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The Effects of Formaldehyde, Hydrogen Peroxide and Trichlorphon Applications on Some Hematological Stress Indicators in Mirror Carp (*Cyprinus carpio* L.)

Aysel ŞAHAN*

Çukurova University, Faculty of Fisheries, Department of Aquaculture, Adana-Turkey

*Corresponding Author: ayselsahan2@gmail.com

Research Article

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Abstract

In this study, the toxic effects of therapeutic doses of formaldehyde (37%), hydrogen peroxide (35%) and trichlorphon (Neguvon), which are commonly used for both prophylactic and treatment purposes in ectoparasites in mirror carp (*C. carpio*), were evaluated in terms of hematological stress indicators. Accordingly, 180 fish (51.13 ± 8.18 g) were kept in eighteen tanks with a volume of 70 L under freshwater conditions at 22±1°C. The study was designed as three different trials and each trial group was compared with its own control group. Formalin administration was performed for five days, as 150 ppm/30 min/day, while hydrogen peroxide administration was performed for two days as 1000 ppm/20 min/day and Trichlorphon administration, was performed for two days as 150 ppm/30 min/day. At the end of the trials, blood and serum samples collected from fish were evaluated in terms of erythrocyte (RBC), leukocyte (WBC), hemoglobin (Hb) and hematocrit (Hct) values, erythrocyte cell indices (MCV, MCH), cell sizes, leukocyte cell types (lymphocytes, monocyte, neutrophil, eosinophil), and glucose and cortisol levels. RBC, Hb, Hct and erythrocyte indices significantly decreased in all three experimental groups compared to those of the control group of each trial group ($p < 0.05$). In addition, percentile values of lymphocyte, monocyte, neutrophil cells, WBC levels, and serum glucose and cortisol levels significantly increased compared to those of the controls. ($p<0.05$). On the other hand, significant increases were determined in the blood cell sizes of the fish in the experimental groups. In the study, it has been reported that the chemotherapeutics in mirror carp caused significant differences in the blood parameters of the stress indicator and that the therapeutics used in the study could be considered as a source of stress in terms of dose and time.

Keywords: Formaldehyde, hematological parameters, hydrogen peroxide, mirror carp, trichlorphon.

Formaldehit, Hidrojen Peroksit ve Trichlorfon Uygulamalarının Aynalı Sazanda (*Cyprinus carpio* L.) Bazı Hematolojik Stres İndikatörleri Üzerine Etkileri

Özet

Bu çalışmada, aynalı sazan (*C. carpio*)'larda gerek profilaktik ve gerekse ektoparazitlerin sağaltımında yaygın olarak kullanılan formaldehit (% 37), hidrojen peroksit (% 35) ve triklorfon (Neguvon)'un tedavi edici dozlarının toksik etkileri hematolojik stres indikatörleri açısından değerlendirildi. 180 adet balık (51,13±8,18 g) 70 L hacimli onsekiz adet tankta, 22±1°C tatlı su koşullarında tutuldu. Üç farklı deneme olarak kurgulanan çalışmada, her deneme grubu kendi kontrol grupları ile karşılaştırıldı. Formalin uygulaması beş gün, 150 ppm/30dk./gün, hidrojen peroksit iki gün 1000 ppm/20 dk./gün ve triklorfon iki gün boyunca, 150 ppm/ 30 dk./gün olarak çalışıldı. Denemelerin sonunda, balıklardan toplanan kan ve serum örnekleri, eritrosit (RBC) ve lökosit (WBC) miktarları, hemoglobin (Hb) ve hematokrit (Hct) değerleri, eritrosit hücre indeksleri (MCV, MCH), hücre boyutları, lökosit hücre tipleri (lenfosit, monosit, nötrofil, eosinofil), glikoz ve kortizol düzeyleri belirlendi. Her üç ayrı deneme grubunda, RBC, Hb, Hct ve eritrosit indeksleri, her deneme grubunun kontrol grupları ile karşılaştırıldığında, istatistiksel olarak, önemli düzeyde azalmalar belirlendi ($p<0,05$). Ayrıca, lenfosit, monosit, nötrofil hücre yüzdeleri ve WBC miktarları ile serum glikoz ve kortizol seviyeleri kontrol ile karşılaştırıldığında istatistiksel olarak önemli düzeyde artış tespit edildi. ($p<0,05$). Diğer yandan deneme gruplarındaki balıkların kan hücre büyüklüklerinde önemli düzeylerde artış belirlendi. Stres göstergesi kan parametrelerinde tespit edilen önemli düzeydeki farklılıklar, aynalı sazanlarda söz konusu kemoterapötiklerin, terapötik doz ve zamanlarda kullanılmasının, stres kaynağı olarak değerlendirilebileceğini gösterdi.

Anahtar kelimeler: Formaldehit, hematolojik parametreler, hidrojen peroksit, aynalı sazan, triklorfon.

INTRODUCTION

In intensive aquaculture, non-optimal conditions, handling, high density stocking rates, transportation, biochemical and physiological changes, applications of disinfectants and different medicaments cause stress in fish at different levels (Yıldız and Karasu, 2001). Formalin (37%), Trichlorphon, an organophosphate compound (75%, Neguvon®Bayer), and Hydrogen peroxide (35%) are commonly used chemicals against parasitic diseases such as *Lernaea* spp., *Argulus* spp., *Costia* spp., *Chilodonella* spp., *Gyrodactylus*, and *Dactylogyrus* spp. infestationsthat cause high stress and mortality (Schmidt et al., 2006; Russo and Yanong, 2007; Jones et al., 2015). Chemicals such as malachite green, methylene blue, formalin, trichlorphon, copper sulphate, chloramine-t are used as chemotherapeutics against parasitic infestations in aquaculture (Powell et al., 1994; Yıldız and Polatsu, 1999). In a study on mirror carp, Neguvon (Trichlorphon) was found to be one of the most effective chemicals used in the treatment of *Larnea* spp. However, it has been reported that organophosphate compounds are acetyl cholinesterase inhibitors, which cause a number of disorders in the nerve cells of the fish, and that the enzyme acetyl cholinesterase plays a decisive role in plasma cortisol levels (Hai et al., 1997). Hydrogen peroxide has also been used in aquaculture as an immersion (bath) treatment against many different disease-causing organisms, including external parasites, bacteria and fungi on different species and life-stages of fish. It has been reported that 250-500 µl/L of hydrogen peroxide were highly toxic and lethal, and denaturated the protein structure in 15 minutes. It has been reported that hydrogen peroxide causes deformations in gills and skin especially in fingerlings with yolk-sac which have started external feeding (Schmidt et al., 2006). Glucose and cortisol are hematological parameters that are the common indicators used to determine the secondary (metabolic) responses in fish. Most of the studies focus on the determination of the appropriate dosage of these chemicals that cause stress during the treatment of parasitic diseases. In the present study, hematological parameters and differences in serum cortisol and glucose levels after formalin, hdrogen peroxide and trichlorphon applications were evaluated as the indicators of the level of stress in fish. The study is of importance to reveal the effects of therapeutic doses of these chemotherapeutic agents on fish metabolism through hematological parameters known as stress indicators, especially serum glucose and cortisol levels.

MATERIAL and METHODS

Experimental Fish and Design

Mirror carps (180 clinically healthy fish, 51.13±8.18 g, 16.71±1.05 cm) were obtained from a commercial aquaculture farm in Adana city, Turkey. During the acclimation period, fish were fed with a commercial diet (%30 crude protein) in two times a day. Feeding was stopped 24 hours before the tests and the weight measurements were performed in deep anesthesia conditions by using quinaldine sulphate. The experiment was conducted in three replicates for six different groups (Formalin, Control-F; Hydrogen peroxide, Control-H; Trichlorphon, Control-T; n=10) and the experiment was set up in 70 L, constantly aerated 18 glass aquariums (100cm x 30cm x 25cm). Water temperature, oxygen, and pH were measured daily by using YSI 6600 multiparameter (USA). During the study, the water temperature was found to be 22±1^{o}C and the oxygen content was 5.5±0.2 ppm. The pH level was in the range of 7.5±0.2.

Applications of the Chemotherapeutics

Commercial Formaldehyde (MERCK Chemicals Co., Germany) (37%), Hydrogen peroxide (Merck) and Trichlorphon (75% Neguvon®Bayer) were obtained from Kutay Chemical Co. Ltd., Turkey. In this study, formalin was administered as 150 ppm/30 min/day for five days, hydrogen peroxide as 1000 ppm/20 min/day for two days and trichlorphon as 150 ppm/30 min/day for two days. All the dosages and exposure periods were determined according to Noga (1996). Formalin -treated fish were analyzed on the fifth day of the experiment. While hdrogen peroxide and trichlorphon-treated fish were sampled for analysis on the second day of the treatment. During all applications and analyses, the fish samples were starved and all the applications were carried out as immersion (bath) treatments (Balta, 2016). Macroscopic examinations on fish were conducted just after tested chemicals (frmalin, trichlorphon and hydrogen peroxide).

Hematological Assay

Blood and Serum Collection

Blood and serum samples were taken from fish at the end of each trial. Blood and serum samples collected after the fish were anaesthetized using quinaldine sulphate (Sigma Chemical Co., Germany) at a dosage of 20 ml/L. for 4-5 minutes (Yanar and Genç, 2004). RBC, WBC, Hb, Hct values were recorded from the blood samples collected from the caudal vein of the anaesthetized fish by using a $1cm^3$ heparinized syringe. Blood smears were also prepared from the heparin-free blood samples to identify the leukocyte cell types. Also, blood samples were taken from fish to heparin-free tubes simultaneously for glucose and cortisol assays.

Blood Analysis

For RBC and WBC analysis, the Natt-Herrick solution was used and transferred to the Thoma slide. Examinations were conducted with an Olympus BX 51 light microscope at 400x magnification. The results are given as $x10^6/mm^3$ for erythrocyte and $x10^3/mm^3$ for leukocyte (Blaxhall and Daisley, 1973). The cyanmethemoglobin method was used to determine haemoglobin amount. The results were given as mg/dl. The microhaematocrit technique was used to determine haematocrit levels. The results were given in percentages (Stollen and FleTrichlorphonher, 1994). Indexes used are as follows; MCV (Mean Erythrocyte Cell Volume); MCH (Mean Erythrocyte Hemoglobin); The formulations for these indexes are as given;

MCV (Mean Erythrocyte Cell Volume) (μ^3) = (Hct) (%) / RBC ($10^6/mm^3$) x10

MCH (Mean Erythrocyte Hemoglobin) (pg) = Hb(g/100 ml)/ RBC ($10^6/mm^3$) x10 (Blaxhall and Daisley, 1973; Kocabatmaz and Ekingen, 1984; Schreck and Moyle, 1990).

Blood smears were dried in air and stained with May Grünwald – Giemsa mixture (Kocabatmaz and Ekingen, 1984). Possible changes in blood cells, structures, and types were photographed with Olympus DPI25 digital camera at 1000x magnification from the blood smears. Cell sizes were measured using a micrometer.

Serum Biochemical Analysis

Blood samples from fish samples were taken to heparin-free tubes, centrifuged at 3500 rpm and the obtained serum was stored at -20 ° C until the time of analysis. Cortisol levels were determined by Radioimmunoassay (RIA) from 100µl serum samples obtained after blood centrifugation at 2000 rpm at $+4^{\circ}C$ for 20 minutes. Accordingly, a 21-thyroglobulin rabbit antiserum was used for determination (Pickering et al., 1987). Glucose levels were measured by a colorimetric method (Burtis and Ashwood, 1999).

Statistical Analysis

Experiments were conducted in triplicates and data obtained from the three replicates were pooled and analyzed with independent sample t-test using SPSS 10.0 software. The level of significance was determined as 0.05 (Hayran and Özdemir, 1995). And also, each treatment was compared to its control group.

RESULTS

Hematological Parameters

The erythrocyte counts, hemoglobin and hematocrit levels decreased in trichlorphon, formalin and hydrogen peroxide -treated fishes. A decrease was determined in MCV and MCH levels of erythrocyte indices in all three trials ($p<0.05$). The decreases observed in RBC, Hb, Hct, MCV and MCH values in all three applications were found to be statistically significant ($p <0.05$), (Table 1).

The cell sizes in three applications are given in Table 2 while the blood photos were taken for each application shown in Figure 1. Comparing each treatment with the control group, statistically significant increases were determined in WBC value, lymphocyte, monocyte cell ratios and erythrocyte, lymphocyte, monocyte and neutrophil cell sizes ($p<0.05$).

Table 1. Hematological parameters of *C.carpio* after **exposure** to formalin, hydrogen peroxide and trichlorphon.

Applications	RBC ($x10^6/mm^3$)	Hb (g/dl)	Hct (%)	MCV ($x10^{-3}$) (μ^3)	MCH ($x10^{-4}$) (mg/cell)
Formalin	0.538.5±13.7*	6.9±1.5*	20.5±3.4*	257.5±91.3*	61.9±17.7*
Control-F	1.120±22.0	10.2±0.3	48.7±1.1	429.5±10.5	104.5±15.8
Hydrogen Peroxide	0.842.3±10.7*	6.1±1.0*	18.2±1.5*	235.2±8.2*	60.5±12.7*
Control-H	1.920.13±19.1	11.3±2.2	39.5±2.1	00.3±7.4	91.7±11.8
Trichlorphon	0.948.05±32.5*	5.9±1.0*	20.5±2.3*	250.5±8.9*	68.06±18.3*
Control-T	1.550±33.9	9.8±1.9	42.5±1.7	399.2±16.3	97.11±21.7

Formalin (F), Hydrogen peroxide (H), Trichlorphon (T), Erythrocyte cell count (RBC), hemoglobin (Hb), haematocrit (Hct) and erythrocyte cell indexes (MCV: Mean Erythrocyte Cell Volume); (MCH: Mean Erythrocyte Hemoglobin). The values in the same column indicated with the superscript differ significantly. *: $p<0.05$

Table 2. Blood cell sizes of *C.carpio* after exposure to formalin, hydrogen peroxide and trichlorphon.

Applications	Lymphocyte (μm)	Monocyte (μm)	Neutrophil (μm)	Eosinophil (μm)	Erythrocyte (μm)
Formalin	14.83±2.99*	11.91±1.11*	-	-	14.30±1.44*
Control-F	9.25±1.83	6.60±1.14	-	7.60±0.89	10.32±1.22
Hydrogen Peroxide	13.09±4.7*	12.58±1.02*	13.50±3.53	9.40±1.94	14.75±2.5*
Control-H	10.01±1.2	6.35±1.5	-	-	10.89±1.0
Trichlorphon	14.0±3.8*	8.14±0.6	14.50±3.21*	-	14.35±1.02*
Control-T	12.0±2.09	7.0±1.0	10.20±1.48	-	11.21±1.20

Formalin (F), Hydrogen peroxide (H), Trichlorphon (T). The values in the same column indicated with the superscript differ significantly. *: $p<0.05$

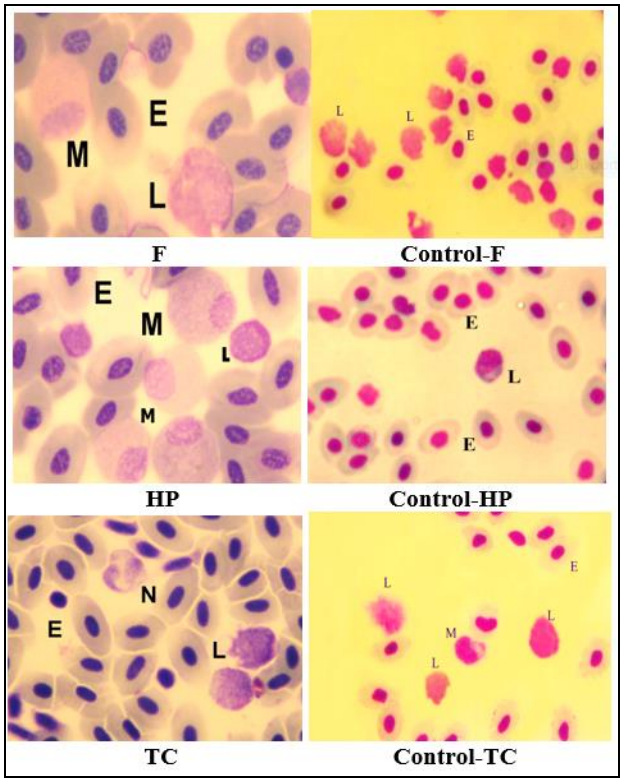

Figure 1. The Effects of Formaldehyde, Hydrogen Peroxide and Trichlorphon Applications on Blood Cells of Mirror Carp (*Cyprinus carpio* L.). Formalin (F), Hydrogen peroxide (HP), Trichlorphon (TC). **E.** Erythrocyte, **L.** Lymphocytes, **M.** Monocyte, **N.** Neutrophil (1000X).

There were significant increases in leukocyte cells after the applications, especially in the percentage values of lymphocytes, monocytes, and neutrophils ($p<0.05$). The most significant increases were observed in leukocyte cell counts and lymphocyte cell ratios. For all three applications, lymphocyte ratios increased by ten units while leukocyte cells increased approximately three-fold, compared to those of the control groups (Table 3). Similarly, increases in monocyte and neutrophil cell ratios between the groups were found to be statistically significant ($p <0.05$) (Table 3).

In the blood smear tests, since no eosinophil cells were detected, they could not be statistically evaluated among the groups (Tables 1, 2 and 3).

Table 3. Leukocyte cells of *C.carpio* after **exposure** to formalin, hydrogen peroxide and trichlorphon.

Applications	WBC ($x10^3/mm^3$)	Lymphocyte (%)	Monocyte (%)	Neutrophil (%)	Eosinophil (%)
Formalin	17.46±5.4*	85±9.0*	13.0±2.5*	12.0±3.4	2.0±0.5
Control-F	6.05±1.4	75±5.2	5.0±1.5	8.0±1.2	-
Hydrogen Peroxide	25.22±6.1*	95.80±2.1*	10.0±6.1*	2.20±1.0	1.0±0.0
Control-H	7.18±1.7	86.40±8.5	3.0±1.0	-	4.0±2.0
Trichlorphon	20.32±7.4*	88±9.3*	16.0±2.1	12.0±2.9*	4.0±1.2
Control-T	6.10±0.2	79±5.4	9.0±1.0	3.0±0.9	-

Formalin (F), Hydrogen peroxide (H), Trichlorphon (T), Leukocyte counts (WBC) and leukocyte cell types (Lymphocyte, Monocyte, Neutrophil, Eosinophil). The values in the same column indicated with the superscript differ significantly. *: $p<0.05$.

Serum Biochemical Parameters

As a result of three chemotherapeutic applications, serum glucose and cortisol values are shown in Table 4. Compared to those of the control group significant increases were determined in serum glucose and cortisol levels($p <0.05$) (Table 4).

Table 4. Serum parameters of *C.carpio* after **exposure** to formalin, hydrogen peroxide and trichlorphon.

Applications	Glucose (mg/dl)	Cortisol (ng/ml^{-1})
Formalin	137.8±16.8*	123.4±7.8*
Control-F	80.6±7.2	79.2±4.2
Hydrogen Peroxide	148.2±12.9*	129.1±5.7*
Control-H	97.2±9.1	70.5±7.7
Trichlorphon	163.2±23.4*	117.2±5.4*
Control-T	100.7±7.5	67.6±3.7

Formalin (F), Hydrogen peroxide (H), Trichlorphon (T). The values in the same column indicated with the superscript differ significantly.*: $p<0.05$

In the macroscopic examination of fish carried out prior to the trials, no significant results were found in the macroscopic health examinations other than local hemorrhage in the gills.

DISCUSSION

The increased fish production in the last two decades has exposed the fish to stressors including the high density stocking rates, handling, transportation, classification, purposes and chemical misuses. While these applications make fish more sensitive to pathogens, they make the diseases the biggest obstacle for fish production. As it's known that fish parasites and diseases have gained importance in aquaculture. On the other hand, misuse of chemicals leads to bioaccumulation and environmental pollution risks (Christybapita et al., 2007).

Therefore, in the present study, formaldehyde, hydrogen peroxide, and trichlorphon were tested in terms of some blood parameters and stress indicators.

In aquaculture, applications cause some changes in hematological parameters of fish (Treves-Brown, 2000; Davis et al., 2008; Tucmechi et al., 2011).

In numerous studies conducted for many years, blood parameters were regarded as a stress indicator. Especially physiological parameters such as serum glucose and cortisol were defined as the best indicators of stress in fish (Omoregie et al., 1998; Yıldız and Karasu, 2001; Kubilay and Uluköy, 2002). An optimal therapeutic dosage should be used for the balanced of the physiological state of fish (Barton et al., 1980; Carragher and Ress, 1994; Jung et al., 2003; Yonar et al., 2014; Bayram and Kocaman, 2017; Kankaya and Kaptaner, 2017). Formaldehyde used in the present study is a disinfectant. Moreover, formaldehyde, which is frequently used against external parasites of the fish, may not always provide the expected effect due to insufficient dose administration and its rapidly degradable structure (Balta, 2016).

Commonly formalin should not be applied more than 40 mg/L in natural environments (lakes-ponds) due to the death of planktons and decrease in dissolved oxygen amount in prolonged baths of fish (1-hour bath for 200-250 mg/L) (Parlar and Kaya, 2011). However, for cultured marine fish 150 ppm formalin /60 minutes dosage increased cortisol and glucose values (Yıldız and Ergönül, 2010). Bayram and Kocaman (2017) stated that the hemoglobin and hematocrit values were increased at the fourth hour at 12.5 mL of formalin dose. It has been reported that formaldehyde which is capable of binding 1 mg/L of oxygen in the water for each 5 mg/L water, reduced the oxygen concentration in the water, triggered the erythropoietin activity in the fish and thus increased the production of erythrocyte, causing the increase of hemoglobin and hematocrit.

In this study, regular oxygen measurements carried out during the study period kept the oxygen levels constant.

A major advantage of hydrogen peroxide as a chemotherapeutic is the minimal environmental impact related to the absence of toxic residues. In water, H_2O_2 decomposes into water and oxygen, and it has also been used as an oxygen source during the transportation of live fish or in ornamental ponds (Innes Taylor and Ross, 1988; Russo and Yanong, 2007). On the other hand, hydrogen peroxide can be toxic to fish, resulting in morbidity (e.g., gill damage) or mortality depending on the size of the fish, fish species, water temperature, concentration of the chemical, and exposure duration (Russo and Yanong, 2007). Tort et al. (2003) studied the effects of hydrogen peroxide on blood parameters in pike-perch. The researchers have reported that 10 mg/L dose was low, and 100-150 mg/L dose could be the effective dose for the treatment of bacterial gill disease. Additionally, subacute hydrogen peroxide toxicity caused anemia by the destruction of the erythrocyte membrane and oxidation of hemoglobin (Metzler, 1977; Newsholme, 1983; Innes Taylor and Ross, 1988). Hydrogen peroxide has also been used in aquaculture as an immersion (bath) treatment against many different disease-causing organisms, including external parasites (Rach et al., 2000a; Montgomery - Brock et al., 2001; Powell and Clark, 2004), bacteria (Speare and Arsenault, 1997; Lumsden et al., 1998; Rach et al., 2000b; Gaikowski et al., 2003) and fungi (Howe et al., 1999; Rach et al., 2004), on different species and life-stages of fish.

Trichlorphon as an organophosphate compound, with a lipolytic structure, can easily pass through the cell membrane and cause a toxic effect. Therefore, in the present study, a 2-day application period for Trichlorphon was applied for safety measurements according to Noga (1996). In a study by Treves-Brown (2000) on dichlorvos, a trichlorphon-like organophosphorus antihelmint, it has been reported that at 20°C, tilapia can survive for 30 minutes when treated with a dosage of 1mg/L dichlorvos while carp can survive at 30 mg/L dichlorvos for 30 minutes.

In the present study, RBC, Hb, Hct and erythrocyte indices significantly decreased compared to the control group in formalin, hydrogen peroxide, and trichlorphon applications. In addition, cell swelling, and cell disintegration were observed with the deterioration of the membrane structure in the cells and it has been reported that the decrease in the number of cells can be caused by this. Decreases on the levels of RBC, Hb, Hct, MCV and MCH were also found similarto the previous studies such as Nussey et al., (1995) and Atamanalp and Yanık (2003).

In the present study, local hemorrhages were observed in gills and significant reductions were determined in erythrocyte cells, Hb and Hct values (Table 1). Similarly, Yang and Chen (2003) explanation, the aggregation of red blood cells in damaged gills after formalin, hydrogen peroxide and trichlorphon exposure.

On the other hand, erythrocyte indexes representing the health status and anemia related to iron deficiency. The mean erythrocyte cell sizes for carp reported as 10-12 mm (Sopinska, 1984; Schindler

and De Vries, 1986; Yang and Chen, 2003). In this study, erythrocyte cell sizes of the control groups were found in the normal range for common carp (Table 2). However, erythrocyte cell sizes in treated groups were found higher and out of the range in comparison to control groups. On the other hand, two-fold differences were determined in terms of leukocyte cell sizes (Table 2).

Compared to the control group, the swelling was observed in blood cells (erythrocytes, lymphocytes, monocytes and neutrophils) in formalin, hydrogen peroxide and trichlorphon applications (Figure 1).

Various researchers have reported that toxic substances might cause morphological anomalies in erythrocytes, including nuclear anomalies, cell deformation, amitosis or hemolysis. Blood cells swell or contract depending on the osmotic pressure in the medium. It has been reported that erythrocytes in a hypotonic solution had swollen by sucking the fluid and were hemolyzed by the breakdown of the cell membrane. Swelling-like growths in the cells which comprise the first stage of cell degeneration were defined as the loss of membrane properties in the cells as the effects of electrolyte imbalance caused by chemotherapeutic agents (Narains and Strivastava, 1989).

Nussey et al. (1995) explained that the increase in the number of leukocytes (leukocytosis) was a normal reaction of the fish body, against foreign substances or chemotherapeutics. Leukocyte cell amounts and leukocyte cell percentages significantly increased in formalin, hydrogen peroxide and trichlorphon treated groups ($p<0.05$). The increase in lymphocyte cells was interpreted as the reaction of the body to the therapeutic doses of formalin, hydrogen peroxide, and trichlorphon. In addition, increases in monocyte and neutrophil cells were also evaluated as an increase in phagocytosis in cells against antigenic agents. Many investigators have demonstrated that the leukocytes of teleosts are extremely sensitive to toxicants, metals, pollutants, foreign substances, pesticides, diseases and acute stress (Nussey et al., 1995; Fast et al., 2006; Çiftçi et al., 2008). The previous study results supported the blood parameter results after the applications in *C. carpio* individuals.

Some studies have reported that significant decreases in neutrophil and monocyte levels were observed after formalin exposure. It is thought that decreases on neutrophil and monocyte levels may depend on the increases of corticosteroids which are known as stress hormones like serum glucose and cortisol. However, increases in these hormones also cause the limitation of the macrophage activity (Mustafa et al., 2000). It has been reported that classical serum indicators of stress conditions were cortisol concentrations, blood glucose concentrations, hemoglobin, and hematocrit (Pickering et al., 1987; Krumschnabel and Lackner, 1993; Tort et al., 2003). Changes in serum corticosteroids such as cortisol as an initial response and serum glucose level in fish as a secondary response can be used as indicators of stress in fish (Barton, 2002; Tucmechi et al., 2011).

Serum cortisol and glucose levels were also analyzed in the present study. Stress-related increases were observed in all three applications compared to the control group. Serum cortisol and glucose levels are widely used as a general indicator of stress conditions in vertebrates, particularly in fish (Carragher and Ress, 1994; Barton, 2002). The increase in blood glucose levels is another indicator used for the determination of stressors, glucose levels in the blood can be easily measured and the procedure relatively costs less. These increases are caused by cortisol induced gluconeogenesis, Furthermore, blood glucose value is also utilized for the indirect measure of altered cortisol secretion (Reddy and Leatherland, 1998). Blood glucose value is the most common parameter to evaluate the stress response in fish (Carragher and Ress, 1994; Tort et al., 1996; Barton, 2002, Kubilay and Uluköy, 2002). Tort et al. (1996) investigated the toxicity level of Cadmium and the effects of stress on the plasma cortisol, metallothionin levels and oxidative state in rainbow trout (*O. mykiss*). As a result, in cases where stress became chronic, there were less insignificant increases in plasma cortisol level whereas significant increases were determined in cortisol levels in case of acute stress.

In the present study, a significant increase on serum cortisol and glucose levels due to the stress were also recorded in formalin, hydrogen peroxide and trichlorphon-treated fish ($p<0.05$), in comparison to the fish in control group (Table 4). However, data obtained from previous studies indicated that the basal cortisol levels in *C. carpio* were higher (<80 ng/ml) than other fish species (Arends et al., 1998; Ruane et al., 1999). Among cyprinid species, higher basal cortisol level (100 ng/ml) was reported in *Leuciscus cephalus*, (Pottinger et al. 2000). An increase in glucose levels can be explained by the necessity of energy depending on the metabolic defense of the fish stressed by the application of chemicals. It has been reported that short-term intensive stress leads to a large increase

in the cortisol concentration followed by a slightly decrease (Barton et al., 1980). Similar studies showed that long-term stress applications result in slightly increases in serum cortisol levels while short term stress applications cause faster increases in cortisol levels (Tort et al., 1996).

Some previous studies conducted on salmon, reduced macrophage activity was reported due to the increasing glucose and cortisol levels. Thus, elevation in cortisol levels cause immunosuppression (Tort et al., 1996; Mustafa et al., 2000). It was noted that stress created in fish did not create any pressure on defense cells, but, on the contrary, it significantly increased leukocyte cell counts and leukocyte cell percentages. Compared with other studies, it was thought that this might be related to the application time and dose. In spite of the decrease in RBC, Hb, Hct and RBC indices, the increase in WBC and leukocyte cell percentages were defined as the defense indicators of stress caused by the applications on fish. However, in the present study, increases in cortisol and glucose levels did not cause repression in the defense cells in carps, on the contrary, they caused increases in defense mechanisms in the cells.

Today, the controlled use of most of the chemotherapeutic agents for all treatments and prophylactic purposes is very important in terms of human health, the health of the living thing on which the chemotherapeutic applied and environmental damage. The fact that the use of formaldehyde in aquaculture as a bath treatment in fish has significant irritation effects in the skin and respiratory system, and therefore causing respiratory problems suggests that chemicals should be preferred at minimum levels for whatever purpose they are used (Bayram and Kocaman, 2017).

CONCLUSION

In the research, the significant decreases in health indicator cells and the increases in the stress indicator parameters indicated that formalin, hydrogen peroxide, and trichlorphon applications can be evaluated as the source of stress in carp in terms of dose and time.

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