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BUTACHLOR INDUCED SERUM BIOCHEMICAL ANOMALIES CORRELATED WITH HISTOPATHOLOGICAL CHANGES IN INTESTINAL TISSUE OF *Clarias batrachus* (LINN.): *In-vivo* STUDY

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AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration among all authors. Author GBC prepared the final draft. Author PV did the histopathological study. Author PS performed the statistical analysis and author SP managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

The present study aimed to investigate the adverse effects of butachlor (2-chloro-2', 6'-diethyl-N -butoxymethyl) acetanilide on serum total protein and glucose level and histo-pathological changes in small intestine especially proximal part (duodenum) of walking catfish *Clarias batrachus* (Linn.). 96 hour LC_{50} of butachlor for the fish was determined by the probit regression and then the experimental fishes were exposed to sub lethal dose (1.5µl/ L i.e. $1/36^{th}$ of LC_{50}) for 7, 14 and 21 days respectively. Blood samples were collected in heparinised syringe by puncturing the caudal vein and the serum was extracted as per standard protocol. The proximal part of intestine was dissected out and fixed in aqueous Bouin's fixative. Paraffin spread sections were double stained in haemotoxylin and eosine, mounted in DPX and viewed under Olympus 2000 trinocular compound microscope. Light microphotographs were taken in canon ISUS 130X digital camera. Serum total protein and glucose estimation was done as per standard protocol. The LM study of intestinal tissues of treated fish showed degeneration in mucosa, sub-mucosa and muscularis mucosa region of duodenum in comparison to that of control fish. After 7 and 14 days exposure of butachlor the degeneration were prominent in tunica mucosa and sub mucosa layer as marked by erosion of brush borders of villi and basal lamina, hypertrophy of goblet cells, appearance of large number of vacuoles in sub mucosa region and migration of lymphoid tissues in the lamina proporia. Exposure of butachlor for longer duration of 28 days resulted in widening of luminal region, necrosis of goblet cells, roblet cells as well as entero-endocrine cells. Besides, extensive haemorrhages

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and congested absorptive region were prominently marked. Biochemical results of treated fish showed significant decrease in serum total protein and abnormal fluctuation in glucose level in contrast to control. A significant ($p<0.05$) consistent decline in serum protein can be correlated with the diminished absorption rate of protein by the altered intestinal enterocytes under butachlor stress. Hence elevated serum protein level correlated with histopathological alteration in the proximal intestine of fish may be considered as bio-indicator of polluted aquatic bodies.

Keywords: Butachlor; *Clarias batrachus*; glucose level; histopathology; small intestine; total protein.

1. INTRODUCTION

Teleosts have successfully adapted themselves to every type of aquatic habitats and constitute an important source of food supplement to the human kind [1]. With the advent of aquaculture sector as industry, employment in fisheries and aquaculture has grown substantially in the last three decades. World aquaculture is dominated by Asia pacific region, which accounts for 89% of the global aquaculture production in terms of quality and 79% in terms of value [2]. The health of fish may be affected by various factors such as aquatic pollutants [3,4,5], nutrition [1] pathogens [6] as well. These xenobiotic factors led to alteration in the serum biochemistry, hormonal metabolism and histology, causing severe pathological implications in fish [7]. Non judicious use of agrochemicals and related aquatic pollution is a regional as well as important global issue [8]. Fishes are either directly exposed to hazardous chemicals resulting from agricultural production via surface runoff or indirectly through the food chain of ecosystem. Hence they can be used as indicator for pollution status of the water bodies.

Like other vertebrates, fish intestine is also a digestive organ responsible for digestion and absorption of dietary nutrients [1]. The digestive process which starts in the stomach gets completed in the fish intestine and the absorption of all nutrients takes place here. Intestinal length is used as a morphological indicator of trophic level in nutritional ecology. Study of anatomical and histological characteristics of fish intestine are expected to be helpful for understanding the digestive and absorptive mechanism related with different feeding habits. It can further be helpful for diagnosing some intestinal diseases and formulating suitable feeds. Intense use of modern agrochemical has generated a lot of stress to the aquatic life. They may affect the normal physiology of fish, resulting in a number of disorders in vital system of their body [9-11].

Butachlor is an herbicide of actanilide class. It is extensively use in India in the form of granules in rice as post emergence herbicide [12]. In spite of known deleterious impact butachlor is consistently being used in paddy fields in Bihar, posing a serious threat to the survival of fishes there. Most of the literatures related to the deleterious impact of butachlor on fish are associated with reproduction and sex steroids [13,14], growth and development [15,16] and genotoxicity [17,18]. In spite of the wide spectrum use of butachlor to the agricultural lands and the notable eco-toxicological impacts associated with its use, there is paucity of information on its impact on the intestinal tissues of air breathing teleosts [1,5].

The present research work was aimed to study the histo-pathological changes induced by an herbicide butachlor on the intestinal tissues of air breathing fish *Clarias batrachus*. To assess any probable biochemical alterations triggered by butachlor in fish, the changes in the serum total protein and glucose were also determined.

2. MATERIALS AND METHODS

2.1 Experimental Animal

Walking catfish *Clarias batrachus* (commonly called Magur) were used in this study. Live and healthy specimen of these animals was collected from different wetlands of North Bihar, India. The standard length and weight of fish were in the range of 18±2 cm and 50±10 gm, respectively. They were brought to the Aquatic Toxicology Laboratory, Department of Zoology, Patna University, Patna, disinfected with 0.01% $KMnO_4$ solution and kept in different sized large plexi glass aquaria. Fishes were acclimated for two week in the laboratory condition before the beginning of the experiment. To maintain normal water temperature, cooler and exhaust were used around the aquarium. The aerated water was changed daily. The animals were fed *ad libitum* daily with feed pellets (mixture of wheat flour + egg + starch as binder) @5% of their body weight.

2.2 Experimental Pesticide

Commercially brand butachlor, (EC50%), manufactured by SEGNOTECH Agro Pvt. Ltd. Lucknow (U.P.) was used in this study. The herbicide was purchased from local supplier. LC_{50} for 96 hours of butachlor for fish was determined as per standard protocol [19] and recorded as 4.2 µl/L. Acclimatized

fishes were divided into four groups and kept in separate aquarium @ six fishes / group. The first group (C) was used as control, while the other three groups (GI, GII and GIII) were considered as treated groups. Accordingly, stock solution was prepared using distilled water, and then fishes were treated @ 1.5µl/ L of butachlor for 7, 14 and 21 days respectively by emersion method. For this 60 µl of butachlor was added to the aquarium of 40 L capacity and mixed thoroughly. The herbicides get their entry in fish body primarily through gills. Another set of six fishes were simultaneously maintained in separate aquarium containing dechlorinated tap water and considered as control. The experiment was set in triplicate. During the entire experimental tenure, the ideal physico-chemical conditions of water were maintained and the water was changed daily. The fishes in the treated aquaria as well as control aquaria were observed for at least two hours after the herbicide was introduced. After schedule exposure period, the specimens were removed from both the treated and control aquaria, anaesthetized with MS222 and were sacrificed.

2.3 Biochemical Investigations

For biochemical analysis, blood sample of both control as well as experimental groups were collected in a heparinized glass culture tube by a puncture in caudal vein, left for 20 minutes and centrifuged at 3000 rpm for 15 minutes for three successive intervals. Clear supernatant serum was decanted in a clean dry vial and stored in deep fridge at -20˚C for biochemical analysis. All the biochemical analysis was done on BT 260 Plus-Semi Automatic Analyser. It is a product of RMD mediads LTD with specifications as- product category: *In vitro* diagnostics; wave length precision ±2 nm; width <=10 nm; absorbance range:0-4.500Abs; carry over<1%; compatibility- Real time curve of 7 wave length; 80 test items pre- programmed; standard filters-340, 405, 492, 510, 546, 578, 630 nm; memory for 10000 samples results; RS232 interface, PC connecting, test mode- kinetic, end point-two point absorbance.

2.3.1 Total protein estimation

It was done by Biuret method [20,21], which is based on the reaction between the peptide bonds of protein and copper (divalent) that produces a blue violet coloured complex in alkaline solution. The intensity of the blue colour is proportional to the protein concentration present in the sample.

2.3.2 Glucose estimation

The glucose estimation was done by GOD-POD method [22], in which glucose is oxidized to gluconoic acid and hydrogen peroxide by the enzyme glucose oxidase. The formed hydrogen peroxide is detected by a chromogenic oxygen acceptor Phenol, 4 amino phenazone (4-AP) in the presence of peroxidise enzyme and produces red colour compound. The intensity of red colour produced is proportional to glucose concentration and is stable for two hours. The colour was measured at 505 nm.

2.4 Histological Investigations

Proximal intestinal tissues of both control and treated groups were carefully removed, cut into small pieces of approximately 5 mm in size with sharp surgical blades, fixed in neutral formalin and embedded in paraffin wax (melting point 57.8^0C). The paraffin spread sections were double stained with haemotoxylin and eosine and mounted in DPX. The permanent double stained sections were viewed under Olympus 2000 tri-nocular compound microscope and microphotographs were taken on Canon ISUS 130X digital camera.

2.5 Statistical Analysis

The data obtained (Mean ± SD) were subjected to Fisher's t-test for the difference between two mean of independent sample. It is further subjected to regression analysis. 5% level of significance (P=0.05) was used to analyse the data. All the statistical analyses were on SPSS (Version 16).

3. RESULTS

3.1 Biochemical Observations

Serum glucose concentration initially declined by 5.82% over the control within 7 days of exposure, but showed a characteristic increase of 25.03% and 4.23% over the control in 14 days and 21 days treated groups respectively (Table 1).

The serum total protein level showed a decreasing trend in all the treated groups. A decrease of 33.46%, 1.46% and 20.41% over the control were marked in 7 days, 14 days and 21 days treated groups respectively (Table 2).

3.2 Histological Observations

The light photomicrographs of proximal (Duodenal) part of intestinal cell of control *C. batrachus* showed basic histology of intestinal wall, consisting of four distinct layers- tunica mucosa, sub mucosa, muscularis mucosa containing longitudinal and circular layers and serosa layers. The tunica mucosa consisted of a mucosal epithelium overlying a layer of

highly vascularised loose connective tissue or lamina propria containing nerves and leukocytes (Figs. 1 and 2). Villi were finger like projections extending into the intestinal lumen, showing decreasing length towards the posterior intestine. They were lined by single layered tall columnar epithelium with basal nucleus and nucleolus (Figs. 1 and 2). Besides, an apical brush border and acidophilic cytoplasm intermingled with intestinal glands, enterocytes and Brunner's glands having distinct Goblet cells and lymphocytes were also clearly marked. The goblet cells are dominant mucous cell types in the intestine of fish were marked by a swollen distal region containing a translucent cytoplasm having mucin like granules (Figs. 1 and 2).

Table 1. Estimation of serum glucose level in normal and butachlor treated fish

Groups	Days of exposure	Mean (mg/l)	SD	Percentage increase (+) or decrease (-) over control
Control (C)	-	238.131	0.280	-
1.5 µl/L butachlor treated (GI,GII,GIII)	7	214.271*	0.196	(-) 5.82%
	14	297.755**	0.585	(+) 25.06%
	21	247.92*	0.850	(+) 4.23 %

*The values are expressed in Mean ± SD of six replicate (n=6) in each case. Paired 't' test have been applied between Group C & Group I, Group C & Group II, Group C & Group III * = significant at P < 0.05** = significant at P < 0.01*

Table 2. Estimation of serum protein level in normal and butachlor treated fish

Groups	Days of exposure	Mean mg/l	SD	Percentage increase (+) or decrease (-) over control
Control(C)	-	6.069	0.126	-
1.5 µl/L butachlor treated (GI,GII,GIII)	7	4.038**	0.004	(-) 33%
	14	5.98*	0.03	(-) 1.46%
	21	4.83**	0.018	(-) 20.41%

*The values are expressed in Mean ± SD of six replicate (n=6) in each case. Paired 't' test have been applied between Group C & Group II, Group C & Group II, Group C & Group III * = significant at P < 0.05** = significant at P < 0.01*

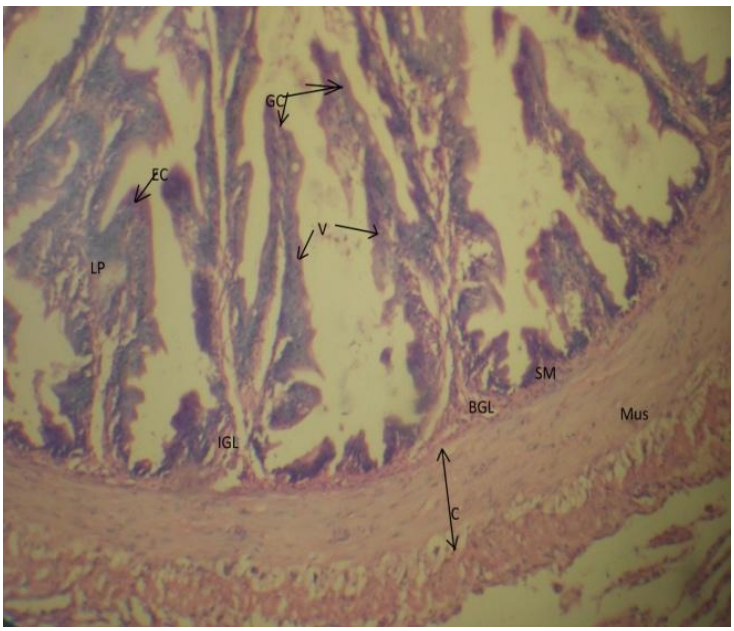

Fig. 1. Portion of duodenum of normal fish stained with haematoxylin and eosine showing distinct mucosa (M), submucosa (SM), muscularis mucosa (Mus), serosa (Ser) & villi (V). The distinct goblet cell (GC), burnner's gland (BGL), intestinal gland (IGL), lamina propria (LP), enterocytes (EC) etc. are marked. X 200

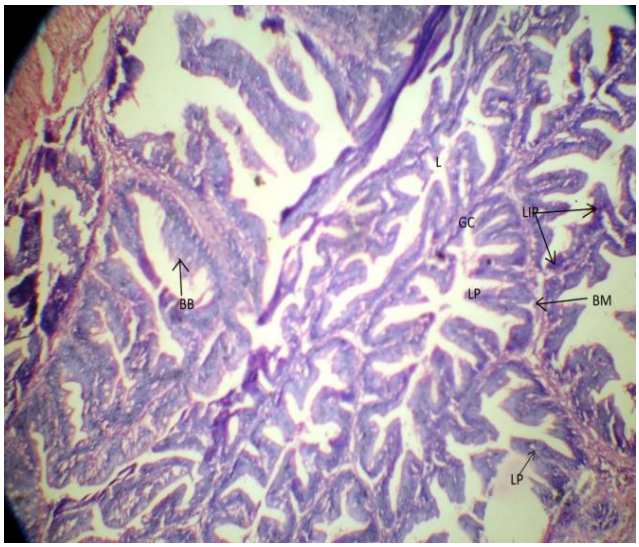

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Fig. 2. Showing distinct normal lumen (L), lamina proporia (LP), Basal lamina (BL), Goblet cells (GC), lipid droplets (LIP) & brush border (BB) X 400

The portion of intestine of Group I (1.5µl/L butachlor treated for 7 days) fishes showed extensive vacuoles in the sub mucosal region and invasion of lymphocytes in the absorptive region of the intestine. An increase in the number of goblet cells and haemorrhagic patches were prominently marked (Figs. 3 and 4).

The intestinal cells of group II (1.5µl/L butachlor treated for 14 days) fishes exhibited increased number of Goblet cells and disrupted basal lamina (Fig. 5). A massive degeneration in brush border area ruptured lamina proporia, high degree of lymphocytic invasion in the mucosa layer, marked erosion of brush border of villi, disrupted basal lamina and appearance of large number of vacuoles in sub-mucosal and muscularis layer etc. were prominent abnormalities incurred in the intestinal tissues of 14 days butachlor treated fish (Fig. 6).

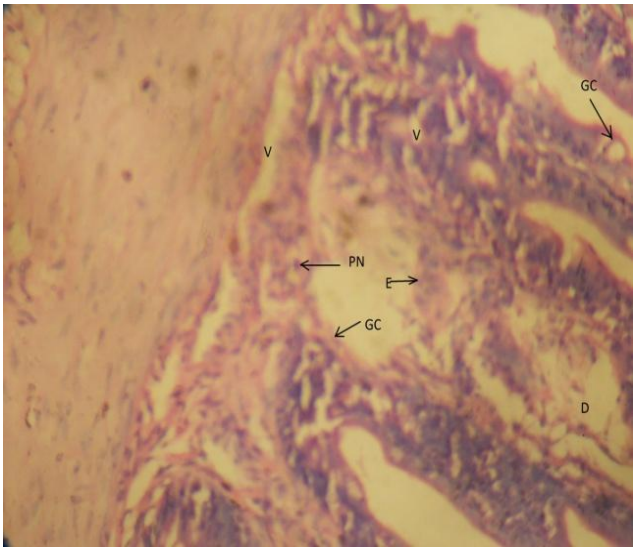

Fig. 3. 7 day butachlor exposed group, showing muscularis mucosa (M), extensive vacuolation in the sub mucosal region (SMR), highly degenerated lamina propria(LP), Increased no. of goblet cells(GC) with pycnotic nuclei (PN). The junction gap between two cells is widened and lumen is congested with invaded lymphocytes X 200

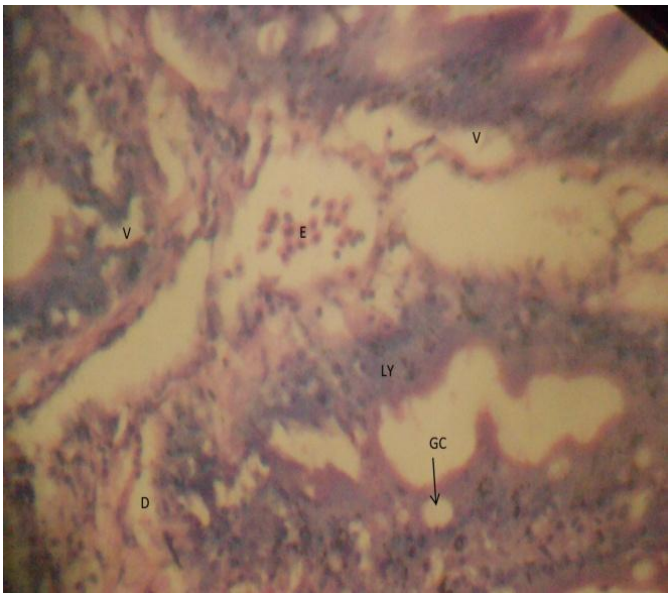

Fig. 4. Magnified view of a portion of submuscularis & muscularis portion of duodenum showing signs of haemorrhages in the lamina proporia (LP) region, hypertrophy of Goblet cells (GC), extensive vacuoles in the submucosa region and degeneration of intestinal gland X 400

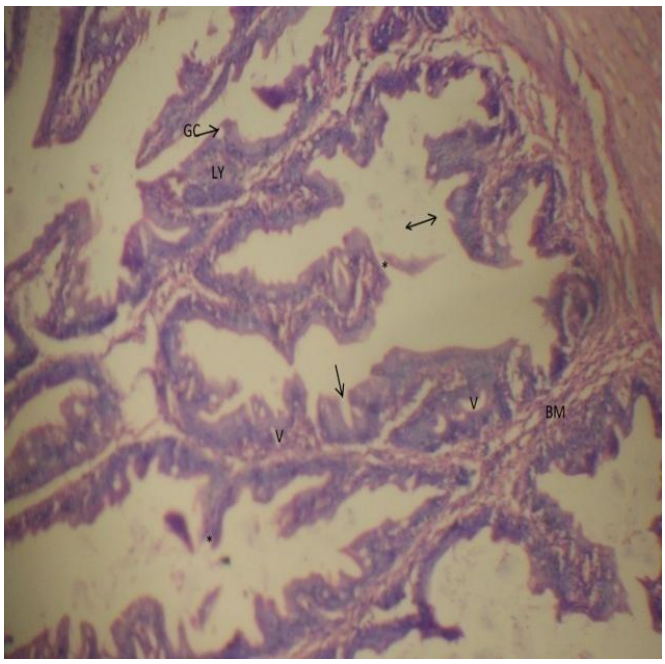

Fig. 5. In 14 days butachlor treated group M & SM portion of duodenum showing high infiltration of lynphocytes, erosion of BB villi, and increased no. of GC, disrupted Basal lamina (BL), vacuolation in enterocytes, and wider lumen (L) than the normal. X 200

After prolonged dose of butachlor (1.5µl/L, 21days) treatment in group III fishes intestinal cells showed severe degeneration of enterocytes and apical brush border along villi (Fig. 7). This degeneration was manifested within prevailing haemorrhage and infiltration of macrophage. A large numbers of hypertrophied goblet cells were also observed at luminal area (Fig. 8).

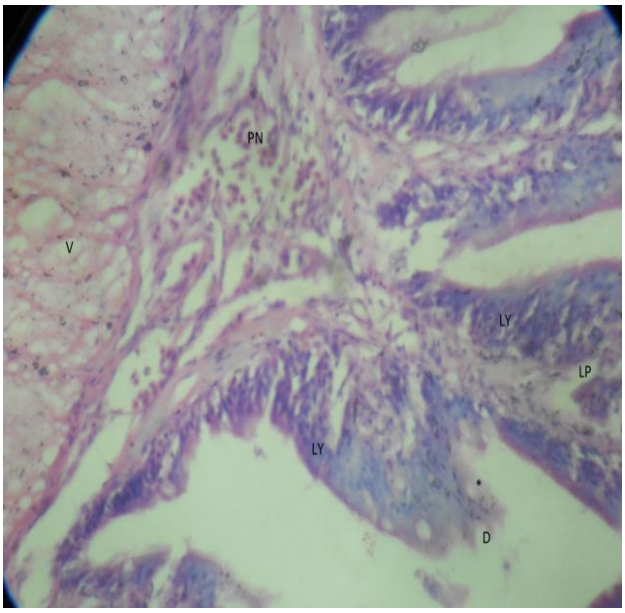

Fig. 6. Magnified view of muscularis, sub muscularis and mucosa region showing extensive vacuoles, pycnotic clumps of lymphocyte, degenerated BB area and ruptured Lamina propria (LP)in muscularis and sub mucosa region X400

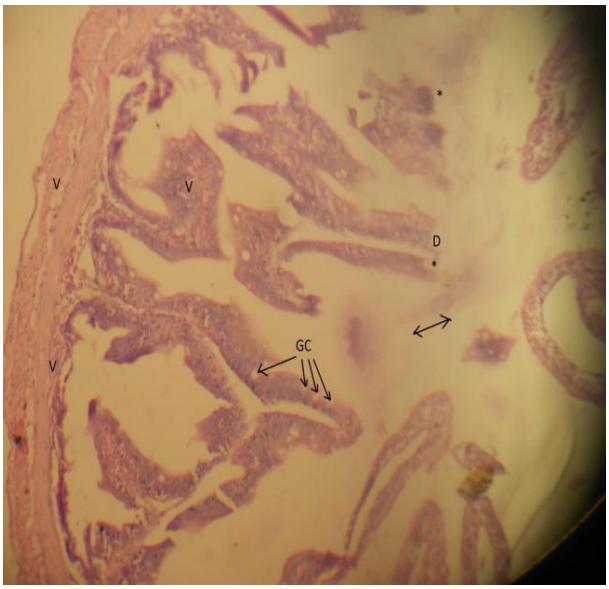

Fig. 7. In 21 days butachlor treated group, muscularis, sub mucosa, mucosa region showing extensive degeneration of enterocytes & BB. A complete erosion of some part of microvilli and large no. of GC is observed, vacuolation is extensive. Lumen area is highly widened. X 200

4. DISCUSSION

Although fishes exhibit a wide variation in the histological details of the intestine based upon their feeding habits [23] but the basic structural plan for the histological details of the digestive tract remains the same [24]. The intestine of air breathing fish *Clarias batrachus*, is composed of basic four layers usually described for vertebrates [25], namely tunica mucosa, sub-mucosa, muscularis mucosa and serosa [26, 27,28].

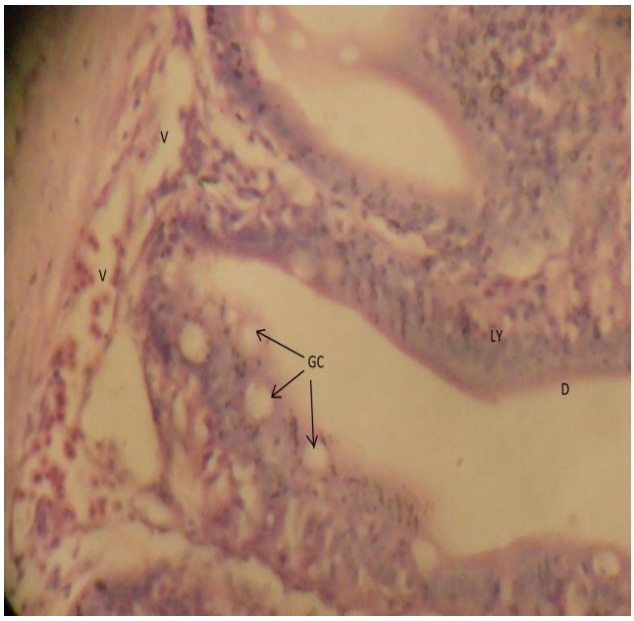

Fig. 8. Magnified view of sub mucosa and mucosa region of duodenum showing extensive vacuoles in submucosal area hypertrophied GC, lymphocytic invasion. Villi regionis marked by complete absence of lipid droplet and degeneration of enterocytes X 400

The tunica mucosa in teleosts comprises of mucosal epithelium overlying a layer of loose connective tissue or highly vascularised lamina proporia containing nerves and leucocytes [29]. The lamina proporia includes stratum compactum of dense connective tissue and stratum granulosum having granular eosinophilic cells [29]. These granular eosinophilic cells are considered to be homologous with the mammalian mast cells and may be involved in mediating inflammatory reactions [30]. Tunica mucosa layer is followed by tunica sub mucosa containing blood vessels , lymphatic tissues and nerve plexi [29]. The tunica muscularis consists of inner circular and outer longitudinal layero0f striated or smooth muscles. In between these two, Aurbech's plexi resides [29]. The tunica serosa is present only in the coelomic cavity and corresponds to mesothelial cells and loose connective tissues containing blood vessels [31].

The intestinal epithelial cells are basically simple or pseudo-stratified and consist of columnar absorptive cells or enterocytes, having a well marked apical free brush border and villi, goblet mucous cells, lymphocytes, rodlet cells and entero-endocrine cells [32,33]. Enterocytes are tall and narrow, having elongated nuclei, mitochondria located in apical and basal regions, a well developed brush borders and lamellar structure parallel to the lateral plasma membrane [1]. Enterocytes express Na^+K^+ ATPase pump, which is essential in driving nutrient uptake and ion regulation [31]. The villi and brush borders contributing up to 90% of total intestinal surface area [34], forms the critical digestive/ absorptive interface and serves as seat for absorption and transport. It provides a functional macro-environment of enzymes like ALP, disaccharidase, amino-peptidases [35]. The intestinal goblet cells in the teleost synthesize neutral and sialo-mucin which lubricates undigested debris for onward progression into the rectum [36,37]. Bozic et al. [38] have observed that starvation induced increase in the number of intestinal goblet cells in carp. The mucus produced by the fish goblet cells protects the mucosa of digestive tract. Sialic acid in fish mucus interferes with the receptor detection by the viruses and thereby protects the mucosa against pathogenic infections [39]. The rodlet cells are ovoid having a basally located nucleus, thick fibrous layer beneath the plasma membrane and elongated rod like cytoplasmic granules. They are mostly involved in electrolyte and water regulation, pH control, lubrication and host defence mechanism in fish against parasitic infection [40]. Amongst different intestinal epithelial cells, a few entero-endocrine cells are present in teleost intestine considered to be as brunner's gland. These cells bear dense core vesicles in their cytoplasm. They may be differentiated on the basis of shape, size and electron density of dense core vesicles and their expression of neuro endocrine substance by immune-histochemistry [41,31]. Here in the experiment, butachlor exposure induces numerous histo-pathological alterations including massive

degeneration in the muscularis mucosa and submucosa of fish intestinal epithelium. These degenerations were more evident in the GIII fishes. These changes were manifested in increased populations of goblet cells, formation of pycnotic nuclei, invasion of lymphocytes and increased haemorrhagic patches in mucosa and sub mucosa region of the duodenum. Increased population of goblet cells in the distal intestine are in accordance with the reports of Cinar and Senols in flower fish *Pseudophoxinus antalyae* [27]. The increased population of goblet cells may imply the need for increased mucosa protection and lubrication for faecal expulsion [42].

Similar hypertrophy of goblet cells, intestinal gland degeneration, and erosion of brush border of villi, basal lamina disruption, necrosis, widening of luminal region were noticed earlier in pesticide treated intestine of fish [43,44]. It has been suggested that the starvation induces an increase in the number of intestinal goblet cells in Carp [38]. In the present study appearance of a large number of goblet cell at longer duration exposure confirmed the starvation condition of fish due to lack of absorption.

The findings of the present results highlighted significant reduction of the protein level due to butachlor exposure. The concentration of the fish serum total protein is an index of the general health condition of the fish. Increased energy demand increases protein consumption and ultimately led to reduction in the level of protein. Ritter [45] reported that the cell necrosis could be either due to progressive degenerative action of intracellular enzyme of the injured cell or to a metabolic disturbance and inhibition of synthesis for the growth and maturation of the cells. Verma and Chand [4] and Verma et al. [46] reported significant decreases in the protein, carbohydrate and lipid content of muscle of *Clarias batrachus* and *Heteropneustes fossilis* at different concentration of endosulfan and rogor exposure.

Furthermore, increase in glucose level in butachlor exposed fish may also be attributed to degranulation and vacuolization of the pancreatic alpha cells in the initial stages and damage of beta cells in later stages. Significant increase of serum glucose concentration in the present study may be correlated with the change in carbohydrate concentration to the indirect response to the internal hypoxia and the great mobilization of liver glycogen into blood glucose.

The findings of the present study signify the direct influence of butachlor on the biochemical indices of the fish, which in turn directly correlated with the altered digestive physiology in fish.

5. CONCLUSION

It is concluded that butachlor exposure poses adverse impact on serum biochemistry and intestinal histology of the fish. Marked necrosis and the occurrence of increased number of vacuoles and goblet cells in butachlor exposed fish, indicates that the duodenal tissue strives hard to get rid of herbicides through their common metabolic phenomenon and undergo starvation condition due to lack of absorption. The histological damage ultimately affects the biochemical metabolism and absorption physiology of duodenum. Prolonged exposure can even lead to the death of the aquatic organism. The study recommends a highly restricted and judicious use of butachlor as agrochemicals. It will help in minimizing the loss in aquaculture sector due to non-judicious use of agrochemicals. In turn it will also raise the socio economic status of the fish farmers.

DECLARATION

The standard guidelines mentioned in CPSCEA were strictly followed.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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