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Effects of Long-term Diazinon Exposure on Some Immunological and Haematological Parameters in Rainbow Trout *Oncorhynchus mykiss* (Walbaum, 1792)

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Abstract

Increased concentrations of various pesticides in surface waters in recent years are a serious threat to survival of fish. Immunosuppression of fish is one of the biggest concerns that endanger their health. This study aimed to assess some immunological and haematological parameters in rainbow trout, *Oncorhynchus mykiss*, after exposure to 0.00, 0.1 and 0.2 mg/L diazinon for 30 day under semi-static conditions. A significant decrease in red blood cells, hematocrit and hemoglobin levels in exposed fish indicates a state of anemia ($p<0.05$). No significant changes were observed in the mean corpuscular volume (MCV), the mean corpuscular hemoglobin (MCH) and the mean corpuscular hemoglobin concentration (MCHC) in fish exposed to diazinon ($p>0.05$). Although, white blood cells, including lymphocytes and monocytes in fish exposed to diazinon were significantly lower than control group ($p<0.05$), a significant increase in the number of neutiphils and thrombocytes were observed in fish after exposure to diazinon ($p<0.05$). The results of the present study showed a significant decrease in ACH50, lysozyme activities, and globulin and total protein levels in plasma of fish exposed to diazinon ($p<0.05$). There were no significant changes in albumin levels in fish treated by diazinon compared with control group ($p>0.05$). However, peroxidase activity levels increased in fish after exposure to diazinon ($p<0.05$). According to the results of the present study, immunosuppression and anemia occurred in fish after long-term exposure to diazinon.

Keywords: Diazinon, Immunological parameters, Hematological parameters, Rainbow trout

Introduction

Diazinon [*O,O*-diethyl *O*-(2-isopropyl-4-methyl-6-pyrimiinyl) phosphorothioate] is a moderately persistent organophosphorus insecticide largely used to control pests in agriculture[1,2]. According to previous studies, the diazinon used in agricultural areas has been found in surface waters and groundwater[3-6]. Because of its continual input into water, diazinon has been reported to be a persistent pesticide in aquatic environments[7,8]. Moreover, long-term exposures to sub-lethal diazinon concentrations have adverse effects on different biological and physiological aspects of aquatic organisms, especially fish[9,10].

Different insecticides at sub-lethal levels have been recognized as stressors causing immune-suppression in fish[11-13]. In addition, some insecticides may exert immunotoxic effects by altering the transcription of important mediators of the fish's immune system[14]. In recent years, effects of various insecticides on fish immune parameters including Interleukin-1β (IL-1β), IL-1β receptor (IL-1R1), Interferon gamma (IFN-γ2b), TNFα, MHCIα, MHCIIα, Mx, TLR9, IγML and C-reactive protein (CRP), TCRα in head- kidney leucocytes, Lysozyme activity, chemiluminuscence (CL) response and immunocompetent cells population size, white blood cells (WBC) and respiratory burst activity, head kidney phagocytes and peripheral blood leucocytes, etc. have been reported by researchers[1,15-19]. The exposure to sub-lethal concentrations of insecticides is what probably makes fish vulnerable to infectious diseases because of their immunodepressive effects[20,21].

Since immune system of fish may be affected by pesticides, the purpose of this study was to determine the effects of sub-lethal concentrations of diazinon on

some immunological and hematological parameters of rainbow trout.

Results and Discussion

No mortality was observed in fish exposed to sublethal concentrations of diazinon and control group during experimental periods. Increased mucus secretion, color changes, behavioral changes such as tremors, lethargy, unbalanced swimming, swimming in surface water and extreme irritability were important changes observed in the some fish exposed to diazinon during experimental periods. These changes intensified at the end experimental periods.

Haematology, based on erythrocyte count, leukocyte count, haemoglobin concentration, and haematocrit has provided valuable information for ichthyologist in the assessment of fish health[1]. In a previous study, Banaee *et al*.[2,9], found that damage to hematopoietic tissues of rainbow trout and common carp can have negative effects on blood cells after exposure to diazinon. Since blood cells are sensitive to oxidative stress induced by diazinon[22], a significant decrease in white blood cells and red blood cells may be caused by damage to the hematopoietic tissues and lipid peroxidation in blood cell membranes. Alterations in hematological parameters of fish exposed to different concentrations of diazinon are presented in Table 1.

Although, no significant changes were observed in red blood cells (RBC) of fish exposed to 0.1 mg/L diazinon when compared with control group on day 30 of trial ($p > 0.05$), RBC of fish significantly decreased following exposure to 0.1 mg/L diazinon on day 7 and 15 of experiment ($p < 0.05$). The results of present study show that RBC significantly decreased in fish exposed to 0.2 mg/L diazinon during experimental periods ($p < 0.05$). Treatment of rainbow trout with diazinon caused a significant decrease in hemoglobin (Hb) and hematocrit (Hct) value ($p < 0.05$). After 30 days of diazinon exposure, no significant changes were observed in the mean corpuscular volume (MCV), the mean corpuscular hemoglobin (MCH) and the mean corpuscular hemoglobin concentration (MCHC) index in fish exposed to diazinon when compared with control group ($p < 0.05$). Reduced hematocrit in fish exposed to diazinon confirms damage to red blood cells and hematopoietic tissues. Decrease in hemoglobin may reduce the efficiency of oxygen delivery by red blood cells to various tissues of body after fish exposure to diazinon. Decrease in red blood cells, hematocrit and hemoglobin are the most common clinical symptoms of anemia. Thus anemia in fish after exposure to diazinon may threaten their survival. Analysis of total leucocytes count and differential leucocyte count revealed leukopenia and lymphopenia in fish after exposure to diazinon. Our result corresponds with the results from other researchers[23-25].

Table 1. Changes in the haematological parameters of fish exposed to diazinon.

Hematological Parameters	Concentrations of diazinon	Periods when the fish were exposed to diazinon		
		7th day	15th day	30th day
RBC (10^6/μL)	0.00 mg/L	1.38 ± 0.14^b	1.35 ± 0.13^b	1.35 ± 0.11^b
	0.1 mg/L	1.10 ± 0.07^a	1.21 ± 0.09^a	1.29 ± 0.11^b
	0.2 mg/L	1.09 ± 0.06^a	1.17 ± 0.04^a	1.18 ± 0.09^a
WBC (10^4/μL)	0.00 mg/L	13.21 ± 0.33^c	12.92 ± 0.81^b	13.22 ± 1.30^b
	0.1 mg/L	12.19 ± 0.62^b	11.67 ± 1.08^a	12.15 ± 0.79^a
	0.2 mg/L	10.98 ± 0.52^a	11.69 ± 0.96^a	11.60 ± 0.93^a
Hb (g/dL)	0.00 mg/L	13.07 ± 1.56^b	11.59 ± 1.05^b	12.43 ± 1.33^b
	0.1 mg/L	10.57 ± 1.28^a	9.91 ± 0.82^a	10.77 ± 1.75^a
	0.2 mg/L	10.32 ± 1.59^a	9.95 ± 1.05^a	10.96 ± 1.06^a
Hct (%)	0.00 mg/L	44.61 ± 2.10^b	42.40 ± 3.52^b	43.78 ± 3.16^b
	0.1 mg/L	38.61 ± 3.88^a	39.17 ± 3.66^a	39.59 ± 3.23^a
	0.2 mg/L	36.64 ± 3.65^a	38.09 ± 2.41^a	40.29 ± 2.77^a
MCV (10^{-4} mm^3)	0.00 mg/L	3.27 ± 0.38^a	3.16 ± 0.28^a	3.26 ± 0.33^a
	0.1 mg/L	3.54 ± 0.50^a	3.25 ± 0.36^a	3.09 ± 0.41^a
	0.2 mg/L	3.39 ± 0.5^a	3.24 ± 0.16^a	3.23 ± 0.29^a
MCH (10^{-5} pg)	0.00 mg/L	9.58 ± 1.46^a	8.67 ± 1.14^a	9.29 ± 1.50^a
	0.1 mg/L	9.63 ± 1.13^a	8.23 ± 0.92^a	8.37 ± 1.48^a
	0.2 mg/L	9.51 ± 1.50^a	8.49 ± 1.03^a	8.71 ± 0.85^a
MCHC (%)	0.00 mg/L	29.33 ± 3.44^a	27.39 ± 2.13^a	28.63 ± 4.58^a
	0.1 mg/L	27.62 ± 4.37^a	25.54 ± 3.38^a	27.56 ± 5.81^a
	0.2 mg/L	28.38 ± 4.98^a	26.22 ± 3.30^a	27.08 ± 2.88^a

Significant differences between values when compared with control groups were characterized by alphabet symbol ($p < 0.05$). Values represent mean ± S.D.

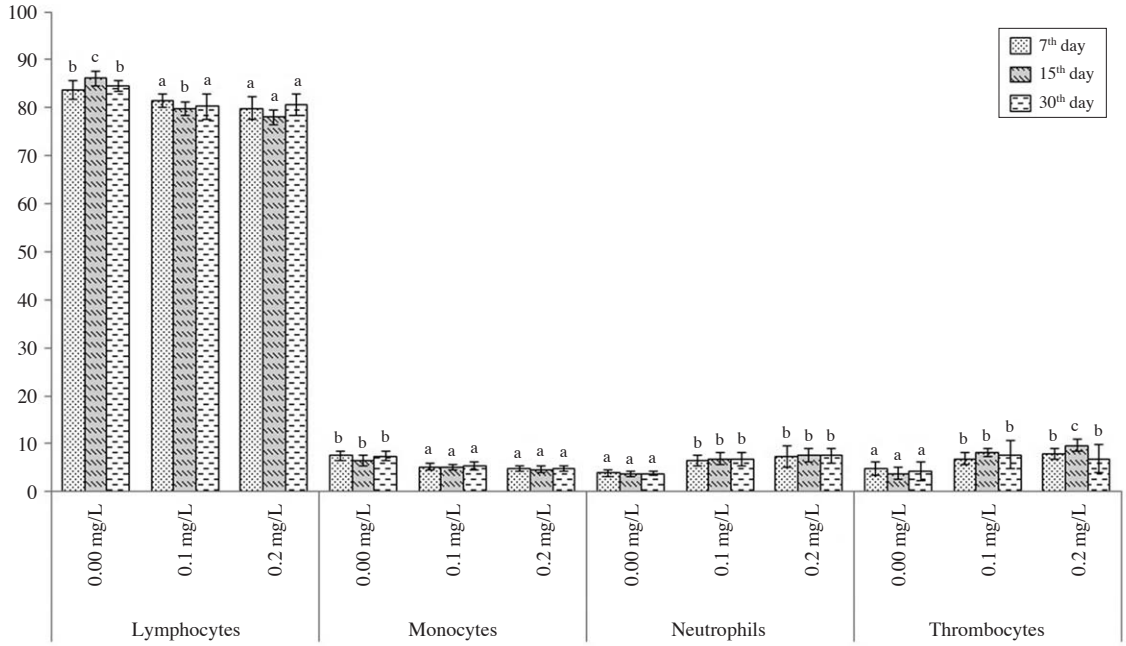

Figure 1. Leukocyte and thrombocyte counts in blood of *O. mykiss* exposed to diazinon.

There was a significant decrease in the white blood cell (WBC) of fish exposed to diazinon during experimental periods ($p < 0.05$). Changes in percentage of different types of white blood cells and thrombocytes in blood of rainbow trout after exposure to diazinon are illustrated in Figure 1. Differential white blood cell count shows that the percentage of lymphocytes and monocytes in fish exposed to diazinon were significantly lower than the control group ($p < 0.05$). Significant increases in neutrophils and thrombocytes were observed in fish exposed to diazinon during experimental periods ($p < 0.05$). Thrombocytes play an important role in blood coagulation in fish and protect an organism from bleeding in the event of injury[26]. Maintaining the fluidity of the blood is of a great importance for the survival of fish[26,27]. Since the coagulation system in fish is highly sensitive to environmental stresses[26,27], any changes in water quality may cause undesirable changes in blood clotting process. The main result of the increased number of thrombocytes is the formation of blood clots in various tissues. So according to our findings, increased thrombocytes may cause blood clots in various tissues of fish exposed to diazinon. Our results are consistent with results of other authors[27].

Changes in the blood immunological parameters of fish exposed to diazinon are presented in Table 2.

After 7 days of fish exposure to 0.2 mg/L diazinon, plasma ACH50 levels were significantly lower than control group ($p < 0.05$). A significant reduction in plasma ACH50 levels was observed in fish exposed to diazinon on day 15 and 30 of the experiment ($p < 0.05$). Complement includes over 20 different plasma proteins that are produced by a variety of cells including hepatocytes, macrophages, and gut epithelial cells. Some complement proteins bind to immunoglobulins or to membrane components of cells. The complement system is an essential and effective part of the innate immune system. It can rapidly distinguish and opsonize bacteria for phagocytosis by specialized phagocytes or destroy them directly by membrane disorder[28,29]. Thus, decrease in plasma ACH50 in fish exposed to diazinon may make them susceptible to pathogens. Similar changes were reported in the alternative complement activity in plasma of fish exposed to cadmium[30], oils and PAHs[31].

There was a significant decrease in lysozyme activity in plasma of fish exposed to diazinon on day 15 and day 30 of trial ($p < 0.05$), however, no significant changes were observed in lysozyme activity in the diazinon-treated fish when compared with control group on day 7 of experiment ($p > 0.05$). Lysozymes are a family of enzymes with antibacterial activity characterized by the ability to damage the cell wall of bacteria[28]. Thus, the significant decrease in lysozyme activity in plasma of fish exposed to diazinon may indicate debility of defence mechanisms against bacterial agents. Redaction in the blood lysozyme activity was observed in various species of fish after exposure to alpha-permethrin[12], tannery effluent[32], diazinon[33],

Table 2. Changes in the blood immunological parameters of fish exposed to diazinon.

Biochemical Parameters	Concentrations of diazinon	Periods when the fish were exposed to diazinon		
		7th day	15th day	30th day
Peroxidase (U/mL)	0.00 mg/L	124.33 ± 12.71^{a}	119.89 ± 15.48^{a}	117.33 ± 10.34^{a}
	0.1 mg/L	130.89 ± 12.38^{ab}	126.56 ± 16.80^{ab}	130.67 ± 18.24^{b}
	0.2 mg/L	139.22 ± 11.34^{b}	140.33 ± 14.35^{b}	137.89 ± 18.24^{b}
ACH50 (U/mL)	0.00 mg/L	318.33 ± 14.02^{b}	319.22 ± 18.15^{b}	320.89 ± 23.97^{b}
	0.1 mg/L	307.78 ± 13.75^{b}	284.89 ± 22.18^{a}	274.67 ± 20.98^{a}
	0.2 mg/L	289.56 ± 23.60^{a}	271.44 ± 26.10^{a}	272.78 ± 22.43^{a}
Lysozyme (U/mL)	0.00 mg/L	118.44 ± 10.99^{a}	121.67 ± 10.78^{b}	114.00 ± 12.80^{b}
	0.1 mg/L	122.11 ± 17.03^{a}	104.56 ± 8.80^{a}	98.33 ± 8.80^{a}
	0.2 mg/L	124.00 ± 16.84^{a}	100.89 ± 14.20^{a}	102.22 ± 7.77^{a}
Protein (g/dL)	0.00 mg/L	4.74 ± 0.69^{b}	4.76 ± 0.62^{b}	4.81 ± 0.34^{b}
	0.1 mg/L	3.76 ± 0.36^{a}	3.54 ± 0.42^{a}	3.67 ± 0.42^{a}
	0.2 mg/L	3.86 ± 0.35^{a}	3.64 ± 0.36^{a}	3.57 ± 0.44^{a}
Albumin (g/dL)	0.00 mg/L	2.17 ± 0.35^{a}	2.06 ± 0.30^{a}	2.28 ± 0.36^{a}
	0.1 mg/L	2.39 ± 0.30^{a}	2.18 ± 0.41^{a}	2.14 ± 0.25^{a}
	0.2 mg/L	2.33 ± 0.41^{a}	2.32 ± 0.43^{a}	2.17 ± 0.26^{a}
Globulin (g/dL)	0.00 mg/L	2.58 ± 0.71^{b}	2.70 ± 0.71^{b}	2.53 ± 0.34^{b}
	0.1 mg/L	1.37 ± 0.37^{a}	1.37 ± 0.26^{a}	1.52 ± 0.36^{a}
	0.2 mg/L	1.52 ± 0.73^{a}	1.32 ± 0.26^{a}	1.40 ± 0.45^{a}

Significant differences between values when compared with control groups were characterized by alphabet symbol ($p < 0.05$). Values represent mean ± S.D.

cypermethrin[17], deltamethrin[34].

Total protein levels in plasma of fish exposed to diazinon were significantly lower than the control group ($p < 0.05$). Although there was no significant change in albumin levels ($p > 0.05$), plasma globulin levels of fish significantly decreased following exposure to different concentrations of diazinon ($p < 0.05$). Liver failure, malnutrition as well as biochemical reaction between diazinon and the amino acid sequences of proteins found in blood may account for lower plasma total protein. Banaee *et al.*[1] and Velisek *et al.*[35] found that levels of total protein are decreased in the carp exposed to diazinon and bifenthrin, respectively. Lower globulin levels may lead to immunosuppression in fish exposed to diazinon. Similar changes were reported in the blood globulin levels of tilapia, *Oreochromis niloticus*[33], beluga sturgeon, *Huso huso*[11] and rainbow trout, *O. mykiss*[2] exposed to diazinon. Banaee[36] believed that diazinon-induced tissue destruction and hepatocyte apoptosis might be the most important agents responsible for reducing the synthesis of total protein and immunoglobulin.

However, the peroxidase activity in plasma of fish significantly rose after exposure to 0.1 mg/L diazinon on day 30 of experiment, the results of present study show that the peroxidase activity significantly increased in blood of fish exposed to 0.2 mg/L diazinon during experimental periods ($p < 0.05$). Peroxidases play an important role in defense system against extracellular bacterial and parasitic pathogens[28]. Myeloperoxidase and eosinophil peroxidase are important active peroxidases in immune system of fish are found in the cytoplasmic granules of neutrophils and eosinophils, respectively[37,38]. Our results show that peroxidase activity in plasma of fish increased after exposure to diazinon. O'Brien[37] and Awad[38] believed that any changes in environmental conditions, exposure to stressful conditions, pathogens and physical damage can be caused by increased peroxidase activities in fish. In first step, peroxidase can oxidized xenobiotic by hydrogen peroxide, but the leukocytes are stimulated and increased in peroxidase activity in blood with increasing levels of bio-accumulation of xenobiotics and their metabolites in blood of animals. In this situation, an increase in peroxidase activity can lead to xenobiotics bind with chlorine or other halogens[37].

In conclusion, immune suppression and hematological changes in fish exposed to diazinon may make them more susceptible to disease agents. Therefore, the presence of diazinon in surface waters is a potential threat to the survival of fish.

Materials and Methods

Fish and Experimental Procedure

Healthy rainbow trout, *Oncorhynchus mykiss* (Walbaum, 1792), average weight 90 ± 15 g were obtained from a private farm (Rainbow trout farm, Kordan village, Karaj, Iran.). They were maintained in closed water recirculating systems (1000 L) at the optimal laboratory conditions (temperature 15 ± 2°C; dissolv-

ed oxygen 6.5 ± 0.5 mg/L; photoperiods: 16 L : 8D; pH 7.4 ± 0.2; water hardness 150 ± 5 mg/L $CaCO_3$) in the Biology Laboratory of Aquaculture Department, Tehran University. After two weeks of acclimation, the fish were randomly divided into three groups by triplicate that each contained 12 fish. Fish were fed commercial diets (Behparvar Co. Karaj, Iran) twice a day, equivalent to 2% of their body weight. Fishes were starved for 1 day before the experiments started, and they were deprived of food for 24 h before sacrifice.

Sub-lethal Toxicity Experiments

For experimental exposure to sub-lethal concentrations of diazinon, 90 healthy fishes were distributed to 1000 L equipped fiberglass tanks with aerator in three groups of 30 individuals each.

Fishes were exposed to diazinon at a nominal concentration of 0.0 mg/L (control group), 0.1 mg/L and 0.2 mg/L, respectively, which were equivalent to approximately 10% and 20% of 96 h LC_{50} value (1.17 mg/L), for 30 days toxicity testing[2]. Test solutions of diazinon were prepared from a commercial diazinon (Partonar Co. Iran), Basudin 60 EM brand, with the active molecule diazinon [*O,O*-diethyl *O*-(2-isopropyl-4-methyl-6pyrimiinyl) phosphorothioate], purity 60% dissolved in 40% organic solvent. The water was changed daily to reduce the build-up of metabolic wastes and to keep concentrations of diazinon near the nominal level. Immunosuppression activity was evaluated on day 7, 15, and 30 of the experimental periods; 12 fish per treatment were captured and anesthetized within aquatic solution of clove powder (as powder of dried flower) (200 mg/L). Fish from each group (experimental and control) were bled from the caudal vein into sterilized glass vials at 4°C containing the anticoagulant (1% EDTA). The blood was centrifuged for 15 min at 4000 × G, 4°C. Plasma were immediately stored at −78°C until biochemistry and immunosuppression activity analysis.

Haematological Parameters

The blood was immediately used to determine the number of red blood cells (RBC) and white blood cells (WBC) by means of a haemocytometer slide at a magnification of 400 ×. Subsequently, blood was diluted to 10^{-2} and 10^{-3} in phosphate buffered saline (PBS), at pH 7.2. Haematocrit (Hct) was determined by the microhaematocrit method. Haemoglobin (Hb) concentration was conducted by using the cyanohaemoglobin method[39,40]. To differentiate blood cell type, blood smears from triplicate samples were prepared according to Banaee *et al.*[2] and examined at a magnification of 400 ×.

The mean corpuscular volume (MCV), the mean corpuscular hemoglobin (MCH) and the mean corpuscular hemoglobin concentration (MCHC) index were calculated according to following formula:

$$\text{MCH}=\frac{\text{Hb}\times 10}{\text{RBC}};\ \text{MCV}=\frac{\text{Hct}\times 10}{\text{RBC}};\ \text{MCHC}=\frac{\text{Hb}\times 100}{\text{Hct}}$$

Alternative Complement Activity

Alternative complement activity (ACH50) was evaluated following the procedure of Yano[41] using rabbit red blood cells (RaRBC). Briefly, RaRBC were washed and adjusted to 2×10^{8} cell · mL^{-1} in ethylene glycol tetra-acetic acid magnesium-gelatine veronal buffer (0.01 M). 100 µL of the RaRBC suspension was lysed with 3.4 mL of distilled water and the absorbance of the haemolysate was measured at 414 nm against distilled water to acquire the 100% lysis value. The test plasma was appropriately diluted, and different volumes ranging from 0.1 to 0.25 mL were made up to 0.25 mL total volume before being allowed to react with 0.1 mL of RaRBC in test tubes. After incubation at 20°C for 90 min with occasional shaking, 3.15 mL of a 0.9% (v/v) saline solution was added to each tube with centrifugation at 1600 × G for 10 min at 4°C. The absorbance (A) of supernatant was measured using a spectrophotometer at 414 nm. A lysis curve was obtained by plotting the percentage of haemolysis against the volume of plasma added. The volume of plasma producing 50% haemolysis (ACH50) was determined and the number of ACH50 units · mL^{-1} was obtained for each fish.

Lysozyme Activity

The turbidimetric assay for lysozyme activity was carried out according to Lange *et al.*[42] with minor modifications. Thus, plasma (50 µL) was added to 2 mL of a suspension of *Micrococcus lysodeikticus* (Actinobacteria: Micrococcaceae) (0.2 mg · mL^{-1}) in a 0.05 M sodium phosphate buffer (pH 6.2). The reaction was carried out at 25°C and absorbance was measured at 570 nm after 0.5 min and 4.5 min by spectrophotometer. PBS was used as the blank. Lysozyme of sample calibrated using a standard curve determined with hen's egg white lysozyme (Sigma) in PBS. The specific activity (units/mL plasma) for lysozyme was determined.

Peroxidases Content

The total peroxidase content present in plasma was measured according to Cuesta *et al.*[43] with modification. Briefly, 10 µL of plasma was diluted with 100 µL of Hank's balanced salt solution (HBSS). Then, 50 µL of 20 mM 3,3′,5,5′-tetramethylbenzidine hydrochloride and 2.5 mM H_2O_2 were added. The colour

change reaction was stopped after 2 min by adding 50 μL of 2 M sulphoric acid and the optical density (OD) was read at 450 nm. Standard samples without plasma were also analyzed. The peroxidase activity (units · mL^{-1} plasma) was determined defining one unit of peroxidase as that which produces an absorbance change of 1 OD.

Blood Biochemical Parameters

Plasma total protein and albumin levels were measured by using the total protein and albumin kit (Parsazmon Co. Iran). Globulin levels were calculated by subtracting albumin values from plasma total protein.

Statistical Analyses

Statistical analyses were performed using SPSS (Release 19) software. Data are presented as mean ± standard deviation. For all data, normal distribution was confirmed by the Kolmogorov-Smirnov test. Data were analyzed by one-way analysis of variance (ANOVA). Means were compared by Duncan test and a $p < 0.05$ was considered statistically significant.

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