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Monitoring Stress Biomarkers Anomalies of *Ctenopharyngodon idella* Valenciennes, 1844) Post-Exposure to Atrazine

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Abstract

Grass carp (*Ctenopharyngodon idella*) were exposed to a sublethal concentration of atrazine, specifically 0.03 μlL^{-1}, for a period of 3, 6, 9, 12, and 15 days. The study measured the DNA damage, cortisol and glucose concentrations, as well as hematological indices of the fish. These observations were compared to a parallel untreated control group to assess the effects of atrazine exposure. Fish showed uncoordinated behavior such as erratic and jerky swimming, frequent surfacing, irregular downward movements, loss of equilibrium, increase in the frequency of opercular movements, becoming exhausted and lethargic and bleeding at the base of the eyeballs. DNA damage and electrolytes (K^+, Cl^-, Na^+) concentrations in gills tissues depends upon the time of exposure as maximum the time of exposure so incremented DNA damage and electrolytes concentration were observed and vice versa. Similarly maximum incline in concentration at all duration of exposure were observed in Hb, RBC, TLC and lymphocyte while upturn in concentration of MCV at exposure for 12 days, MCH and MCHC at exposure for 9 and 12 days were reported. Decrement in concentration of platelet, neutrophils, monocytes and eosinophils against all groups were noticed with the gradually increment at exposure period of 12 and 15 days respectively. In conclusion, short and long-term exposure to atrazine at sub-lethal concentrations induces severe physiological alterations in *C. idella* that may potentially disrupt their survival in their natural habitat.

Keywords: Gras Carp; Hematology; Toxicological; RBC; WBC; TLC

Introduction

Herbicides utilization is an important part of the modern agriculture for the gaining quality and quantity products of agriculture to overcome the need of the human beings but due to injudicious and unpredictable utilization of herbicide in crops field, remaining residues of these chemicals in soils shall be carried to aquatic bodies through rain water due to its high mobility through soil, thus have toxic impact on a variety of non-target life forms including fish which is an issue of worldwide significance [1-3].

Atrazine class herbicides are broadly being utilized in horticulture and record for around half of the worldwide herbicide use [4]. Atrazine is triazine class herbicide that is utilized for to stop the pre-development broadleaf weeds in crops, for example, maize (corn) and sugarcane and on turf, for example, golf courses and residential lawns. After application, atrazine effectively washes into surface water and at last reaches to lakes, streams and rivers. Previous investigations have confirm quick debasement of atrazine however regular utilization of atrazine herbicide recorded presence in the aquatic bodies for long duration and ends in creating stress in aquatic ecosystem [5].

Hematological and histological biomarkers can be distinguished quickly and documented as critical to sub-lethal grouping of various environmental stressors. Therefore, the alterations in concentrations of these parameters against the toxic chemical can be utilized for forecast and analysis of

herbicides poisonousness [6].

Hussein SY, et al. [7] noticed that application of atrazine without the proper management ends it travelling to aquatic bodies. It has been documented that the natural and surface water recorded 0.1 gl -1 concentration of atrazine which is exceeding with the passage of time because of thoughtless applications of this chemicals and thus aggregate in various tissues of fish which furthermore have also adverse impact on human beings. Different authors have revealed the effect of atrazine on the physiology and metabolism of aquatic living beings especially on fishes [7,8].

Fish are one of the most generally appropriated organism in aquatic ecosystem and being helpless to natural tainting may reflect the degree of the organic impacts of ecological contamination in waters. In Pakistan, atrazine still is one of the most generally utilized herbicides controlling expansive leave weeds and grasses. The potential impacts of atrazine on aquatic biological system have stimulated studies to understand the mechanisms and measurement of the harmful impacts of it to aquatic life forms. Keeping in view the above stated facts, the present study was designed to assess the deleterious effect of atrazine on fresh water fish grass carp (*C. idella*), through analyzing intense impact of atrazine as a herbicide on hematological, DNA damage , cortisol and glucose concentrations.

Materials and Methods

Chemical, Test Animal and Study Duration

Fingerlings of grass carp (3.23 ± 0.34g) were packed in oxygenated bags and transported from Mardan and Peshawar carp hatcheries to Fisheries and Aquaculture research lab, Department of Biology, University of Haripur. Before experimentation fish were acclimatized for two weeks in aquarium having tap water, 500L and were fed properly with commercial pellets carp diet. Air pumps were installed in aquarium for purpose to aerate the aquarium water so that all the water quality parameters should be adjusted to normal range particularly dissolved oxygen which is important parameters that plays critical role in fish survival. The hardness and pH of water were 250 mg $CaCO_3$ L^{-1} and 7.4 ± 0.01, individually. The medium utilized was filtered and the degrees of NH_4, NO_2 and NO_3 in the water recorded to be inside 0.1, 0.1 and 20 mg L^{-1}, separately. After stipulated duration of acclimatization, randomly fish were transported to experimentation aquarium having 100L water. These tanks were also having the air pumps to aerate the water and to maintain the aquarium environment suitable for fish. During the experiment, grass carp behavior rate were also recorded.

Chemical selected for the current study was herbicide named as atrazine which is the most important part of modern agricultural setup because it is used as extensively against the herbs although it extensive utilization has adverse impact on the surrounding ecosystem. In the current study, atrazine herbicide (99.5 % purity, Chem-service, USA) was introduced at sub lethal concentration i.e. 0.03 μlL^{-1}, for a period of 3,6,9,12 and 15 days respectively against *C. idella*.

DNA Damage Analysis

DNA damage was analyzed in fish blood through the process of comet assay according to Singh, et al. with minor modifications. Fish blood were immobilized on a clean clear slide through agarose gel, then were lysed with lysis buffer, stained with ethidium bromide and analyzed through TriTek Comet Score that is classified into five categories (measured) starting from Type 0 (undamaged) to Type IV (complete damage. Comet Software was used to measure the comet tail length of damaged cells and cumulative tail length of all examined cells (n=50 per replicate).

Biochemical Indices Analysis

Cortisol hormone levels contents were assessed using the radioimmunoassay methods as described by Waring CP, et al. [2]. Electrolytes and glucose levels were assessed using the flame emission method according to Lerner, et al. The concentrations of Cl^-, Ca^{2+}, and glucose were determined using a semi-automated clinical chemistry analyzer Pictus B (Diatron, Hungary).

Blood Analysis

The fresh whole blood collected in EDTA tubes was instantly subjected to analyse the blood indices through a hematological analyzer (PocH-1001V, Sysmex Corp., Kobe, Japan).

Statistical Analysis

One-way ANOVA test (IBM SPSS Statistics 20) were used to determined significant differences between the untreated and treated variables.

Results

Various parameters including hematology, biochemistry and DNA damage of grass carp were observed against the specific sub lethal dose of atrazine herbicide for various time periods. Behavior of fish was also under observation throughout the experimentation.

Behaviorally Observations

Behavior of fish were observed regularly during the experimentation and noted that atrazine poisoning in fish ends in high rate of breathing due to suffocation that was observed by maximum frequency of opercular movement, loss of body balance, includes erratic and jerky swimming, frequent surfacing, becoming exhausted and lethargic and bleeding at the base of the eyeballs were documented which in line with the findings of Velisek J, et al. [9].

Hematological Indices Analysis after 3 Days

The hemoglobin concentration (g dl^{-1}) mean value 9.8 ± 0.680 in comparison to the mean value of 12.12± 0.360 for the control group showed a significant declined ($p < 0.05$) after the exposure for 3 days. Similarly declined in concentration of red blood cells ($millcm^{-1}$) 1.82 ± 0.104 were observed in comparison of control group having mean value of 2.92± 0.088. In stress environment, the total leukocyte counts (cmm^{-1}) of *C. idella* were significant incremented ($p < 0.05$) against the atrazine having mean value 61350 ±17065.1 in comparison with control group mean value of 8100±2066.39 respectively, while significant decrease ($p < 0.05$) in platelets counts ($lakscu^{-1}$) 5666 ± 2081.66 were noticed in comparison with the reference group 8100±2066.39. Except the mean concentration of MCV(Mean corpuscular volume, fl^{-1}) 97.6 ± 7.879, the mean concentration of MCH (Mean corpuscular hemoglobin, pg^{-1}) and MCHC (Mean corpuscular hemoglobin concentration, gdL^{-1}) of treated group, 54.1 ± 1.242 and 55.6 ± 3.51 showed significant elevation ($p < 0.05$) in contrast to control group mean value 47.8±0.665 and 47.3±0.577 respectively. To cope with the stressful surroundings as imposed by the toxicity of atrazine the *C. idella* increased the immunity system that is denoted by the increase in the mean concentration of lymphocytes (%) 87.3± 6.35 in comparison with control group mean concentrations as 76.6±2.88, while significant decline ($p < 0.05$) in concentration of neutrophiles, monocytes and eosinophiles (%) were recorded 1 ± 1.154, 6.3 ±2.30 and 4.6 ± 2.88 respectively in contrast to untreated group mean concentrations as 10±2.645, 7.6±2.309 and 6±1.73 respectively while no data were recorded for the basophiles in all treated groups.

Hematological Indices Analysis after 6 Days

Similarly the hemoglobin concentration (g dl^{-1}) mean value 10.03± 0.383 in comparison to the mean value of 12.12± 0.360 for the control group showed a declined after the exposure for 6 days. Similarly declined in concentration of red blood cells ($millcm^{-1}$) 1.8±0.166 were observed in comparison of control group having mean value of 2.92± 0.088 while non-significant difference were seen in the total leukocyte counts (cmm^{-1}) of *C. idella* against the atrazine having mean value 81600±6077.00 in comparison with control group mean value of 8100±2066.39 respectively. Significant decrease ($p < 0.05$) in platelets counts ($lakscu^{-1}$) 5666±1154.7 were noticed in comparison with the reference group 8100±2066.39. The mean concentration of MCV(Mean corpuscular volume, fl^{-1}), MCH (Mean corpuscular hemoglobin, pg^{-1}) and MCHC (Mean corpuscular hemoglobin concentration, gdL^{-1}) of treated group 89±3.360, 53±3.36 and 59.6±0.577 showed variations of small ranges in contrast to control group mean value 101.03±1.184, 47.8±0.665 and 47.3±0.577 respectively. Increased in the mean concentration of lymphocytes (%) 82.6±2.516 and Monocytes (%) 8.66±2.516 in comparison with control group mean concentrations as 76.6±2.88 and 7.6±2.309 respectively were noticed. While significant decline ($p < 0.05$) in concentration of neutrophiles and eosinophiles (%) were recorded 3.33±2.88 and 5.3±1.154 respectively in contrast to untreated group mean concentrations as 10±2.645 and 6±1.73 respectively while no data were recorded for the basophiles in all treated groups.

Hematological Indices Analysis after 9 Days

After exposure duration of 9 days, decline in the hemoglobin concentration (g dl^{-1}) mean value 10.7±0.665 in comparison to the mean value of 12.12± 0.360 for the control group were recorded. Similarly declined in concentration of red blood cells ($millcm^{-1}$) 1.91±0.150 were observed in comparison of control group having mean value of 2.92± 0.088 while significant difference ($p < 0.05$) were seen in the total leukocyte counts (cmm^{-1}) of *C. idella* against the atrazine having mean value 64866±13855.08 in comparison with control group mean value of 8100±2066.39 and significant decrease ($p < 0.05$) in platelets counts ($lakscu^{-1}$) 10333±13576.94 were noticed in comparison with the reference group 8100±2066.39. The mean concentration of MCV (Mean corpuscular volume, fl^{-1}) showed significant variation 89±3.874 in contrast to control group mean concentration 101.03±1.184 while MCH (Mean corpuscular hemoglobin, pg^{-1}) and MCHC (Mean corpuscular hemoglobin concentration, gdL^{-1}) of treated group 56.3±1.040 and 63.3±4.932 showed variations of small ranges in contrast to control group mean value 47.8±0.665 and 47.3±0.577 respectively. Mean concentration of lymphocytes (%) 89.6±2.51 in comparison with control group mean concentrations as 76.6±2.88 increase to adjust the body immunity with the surrounding environment While significant decline ($p < 0.05$) in concentration of neutrophiles, monocytes and eosinophiles (%) were recorded 1.3±0.577, 4.6±0.577 and 4.3±1.52 respectively in contrast to untreated group mean concentrations as 10±2.645 and 6±1.73 and 6±1.732 respectively while no data were recorded for the basophiles in all treated groups.

Hematological Indices Analysis after 12 Days

After exposure duration of 12 days, the hemoglobin concentration (g dl^{-1}) mean value 9.1± 0.346 in comparison to the mean value of 12.12± 0.360 for the control group showed a significant declined ($p < 0.05$,). Likewise declined in concentration of red blood cells (millcm^{-1}) 2.06± 0.1552 were observed in comparison of control group having mean value of 2.92± 0.088 while significant decline; $p < 0.05$,were seen in the total leukocyte counts (cmm^{-1}) of *C. idella* against the atrazine having mean value 59733± 15992.92 in comparison with control group mean value of 8100±2066.39 and significant decrease ($p < 0.05$) in platelets counts (lakscu^{-1}) 6666± 4618.802 were noticed in comparison with the reference group 8100±2066.39. The mean concentration of MCV(Mean corpuscular volume, fl^{-1}), MCH (Mean corpuscular hemoglobin, pg^{-1}) and MCHC (Mean corpuscular hemoglobin concentration, gdL^{-1}) of treated group concentrations 105.9± 8.075, 47.5± 2.157 and 45.1± 5.157 showed non-significant variation in contrast to control group mean concentration 101.03±1.184, 47.8±0.665 and 47.3±0.577 respectively. Mean concentration of lymphocytes and eosinophiles (%) 83.6± 3.577 and 8.6± 2.516 in comparison with control group mean concentrations as 76.6±2.88 and 6±1.732 increase to adjust the body immunity with the surrounding environment While significant decline ($p < 0.05$) in concentration of neutrophiles and monocytes (%) were recorded 7.6± 0.577 and 5.6± 0.577 respectively in contrast to untreated group mean concentrations as 10±2.645 and 7.6±2.309 respectively while no data were recorded for the basophiles in all treated groups.

Hematological Indices Analysis after 15 Days

The hemoglobin concentration (g dl^{-1}) mean value 10.2± 0.655 in comparison to the mean value of 12.12± 0.360 for the control group showed declined after the exposure for 15 days. Similarly declined in concentration of red blood cells (millcm^{-1}) 1.87±0.1357 were observed in comparison of control group having mean value of 2.92± 0.088 while significant inclined ($p < 0.05$) in concentration were seen in the total leukocyte counts (cmm^{-1}) of *C. idella* against the atrazine having mean value 32133±3716.62 in comparison with control group mean value of 8100±2066.39 and significant decrease ($p < 0.05$) in platelets counts (lakscu^{-1}) 10333±2516.61 were noticed in comparison with the reference group 8100±2066.39. The mean concentration of MCV (Mean corpuscular volume, fl^{-1}) and MCH (Mean corpuscular hemoglobin, pg^{-1}) of treated group 115.7±4.80 and 54.9±2.218 were significantly increased ($p < 0.05$) in contrast to control group concentration 101.03±1.184 and 47.8±0.665 while MCHC (Mean corpuscular hemoglobin concentration, gdL^{-1}) of treated group concentrations 47.6±1.154 showed non-significant variation in contrast to control group mean concentration respectively. Mean concentration of lymphocytes, monocytes and eosinophiles (%) 82±11.357, 12.6±0.577 and 7±01 in comparison with control group mean concentrations of control group as 76.6±2.88, 7.6±2.309 and 6±1.732 increase to adjust the body immunity with the surrounding environment While significant decline ($p < 0.05$) in concentration of neutrophiles (%) were recorded 8.3±1.527 respectively in contrast to untreated group mean concentrations as 10±2.645 respectively while no data were recorded for the basophiles in all treated groups.

Electrolyte Concentration

Electrolyte concentrations (K^+, Cl^-, Na^+) along with glucose concentration were documented against the atrazine specific concentration and were compare with the control group and it was observed that all the parameters were altered in a way that maximum incremented ($p < 0.05$) concentration were observed against the atrazine. Chloride concentrations were effected maximum that unveiled the alteration in osmoregulation of fish because of the toxic surrounding followed by glucose, potassium and sodium.

Discussion

Assessing contaminants toxicity in fish is of extraordinary concern because of their expected unfavorable effect on human being after utilization in food. Hence toxicity study are fundamental for decide sensitivity of animals and human beings to poisons and furthermore helpful for assessing the level of harm to target organs and the consequent physiological, biochemical and behavioral disorders [10]. In the current experimentation, during toxicity period, different behaviorally irregularities like expanded opercula, mucous discharge, jerky development, drifting on the sides, hypersensitivity showing violent erratic and quick swimming and so on have been seen which demonstrates the harmful impact of atrazine on central nerves framework and cardiovascular framework [11].

Chemical contamination of aquatic ecosystem can influence the balance of fish and can be utilized as a index of toxic stress [12]. By adapting various behaviorally movements, fish attempt to decrease the impacts of pesticides enter in to the body from the surrounding medium or to limit the harm of their body tissues. In the current examination, treated fish documented disquiet and lazy swimming which is as per the report of Bradbury and Coats [13]. As indicated by Marler and Hamilton, changes in behavior of *Labeo rohita* may be because of the impact of pesticides on the central nervous system or the aggravations in physiological component [14]. Comparable behavioral adaptations have likewise been accounted for in *Cyprinus carpio* against Diazinon and

in *Labeo rohita* against cypermethrin and diazinon toxicity [15].

Among physiological disturbance against the toxicity, hematological indices are viewed as likely biomarkers of toxicity as incline or decline in the different hematological indices can be observed [16]. In comparison with the control group , the hematological indices of *C. mrigala* exposed to 0.815 mg/L and 1.63 mg/L of diazinon for 30 days was recoreded a huge decline in RBC, Hb, HCT, MCV, MCH and WBC count. The decrementing in in RBC, WBC counts and upsides of other hematological indices in *C. mrigala* can be credited to concealment of hematopoietic system of the fish, brought about by longterm toxicity to diazinon. These outcomes are reliable with the findings of different examinations exploring hematological indices of various fish species exposed to other organophosphate pesticides as well similarly as with the current observation of atrazine against grass carp. In this association, changes in hematological lists were seen after exposure to malathion in *C. gariepinus*, phosalone in *O. mossambicus* [17], and trichlorfon in *C. carpi*o [18]. Comparative decline in RBC count, WBC count, hemoglobin and hematocrit levels have been accounted for in male brood stock, *Rutilus frisii kutum* [19] and grass carp, *Ctenopharyngodon idella* after long term exposure to sub-deadly centralizations of diazinon. Adedeji OB, et al. [1] and Khoshbavar-Rostami H, et al. [20] additionally detailed declined in Hb, RBC and WBC count and expanded upsides of MCV and MCH after diazinon exposure in monster sturgeon (huso) and African catfish, *C. gariepinus* separately.

Red blood cells concentration was declined in fish exposed to toxicant surroundings. Downturn in the erythrocyte profile ends in oxygen deficiency in the body because of gill harm due to contaminants [21]. Restraint of erythropoiesis and expansion in the rate of erythrocyte obliteration in hematopoietic organs is the reason for decline in RBC count [22]. In the current study, the critical declined in RBCs and hemoglobin content could have resulted because of the declining of the oxygen content of the water because of the presence of atrazine in the surrounding ecosystem. Leucocytes are engaged with the regulations of immunological capacity and their concentration increment as defensive reaction in fish against toxic media. Such an expansion leucocyte count (TLC) happens by the increment in lymphopoiesis and additionally upgraded release of lymphocytes from lymphoid tissues [23]. Khan revealed that WBC increment could in connection of an induced proliferation because of the herbicide toxicity.

Inclined in concentration of lymphocytes and monocytes could be brought about by high phagocytic exercises because of the harmful chemicals in surrounding. Fluctuation in concentration of neutrophils and monocytes in the current work have been resembled to the first and second line of defense in light of cell harm brought about by chemical exposure [24]. Besides, neutrophilis concentration in comparison to the current findings was accounted for in Hoplias malabaricus presented to poisonous metal and could be the outcome of tissue harm attributed to impacts of harmful metals. Likewise, a similar monocyte percentage was determined in *Astyanax gr. bimaculatus* giving significant greater values of the exposed (25.00 ± 0.83, 27.40 ± 0.76) group as compared with the unexposed group (19.47 ± 0.82). Besides, like the results of the present study, fluctuated concentration of monocytes, neutrophils and esinophiles were determined in gray mullet (2.00 ± 1.41, 1.50 ± 0.71, and 1.50 ± 0.71 for 14, 21, and 28 days, respectively) as compared with the values of the control group (0.10 ± 0.06) after exposition to different lead concentrations [25].

Past findings detailed that electrolytes levels commonly diminished in like common carp presented to cypermethrin. Interestingly, an increment in electrolyte levels might be credited to increased passive influx of ions across the gill related with poison initiated harm to chloride cells. As was referenced, the osmoregulatory capacity of cortisol includes the expansion in the quantity of chloride cells and Na^+/K^+ ATPase movement. In this way, our outcomes might propose that the atrazine-instigated expansion in cortisol levels and the ensuing expansion in chloride cells number. The obtained results in the current research demonstrated that increment in K^+ and Cl^- levels may be extraordinarily impacted by atrazine. This request was additionally upheld by a few scientists [26]. The outcomes accomplished in the current review could likewise be connected with the observed significant increase of chloride cells in gills ($p < 0.05$) [27].

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Conflict of interest

All the authors confirmed that the content of this manuscript has no conflict of interest.

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