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Macrophages and Lipid Peroxides Concentration of the Tissues: A Bio-Indicator of Ecological Hazard

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Abstract

A comprehensive study on the reproductive system of air breathing catfishes was undertaken to evaluate the effect of environmental toxicants on the breeding capacity of the catfishes, *Heteropneustes fossilis*. Histopathological examination of ovary by standard error of mean along with oxidative stress estimation measuring lipid peroxides and protein peroxides was carried on the ovary and the vital organs of 125 fishes from a polluted pond near Hindon river in Ghaziabad, Uttar Pradesh, India. Depending on the physico-chemical parameter of the polluted pond near Hindon river, it was found unhealthy and designated as Type E. Fresh fishes were taken as control from Gazipur, fish farm. Comparative study of ovarian follicles of control and polluted pond fishes was conducted to estimate the histopathology of the reproductive organ. Polluted pond fishes ovaries showed degenerative changes such as prominent longitudinal zig-zag folds, with eroded surface forming convex region on the margin of developing ovaries. Phagocytic macrophages were also seen present on the surface epithelium of the ovary. It was probably due to stressful condition induced by xenobiotics in the pond. Lipid peroxidation in different tissues was found more consistent in their action as compared to the protein peroxidation due to free radical generation by xenobiotics present in the pond. Thus, excess of macrophages and high level of lipid peroxidation of the tissues can be taken as bio-indicator of ecological hazards in an aquatic ecosystem.

Keywords: *Heteropneustes fossilis*, Lipid Peroxides, Macrophages, Malathion, Oocytes, Polluted Pond, Protein Peroxides, Standard Error of Mean, Ultrastructural

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1. Introduction

Anthropogenic environmental degradation is a major concern as it is damaging the water reservoir and causing stress to the aquatic ecosystem. Due to societal, agricultural, municipal, and factorial sewage, harmful chemicals are continuously getting mixed in the water bodies.

One of the major agricultural chemical groups is pesticide, weedicides, herbicides, which play an important role in increasing agricultural productivity through controlling pest and unwanted plants. But on the other hand, they cause much damage to the nontarget organisms both in terrestrial and aquatic environment. Besides, heavy metal toxicity is another problem to tackle with, which have posed potential health hazard not only to livestock and wild life but also to fish, birds, mammals, and even human beings. Aquatic organisms, including fish, accumulate pollutants directly from contaminated water and indirectly via food chain (Sasaki *et al.*, 1997). The aquatic environment is continuously being contaminated with toxic chemicals from industrial, agricultural, and domestic activities. In India, pesticides and heavy metals are one of the major classes of toxic substances polluting air, water, and soil. The surface runoff from treated areas enters the river and aquaculture ponds that are supplied by rivers. Water pollution due to pesticide and heavy metals is posing intricate problems that need our immediate attention. Agricultural runoff (pesticides, weedicides fungicides, and insecticides) and heavy metal has reported to cause degenerative changes in the ovary of teleost (Singh and Sahai, 1985). Mani and Saxena in 1985;

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Saxena and Greg in 1978; Pandey and Shukla in 1985; and Ojha *et al*., in 1992 have reported partial sterility in the mice due to malathion exposure. Chowdhary *et al*., in 1994 reported that malathion toxicity directly interferes with spermatozoa tubulin assembly. Nath and Kedia in 1997 have also observed numerous histopathological changes in the spermatozoa of *Heteropneustes fossilis* due to malathion exposure. Arsenic, fluoride, and other heavy metal contamination of water bodies has also been reported by many scientists (Singh *et al.,* 2014; Sehgal *et al.,* 2012; Sen *et al.,* 2011).

Histopathology deals with the study of pathological changes induced in the microscopical structure of body tissue. Any alteration in the normal structure of tissue indicates the presence of disease or the effect of toxic substances such as heavy metal and pesticides. Sprague in 1973 described histopathology as an important tool for evaluating the action of any toxicant at tissue level. Histopathology basically provides data concerning tissue damage. Histopathological alterations can be used as indicators for the effect of various anthropogenic pollutants on organisms and a reflection of overall health of the entire population in the ecosystem. These histopathological biomarkers are closely related to the other biomarkers of stress since many pollutants have to undergo metabolic activation to be able to provoke cellular change in the affected organism (Ismail *et al.,* 2009).

The present investigation has been undertaken to predict oxidative stress and histopathology induced in the reproductive organs of H. fossilis. Lipid peroxidation and protein peroxidation has been considered to estimate oxidative stress, and histological examination of oocytes was done by using compound microscope and Standard Error of Mean (SEM) for pathological estimation.

2. Materials and Methods

The experiments were done in two groups. First group comprises 125 live female *H. fossilis*, average size 5" to 8" and average weight of 30-40 g and were collected from the polluted pond near Hindon river, Ghaziabad, Uttar Pradesh.

In the second group of experiment, a fresh batch of 100 healthy female *H. fossilis* of average 5" to 8" length and average weight of 30-40 g were collected from Gazipur fish farm which were isolated from any industrial, agriculture, domestic, and municipal sewage effluent discharge.

The physico-chemical parameter of the pond is done following the procedures given in APHA-AWWA-WPCF (1985). The sample of surface water was collected in polythene bag. Dissolved oxygen and free CO_2 test were done on spot. The water is then preserved with a few drops of chloroform and transferred to laboratory for further physico-chemical analysis.

For zooplankton study, initially, 200 L of water were filtered through a plankton net at five different sites of polluted pond. Finally, the sample was concentrated to 50 mL and preserved with 4% formalin and counting was done with the help of Sedgewick Rafter counting chamber.

Local agriculturists and fish farmers reported the use of pesticide containing organophosphate mainly malathion, parathion, rotenone, and endosulfan in the agricultural field. By studying the physico-chemical parameter at five sites in the pond and depending on its color, the water quality of the pond is designated as E (Gopal and Asthana, 1991).

The fishes of first and second group were sacrificed and brain, liver, kidney, gills, muscles, and ovary from female specimen were dissected out in normal physiological saline, thoroughly washed, and then ovary was fixed in chilled 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH = 7.4) at 4°C for 2 h followed by its dehydration in graded alcohol and amyl acetate. Ovary was further dehydrated by critical point drying at 350°C and 65–67 kgf/cm^2 for 30-40 min by Hitachi HCP-2 CP drier. After C.P.D, tissues were gold sputtered by EIB-2 gold coating machine. The tissues were then observed in HITACHI S-530 Model SEM at the Department of Anatomy, AIIMS, New Delhi.

Lipid peroxides (LPO) were estimated of all the tissues in both control and polluted pond fishes. The assay of LPO was done by the thiobarbituric acid reaction with malondialdehyde (MDA), a product formed due to the peroxidation of lipids (Ohkawa *et al.,* 1979). The ovary, brain, liver, kidney, gills, and muscle tissues were homogenized in ice-cold 0.15 M phosphate buffer (pH 7.4, 10% wt./vol.) and the homogenates were centrifuged at 5000 rpm for 20 min. The amount of MDA formed was measured by using the molar extinction coefficient for MDA at 530 nm. (Ohkawa *et al.,* 1979) Protein Peroxides (PPO) were estimated in both control and polluted pond fishes tissues such as the LPO.

The assay of PPO was done by following the method of Gay *et al*., in 1999. For this tissue, homogenate was mixed with equal amount of 10% cold Trichloroacetic Acid

(TCA) and precipitate was collected by centrifugation. The collected precipitate was washed 3 times with 10% cold TCA and resuspended in 25 mM sulfuric acid for the determination of hydroperoxides. The sulfuric acid suspended precipitate was then mixed with 5 mM ferrous ammonium sulfate and equal amount of xylenol orange and incubated in room temperature for 30 min. The resultant ferric ions are measured as the xylenol orange complex at 560 nm. The results were analyzed statistically using Student's *t*-test.

3. Results

The principal zooplanktons present in pond belong to America radiosa, *Arcella discoides*, Cyclops, Brachionus, etc.

A comparative physico-chemical parameter of pond near Gazipur farm and polluted pond near Hindon river, Ghaziabad, observed is shown in Table 1.

Microphotographs of the ovaries of controlled fish group showed smooth, regular, and spherical oocytes.

Table 1. A comparative physic-chemical parameter of pond near Gazipur farm and polluted pond in Ghaziabad, observed

	Pond A near Gazipur farm (free from industrial/agricultural effluents)		Pond B near Mohan Nagar Industrial area, Ghaziabad, Uttar Pradesh	
	Mean	SD (:f:)	Mean	SD (:f:)
Water temperature	22.5°C	0.2	24.3°C	0.5
pH	7.9	1.1	8.4	0.7
Dissolved oxygen	7.5	1.2	2.5	0.2
Free CO_2 (mg/L)	18.5	1.5	22.8	1.7
Total alkalinity	190	5.6	325	6.0
Total hardness (as $CaCO_3$)	195	3.9	295	4.7
BOD (mg/L)	4.97	1.2	.40	3.5
COD (mg/L)	52	1.5	21	3.5
Ca^{++} (mg/L)	39.5	1.7	29.6	3.5
Mg^{++} (mg/L)	18.4	1.2	22.0	1.6
Cl^{-} (mg/L)	18.4	1.8	30.7	2.4
Nitrate nitrogen (mg/L)	0.12	0.01	0.26	0.03
Phosphate, phosphorous (mg/L)	4.6	0.8	11	1.3
SO_4^{--} (mg/L)	10.4	1.2	55.5	2.9

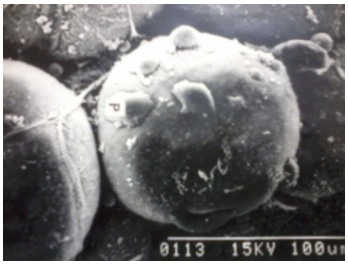
SD=Standard deviation, BOD=Biochemical oxygen demand, COD=Chemical oxygen demand

Table 2. Comparative account of lipid peroxidase content (nmol/mg tissue) and protein peroxidase content of control and polluted pond cat fishes

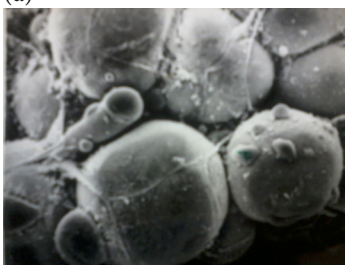
Tissues	Control LPO	Polluted pond fish LPO	Control PPO	Polluted pond fish PPO	Percentage deviation of mean value of LPO and PPO
Ovary	1.86±0.13	2.68*±0.19	0.64±0.07	2.31*±0.01	LPO - 22.05 PPO - 73.87
Brain	2.65±0.12	3.25*±0.15	0.90±0.07	1.43*±0.10	LPO - 21.15 PPO - 76.05
Liver	1.43±0.07	2.19*±0.01	1.19±0.05	2.34*±0.12	LPO - 51.01 PPO - 96.23
Kidney	1.70±0.08	2.86*±0.12	1.17±0.06	1.75*±0.09	LPO - 77.95 PPO - 44.92
Gills	1.54±0.10	2.34*±0.10	1.35±0.09	2.38*±0.12	LPO - 53.84 PPO - 75.26
Muscles	0.38±	0.98*±0.06	0.56±0.04	2.15*±0.06	LPO - 162.75 PPO - 118.42

n=125, data are mean±SEM. *P<0.001. LPO-Lipid peroxidase content (nmol/mg tissue), PPO=Protein peroxidase content (nmol/mg tissue), SEM=Standard error of mean

The developing oocytes showed uniform thickness of follicular epithelium. Micro granules were found densely scattered all over the surface of oocytes [Figure 1(a) and (b)].



(a)



(b)

Figure 1. **(a)** Microphotographs of the ovaries of controlled fish group showed smooth, regular, and spherical oocytes. The developing oocytes showed uniform thickness of follicular epithelium. Microgranules were found densely scattered all over the surface of oocytes. **(b)** Control oocytes.

Polluted pond fishes ovaries showed degenerative changes such as cracks on the surface, prominent longitudinal zigzag folds with eroded surface forming convex ridges on its margin [Figures 2 and 3]. Outer surface of the oocytes was fully wrinkled with many pits showing numerous cracks and furrows. The fusion of growing oocytes is quite prominent. Besides the appearance of dense osmiophilic granules, macrophages of phagocytic nature were also very common.



Figure 2. Cracks on the surface of polluted pond ovary.

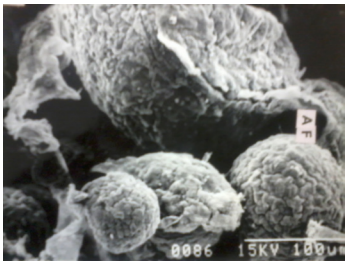


Figure 3. Zig-zag fold on the surface of the ovary.

Normal oocytes show symmetry and dense osmiophilic granules are not present in it (left) [Figure 4]; however, polluted pond oocytes showed numerous osmiophilic granules (right) [Figure 5].

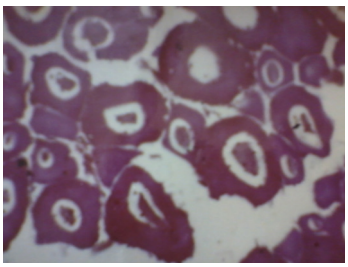


Figure 4. Normal oocytes showing regular symmetry.

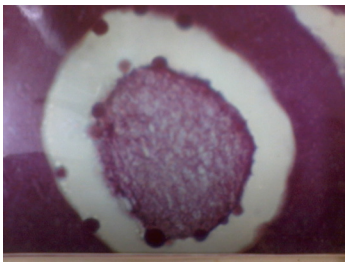


Figure 5. Dense osmiophilic granules in the polluted pond ovary.

The micrographs of oocytes of the ovaries of fish collected from polluted stagnant water near Hindon river showed the presence of amoeboid cells such as macrophages. The cells have collar-like structure at one end. Collar consists of number of finger-like projections, protruding toward the center which has an opening. Few ova are observed near them as if they are being engulfed by them [Figures 6 and 7].

The changes in the LPO status in muscle of polluted pond showed significant response ($P < 0.001$) than the control fishes. LPO of gill tissue showed significant

increase. The increase in LPO level can be correlated with the concentration of xenobiotics present in the pond water. In liver, the LPO content was found significantly increased from the control group. Similarly, in brain and ovary, the mean values of LPO were found increased from control which was about 45.51% and thus the mean value was higher than the control group. Similar results were observed in kidney tissue where LPO level was observed high in the polluted pond group.

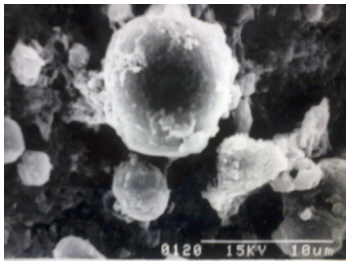


Figure 6. Macrophages in the polluted pond ovary.

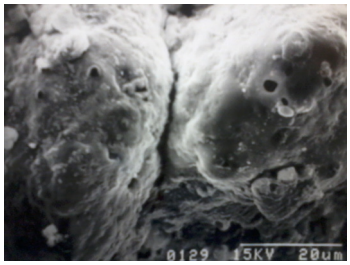


Figure 7. Numerous finger-like structures on the surface of oocytes.

In general, the PPO contents of all the tissues of polluted pond group were found increased than the control group.

4. Discussion

Ovary consists of a series of ovarian lamelle, radially oriented toward the lumen and containing oocytes at different stages of development in accordance to the maturation stages of the ovary. Control group showed well developed Stage IV oocytes with properly distributed provitelline nucleoli. Oocytes showed mature nucleus, nucleolus, and oocytes were full of yolk granules; however, polluted pond oocytes microphotograph showed primary follicle adhesion, cytoplasmic retraction, and atretic oocytes. Partial destruction of the ovigerous lamelle and vitellogenous membrane was also observed. Many oocytes had increased intrafollicular spaces, vacuolated cytoplasm, and extrusion of the karyoplasm. Some showed necrosis in the cytoplasm and total loss of the internal symmetry. Detail histopathology suggest disturbance in endocrine hormonal imbalance. Rosette structure on the surface of the oocytes was also observed. Many macrophages were observed in the ovary showing cytoplasmic protrusion. Some macrophages were seen engulfing the atretic oocytes. Similar observation in the oocytes by malathion (organophosphate) treated fish is also reported by Dutta *et al*., in 1994 as follicular atresia, clumping of cytoplasm, increased number of nucleoli, shrunken nuclear material, fusion of oocytes, well supported by low serum estrogen level. The rosette structure forming lattice is probably the intermediate in transporting protein from the Golgi to the cell surface which would be expected to have considerable plasticity to allow passage to cell particle such as membrane vesicle (Darnell *et al.,* 1986). Pareda *et al*., have also reported the presence of similar rosette structure as macrophages in human ovary which can be reversed on elimination of stress.

Mono nuclear phagocytes with large eccentric nucleus, vacuolated amoeboid cytoplasm which often contains phagocytosed inclusion were also seen. Free macrophages, which have appeared on the oocyte surface of *H. fossilis* of polluted pond are supposed to be originated in spleen, liver, and bone marrow from fixed macrophages through contraction and isolation. Macrophages are reported to be acting as antigen processor and presenting cell, thereby involved in all steps for immunological responses, free macrophages appears at subcellular level in certain diseased conditions, especially those of septic nature. The appearance of macrophages in pathological condition or during stress is reported to be an agonal phenomenon and referred in the name of "hemohistoblast" in leukemia (Bloom and Fawcett, 1962). In the testes of amphibia, the appearance of some phagocytic cells other than germ cells have been reported by Nath and Jamuar (1992). Besides marked increase in acid phosphatase activity of macrophages in spleen and kidney of $HgCI_2$ treated in air breathing fish Channa punctatus has also been explored by Dutta Munshl *et al*., in 1990. Nath and Chand in 1997 have also reported similar malathion-induced spermiophagy in the testes of *H. fossilis* showing its macrophagic action. The engulfed protein and DNA of testicular cell will finally recycle and provide nutrient to growing testicular cell. Azmi *et al*., in 1986 have reported that macrophages are specially designed cell evolved in the phagocytosis and

elimination of degenerated testicular cells. Spermiophagy in the tests of cancer, patient of the prostate by similar macrophages are well reported by Murakami *et al*., in 1986. Motta and Makabe in 1986 have also revealed that some primitive germline cells present in cortical areas of ovary possibly migrate to surface coelomic epithelium and show macrophagic features. While the germ cells which are deeply located in ovarian tissues during embryonic conditions are associated with proliferating primitive follicle in human developing ovary, Giri *et al*., 2000 have reported histoanatomical insult to the ovarian tissues by xenobiotic and reduction in the reproductive capacity of *H. fossilis*. The possible role of macrophages for defensive purposes against stress in major carps and air breathing fish is also illustrated by Saha *et al*., in 1993. Srivastav *et al*., in 2008 also reported gonadal impairment due to pesticide-induced toxicity fishes.

The present investigation correlates the appearance of macrophages in the oocytes of the fish collected from polluted pond near Hindon river, Ghaziabad. Though appearance of macrophages in aforesaid polluted pond might be results of synergistic effects, local information about the recurrent use of pesticides in abundance and physico-chemical analysis of water body have shown the presence of pesticides and heavy metal in it. Under stressful condition, fishes exhibit several histopathological immune responses for defense purposes. The appearance of macrophages is seems to be pioneer among them, which probably engulf dead and infected decaying oocytes. The peroxidation of lipid/proteins in different tissues on exposure to sublethal concentration of xenobiotics for a prolong period of time can slowly damage the tissues in long run.

During the study, it has been observed that out of the six tissues, muscle is the tissue most affected by peroxidation and brain is the least affected tissue. The lipids of gill, liver and kidney tissues are peroxidised moderately occupying an intermediate position, liver being at the center of the peroxidation affected tissues. The maximum involvement of muscle lipids may be due to a relatively weak free radical scavenging system in the muscle tissue (Kale and Sitaswad, 1990). The minimum peroxidation of brain lipids even with maximum peroxidizable lipids may be due to the protected position of the brain exerted by the blood-brain barrier. It is also observed that a tissue with the least amount of peroxidised lipid responds by forming the highest amount of LPO and the tissue with highest amount of peroxidised lipid responds with formation of a minimum amount of LPO to xenobiotic exposure.

The trend of protein peroxidation in different tissues was observed to be relatively more persistent than the peroxidation of lipid on corresponding tissues. Such kind of generation of free radicals associated with xenobiotic has also been reported by Bagchi and Stohs in 1993 and Thomas and Wofford in 1993, the toxic effects of xenobiotics observed with increased level of tissue peroxides in the present study is thought to be due to the result of pesticide and heavy metal induced free radical generation. Kale and Sitaswad in 1990 has also reported free radical damage to the membrane. Thus the higher concentration of LPO and the presence of macrophages in the tissues of fish may serve as bio-indicator of ecotoxicological hazards in aquatic ecosystem. There is a need to take safeguard against Industrial, agriculture and domestic effluent discharge in the aforesaid polluted pond with the help of local authority and Pollution Control Board. It will definitely contribute a little towards bioconservation of the aquatic organism specially air breathing fish with high nutritive index.

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