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Serum biochemical characteristics of *Carassius auratus* (L) following short-term formalin or NaCl treatment

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Abstract: Goldfish (*Carassius auratus*) were subjected to either formalin (250 ppm) or NaCl (10 ppt), over a 0.5-h period followed by 24-h freshwater exposure. Serum biochemistry was monitored before exposure (0), immediately after (0.5) plus 3 and 24 h after exposure. Results showed that both formalin and NaCl treatments caused rapid increase in cortisol with a peak at 3 h, which did not recover until 24 h. Likewise, glucose showed similar patterns, however, returned to initial levels at 24 h after exposure. Formalin caused significant decrease in sodium and chloride levels which returned to initial levels at 24 h after exposure. Both formalin and NaCl caused calcium and total protein elevation at 3 and 24 h after exposure. Albumin and globulin levels were significantly affected by formalin and NaCl at 3 and 24 h after exposure. It is suggested that formalin and NaCl at the therapeutic concentrations cause rapid stress in goldfish which is eliminated after 24 h in freshwater. In addition, formalin causes slightly osmotic disturbance which is eliminated after 24 h recovery in freshwater. Both formalin and NaCl cause serum calcium and protein alteration after a while, which lasts until, at least, 24 h. More studies are needed to explain underlying mechanisms. Formalin and NaCl treatment, although advantageous in ecto-parasite removal, are stressful in goldfish, which should be considered if they are going to be used. Since formalin causes osmotic disturbance and more stress response, NaCl treatment is suggested as an alternative.

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Introduction

Goldfish is an ornamental fish and the most common one in Iran. Breading and rearing of this species is performed by locals in some regions of Iran (Hoseini and Tarkhani, 2012).

Ecto-parasites are common in fish culture activities, including goldfish culture, causing huge economic loss. Certain chemical compounds are used as therapeutic agents to control ecto-parasits infection. Formalin and NaCl are two effective therapeutics to control ecto-parasites. 250 ppm formalin and 10 ppt NaCl over a 30-min is effective to remove ecto-parasites in fish (Klinger and Francis-Floyd, 1998). Beside the anti-parasitic effects of these therapeutics, they may be used as a prophylactic agents in healthy fish.

Although therapeutics have the health benefit for fish, they might cause adverse effects on fish. For instance, formalin is a reducing agent forming methylene cross-link in proteins (Chang and Gershwin, 1992). Also, formalin treatment causes pathological symptoms in fish gills (Wedemeyer, 1971). Similarly, NaCl exposure causes osmotic disturbance and stress in freshwater fish.

There are several studies on the effect of formalin treatment (Wedemeyer 1971; Nieminen et al., 1983; Powell et al., 1996; Sykes et al., 2011) and NaCl exposure on freshwater fish (Bœuf and Payan, 2001; Gupta and Hanke, 1982; Hoseini and Hosseini, 2010). However, no study has investigated the effect of therapeutic dose of formalin and NaCl on stress response and serum characteristics in goldfish. Thus, in the present study, adult goldfish were subjected to 250 ppm formalin or 10 ppt NaCl over a 30-min period (Klinger and Francis-Floyd 1998) and

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changes in their serum characteristics were investigated.

Materials and Methods

Fish and maintenance conditions: A total number of 90 fish [90.3 ± 3.1 g (mean ± SD)] were randomly distributed into 9 glass aquaria (1 × 0.4 × 0.5 m) filled with 200 L well water. Fish were fed twice a day at a rate of 2% of body weight. All aquaria were aerated continuously and 90% of the water was exchanged daily. Water temperature, pH, total hardness and dissolved oxygen were 26 ± 1°C, 7.05 ± 0.1, 190 ± 7.1 ppm and 6 ± 0.71 ppm (mean ± SD). Fish were maintained under these conditions for 1 mouth to acclimate experimental condition.

Experimental design and sample collection: Feeding was ceased 24 h before the first blood sampling. One fish was removed from each aquarium (three samples per treatment) for blood sampling. After the initial sampling, the aquaria were assigned as: control, formalin-treated (FT) and NaCl-treated (ST) with three replicates. FT and ST groups were exposed to 250 ppm formalin (Merck, Frankfurter, Germany) or 10 g L^{-1} NaCl (purchased from local supplier; 95% purity) over a 0.5 h period (Klinger and Francis-Floyd, 1998), respectively. After 0.5 h, second blood samples were collected from all groups. 3 and 24 h after exposure, further blood samples were collected from all groups. For blood sampling, fish were anesthetized using 3000 ppm clove solution over less than 1 min (Hoseini et al., 2011). Thereafter, blood samples (0.7 ml) were collected by caudal severance. Blood samples were poured in non-heparinized plastic tubes and remained at 4°C for 2 h prior to centrifugation (2000 × g, 6 min). All serum samples were stored at -20°C until further analyses.

Serum analyses: Serums were analyzed for cortisol using ELISA method (Ruane et al., 2002) using commercial kite (IBL, Gesellschaft für Immunchemieund Immunbiologie, Germany). Inter assay coefficient of variation was found to be 9.2% calculated by measuring three known concentrations of cortisol for 5 times. Glucose (glucose oxidase method), calcium (cresolphethalein complexone method), total protein (biuret method) and albumin (bromocerol green method) were determined according to Thomas (1998) using commercial available kits (Pars Azmun Co. Ltd, Tehran, Iran). Chloride levels were measured spectrophotometerically via thiocyanate method (Thomas, 1998) using available kit (Zist Chem Co. Tehran, Iran). Sodium was measured using flame photometer (SEAC, Florence, Italy; Hoseini and Hosseini, 2011). Globulin levels were calculated by subtraction of albumin from total protein.

Statistical analyses: Data were examined for normality and homogeneity of variances using the Shapiro-Wilk's and Levene's test, respectively. Accordingly, cortisol and glucose values were log-transformed. All data were analyzed using 2-way ANOVA and LSMeans' test with treatment (control, FT and ST) and time (0, 0.5, 3 and 24 h) as factors. Data are presented as the mean ± standard deviation.

Results

Treatment and sampling point had a significant effect (P<0.0001) on cortisol level (Fig. 1). In the control group, cortisol had no significant difference among 0, 0.5 and 3 h, however, at 24 h, it was significantly lower than 0.5 and 3 h (Fig. 1). In both FT and ST groups, cortisol elevated significantly (~ 20 folds) at 0.5 h compared to 0 h and stabled at 3 and 24 h, despite the significant decrease compared to 0.5 h (Fig. 1). Both FT and ST groups, showed significantly higher cortisol level compared to that of control at 0.5, 3 and 24 h (Fig. 1).

There was no significant difference in glucose level of control group at any sampling point (Fig. 2). Both FT and ST groups showed significant elevation in glucose levels at 0.5 and 3 h which returned to 0 h levels at 24 h. There was no significant difference between 0 and 24 h values among the groups. FT group showed significantly higher glucose levels at 0.5 and 3 h compared to control, while, ST group showed significantly higher levels only at 3 h.

Sodium and chloride levels did not show any changes over the time in the control and ST groups

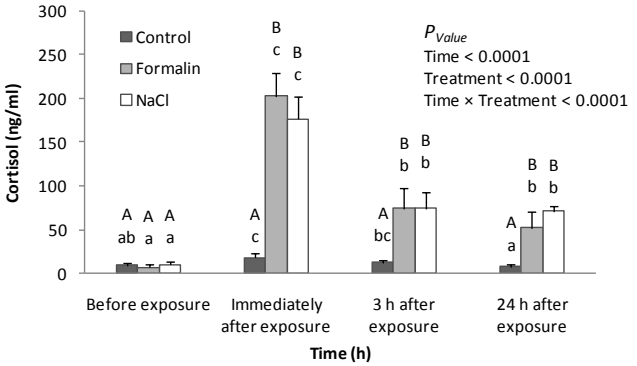

Figure 1. Effect of formalin and NaCl treatments on serum cortisol levels of goldfish *C. auratus* over the time. Different lowercase letters over the bars show significant difference in cortisol levels over the time in each treatment, separately. Different uppercase letters over the bars show significant difference in cortisol levels between the treatments at each time point, separately.

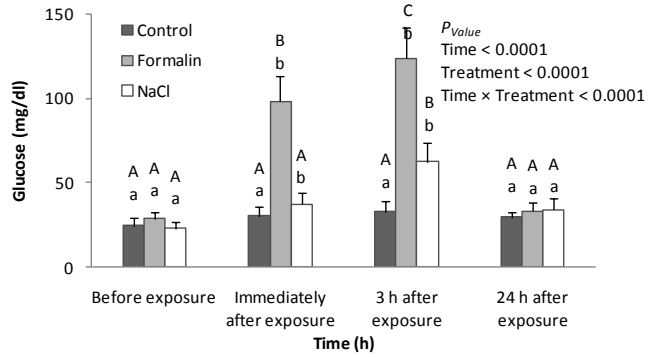

Figure 2. Effect of formalin and NaCl treatments on serum glucose levels of goldfish *C. auratus* over the time. See figure 1 for elucidation.

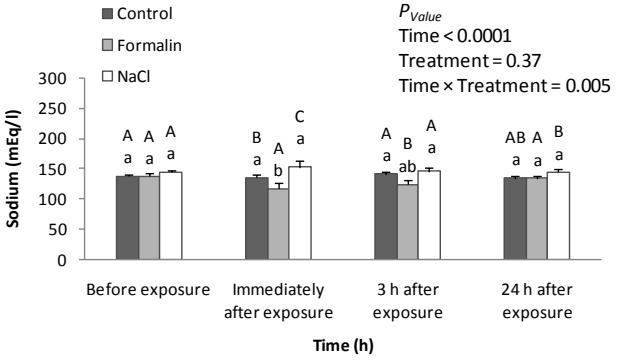

Figure 3. Effect of formalin and NaCl treatments on serum sodium levels of goldfish *C. auratus* over the time. See figure 1 for elucidation.

(Fig. 3 and 4). Conversely, in the FT group, sodium levels was significantly lower at 0.5 h compared to 0 h, however, increased at 3 and 24 h and reached the value of 0 h (Fig. 3). Chloride levels were significantly low at 0.5 and 3 h compared to 0 and

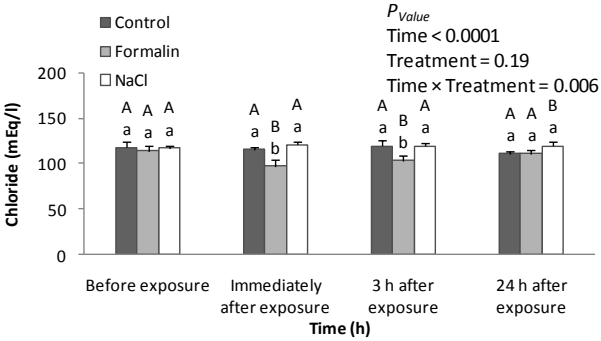

Figure 4. Effect of formalin and NaCl treatments on serum chloride levels of goldfish *C. auratus* over the time. See figure 1 for elucidation.

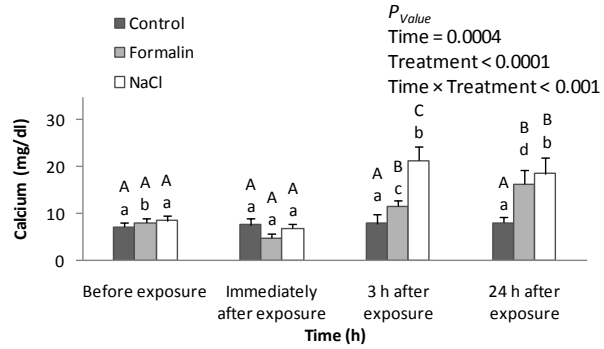

Figure 5. Effect of formalin and NaCl treatments on serum calcium levels of goldfish *C. auratus* over the time. See figure 1 for elucidation.

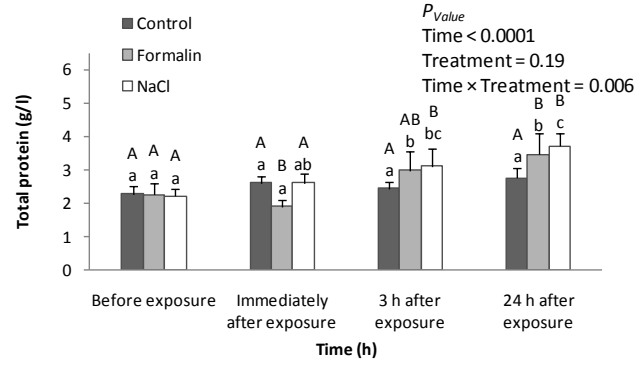

Figure 6. Effect of formalin and NaCl treatments on serum total protein levels of goldfish *C. auratus* over the time. See figure 1 for elucidation.

24 h, in FT group (Fig. 4). There was no significant change in sodium and chloride levels at 0 h among the treatments (Fig. 3 and 4). The FT group had the lowest level of sodium, then, the control and ST groups, at 0.5 h. The chloride levels of FT group were lower than those of the control and ST groups, at 0.5 h. FT group had lower sodium and chloride levels than the control and ST group, at 3 h. The sodium levels of ST group were significantly higher

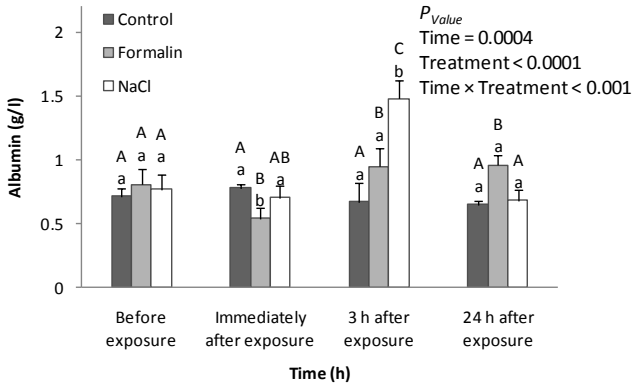

Figure 7. Effect of formalin and NaCl treatments on serum albumin levels of goldfish *C. auratus* over the time. See figure 1 for elucidation.

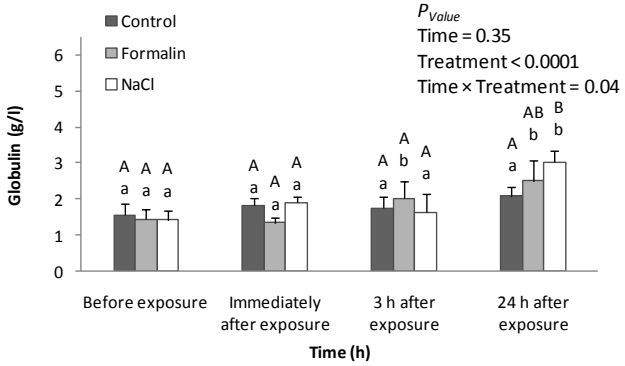

Figure 8. Effect of formalin and NaCl treatments on serum globulin levels of goldfish *C. auratus* over the time. See figure 1 for elucidation.

than that of FT group, at 24 h, however, chloride levels were significantly higher in the ST group than both control and FT groups, at this point.

Calcium levels did not change significantly over time, in the control group (Fig. 5). FT group showed a significant decrease at 0.5 h followed by an increase at 3 and 24 h, compared to 0 h values. ST group showed significant increase at 3 and 24 h compared to 0 and 0.5 h. There were no significant differences among the groups at 0 and 0.5 h. However, at 3 h, the levels of calcium significantly increased in FT group followed by an increase in ST group, compared to control. At 24 h, both FT and ST groups had significantly higher levels compared to control.

Serum levels of total protein are shown in the Fig. 6. Control group showed no significant change over time. FT group showed significant increase at 3 h which remained elevated until 24 h. ST group showed steady increase in total protein over time. There was no significant difference among the groups at 0 h, however, FT group showed significantly lower values at 0.5 h compared to both control and ST groups. At 3 h, ST group had higher levels compared to control and at 24 h both FT and ST groups showed higher levels compared to control.

Albumin levels had no change over time, in control group (Fig. 7). FT group showed significantly lower values at 0.5 h compared to the other sampling points. ST group showed ~ 2 folds increase at 3 h compared to the other sampling points. There was no significant difference among groups at 0 h. At 0.5 h, FT showed significantly lower levels compared to control. At 3 h, both FT and ST groups showed significantly higher levels compared to control, furthermore, the values of ST group was significantly higher than FT. Control and ST groups showed similar albumin levels which were significantly lower than FT group, at 24 h.

There was no significant difference in globulin levels over the time in the control group (Fig. 8). Globulin levels at 3 and 24 h were significantly higher than 0 and 0.5 h in the FT group. ST group showed significantly higher globulin levels at 24 h compared to the other sampling points. There were no significant differences among the groups at 0, 0.5 and 3 h, however, ST group showed significantly higher levels compared to the control, at 24 h.

Discussion

Chemicals may be stressful for fish being reflected in changes of serum biochemical's levels (Hoseini and Tarkhani, 2012). Serum cortisol elevation has been known as a primary stress response which increases rapidly after stress and cortisol is routinely used as an indicator of the stress in fish (Wendelaar Bonga, 1997; Barton, 2002). Hyperglycemia is secondary stress response, which is stimulated by primary stress responses (release of catecholamines and corticosteroids in to the circulation) to supply energy needed to cope with stress (Wendelaar Bonga, 1997; Barton, 2002). Formalin was found to

damage gill tissue (Wedemeyer, 1971; Smith and Piper, 1972) which could cause respiratory distress. The present results demonstrated that formalin and NaCl exposure over 0.5 h were stressful for goldfish, since near 20 folds increase in circulating levels of cortisol was observed after treatment (0.5 h). It seems that FT group experienced severe stress compared with the ST group because of higher glucose levels than ST group at 0.5 and 3 h. Also, results showed that FT and ST groups did not recover from stress at 3 h, cortisol levels did not reach the pre-treatment levels at this point, despite a marked decrease. On the other hand, glucose levels remained high, particularly FT group which showed peak at this point. The higher cortisol level at 24 h in both FT and ST groups seems not to be attributed to stress, since glucose levels reached pre-treatment levels at this point. Glucose levels remain higher during stress (Wendelaar Bonga, 1997; Barton, 2002) to ensure energy supply to cope stress. On the other hand, it was found that glucose levels remained elevated for a while even after stress termination (Ruane et al., 2002; Hoseini, 2010). Higher cortisol levels in FT and ST groups at 24 h might suggest that the fish were stressed as a result of formalin and NaCl treatment. Present results are in agreement with the previous studies (Nieminen et al., 1983; Kakuta et al., 1991; Sanchez et al., 1997; Hoseini and Hosseini, 2010) which showed stress elevation as a result of formalin or NaCl treatment in rainbow trout *O. mykiss* (Walbaum) and common carp *Cyprinus carpio* L.

Sodium and chloride are the most abundant blood ions. Maintenance of hydromineral balance is crucial in fish. There are many studies reporting increase in sodium and chloride concentrations as a result of NaCl exposure (Abohegab and Hanke, 1984; Van der Linden et al., 1999; Hoseini and Hosseini, 2010; Hosseini and Hoseini, 2010). Passive ion transfer into and from the body lead to hemoconcentration during NaCl exposure. No significant change in sodium and chloride levels in ST group might be due to low salinity and short-term exposure. The results suggest osmotic disturbance occurrence due to formalin treatment since decrement in both ions were observed at 0.5 and 3 h. Previous studies showed formalin treatment led to gill damage (Wedemeyer, 1971; Smith and Piper, 1972) and gill is the most important organ in ion transport. The sodium and chloride levels returned to initial levels at 24 h, could suggest that gill damage was not irreversible over this period. On the other hand, stress might cause ion loss in freshwater fish. Under stressful condition, fish need more oxygen, increase the respiration rate, which increases the gill permeability and passive ion loss (Wendelaar Bonga, 1997). Thus, stress caused by formalin exposure could be, at least in part, responsible for lower sodium and chloride levels in FT group.

Calcium levels showed different patterns compared to sodium and chloride, after formalin and NaCl treatment. It was expected that ions show similar pattern as a result of gill potential damage or stress. Thus, the reason of the change in calcium levels is different of that of sodium and chloride. Based on the measured parameters in this study, it is not clear why calcium showed such patterns. However, prolactin and somatolactine as well as corpuscles of Stannius are involved in calcium regulation in fish (Kaneko and Hirano, 1993). Likewise, Flik and Perry (1989) found hypercalcemic effect of cortisol via act on calcium pump in fish gill. Measurement of prolactin, somatolactin and calcium pump activity might explain the observed changes in calcium levels.

Serum levels of protein change as a result of hemoconcentration or hemodilution (Wood et al., 1983) as well as stress (Almeida et al., 2005). However, present results are hard to interpret precisely. In FT group, decrease in protein at 0.5 h might be due to hemodilution, as the levels of sodium and chloride were significantly low at this point. However, increase in protein levels of both FT and ST group, might be as a result of stress, as cortisol and glucose levels were high at this point. Amino acids are an important source for gluconeogenesis in fish and the levels might increase during stress, although previous work showed inconstancy in total protein change during stress

(Wells et al., 1986; Laidley and Leatherland, 1988; Pickering and Pottinger, 1995; Di Marco et al., 2008). Surprisingly, protein levels in both Ft and ST group were high at 24 h, when serum glucose, sodium and chloride levels had returned to initial levels. So that this elevation in serum protein might be due to another factor rather than stress or osmotic disturbance, which needs further research. On the other hand, patterns of albumin and globulin did not follow the pattern of total protein, suggesting change serum protein profile and alteration in liver protein synthesis. Further work on serum protein profile and liver protein synthesis might illustrate the change observed in this study.

In conclusion, formalin and NaCl treatment at therapeutic concentrations caused rapid stress response in goldfish which was eliminated after 24 h recovery in freshwater, however, treated fish might be stressed at this point. Formalin treatment, in addition, causes slightly osmotic disturbance which was eliminated after 24 h recovery in freshwater, too. However, formalin and NaCl treatments cause serum calcium and protein profile alteration after a while, which lasted until, at least, 24 h and more detailed studies are needed to explain underlying mechanisms. Formalin and NaCl treatment, although advantageous in ecto-parasite removal, are stressful in goldfish which should be considered when are used. Likewise, since formalin causes osmotic disturbance and more stress response (glucose levels), NaCl treatment is suggested as alternative.

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