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Effects of chronic exposure to carbamazepine on hematological parameters in *Cyprinus carpio*

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Abstract

Tests on pysicochemical parameters in the recent years have confirmed the existence of drug residues and their metabolites in different parts of aquatic environments the permanent release of which has led to micro-persistent pollution. The drugs are designed in a way to be chemically stable, resistant to degradation and to survive by using their biological effects on the organisms. In the present research study, toxicity and the effect of carbamazepine on *Cyprinus carpio* was examined using blood responses. Thus, the effects of three different concentrations of carbamazepine (1.25, 2.5 and 5 mg L^{-1}) on the changes of blood factors such as red blood cell (RBC), hemoglobin (Hb), hematocrit (Hct), mean cellular volume (MCV), mean cellular hemoglobin (MCH), mean cellular hemoglobin concentration (MCHC), white blood cell (WBC), lymphocyte (Lymph), neutrophil (Neut) and monocyte (Mono) were studied on blood samples collected on days 7, 14 and 21 after exposure to the drug. The results showed that RBC, WBC and lymph counts were reduced in fishes treated with carbamazepine (CBZ). In contrast, the value of Hb, Hct, MCV, MCH, Neut and Mono were increased after exposure to carbamazepine. No significant difference was observed in the MCHC levels in all concentrations. Changes in hematological parameters can act as a biomarker in testing the toxicity of CBZ in aquatic environments. However, detailed studies regarding the application of special biomarkers for assessing human drugs is required.

Keywords: Carbamazepine, Hematological parameters, *Cyprinus carpio*

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Introduction

Nowadays, a large number of drugs are used for preventing, detecting and treating the diseases in animals and human beings. A large number of these drugs are not completely decomposed after application (Bound and Voulvoulis, 2004). As a result, the metabolites of drugs and some unchanged forms of these compounds are excreted at first and then, they enter the ecosystem (Fent *et al*., 2006).

Carbamazepine was one of the most frequently used drugs in aquatic systems that was recognized as an anthropogenic marker in water bodies which is due to its stability in surface waters and sewages (Clara *et al*., 2004). However, its detrimental ecological effects on aquatic organisms especially fishes are less recognized.

Carbamazepine is usually used to control seizures disorders, neurological pains and for a variety of mental disorders (Jones *et al*., 2002; RxList, 2006). The physiological rule of CBZ is to stabilize the inactive status of sodium channels so that the brain cells are less stimulated (Contardo-Jara *et al*., 2011). CBZ is a relatively lipophilic compound (K_{ow}=2.2) (Gagn**é** *et al*., 2006). CBZ has been assessed as a toxic compound for aquatics such as bacteria, seaweeds, invertebrates and fishes (Ferrari *et al*., 2003; Li *et al*., 2011b).

It is estimated that 1014 tons of Carbamazepine is used each year all over the world (Zhang *et al*., 2008). According to the findings of Food and Drug Organization of the Ministry of Health, Treatment and Medical Education in Iran, the production of the aforementioned drug in 2014 was equal to 35 tons.

The fishes were selected amongst the other aquatic organisms for examining the effects of environmental pollution on aquatic ecosystems. In recent years, sensitive biomarkers have been extensively used to test the probable dangers for the aquatic organisms. In general, the changes of these biomarkers in blood play an effective role in detecting the structural and functional status of the organisms confronted with toxic materials and also monitoring the aquatic environment (Van der Oost *et al*., 2003; El-Sayed *et al*., 2007).The interaction of a toxic material and a biological system can be examined using hematological biomarkers (Li *et al*., 2010). The changes in hematological biomarkers (RBC and WBC count, Hb, Hct, MCV, MCH and MCHC) are extensively used for examining toxic stress, and integrity of the immune system (Talas and Gulhan, 2009; Kavitha *et al*., 2010). The changes of these biomarkers, are sensitive to the environmental and physiological changes and are easily measurable and they analyze the physiological changes in organisms (Remyla *et al*., 2008)

In the northern coast of Iran, factors such as high density of human population, the lack of an appropriate wastewater treatment system and the existence of urban and hospital wastewater, have allowed pharmaceutical pollutants to enter the Caspian Sea and thus, pharmaceutical pollution is recognized as a significant

dangerous factor that threatens the biology and the survival of the organisms.

The impact of drugs on the physiology of aquatic organisms has been investigated in several studies. For example, Li *et al*. (2010) examined hepatic liver antioxidants and hematological parameters in *Oncorhynchus mykiss* exposed to carbamazepine. The fish were exposed to sublethal concentrations (1.0 µg L^{-1}, 0.2 µg L^{-1} and 2.0 µg L^{-1}) of carbamazepine for 7, 21 and 42 days. Compared to the control group, the fish exposed to higher concentration of CBZ showed higher levels of hemoglobin. Saravanan *et al*. (2011) investigated the ecotoxicological impact of clofibric acid and diclofenac on hematological, biochemical, ionoregulatory and enzymatic responses in *Cyprinus carpio*. The fish were exposed to different concentrations (1, 10, 100 µg L^{-1}) for 96 hours. At all concentrations, the level of red blood cells reduced in the fish treated with diclofenac and clofibric acid. On the contrary, the level of white blood cell increased in the fish. However, a mixed trend was observed in hemoglobin, hematocrit, mean cellular volume, mean cellular hemoglobin, and mean cellular hemoglobin concentration. In another study Li *et al*. (2011a) investigated the acute toxicity of carbamazepine in *O. mykiss*. The fish exposed to CBZ, showed higher levels of Er, Hb, MCHC, monocytes, granulocytes neutrophil and lower MCV and lymphocytes, compared to the control group.

The present study aims to study the effects of carbamazepine on *C. carpio*, using hematological parameters in order to evaluate the probable dangers of drugs transferred from water in the survival and physiology of aquatics and contribute to risk management decisions in the future for this emerging contaminant in water.

Materials and methods

Chemicals

Carbamazepine and other chemicals were obtained from Sobhan Darou Corporation (Iran). The CBZ was dissolved in dimethyl sulfoxide (DMSO) in order to make a stock solution at a concentration of 100 mg ml^{-1}.

Fish

C. carpio, weighing 25 to 35g (mean±SD), were obtained from a local commercial hatchery (Golestan, Iran). They were held in aquaria containing 160 liters of freshwater continuously aerated to maintain dissolved oxygen values of 7.5–8.0 µg L^{-1}. Temperature and pH were 26±1 ◦C and 7.1±0.2 respectively. Photoperiod was a 12:12 light–dark cycle. Water was renewed (50%) daily to avoid accumulation and contamination to excretory materials. In this study, tap water -chlorine free- was used and the physicochemical characteristics of tap water such as temperature, pH, dissolved oxygen, total alkalinity and total hardness were measured according to APHA (1998) and maintained during the study. Fish were acclimatized for 14 days before the beginning of the experiment and

were fed commercial fish food. The fish were starved for 24 h prior to sampling to avoid prandial effects during the assay.

Exposure to CBZ

A 140 l semi-static system was used in which 20 fish were distributed to each of the 15 aquaria randomly. Given that LC_{50} levels in the *C. carpio* fish was determined as 59.70 µg L^{-1} for 24 hours (Malarvizhi *et al*., 2012), and the fish were exposed long term to drug, the test concentrations were considered less than 1.4 LC_{50}. The nominal used concentrations of CBZ were 1.25 mg L^{-1}, 2.5 mg L^{-1} and 5 mg L^{-1}.

Carbamazepine was dissolved in DMSO with a final concentration of less than 0.05%. Two other groups were used as contrast groups, a control group exposed to clean freshwater and a DMSO group exposed to the volume of DMSO (v/v, 0.05%) used for the highest CBZ concentration. Each experimental condition was run in triplicate. The feeding of fish was performed daily, with commercial fish pellets at 1% total body weight at a fixed time and the extra food was removed. Fifty percent of the exposed solution was renewed each day after 2 h of feeding to maintain the appropriate concentration of CBZ and DMSO and water quality. The test equipment was cleaned every 14 days. Since the effect of chronic toxicity of carbamazepine was investigated in the present study, sampling was performed at 7, 14 and 21 day intervals in order to identify the effects of sublethal concentrations in fish and prevent death of fish due to side effects. Experiments were done in accordance with the European Communities Council Directive (86/609/EEC).

Collection and preparation of blood samples

Blood samples were taken from each fish by caudal venipuncture using a syringe heparinized at a concentration of 5000 IU heparin sodium salt in 1ml. An aqueous solution of heparin sodium salt at 0.01 ml/1 ml blood was used to stabilize the samples.

Hematological analysis

Hb was estimated by the cyanmethaemoglobin method (Drabkin, 1946). RBC and WBC were counted by the method of Rusia and Sood (1992). Hct was estimated according to Nelson and Morris (1989). Differential white blood cells were counted following the method of Svobodova *et al*. (1991). Erythrocyte indices like MCV, MCH and MCHC were also calculated according to standard formulae as follows:

$$\text{MCV (fl)} = \frac{\text{Hct(\%)} \times 10}{\text{RBC count in millions/mm}^3}$$

$$\text{MCH(pg)} = \frac{\text{Hb(g/dl)} \times 10}{\text{RBC count in millions/mm}^3}$$

$$\text{MCHC(g/dl)} = \frac{\text{Hb(g/dl)}}{\text{Hct(\%)}} \times 100$$

Statistical assays

All values were expressed as mean±SD and analyzed by SPSS software. One-way repeated measure ANOVA following S-N-K's test was used to

determine whether results of treatments were significantly different from the control group (p<0.05).

Results

Hematological parameters of *C. carpio* that were exposed to CBZ showed changes in comparison with control groups. The results showed the reduction of RBC in the treated fish exposed to the drug on the 14th and 21st days. Hb and Hct contents were increased in the different drug concentrations. Also, MCV, MCH values were increased in fishes treated with drugs which corresponded with increasing periods for exposure to the drug. No significant difference was seen in the values of MCHC in all the concentrations. The WBC count and the percentage of lymphocytes in treated fishes exposed to drug on the 14th and 21st days were reduced and conversely, the percentage of neutrophil on the 21st day and monocytes on the 14th and 21st days, in treated fishes exposed to carbamazepine increased.

In Figs. capital letters represent significant differences between treatments in terms of time.

Lowercase letters represent significant differences between treatments in terms of the concentration factor.

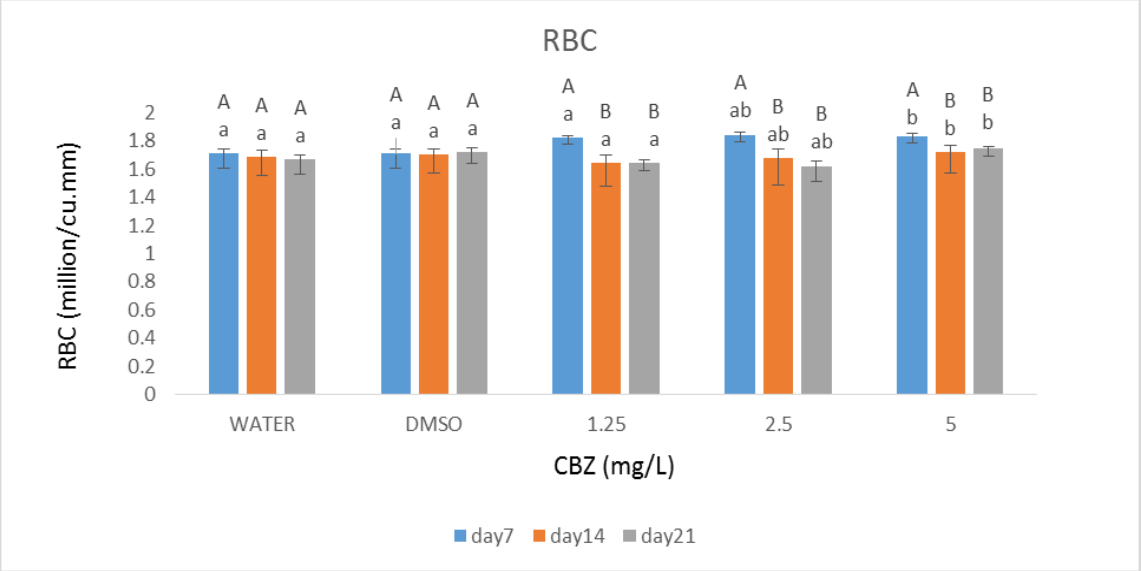

Figure 1: Changes in the RBC count in *Cyprinus carpio* exposed to CBZ

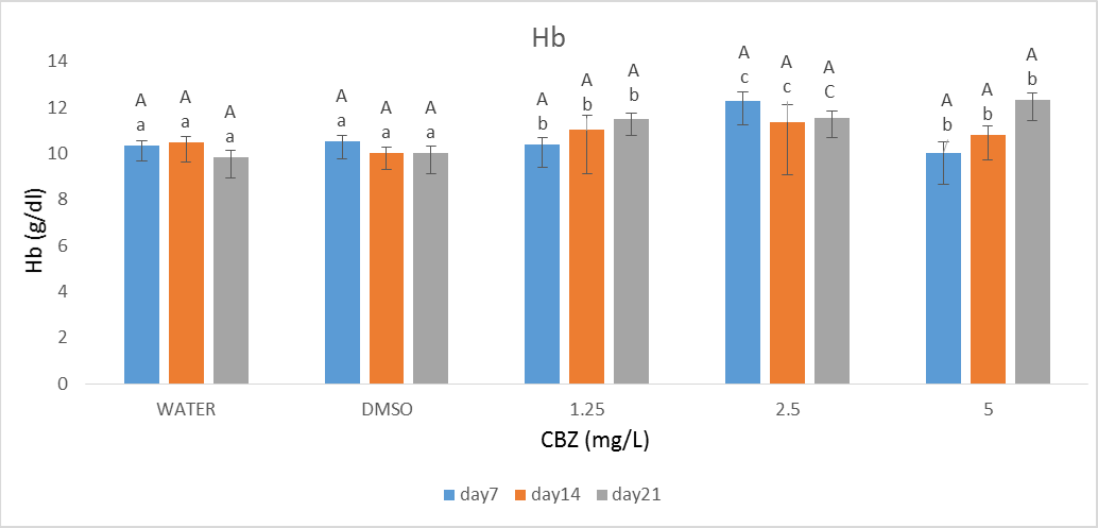

Figure 2: Changes in the Hb values in *Cyprinus carpio* exposed to CBZ.

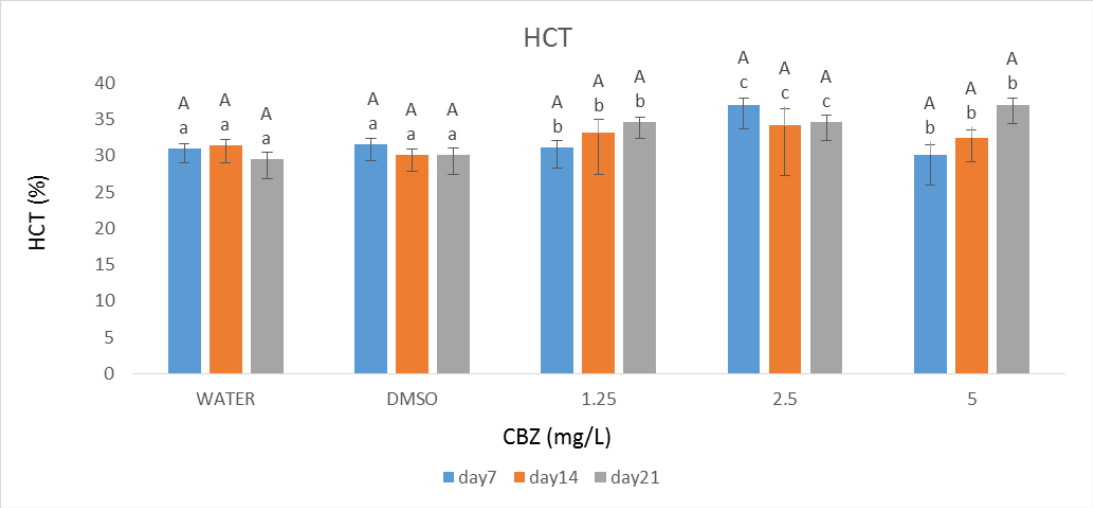

Figure 3: Changes in the Hct values in *Cyprinus carpio* exposed to CBZ.

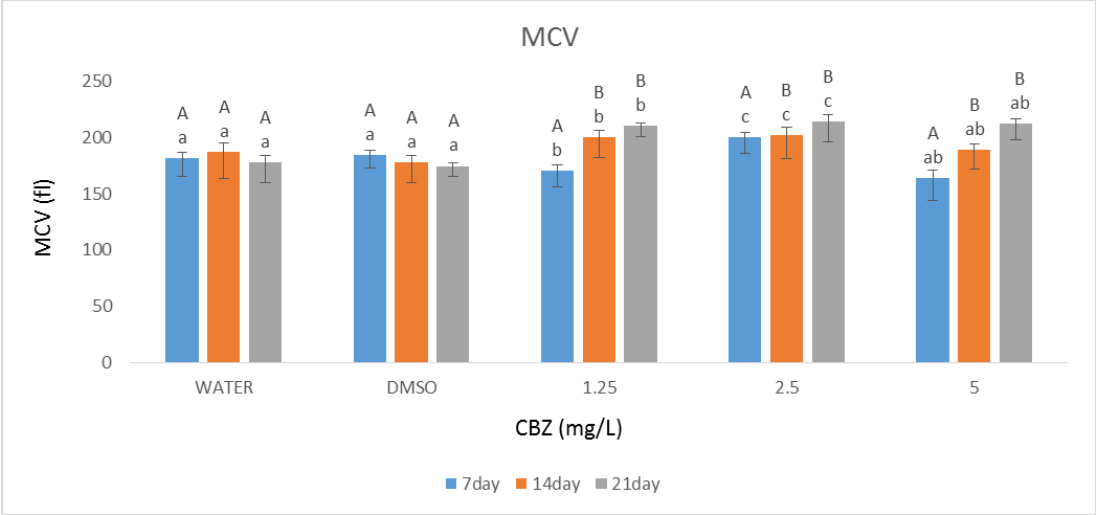

Figure 4: Changes in the MCV values in *Cyprinus carpio* exposed to CBZ.

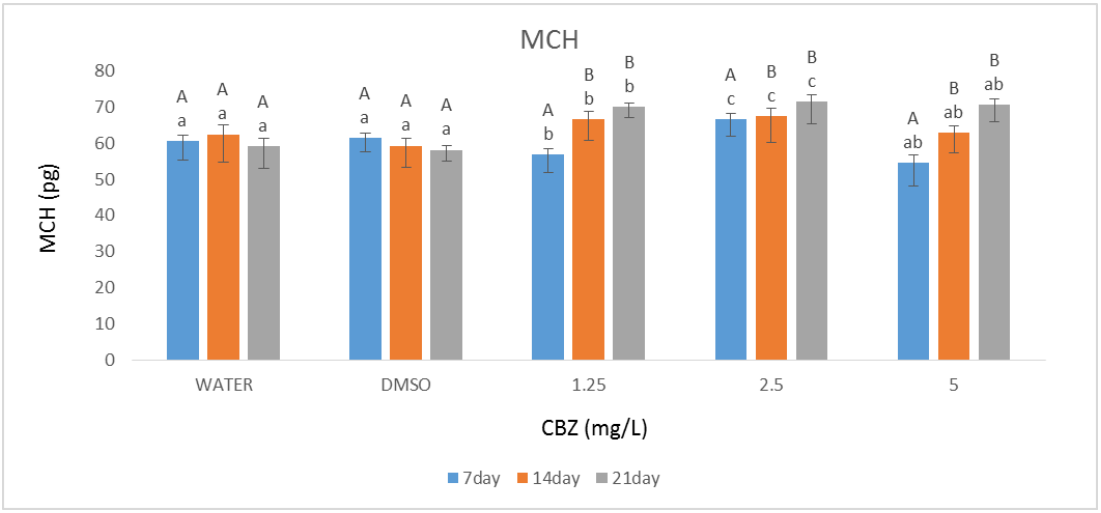

Figure 5: Changes in the MCH values in *Cyprinus carpio* exposed to CBZ.

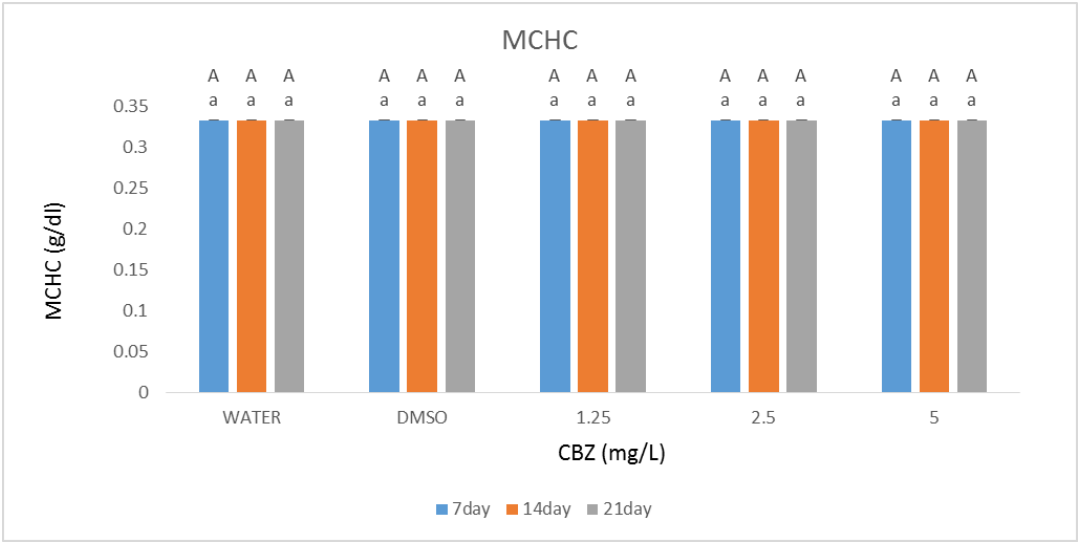

Figure 6: Changes in the MCHC values in *Cyprinus carpio* exposed to CBZ.

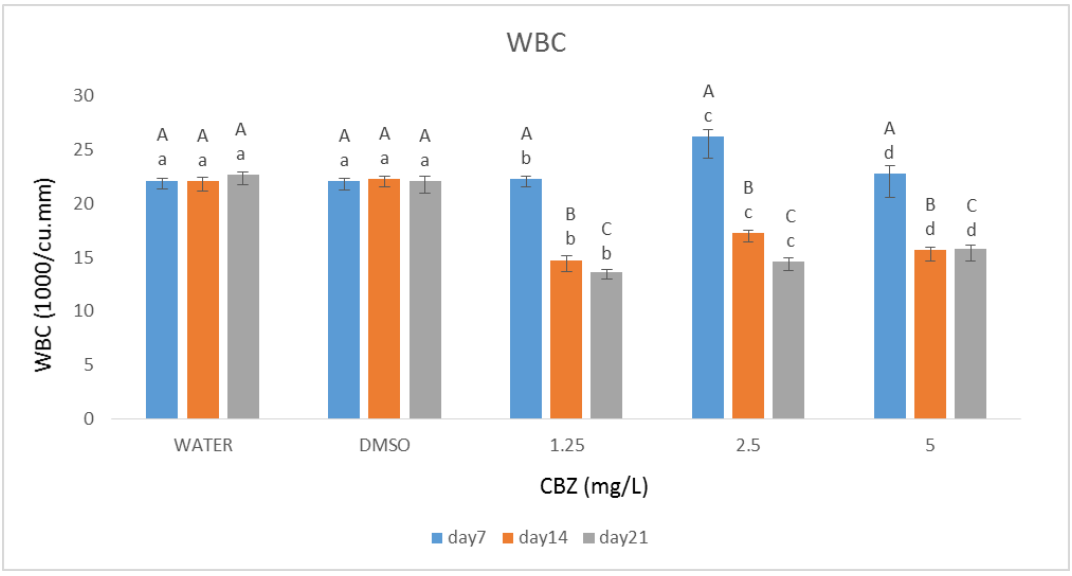

Figure 7: Changes in the WBC count in *Cyprinus carpio* exposed to CBZ.

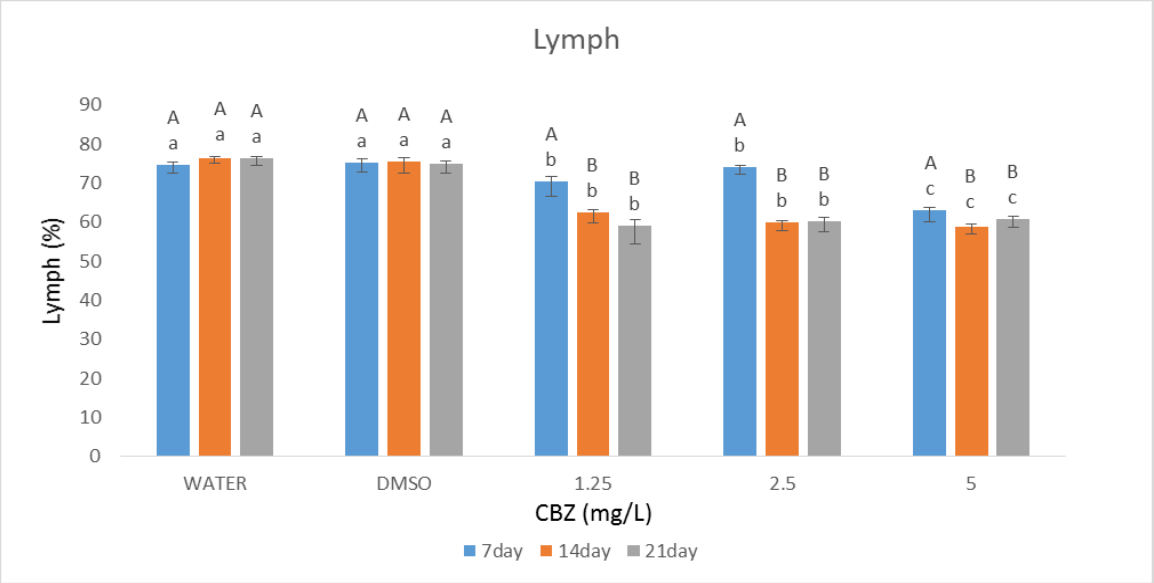

Figure 8: Changes in the Lymph percentage in *Cyprinus carpio* exposed to CBZ.

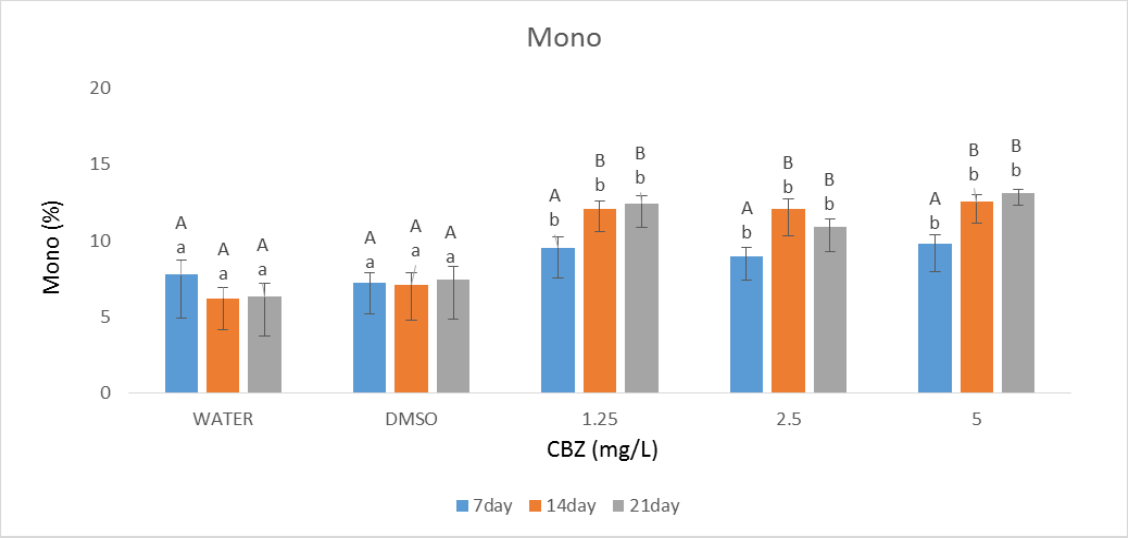

Figure 9: Changes in the Mono percentage in *Cyprinus carpio* exposed to CBZ.

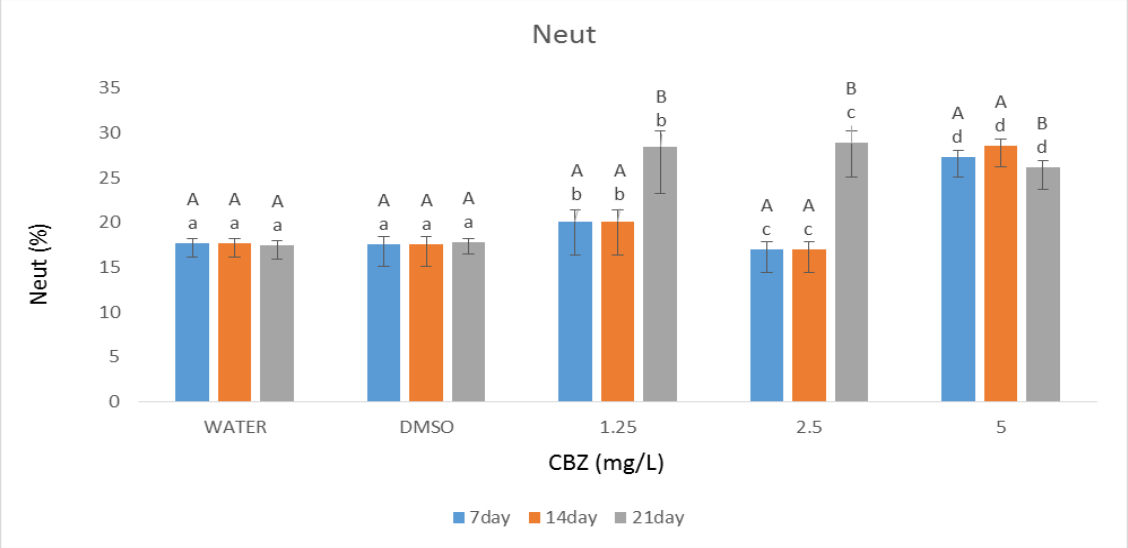

Figure 10: Changes in the Neut percentage in *Cyprinus carpio* exposed to CBZ.

Discussion

Toxicity tests were used extensively in order to evaluate the effect of the toxicity of the chemical materials on non-target organisms (Quinn *et al*., 2008; Ginebreda *et al*., 2010; Santos *et al*., 2010). Pharmaceuticals are active biologically and the persistent particles enter the aquatic environments through waste water treatment plant (WWTP) and they may affect the aquatic organisms due to their innate biological activities, both in acute and chronic concentrations. Pharmaceuticals can even agitate their toxicity effects on non-target organisms even in low concentrations and thus, studying the toxicity of these compounds can help the activities and the toxicity of the pharmaceuticals (Hoeger *et al*., 2008; Malarvizhi *et al*., 2012).

The results of present research on blood parameters showed a reduction in the count of red blood cells in fishes exposed to pharmaceuticals on the 14^{th} and 21^{st} days.

This may have resulted from the hemolysis of the red blood cells by carbamazepine. It may also be due to the destruction of hematopoietic centers by CBZ. Finally we also observed a reduction of RBC.

Saravanan *et al*. (2011) investigated the ecotoxicological impacts of clofibric acid and diclofenac in common carp (*C. carpio*) and demonstrated a reduction in RBC count caused either by the inhibition of erythropoiesis or by the destruction of red blood cells by the drugs CA and DCF.

In another research by Ambili *et al*. (2013) on the effects of the antibiotic oxytetracycline on Indian major carp *Labeo rohita* it was reported that due to prolonged exposure of the antibiotic, the gill region was affected, resulting in impaired osmoregulation and anemia and leading to a decrease in RBC count. The results showed that the values of Hb and HCT were increased after exposure to the drug. Despite the reduction of RBC, Hb was increased. This may be because the red blood cells swell and as a result, the values of Hb increase which was due to the presence of more oxygen in response to hypoxia induced by the drug.

In a study by Li *et al*. (2010), rainbow trout (*O. mykiss)* was exposed to different concentrations of carbamazepine (1 ug L^{-1}, 0.2 mg L^{-1}, 2 mg L^{-1}). In comparison with the control group, fishes exposed to higher concentrations of CBZ, showed higher level of hemoglobin. They showed that the significant high percentage of Hb and PCV were perhaps attributable to the erythropoiesis reactivation mechanism induced by the spleen and liver to compensate for the cerebral hypoxia induced by environmental stress.

Saravanan *et al*. (2011) reported that the elevated level of Hb content in *C. carpio*, exposed to clofibric acid and Diclofenac might have resulted from replacement of oxidized denatured Hb and to supply more oxygen to tissues. Swelling of RBCs due to stress and disorders of respiratory capacity, caused by the damage in the gill, may also increase Hct level.

MCV, MCH, MCHC are widely used for determining the size, content and density of Hb in red blood cells. (Ambili *et al*., 2013). The increase of MCV and MCH were seen in CBZ-treated fish which was simultaneous with time increase but no significant difference was seen in the value of MCHC of treatments in all the concentrations. With regard to the reduction of red blood cells and the increase of Hb and HCT by exposure to drug, may be probably due to the swell of RBCs and the signs of macrocytic anemia.

Saravanan *et al*. (2012) also suggested that increase in MCV and MCH along with the reduction in MCHC shows that the type of anemia is macrocytic.

Changes in the level of MCV, MCH and MCHC with lower and higher concentrations of drugs may be the response of stress to the drugs. The increase in MCV may be due to the increase of immature RBC. In addition, anemia due to disorders in gas exchange across the gills may lead to the increase of MCH and MCV (Caravalho and Fernandes, 2006).

An increase in MCV and MCH levels also indicates the swelling of RBCs which is caused by the toxicity of the drug. (Li *et al*., 2011b).

WBCs play a significant role in regulating the immunological function in organisms and changes in the number of WBC to pollutants, signifying the reduction of the non-specific immunity of the fishes (Kavitha *et al*., 2010).

A significant difference was seen in the exposed fish between the first week and the other days. A significant reduction was observed in the number of white blood cells and the percentage of lymphocyte on days 14 and 21. With regard to long-term exposure to carbamazepine, the hematopoietic centers may have been destructed and as a result WBC reduced.

The toxicity data in the present study also show the increase of neutrophil on the 21st day and the increase of monocytes on the 14th and 21st days in fish exposed to carbamazepine because of the neutrophils and monocytes infiltrating cells in the inflammatory response of cells to prevent their damage.

Li *et al*. (2011a) noted that generally, a decrease in lymphocyte numbers and a concurrent increase in monocytes and neutrophilia occur in response to a stressor. The results of this study were similar to our findings with regard to the changes of differential white blood cells.

Unlike the results of this study, Saravanan *et al*. (2012) reported an increase in the WBC count in fishes treated with Ibuprofen for 35 days showing that the stimulatory effect of the toxicant on the immune system and release of lymphocyte from lymphomyeloid tissue act as a defense mechanism which may increase WBC count in fishes. It seems that the reduced count of white blood cells is mainly caused by reduction in the lymphocyte count. Defects in the construction of white blood cells caused by the stress resulting from the introduction of drugs into the fish habitat, affect the hematopoietic tissues including the kidney which leads to a

decline in the production of white blood cells.

In general, changes in hematological parameters in fish exposed to pharmaceuticals indicate a compensatory response to maintain gas exchange (Li *et al*., 2011b), and this may vary in relation to the toxicant, concentration, exposure period and the tested species (Borges *et al*., 2007).

In the present study, it is concluded that CBZ with impact on hematological profiles of common carp (*C. carpio*) is toxic to aquatic organisms. These parameters can effectively be used as potential biomarkers of CBZ toxicity in the evaluation of environmental biomonitoring and it can also act as an early warning signal for pharmaceutical exposure to aquatic organisms. However, more detailed studies on the application of special biomarkers for biomonitoring human drugs are necessary.

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