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ORIGINAL PAPER

Analysis of primary and secondary stress responses in bighead carp (*Hypophthalmichthys nobilis*) by anesthetization with 2-phenoxyethanol

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Abstract The main objective of the present study was to shed light on the effects of 2-phenoxyethanol on possible primary (cortisol level) and secondary (hematological indices and glucose level) stress responses and changes in the activity of metabolic enzymes (AST, ALT and ALP) in bighead carp (*Hypophthalmichthys nobilis*). The fish were exposed to 0.1, 0.3, 0.5, 0.7 and 0.9 ml L^{-1} of 2-phenoxyethanol. 2-Phenoxyethanol induction and recovery times were 115–595 and 29.66–179.3 s, respectively. At a concentration of 0.1 ml L^{-1}, the anesthetic failed to induce deep anesthesia. The lowest effective concentration of 2-phenoxyethanol was determined to be 0.7 ml L^{-1}, whereas 0.9 ml L^{-1} was found to be the most effective one. Data showed that RBC, WBC, hemoglobin and hematocrit values were significantly high in some treatments compared to the control. MCV and MCH contents decreased significantly. MCHC, ALP, AST and ALT did not vary significantly. Plasma glucose and cortisol levels were significantly high compared to the control (in some doses). Moreover, Na^+, K^+, Ca^{2+}, Mg^{2+} and Cl^- did not vary significantly. Findings suggest that blood parameters were affected by anesthetizing bighead with 2-phenoxyethanol at 0.9 ml L^{-1} to the lowest extent.

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Introduction

There are several reports on anesthesia or sedation of fish species in aquaculture. Many agents are used as anesthetics in order to cope with stress and physical injuries caused by sorting, artificial reproduction, tagging, surgery, transporting and handling (Roubach et al. 2005; Velíšek et al. 2005). During experiments, anesthetics are applied under field or laboratory conditions, and this is the case also for commercial fish culture (Marking and Meyer 1985). Modern anesthetics are secure and highly soluble in water and have rapid response time and no residue accumulation in target organisms (Broîová and Svobodová 1986; Brown 1988; Ross and Ross 1999). Moreover, efficiency and cost-effectiveness are determinant criteria in choosing an anesthetic among others. According to previous studies, tricaine methanesulfonate (MS-222) (Congleton 2006; Carter et al. 2011), clove oil (Waterstrat 1999; Ucar and Atamanalp 2010), quinaldine sulfate (Munday and Wilson 1997), metomidate benzocaine (Ferreira et al. 1984), and 2-phenoxyethanol (ethylene glycol monophenyl ether) (2-PE) (Hseu et al. 2000; Jahanbakhshi et al. 2012; Javadi Moosavi et al. 2014) are found to be the most commonly used anesthetics in aquaculture.

2-PE is one of the anesthetics recently used in aquaculture. The main reasons behind 2-PE use are its fast effect and quick recovery time (Shaluei et al. 2012; Javadi Moosavi et al. 2014) because a good anesthetic should induce anesthesia quickly (in 180 s or less) while allowing fast recovery (in 300 s or less) with no toxicity to fish (Iwama and Ackerman 1994; Marking and Meyer 1985;

Mylonas et al. 2005). Moreover, easy preparation and cost-effectiveness make this agent very popular in aquaculture operations (Javadi Moosavi et al. 2014). In addition, bactericidal and fungicidal features have made it a useful anesthetic during surgery (Jolly et al. 1972).

The efficacy and anesthetic effects of 2-PE have been proved for different fish species including silver carp (McCarter 1992), platy fish (Guo et al. 1992), goldfish (Kaiser and Vine 1998), kutum (Javadi Moosavi et al. 2014), rainbow trout (Velíšek and Svobodova 2004b), common carp (Velíšek and Svobodova 2004a; Velíšek et al. 2007a) and great sturgeon (Shaluei et al. 2012). Nevertheless, before using 2-PE for any fish in large scales, it would be better to calibrate it in a laboratory scale.

An attractive tool to specify the condition of an aquatic organism is blood parameters (Bahmani et al. 2001). Analysis of blood parameters not only can provide important information about the internal environment of the organism, but also is among the most worthwhile methods available for modern diagnostics (Anver Celik 2004; Kristan et al. 2012). Hematological and biochemical profiles are regularly utilized for assessing the effect of anesthetics (Iwama et al. 1989, Jahanbakhshi et al. 2012; Kristan et al. 2012; Shaluei et al. 2012; Javadi Moosavi et al. 2014). Enzymatic activity in blood plasma is found to be primarily a stress indicator (Velíšek et al. 2011). Stress hormones (such as cortisol) activate a number of metabolic pathways, resulting in change in blood chemistry and hematology parameters (Iwama 1998).

To the best of our knowledge, there is paucity of the literature dealt with species-specific protocols for anesthetics about bighead carp with outstanding important role in Asian aquaculture. Moreover, bigheaded carp is one of the most highly cultured and consumed fishes worldwide (Michielsens et al. 2002).

Therefore, the present research aimed to evaluate changes in some hematological indices [red blood cell (RBC), white blood cell (WBC), hemoglobin (Hb), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC)] and biochemical blood profile [alkaline phosphatase (ALP), aspartate transaminase (AST), alanine transaminase (ALT), chloride (Cl^-), sodium (Na^+), potassium (K^+), calcium (Ca^{2+}), magnesium (Mg^{2+}), glucose and cortisol] values of bighead carp having been anesthetized under various concentrations (0.1, 0.3, 0.5, 0.7 and 0.9 ml L^{-1}) (according to Shaluei et al. 2012; Jahanbakhshi et al. 2012; Javadi Moosavi et al. 2014) of 2-PE and to determine the effective and safe concentrations based on blood parameters and induction and recovery times. This study was conducted in February 2015 in the Aquaculture Research Center of Gorgan University of Agricultural Sciences and Natural Resources (Gorgan, Iran).

Materials and methods

Fish and experimental conditions

A total of 180 bighead carps (mean weight 65.4 ± 5 g) were purchased from a farm in Golestan Province (Iran). Fish were transported to Aquaculture Research Center of Gorgan University of Agricultural Sciences and Natural Resources (Gorgan, Iran) with a tank equipped with oxygen injection and immediately were acclimatized for 14 days in 14,500 L.

During the adaptation period, all tanks were aerated continuously. The fish were fed twice a day with commercial diet (by-products from grain processing and organic detritus). During this period, photoperiod regimen was 12L:12D (Jahanbakhshi et al. 2012). Water temperature, pH and dissolved oxygen were assessed daily and estimated to be 26 ± 1 °C, 7.1 ± 0.05 and 5 ± 0.2 mg L^{-1}, respectively. Moreover, water exchange was 70 % a day. Feeding was stopped 24 h before the test began.

2-PE characteristics

Through dissolving pure 2-PE (99.5 %, $d = 1.107$–1.108 g l^{-1}; Sigma Chemicals, St. Louis, MO, USA) in 95 % ethanol, 2-PE stock solution was prepared by 1:10 ratio of 2-PE to ethanol.

Anesthesia test

Concentrations of 0.1, 0.3, 0.5, 0.7 and 0.9 ml L^{-1} of 2-PE were added to 15,100-L tanks. Up to 2 min (Jahanbakhshi et al. 2012) before the tests started, tanks were continuously aerated. Thereafter, 20 fish were randomly exposed to each dose. Subsequently, according to Yoshikawa et al. (1988), a chronometer was considered to record induction times. The fish were transferred to fresh and highly aerated tanks (9 ± 0.1 mg L^{-1}) as soon as they subjected to deep anesthesia phase, and their recovery time (time required to return fish to normal condition of swimming) was recorded.

Behavioral observations during anesthetic exposure

To monitor behavioral responses, fish were kept in anesthetic tanks. Anesthesia (A) and recovery (R) phases are summarized in Table 1.

Hematological and biochemical blood plasma profile of fish exposed 2-PE

In order to find changes in hematological and biochemical blood parameters, 10 fish randomly were exposed to the

Table 1 Behavioral observation of different anesthesia stages in bighead carps

Stages of anesthesia	Exhibited behavior	Description
A1	Light sedation	Decrease in reactivity to exogenous stimuli, slight reduction in opercular rate
A2	Deep sedation	Loss of large part of reactivity to external stimuli, further reduction in opercular rate
A3	Slight loss of equilibrium	Increased opercular rate and unbalanced swimming
A4	Complete loss of equilibrium	Almost horizontal position
R1	Regain of equilibrium	Normal respiratory rate
R2	Reaction to stimuli	Normal swimming

effective concentrations (0.3, 0.5, 0.7 and 0.9 ml L^{-1} 2-PE). At the same time, 10 fish were kept in 2-PE-free freshwater as control group. When fish were subjected to deep anesthesia, midline posterior and anal fins of the fish were cleaned using paper (since water may cause some contaminations). Blood samples were taken from the caudal peduncles via syringes and transferred to heparinized (under 5000 IU heparin sodium salt concentration in 1 ml) (for hematological assessments) and non-heparinized (for biochemical assessments) tubes. As for control group, to reduce stress, a wet cloth was used to cover their heads.

Hct values were determined according to microhematocrit method (at 5000 rpm for 5 min) (Goldenfarb et al. 1971). RBC and WBC were counted using the Neubauer hemocytometer (Smit and Hattingh 1980), while as for Hb values, cyanomethemoglobin method was considered (Wedemeyer and Yasutake 1977). Standard formula was adopted to compute erythrocyte-related indices (MCHC, MCV and MCH) from RBC, Hct and Hb values (Decie and Lewis 1991). A flame photometer (SEAC, Florence, Italy) was utilized to the assessment of Na^+ and K^+ values. For other electrolytes (Ca^{2+}, Mg^{2+} and Cl^-), the spectrophotometric method (WPAS2000-UV/VIS, Cambridge, UK) and standard analysis kits were used (Pars Azmoon, Tehran, Iran) (Shaluei et al. 2012).

Estimation of plasma glucose, cortisol, ALP, AST and ALT

Blood prepared in non-heparinized vials was sent to biochemical analysis. At first, the blood samples were allowed to be clotted (for half an hour at room temperature) (Tahmasebi-Kohyani et al. 2012) and subsequently centrifuged (at 3000 rpm for 10 min) (Wood et al. 1996). Finally, blood plasma was separately stored and frozen at −80 °C for following analysis (Lepic et al. 2014). Glucose plasma level was determined by spectrophotometer (WPAS2000-UV/VIS, Cambridge, UK) using commercial kits (Pars Azmoon, Iran) (Shaluei et al. 2012). In order to measure the cortisol value, ELISA kit (DRG Diagnostics, Mountainside, NJ, USA) was used (Shaluei et al. 2012). ALT, AST and ALP values were determined by the method described by Peyghan and Takamy (2002).

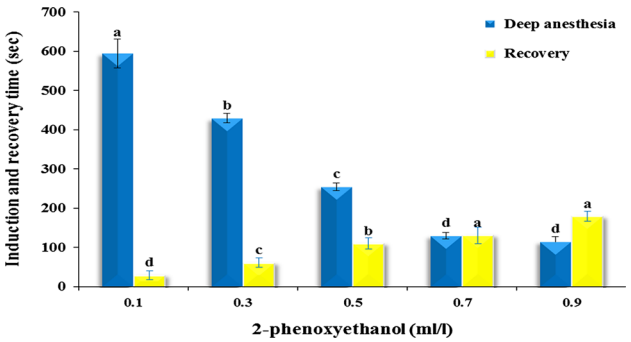

Fig. 1 Induction and recovery times for bighead carp deeply anesthetized with various concentrations of 2-PE. Data are given as mean ± SD ($n = 20$). Diverse little letters (*a* and *b*) above *bars* indicate significant differences ($\alpha = 0.05$ level) between concentrations in induction time as well as recovery

Statistical analysis

Kolmogorov–Smirnov and Levene's tests were applied to check data normality and homogeneity of variances, respectively. The effects of various concentrations of 2-PE on blood parameters (hematological and biochemical indices) were evaluated once all data were statistically processed using means of the analysis of variance (one-way ANOVA) followed by Tukey's test at the significance level of 5 %. All data were analyzed using statistical software SPSS, version 16. Data were reported as mean ± SD.

Results and discussion

Anesthetics has many applications in aquaculture. As fish differ in their response to the same anesthetic, use of different agents characterized with various concentrations seems to be necessary (Velíšek et al. 2007a). Here, induction and recovery times upon applying foregoing concentrations are shown in Fig. 1. During anesthesia, no mortalities were resulted upon applying 2-PE on any of the exposure groups. Recovery time and concentrations of the

anesthetics were found to be related positively (Limsuwan et al. 1983; Velíšek et al. 2005; Gullian and Villanueva 2009). As data illustrated, since 2-PE can induce rapid induction and recovery in a short period of time, it can be perceived as an impressive anesthetic for bighead carp. In the current study, 0.1 ml L^{-1} of 2-PE was unable to stimulate deep anesthesia, so 0.3 ml L^{-1} was considered as least effective concentration among others. Given that concentrations 0.7 and 0.9 ml L^{-1} may induce anesthesia in <180 s under recovery time which was <300 s, they are regarded as suitable effective doses (Marking and Meyer 1985). Nonetheless, 0.9 ml L^{-1} had no significant effect on most of the blood parameters (RBC, WBC, Hb, Hct and MCHC) compared with control fish, so it would be the best concentration for deep anesthesia of bighead carp in the size ever been. According to Shaluei et al. (2012), sedation is a stage that fish has not yet lost its equilibrium and so it can be useful for fish transportation. Here, 0.1 ml L^{-1} of 2-PE could be regarded as a perfect concentration for sedation induction in bighead carp.

As it can be obvious, 2-PE caused significant differences in hematological indices for bighead carp (except 0.9 ml L^{-1}, as shown in Table 2). The RBC count and Hb and Hct values were significantly high in some groups (RBC in 0.1 and 0.3 ml L^{-1}, Hb in 0.1, 0.3 and 0.5 ml L^{-1}, Hct in 0.1, 0.3 and 0.5 ml L^{-1}). Javadi Moosavi et al. (2014), Jahanbakhshi et al. (2012) and Shaluei et al. (2012) reported an increase in RBC count, Hb and Hct levels upon anesthesia under 2-PE in kutum (*Rutilus frisii kutum*), Persian sturgeon (*Acipenser persicus*) and great sturgeon (*Huso huso*), respectively. The primary reason for the observed increases in RBC count is the diffusion of immature erythrocytes from hematopoietic organs in order to supply oxygen under stress conditions (Bonga 1997). In this study, increased Hct value can be explained by increased number and/or the presence of larger erythrocytes (Gomułka et al. 2014). MCV, MCH and MCHC showed a wide range of variation. In the current study, compared to control group, MCV and MCH levels were significantly lower in fish anesthetized by 2-PE. Shaluei et al. (2012) reported no significant changes in MCV, MCH and MCHC values of great sturgeon (*Huso huso*) anesthetized by 2-PE and stated that alternation in MCV, MCH and MCHC values could be distributed to changes in Hct, Hb values and RBC counts.

In the present research, an increase in the number of leukocytes was observed. Ucar and Atamanalp (2010) reported an increase in the number of leukocytes in brown trout (*Salmo trutta fario*) upon anesthesia with 2-PE (0.2 ppm of 2-PE). In contrast, Velíšek et al. (2007b) and Shaluei et al. (2012) studied the effects of 2-PE anesthesia on sheatfish (*Silurus glanis* L.) and great sturgeon (*Huso huso*), respectively, where there were no significant

Table 2 Derived hematological indices in bighead carp, deeply anesthetized under various doses of 2-PE

Parameters	Concentrations (mean ± SD)						*P* value
	Control (0 ml L^{-1})	0.1 ml L^{-1}	0.3 ml L^{-1}	0.5 ml L^{-1}	0.7 ml L^{-1}	0.9 ml L^{-1}	
RBC ($\times 10^6$ cells/mL)	2.255 ± 0.01^{a}	2.326 ± 0.03^{bc}	2.362 ± 0.02^{c}	2.294 ± 0.00^{ab}	2.292 ± 0.00^{ab}	2.254 ± 0.00^{a}	0.00
WBC ($\times 10^3$ cells/ml)	6.34 ± 0.1^{a}	6.966 ± 0.02^{c}	6.806 ± 0.06^{c}	6.733 ± 0.15^{bc}	6.493 ± 0.09^{ab}	6.326 ± 0.09^{a}	0.00
Hb (g/dL)	8.033 ± 0.11^{a}	8.666 ± 0.25^{b}	8.766 ± 0.15^{b}	8.623 ± 0.15^{b}	8.133 ± 0.15^{a}	8 ± 0.2^{a}	0.00
Hct %	29.266 ± 0.57^{a}	30.633 ± 0.25^{c}	30.766 ± 0.11^{c}	30.266 ± 0.15^{bc}	30 ± 0.1^{abc}	29.8 ± 0.2^{ab}	0.00
MCV (fL)	135.105 ± 1.48^{a}	131.42 ± 0.07^{b}	131.52 ± 0.03^{b}	130.81 ± 0.20^{bc}	128.34 ± 0.10^{d}	129.64 ± 0.11^{cd}	0.00
MCH (pg)	39.87 ± 0.46^{a}	39.48 ± 0.27^{ab}	40 ± 0.29^{a}	39.15 ± 0.05^{b}	38.17 ± 0.06^{c}	38.09 ± 0.08^{c}	0.00
MCHC %	29.16 ± 0.30	29.2 ± 0.1	29.2 ± 0.1	28.87 ± 0.02	29.26 ± 0.30	29.2 ± 0.4	0.43

Data with different superscript letters (a, b, c and d) are significantly different $n = 10$, ($\alpha = 0.05$)

changes in the number of leukocytes compared to the control group. Playing an important role in body defensive system (Desai and Parikh 2012), an increase in the number of leukocytes is a symptom of an unfavorable condition under improper concentrations of 2-PE. Simultaneously, cortisol as a stress hormone has the potential of activating a number of metabolic pathways which in turn results in change in hematology indices (Iwama 1998).

Table 3 and Figs. 2 and 3 show effects of concentrations of interest of 2-PE on the biochemical indices of bighead carp. The physiological and blood parameters of fish are affected by the anesthetic used (Bolasina 2006; Hoseini et al. 2011). Results showed significantly high cortisol levels in fish exposed to 2-PE at 0.1, 0.3 and 0.5 ml L^{-1}. As Shaluei et al. (2012) reported, cortisol levels are increased upon anesthesia with 2-PE in great sturgeon (*Huso huso*), suggesting the activation of hypothalamus–pituitary–interrenal (HPI) axis due to different stressors, and it may be considered as a reason for a cortisol release. These results are in line with those on Persian sturgeon (*Acipenser persicus*) (Jahanbakhshi et al. 2012) and sea bass (*Lates calcarifer*) (Hseu et al. 2000) anesthetized using 2-PE. On the contrary, King et al. (2005) have reported no significant changes in cortisol levels over the 10–30 min exposure to 300 mg L^{-1} of 2-PE. Such differences can be attributed to experimental condition and differences between species. Anesthetics may lead to changes in plasma glucose levels; however, these changes are not the same in different experiments. Many authors have reported increased glucose levels (Hoseini et al. 2011; Weber et al. 2011; Shaluei et al. 2012; Javadi Moosavi et al. 2014). As per the report by Javadi Moosavi et al. (2014), significant increase in plasma glucose levels upon anesthesia by 2-PE in kutum (*Rutilus frisii kutum*) was found. Similar anesthetic-induced increases in plasma glucose are reported in red drum (*Sciaenops ocellatus*) during 10–15 min of exposure to immobilizing doses of quinaldine sulfate and MS-222 (Thomas and Robertson 1991). The release of catecholamines and glucocorticoids from the adrenal is a major reason for the observed increase in plasma glucose level (Bonga 1997).

Enzyme analysis showed no significant difference in the activity of ALT, AST and ALP enzymes (Table 3). Similarly, Shaluei et al. (2012) reported no significant changes in the activity of ALP and ALT enzymes in great sturgeon (*Huso huso*) due to anesthesia by 2-PE. In contrast, Congleton (2006) reported elevated activity of AST and ALT in Brazilian codling (*Urophycis brasiliensis*) due to anesthesia with MS-222, whereas Velíšek and Svobodova (2004a) found high ALT activity in common carp (*Cyprinus carpio*) anesthetized with 2-PE and Velíšek and Svobodova (2004b) similarly reported low AST activity in

Table 3 Derived biochemical indices in bighead carp, deeply anesthetized with various doses of 2-PE, $n = 10$

Parameters	Concentrations (mean ± SD)						*P* value
	Control (0 ml L^{-1})	0.1 ml L^{-1}	0.3 ml L^{-1}	0.5 ml L^{-1}	0.7 ml L^{-1}	0.9 ml L^{-1}	
ALP (unit/L)	273.34 ± 0.97	273.37 ± 0.79	273.13 ± 0.59	272.15 ± 0	271.39 ± 1	272.49 ± 0.8	0.054
AST (unit/L)	254.18 ± 4.31	252.7 ± 1.47	250.67 ± 1.20	249.83 ± 0.84	250.37 ± 0.1	249.9 ± 1.72	0.24
ALT (unit/L)	45.523 ± 1.07	45.566 ± 0.65	45.366 ± 0.66	45.8 ± 0.61	45.666 ± 0.51	45.566 ± 0.55	0.98
Chloride (mmol/L)	82.45 ± 4.00	86.41 ± 3.10	80.12 ± 2.50	83.26 ± 4.40	83.85 ± 5.50	82.45 ± 4.00	0.80
Sodium (mmol/L)	113.25 ± 3.01	105.15 ± 2.01	115.27 ± 4.21	112.19 ± 6.01	114.35 ± 3.31	113.25 ± 3.01	0.52
Potassium (mmol/L)	3.67 ± 1.21	3.27 ± 1.42	3.17 ± 0.45	4.11 ± 0.81	3.91 ± 1.37	3.67 ± 1.21	0.61
Calcium (mmol/L)	7.43 ± 0.35	7.85 ± 0.17	7.93 ± 0.41	7.13 ± 0.12	7.51 ± 0.45	7.43 ± 0.35	0.22
Magnesium (mmol/L)	2.11 ± 0.27	2.18 ± 0.42	2.71 ± 0.14	2.11 ± 0.27	2.07 ± 0.10	2.11 ± 0.27	0.59

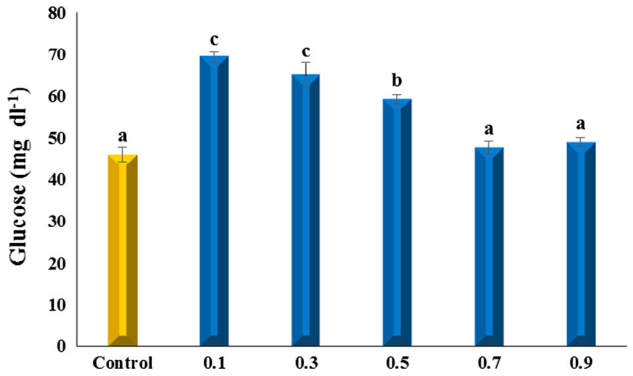

Fig. 2 Plasma glucose levels in bighead carp exposed to different doses of 2-PE. Data are given as mean ± SD ($n = 10$). Diverse little letters (*a*, *b* and *c*) above *bars* indicate significant differences ($\alpha = 0.05$ level) between concentrations

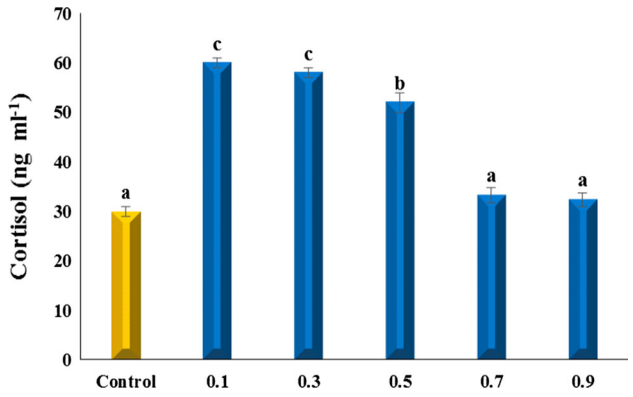

Fig. 3 Plasma cortisol levels in bighead exposed to different doses of 2-PE. Data are given as mean ± SD ($n = 10$). Diverse little letters (*a*, *b* and *c*) above *bars* indicate significant differences ($\alpha = 0.05$ level) between concentrations

rainbow trout (*Oncorhynchus mykiss*) after 2-PE anesthesia.

The increased AST and ALT activities occur following stress condition to supply gluconeogenesis substrates (lactate and amino acids). Therefore, short period of anesthesia-induced stress could be expressed as a logical reason for the lack of significant changes in the activity of these enzymes. No significant changes were observed in the Na^+, K^+, Ca^{2+}, Mg^{2+} and Cl^- values compared with the control (Table 3).

Conclusion

Finally, it is clear that 2-PE can be considered as an anesthetic which is somewhat safe for bighead carp under the concentration of 0.9 ml L^{-1}. Moreover, rapid anesthesia with the highest concentration of 2-PE (0.9 ml L^{-1}) has the least effect on physiological stress and could not be considered as a dangerous material. Therefore, 2-PE can be used in the mentioned concentration as a promising agent to reduce fish stress during handling fish and other aquaculture applications. Moreover, blood parameters assessment can be effective in determining the propitious concentrations of an anesthetic.

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