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ARTICLE

Ecotoxicological impacts of isoprothiolane on freshwater fish *Cyprinus carpio* fingerlings: a multi-biomarker assessment

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Abstract Extensive use of fungicides in agriculture has led to the detrimental effects on aquatic ecosystems and organisms even at very low concentrations. In this respect, the present study was aimed to investigate the impact of environmentally relevant concentrations of fungicide isoprothiolane (2.7 and 27 μg/L; reported concentration in Yung San River, Korea) on a freshwater fish *Cyprinus carpio* for a short-term period of 96 h using various biomarkers such as hematological, ionoregulatory, biochemical, and enzymological parameters. At both concentrations, hemoglobin, hematocrit, red blood cell, serum glucose, cholesterol, triglycerides, aspartate aminotransferase, and gill Na^+/K^+-ATPase levels were significantly ($p < 0.05$) decreased in fish treated with isoprothiolane. Contrastingly, white blood cell, serum sodium (Na^+), chloride (Cl^-), and albumin levels were increased ($p < 0.05$). However, the short-term exposure of isoprothiolane in carp resulted in slight alterations in mean cellular volume, mean cellular hemoglobin, mean cellular hemoglobin concentration, serum potassium (K^+), protein, globulin, and alkaline phosphatase levels. The present study concludes that alterations of hematological, ionoregulatory, biochemical, and enzymological parameters can be used as efficient biomarkers in monitoring the toxicity of isoprothiolane as well as other pesticides in aquatic organisms. Further, more detailed studies on using of specific biomarkers to monitor the long-term effects of isoprothiolane are needed.

Keywords *Cyprinus carpio* · Enzymes · Hematology · Ionoregulation · Isoprothiolane · Toxicity

Introduction

Korea is one of the major rice-producing countries in Asia (Jeong et al. 2012). The usage of pesticides for the control of pests in agriculture has been steadily increasing during the last 50 years (Korea Crop Protection Association 2012; Cha et al. 2014). In Korea, the insect pests of rice are usually controlled by spraying insecticides and fungicides than herbicides (Ha et al. 2012). Fungicides are mostly used in terms of volume next to insecticides. Among the fungicides, isoprothiolane is ranked in the 23 place in the top 50 pesticides according to quantity of use in Korea (Cha et al. 2014). Isoprothiolane is used as a dithiolane fungicide and also as an insecticide in rice fields to control rice blast fungus *Magnaporthe oryzae* and to reduce the population of brown plant hoppers and leaf hoppers (Ishizuka et al. 1998; Uesugi 2001; Hu et al. 2014). Typically, the mode of fungicidal action of isoprothiolane is inhibition of choline biosynthesis (Arul Selvi et al. 2013). It can reach various segments of the aquatic environment either via transfer of the chemicals from soil or by spraying against target organisms (Oruc et al. 2002). Thereby, the levels of isoprothiolane residues were found to be more in aquatic environment rather than other environment (Kilgore and Li

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1975) and become hazardous to the aquatic life (Prusty et al. 2011).

Some of the previous studies reported the occurrence of isoprothiolane and their residues in water, soil, and agricultural products in Korea. For instance, Park et al. (2007) reported that the isoprothiolane was found at 3.2 μg/L in water of Bokpocheon, Namhan River. Lee et al. (2011) detected at an average value of 0.092 μg/L in six major rivers namely, Han, Geum, Mankyung, Youngsan, Nakdong, and Seomjin in Korea. The national institute of environmental research (NIER) reported a maximum concentration of isoprothiolane (26.3 μg/L) in Yung San River, Korea (NIER 2009). In soil, it was found to be 0.01–0.06 and 0.075–0.810 mg/kg in paddy field and also 0.009–0.208 mg/L was in greenhouse soils (Park and Lee 2011). Isoprothiolane residues in water and soil are of concern as their uptake can lead to accumulation (Fushiwaki et al. 1993) that results toxic effects to the non-target species (Worthing 1991) through food chain (Arul Selvi et al. 2013). Lee et al. (2011) suggested that an integrated ecological risk assessment of isoprothiolane and their residues using various biological species is urgently needed. However, the knowledge on the toxicity and effects of isoprothiolane on aquatic organisms are meager.

Ecotoxicological effects of pesticides in aquatic ecosystem are mainly derived from the toxic effects to non-target organisms like fish (Golow and Godzi 1994). Fish are used as excellent indicator of aquatic pollution due to their high sensitivity to environmental contaminants (Mahttiessen et al. 1995). Blood parameters such as hematological, ionoregulatory, biochemical, and enzymological responses have been routinely used as sensitive toxicity indicator to different types of pesticides (Handy and Depledge 1999; Sancho et al. 2000; Singh et al. 2008) as well as valuable biomarkers to assess the functional status of the animal health (Cajaraville et al. 2000; Adhikari et al. 2004; Wu et al. 2014). To our best knowledge, there are no studies on environmentally relevant concentrations of isoprothiolane on the physiology of freshwater fishes. Consequently, the present study was aimed to evaluate the ecotoxicological impacts of environmentally relevant concentrations of isoprothiolane in *Cyprinus carpio* using various hematological, ionoregulatory, enzymological, and biochemical responses as biomarkers.

Materials and methods

Fish and water

The fish *C. carpio* fingerlings (mean length: 7.5 ± 0.3 cm and weight: 5.8 ± 0.8 g) obtained from local fish farm in Gyeonggi-Do, Yongin-Si, South Korea were transferred to the laboratory. Fish were acclimatized to laboratory conditions in glass aquaria (1200 L capacity) for 20 days before exposure. During this period, fish were fed commercial food and the dechlorinated tap water was changed daily to remove the excretory wastes. The physico-chemical characteristics of water used in the present study were measured using the method of APHA (1998), which were temperature (25.9 ± 0.07 °C), pH (7.6 ± 0.04), salinity (0.7 ± 0.00 ‰), and dissolved oxygen (8.51 ± 0.08 mg/L).

Isoprothiolane exposure for 96-h short-term study

The fungicide isoprothiolane (diisopropyl-1,3-dithiolan-2-ylidenemalonate) was purchased from the Dong Bu Han Nong Ltd, Seoul, South Korea, and all the chemicals used were of analytical grade and obtained from Sigma-Aldrich Chemie GmbH, Germany. In this study, the range of exposure concentrations (2.7 μg/L as low and 27 μg/L high concentrations) was selected based on the information from the reports of NIER (2009). Fifteen healthy fish from the stock which already withheld from feeding for 48 h were collected and introduced into each aquarium (70 cm × 45 cm × 32 cm) of 30 L of water capacity. Then, based on the information from the reports of NIER (2009), two different concentrations of isoprothiolane (2.7 and 27 μg/L) were added to each glass aquaria. For each concentration, three replicates were also maintained. Simultaneously, control groups were also maintained. At the end of 96 h, fish from control and isoprothiolane treatments were taken for analyses. The test water was renewed (1/3) every 24 h and freshly prepared solution was added to maintain the concentration of isoprothiolane at a constant level. No mortality was observed in this study.

Hematological studies

At the end of 96 h, 15 fish were collected from control and experimental aquaria and sacrificed without being anesthetized. Blood samples were collected by heart puncture using plastic disposable syringes fitted with 26 gage needles. The collected blood was transferred into small vials. The fresh blood was immediately used for RBC and WBC counts (Rusia and Sood 1992). Hb content was estimated by Cyanmethemoglobin method (Drabkin 1946) and Hct by microhematocrit (capillary) method (Nelson and Morris 1989). Further, the hematological indices (MCV, MCH and MCHC) were also calculated according to standard formulas as MCV (fl) = Hct (%) × 10/RBC (millions/cu.mm × 10^6), MCH (pg) = Hb (g/dl) × 10/RBC (millions/cu.mm × 10^{-6}) and MCHC (%) = Hb (g/dl)/Hct (%) × 100.

Biochemical and electrolytes analysis

The remainder of the blood samples was left to coagulate for 10 min. After coagulation, blood samples were centrifuged at 3000 rpm (842 g) at 5 °C over 20 min to separate serum using polyethylene eppendorf test tubes, which was used for the estimation of electrolytes, biochemical parameters and enzymes. In this study, levels of electrolytes (Na^+, K^+, and Cl^-) were examined by the Electrolyte Analyzer (RapidChem, M-744; Siemens). Biochemical parameters (glucose, total protein, cholesterol, albumin, and triglycerides) were analyzed using DIRUI CS-T240 auto-chemistry analyzer (China) following the manufacturer's instructions. The globulin level was calculated from the total protein and albumin using standard formula (globulin (g/dL) = total protein − albumin).

Enzyme assay

Serum AST activity was estimated by the method of Reitmen and Franckel (1957) using a diagnostic kit (Asan Pharm. Co. Ltd., Korea). The activity of AST was measured by a NEOSYS-2000 UV/vis spectrophotometer (Scinco), at 505 nm. Activity of serum ALP was measured by DIRUI CS-T240 auto-chemistry analyzer (China) following the manufacturer's instructions. For the estimation of gill Na^+/K^+-ATPase activity, gills were isolated from the control and isoprothiolane treated fish and 100 mg of each tissue was weighed and homogenized with Teflon homogenizer along with 1 mL of 0.1 M Tris–HCl buffer adjusted to pH 7.4. The homogenates were centrifuged at 93.9 g at 4 °C for 15 min, and the clear supernatant was used following the method of Shiosaka et al. (1971) using a NEOSYS-2000 UV/vis spectrophotometer (Scinco) at 680 nm.

Statistical analysis

Results are given as mean ± SEM. All data from different treatments were compared by a one-way analysis of variance (ANOVA) and statistically different treatments were identified by Tukey's test. All differences were considered significant at $p < 0.05$.

Results

Hematological studies

Changes in hematological profiles of fish *C. carpio* exposed to two different concentrations of isoprothiolane (2.7 and 27 µg/L) for 96 h are given in Table 1. A significant ($p < 0.05$) decrease in Hb, Hct, and RBCs levels were observed in both the concentrations when compare to that of their control groups. In contrast, WBC counts were increased at significant ($p < 0.05$) level in both concentrations comparing to the control one. The other hematological indices such as MCV, MCH, and MCHC were slightly altered in comparison to control groups which are found to be not significant.

Ion regulation

The results of the present study revealed that both the concentrations of isoprothiolane caused significant ($p < 0.05$) increase in blood serum Na^+ and Cl^- levels compared to control groups. However, there were no significant changes in serum K^+ levels (Fig. 1).

Biochemical parameters

The results obtained in this study show significant ($p < 0.05$) decrease in glucose, cholesterol, and triglycerides levels in both isoprothiolane concentrations (Table 2). In contrast, an increase in serum protein, albumin, and globulin levels were noted. Among the biochemical parameters, serum albumin alone elevated at significant ($p < 0.05$) level when compare to that of their control groups.

Table 1 Changes in the hematological parameters in the freshwater fish *C. carpio* treated with different concentrations of isoprothiolane (2.7 and 27 µg/L) for a period of 96 h

Parameters	Control	2.7 µg/L	% Change	27 µg/L	% Change
Hematological parameters					
Hb (g/dL)	4.916 ± 0.267[a]	4.149 ± 0.430[b]	−15.60	2.961 ± 0.208[c]	−39.76
Hct (%)	14.475 ± 0.837[a]	12.450 ± 1.222[b]	−13.98	8.725 ± 0.677 [c]	−32.73
RBC (million/cu mm)	0.757 ± 0.025[a]	0.637 ± 0.010[b]	−15.85	0.497 ± 0.023[c]	−34.35
WBC (1000/cu mm)	30.366 ± 1.264 [c]	56.075 ± 5.213[a]	+84.66	54.453 ± 2.081[b]	+79.32
MCV (fl)	191.350 ± 11.118[a]	198.238 ± 18.921[a]	+3.59	174.736 ± 9.409[a]	−8.68
MCH (picograms)	64.978 ± 3.469[a]	65.270 ± 7.250[a]	+0.45	59.337 ± 2.830[a]	−8.68
MCHC (g/dL)	33.985 ± 0.197[a]	33.310 ± 0.692[a]	−1.98	33.991 ± 0.341[a]	+0.08

Means in a column followed by common superscript are not significantly different ($p < 0.05$)

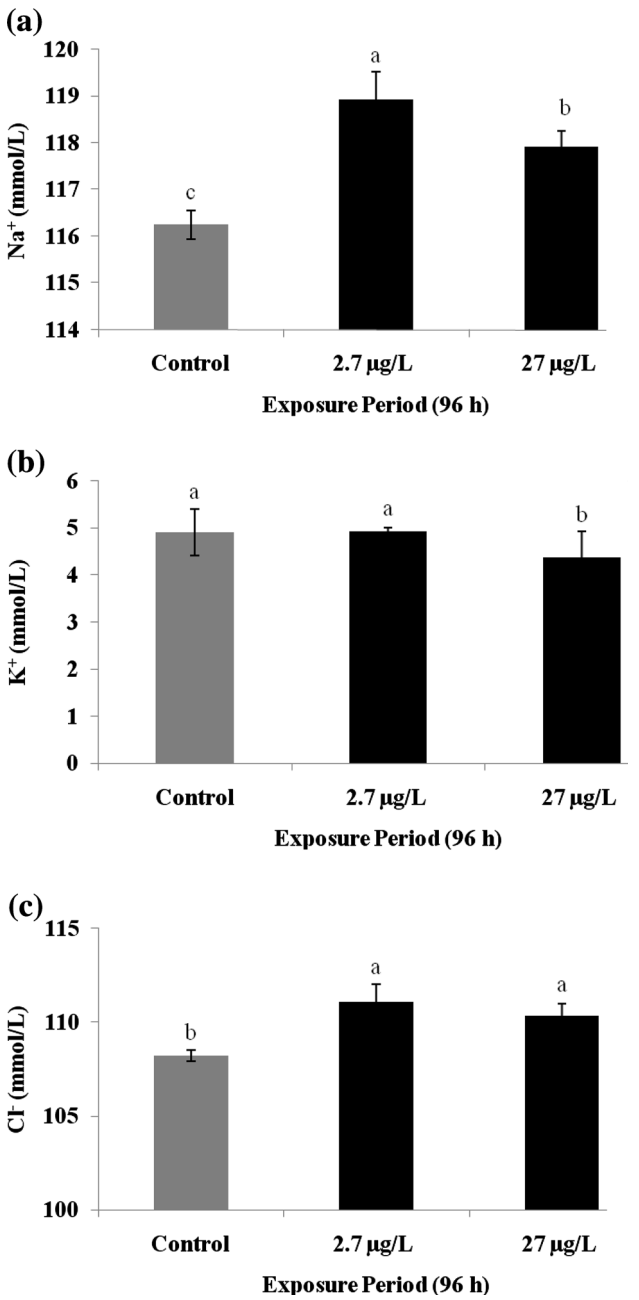

Fig. 1 Changes in the serum Na^+ (**a**), K^+ (**b**), and Cl^{-+} (**c**) levels in the freshwater fish *C. carpio* after 96 h exposure to different concentrations of isoprothiolane (2.7 and 27 µg/L)

Enzyme assay

The exposure of *C. carpio* to isoprothiolane caused a significant decrease ($p < 0.05$) in serum AST and gill Na^+/K-ATPase activities, while a slight change in ALP activity was observed in comparison with their control groups (Fig. 2).

Discussion

Due to extensive and continues use of various pesticides, their residues have been detected in waters and affected the environment adversely (Bijoy Nandan and Nimila 2012). The present study examined alterations of hematological, ionoregulatory, biochemical, and enzymological responses of *C. carpio* after exposure to environmentally relevant concentrations of isoprothiolane. These responses are strongly recommended for biomotoring of any pesticide toxicity. In toxicological studies, such responses are widely used as biomarkers to evaluate the impacts of any environmental contaminants exposure on organisms. Hematological parameters such as Hb, Hct, RBC, WBC, and hematological indices like MCV, MCH, and MCHC are extensively used to evaluate the toxic stress of environmental contaminants (El-Sayed et al. 2007; Saravanan et al. 2011c). In this study, the lower level of Hb, Hct, and RBCs may be attributed to the inhibition of erythropoiesis and lysing of RBCs due to isoprothiolane toxicity (Svobodova et al. 1997; Kori-Siakpere and Oghoghene 2008). Furthermore, impairment of osmoregulation due to accumulation of isoprothiolane in gills may also results in a lower value of hematological parameters. Alterations in these hematological parameters are the possible reasons for the observed anemic condition of fish (Zhang et al. 2007). Similar to our results, decreased levels of Hb and RBCs were found in *C. carpio* after short-term exposure to diazinon (Svoboda et al. 2001) and chlorpyrifos (Ramesh and Saravanan 2008). Typically, WBCs play an important role in immune system as a regulator (Gaber et al. 2013) and an increase of WBCs in fish exposed to any toxic stress is due to leucocytosis (Dick and Dixon 1985). Data of the present work revealed that a significant increase of WBCs in *C. carpio* after exposure to isoprothiolane is a generalized immune response to isoprothiolane toxicity. In addition, an enhanced release of lymphocytes from lymphomyeloid tissue may lead to an increase in WBC numbers (El-Sayed et al. 2007). The results of this study agree with the previous reports of lindane-treated fish *C. carpio* (Saravanan et al. 2011b) and *Etroplus maculatus* (Bijoy Nandan and Nimila 2012), and in endosulfan-exposed fish *Labeo fimbriatus* (Saravanan et al. 2011a). The levels of MCV, MCH, and MCHC were not significantly affected in fish exposed to 2.7 µg/L of isoprothiolane. Velíšek et al. (2007) and Li et al. (2011) found the same trend in *Oncorhynchus mykiss* exposed to deltamethrin and propiconazole, respectively. Harabawy and Ibrahim (2014) found similar responses in hematological parameters of *Clarias gariepinus* exposed to different

Table 2 Changes in the biochemical responses of the freshwater fish *C. carpio* treated with different concentrations of isoprothiolane (2.7 and 27 μg/L) for a period of 96 h

Parameters	Control	2.7 μg/L	% Change	27 μg/L	% Change
Biochemical parameters					
Glucose (mg/dL)	52.000 ± 3.488[a]	44.750 ± 0.750[b]	−13.94	36.250 ± 4.956[c]	−30.28
Protein (g/dL)	3.250 ± 0.517[a]	4.200 ± 0.367[a]	+29.20	3.800 ± 0.212[a]	+16.92
Cholesterol (mg/dL)	187.780 ± 15.418[a]	141.280 ± 2.654[b]	−24.76	121.400 ± 4.324[c]	−35.46
Albumin (g/dL)	1.325 ± 0.110[c]	1.700 ± 0.090[a]	+28.30	1.400 ± 0.070[b]	+5.66
Globulin (g/dL)	1.925 ± 0.426[a]	2.500 ± 0.308[a]	+29.87	2.400 ± 0.282[a]	+24.67
Triglycerides (mg/dL)	41.250 ± 5.588[a]	27.500 ± 3.278[b]	−33.33	18.250 ± 0.478[c]	−55.75

Means in a column followed by common superscript are not significantly different ($p < 0.05$)

concentrations of carbofuran which corroborates our findings on hematological studies.

Fish gills play a vital role in transport of ions like Na^+, K^+, and Cl^- to maintain acid base balance, osmotic pressure, and regulation of water influx and ion efflux (Mayer et al. 1989). Serum electrolyte levels are commonly measured as indicators of ionoregulatory performance in fishes. Therefore, measurement of such specific ions Na^+, K^+, and Cl^- are considered as sensitive biomarkers to aquatic pollution (Saravanan et al. 2011c). Elevated levels of Na^+ and Cl^- ions may be due to toxic effects of isoprothiolane on gill structures that are responsible for ion homeostasis (Wendelaar Bonga and Lock 1991). An elevated concentration of ion levels may be due to transportation of ions from other tissues into blood (Suvetha et al. 2010). Fletcher (1975) suggested that loss of water from the circulation may also lead to significant rise in electrolytes during stress condition. Besides, Hilmy et al. (1987) reported that the increase of both Na^+ and Cl^- ions in fish blood may be due to changes in the water ion equilibrium that occurred in inner- and inter-cells under the toxic effect. Similar to our findings, Nieves-Puigdoller et al. (2007) reported that atrazine caused significant elevation in Na^+ and Cl^- levels in the blood of *Salmo salar,* and Katuli et al. (2014) found significant alterations on ionoregulatory responses of *Rutilus rutilus* fingerlings after short-term diazinon exposure. However, the level K^+ in blood serum was not significantly altered by isoprothiolane when compared to the control group.

Biochemical parameters are commonly used to evaluate the toxic effects of environmental pollutants on animal health (Korkmaz et al. 2009; Saravanan et al. 2013). In the present investigation, a significant decrease in serum glucose level indicates the hypoglycemic response (low blood sugar) in *C. carpio* due to rapid utilization of blood glucose and enhanced energy demand after isoprothiolane exposure (El-Sayed et al. 2007). Moreover, the isoprothiolane might have caused adverse effects in the glycogenolysis process. Cholesterol, and triglycerides level is used to evaluate nutritional status and lipid metabolism (Agrahari et al. 2007). The present study reveals that the *C. carpio* exposed to isoprothiolane exhibited a significant depletion in blood cholesterol level. Results of this study were in the conformity with Agrahari et al. (2007) who found hypocholesteremia in monocrotophos-treated fish *Channa punctatus.* Likewise, the decline of serum triglycerides levels in *C. carpio* exposed to isoprothiolane might be due to liver damage or to meet extra energy demand. The results of this study coincide with the work of Lal and Singh (1987) in *Clarias batrachus* and Yang and Chen (2003) in *C. carpio* suggesting that the significant decrease in triglycerides contents is an indication of liver damage. Typically, albumin and globulin are two major components of blood proteins. Albumin and globulin are used as biochemical indicators to monitor the immune disorders, liver dysfunction, and impaired kidney activity (Isik and Celik 2006; Sunmonu and Oloyede 2007; Banaee et al. 2008). In the current study, the serum albumin was significantly increased to isoprothiolane exposures which are thought to be associated with a stronger innate response of fish (Wiegertjes et al. 1996). However, there were no significant changes in serum protein and globulin contents. Similar findings were observed in the serum protein of *C. carpio* exposed to endosulfan (Jenkins et al. 2003) and cyfluthrin (Sepici-Dinçel et al. 2009), and also in the serum globulin of *L. rohita* fingerlings under short-term exposure of fenvalerate (Prusty et al. 2011).

The AST and ALP regulate a number of essential functions in fish which are often used as sensitive stress biomarkers (Atli and Canli 2007; Gholami-Seyedkolaei et al. 2013). In the present work, there was a significant inhibition in AST in serum of *C. carpio* exposed to isoprothiolane. The lower level of AST in blood serum indicates impairment of liver and a reflection of tissue damage (Nemcsok and Benedeczky 1990). Since isoprothiolane residues have the ability to accumulate in living tissues

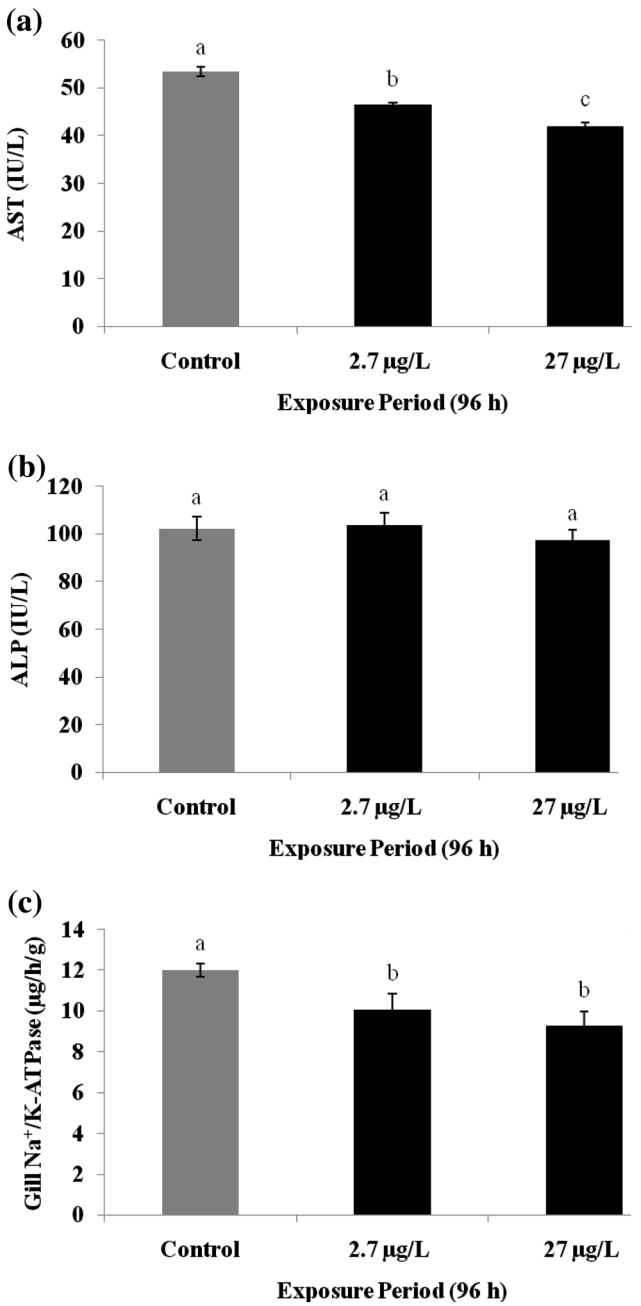

Fig. 2 Changes in the serum AST (**a**), ALP (**b**), and gill Na^+/K^+-ATPase (**c**) activities in the freshwater fish *C. carpio* after 96 h exposure to different concentrations of isoprothiolane (2.7 and 27 µg/L)

(Fushiwaki et al. 1993), it may damage the liver cells. Recently, Sapana Devi and Gupta (2014) reported similar changes of AST activity in liver tissues of *Anabas testudineus* treated with deltamethrin and permethrin. However, the activity of ALP enzyme was not significantly altered by isoprothiolane which shows a similar trend to control group. Velíšek et al. (2006) reported a similar observation in *C. carpio* exposed to deltamethrin. Gills are generally considered as a good indicator of water quality (Shakoori et al. 1996) because they are the primary route for the entry of pesticides and other chemicals. Analysis of enzymes in gills is mainly used to determine the cellular changes in fish exposed to toxicants. Na^+/K^+-ATPase is present in the basolateral membrane of gill epithelial cells and actively involved in the electrolyte transport across the gills (Parvez et al. 2006). Gill Na^+/K^+-ATPase activity is a widely used marker for pollutant-induced osmoregulatory changes (Stagg et al. 1992) and for tolerable levels of a large group of environmental contaminants (Oruc et al. 2002; Mathan et al. 2010). Watson and Beamish (1981) reported that xenobiotics can alter Na^+/K^+-ATPase activity due to disruption of energy producing metabolic pathways. In this study, the decrease of Na^+/K^+-ATPase activity in gill might have resulted from the direct toxicity of the isoprothiolane on ATPase function. The result of our study is in agreement with the findings of Suvetha et al. (2010) who observed inhibition of the gill ATPase activity in *C. carpio* exposed to cypermethrin. A similar observation was also made in *S. salar* treated with atrazine (Waring and Moore 2004), and in *Ancistrus multispinis* (de Assis et al. 2009) and *Catla catla* exposed to deltamethrin (Vani et al. 2011). Yang et al. (1992) confirmed that isoprothiolane induces inhibition of enzymes in the fish *C. carpio*.

The present study concludes that both the isoprothiolane concentrations caused significant alterations in hematological, ionoregulatory, biochemical, and enzymological parameters of *C. carpio*. Furthermore, the observed hemato-biochemical parameters were high at 27 µg/L when compared to 2.7 µg/L of isoprothiolane concentrations. The results of this study suggest that the presence of isoprothiolane even at very low concentrations in the aquatic environments can cause harmful effect on aquatic organisms. This study will be very helpful for the assessment of ecotoxicological risks of isoprothiolane and other related fungicides in the rivers of South Korea. Further, more detailed studies on using of specific biomarkers to monitor the long-term effects of isoprothiolane need to be investigated in the future studies.

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