



Alteration in Oxidative Stress Biomarkers and Cytoarchitecture of Hepatic Tissues in Freshwater Fish *Clarias batrachus* (Linn.) under Sub lethal Butachlor Stress: Spectrophotometric and TEM Study

Gyanendra Bahadur Chand*, Prakriti Verma and Prakash Singh

Department of Zoology, Patna University, Patna – 800005, Bihar, India; gbchand@patnauniversity.ac.in

Abstract

The present study addresses the deleterious impact of sub lethal exposure of butachlor 2-chloro-N-(2-6-diphenyl) acetamide on hepatic cells of air breathing fish *Clarias batrachus* (Linn.) based on light and transmission electron microscopy and estimation of oxidative stress biomarker enzymes viz. reduced glutathione and lipidperoxidase. Fishes were exposed to 1.0µl/L butachlor for 5, 10 and 15 days respectively. After schedule exposure, blood sample were collected and extracted serum were analyzed for quantitative estimation of serum reduced glutathione and lipid peroxidase activity by spectrophotometer. The liver tissues were processed for light and electron microscope. Light photomicrographs of hepatic cells reveal dose related abnormalities which increases with the duration of exposure. Major changes attributed to the hepatic cell were parenchyma degeneration, necrosis along with fibrosis, widening of sinusoids, vacuolation, and infiltration of eosinophilic inclusion, karyolysis, pyknosis and perivenular congestion. Transmission electron microscopy of hepatic cells also revealed degenerated hepatic parenchyma, accumulation of lipid and electron dense material, degenerated mitochondria, nuclear shrinkage and enlarged lysosomes engulfing cytoplasmic particles in contrast to control fish. On prolonged exposure, the most frequent pathological modifications were mitochondrial swelling with regression of cristae and giant lysosome with myelinated phospholipid membrane pointing towards phospholipidosis. The activities of all the marker enzymes showed high fluctuation indicating significant imbalance in comparison to control. The study highlights the oxidative stress caused by butachlor correlated with histopathological anomalies of hepatic cells. It can be used as sensitive index for assessing the magnitude of oxidative damage and physiological dysfunction of experimental fish under laboratory condition.

Keywords: Butachlor, *Clarias batrachus*, Histopathology, Liver Cell, Lipid Peroxidase, Reduced Glutathione

1. Introduction

Modern agrochemicals, synthetic fertilizers and pesticides are indispensable since last four decades increasing food production by controlling agricultural pests and reducing vector borne diseases. Aquatic Ecosystem has been subjected to a vivid array of xenobiotics resulting in its deterioration. The toxic impact of pesticides on fish and other aquatic organisms have been documented¹⁻³. Butachlor, an important chloro-acetanilide herbicide is used primarily against annual grasses and many broad leaf weeds in rice fields in India. It has been reported as a proven carcinogen⁴, affecting the normal functioning of thyroid, level of sex steroid hormones and reproductive process in Zebra fish⁵. The biochemistry,

physiology and metabolism of fish have been reported to be adversely affected by butachlor⁶⁻¹⁰. Ishizuka *et al.*¹¹ have reported adverse effect of butachlor on rat's hepatic xenobiotic metabolizing potential.

Lipid peroxidation is the principal oxidative biomarker and primarily involved in generating oxidative stress induced cellular injuries in both plants and animals. It refers to the oxidative degeneration of lipid and lead to the generation of reactive carbonyl compounds. Malondialdehyde (MDA) and 4 hydroxyalkenals (HAE) are the principal components of the decomposition of poly unsaturated fatty acids (PUFA). Quantitative assessment of MDA alone (in hydrochloric acid) or MDA in combination with 4 hydroxyalkenals has consistently been used for assessing the extent of lipid

*Author for correspondence

peroxidation. Reduced glutathione (GSH) is a dominant intracellular compound involved in cellular defense against oxidative injury^{12,13}. Any alteration in GSH level indicates the interference of pesticides on the metabolism of fish.

The present research work emphasizes on the systematic deleterious effects of butachlor on the oxidative biomarkers viz. lipid peroxidase and reduced glutathione. Toxicants exert their first sign of damage at cellular and sub cellular entities, before being apparent in the morphology of the fish. Liver, being the principal detoxifying organ of fish body, are highly susceptible to any xenobiotic exposure to the fish. Hence the transmission electron microscopic study of the hepatic cells of the treated fish has been done in the present study.

2. Materials and Methods

2.1 Experimental Animal

The experimental animal considered in the present study was fresh water walking cat fish *Clarias batrachus* (Linn.), commonly called 'Magur'. Fishes were procured from few selected wetlands of Saharsa and Supaul districts of North Bihar. They were transported to Aquatic Toxicology Laboratory, Department of Zoology, Patna University, Patna, kept in plexiglass aquaria and treated with 0.05% KMnO₄ solution. The fishes were acclimatized for 15 days under ideal physico-chemical conditions. Fishes of standard length (18±2 cm) and weight (50±10 gm) were considered for further experimentations. The aerated water was changed daily. During acclimated period they were fed *ad libitum* with feed pellet containing a mixture of wheat flour, egg and starch (as binder) @5% of their body weight and water was changed daily. After acclimatization, fishes were divided into four groups of eighteen fishes each as *Group I* – Normal/control, *Group II* – Butachlor treated for 5 days, *Group III* – Butachlor treated for 10 days and *Group IV* – Butachlor treated for 15 days respectively.

2.2 Pesticide Used

The test chemical used in the experimental protocol was commercial grade Butachlor (EC 50%). The 96 hrs and 48 hrs LC₅₀ of butachlor for fish were determined as 4.2 µl/L and 5.5µl/L respectively by standard protocol¹⁴. In the present study a lower sub lethal dose of 1.0µl/L of butachlor was selected for administration in fish for 15 days. Stock solution was prepared and its exact amount, as per the volume of water taken in the aquaria, were poured into the aquarium and mixed thoroughly. The solution was changed regularly in the morning hours exactly after completion of 24 hours.

2.3 Collection of the Samples

After schedule exposure blood samples of both control and treated groups of fishes were collected in heparinized syringe by pricking caudal vein. A considerable part of blood was kept at -20°C for estimation of blood GSH. Rest amount of blood samples were centrifuged at 4°C for 10 minutes at 5000 rpm and clear supernatant serum was stored at -20°C for estimation of serum LPO. For histological studies, autopsy was done after extraction of blood. Hepatic tissues of both control and treated groups were cut into 5mm thick sections, fixed in neutral formalin and processed for microtomy. The paraffin spread sections were double stained in Delafield's haematoxylin and eosin, mounted in DPX and viewed in Labomed CXRIII trinocular light microscope.

For Transmission Electron Microscopy (TEM) the small pieces of tissue (approx. 2 mm in size) were fixed in chilled (4°C) phosphate buffered (pH-7.2) 2.5% gluteraldehyde. Ultra thin sections of 60-90 nm embedded on copper grid were stained in the uranyl acetate and lead citrate and viewed under Philip's CM-10 TEM at Sophisticated Analytical Instrument Facility-Electron Microscopic Unit, Department of Anatomy, All India Institute of Medical sciences, New Delhi.

2.4 Biochemical Analysis

2.4.1 Method of Glutathione (GSH) Estimation

For the biochemical analysis of glutathione, blood samples were obtained from the fishes of both the groups and it were mixed with EDTA. 0.2ml of mixed EDTA blood was added to 1.8ml lysine solution (disodium EDTA 1g/lit) and kept at 35-37 °C for 5 minutes. To this solution 3ml precipitating reagent (Metaphosphoric acid + EDTA + NaCl) was added, mixed well and kept at room temperature 35-37 °C for 5 min. The mixture was filtered. 1ml of clear filtrate was mixed with 4ml freshly prepared disodium phosphate. This mixture was labeled as TA₁ and was observed photo-metrically at 412nm. Another solution was prepared same as TA₁. 0.5 DTNB reagent (trisodium citrate + DTNB) was added to the solution, mixed well and kept at room temperature for 10min. This mixture was labeled as TA₂. The TA₂ is observed in spectrophotometer at 412nm. Then the standard was prepared by mixing 4ml of sodium hydrogen phosphate to 1ml of filtrate. This sodium was labeled as SA₁. Another solution was prepared same as SA₁ in which 0.5ml of DTNB reagent was added, mixed well and kept at room temperature for 10 minutes. This solution was labeled as SA₂. The reading was taken at 412nm. The activity of GSH was determined in mg/dl. All spectrophotometric observations were done on UV-VIS Spectrophotometer LAMBDA 25, manufactured by Perkin, Elmer.

2.4.2 Methods of Lipid Per-Oxidase (LPO)

Estimation

In this assay procedure, serum was extracted and maintained at 4°C. 0.5 ml of serum was mixed with 1ml of TCA and 1 ml of TBA (0.1M). The serum homogenate along with TBA and TCA was mixed thoroughly and was kept in boiling water bath for 20 minutes. The mixture was removed from the water bath and centrifuged at 1000g for 10 minutes. After centrifugation the filtrate was taken out in a cuvette and absorbance was read at 535 nm against blank. This gives the level of MDA in the sample which in turn indicates the level of LPO. MDA equivalent of the sample were calculated using an extinction coefficient of $1.56 \times 10^5 \text{ m}^{-1} \text{ cm}^{-1}$ Blank -1ml TCA + 1ml TBA.

2.5 Statistical Analysis

Six observations were taken randomly in each case and the Mean, standard deviation and standard error of mean was calculated. Unpaired 't' test and One way analysis of variance (ANOVA) were applied to the obtained data to depict any statistical significance at $p < 0.05$ and $p < 0.01$ between control and treated groups. All statistical calculations were done on SPSS version 16.

3. Results

3.1 Light Microscopy of Hepatic Cells (Control and Pesticide Treated)

3.1.1 Control Fish

Light microscopy of control test fish (*Group I*) showed normal architecture of hepatic tissues in which radiating chords of hepatocytes were draining into the central vein. The hepatocytes were prominent with homogenous cytoplasm and large nucleus. Portal vein and hepatic artery were almost normal (Figure 1).

3.1.2 Butachlor Treated Fish

The liver cells of test fish after 5 days of butachlor administration (*Group II*) showed widening of central vein, swelling of hepatic parenchyma, increased vacuoles and clumps of pyknotic nuclei (Figure 2). After 10 day of exposure (*Group III*), hepatic tissues showed invasion of eosinophilic inclusions in the portal vein leading to its massive congestion. Besides, portal tract with mild periportal fibrosis, necrosis, and nuclear pyknosis were

prominently marked (Figure 3). After 15 days exposure of butachlor (*Group IV*), liver cells were marked with presence of extensive hemorrhagic patches, congestion and necrosis of hepatic chords, enlargement of sinusoids and invasion of lymphocytes in it (Figure 4).

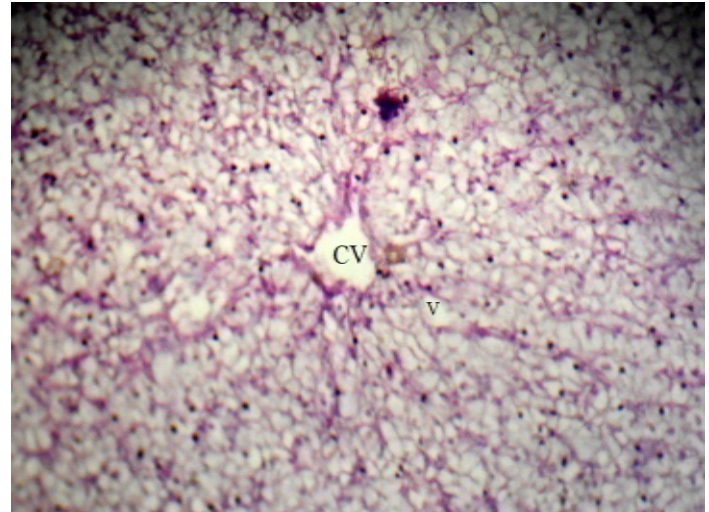


Figure 1. Liver of control fish showing hepatic parenchyma, distinct central vein (CV), intact lining of endothelial cells, sinusoidal space, and nucleus. X 200.

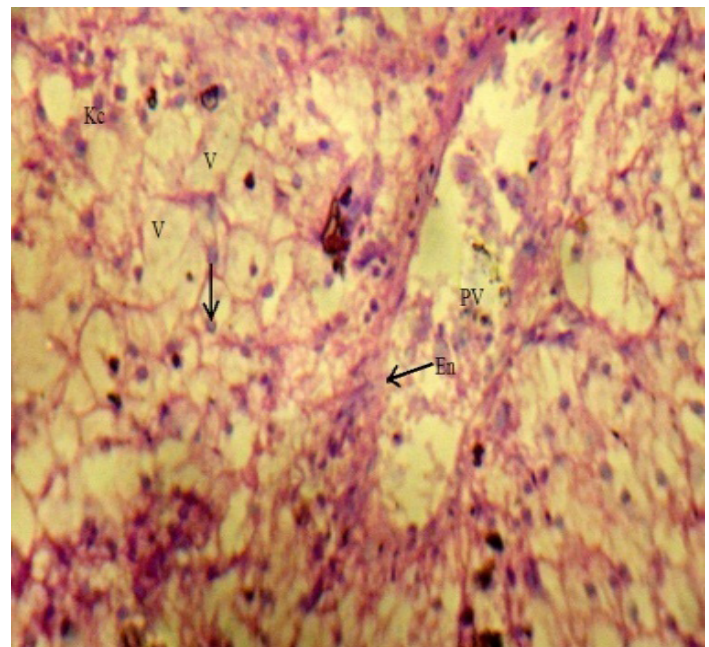


Figure 2. After 5 days butachlor treatment hepatic tissues of fish showing widening of central vein (CV), swelling of hepatoplasm, increased vacuoles, nuclear pyknosis (N). X 200.

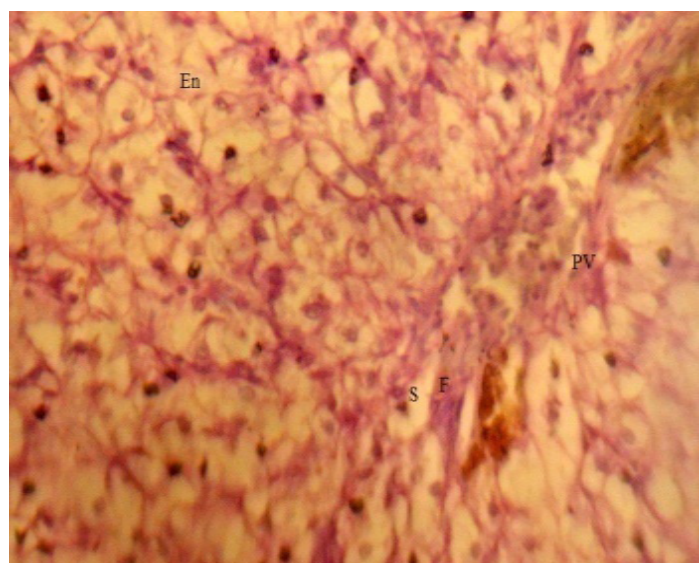


Figure 3. After 10 days butachlor treatment hepatic tissues of fish showing infiltration of eosinophilic inclusion, increasing pycnotic nuclei, congestion of portal triad and increased fibrosis (F). X 200.

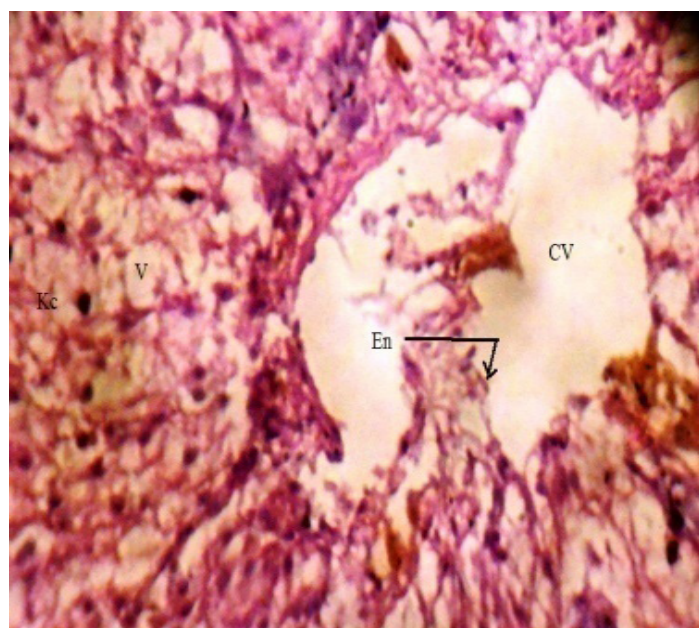


Figure 4. After 15 days butachlor exposure portion of portal vein showing massive vacuoles and swelling, X 200.

3.2 Transmission Electron Microscopy of Hepatic Cells

3.2.1 Control Fish

The transmission electron microphotographs of the hepatocytes of control fish (*Group I*) showed normal hepatic parenchyma, hepatoplasm, uniformly arranged stacks of endoplasmic reticulum, normal functional mitochondria, golgi bodies, microsomes, peroxisomes and nucleus with intact nuclear membrane and nucleolus (Figure 5).

3.2.2 Butachlor Treated Fish

Butachlor exposed fish (*Group II*) revealed distorted hepatic parenchyma, hyperactivity of cytoplasmic organelles with less regular compartmentalization of different components (Figure 6). The hepatocytes of Group III fishes showed consistent loss of structural integrity of rough endoplasmic reticulum, marked by the presence of dilated cisternae, nuclear shrinkage and enlarged lysosome with digested cytoplasmic particles (Figure 7). In Group IV fishes more frequent pathological changes observed were increased heterogeneity in the lysosomal matrix which showed increased phagocytosis of degraded cell organelles and accumulation of lipid and electron-dense materials. The presence of giant lysosomes with myelinated phospholipid membrane pointed towards phospholipidosis induced by butachlor (Figure 8).

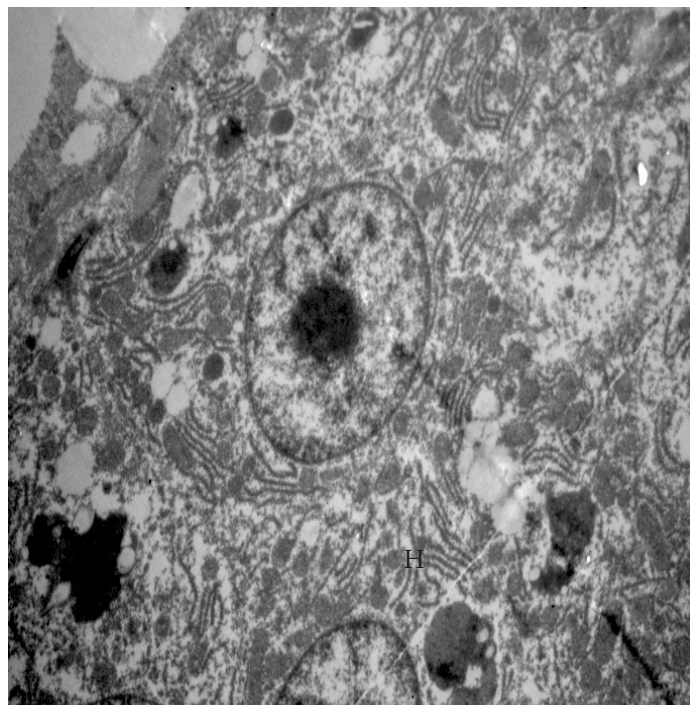


Figure 5. Transmission electron micrograph of a portion of hepatic cells showing normal hepatic parenchyma, hepatoplasm (H), uniformly arranged stacks of endoplasmic reticulum (ER), normal mitochondria (M), nucleus (N) and golgi bodies (GB), microsomes and peroxisomes (P). X 15500.

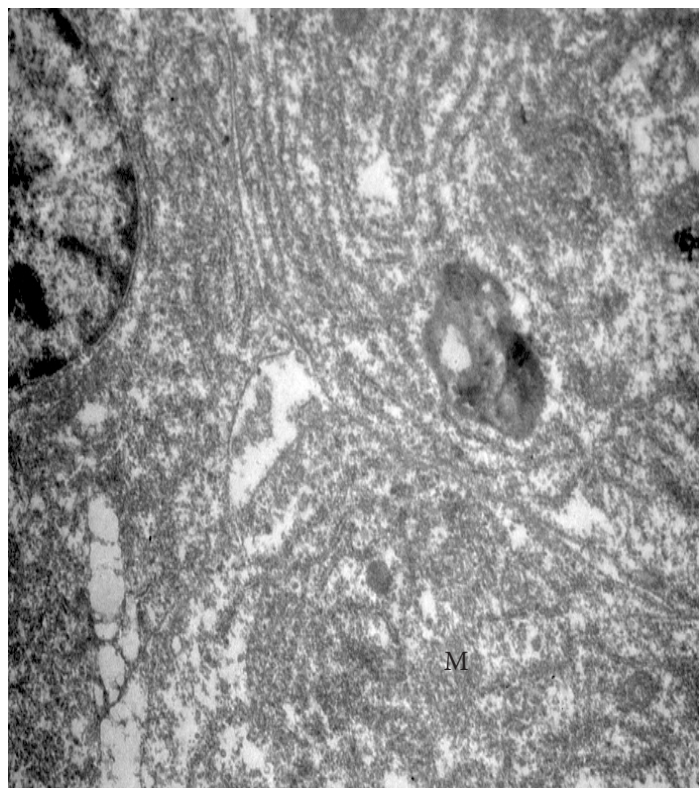


Figure 6. After 5 days butachlor treatment fish hepatocytes marked with distorted hepatic parenchyma (dH), hyperactive cytoplasmic organelles, nuclear shrinkage (N) and lysosome with digested cytoplasmic particles (L). X 21000.

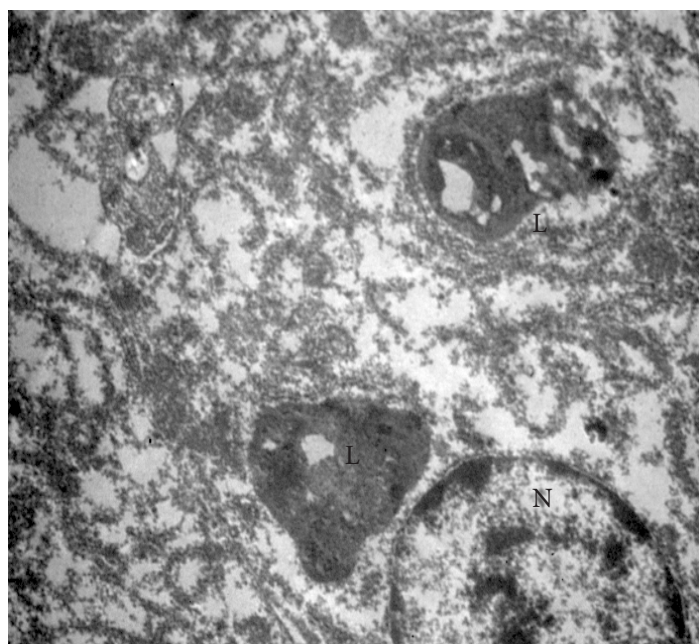


Figure 7. After 10 days butachlor exposure, fish hepatocytes are marked with increased lysosomal activities (L) distorted hepatic parenchyma (Dh), nuclear shrinkage (N), and altered ultrastructure. X 21000.

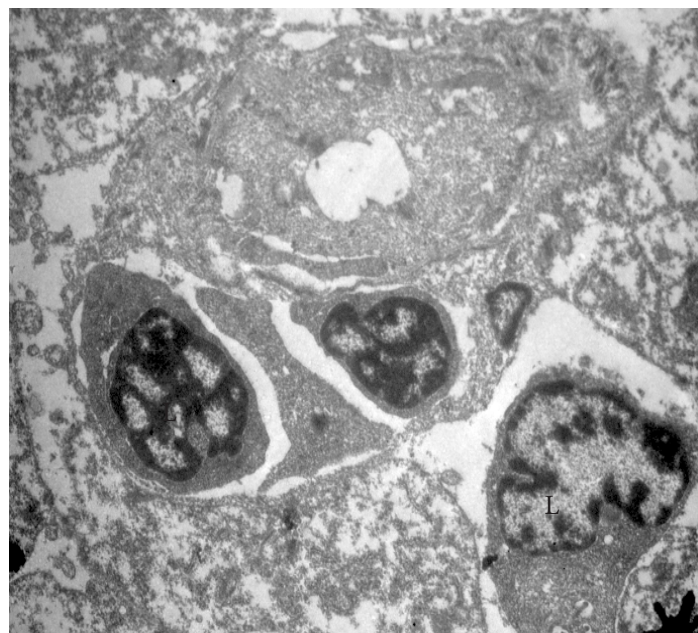


Figure 8. After 15 days butachlor exposure portion of hepatic cell showing accumulation of lipid and electron –dense materials, giant lysosome with myelinated phospholipid membrane pointing towards phospholipidosis (L)induced by butachlor. X 21000.

3.3 Spectrophotometric Analysis of GSH and Lipid Peroxidase Activity

The level of reduced glutathione (GSH) in control and experimental groups were shown in Table 1. Blood GSH in control fish was recorded as 16.48±0.96 mg/ml.

Table 1. Estimation of serum glutathione level in normal and butachlor exposed test fish

Groups	Days of Exposure	Mean (mg/l)	±SEM	Percentage increase (+) or decrease (-) over control
Control (Group I)	-	16.48	0.96	-
Group II (1.0 µl/L butachlor treated)	5	14.87 ^{NS}	0.944	(-)9.769%
Group III (1.0 µl/L butachlor treated)	10	22.88 ^{**}	0.99	(+) 38.83%
Group IV (1.0 µl/L butachlor treated)	15	15.76 [*]	0.74	(-) 4.36%

For each parameter values are expressed as mean ±SEM of 6 replicates (n=6). All the treated groups were compared to control groups. Significance level is marked as *= $p < 0.05$, **= $P < 0.01$, NS= not significant.

Table 2. Estimation of serum Lipid peroxidase activity in normal and butachlor exposed test fish

Groups	Days of Exposure	Mean (mg/l)	±SEM	Percentage increase(+) or decrease(-) over control
Control (Group I)	-	0.927	0.44	-
Group II (1.0 µl/L butachlor treated)	5	1.093*	0.315	(+) 17.90%
Group III (1.0 µl/L butachlor treated)	10	1.53**	0.50	(+) 65.04%
Group IV (1.0 µl/L butachlor treated)	15	2.16**	0.686	(+) 133.0%

For each parameter values are expressed as mean ±SEM of 6 replicates (n=6). All the treated groups were compared to control groups. Significance level is marked as *= $p < 0.05$, **= $P < 0.01$, NS= not significant.

The level of serum LPO in control and different experimental groups were shown in Table 2. Serum LPO in control fish was determined as 0.927 ± 0.44 mg/ml and it showed a consistent increasing trend in all the treated groups.

4. Discussion

The present experiment emphasizes on the deleterious impacts of butachlor on the oxidative biomarkers and its correlation with the histopathological changes in the hepatic tissues in fresh water air breathing fish *Clarias batrachus*. Xenobiotics impose some sort of stress in fish leading to few stress responses at repercussions. In the present study, blood GSH level showed a marked fluctuation in butachlor treated fish when compared with control. In due course of detoxification mechanism, GSH is converted to GSSG to neutralize the free radical species. The decreased level of GSH (9.769% over the control) after 5 days exposure clearly reveals that earlier accumulated GSH get exhausted in counteracting the free radicals generated by the pesticide. The results are similar to Jin *et al.*¹⁵ who also reported a consistent decline in GSH level in adult female zebra fish after atrazine exposure. A sharp increase of 22.88% over the control in 10 days butachlor treated fish indicates that the fish immune system counter balances the oxidative stress but a sharp decline of 4.36% over the control after 15 days exposure suggests that the fish antioxidant system fails to counteract the free radicals generated after prolonged exposure of butachlor.

A consistent significant increase in serum LPO level of treated fish has been marked at all the durations of butachlor exposure when compared with the control group. The serum

LPO showed a consistent increase of 17.90%, 65.04% and 133.0% over the control in group II, III and IV respectively. The results of the present study coincide with the findings of Xing *et al.*¹⁶ who also reported a sharp increase in hepatic tissues LPO content with respect to control groups. Butachlor exposure induces the level of MDA as implications of ROS generation that enhances the oxidation of PUFA leading to lipid peroxidation¹⁷. Lipid peroxidation induced by free radical generation has been considered as principal toxico-kinetics of sub chronic toxicity of malathion in blood and liver of rats¹⁸. Biochemical impact of some pesticides on lipid peroxidation and free radical scavengers were also reported by Banerjee *et al.*¹⁹.

Results of the present investigation show a direct correlation amongst duration of exposure, level of toxicity and degeneration in hepatic tissues. Butachlor leads to prominent alterations in liver cells of *Clarias batrachus*. In the present study, a close examination of the hepatic cells of fish treated with 1.0µl/l butachlor for 5 days showed widening of central vein, swelling of hepatic parenchyma, increased occurrence of vacuoles and pyknotic nucleus, which is further increased after 10 days exposure. Infiltration of eosinophilic inclusions and massive congestion in the portal vein and bile duct, portal tract with mild periportal fibrosis, necrosis, increased nuclear pyknosis and vacuoles *etc.* contribute to the parenchymal deformities at longer duration of butachlor exposure. Similar kind of cellular alterations in the liver of *Clarias batrachus* have been reported earlier^{8,20-23}. Hameeda *et al.*²⁴ have evaluated similar impact of endomethacin, an anti-inflammatory drug on the liver of mice. Joshi *et al.*²⁵ also reported abnormal changes in liver like vacuole formation, necrosis and fibrosis in *Heteropneustes fossilis* exposed to cypermethrin. The appearance of inflammations in the present study after 15 days of butachlor exposure results in movement of fluid and leucocytes from blood circulation to tissues^{26,27}. Moreover, the hepatic necrosis and the loss of parenchymal cell membrane may be a consequence of increased oxidative stress in the liver due to butachlor exposure in fish. Cells of various tissues are consistently being protected from xenobiotic induced oxidative injuries by extracellular GSH. Besides it constitutes a major transportable route of cystein amino acid within the cells. Being one of the dominant intracellular thiol, GSH play a significant role in the cellular defense against any kind of oxidative injury²⁸. Various metabolizing enzymes produce a series of electrophiles which are implicated in several immunological disorders in vertebrates. Basically neutralization of such electrophilic xenobiotics takes place by conjugating itself with GSH. GSH plays a significant role in the initiation and progression of lymphocyte activation which is one of the essential requisites for mounting active immunity. GSH is critical for the function of natural killer cells and for

lymphocyte-mediated cytotoxicity, as it has been shown that depletion of intracellular GSH inhibits lymphocyte activation by mitogens²⁹. A significant decrease in GSH level was seen on 10th day and 15th day which is consistent with the findings of Soliman *et al.*³⁰ who studied the effect of abamectin on liver function and lipid peroxidation, and reported decline in blood GSH at both high and low doses of abamectin. A considerable decline in blood GSH in the present study can be correlated with the fact that most of the GSH got depleted in scavenging free radical, resulting in their conversion to the oxidized form GSSG³¹.

5. Conclusions

In conclusion, the present study indicated that butachlor at sub-lethal levels under laboratory condition has the capacity to alter the normal physiological function of cat fish *C. batrachus*. It also increased oxidative stress in fish and the later failed to counterbalance the generated reactive free radicals leading to corresponding damages in the cyto-architecture of their tissues. The liver of butachlor treated fish ultimately ends with impaired metabolism. Reduced glutathione and lipid peroxidase activity may serve as monitoring tool as oxidative stress biomarkers and histopathology of fish liver allows the assessment of extent of environmental stressor effect on fish.

6. Acknowledgements

Authors are thankful to (WOS-A) Scheme (No-SR/WOS-A/LS-77/2012) SERC Division, DST, New Delhi for financial assistance and Department of Zoology for providing infrastructural facilities.

7. References

- Ateeq B, Farah M, Ali M, Ahmad W. Induction of micronuclei and erythrocyte alteration in catfish *Clarias batrachus* by 2, 4-dichlorophenoxy acetic acid and Butachlor. *Mutation Research*. 2002; 518:135-144. [https://doi.org/10.1016/S1383-5718\(02\)00075-X](https://doi.org/10.1016/S1383-5718(02)00075-X).
- Peebua P, Kosiyachinda P, Pokethitiyook K. Evaluation of alachlor herbicide impacts on Nile tilapia (*Oreochromis niloticus*) using biochemical biomarkers. *Bulletin of Environ. Conta. and Toxicol.* 2007; 78(2):138-141. <https://doi.org/10.1007/s00128-007-9027-8>. PMID:17435964.
- Geng J, Nair U, Yorimitsu KY. Golgi secretory proteins are required for autophagy in *Saccharomyces cerevisiae*. *Mol. Biol. Cell*. 2010; 21(13):2257-2269. <https://doi.org/10.1091/mbc.e09-11-0969>. PMID:20444978 PMCID:PMC2893989.
- Ou YH, Chung PC, Chang YC, Nago FQ, Hsu KY, Chen FD. Butachlor, a suspected carcinogen, alters growth and transformation characteristics of mouse liver cells. *Chem. Res. Toxicol.* 2000; 13:1321-1325. <https://doi.org/10.1021/tx990076c>. PMID:11123974.
- Chang J, Wang M, Gui W, Zhao Y, Yu L, Zhu G. Changes in Thyroid hormone levels during zebra fish development. *Zoolog. Sci.* 2012; 29:181-184. <https://doi.org/10.2108/zsj.29.181>. PMID:22379985.
- Geng BR, Yao D, Xue QQ. Acute toxicity of the pesticide dichlorvos and herbicide butachlor to the tadpoles of four anuran species. *Bull. Environ. Conta. Toxicol.* 2005; 75:343-349. <https://doi.org/10.1007/s00128-005-0759-z>. PMID:16222508.
- Farombi EO, Ajimoko YR, Adelewo OA. Effect of Butachlor on antioxidant enzyme status and lipid peroxidation in fresh water catfish (*Clarias gariepinus*). *International Journal of Environmental Research and Public Health*. 2008; 5(5):423-427. <https://doi.org/10.3390/ijerph5050423>. PMID:19151438 PMCID:PMC3700003.
- Verma P, Chand GB. Oxidative stress response, hormone imbalance and gonado-toxicity in *Clarias. batrachus* (Linn.) exposed to Butachlor. *Recent Life Science Mirror*. 2014; 3(1&2):23-28.
- Verma P, Chand GB. Butachlor induced oxidative imbalance correlated with gonadal toxicity in fish: In vivo Enzymatic and Ultrastructural study. *World Journal of Pharmaceutical Research*. 2015; 4(8):2327-2340.
- Peixoto Francisco P, Carrola Joao, Coimbra Ana Maria, Fernandes Conceicao, Trixeira Paulo, Coelho Luies, Conceicao Ivo, Oliveira Maria Manuel, Fernandes Antonio Fontainhas. Oxidative stress responses and histological hepatic alterations in Barbel, *Barbus bocagai* from Vigela River Portugal. *Revista Internacional de Contaminacion Ambiental*. 2013; 29(1):29-38.
- Ishizuka M, Iwata H, Kazusaka A, Hatakeyama S, Fujita S. Effects of the agrochemicals Butachlor, pretilachlor and isoprothiolane on rat liver xenobiotic-metabolizing enzymes. *Xenobiotica*. 1998; 28(11):1029-1039.
- Meister A, Anderson ME. Glutathione. *Ann. Rev. Biochem.* 1983; 52:711-760. <https://doi.org/10.1146/annurev.bi.52.070183.003431>. PMID:6137189.
- Numan L, Hassan M, Stohs S. Endosulfan induced depletion of glutathione and inhibition of glutathione peroxidase activity in rats. *Gen. Pharmacol.* 1990; 21(5):625-628. [https://doi.org/10.1016/0306-3623\(90\)91008-F](https://doi.org/10.1016/0306-3623(90)91008-F).
- APHA. Standard methods of examination of water and waste water 21sted. APHA, AWWA and WPCF, American Public Health Association, Washington DC.US; 2005.
- Jin Y, S Zheng, Y Pu, L Shu, L Sun, W Liu, Z Fu. Cypermethrin has the potential to induce hepatic oxidative stress, DNA damage and apoptosis in adult zebrafish (*Danio rerio*). *Chemosphere*. 2011; 82:398-404. <https://doi.org/10.1016/j.chemosphere.2010.09.072>. PMID:20965546.
- Xing H, Wang X, Sun G, Gao X, Xu S, Wang XL. Effects of atrazine and chlorpyrifos on activity and transcription of glutathione S-transferase in common carp (*Cyprinus carpio* L.). *Environmental Toxicology and Pharmacology*. 2012; 33(2):233-244. <https://doi.org/10.1016/j.etap.2011.12.014>. PMID:22236720.
- Liu X, Hua X, Guo J, Qi D, Wang L, Liu Z. Enhanced tolerance to drought stress in transgenic tobacco plants over expressing VTE1

- for increased tocopherol production from *Arabidopsis thaliana*. *Biotechnol. Lett.* 2008; 30:1275-1280. <https://doi.org/10.1007/s10529-008-9672-y>. PMID:18317702.
18. Akhgari M, Abdollahi M, Kebryaezadeh A, Hosseini R, Sabzevari O. Biochemical evidence for free radical induced lipid peroxidation as a mechanism for subchronic toxicity of malathion in blood and liver of rats. *Human Experimental Toxicology.* 2003; 22:205-211. <https://doi.org/10.1191/0960327103ht346oa>. PMID:12755471.
 19. Banerjee B, Seth V, Bhattacharya A, Pasha S, Chakraborty A. Biochemical effects of some pesticides on lipid peroxidation and free radical scavengers. *Toxicology Letters.* 1999; 107:33-47. [https://doi.org/10.1016/S0378-4274\(99\)00029-6](https://doi.org/10.1016/S0378-4274(99)00029-6).
 20. Hjeltnes Brit, Samuelsen Ole bent, S Vardal Asbjorn Martin. Changes in plasma and liver glutathione levels in Allantic Salmon *Salmo salar* suffering from infections salmon anemia (ISA). 1992; 14:31-33. https://agris.fao.org/agris-search/search.do;jsessionid=5C3DCE660C205ED524541513679618C8?request_locale=es&recordID=AV2012065888&sourceQuery=&query=&sortField=&sortOrder=&agrovocString=&advQuery=¢erString=&enableField=&aggregatorField=. <https://doi.org/10.3354/dao014031>.
 21. Huxley PM. Mode of carcinogenic action of pesticide inducing thyroid follicular cell tumors in rodents. *Environ. Health Perspect.* 1998; 106(8):783-798.
 22. Tripathi G, Verma P. Endosulfan-mediated biochemical changes in the fresh water fish *Clarias batrachus*. *Biomedical Environmental Science.* 2004; 17:47-56.
 23. Fontainhas A, Peixoto FP, Carrola J, Coimbra AM, Fernandes C, Teixeira P, Coelho L. Oxidative stress responses and histological hepatic alterations in Barbel, *Barbus bocagai* from Vigela River Portugal. *Revista Internacional de Contaminacion Ambiental.* 2013; 29(1):29-38.
 24. Hemieda FAE, Abdel H. Physiological and Histopathological changes in male rats treated with indomethacin. *E.S.K.J. Egypt. Ger. Soc. Zool.* 1998; 26(C).
 25. Joshi N Dharmalata, Sahu AP. Histopathological changes in liver of *Heteropneustes fossilis* exposed to cypermethrin. *J. Environ. Biol.* 2007; 28:35-37.
 26. Roberts RJ. *Fish Pathology*, 3rd Edition. W.B. Saunders, Philadelphia; 2001. p. 367.
 27. Rubin GP, Hungin AP, Kelly PJ, Ling J. Inflammatory bowel disease: Epidemiology and management in an English general practice population. *Alimentary Pharmacology and Therapeutics.* 2000; 14(12):1553-59. <https://doi.org/10.1046/j.1365-2036.2000.00886.x>. PMID:11121902.
 28. Chan yu-jian, Ming Jiang Zhu Min-li De. Glutamine: A precursor of glutathione and its effect on liver. *World J. Gastroenterol.* 1999; 155(2):143-146. <https://doi.org/10.3748/wjg.v5.i2.143>. PMID:11819414 PMCid:PMC4688527.
 29. Darnell JH, Lodish, Baltimore *Molecular Cell Biology*, Sci. Am Books, New York, USA; 2000.
 30. Soliman EM, Mahmoud ME, Ahmad SA. Reactivity of thioglycolic acid physically and chemically bound to silica gel surface a new selective solid phase extracts for removal of heavy metals ions from natural water sample. *Int. J. Anal. Chem.* 2009; 82. <https://doi.org/10.1080/03067310290007831>.
 31. Bamela CC, Richard AH. *Libbincott's Illustrated Reviews Biochemistry.* 2nd ed., Washington, Philadelphia, Pennsylvania; 1994. p.113-114.