and there has been a considerable decrease from 33.3% to 23.5% in the toxin injected fish when compared to normal *Clarias batrachus*.

4.2.5 Erythrocyte Sedimentation Rate (ESR)

Erythrocyte Sedimentation Rate in normal fish was 0.2 mm/hour and an increase was observed to 3.625 mm/hour in toxin injected fish (Fig.5).

4.2.6 Differential Count

The differerntial count of both granulocytes and agranulocytes cells were observed Neutrophils, Eosinophils were included in granulocytes and Lymphocytes, Monocytes among non- granulocytes. Basophils were not observed in the blood film. The average differential count of leucocytes observed in the normal fish were Lymphocytes 75%, Monocytes 8%, Neutrophils 8%, Eosinophils 9%. After injecting *vibrio* toxin it was observed that Lymphocytes and Monocytes were increased to 80% and 14% respectively but among granulocytes neutrophils and eosinophils has decreased to 3% and 2% . One percent Macrophages were observed in toxin injected fish (Figure 6 and plates 1-4).

FIG. 3: HAEMOGLOBIN VALUES IN NORMAL AND *Vibrio* TOXIN INJECTED *C.batrachus*

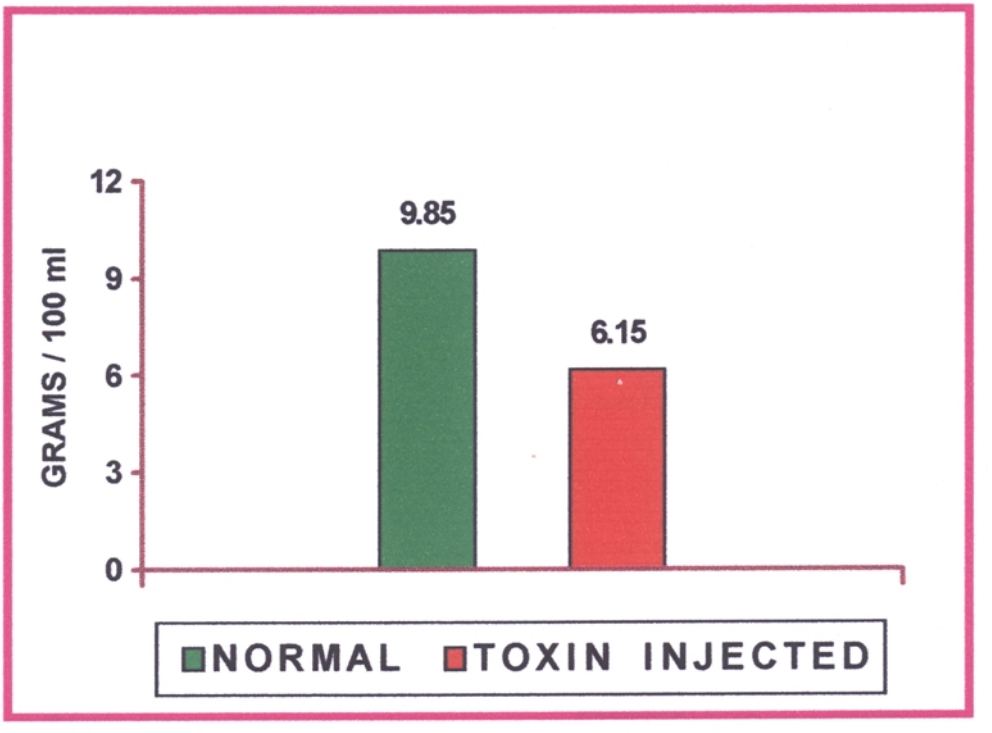


FIG. 4: HAEMATOCRIT VALUES IN NORMAL AND *Vibrio* TOXIN INJECTED *C.batrachus*

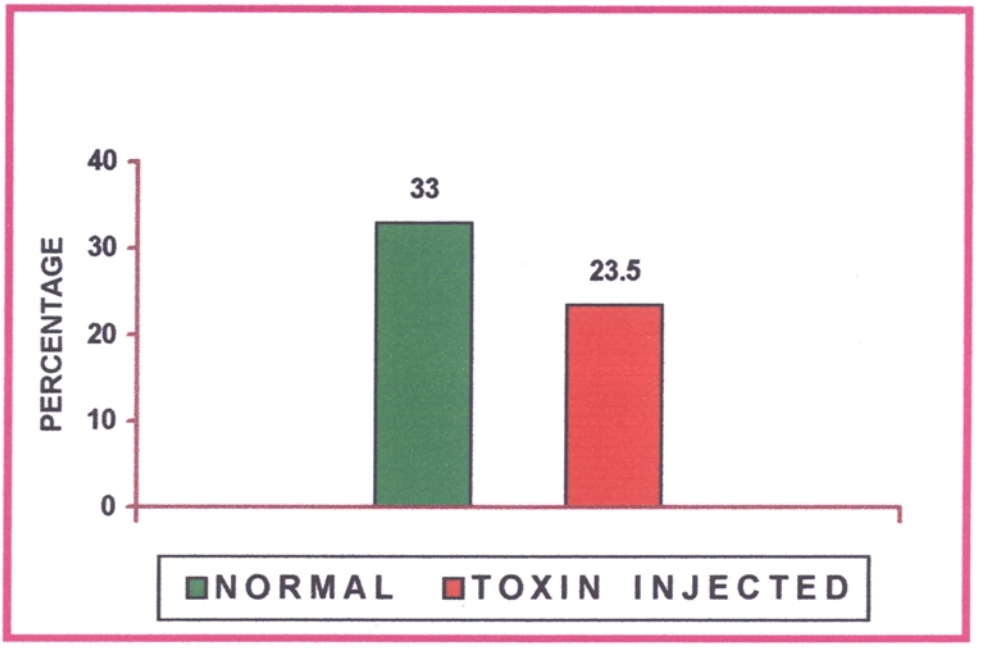


FIG. 5: ERYTHROCYTE SEDIMENTATION RATE IN NORMAL AND *Vibrio* TOXIN INJECTED *C.batrachus*

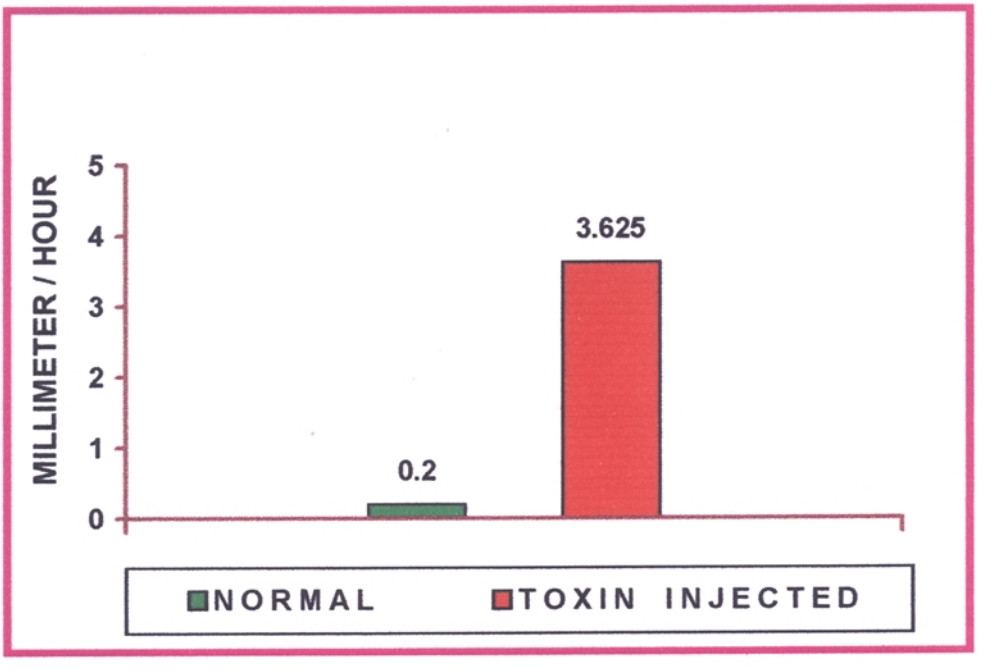
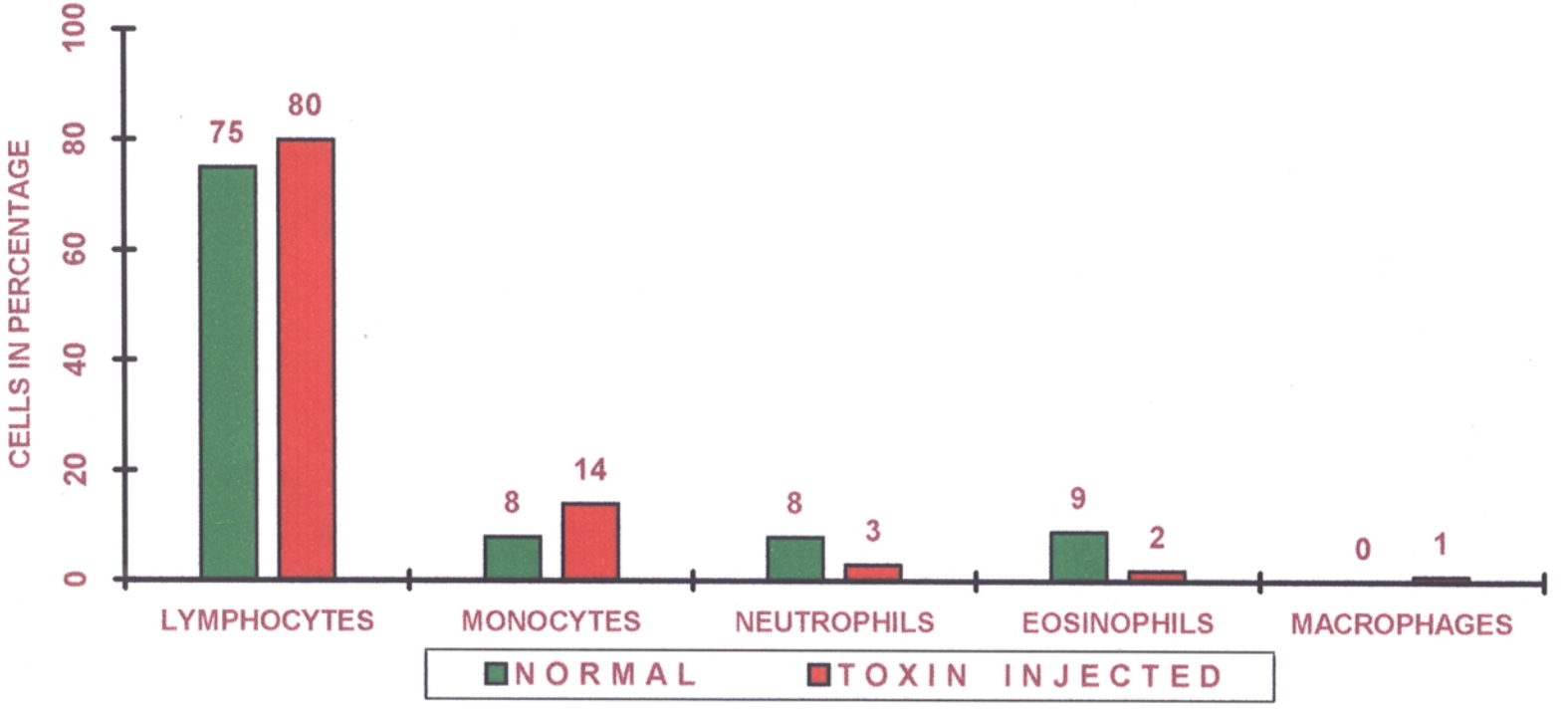


FIG. 6: DIFFERENTIAL COUNT IN NORMAL AND *Vibrio* TOXIN INJECTED *Clarias batrachus*



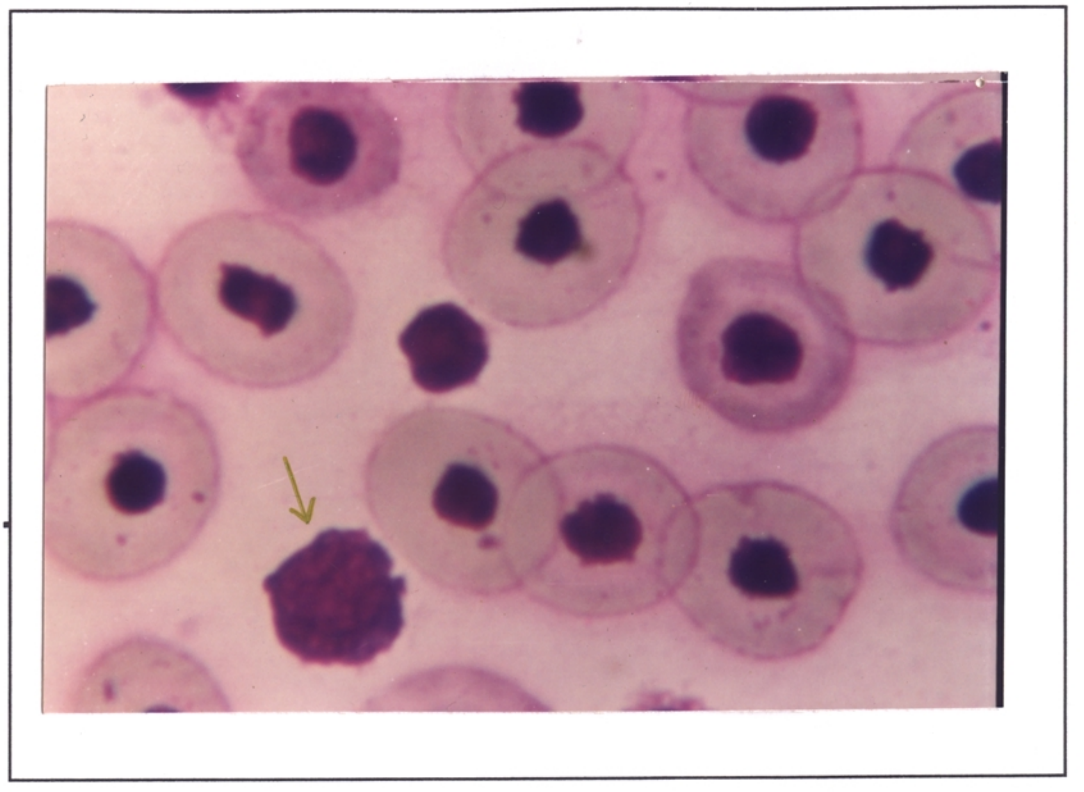


PLATE 1 LYMPHOCYTE OF *Clarias batrachus* (100 X)

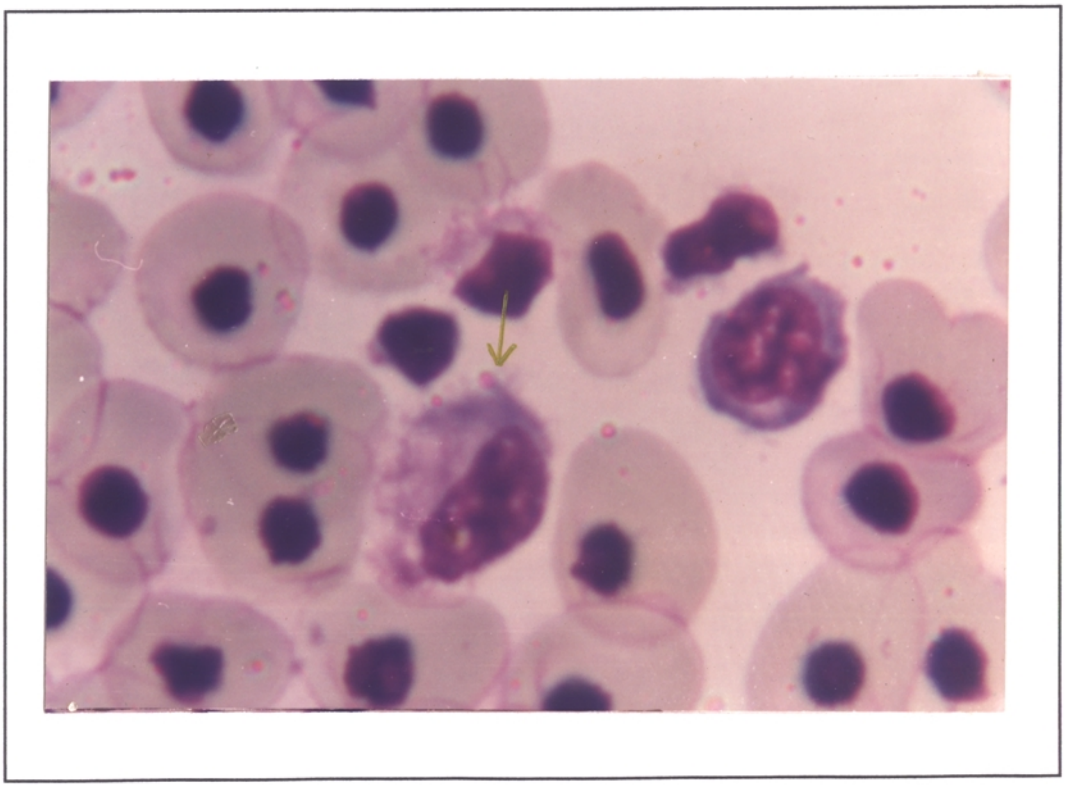


PLATE 2 MONOCYTE OF *Clarias batrachus* (100 X)

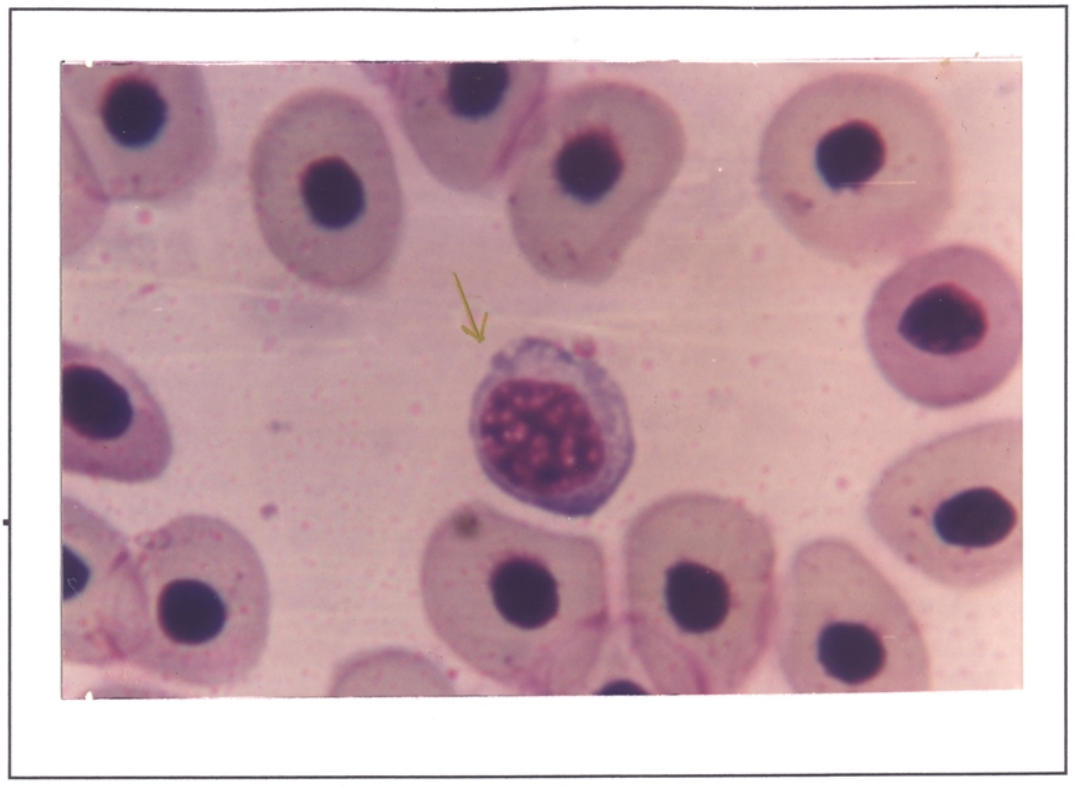


PLATE 3 EOSINOPHIL OF *Clarias batrachus* (100 X)

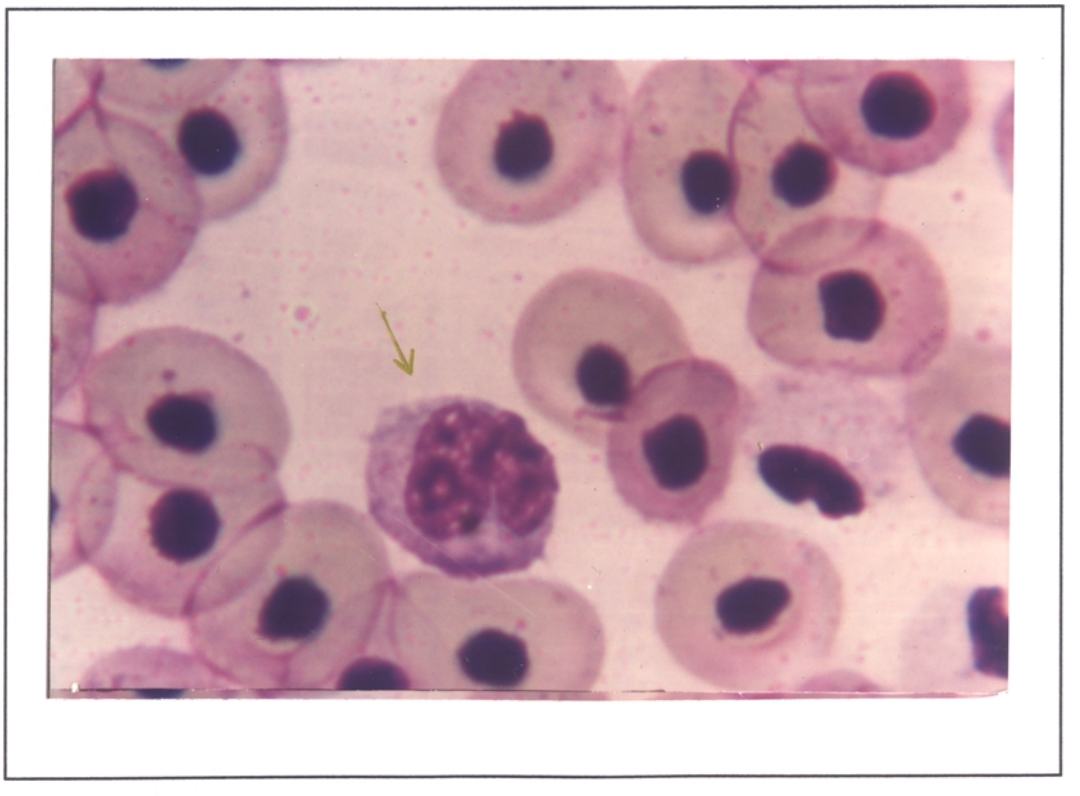


PLATE 4 NEUTROPHIL OF *Clarias batrachus* (100 X)

4.2.7 Red Blood Cell Indices

The calculated values of MCV, MCH and MCHC in normal fish were 168.6 fl, 55.40 pg and 29% respectively. In case of toxin injected fishes there is decrease in MCH and MCHC to 50.805 pg and 25% respectively but MCV has increased to 204 fl. (Table 3).

4.3 BLOOD CHEMISTRY

Concentration of creatinine, uric acid, triglyceride, glutamic pyruvate transaminase and glutamate oxalacetic transaminase in blood serum were estimated for normal fish and as well as for toxin injected fish. Lot of variations were estimated for normal fish and toxin injected fish. Creatinine, SGPT, has increased to 1.92 mg/dl and 33.31 IU/L respectively from the normal values 1.66 mg/dl and 23.4316 IU/L.

Triglycerides, uric acid, SGOT values were decreased for toxin injected fish to 25.44 mg/dl, 1.717 mg% and 103.699 IU/L, whereas normal values estimated were 33.9855 mg/l, 1.93055 mg% and 155.6495 IU/L. (Figure 7-10)

4.4 EXTERNAL SIGNS OF TOXIN INJECTED FISH

The signs observed in *Clarias batrachus* intravenously injected with 1 ml vibrio toxin were

1. Lethargic
2. Offfeed
3. Necrosis of dorsal and caudal fins (Plate 5)
4. Haemorrhages on the mouth (Plate 6)

FIG. 7: CREATININE LEVEL IN SERUM OF NORMAL AND *Vibrio* TOXIN INJECTED *C.batrachus*

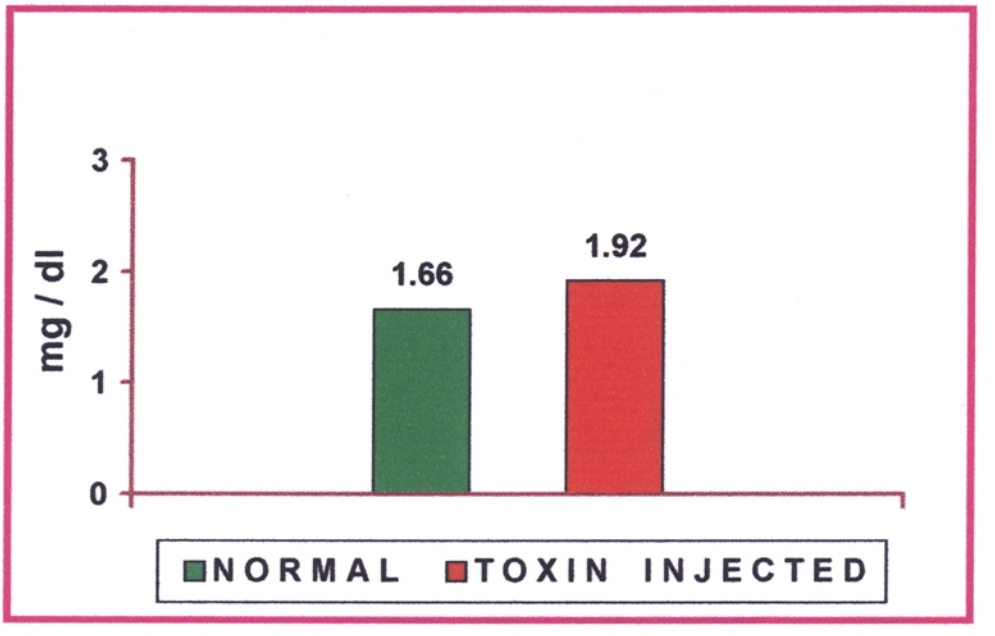


FIG. 8: TRIGLYCERIDES LEVEL IN SERUM OF NORMAL AND *Vibrio* TOXIN INJECTED *C.batrachus*

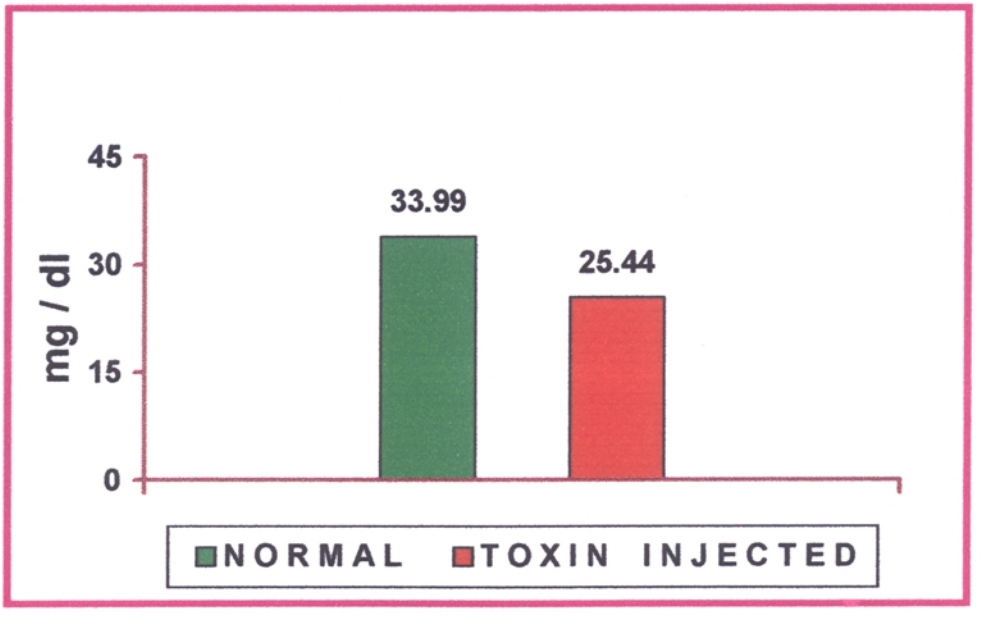


FIG. 9: SGOT AND SGPT LEVELS IN SERUM OF NORMAL AND *Vibrio* TOXIN INJECTED *C.batrachus*

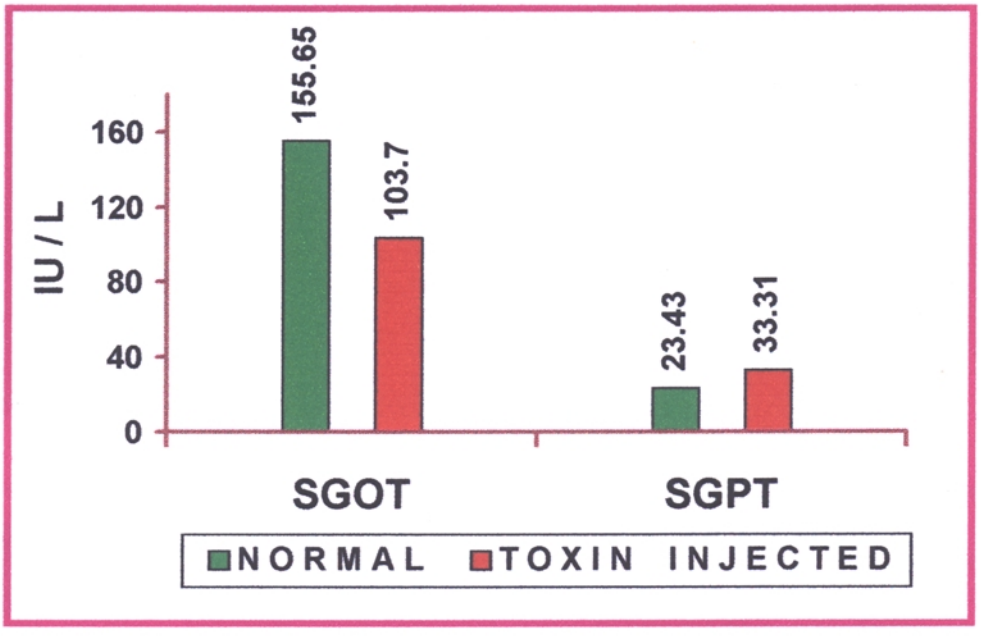


FIG. 10: URIC ACID LEVEL IN SERUM OF NORMAL AND *Vibrio* TOXIN INJECTED *C.batrachus*

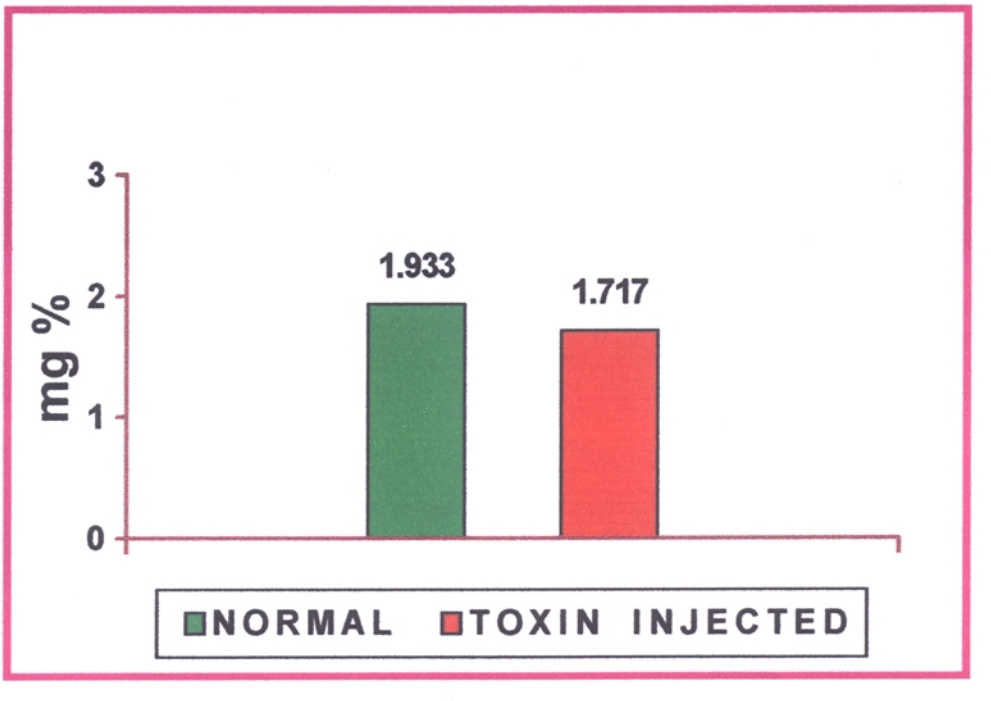


PLATE 5 NECROSIS OF DORSAL AND CAUDAL FINS IN *Vibrio* TOXIN INJECTED *Clarias batrachus*

PLATE 6 HAEMORRHAGES ON THE MOUTH OF *Vibrio* TOXIN INJECTED *Clarias batrachus*

4.5 HISTOPATHOLOGICAL STUDIES

Histopathology of the different organs of the toxin injected fish are

4.5.1 Heart

Significant changes were seen in the heart muscle. Extensive vacuolation was observed (Plate 7 and 8) in toxin injected fishes.

4.5.2 Liver

In the toxin injected fishes necrosis of hepatic cells and stagnation of bile were observed (Plate 9 and 10)

4.5.3 Kidney

The gap between glomerulus and Bowmans capsule was reduced. There was increase in infiltration of leucocytes, tubular necrosis as well as haemopoietic necrosis were observed (Plate 11 and 12).

4.5.4 Gill

There was no change observed in the gill filaments (Plate 13).

4.5.5 Intestine

There is increased infiltration of leucocytes was observed in the intestinal wall (Plate 14).

4.5.6 Stomach

Atrophy of gastric gland was observed (Plate 15 and 16).

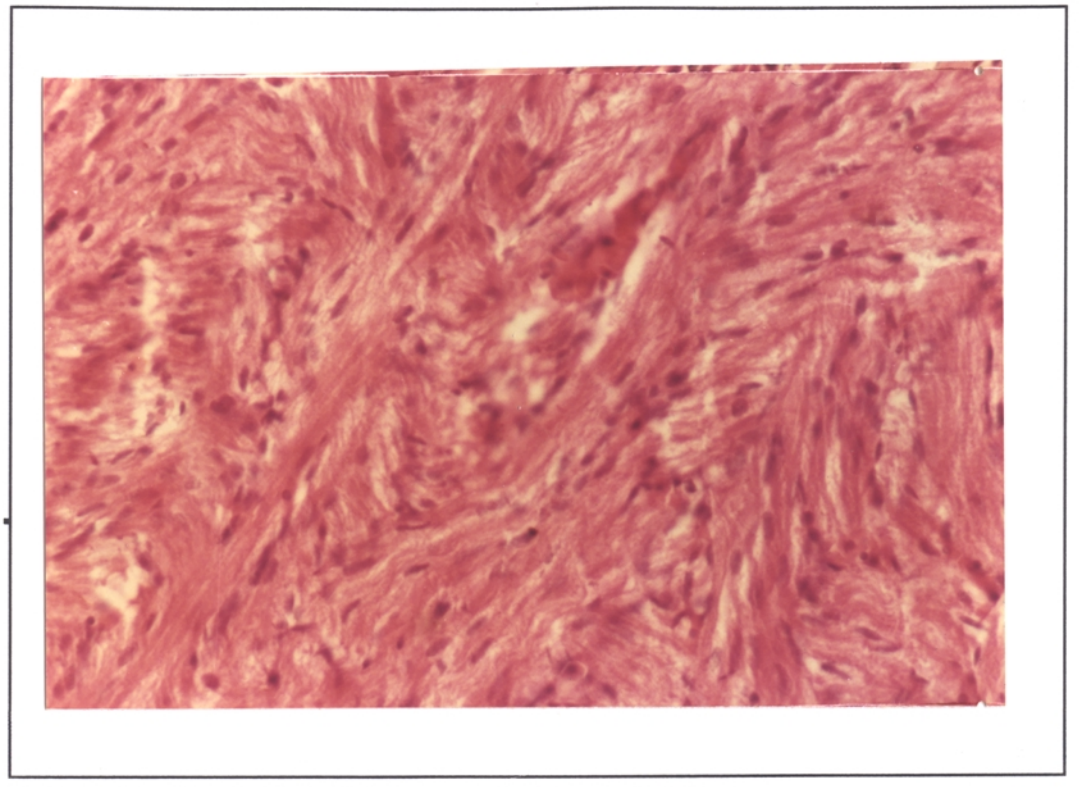


PLATE 7 **CROSS SECTION OF HEART OF NORMAL *Clarias batrachus* (Haematoxylene & Eosin X 400)**

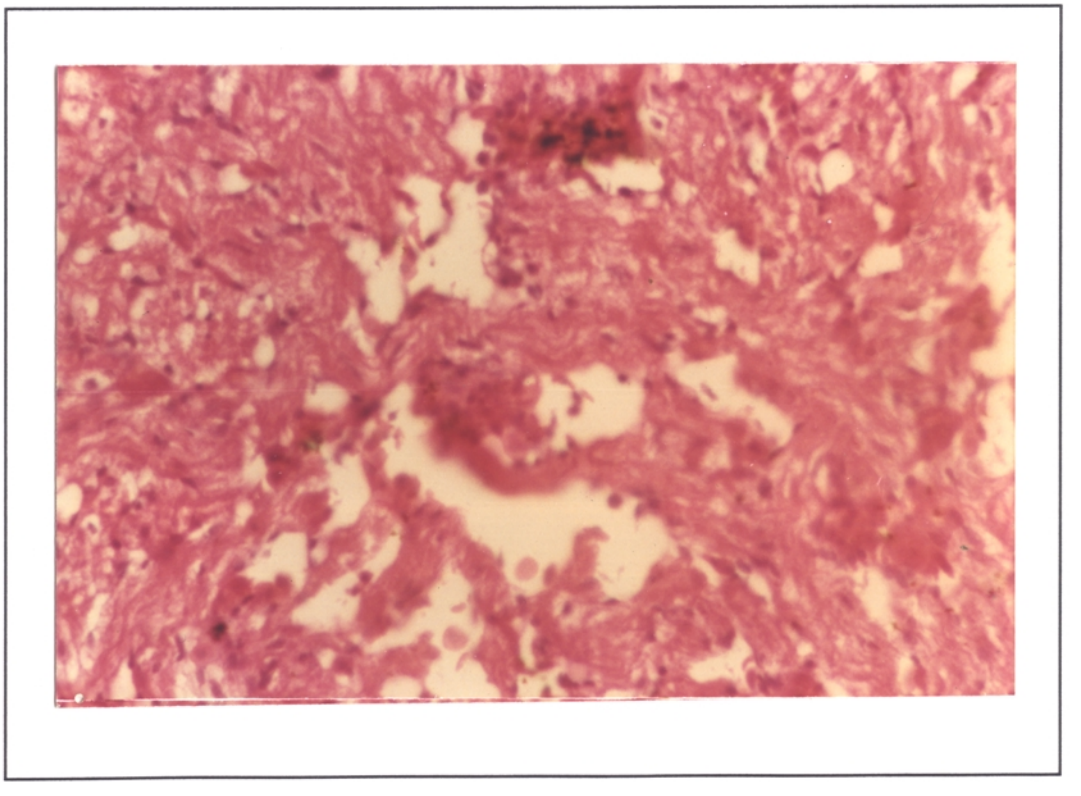


PLATE 8 **VACUOLATION OF CARDIAC CELLS IN *Vibrio* TOXIN INJECTED *Clarias batrachus* (Haematoxylene&Eosin X 400)**

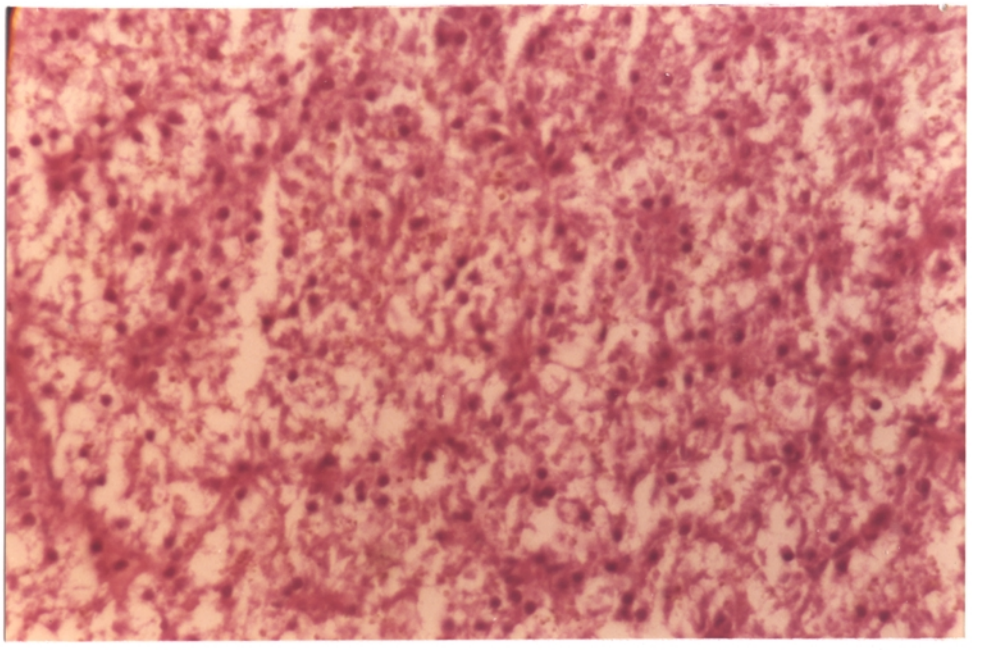


PLATE 9 **CROSS SECTION OF LIVER OF NORMAL *Clarias batrachus* (Haematoxylene & Eosin X 400)**

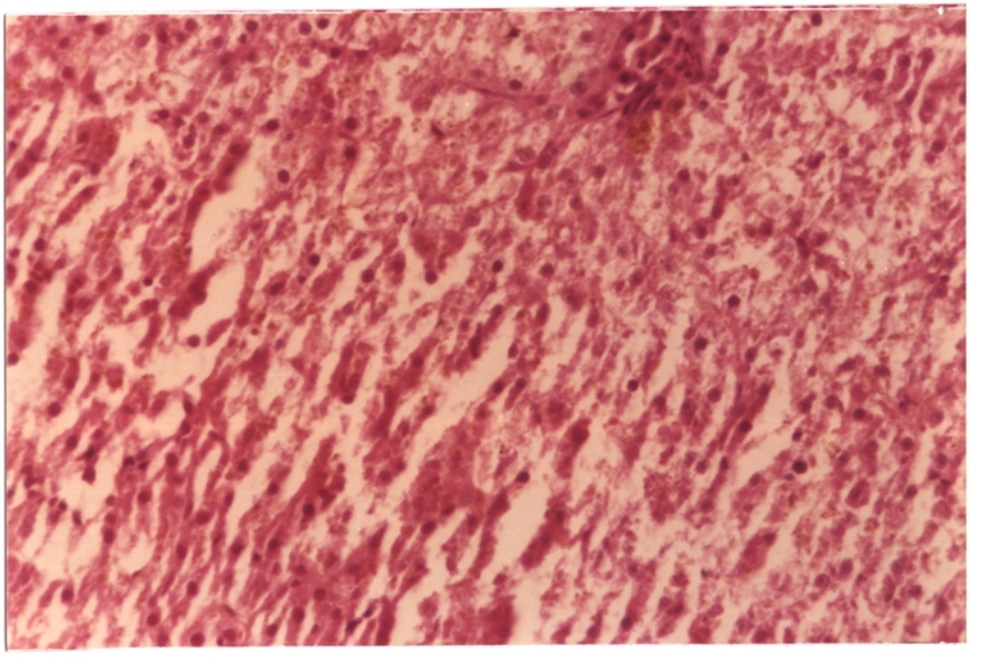


PLATE 10 **NECROSIS OF HEPATIC CELLS IN *Vibrio* TOXIN INJECT *Clarias batrachus* (Haematoxylene & Eosin X 400)**

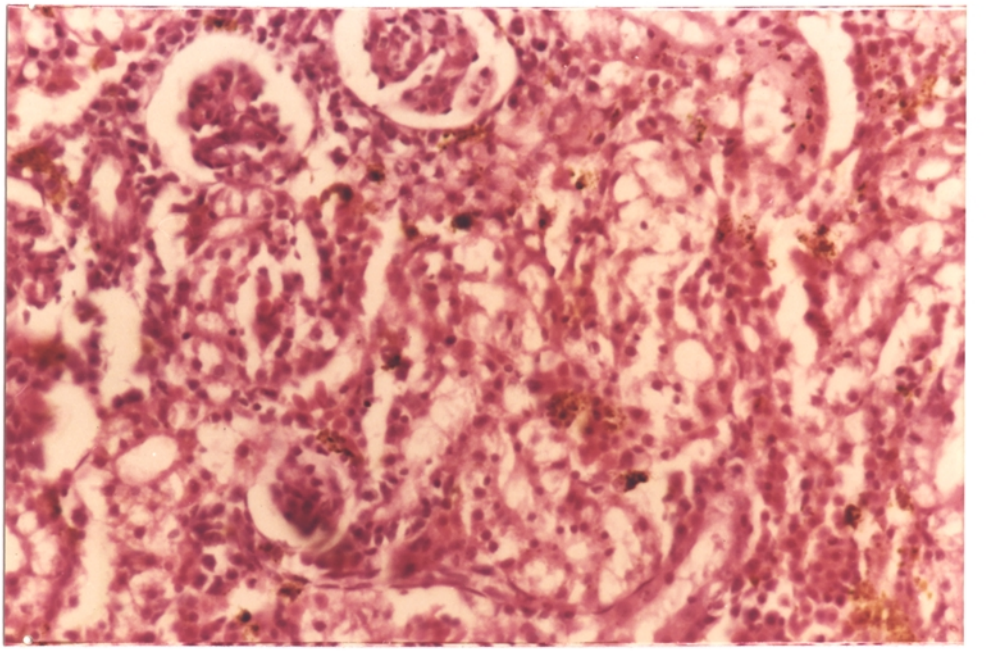


PLATE 11 **CROSS SECTION OF KIDNEY OF NORMAL *Clarias batrachus* (Haematoxylene & Eosin X 400)**

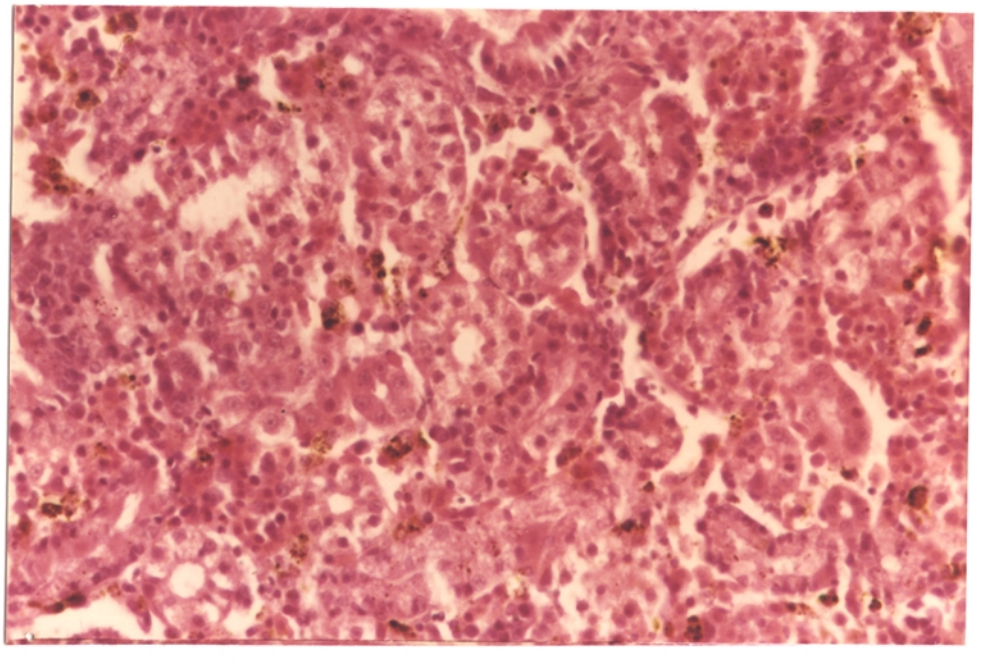


PLATE 12 **ACCUMULATION OF LEUCOCYTES IN KIDNEY OF *Vibrio* TOXIN INJECTED *Clarias batrachus* (Haematoxylene & Eosin X 400)**

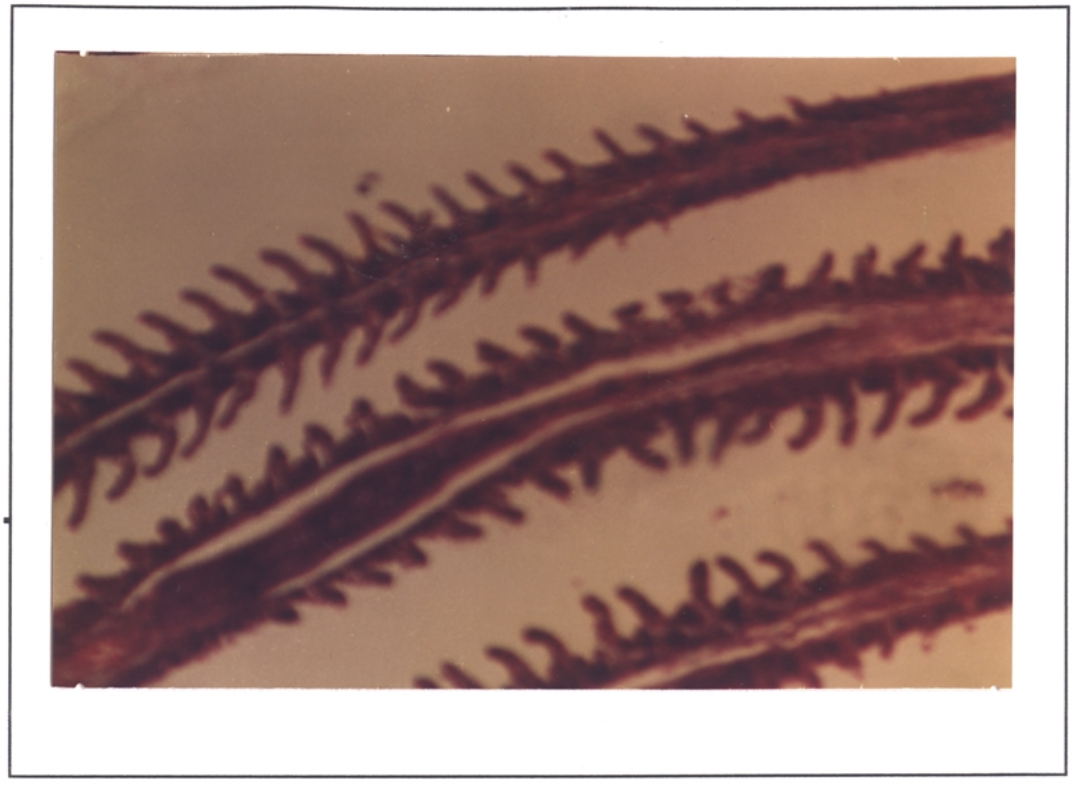


PLATE 13 **CROSS SECTION OF GILL OF NORMAL *Clarias batrachus* (Haematoxylene & Eosin X 400)**

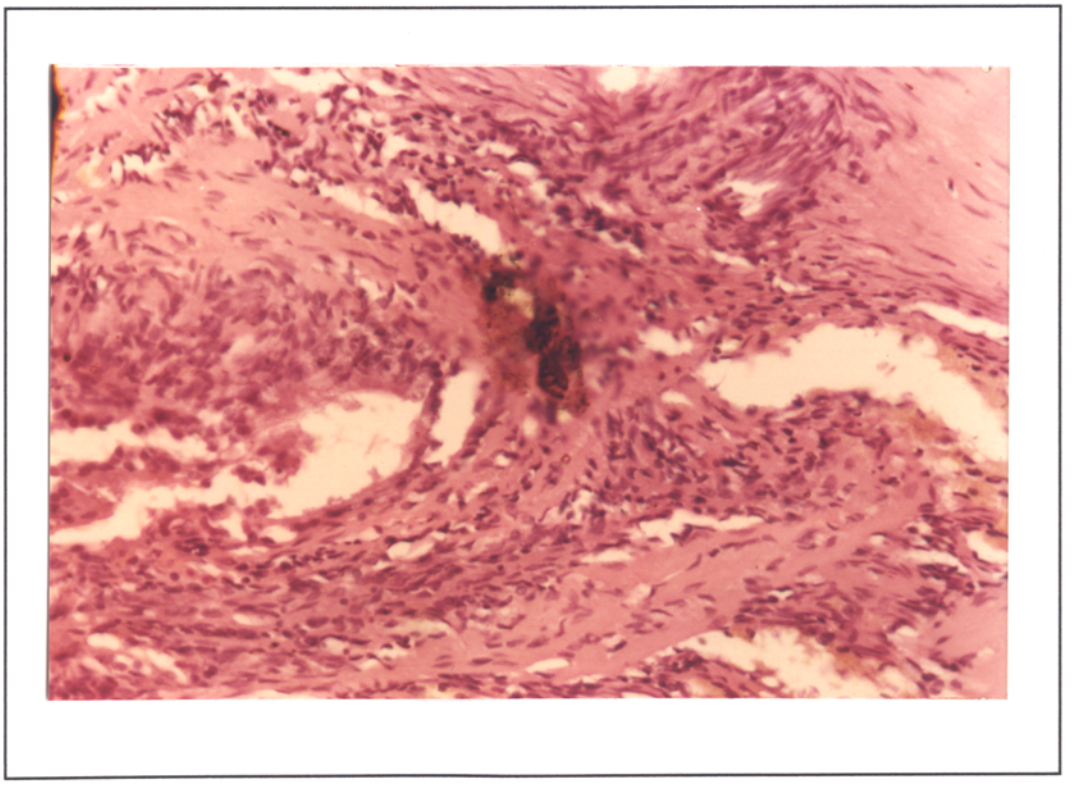


PLATE 14 **INFILTRATION OF LEUCOCYTES IN INTESTINE *OF Vibrio* TOXIN INJECTED *Clarias batrachus* (Haematoxylene & Eosin X 400)**

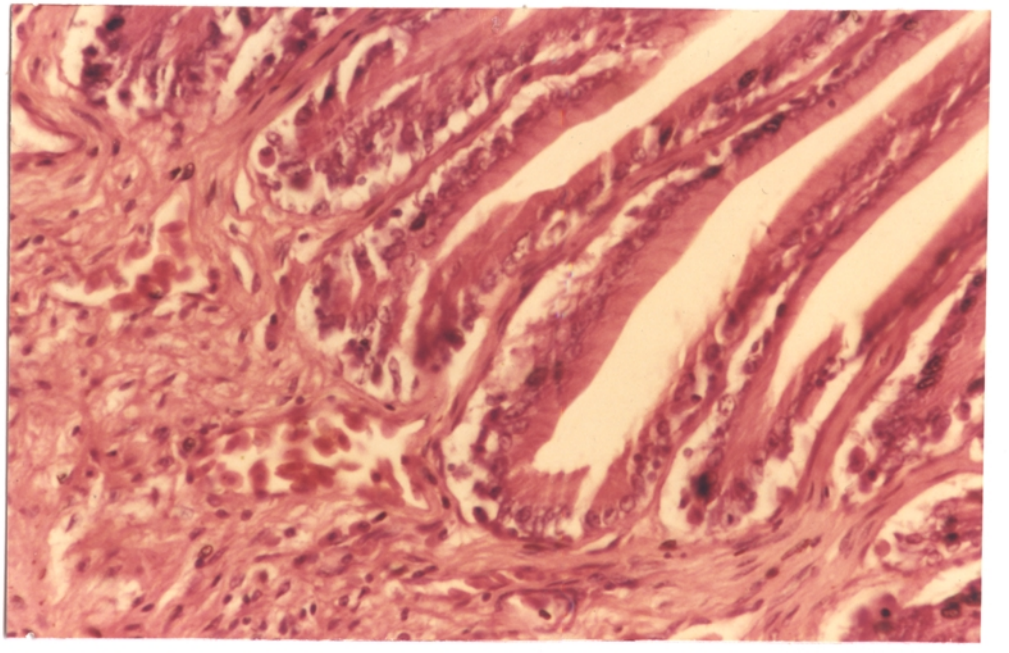


PLATE 15 CROSS SECTION OF STOMACH OF NORMAL *Clarias batrachus* (Haem atoxylene & Eosin X 400)

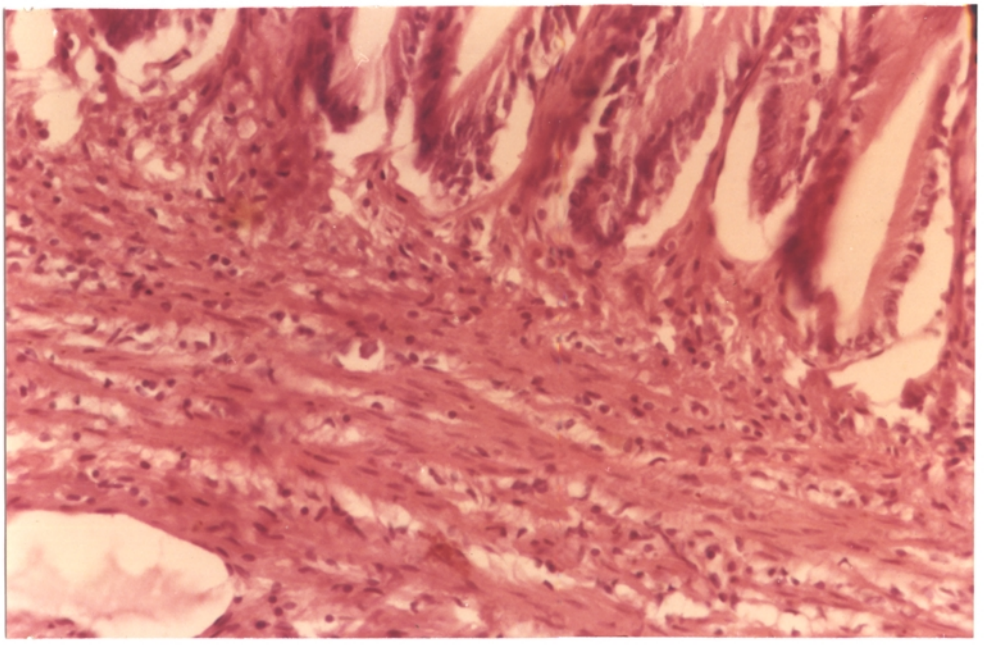


PLATE 16 ATROPHY OF GASTRIC GLANDS IN STOMACH OF *Vibrio* TOXIN INJECTED *Clarias batrachus* (Haematoxylene & Eosin X 400)

DISCUSSION

5 DISCUSSION

Haematology, blood chemistry and histopathology plays an important role in disease diagnosis. Haematological parameters such as RBC count, WBC count, Haemoglobin concentration, Haematocrit value and Erythrocyte Sedimentation Rate are highly sensitive to physiological condition of the fish. The Haematological and histopathological studies can be used as an important tool to obtain information about internal disturbance before fish shows any external symptoms.

Vibrios are ubiquitous, especially more frequent where organic loads are high but only certain species are pathogenic and some are innocuous. Since *Vibrios* are gram negative bacteria, it produces endotoxin, that damage the cells of the host. *Vibrio* endotoxin is a lipopolysaccharide-protein complexes, derived from the outer layers of the cell walls of bacteria.

5.1 HAEMATOLOGICAL STUDIES

5.1.1 Enumeration of Red Blood Cells

The average RBC count in normal *Clarias batrachus* used in the present study was 2×10^6 /mm^3 of the fish blood. The total RBC count determined for this fish closely agrees witht the values reported by Ahmad (1982), 1.63-2.02 $\times 10^6$/mm^3 and Srivastava (1968b), 1.58 - 2.59 $\times 10^6$/ mm^3 in the same fish. 48 hours after of injecting *Vibrio* toxin, the RBC count decreased to 1.18 $\times 10^6$/ mm^3. Tripathi *et al.* (1979) reported an increase level of RBC count in *Clarias batrachus*

when they were kept under submerged and exclusive air-breathing conditions. The values were increased from 2.26×10^6/ mm^3 to $2.78 \times 10^6/mm^3$ and $3.14 \times 10^6/mm^3$ respectively, after 18 hours of infection with *Vibrio* toxin.

In the present study the RBC count decreased as the toxin may be hemolytic to fish RBC. The *Vibrio* toxin may also be related to affect the iron levels due to its high requirement of iron and is in confirmation to the studies of Roberts (1975) (Figure 1).

5.1.2 Enumeration of White Blood Cell

The average WBC count in *Clarias batrachus* used in the present study was 33×10^3/ mm^3 of the blood. The total WBC count determined for the fish are in confirmation with values of male *Clarias batrachus* reported by Ahmad (1982) 29.38×10^3/ mm^3 and for female fish 33.66×10^3/ mm^3. The total WBC count in present study increased to 54.4×10^3/ mm^3 when fish was injected with *Vibrio* toxin.

The leucocytosis condition was observed as the fish was under stress due to toxin injection (Figure 2)

5.1.3 Haemoglobin concentration

The average haemoglobin concentration of the blood in *Clarias batrachus*, used in the present study was 9.85 g/100ml. Earlier reports of Ahmad (1982) were 7.5 - 7.8 gm/100ml and by Srivastava (1968a) 10.2 - 18.8 g/100ml are in confirmity to the present study.

The total haemoglobin concentration reduced to 6.15g/100ml in toxin injected fish.Tripathi *et al.* (1979) reported that in submerged and exclusive air-breathing condition the Haemoglobin value increased from 12.6 gm/100ml to 14.2gm/100ml and 15.8gm/100ml.

The decrease in haemoglobin due to iron deficiency in the fish body (Figure 3).

5.1.4 Haematocrit value

The average Haematocrit value of the blood in normal *Clarias batrachus* used in the present study was 33.3%. The values obtained for the fish closely agrees with the values reported for the same fish by Ahmad (1982) 26.0 - 36% and by Srivastava (1968) 20.4 -35.2%.

The Haematocrit value in toxin injected fish was decreased to 23.5%. Tripathi also reported the increasing trend in haematocrit value in *Clarias batrachus* in submerged an exclusive air-breathing condition the haematocrit value was increased from 37.3% - 43.7 and 48.1%. The haematocrit value decreased in case of toxin injected fish due to deficiency of iron (Figure 4).

5.1.5 Erythrocyte Sediment ation Rate

The value of ESR of the normal blood in *Clarias batrachus* used in present study was 0.2 mm/ hour. Srivastava (1968) reported the ESR value for normal *Clarias batrachus* was 2.0 - 3.4 mm/hour.

The ESR has increased to 3.625 mm/hour after 48 hours of injecting the *Vibrio* toxin to the same fish. Schumacher *et al.* (1956) reported increased ESR in brook trout infected by furunculosis. Murad

et al. (1988) found a similar response in cat fish (*Heteropneustes fossilis)* parasitised by metacercariae and also reported high ESR in fishes. The present study also indicates the *Vibrio* toxin infection and the increase in ESR was in confirmation to earlier reports (Figure 5).

5.1.6 Diffrential Count

The average differential leucocyte counts in normal *Clarias batrachus* used in present study were lymphocytes 75%, Monocytes 8%, neutrophil 8% and Eosinophil 9%. Ahmad (1982) reported about differential counts of leucocyte in *Clarias batrachus* male agranulocyte and Granulocyte % was 49.99% and 50.01%. For female fish it was reported agranulocyte 50% and Granulocyte 50%.

In toxin injected fish the differential leucocytes counts were Lymphocyte 80%, Monocytes 14%, Neutrophils 3%, Eosinophils 2% and Macrophages 1%. The increasing level of Lymphocyte and Monocytes and occurance of Macrophages is led to develop defence mechanism by phagocoytically active leucocytes to destroy toxin (Figure 6 and Plates 1- 4)

5.1.7 Red Blood Cell Indices

The average Red Blood Cell Indices in the normal fish, *Clarias batrachus* in the present study were calcualted to be MCV - 168.6 fl., MCH - 55.40 p.g. and MCHC - 29%. The values determined for the Red Blood Cell Indices closely agrees with the values reported by Ahmad (1982) MCV 150.46 - 214.07 fl, MCH - 41.02 pg, MCHC - 23.09% Srivastava (1968) reported values of Red Blood Cell Indices

as, MCV - 87.81 - 150.0, MCH - 30.73 - 52.87 pg, MCHC - 33.55% - 38.62%.

In toxin injected fish the MCV was increased to 204 fl and MCH, MCHC decreased to 50.805 pg, 25% respectively.

Increasing of MCV is a sign of macrocytosis is related to anaemia associated with iron deficiency. Decreasing the value of MCH and MCHC was indicated microcytic and macrocytic anaemia.

5.2 BLOOD CHEMISTRY

The Average Value of Uric Acid, Creatinine and Triglyceride level in normal *Clarias batrachus* used in present study were 1.637 mg/dl, 1.93055 mg% and 33.9855 mg/dl respectively. Sandnes *et al.* (1988) reported the normal range of Creatinine level in African lung fish 10-80 µM. Hille (1982) reported normal value of uric acid in blood plasma of rainbow trout was 40 -100µM. Zammit and Newsholme. (1979) reported plasma Triglyceride level in normal spotted and spiny dogfish was 30 - 120mg/dl. The average value of SGPTand SGOT in normal *Clarias batrachus* was found 23.36 37 IU/L and 155.5488 IU/L.

After injecting *Vibrio* toxin to the fish it was observed that the plasma Triglyceride and uric acid level was decreased to 25.44 33 mg/dl and 1.93055 mg% respectively. It may due to dysfunction of cardio vascular muscle and kidney. This was in confirmity due to the necrosis of heart muscle and damage to the kidney.

After injecting Vibrio toxin it was observed that the level of SGPT and SGOT in blood serum increased to 33.31 IU/L and decreased to 103.69 96 (Figure 7-10)

Fluctuation of the concentration of the SGPT and SGOT level in blood serum associated with dysfunction of liver and was also confirmed by histopathology.

5.3 EXTERNAL SYMTOMS

Toxin injected fishes showed several external symptoms that are coincide with *Vibrosis*. Haemorrhagic ulcers on the mouth or skin surface, necrosis of dorsal and caudal fin visualised (Plates 5 and 6).

5.4 HISTOPATHOLOGY

A comparative study of histology was done on normal and toxin injected fish, *Clarias batrachus*.Some distinct changes were observed in specific organs like heart, liver and kidney. Vacuolation of cardiac muscle was observed in the heart of toxin injected fish which is closely agrees with Frerichs and Roberts (1989) report. Frerichs reported extensive vacuolation of the sarcoplasm of the atrium of Dover sole with small foci of inflammatory cells and hyperaemia of the paricardium with peracute *Vibriosis*.

In kidney it was observed the reducing gap between bowman's capsule and glomerulus. Tubular necrosis of the kidney was also observed. Same report was given by Inglis (1993) about tubular necrosis as well as haemopoietic depletion and necrosis on the mid-kidney of Salmon with acute *Vibriosis*.

Accumulation of leuco yte in the epithelium of Intestine and stomach was visualised which was evident of infiltration of blood.

In liver necrosis of hepatic cell and bile stagnation was observed. There was no changed in gill observed for both the normal and toxin injected fish.

The present study gave an insight into the significance of Haematology and histopathology on the status of fish *Clarias batrachus*, health condition in normal and in *Vibrio* toxin infected fishes (Plate 7 to 16).

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