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2-Chloro-N-(2-6-Diphenyl) Acetamide Induced Imbalance Of Serum Cortisol Level Correlated With Histopathological Alterations On The Spleen Cells Of Air Breathing Fish *Clarias Batrachus(Linn.)* : As Bioindicators Of Stress

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ABSTRACT: The present study was carried out to investigate physiological stress induced by Butachlor on serum cortisol level and histopathological alterations in spleen cells of air breathing fish *Clarias batrachus(Linn.*). Fishes were collected from different wetlands of North Bihar and acclimatized for fifteen days in the Laboratory. The LC50 of Butachlor (EC 35%) was calculated as per standard APHA method, the fishes were then exposed to sub lethal concentration of (1.5µl/L) for a period of 5, 10 and 15 days. After termination of each exposure period, blood was collected from control and pesticide exposed fish, serum was separated and then fishes were sacrificed. The results showed a significant increase in serum cortisol level. A steep decline was observed in the mean value of cortisol level (198.833±1.471) IU on 5 day of exposure as compared to the control fish (201.33 ±5.0) IU. Again on 10th day there was sharp increase (393.5±1.048) IU and after 15th day it showed more than two fold increase (4017.5±1.87) IU than controlled fish. Light photomicrographs of spleen cells showed massive damage to red pulp and white pulp and numerous melanomacrophage centers. Spleen cell also showed vacuolations and necrosis along with fibrosis, and widening of trabaculae.
KEY WORDS: Butachlor, cortisol, fish, spleen cells

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I. INTRODUCTION

Pesticide poisoning in fish is considered to be very serious as fish form a major food resources for mankind affecting the consumer's health (Dubois, 1971) and may also adversely affect the yield of fish. Their indiscriminate use results in oxidative damage to aquatic organisms (Verma 2014). Reactive oxygen species (ROS) produced during the pesticide detoxification process in liver tissue may react with vital macromolecules such as lipid, protein, carbohydrate and nucleic acid. The oxidative damage and cellular deformities leading to dysfunction of physiological system may be assured through histological examination of tissues (Roberts, R.J. 2001., Norris 1997, Mommsen *et al*.1999). Since, fish dwell in water, they are exposed to many aquatic pollutants which are continually added to the water. The physiological response to stress is well described in freshwater fish, particularly salmonids (Pickering, 1993). Pesticides are toxicants that can activate the stress response in fish, but some can also inhibit cortisol secretion by disrupting the signaling pathways (Leblond & Hontela 1999, Leblond *et al*. 2001, Bisson & Hontela 2002).The physiological stress response is mediated by adrenal system in all the vertebrates causes secretion of stress hormone corticosteroids especially cortisol from the adrenals via hypothalmo-pituitary-interrenal (HPI) axis (Hontela, 2005).

The present study thus aimed to determine the toxicity of butachlor to the freshwater fish *Clarias batrachus* (Linn.) on histopathogical alterations in the spleen cells and physiological stress response indicator, serum cortisol level. The air breathing teleost, *Clarias batrachus* (Linn.) has been selected as a test species because it is the most important fresh water fish species found in wetlands of North Bihar and the worst sufferer of these anthropogenic stresses.

II. MATERIALS AND METHODS

Experimental animal: Air breathing fish, *Clarias batrachus* (commonly called Magur) were obtained 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 laboratory, disinfected with 0.01% $KMnO_4$ solution and kept in different sized large plexy glass aquarium and plastic pools. Fishes were acclimated for 15 days in the laboratory condition. To maintain normal water temperature, cooler and exhaust were used around the

aquarium. The aerated water was changed daily. During acclimated period they were fed pelleted feed made in laboratory (mixture of wheat flour + egg + starch as binder) @5% of their body weight and water was changed daily. After acclimatization, fishes were divided into two groups. Each group contained 18 fishes, Group I – Normal/control, Group II – Butachlor treated.

Pesticide selection - Commercially brand "Butachlor (EC 50%)" manufactured by SEGNOTECH Agro PVT. LTD. Lucknow (U.P.). Here in the experimental protocol, the LC_{50} of Butachlor for fish was performed by the technique described in the standard methods APHA (2005). The LC50 value for 96 hours of Butachlor exposure for *C. batrachus* was calculated as 4.2 µl/L while LC50 value for 48hours of Butachlor exposure was calculated as 5.5µl/L. For the present experiment the dose selected was 1.5µl/ L (sub lethal). Accordingly stock solution was prepared using distilled water, and then fishes were treated with 1.5µl/L of Butachlor for 15 day respectively. The solution was changed regularly.

Collection of the samples

On the termination of exposure day, blood sample of both control as well as experimental group were collected in a heparinized glass culture tube syringe from caudal vein. The serum was separated by centrifuging blood at 5000 rev. /min for 10 min at 4°C and stored at -20°C for further hormone Assay. Fishes were then anaesthetized with MS222 and the spleen tissues were carefully removed and cut into small pieces with sharp surgical blades.

Hormone Analysis:-All the reagents and kits were of analytical grade

The cortisol (Antigen) in the sample competes with horseradish peroxide-cortisol (Enzyme-labelled antigen) for binding to the limited no. of anticortisol (antibody) sites on the micro plates (solid phase). After incubation, the bound/free separation is performed by a simple solid-phase washing. The in the bound fraction reacts with the substrate (H_2O_2) and the TMB substrate & develops a blue colour that changes into yellow when the stop solution (H_2SO_4) is added. The colour intensity is inversely proportional to the Cortisol concentration in the sample. The absorbance is measured at 450 nm against blank.

Assay procedure:

All materials and prepared reagents were equilibrated to room temperature prior to use. All standards, controls and samples are assayed in duplicate.All reagents, working standards, and samples are prepared. Excess microplate strips are removed from the plate frame, returned to the foil pouch containing the desiccant pack, resealed and returned to 4°C storage.Added 20 µL standard, control or sample into their respective wells and 200 µL Cortisol-HRP Conjugate is added to each well. A blank well is left for substrate blank. Wells are covered with the foil supplied in the kit and Incubate for 1hourat37°C.When incubation has been completed, the foil is removed, the content of the wells are discarded and each well is washed three times with 300 µL diluted washing solution. Overflows from the reaction wells checked. The soak time between each wash cycle was more than 5 seconds. At the end remaining fluid is carefully removed by tapping strips on tissue paper .100 µL TMB Substrate Solution was added into all wells.Incubation was done for exactly 15 minutes at room temperature in the dark.100 µL Stop Solution was added into all wells in the same order and at the same rate as for the TMB Substrate Solution. The microplate was gently shaked. Blue color developed during the incubation turned into yellow. The absorbance of the sample was measured at 450 nm within 5 minutes of addition of the Stop Solution. The reading was depicted in. (Table-1)

Statistical analysis- Details of histopathological observations based on Light Microscopy of the spleen cells, and Enzyme Linked Immune Sorbent Assay of serum cortisol level have been mentioned in observations. Six observations have been taken in each case, the statistical analysis include Mean and Standard deviation. In case of serum cortisol assay, the data were analyzed for statistical significance between control and experimental groups with an analysis of variance (Two way ANOVA) and't' test. After applying't test the calculated values were referred to fisher's table to see level of significance at ($P<0.05$) and ($P<0.01$)

III. OBSERVATIONS

Spleen- General Histomorphology

Normal/controlled: Unlike other teleost, the spleen of *Clarias batrachus* is also lymph node like structure and distinct. It is more or less divided into a red, outer cortex and white inner pulp, the medulla. 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 centers (MMC).

Light Micrograph of Spleen of normal/controlled *Clarias batrachus* shows different components of spleen parenchyma, red pulp and white pulp encircled by a capsule. Distinct trabeculae, blood vessels and few scattered melanomacrophage centres (PLATE-I, Fig-1). 1.5µl/L Butachlor treated *C.batrachus* , spleen after 5 days shows slight dissolution of red pulp and white pulp, increased vacuolation in the splenic parenchyma with increased aggregation of melanomacrophage granules. Large melanomacrophage centres at the side of blood

vessels are seen with increased vacuolation (PLATE-I, Fig-2). After 10 days, shows spleen parenchyma with no demarcation of red pulp and white pulp, increased vacuolation, trabecular spaces and increased melanomacrophage centre (PLATE-I, Fig-3). After 15 days, showing increased thickness of splenic capsule, increased vacuolation in the splenic parenchyma with large patches of melanomacrophage centres (PLATE-I, Fig-4).

IV. SERUM HORMONE OBSERVATIONS:

TABLE-1

Fluctuation of serum cortisol level in test fish after exposure to 1.5µl/L of Butachlor for continuous fifteen days.

Sl. No.	Parameter	Period of exposure of Butachlor on experimented fishes in days			
		control	5^{th} days	10^{th} days	15^{th} days
	Cortisol (UI/ml)	201.33 ± 5.0	198.833± 1.47	393.5 ± 1.08*	417 ± 1.87*

Values are expressed in Mean±SE of 6 individuals in each case

* (Significant at $P < 0.05$, Compared to control group)

** (Significant at $P < 0.01$, Compared to control group)

A steep decline was observed in the mean value of cortisol level (198.833±1.471)IU on 5 day of exposure as compared to the control fish.(201.33 ±5.0)IU. Again on 10th day there was sharp increase (393.5±1.048) IU and after 15^{th} day it showed more than two fold increase (4017.5±1.87) IU than controlled fish.

V. RESULTS AND DISCUSSION

Here in the present work, sublethal exposure of butachlore induces many histopathological anomalies in the spleen. The normal histological structure of fish spleen has been greatly damaged after butachlor exposure. Major histopathological changes in spleen included Dissolution of the white pulp, aggregation of more melanomacrophage , autolysis of RBCs and loss of cellular architecture. Marked dissolution of white pulp and increased, eosinophiles at the red pulp region marks the stressful condition of fish. Aggregation of patches of melanomacrophage showed the extreme pathological condition.The histopathological findings are much in consequence to other investigation. Schwaiger *et al*., (1996) have shown, white pulp proliferation, lymphocyte depletion, as well as an increase in the size of spleen. Haemosiderosis and increase in melanomacrophage centers has often been associated with environmental contamination (Gogal *et al*., 1999; Montero *et al*., 1999; Garcia-Abiado *et al*., 2004). One of the important physiological features is melanomacrophage centers (MMC) which are seen in the fish spleen (Agius and Roberts, 2003). They are assumed to be the functional substitutes of the germinal centers of spleen (Ellis, 1980). MMC may contain four types of brown pigments: melanin, lipofuscin, ceroid and hemosiderin (Couillard *et al*., 1999). Stressful conditions to the animal often result in increased number of its splenic MMC's (Montero *et al*., 1999), 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 butachlore The present investigation is also in agreement with the findings of Fournie *et al*. (2001) 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*. (1992) who also observed vacuolation in splenic tissue of European eel *Anguilla anguilla* following stress and resulting in impairment of normal physiology of fish.

The present experiment also reveals the effect of physiological stress response on spleen cells imposed by Butachlor intoxication to *Clarias batrachus*..1.5µl/l Butachlor treated fish showed a rise in serum cortisol level throughout the experimental period.The main effect of cortisol is energy mobilization by the metabolism of carbohydrates, proteins, amino acids and lipids (Norris 1997). The metabolic actions of cortisol were reviewed by Mommsen et al. (1999). Cortisol also interacts with other hormones, including thyroid hormones, growth hormone and catecholamines (Hontela 1997), particularly during exposures to stressors. Cortisol production has a negative influence on the immune system in most teleost fish (Barton & Iwama 1991, Hontela 1998, Schreck et al. 2001). Cortisol also inhibits the ability of the fish to resist disease or pathogens. High levels of cortisol are known to suppress the ability of fish to form antibodies because the production of leukocytes is depressed (Barton & Iwama 1991, Hontela 1997, Schreck et al. 2001). Studies in brown trout (*Salmo trutta*) have shown that handling stress can cause a significant decrease in leukocytes (Barton & Iwama 1991). Anderson *et al*. (1989) who reported the study on immune-suppression in splenic section of rainbow trout upon exposure to different concentrations of chemical toxicant. Thus under pesticide stress the increased cortisol level as well as histopathological alteration in spleen results in suppressed immune responses. The study suggests the histopathological alterations in spleen may be observed as pathological biomarker and imbalance in cortisol level as physiological stress marker to pollutant toxicity.

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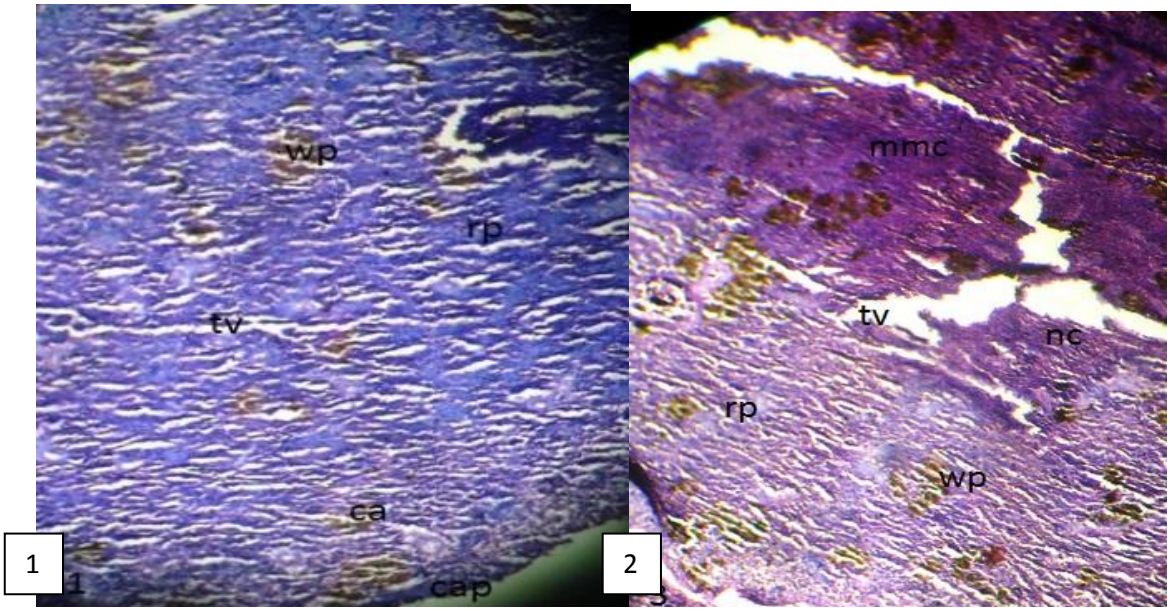
PLATE-I



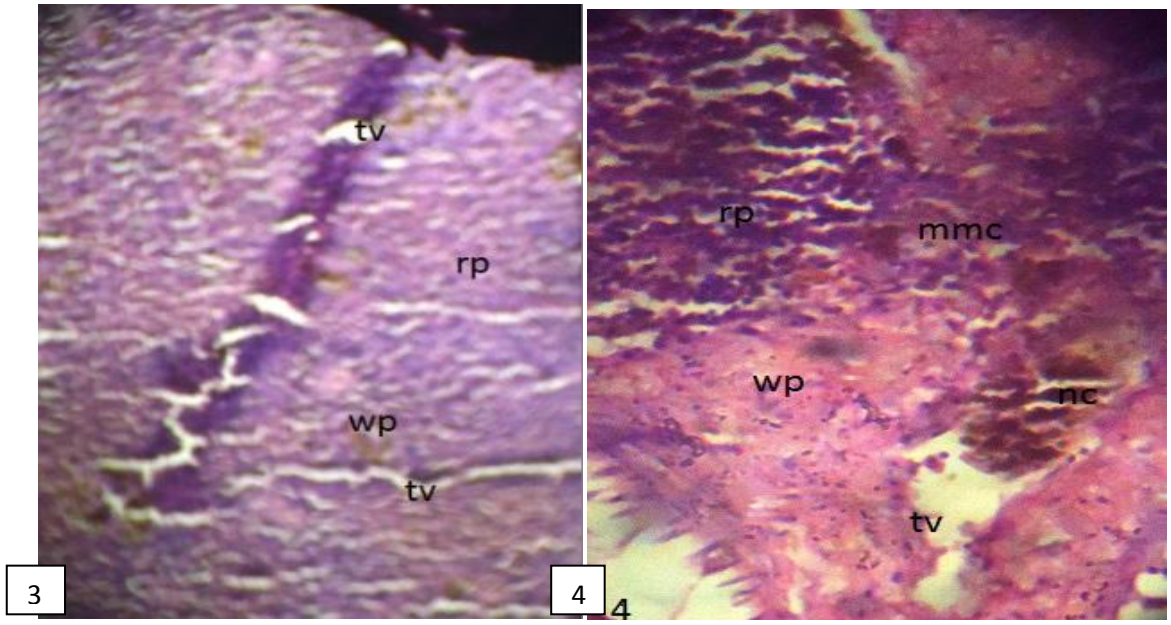


Fig 1 - The transverse section of normalcontrol spleen of *Clarias batrachus*, histoarchetecture of spleen showing distinct red & white pulp. x 200.Fig 2..1.5µl/l butachlor exposed 5day *Clarias batrachus* spleen showing starting of dissolution at the white pulp region (WP).Note –increased melanomacrophage centers.x 400Fig.3.After 10 days showing melanomacrophase centers (MMC).X 200 Fig 4 - After 15 days showing increased necrosis both in red pulp (RP) and white pulp (WP) and vacuolations and patches of melanomacrophase centers (MMC). X 400

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