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Effects of formalin on haematological and blood chemistry in olive flounder, *Paralichthys olivaceus* (Temminck et Schlegel)

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Abstract

Erythrocytes of olive flounder, *Paralichthys olivaceus* (Temminck et Schlegel), were treated with serial concentrations of formalin (37% formaldehyde) to investigate *in vitro* haemolysis and methaemoglobin formation. In addition, the short-term toxicity of formalin concentrations of 0, 100, 212 and 300 ppm was also studied by clinical tests in which fish were subjected to 3-h bath exposure. There was no haemolysis of fish erythrocytes exposed to formalin concentrations ranging from 31.3 to 2000 ppm. Methaemoglobin formation, however, was induced at concentrations greater than 500 ppm. Red blood cell count, haemoglobin, haematocrit, mean corpuscular haemoglobin concentration and percentage of immature erythrocytes were also markedly elevated in all formalin-exposed groups ($P < 0.05$). Formalin exposure also caused significant increases in alkaline phosphatase, aspartate aminotransferase, lactate dehydrogenase, potassium, chloride, magnesium and inorganic phosphorus ($P < 0.05$). However, total protein decreased significantly in the formalin-exposed groups ($P < 0.05$). No significant differences in white blood cell count, mean corpuscular volume, mean corpuscular haemoglobin, albumin, glucose, total cholesterol, high-density lipoprotein cholesterol, free cholesterol, alanine aminotransferase, calcium, creatinine and total bilirubin were observed in the formalin-exposed groups ($P > 0.05$).

Keywords: olive flounder, formalin, haemolysis, methaemoglobin, haematological parameters, serum chemistry

Introduction

Formalin is a liquid formulation of 37% formaldehyde gas dissolved in an aqueous solution. It has been employed to control external parasitic protozoans and monogenetic trematodes in fish by bath exposures ranging from 167 to 250 ppm formalin for 1 h (Roberts 1978; Schnick 1988; FDA 1992, 1998). Formalin is also used for the control or prevention of mortalities associated with fungal infections on fish eggs at 1000–2000 ppm for 15 min. It is approved for use as an aquatic chemotherapeutant in both the US and Canada (Schnick, Alderman, Armstrong, Gouvello, Ishihara, Lacierda, Percival & Roth 1997), and is customarily used in Asia (Main & Rosenfeld 1996). The US Food and Drug Administration (FDA) approved three commercial formaldehyde products for use in US aquaculture to control fungi on all fish eggs and external parasites on all fish. The three products have the same formulation of about 37% formaldehyde. The withdrawal time for these products is zero when use is consistent with the label recommendations. However, regulation on the therapeutic usage of formalin toward the aquaculture industry and also guide directions to fish farmers are imperfect in Asia.

Currently, concern has been raised regarding the high concentrations (800–1000 ppm) of formalin solution that are used to control external parasites of tiger puffer (*Takifugu rubripes*) reared in cages in Japan (NRIA 1998).

Olive flounder *Paralichthys olivaceus* is the most important marine cultured finfish in Korea, and its culturing production in 2002 was about 44 172 metric tonnes or 43% of the total marine culturing production of 102 533 metric tonnes. Olive flounder have been cultured in land-based tanks and are valuable food species in Korea. Nowadays, the aquaculture industry of olive flounder can be classified into fertilized egg production, seed production, and on-growing business sectors. With the increased production of cultured olive flounder, the application of toxic chemicals, including antibiotics, to diseased fishes became more frequent. Formalin is usually used to control parasitic infections such as *Trichodina*, *Ichthyobodo*, *Chirodonella* and *Sucuticociliata* in fish. A short-term bath in the range of 100–300 ppm of formalin for 1 h is generally recommended (Chun 1992). In practice, some fish farmers apply formalin for periods exceeding the recommended levels. Therefore, these abnormal culture practices resulted in biological system damage of cultured fish, which could lead to impaired growth and delayed mortalities.

Red blood cell (RBC) counts, haemoglobin (Hb), haematocrit (Ht), immature erythrocytes and some haemochemical parameters are suitable measures of the physiological damages of organs in human clinical medicine as well as in fish. Therefore, blood values have been used to demonstrate the toxic effects of formalin exposure in many species of freshwater finfish (Wedemeyer 1971; Smith & Piper 1972; Wedemeyer & Yasutake 1974; Williams & Wootten 1981; Beevi & Radhanrishnan 1987; Kakuta, Namba, Uematsu & Murachi 1991; Yamamoto 1991; Omoregie, Eseyin & Ofojekwu 1994). However, changes in Hb, plasma chloride (Cl^-) and calcium (Ca^{2+}) after formalin exposure have not been consistent even in the same fish species such as carp (Kakuta *et al.* 1991; Yamamoto 1991), and trout and salmon (Wedemeyer 1971; Wedemeyer & Yasutake 1974).

Although the appearance of abnormal RBC increased as a result of formalin exposure in olive flounder, *P. olivaceus* (Cho, Chun & Yang 1997), nothing is known about the influence of formalin on haematological and haemochemical changes in olive flounder. Therefore, the aim of the present study was to evaluate adverse effects of the therapeutic usage of formalin on certain haematological and serum chemistry parameters in cultured *P. olivaceus*, using a clinical approach. Furthermore, the present study is a continuation of the evaluation revealing that 1-h exposure of 100–300 ppm formalin had no effect on the fish.

Materials and methods

Test fish

Healthy, cultured olive flounders weighing 390–480 g were selected from a local fish farm in Koje, Korea. They were acclimated for a month to 400-L aquaria with flow-through filtered seawater at the Disease Laboratory of the National Fisheries Institute, Busan, Korea. During this conditioning period, they were fed commercial olive flounder pellets (Jeilfeed, Daejeon, Korea). They were starved for 48 h before blood sampling. The water temperature, pH, salinity and dissolved oxygen were maintained at 19.5 ± 1 °C, 7.99, 34.8‰ and 7.5 ppm respectively.

In vitro exposure of blood to formalin

Blood was sampled from the caudal vessel of the fish using a heparinized syringe (23-G needle) after the fish were anaesthetized with 0.015% 3-aminobenzoic acid ethyl ester (Sigma Chemical, St Louis, MO, USA). Per cent haemolysis and methaemoglobin formation were determined using blood treated with formalin (37% formaldehyde, Kishida Chemical, Osaka, Japan) at concentrations ranging from 31.3 to 2000 ppm. In the present study, 37% formaldehyde was regarded as 100% formalin. The procedure followed that of Kawatsu, Yamazaki & Miyamori (1991).

Blood examination after formalin exposure

Four tanks (400 L) were filled with filtered seawater and a net cage was placed within each tank. Formalin was added to three tanks at 100, 212 and 300 ppm. The fourth tank, without formalin, was used as a control tank. Four groups of fish, each consisting of 20 fish, were placed in the net cages for 3 h. Blood was taken immediately after the exposure to minimize the stress of sampling time. For the haematological tests, 0.5 mL of blood was taken from the caudal vessel of 10 fish after the fish were anaesthetized at the end of the exposure. Each blood sample was placed in a tube on ice. Also, approximately 2 mL of blood was collected from another 10 fish with an unheparinized syringe for blood serum chemistry. Each of these samples was transferred into a glass test tube

and allowed to clot at room temperature for 1 h. Serum was obtained by centrifugation at 720 *g* for 15 min and stored at −80 °C for 2 or 3 days until analysed. Blood smears were also prepared immediately after the blood was withdrawn, and were stained with Giemsa for the observation of white blood cell (WBC) count, immature erythrocytes and erythrocyte size. The experiment was conducted in duplicate.

Haematological tests

The percentage of immature erythrocytes and the WBC count were calculated as previously described (Kawatsu 1980). The Price–Jones curve was the same as that used for erythrocyte size determined by measuring 200 cells, except for immature cells. Haematological tests were completed using the heparinized blood. RBC count and Hb were determined according to the procedure of Kawatsu (1980). Ht was measured by microhaematocrit method. Mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) associated with RBC indices were calculated by standard methods in clinical haematology.

Analysis of blood serum chemistry

Serum chemistry was analysed by spectrophotometry (DMS 80 UV visible, Varian, England) using clinical test kits (Asan Pharm., Seoul, Korea). Total protein, albumin, aspartate aminotransferase (AST) and alanine aminotransferase (ALT), inorganic phosphorus (IP), Ca^{2+}, magnesium (Mg^{2+}), alkaline phosphatase (ALP), total bilirubin and creatinine were measured using the biuret method, BCG method, Reitman–Frankel method, molybden blue method, OCPC method, xylidyl blue method, Kind–King method, AAB method and Jaffe method, respectively. Glucose, total cholesterol, high-density lipoprotein (HDL) cholesterol, free cholesterol and lactate dehydrogenase (LDH) were measured using the pyruvate substrate method. Potassium (K^+) and Cl^- were measured using an automated electrolyte analyser (Hitachi 4375, Tokyo, Japan).

Statistical analysis

Data obtained for the haematological tests and serum chemistry were analysed by one-way analysis of variance (ANOVA) and Tukey's multiple comparison test using Microsoft Excel 2000 (Redmond, WA, USA). Differences were reported as statistically significant when $P < 0.05$ (Zar 1984).

Results

In vitro haemolysis and methohaemoglobin formation

Figure 1 represents *in vitro* haemolysis and methaemoglobin formation in the RBCs of the fish treated with different concentrations of formalin. Haemolysis was not observed even at the highest concentration of 2000 ppm. In contrast, the methaemoglobin values gradually increased at 500 ppm (13%) and reached approximately 30% at 2000 ppm.

Haematological tests

Table 1 summarizes the results of the haematological tests after formalin exposure. Hb and Ht significantly increased in all test groups, and RBC count at the highest concentration of 300 ppm was significantly higher than the control ($P < 0.05$). The MCHC increased significantly at the highest concentration, whereas MCV and MCHC did not show any significant difference in any of the treated groups ($P > 0.05$). The characteristics of MCV were reflected in the Price–Jones curves of major diameter of erythrocytes (Fig. 2). In the control group, the major diameter of RBCs was 8.99 ± 0.33 μm. The order of size distribution was 8–9 μm (55.9%) > 9–10 μm (34.5%) > 10–11 μm (6.6%) > 7–8 μm (2.6%) > 11–12 μm (0.4%). In the treated groups, percentages of 7–8 and 8–9 μm slightly increased and those of 9–10 and 10 ~ 11 μm decreased a little, while the base of curves in treated groups did not shift toward the left or right as

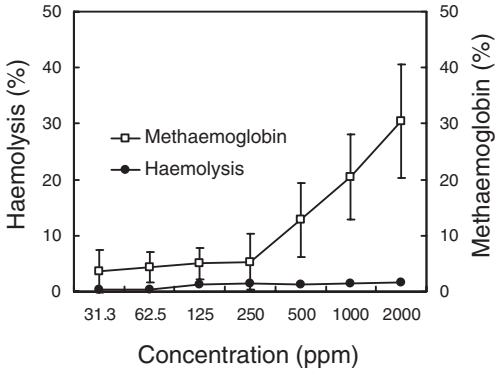

Figure 1 *In vitro* haemolysis and methaemoglobin formation in olive flounder erythrocytes at different concentrations of formalin. Each symbol with vertical bar represents mean ± SD (five samples).

Table 1 Changes of parameters of haematological tests in olive flounder after 3-h formalin exposure at different concentrations

		Formalin (ppm)		
	Control	100	212	300
Body weight (g)	422±31	437±32	429±50	426±25
Red blood cell count ($\times 10^4$ mm^{-3})	273±40^a	320±51^{ab}	315±52^{ab}	337±36^b
Haemoglobin content (g dL^{-1})	5.5±0.6^a	7.4±1.0^b	7.2±1.1^b	7.4±1.3^b
Haematocrit value (%)	29.1±2.9^a	35.8±3.4^b	35.0±3.7^b	36.2±3.3^b
Immature red blood cell count (%)	0.6±0.4^a	1.2±0.2^b	1.0±0.2^b	1.1±0.3^b
White blood cell count ($\times 10^4$ mm^{-3})	6.5±2.4	6.5±1.6	8.2±2.2	9.2±3.8
Red blood cell indicies				
Mean corpuscular volume (fL)	107.7±8.4	114.1±0.7	112.2±11.8	107.4±6.9
Mean corpuscular haemoglobin (pg)	20.5±3.2	23.3±2.5	23.1±1.5	21.8±2.4
Mean corpuscular haemoglobin concentration (%)	19.2±2.4^a	20.7±2.2^{ab}	19.8±0.9^a	22.6±1.3^b

The values are mean±SD of 10 fish. Values in rows with the same superscripts are not significantly different ($P<0.05$).

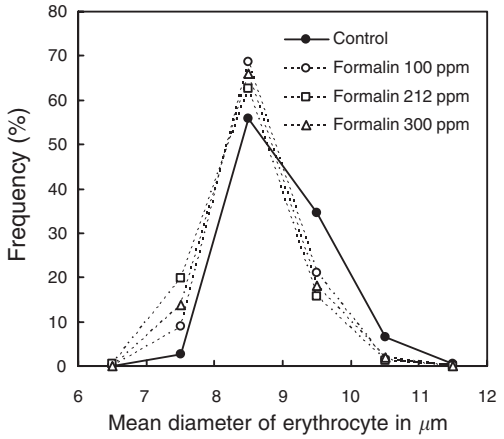

Figure 2 Price–Jones curve in olive flounder after 3-h formalin exposure at different concentrations.

compared with the control. The number of immature erythrocytes increased slightly in all treated groups ($P<0.05$). WBC count increased at concentrations of 212 and 300 ppm, but this was not significant ($P>0.05$).

Blood serum chemistry

Table 2 shows the changes in serum chemistry after formalin exposure. Total protein significantly decreased in all the treated groups ($P<0.05$), while albumin did not show any significant difference. Glucose appeared to decrease, but there was no significant difference ($P>0.05$). Significant increases were observed in Mg^{2+} in all the treated groups in Cl^- at concentrations higher than 212 ppm ($P<0.05$), and K^+ at the highest concentration of 300 ppm Ca^{2+} did not show any recognizable changes. IP significantly increased at a concentration of 212 ppm ($P<0.05$). Total cholesterol and HDL cholesterol seemed to increase in all the treated groups, but the differences were not significant ($P>0.05$). In contrast, free cholesterol appeared to decrease, but the decrease was not significant.

Among enzyme activities, LDH and ALP increased significantly at a concentration of 212 ppm ($P<0.05$), whereas no significant difference was observed in AST and ALT ($P>0.05$). No significant change was observed in total bilirubin and creatinine ($P>0.05$).

Discussion

Haemolysis is associated with the destruction of RBCs, and the formation of methaemoglobin indicates a change to the ferric (Fe^{3+}) state. Both haemolysis and methaemoglobin formation diminish the oxygen-carrying capacity of blood. Little is known about the toxic effect of parasiticides in relation to cell damage associated with RBCs in fish. Hydrogen peroxidase (H_2O_2), which is used to control the monogenetic trematode, *Benedenia seriolae*, on yellowtail (Sako 1995), induced *in vitro* haemolysis of 75% and methaemoglobin of 60% at low concentrations of 83 ppm (Kawatsu *et al.* 1991) in common carp (*Cyprinus carpio*). In the present study, formalin did not induce *in vitro* haemolysis at any of the test concentrations, but methaemoglobin formation was observed at concentrations above 250 ppm. This suggests that the mechanism of cell damage caused by formalin might be different compared with that caused by H_2O_2.

Table 2 Changes of parameters of blood serum chemistry in olive flounder after 3-h formalin exposure at different concentrations

		Formalin (ppm)		
	Control	**100**	**212**	**300**
Total protein ($g\,dL^{-1}$)	5.8 ± 0.7^{a}	4.4 ± 0.9^{b}	4.2 ± 1.1^{b}	4.3 ± 1.0^{b}
Albumin ($g\,dL^{-1}$)	1.2 ± 0.4	1.4 ± 0.4	1.3 ± 0.4	1.2 ± 0.3
Glucose ($mg\,dL^{-1}$)	57.1 ± 14.6	49.2 ± 13.8	46.7 ± 16.4	47.4 ± 20.9
Total cholesterol ($mg\,dL^{-1}$)	171 ± 60	235 ± 90	213 ± 82	234 ± 42
High-density lipoprotein cholesterol ($mg\,dL^{-1}$)	107 ± 33	115 ± 40	156 ± 81	170 ± 70
Free cholesterol ($mg\,dL^{-1}$)	88.3 ± 17.6	71.3 ± 16.3	79.2 ± 25.8	65.8 ± 20.5
Aspartate aminotransferase (Karmen unit)	33 ± 9.6^{a}	33 ± 6.9^{a}	42 ± 5.4^{ab}	46.3 ± 13.8^{b}
Alanine aminotransferase (Karmen unit)	14 ± 4.8	14 ± 3.8	16 ± 4.2	15 ± 9.1
Inorganic phosphorus ($mg\,dL^{-1}$)	12.6 ± 1.5^{a}	13.0 ± 3.0^{a}	17.3 ± 1.4^{b}	14.6 ± 2.1^{ab}
Calcium ($mg\,dL^{-1}$)	12.8 ± 2.2	12.8 ± 2.2	12.8 ± 2.2	13.1 ± 2.3
Magnesium ($mg\,dL^{-1}$)	3.4 ± 0.4^{a}	7.7 ± 2.8^{b}	5.9 ± 1.6^{b}	6.4 ± 0.6^{b}
Potassium ($mEq\,L^{-1}$)	8.0 ± 0.8^{ab}	8.0 ± 0.9^{ab}	7.6 ± 0.8^{a}	8.9 ± 1.1^{b}
Chloride ($mEq\,L^{-1}$)	139 ± 1.0^{a}	140 ± 2.7^{a}	145 ± 4.5^{b}	150 ± 1.1^{c}
Alkaline phosphatase (King–Armstrong unit)	6.0 ± 1.7^{a}	7.0 ± 1.8^{ab}	9.2 ± 1.8^{b}	8.1 ± 1.8^{ab}
Total bilirubin ($mg\,dL^{-1}$)	0.57 ± 0.22	0.57 ± 0.24	0.44 ± 0.23	0.44 ± 0.23
Creatinine ($mg\,dL^{-1}$)	0.70 ± 0.16	0.74 ± 0.26	0.61 ± 0.11	0.56 ± 0.11
Lactate dehydrogenase (Wroblewski unit)	144 ± 45^{a}	177 ± 25^{ab}	236 ± 36^{b}	217 ± 83^{b}

The values are mean $\pm$ SD of 10 fish. Values in rows with the same superscripts are not significantly different ($P<0.05$).

Acute toxicity of formalin to olive flounder has been studied previously. Park, Kim, Kim & Park (1995), using olive flounder with a mean total length of 6.1 cm, reported a 1-h LC_{50} of 2500 ppm, a 2-h LC_{50} of 1610 ppm and a 4-h LC_{50} of 870 ppm. At 24, 48, 72 and 96 h, the LC_{50} values of formalin for the fish (4.7 ± 0.4 cm; mean $\pm$ SD) were 209, 182, 158 and 141 ppm respectively (Jung & Kim 1998). Ryu, Bang, Lee, Shim & Kim (1998) also reported that the 24-h LC_{50} of formalin for the fish (17.6 ± 1.0 cm; mean $\pm$ SD) was 321.65 ppm. Jung & Kim (1998) reported 200-ppm LT_{50} to be 41 h, 225 ppm LT_{50} to be 11 h and 300-ppm LT_{50} to be 9 h, while the LT_{50} of 75–150 ppm formalin was more than 96 h. In their report, there was no cumulative mortality formalin-exposed fish to 300 ppm until 4 h after the start of the exposure.

We examined the effects of standard 1-h treatment of formalin in olive flounder based on the recommended concentration used for the removal of external parasites. However, we did not find significant changes in haematological and haemochemical values in fish after 100 to 300 ppm formalin treatment for 1 h. Therefore, olive flounder were exposed to formalin, concentrations of 100–300 ppm for 3 h to investigate the adverse effects of formalin. No fish deaths were observed during the formalin exposure. Fish were transferred to recover in a tank supplied with aerated filtered seawater after formalin exposure. The general condition and behaviour of the fish were normal during recovering days. Unfortunately, we did not measure on how long the fish needed to recover from the effects of 3-h formalin treatment.

Haematological parameters of RBC, Hb and Ht have been used as parameters of physiological stress by toxic chemicals in fish (Kawatsu 1980; Chandrasekar & Jayabalan 1993; Sampath, Velammal, Kennedy & James 1993). Increases in Hb, Ht and immature erythrocytes in carp *Cyprinus carpio* and rainbow trout *Oncorhynchus mykiss* have been reported as a result of formalin exposure (Smith & Piper 1972; Williams & Wootten 1981; Yamamoto 1991; Kakuta *et al.* 1991). These results were consistent with increases in immature erythrocytes, Hb and Ht in the present study. However, Wedemeyer & Yasutake (1974) and Kakuta *et al.* (1991) reported that Hb did not change significantly in carp, chinook salmon *O. tshawytscha* or steelhead trout *O. mykiss* post-formalin exposure. Hb is generally equivalent to the oxygen-carrying capacity of the blood (Ikeda, Ozaki & Sezaki 1986). Meanwhile, Beevi & Radhanrishnan (1987) reported a decrease in RBC, and an increase in Hb and Ht in formalin-treated tilapia (*Sarotherodon mossambicus*). It is thought that the increased Hb and Ht in tilapia were due to swelling of the RBCs. RBC size as revealed by the Price–Jones curve and values for MCV and MCHC are useful in the differential diagnosis of anaemia in fish (Kawatsu 1968, 1969, 1971, 1980; Maita, Shomitsu & Ikeda 1984). In the present study, morphological changes of erythrocytes induced by formalin were

categorized as normocytic because anisocytosis did not appear in the Price–Jones curve and there was no significant change in the MCV. Furthermore, it was slightly hyperchromic because of an increase in MCHC. Therefore, increases in Hb and Ht in the present study were due to only an increase in RBC size because there was no expansion of RBC based on the results of the MCV value and the Price–Jones curve.

Plasma glucose increased in formalin-treated carp and Nile tilapia *Oreochromis niloticus* (Kakuta *et al.* 1991; Omoregie *et al.* 1994), but serum glucose did not increase significantly in the present study. White & Fletcher (1989) suggested that glucose had been utilized faster than it was produced in plaice *Pleuonectes platessa* because of naturally low glycogen reserve in the liver of plaice. The glucose level is generally low in slower-moving bottom fish than more active species, such as yellowtail *Seriola quinqueradiata* or sea bass *Lateolabrax japonicus* (Ikeda *et al.* 1986). We have observed that, in olive flounder, the values were approximately two or three times lower than those of yellowtail or sea bass. We suggest that the small decrease in glucose in the present study may be related to the hypothesis of White & Fletcher (1989).

The changes in the enzyme activities of AST (glutamic oxaloacetate transaminase – GOT), ALT (glutamic pyruvate transaminase – GPT), ALP and LDH have been used for demonstrating liver damage caused by toxic chemicals in fish (Joshi & Desai 1984; Asztalos, Nemcsok, Benedeczky, Gabriel, Szabo & Refaie 1990; EL-Deen & Rogers 1993; Jyothi & Narayan, 1997). In general, there are significant increases in these activities in fish exposed to various chemical toxicants. In formalin-treated rainbow trout, AST was elevated significantly but both ALT and LDH were not altered significantly (William & Wootten 1981). The present results agreed with those on AST and ALT, but differed from that of LDH. Therefore, it is suggested that the significantly increased activities of AST, LDH and ALP in the present study reflect some damage in the liver of olive flounder exposed to formalin.

Differences in the sensitivity of rainbow trout and salmon to chemical treatment have been demonstrated for formalin. Wedemeyer (1971) reported hypochloraemia, hypocalcaemia and elevated bilirubin in formalin-treated rainbow trout, and no changes in formalin-treated coho salmon. Also, Wedemeyer & Yasutake (1974) reported hypochloraemia, hypercholesterolaemia and hypercalcaemia in formalin treated steelhead trout, and hypochloraemia, hypocholesterolaemia and no change in Ca^{2+} levels in formalin treated chinook salmon. Except for an increase in Cl^- levels, Ca^{2+} levels, total cholesterol and total bilirubin were not changed significantly in the present study by formalin exposure.

Casillas, Myers & Ames (1983) stated that increases in serum chemistry parameters, such as creatinine, Mg^{2+}, IP and Ca^{2+}, were potentially indicative of kidney damage in fish. Clinically, electrolytes of Mg^{2+}, IP, Ca^{2+}, Cl^- and K^+ are well correlated with the dysfunction of renal tubules and glomerula in kidney. The kidney plays an important role in regulating the Na^+, K^+ and Cl^- contents of body fluids. Alteration of these electrolytes may be the result of a complicated renal damage. Although there were no significant changes in Ca^{2+} and creatinine, increases in other electrolytes of Mg^{2+}, IP, Cl^- and K^+ in the present study showed that the kidney of olive flounder was affected by formalin exposure.

In conclusion, increases in immature erythrocytes, RBC, Hb and Ht in the present study demonstrated that formalin might have inhibited oxygen transfer by blood of the olive flounder. Moreover, the classically changed parameters of blood serum chemistry, AST, LDH, ALP, K^+, Mg^{2+}, IP and Cl^-, could reflect that some of the damage in the liver and kidney of olive flounder was affected by short-time formalin exposure.

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