UTTAR PRADESH JOURNAL OF ZOOLOGY

41(15): 1-11, 2020
ISSN: 0256-971X (P)

HISTOPATHOLOGICAL CHANGES OBSERVED IN THE SPLENIC TISSUES OF THE FISH *Clarias batrachus* on EXPOSURE TO THE PESTICIDE ROGOR

NANDINI SHARAN[1*] AND PAWAN KUMAR[1]
[1]Magadh University, BodhGaya, India.

AUTHORS' CONTRIBUTIONS
This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

Editor(s):
(1) Dr. Telat Yanik, Professor, Atatürk University, Turkey.
Reviewers:
(1) Burak Kaptaner, University of Van Yuzuncu Yil, Turkey.
(2) Hamdy M. A. Hassanein, Sohag university, Egypt.

Received: 18 July 2020
Accepted: 22 October 2020
Published: 22 October 2020

Original Research Article

ABSTRACT

Present study focuses on the effect of Rogor induced histological alteration in the spleen tissues of freshwater fish *Clarias batrachus* more commonly called Mangur. The lethal concentration50 of the pesticide Rogor was calculated which came out to be 4 µl/L. Then, solution of the two sub lethal concentration were prepared in distilled water .The lower and upper sub lethal concentrations were taken as 1 µl/L and 2.5 µl/L respectively. Fishes were grouped in three; first were exposed to lower sub lethal concentration, the second were exposed to higher sub lethal dose and the third were the controlled fishes. Groups consisted of 10 fishes each. Fishers were sacrificed for the experimental protocol on the 5th, 10th and 15th day of exposure to the pesticide. The splenic tissues of fishes from all three groups were dissected and then kept in 10% formalin. They were cut in small pieces and were fixed in neutral formalin solution and light microscopy was performed. The light micrograph was stained with hematoxylin and eosin that showed its parenchymatic components. Seven plates were observed under a low and high power.1st plate was of tissues of controlled fishes. Three plates were of concentration 1 µl/L and three were of 2.5 µl/L each, observed on the 5th, 10th and 15th day of exposure. In controlled fishes under lower and higher power, the red and white pulp enclosed in a capsule, melanomacrophages, distinct trabaculae and blood vessels were observed .In the present work, sub lethal exposure of rogor induced many histopathological anomalies in the spleen, viz. Dissolution of the white pulp, aggregation of more melanomacrophage , autolysis of RBCs and loss of cellular architecture and matrix and distinct ridge formation. Hence, the experimental protocol displayed the alteration of the normal tissues of spleen of fish thereby showing the toxic impact of the pesticide.

Keywords: Splenic tissue; *Clarias batrachus*; Rogor; toxic nature of pesticide.

**Corresponding author: Email: nandinisharan21080@gmail.com;*

1. INTRODUCTION

We are living in a fast world where the pace of development in every field had become very fast. With the developing technology we are getting introduced to several artificially made equipments and substances which are brought to use in order to get more productivity in everything. In agriculture a lot of artificially designed chemical fertilizers, pesticides, insecticides etc. are used. The pesticides , insecticides, etc. not only kills the wanted pests but also reaches the water bodies through leaching , during times of heavy rainfall and kill the aquatic flora and fauna ,which were not supposed to be affected in any of the way. Specially the uncontrolled use of such chemicals are posing a serious threat to the aquatic life. It is widely used so as to increase the economy but the amount of its negative impact, that is the reduction in number of the important aquatic flora and fauna due to its toxicity, is overtaking the positive effects of such chemicals, Hence a large number of countries have banned the user of such chemical pesticides, though, they are being frequently used by the developing countries to increase their agricultural economy. But it is high time for these countries to realise that they are facing a lot of losses when it comes to export of fishes, other aquatic animals and plants along with damaged coral reefs which are a great tourist spots in many of the countries and is a great source of income. In 2017 , India earned 1.1 trillion rupees from fishing and aquaculture(ref. Fishery and Aquaculture Country Profiles - The Republic of India (www.fao.org › FAO Home › Fisheries & Aquaculture)) [1].

Natural water bodies receive different types of pollutant from different source like agricultural pesticidal residue, which leads to the physico-chemical alteration of water bodies. These changes in water bodies adversely affect the aquatic biota specially and cause mass mortality of fishes. By reduction in number of aquatic life will result is less yield, which, in turn will lead to decline of such economic progress.

Organochlorine pesticide, persists in the environment and are not easily biodegradable. DDT is an organochlorine pesticide which once introduced in the environment, keeps circulating for many years. The biological action of DDT on the environment has been most extensively studied. The central nervous system is the target organ of DDT. It dissolves in the lipid tissue and accumulates in fatty membrane surrounding nerve. Yeager and Munson [2]

Malathion is an organophorous insecticide also having cholinesterase inactivity. It has also low mammalian toxicity, Lin et al. [3]. Endosulphan is more toxic in comparison to malathion to fish channa punctatus. Kulkarni & rafique [4].

Rogor, an organophosphate pesticide, also called dimethoate is a biodegradable pesticide used to kill mites, insects and other pests when they come in contact. Like other organophosphates, dimethoate is an acetylcholinesterase inhibitor which disables cholinesterase, an enzyme essential for central nervous system function. Cyanamid (1950) [5].

Some of the pesticides directly affect the different organ of fishes like ovary, testis, gill, skin, muscle, liver, spleen etc. which leads to the adverse effect on growth and survival of fishes. Dimethoate and Endosulfan are two harmful pesticides that effect biochemical composition of fish [6]. Pesticides accumulated in tissues leads to many physiological and biochemical changes thereby influencing the activities of several enzymes and metabolites and finally causes the entire metabolic process disturbed (Scott, 1967) [7]; (Jackson, 1968) [8]; (Mukesh, 2013) [9].

In teleost fish, the immune organs consist of the spleen, pronephros and thymus. Since teletost fish have no modularly cavity in their bones, the spleen and kidney serve as the primary haemopoitic organs [10]. As fish have no lymph nodes, the spleen alone plays an essential role in antigen trapping [11]. The spleen is the major peripheral lymphoid organ [12].

The spleen is covered by a thin fibrous capsule with little evidence of contractile ability [13]. Red pulp is an extensive, interconnecting system of splenic cords and sinusoid capillaries. White pulp, consisting mainly of sinusoid capillaries. lymphoid cells typically surrounds arterial vessels, melanomacrophage centres [13]. Melanomacrophage centers (MMC) are physiological features in fish spleen and kidney [10]. They are believed to be functional equivalents of the germinal centres of spleen and lymph nodes in mammals [14]. MMC may contain four types of brown pigments: melanin, lipofuscin, ceroid and hemosiderin [15]. Wolke et al. [16] first suggested MMC as potential monitors of fish health. Montero et al. [17] found that stressful situations related to aquaculture practices have resulted in increased numbers of splenic and kidney MMC. Lymphocytes and the macrophages are the mainly concentrated areas in earlier histopathoogical studies as they are important for the defence system of the fishes [18] (Kurtovic et al., 2008) [19].The spleen plays an important role in filtration from the blood stream of all foreign matter, including obsolescent and damaged blood cells, and participation in the immune

response to all blood-born antigens. Designed ingeniously for these functions, the spleen is a major repository of mononuclear phagocytic cells in the red pulp and of lymphoid cells in the white pulp. (ref - https://teachmephysiology.com/gastrointestinal system/ spleen/function-of-spleen/) [20]. Although spleen is known for its vital role in the immune system regulation, comparatively, less attention is attributed to its structure and microanatomy.

Changes in spleen of any aquatic fish is a key tool to study the toxic nature of any toxicant. Therefore, taking all these under consideration the present research work is done to calculate the LC50 of the pesticide and study the negative toxic impact of the exposure of the pesticide in the fish.

2. MATERIAL AND METHODOLOGY

Healthy *Clarias batrachus*, more commonly called "mangur" of 50-80 gm were brought from Dighadiyara, Patna, Bihar. They Were disinfected with 0.1% KMnO4 solution prepared in distilled water and were kept in standard laboratory condition in Plexiglas aquaria or plastic pools. To maintain normal water temperature, cooler and exhaust were used around the aquarium. The aerated tap water was changed daily. After 48 hours, fishes were fed with pellets of wheat flour & egg at 5% of their body weight. After, two weeks of properly acclimatization fishes were grouped in many sets of ten each for lower sub lethal, higher sub lethal concentrations and controlled fishes.

Rogor , an organophosphate pesticide which is widely used by the farmers of Bihar as well as in many parts of India in agriculture was selected as the pesticide due to its wide demand and affordability. LC_{50} of Rogor for fish was performed by the technique described in the standard methods APHA (2005) [21]. For this, 10 fishes of some size and weight were used for each concentration of the pesticides and simultaneously 10 fishes were kept in controlled condition.

The number and percentage of surviving and dead fished were noted at the end of every 24 hours of pesticide exposure. Dead fish were removed to check the contamination. The upper and lower limit of pesticides at which 100% and 0% fish mortality could be seen were calculated by pilot test using 5 or more concentration of pesticides for 48 hours and 96 hours, the logarithmic intermediate concentration were selected as LC_{50} of Rogor.

Determination of LC_{50} for the corresponding mortality of fish at different concentration of Rogor

Aquarium Number	No. of fish (*Clarias batrachus)*	Concentration of toxicants (µI/L	Mortality			
			48 hrs.		96 hrs.	
			Number	%	Number	%
1	10	1.00	0	0	0	0
2	10	3.00	1	10	2	20
3	10	4.00	3	30	5	50
4	10	6.00	7	70	9	90
5	10	8.00	10	100	10	100

Here in the experimental protocol, the 96 hr. LC_{50} of rogor for *Clarias batrachus* was calculated as 4 µI. The two dose considered in the experimental protocol was, 1 µ1/L of rogor and one does of 2.5 µ1/L of Rogor and accordingly stock solution of rogor were prepared using distilled water. Fishes were then treated with 1.µ0l/L (lower) and 2.5 µI/L (upper sub lethal) for 15 days. The fishes were slaughtered for observing them on the 5^{th}, 10^{th} and 15^{th} day of exposure.

Spleen tissues of both control and treated group were dissected out and kept in 10% formalin for light microscopy. Tissues were cut into small pieces with sharp surgical blades, and were fixed in neutral formalin for histological and histochemical studies and processed for Light Microscopy. The details of histopathological observations based on Light Microscopy (LM) and alteration in normal functioning of the spleen have been mentioned in results and discussion.

2.1 METHOD FOR LIGHT MICROSCOPY

A. Fixation:

Small pieces of spleen tissues were fixed in the following fixatives for subsequent histopathological studies :-

Neutral formalin: In a clean dry bottle following chemicals were mixed properly to prepare neutral formalin –

i. Formalin (40%) -10 ml
ii. Distilled water- 90 ml

PROCEDURE

- The tissues were fixed for 5 days and washed overnight under running tap water.
- Tissues were dehydrated in graded series of alcohol for 2 hours in each grade with changes in between.
- Cleaned in absolute alcohol and xylene (1:1) and taken in pure xylene for 1 hour with a change in between.
- Embedded in molten paraffin wax with cerasine (Malting point 58°C to 68°C) after passing through xylene and molten wax (1:1).
- Block were prepared and sections were cut in semi-thin microtome at 4-6 micrometer and then stained for histological slide preparation.

B. STAINING

a. 4-5 micrometer thick section were fixed on albumen rubbed on clean glass slide and deparaffinized in xylene and hydrated through descending series of alcohol upto watter.
b. These hydrated sections were stained in Delafields haemotoxylene for ten minutes.
c. Washed the section in running tap water at least for one hour and then rinsed in distilled water.
d. Sections were dehydrated in ascending series of alcohol up to 70% counter stained in Eosin and dehydrated further in 90% and absolute alcohol.
e. The sections were cleared in xylene and mounted in DPX with clean glass cover slip.

All light microscopic photography was done in Laboratory itself.

3. RESULTS AND DISCUSSION

3.1 Light Microscopy Observation of Spleen Cells of *Clarias batrachus*

Normal Spleen Cells of *Clarias batrachus*: The spleen is distinct. It is more or less divided into a red, outer cortex and white inner pulp, the medulla. It is a slightly ovate organ. The main elements of the spleen parenchyma are white and red pulp The white pulp is composed of lymphoid tissue, surrounding small arteries and diffusely intermeshing with the red pulp. The red pulp is composed of a reticular cell network and supporting blood-filled sinusoids that hold diverse cell populations, including macrophages and lymphocytes. Scattered through the parenchyma are numerous accumulations of the pigmented macrophages, i.e. melanomacrophage centres (MMC) were found. They appear as poorly organized, irregular cell clusters which may be located in the white, as well as in the red pulp, usually concentrated in a large amount around the blood vessels. In the hematoxylin and eosin stained sections, the MMC were packed with dark brown-black or brown-yellow deposit.

PLATE-I: Shows Light Micrograph of Spleen of normal/controlled *Clarias batrachus* stained with hematoxylin and eosin, illustrating components of spleen parenchyma. Under low power, Red pulp (RP) and white pulp (WP) encircled by a capsule (C) is seen. Distinct trabeculae (TV), blood vessels (BV) and few scattered Melanomacrophage centres are also observed. Under high power, distinct splenic trabeculae, Red pulp and white pulp are observed.

The pictures of the plate is shown below:

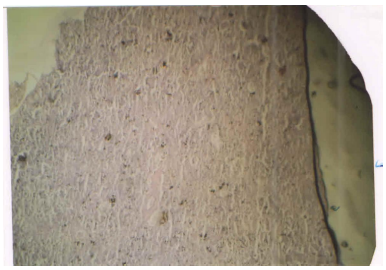
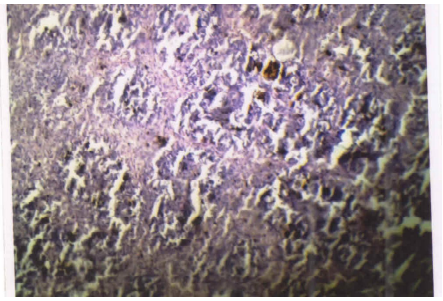


PLATE- I Light Micrograph of spleen of the normal *C.batrachus* stained with hematoxylin and eosin illustrating components of the spleen parenchyma, Red pulp (RP) and White pulp (WP) enclosed by a capsule (C) ,distinct trabaculae (TV) and blood vessels. Note: Few scattered Melanomacrophage centres are also seen. (H&E) 10X

Control Group Under Low Power.



High Power showing

Distinct splenic trabaculae(TV),Red Pulp (RP), white pulp (WP). (H&E)-40X.

Control Group Under High Power.

PLATE-II: Light micrograph of spleen of 1µl/L rogor treated *C. batrachus*, stained with hematoxylin and eosin, after 5 days shows slight dissolution of red pulp and white pulp, increased vacuolation in the splenic parenchyma with increased aggregation of melanomacrophage granules. Under high power, large melanomacrophage centres at the side of blood vessels are seen with increased vacuolation.

The picture of the plate is shown below:

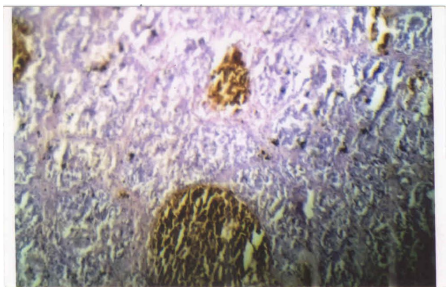
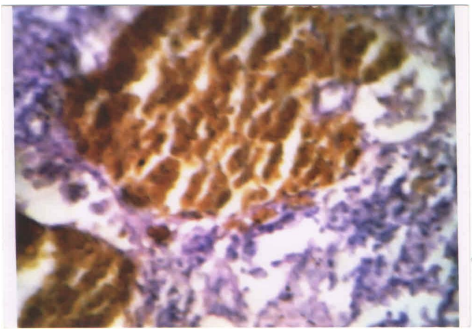


PLATE- II Light Micrograph of spleen of 1 µl/l Rogor treated *C. batrachus* after 5thday stained with hematoxylin and eosin shows slight dissolution of red pulp and white pulp, increased vacuolations in the spleen parenchyma with increased aggregation of the melanomacrophage granules(MG).X40

LOW POWER



High Power showing
Large melanomacrophage centres at the side of blood vessels.
Note: Increased vacuolations. 100X

HIGH POWER

PLATE –III : Light micrograph of spleen of 1µl/L rogor treated *C.batrachus,* stained with hematoxylin and eosin ,after 10 days, shows spleen parenchyma with no demarcation of red pulp and white pulp, increased vacuolation, trabecular spaces and increased melanomacrophage centres. In high power enlarged melanomacrophage centres are seen.

The picture of the plate is given below:

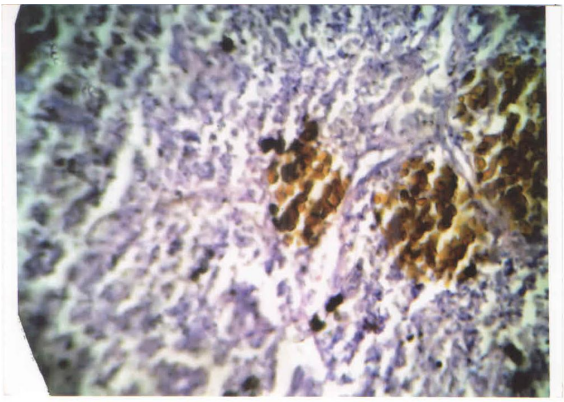
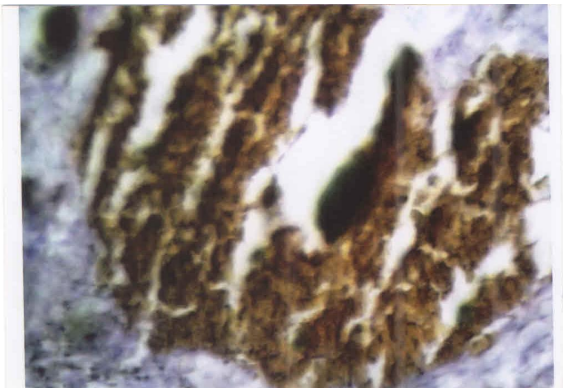


PLATE -III Light Micrograph of spleen of 1µl/L rogor treated *C. batarachus* after 10 days showing spleen parenchyma with no demarcation of red pulp (RP) and white pulp (WP),increased vacuolation (V), trabacular space (TV) and increased melanomacrophage(MG) centres. 40X

LOW POWER



High power showing large melanomacrophage centres.

HIGH POWER

PLATE – IV: Light micrograph of spleen of 1µl/L rogor treated *C.batrachus,* stained with hematoxylin and eosin, after 15 days, showing increased thickness of splenic capsule , increased vacuolation in the splenic parenchyma with increased trabeculae and increased melanomacrophage centres. In high power, complete dissolution of splenic parenchyma is seen with abnormally enlarged melanomacrophage centres.

The picture of the plate is given below:

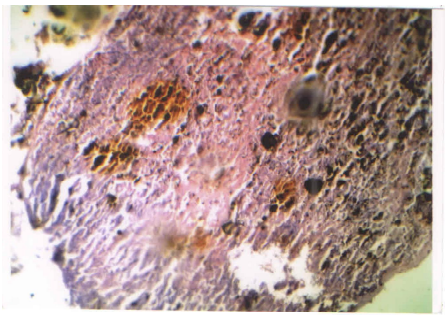
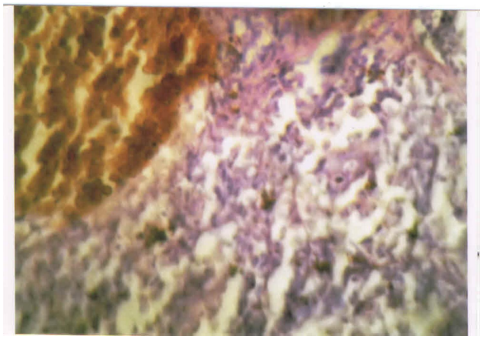


PLATE- IV Light micrograph of spleen of 1µl/L rogor treated *C.batrachus* stained with H & E after 15 days shows increased thickness of splenic capsule, increased vacuolation ,increased trabeculae and increased melanomacrophage centre

LOW POWER



In high power complete dissolution of splenic parenchyma is seen with abnormally large melanomacrophage centre.

HIGH POWER

PLATE – V: Light micrograph of spleen of 2.5µl/L rogor treated *C.batrachus,* stained with hematoxylin and eosin after 5 days, shows dilation of blood vessels, splenic parenchyma is largely occupied by melanomacropahge granules (pale coloured), debris of RBCs, dissolution of red pulp and white pulp. Under high power, dissolution of splenic parenchyma, large trabecular space and large patches of melanomacrophage centres are seen.

The picture of the plate is given below:

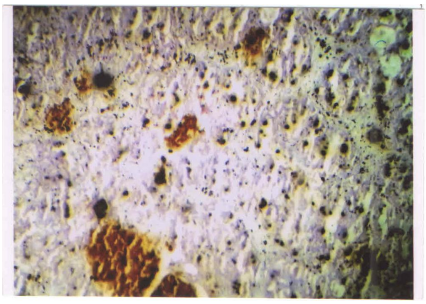
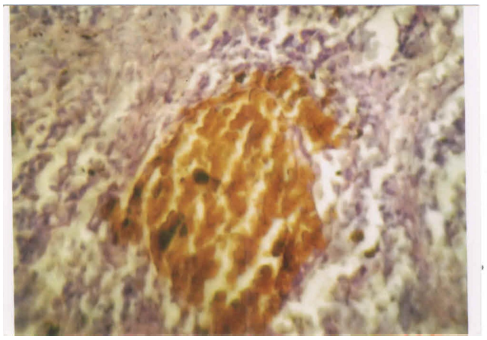


PLATE – V Light micrograph of spleen of 2.5µl/L rogor treated *C.batrachus* stained with H & E after 5 days, shows dilation of blood vessels, splenic parenchyma largely occupied by melanomacrophage (pale colour),RBC debris, dissolution of red pulp & white pulp

LOW POWER



In high power dissolution of splenic parenchyma, large trabecular space and large patch of MMC is seen.

HIGH POWER

PLATE – VI : Light micrograph of spleen of 2.5µl/L rogor treated *C.batrachus,* stained with hematoxylin and eosin after 10 days, shows much increased trabecular space, increased vacuolation and enlarged blood vessels . At one corner deeply stained melanomacrophage associated with blood vessels are observed. High power shows very enlarged trabecular space representing cracking of the splenic parenchyma.

The picture of the plate is given below:

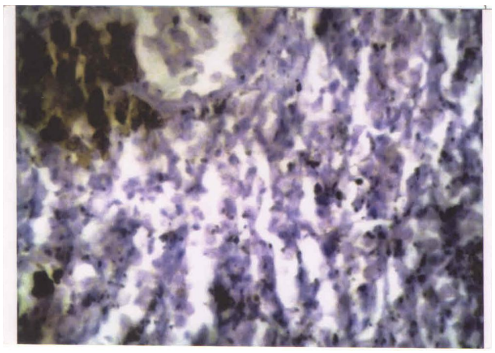
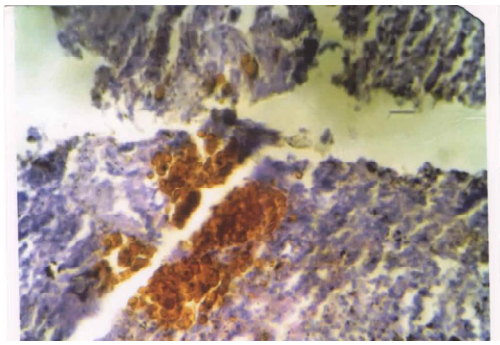


PLATE – VI Light micrograph of spleen of 2.5µl/L rogor treated *C.batrachus* stained in H&E after 10 days shows much increased trabecular space, increased vacuolation and enlarged blood vessels .At one corner deeply stained melanomacrophage associated with blood vessel is seen

LOW POWER



High power shows very enlarge trabecular space representing cracking of the splenic parenchyma.

HIGH POWER

PLATE – VII: Light micrograph of spleen of 2.5µl/L rogor treated *C.batrachus,* stained with hematoxylin and eosin after 15 days, shows complete autolysis of splenic parenchyma, large patches of melanomacrophage at the side of highly dilated blood vessels are seen. Under high power a portion showing complete dissolution of splenic parenchyma is seen.

Thus various findings like enlarged patches of melanomacrophage centers (MMC), vacuolation, degeneration of red blood cells and white pulp and increased trabecular space were observed in the spleen sections of all fish exposed to both the doses of rogor.

The picture of the plate is given below:

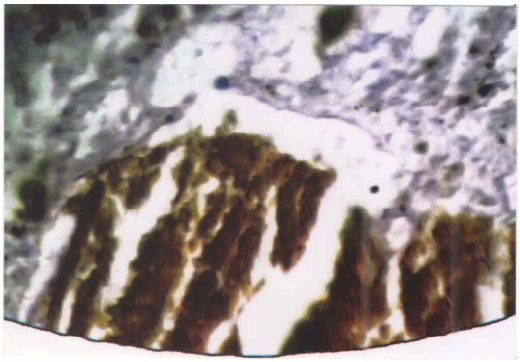
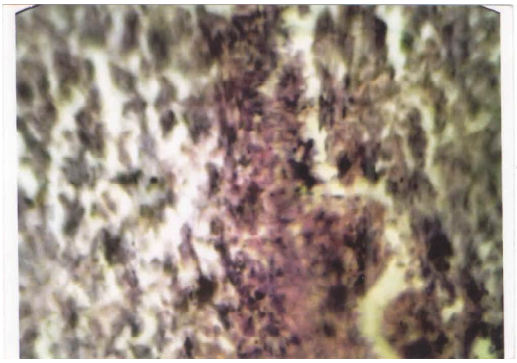


PLATE – VII Light micrograph of spleen of 2.5µl/L rogor treated *C.batrachus* stained with H&E after 15 days shows complete autolysis of splenic parenchyma, large patches of melanomacrophage at the side of highly dilated blood vessels are seen

LOW POWER



High power shows complete dissolution of splenic parenchyma is seen.

HIGH POWER

3.2 HISTOPATHOLOGICAL CHANGES OBSERVED IN THE SPLENIC TISSUES OF THE FISH

The exposure of fish to chemical pesticide is likely to induce a number of lesion in different organs [22]. Here in the present work, sub lethal exposure of rogor induces many histopathological anomalies in the spleen, viz. Dissolution of the white pulp, aggregation of more melanomacrophage, autolysis of RBCs and loss of cellular architecture and matrix and distinct ridge formation. Dilution and fibrosis of sinusoids, one of the demarcated finding is ellipsoidal necrosis , which has also been observed by (Klontz et.al. 1966)[23], [24]. Marked dissolution of white pulp and increased eosinophiles at the red pulp region and fibrosis of sinusoids marks the stressful condition of fish. Aggregation of more melanomacrophage shows the extreme pathological condition. In spleen of fish, white pulp proliferation, lymphocyte depletion, as well as an increase in the size of spleen, haemosiderosis and increase in melanomacrophage centres has often been associated with environmental contamination [17]. One of the important physiological features is melanomacrophage centres (MMC) which are seen in the fish spleen [10]. They are assumed to be the functional substitutes of the germinal centres of spleen [14]. MMC may contain four types of brown pigments: melanin, lipofuscin, ceroid and hemosiderin [15]. Stressful conditions to the animal often result in increased number of its splenic MMC's [17], which is in agreement with the present investigation as a large number of MMC's are observed in splenic sections of fish exposed to sub lethal concentration of Rogor.

Haemosiderin is one of the breakdown products of Hb from senescent and degenerated erythrocytes [25]. Haemosiderosis is a pathological condition occurring due to the deposition of haemosiderin. Haemosiderosis is related to an increased rate of erythrocyte destruction in the spleen [26] which in present case is perhaps a consequence of sub lethal rogor exposure to the fish. Kaleeswaran *et al*. [27] suggested the increased severity in the MMC as a homeostatic mechanism of the fish spleen to phagocytose the increasing deposits of haemosiderin and other debris resulting from the destruction of tissues. This matches with the present study wherein the occurrence of MMC could be seen in splenic sections of exposed fish. The present investigation is also in agreement with the findings of Fournie et al. [18] who associated the density of splenic macrophage aggregates in estuarine fishes to exposure to degraded environments.

The vacuolation obtained in the present investigation are in agreement with reports of Spazier *et al*. [28] who also observed vacuolation in splenic tissue of European eel *Anguilla anguilla* following stress and resulting in impairment of normal physiology of fish. Histopathological study of spleen may prove to be an important biomarker to characterize the toxicological impacts in the environment.

4. CONCLUSION

Pesticides cause significant variation in structural, histological and biochemical changes in the aquatic fauna, particularly in fish. Present study focuses on the effect of Rogor induced histological alteration in the spleen tissues of freshwater fish *Clarias batrachus*. The details of histopathological observations based on Light Microscopy (LM) as well as hormonal imbalance in the spleen had been observed. Pesticidal impact on the spleen tissues were also done. Under low power, Red pulp (RP) and white pulp (WP) encircled by a capsule (C) was seen. Distinct trabeculae (TV), blood vessels (BV) and few scattered Melanomacrophage centres were also observed. Under high power, distinct splenic trabeculae, Red pulp and white pulp were observed.

Light micrograph of spleen of rogor treated *C. batrachus,* stained with hematoxylin and eosin after 15 days, shows complete autolysis of splenic parenchyma, large patches of melanomacrophage at the side of highly dilated blood vessels were seen. Under high power a portion showing complete dissolution of splenic parenchyma is seen. Exposure to sub-lethal concentrations of rogor resulted in significant histopathological alterations in the spleen . Tissues injuries and damages in spleen can result in the reduced immunity and may lead to reduced survivorship, growth and fitness, the low reproductive success or increase of susceptibility to pathological agents. On the other hand, these changes may be potentially disruptive for the survivability of *Clarias batrachus* in aquaculture farms.

The adrenal tissue can lose the ability to produces hormones such as cortisol which help fish to properly cope with stressful situations. Excessive stress or an impaired stress response can lead to decreased reproduction, growth, fitness and survivorship. Hence, from the experimental protocol we can conclude that exposure to sub-lethal concentrations of rogor resulted in significant histopathological alterations in the spleen . Tissues injuries and damages in spleen can result in the reduced immunity and may lead to reduced survivorship, growth and fitness, the low reproductive success or increase of susceptibility to pathological agents. On the other hand, these changes may be potentially disruptive for the survivability of *Clarias batrachus* in aquaculture farms. Histological alterations also could be used as meaningful indicators of pesticide pollution. Accumulation of pesticides in the water body primarily affects the non-target organism especially fish and get deposited. These fish through food chain affects humans and causes deleterious effects. Hence, the usage of the pesticide should be restricted to have a healthy ecology.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Available:www.fao.org › FAO Home › Fisheries & Aquaculture): Fishery and Aquaculture Country Profiles - The Republic of India
2. Yeager and Munson; 1945.
   Article on DDT at www.wikipedia.com
3. Kulkarni, Rafique. Comparable toxicity study of two heavy metals and pesticides under individual combination exposure on the fish *C.puntata.* Indian Zoologist. 1992;16(1&2):69-71.
4. Lin et al. Kim CT, Tsung SH, Peoples JB. Malignant fibrous histiocytoma of ileum . Dis. Colon Rectum. 1983;26:335-338
5. Cynamid; 1950.
   Article on dimethoate at www.wikipedia.com
6. Kumble, Muley. Effect of acute exposure of endosulfan and chloropyrifos on the biochemical composition of the freshwater fish (Sarotherodon mossambicus). Ind. J. Environ. Sci. 2000;4:97-102 .
7. Scott. Pesticides toxic to vertebrates. Vet. Res. 1967;80:168-173.
8. Jackson GA. Biological half-life of endrin channel cat fish tissues. Bull. Environ. Contam. Toxicol. 1968; 16:505-507.
9. Mukesh KN. The effect of pesticides on fish fauna of Bhopal lower lake (M. P.). African Journal of Environmental Science and Technology. 2013;7(7):725-727.
10. Agius, Roberts. Melano-macrophage centres and their role in fish pathology. J. Fish Dis. 2003;26:499-509
11. Press C. McL., Dannevig BH, Landsverk T. Immune and enzyme histochemical phenotypes of lymphoid and nonlymphoid cells within the spleen and head kidneyof Atlantic salmon (*Salmo salar L.*). Fish Shellfish Immunol. 1994;4:79-93.

12. Hansen JD. Characterization of Rainbow trout terminal deoxynucleotidyl transferase structure and expression. TdT and RAG1 co-expression define the trout primary lymphoid tissues. Immunogenetics. 1997;46:367-375.
13. Genten, Terwinghe Atlas of fish rainbow trout (*Oncorhynchus mykiss*). Cell and histology. Science Publishers. 2009;57.
14. Ellis. Antigen-trapping in the spleen and kidney of the plaice, *Pleuronectes platessa* (L.). J. Fish Dis. 1980;3:413-426.
15. Couillard et al. Histopathological evaluation of Atlantic tomcod (*Microgadus tomcod*) collected at estuarine sites receiving pulp and paper mill effluent. Aquat.Toxicol. 1999;44:263-278.
16. Wolke REG, GEORGE CJ, Blazer VS. Pigmented macrophage accumulations (MMC, PMB): possible monitors of fish health. In: W.J. Hargis (Editor). Washington D.C., NOAA Technical Report NMFS. 1985;25:93-97.
17. Montero D, Blazer VS, Socorro J, Izquierdo MS, Tort L. Dietary and culture influences on macrophage aggregate parameters in gilthead seabream (*Sparus aurata*) juveniles. Aquaculture. 1999;179:523-534.
18. Fournie et al. Utility of splenic macrophage aggregates as an indicator of fish exposure to degraded environments. J.Aquat. Anim. Health. 2001;13:105-116.
19. Kurtovic. Histological comparison of spleen and kidney tissue from farmed and wild European sea bass (*Dicentrachus labrax* L.). Acta adriat. 2008;49(2):147-154.
20. Available:https://teachmephysiology.com/gastrointestinal-system/spleen/function-of-spleen/) : Functions of Spleen.
21. APHA. Standard methods for the examination of water and wastewater (21st Edn.). Washington, D.C.: American Public Health Association; 2005.
22. Bucke. Common diseases and parasites of fish in the North Atlantic: Training guide for identification international council for the exploration of the sea techniques in marine environmental sciences, Copenhagen; 1996.
23. Klontz. Bacterial diseases of the salmonidae in the western United States: Pathogenesis of furunculosis in rainbow trout. Am. J. Vet. Res. 1966;27:1455-1460.
24. Thorpe JE, Roberts RJ. An aeromonad epidemic in the brown trout. Salmo trutta. L. J. Fish Biol. 1972;4: 441-451.
25. Zapata and Cooper. Histological and Histochemical Studies on the Early Developmental Stages of the Egyptian Toad Bufo regularis Reuss ; Open Journal of Animal Sciences. 1990;5(2).
26. Hibiya. Changes in Muscle and Collagen Fibers of Fish after Exposure to Urban Pollutants and Biodegradable Detergents; Microscopy Research. 1982;3(3).
27. Kaleeswaran et al. Changes in biochemical, histological and specific immune parameters in Catla catla (Ham.) by *Cynodon dactylon* (L.) Journal of King Saud University-Science. 2010;24(2):139-152.
28. Spazier et al. Cytopathology of spleen in eel Anguilla Anguilla exposed to a chemical spill in the Rhine River .Diseases of Aquatic Organisms. 1992;14(1):1-22.