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ORIGINAL PAPER

Effects of waterborne selenium on toxic and physiological stress response in goldfish, *Carassius auratus*

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Abstract Selenium (Se) is an essential element, known to bioaccumulate to toxic levels in aquatic environments. Here, we tested the toxic and physiological stress response of goldfish, *Carassius auratus* exposed to different concentrations of Se for 120 h. At the end of the experiment, the toxic stress indicators [metallothionein (MT), aminotransferase/aspartate (AspAT and AlaAT), and gill Na^+/K^+-ATPase] and other stress-related parameters [corticotropin releasing hormone (CRH), adrenocorticotropic hormone (ACTH), and glucocorticoid receptors (GRs) mRNA and activity and/or plasma ACTH, cortisol, glucose, hydrogen peroxide (H_2O_2), and muscle lipid peroxidation (LPO)] were significantly higher in specimens exposed to 3 and 4 mg/L of Se than in those exposed to lower concentrations (0 and 2 mg/L). These results indicated that 3 and 4 mg/L Se concentration can induce an acute toxic and physiological stress response in aquarium conditions.

Keywords Cortisol, HPI axis, Metallothionein, Physiological stress response, Selenium, Toxicity

Selenium (Se) is a trace element, essential for several physiological functions, including normal growth, antioxidant response, and immune response in teleost fishes, and is thus required in the diet[1,2]. Se can be accumulated within organisms, although the homeostatic function found in teleosts can help maintain Se at the correct concentration. However, bioaccumulation of Se at concentrations slightly above homeostatic requirements can be toxic[3,4].

Se can be found at high concentrations in soils derived from black shale and phosphate rocks[5]. Agricultural drain water, sewage sludge, airborne ashes from coal-fired power plants, oil refineries, and mining activities related to the extraction of phosphates and metal ores are known to represent exogenous sources of contamination, increasing the concentration of Se in aquatic environments[6,7]. Organisms can take up Se through the water or as part of the diet. In fish and other wildlife, water-soluble selenium can enter the organism through the gills, epidermis, or gut. However, dietary exposure to selenium is generally the most common pathway for animals, as they often occupy higher trophic levels in aquatic and terrestrial food webs[8,9]. Se occurs in several different oxidation states in aquatic environments, and previous study reported that high concentration of Se act the toxic in fish and causes a deformity of juvenile[10-13]. Other documented effects in fish include the presence of skin lesions, cataracts, swollen gill filament lamellae, myocarditis, and liver and kidney necrosis[11,14].

Typically, exposure to toxic levels of natural and anthropogenic stressors in vivo disrupts the organism's homeostasis and can represent a life-threatening situation. Organisms have evolved a suite of mechanisms, collectively known as "integrated stress response", which are crucial to regain homeostasis upon exposure to stressors[15]. The hypothalamus-pituitary-interrenal axis of teleosts can be activated by abiotic and biotic stressors[16,17], promoting the release of hypothalamic corticotropin-releasing hormone (CRH) and pituitary

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adrenocorticotropic hormone (ACTH), to subsequently stimulate the synthesis and release of cortisol by the pronephros interrenal tissue[15,18].

The levels of catecholamine in blood are rarely used as stress indicators, because they are not easy to determine and they quickly disappear from the blood stream[15]; however, high levels of cortisol in plasma have been widely used both as a short-term and a long-term stress indicator. In addition, other physiological consequences related to high cortisol levels, such as alteration in blood cell count, glycaemia, and plasmatic lactate are also useful stress indicators. In fish, cortisol binds and activates the intracellular glucocorticoid receptors (GRs), which promote the expression of target genes using ligand-dependent transcription factors[19]. In teleosts, GR is known to present two isoforms, GR1 and GR2, with high nucleotide sequence homology and similar physiological function[20]. Stress can increase the level of cortisol in plasma; in addition, it is also known to typically increase plasma glucose and lactate levels. Elevations in plasma glucose levels are generated by catecholamine-mediated glycogenolysis initially, and by cortisol-mediated gluconeogenesis at later stages. The increase in lactate levels, typically observed following a stress response, is associated with the release of lactate produced in muscles under anaerobic conditions[21,22].

Fish gills are important organs in this group, fulfilling a number of essential body functions, including gas exchange, regulation of osmotic pressure, and maintenance of the acid-base balance, ion transport, and excretion of nitrogenous waste. These functions are mainly performed by the chloride or the epithelial cells in the gill[23]. In the chloride cells, the Na^+/K^+-ATPase (NKA) is responsible for the ion exchange functions. On the other hand, energy conversion occurs through their system of membrane channels. The gills are also the primary target of environmental pollutants. Once the gills are damaged, gas exchange and osmotic pressure regulatory functions are also affected, potentially compromising the fish's health[24-26]. Thus, NKA is also often used as an indicator of the severity of damage to the organism caused by the presence of toxins.

Metallothioneins (MTs) have a high affinity to metals and are easily induced by their presence; therefore, they are widely regarded as useful biomarkers to test for metal exposure, particularly in the marine environment[27,28]. Aspartate aminotransferase (AspAT) and alanine aminotransferase (AlaAT) are enzymes also frequently used in the diagnosis of damage caused by the presence of environmental toxins in various tissues, including liver, muscle, and gills[29].

Previous research related to teleost ecotoxicology have reported the use of different biomarkers[26,27], in particular, there are reports on the effect of Se on fish reproductive biology[30,31]. However, only limited knowledge is available regarding the physiological response and toxicity of increased levels of Se.

Here, we investigated the effect of increasing Se concentration and the toxicity threshold, by exposing in goldfish, *Carassius auratus* to 2, 3, or 4 mg/L of sodium selenite (Na_2SeO_3) for 0, 6, 12, 24, 72, and 120 h. We assessed sodium selenite toxicity by directly measuring the level of expression of MT mRNA, and MT protein expression, alterations in AspAT, AlaAT, and NKA activity, and variations in NKA protein expression. We also determined fish physiological response to the presence of Se by measuring changes in HPI axis gene expression (mRNA), including CRH, ACTH, and GRs, and levels of plasma ACTH, cortisol, glucose, and H_2O_2, and lipid peroxidation (LPO).

MT expression levels following exposure to Se (in vivo and in vitro)

We used QPCR to examine MT expression MT in liver and cultured liver cells following Se exposure (Figure 1B, C). Western blot analysis revealed a protein with MT enzyme-specific immunoreactivity, and a mass that corresponded to the predicted mass of goldfish MT (15 kDa; Figure 1A). MT mRNA expression in liver was significantly higher after exposure to 3 and 4 mg/L of Se after 72 h (approximately 16.8- and 23.7-fold higher, respectively) compared to the control and the 2 mg/L Se treatment groups (Figure 1B). The MT protein expression pattern resembled the expression pattern of MT mRNA in goldfish liver (Figure 1A). In addition, exposure to 50 and 100 μM of Se significantly increased MT mRNA expression levels of the MT in cultured liver cells (Figure 1C). In particular, the MT mRNA expression after 120 h of exposure was significantly increased, reaching values approximately 8.9- and 11.4-fold higher than the control and the 1 μM treatment group, respectively.

Levels of plasma AspAT and AlaAT in response to Se treatment

Figure 2 shows the variation of plasma AspAT and AlaAT following exposure to Se. Initial plasma AspAT levels were 552.5 ± 50.2 U/L; however, they significantly increased after exposure to 3 and 4 mg/L Se. In particular, AspAT increased significantly by 3.97- and 5.19-fold after 72-h exposure to 3 and 4 mg/L Se (2305.3 ± 70.8 and 3000.2 ± 50.3 U/L, respectively) compared to the control and 2 mg/L Se groups (552.5 ± 50.2 and 612.2 ± 60.8 U/L, respectively; Figure 2A). Similarly, AlaAT levels significantly increased after 72-h exposure to 3 and 4 mg/L Se (approximately 3.51-

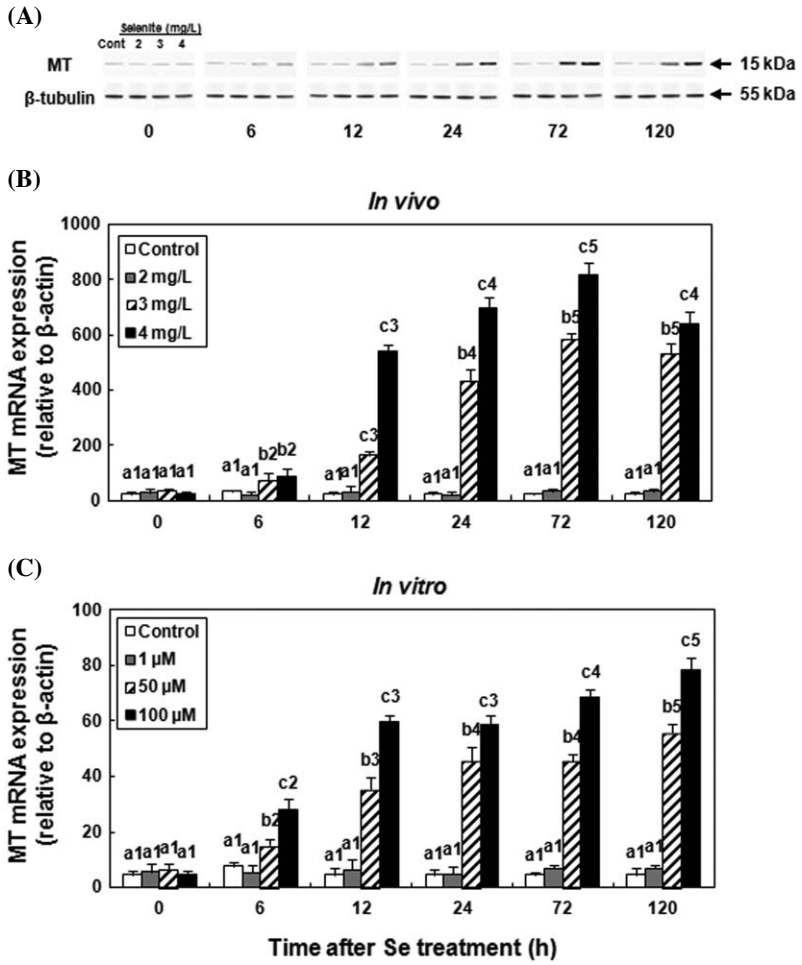

Figure 1. Variation in MT mRNA expression in liver (B, *in vivo*) and cultured liver cells (C, *in vitro*) during exposure to Se in goldfish. MT mRNA expression levels are expressed in relation to β-actin mRNA levels in goldfish liver after Se treatment, based on quantitative real-time PCR data. We reverse-transcribed 3 μg of total RNA, prepared from liver samples, and amplified it using gene-specific primers. The results are expressed as normalized fold expression (relative to the control data) in relation to β-actin levels for the same sample. (A) Western blot of MT (15 kDa) protein expression in goldfish liver after Se treatment; β-tubulin (55 kDa) was used as an internal control. The numbers indicate significant differences among the different time points at the same Se concentration ($P<0.05$). The lower-case letters indicate significant differences among the different Se concentrations at each time point (h) ($P<0.05$). All values are means ± SE ($n=5$).

and 4.15-fold, respectively) compared to the control and 2 mg/L Se groups (9.5 ± 1.2 and 10.2 ± 0.8 U/L) (Figure 2B), to subsequently decrease.

NKA activity levels in gills following Se exposure

Figure 3 shows the variation in NKAα protein expression and NKA activity in fish gills following exposure to Se (Figure 3). Western blot analysis revealed a protein with NKAα enzymes-specific immunoreactivity and a mass that corresponded to the predicted mass of goldfish NKAα (100 kDa; Figure 3A). NKAα protein expression in gills significantly increased following 72-h exposure to 3 and 4 mg/L of Se compared to the control and 2 mg/L Se treatment groups. NKAα protein expression patters resembled the pattern of activity in goldfish gills. The level of gill NKA activity in the control group was 24.0 ± 2.6 μmoles ADP/mg protein/h; however, after exposure to 3 and 4 mg/L Se gill NKA activity significantly increased to 52.8 ± 2.9 and 61.2 ± 2.5 μmoles ADP/mg protein/h (after 72 h of exposure to Se), respectively, and then decreased to 42.6 ± 3.9 and 48.2 ± 2.7 μmoles ADP/mg protein/h (after 120 h of exposure to Se; Figure 3B).

CRH and ACTH expression following Se exposure

CRH mRNA expression in brain tissue significantly increased following 72-h exposure to 3 and 4 mg/L of Se (approximately 12.3 and 20.3-fold higher, respec-

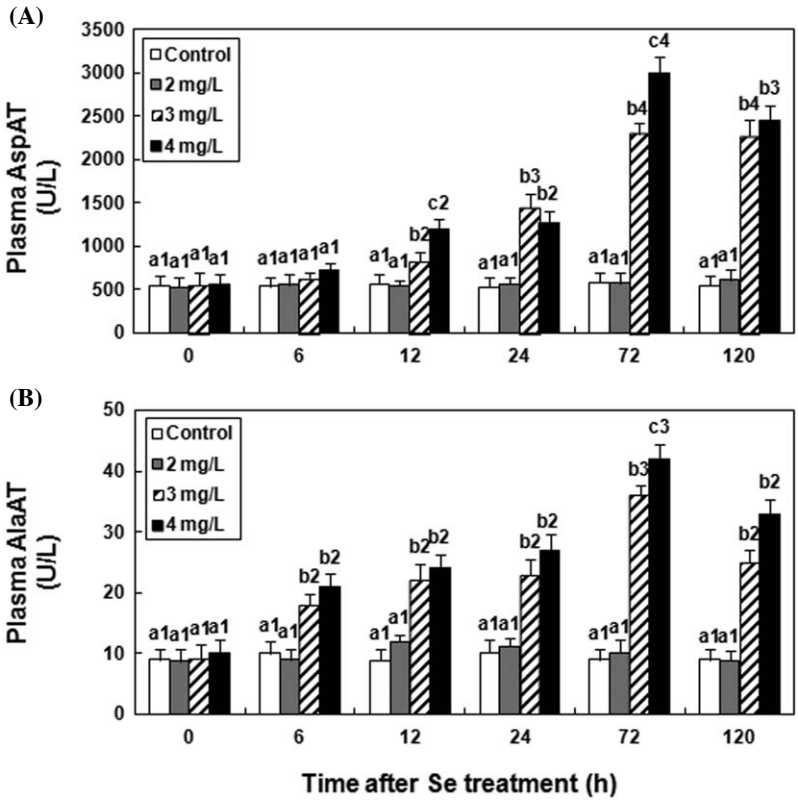

Figure 2. Variation in plasma AspAT (A) and plasma AlaAT (B) levels during exposure to Se in goldfish. The numbers indicate significant differences among the different time points at the same Se concentration ($P<0.05$). The lower-case letters indicate significant differences among different Se concentrations at each time point (h) ($P<0.05$). All values are means ± SE ($n=5$).

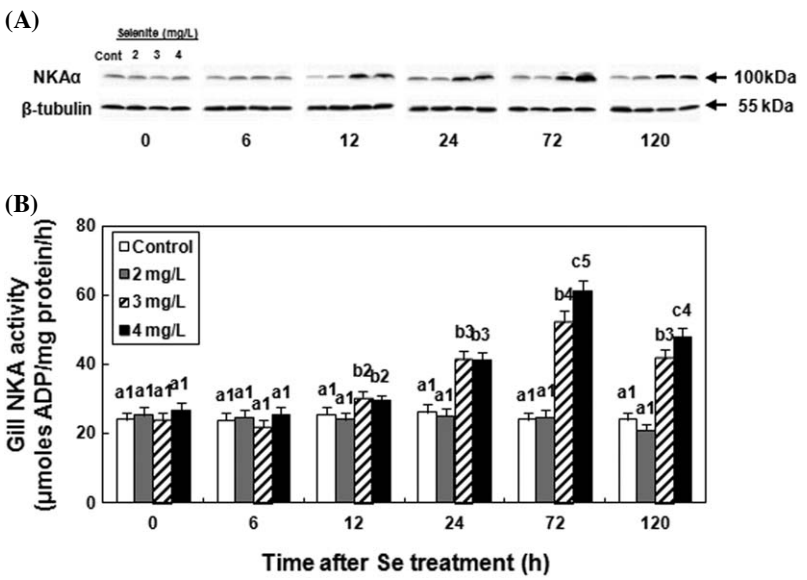

Figure 3. Variation in NKAα protein expression (A) and gill NKA levels (B) during exposure to Se in goldfish. (A) Western blot of NKAα (α5; 100 kDa) protein expression in goldfish gills after Se treatment; β-tubulin (55 kDa) was used as the internal control. The numbers indicate significant differences among different time points at the same Se concentration ($P<0.05$). The lower-case letters indicate significant differences among different Se concentrations at each time point (h) ($P<0.05$). All values are means ± SE ($n=5$).

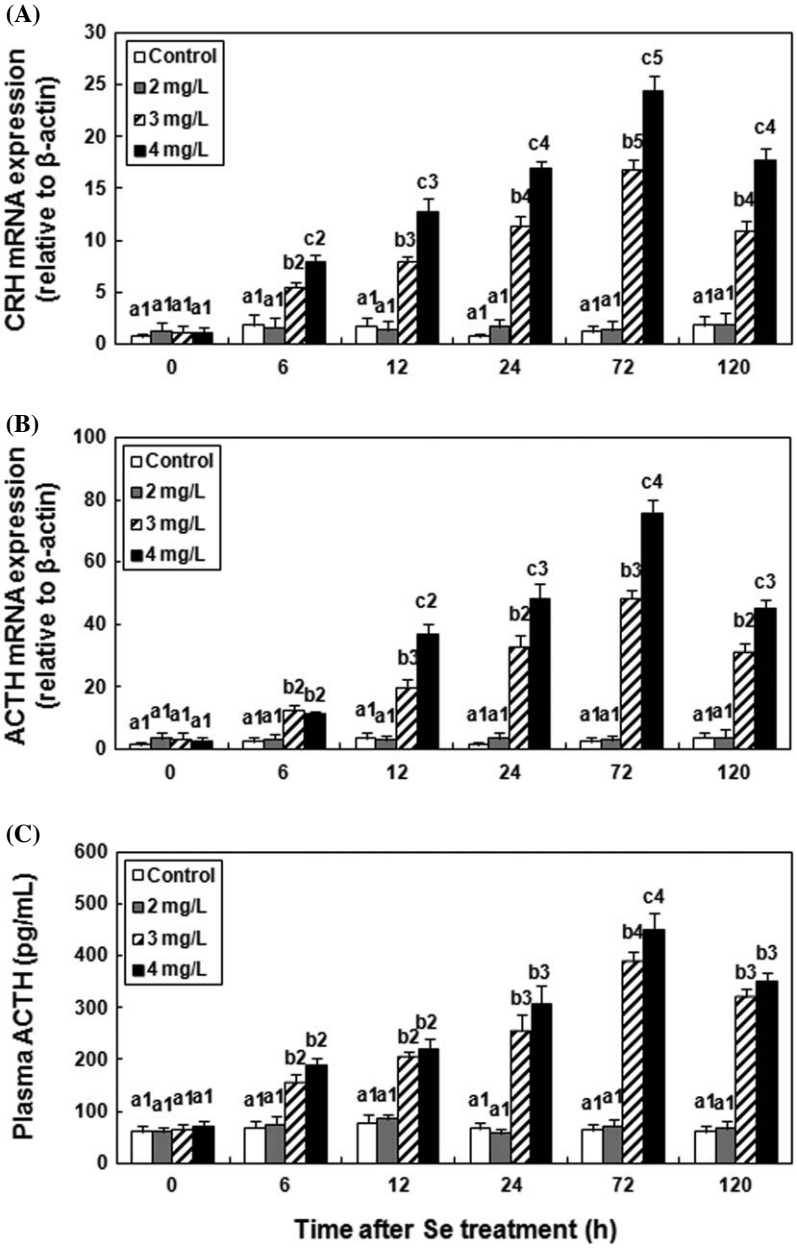

Figure 4. Variation in CRH and ACTH in the brain and pituitary gland during exposure to Se in goldfish. CRH (A) and ACTH (B) mRNA levels are relative to β-actin mRNA levels in goldfish liver after Se treatment, based on quantitative real-time PCR data. (C) The level of ACTH in plasma during Se treatment was analyzed using a plate reader. The numbers indicate significant differences among different time points at the same Se concentration ($P<0.05$). Lower-case letters indicate significant differences among different Se concentrations at each time point (h) ($P<0.05$). All values are means ± SE ($n=5$).

tively) compared to the control and 2 mg/L groups, to subsequently decrease (Figure 4A).

In addition ACTH mRNA expression increased after exposure to 3 and 4 mg/L Se, compared to the control and 2 mg/L groups (Figure 4B). In particular, ACTH expressions increased by approximately 27.3- and 28.1-fold after 72-h exposure to 4 mg/L Se compared to the control and 2 mg/L groups, respectively. Moreover, plasma ACTH level was 61.6 ± 8.2 pg/mL at the start of the experiment (Figure 4C); however, levels notably increased to 390.7 ± 19.5 and 450.5 ± 20 pg/mL following 72-h exposure to 3 and 4 mg/L of Se, respectively (Figure 4C).

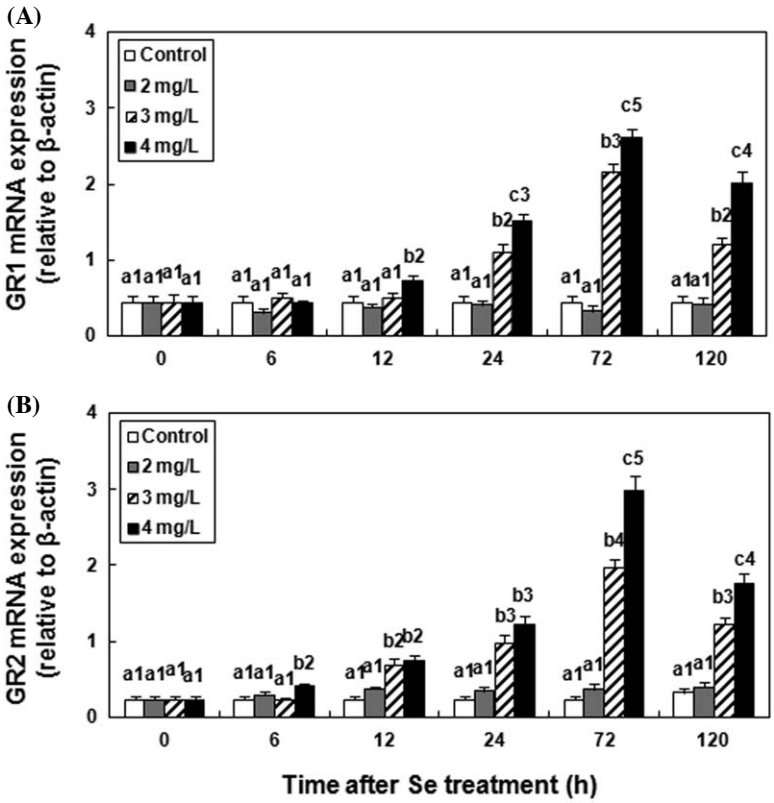

Figure 5. Variation in GR1 (A) and GR2 (B) mRNA in kidney during exposure to Se in goldfish. The numbers indicate significant differences among different time points at the same Se concentration ($P<0.05$). The lower-case letters indicate significant differences among different Se concentrations at each time point (h) ($P<0.05$). All values are means ± SE ($n=5$).

GR mRNA expression in response to Se exposure

To further elucidate the regulatory effects of Se, we measured expression levels of GRs (GR1 and GR2) mRNA in kidney tissue following Se treatment in goldfish (Figure 5). GR mRNA expression in kidney tissue significantly increase following 72-h exposure to 3 and 4 mg/L of Se (Figure 5A, showing a 4.9- and 7.3-fold increase in GR1 expression after 3 and 4 mg/L exposure, respectively; Figure 5B, showing a 8.3- and 9.2-fold increase in GR1 expression after 3 and 4 mg/L exposure, respectively) compared to the control and 2 mg/L Se treatment groups.

Levels of cortisol and glucose in plasma following Se exposure

At the start of the experiment, cortisol and glucose levels in plasma were 6.2 ± 0.5 ng/mL and 24.7 ± 3.6 mg/dL, respectively. In those groups exposed to 3 and 4 mg/L of Se, the levels of cortisol and glucose in plasma significantly increased and peaked after 72 h exposure to 3 and 4 mg/L Se (42.3 ± 3.1 and 50.6 ± 2.5 ng/mL for cortisol, Figure 6A; 60.5 ± 3.3 and 90.4 ± 2.9 mg/dL for glucose, Figure 6B, after exposure to 3 and 4 mg/L, respectively).

Levels of H_2O_2 and LPO in plasma following Se exposure

The levels of H_2O_2 in plasma were 39.7 ± 3.7 nmole peroxide/mL at the start of the experiment (Figure 7A); however, they significantly increased after 72-h exposure to 3 and 4 mg/L of Se (approximately by 1.21- and 1.67-fold compared to the control group) and then decreased. On the other hand, H_2O_2 levels significantly decreased in those individuals exposed to 2 mg/L Se (approximately 0.63-fold compared to the control group). H_2O_2 significantly increased and peaked after 72-h of exposure to 3 and 4 mg/L Se (47.8 ± 3.1 and 66.6 ± 3.7 nmole peroxides/mL, respectively).

LPO levels were 24.3 ± 0.7 nM/g at the start of the experiment (Figure 7B), although they significantly increased after exposure to 3 and 4 mg/L of Se (approximately 1.29- and 1.31-fold compared to the control), and then decreased. Specifically, LPO levels significantly increased and peaked after 24-h and 72-h exposure to 3 and 4 mg/L Se (31.1 ± 1.1 and 33.5 ± 0.7 nM/g, respectively).

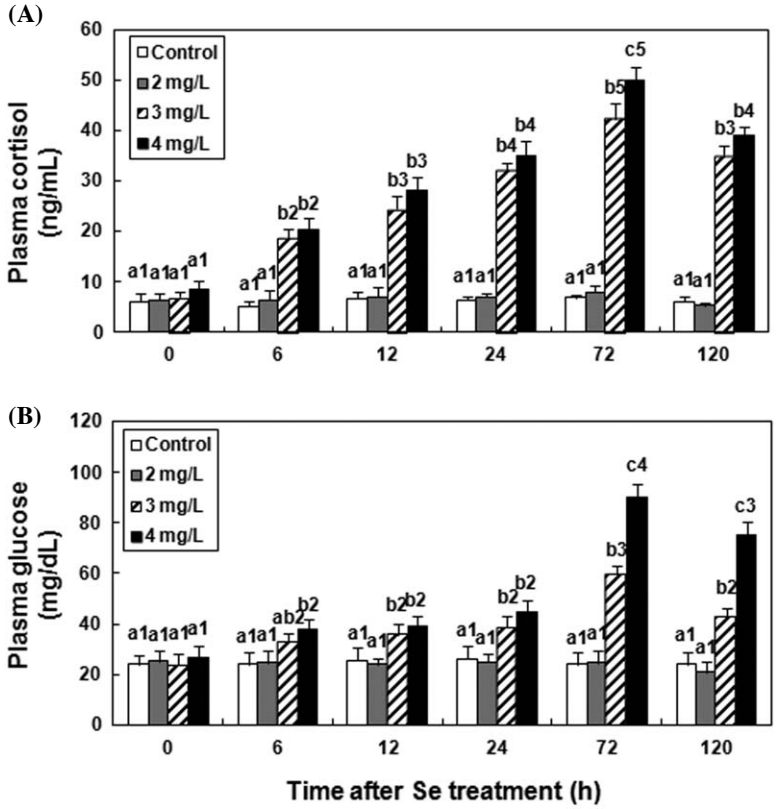

Figure 6. Variation in the levels of cortisol (A) and glucose (B) in plasma during exposure to Se in goldfish. The numbers indicate significant differences among different time points at the same Se concentration ($P<0.05$). The lower-case letters indicate significant differences among different Se concentrations at each time point ($P<0.05$). All values are means ± SE ($n=5$).

Discussion

Here, we investigated the effect of toxic concentrations of waterborne Se and the physiological response in goldfish, by experimentally exposing live specimens to different Se concentrations (2, 3, and 4 mg/L) for 0, 6, 12, 24, 72, and 120 h.

We found that, in goldfish, MT mRNA and protein expression varied with the level of sodium selenite present. In particular, MT mRNA and protein expression increased after 6 h of exposure to 3 and 4 mg/L Se, and then decreased approximately 72 h after, and it was significantly higher after exposure to 3 and 4 mg/L Se than that of the control and 2 mg/L Se treatment groups. According to Hamza-Chaffai *et al.*[32] and Giguére *et al.*[26], the cysteine-rich MT protein plays a detoxifying role in the cell, by removing SH groups structurally bound to metals. Therefore, the increased expression of MT mRNA detected in goldfish could play a protective role in teleosts, reducing the toxicity induced by exposure to 3 and 4 mg/L of Se. These results are also consistent with those of Cleveland *et al.*[33], who reported that mortality of bluegill, *Lepomis macrochirus*, increases with increasing Se concentration (from 0.16 to 2.8 mg/L) following 60 days of exposure to waterborne Se.

Furthermore, we also investigated changes in MT mRNA expression in cultured hepatocytes by Se treatment; the observed patterns were similar to those found in *in vivo* experiments. MT mRNA expression significantly increased in hepatocytes treated with different Se concentrations (0, 1, 50, and 100 μM), and the increase was proportional to the exposure time for the higher concentrations (50 and 100 μM). Similarly, Wu *et al.*[34] reported that MT protein levels increase in cultured hepatocytes of tilapia, *Oreochromis mossambicus*, after exposure to 100 μM of Cu, a heavy metal. Therefore, we have suggested that Se directly induced toxicity on hypatocytes.

AspAT, AlaAT, and NKA activity, and the expression level of NKA protein increased significantly after exposure to 3 and 4 mg/L of Se. Nemcsók and Benedeczky[29] reported that plasma AspAT and AlaAT activity increases following exposure to phenol, a toxic substance, in the carp, *Cyprinus carpio* L. Miller *et al.*[35] also reported that exposure to high concentrations (2.52

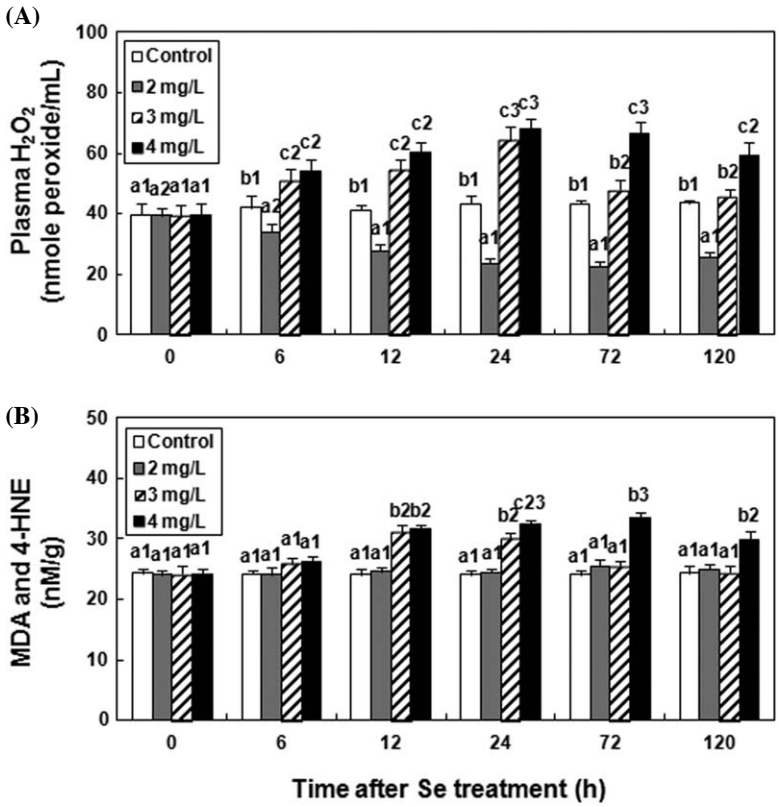

Figure 7. Variation in the levels of H_2O_2 (A) and LPO (B) in plasma during exposure to Se in goldfish. The numbers indicate significant differences among different time points at the same Se concentration ($P<0.05$). The lower-case letters indicate significant differences among different Se concentrations at each time point ($P<0.05$). All values are means ± SE ($n=5$).

and 3.60 mg/L) of Se causes acute toxicity and increases NKA activity in the rainbow trout, *Oncorhynchus mykiss*. Therefore, the increase in AspAT and AlaAT and NKA activity and in the expression of NKA protein observed here after exposure to 3 and 4 mg/L of Se, to be an indicator of the toxicity of Se to goldfish.

During stress and toxicity response in vertebrates, the neuroendocrine stress axis is activated by the hypothalamus-pituitary-interrenal axis (HPI axis), which plays an important role in the adaptation to stress[15,18].

In this study, we assessed the changes in HPI activity levels observed in response to exposure to Se, to confirm its role in the toxic stress response. CRH, ACTH, and GRs mRNA significantly increased after 72 h exposure to 4 mg/L Se. ACTH levels also increased with exposure time and Se concentration, but decreased again after 120 h.

In a similar study, Pepels *et al*.[36] showed that stress levels increase with capture time in tilapia, detected as the increase cortisol and CRH in plasma. An *et al*.[37] reported an increase in GR mRNA expression in gills, kidney, and intestine, and an increase in the levels of cortisol in plasma following temperature and salinity disturbances in the black porgy, *Acanthopagrus schlegeli*. On the other hand, here we found that exposure to 3 and 4 mg/L of Se activated the HPI axis, which was related to Se toxicity, and induced stress in goldfish.

Levels of cortisol and glucose in plasma also increased with exposure time and Se concentration, but decreased again after 120 h. Mommsen *et al*.[21] and Begg and Pankhurst[22] reported that stress-induced catecholamine secretion promoted the degradation of glucose; however, in order to maintain glucose homeostasis in vivo, cortisol promotes the synthesis of glucose, leading to a subsequent increase in plasma glucose. Therefore, the changes in the concentrations of glucose and cortisol in plasma were also associated with the toxic stress response induced by exposure to 3 and 4 mg/L of Se. Additionally, levels of cortisol and glucose decreased after 120 h exposure to Se, we have considered that goldfish was adapted the Se toxic after 120 h.

Physiological stress induced by toxicity directly increases reactive oxygen species (ROS), including superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), and singlet oxygen ($^1O_2$)[38,39]. Overpro-

duction of ROS linked to environmental stress can also increase lipid peroxidation (LPO), the oxidation of nucleic acids and proteins, and DNA damage. It can also affect cell viability by causing membrane damage and enzyme inactivity, and can accelerate cell senescence and apoptosis[40,41]. To confirm the extent of LPO and production of ROS caused by toxic stress, we investigated the levels of H_2O_2 and LPO in plasma. Levels of H_2O_2 and LPO increased following exposure to 3 and 4 mg/L of Se compared to the control and 2 mg/L Se treatment groups. In a similar study, Choi *et al*.[42] reported that Se treatment effectively suppresses oxidative stress and enhances immunity and antioxidant function is goldfish following exposure to different environmental concentrations of Se (1 and 2 mg/L Se). So, we suggest that 2 mg/L Se concentrations are optimal concentration intervals that can effectively suppress oxidative stress and H_2O_2 levels by a powerful antioxidant. In addition, another previous study showed that, in rainbow trout, exposure to 2.52 mg/L of Se reduced the level of LPO, although LPO levels increased if the rainbow trout was exposed to 3.60 mg/L Se, indicating that this higher concentration was sufficient to induce lipid damage through peroxidation[35]. In our study, exposure to 3 and 4 mg/L of Se induced acute toxicity in goldfish, and we believe that the ROS generated by the toxic stress response led to the observed increase in LPO and H_2O_2 in goldfish.

In conclusion, here we confirm that Se at high concentrations of 3 and 4 mg/L can effectively cause acute toxicity in goldfish, and induce physiological stress. Furthermore, HPI axis activity increased due to the activation of stress response mechanisms in goldfish. Thus, the adaptive response of goldfish to toxic stress involved the activation of the HPI axis after 72 h of exposure to Se.

Thus, our results showed the concentration range at which Se induces a toxic response in the organism, as an important guideline to establish environmental standards for Se concentration. Furthermore, our results might be also useful as background information in future studies assessing fish physiological response to Se.

Materials & Methods

Experimental fish

Goldfish [n=120, average length, 11.0±0.2 cm; mass, 12.1±0.6 g] were purchased from a commercial fish farm (Busan, Korea), and maintained with five100-L circulation filter tanks prior to experiments in the laboratory for 2 weeks. The fish were exposed to a simulated natural photoperiod. The water temperature and photoperiod were 22±1°C and 12-h light: 12-h dark period (lights on 07:00 h and light off 19:00 h), respectively.

Se treatment and sampling

To investigate the effects of Se on antioxidant and immunity in goldfish, fish were treated with waterborne sodium selenite (Se, Na_2SeO_3; Sigma, St Louis, MO, USA) at 0, 2, 3, and 4 mg/L. The tissues (pituitary, brain, liver, and kidney) were sampled from five different fish at each of the following time periods: 0, 6, 12, 24, 72, and 120 h. All tissues were collected, immediately frozen in liquid nitrogen, and stored at −80°C until total RNA was extracted. Also, blood was collected from the caudal vein using a 3-mL syringe coated with heparin. Plasma samples were separated by centrifugation (4°C, 12,000 × g, 10 min) and stored at −80°C.

Liver cell culture

Livers were removed from 10 goldfish, and cut using a scalpel into 1-3 mm^3 sections, which were subsequently weighed and placed in a 24-well culture plate. Under a sterile hood, the sections were repeatedly washed with culture medium solution (M199, Invitrogen, Carlsbad, CA, USA). Approximately 50 mg of each of the liver sections was deposited in each of the wells of the 24-well plate. In addition, 2 mL of fresh culture medium was added to each well. The liver pieces were left exposed to ambient conditions and allowed to acclimatize to room temperature for 2 h. Following this period, 1, 50, and 100 μM of Se were added to different treatment groups. Equal volumes of distilled water (dH_2O) were added to the control group. The liver sections were then incubated for 0, 6, 12, 24, 72, or 120 h at 28°C, 100% humidity, and 5% atmospheric CO_2. Following incubation, each sample was centrifuged (20°C, 10,000 × g, 15 s), and the supernatant was removed and stored in individual microcentrifuge tubes at −80°C for subsequent use.

Quantitative PCR (QPCR)

QPCR was conducted to determine the relative expression of MT, CRH, ACTH, and GRs mRNA using total RNA extracted from goldfish tissue. Known goldfish DNA sequences were used to design the QPCR primers, which are shown in Table 1. These primers were designed for each gene using the Beacon Designer software (Bio-Rad, Hercules, CA, USA). Primer alignments were performed with the BLAST database to ensure the specificity of primers. QPCR amplification

Table 1. Primers used for QPCR amplification.

Genes	Primer	DNA sequences
MT (X97271)	Forward Reverse	5′-GAA GAG TTG CTG TTC TTG TTG-3′ 5′-CAG GAA TTG CCC TTA CAC A-3′
CRH (AF098629)	Forward Reverse	5′-CCC GAG ACA TCC CAG TAT T-3′ 5′-GTA ATT GCC ATC CAA GCG A-3′
ACTH (AJ431209)	Forward Reverse	5′-ATC TTC TTC GTT GTG CTC TT-3′ 5′-GTG AAG TCC TGG CAT AGA-3′
GR1 (HQ656017)	Forward Reverse	5′-CTC CTG GCT CTT CCT CAT-3′ 5′-TCT GGT TAC ACT GGT CAC T-3′
GR2 (HQ656018)	Forward Reverse	5′-GCC GAC TTC AAT AGC ACT T-3′ 5′-GGT CTA TAT CGC CTT TAT CCA TT-3′
β-actin (AB039726)	Forward Reverse	5′-TTC CAG CCA TCC TTC CTA T-3′ 5′-TAC CTC CAG ACA GCA CAG-3′

was conducted using a BIO-RAD CFX96™ Real-Time System (Bio-Rad) and an iQ™ SYBR Green Supermix (Bio-Rad), according to the manufacturer's instructions. QPCR included the following steps: 1 cycle of denaturation at 95°C for 5 min, 35 cycles each consisting of denaturation at 95°C for 20 s, and annealing and extension at 55°C for 20 s. After PCR, the QPCR data from three replicate samples were analyzed using the analysis software specific for the hardware (Bio-Rad), and used to estimate the number of copies of each transcript found in each sample. The efficiencies of the reactions were determined by performing the QPCR. The efficiencies were found to be as follows: β-actin=94.8%, MT=94.3%, CRH=95.3, ACTH=95.7%, GR1=94.9%, and GR2=95.1%. As an internal control, β-actin was also amplified in each sample, and all data were expressed as the change with respect to the corresponding estimated β-actin threshold cycle (Ct) levels. All the analyses were based on the Ct values of the PCR products. The Ct was defined as the PCR cycle at which the fluorescence signal crossed over a threshold corresponding to the exponential phase of the amplification curve. The calibrated ΔCt value (ΔΔCt) for each sample and the internal controls (β-actin) was estimated as $\Delta\Delta Ct = 2^{-(\Delta Ct_{sample} - \Delta Ct_{internal\ control})}$.

Western blotting

Following Se exposure, total protein content was extracted from goldfish liver and gills in a protein extraction buffer (5.6 mM Tris, 0.55 mM EDTA, 0.55 mM EGTA, 0.1% SDS, 0.15 mg/mL PMSF and 0.15 mg/mL leupeptin), subsequently sonicated, and quantified using the Bradford method (Bio-Rad). Total protein was loaded onto a 4% acrylamide stacking gel (30 μg per lane) and a 12% acrylamide resolving gel. The gels were immediately transferred to a 0.2-μm polyvinylidene diflouride membrane (Bio-Rad), and run at 85 V for 1.5 h at 4°C. Thereafter, the membranes were blocked with 5% milk in Tris-buffered saline (TBS) for 45 min, and then washed with TBS. The membranes were incubated with MT (1 : 2000 dilution; MA1-25479; Pierce Biotechnology, Rockford, IL, USA) and NKAα (dilution 1 : 2000; monoclonal mouse antibody to α-subunits of chicken, α5; Development Studies Hybridoma Bank, USA), followed by horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (1 : 5000; Bio-Rad) and anti-mouse IgG secondary antibody (dilution 1 : 2000; Bio-Rad) for 60 min. β-tubulin (1 : 5000; ab6046, abcam, Cambridge, UK) was used as internal control, followed by horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (1 : 5000; Bio-Rad) for 60 min. Bands were detected using a WesternBright™ ECL (Advansta, Menlo Park, CA, USA) and exposure for 30 s with a Molecular Imager®ChemiDoc™ XRS+ Systems (Bio-Rad). The images of the membranes were scanned using a high-resolution scanner, and the band density was estimated using Image Lab™ Software (version 3.0, Bio-Rad).

Plasma parameter analysis

Plasma AspAT, AlaAT, and glucose levels were measured using a dry multiplayer analytic slide method in a biochemistry autoanalyzer (Fuji Dri-Chem 4000; Fujifilm, Tokyo, Japan).

A plate reader was used to observe variations in cortisol and ACTH levels in plasma during Se exposure. The levels of cortisol in plasma were analyzed using an ELISA immunoassay, according to the manufacturer's instructions (E08487f; Cusabio Biotech, Hubei, China).

The levels of ACTH in plasma were analyzed using

an ELISA immunoassay, according to the manufacturer's instructions (E15926Fh; Cusabio Biotech, Hubei, China).

NKA activity of gills

Following Se exposure, NKA activity was measured from goldfish gills. Gill samples (approximately the 4-6 primary gill filaments immediately above the septum) were collected from anesthetized fish, immersed in 100 μL of ice-cold SEI buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3), and frozen at −80°C for subsequent analysis. The filaments were thawed, homogenized in SEI buffer containing 0.1% deoxycholic acid, and centrifuged at 5,000 × *g* for 30 s to remove any trace of insoluble materials. NKA activity and protein content was measured from the supernatant. The homogenate samples (10 μL) were added to a 200-μL assay mixture, with or without 0.5 mmol/ Louabain, in 96-well microplates at 25°C and read at 340 nm for 10 min with intermittent mixing. The protein content of each sample was determined using a Protein Assay Standard II (Bio-Rad). NKA activity was estimated as the difference in ATP hydrolysis detected in the presence and absence of ouabain, and expressed as μmoles of ADP/mg protein/h.

H_2O_2 and LPO assay

A plate reader was used to observe the levels of H_2O_2 and LPO in plasma during Se. H_2O_2 concentrations were measured using the Peroxide Detect kit (Sigma). Absorbance was read at 560 nm, and the concentration of H_2O_2 was interpolated from a standard curve. The concentrations are expressed as nM/mL.

LPO was quantified by measuring the quantity of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), products of the degradation of polyunsaturated fatty acids (PUFAs) hydroperoxides (Esterbauer *et al*., 1991), and measured according to the manufacturer instructions (Lipid Hydroperoxide Assay Kit, Cayman Chemical, Ann Arbor, MI, USA). The absorbance was read at 500 nm using a plate reader. LPO was expressed as nM of MDA and 4-HNE/g of protein.

Statistical analysis

All data were analyzed using the SPSS statistical package (version 10.0; SPSS Inc., USA). One-way ANOVA followed by Tukey *post hoc* test was used to compare differences in the data. All statistical significance tests were done with a one-tailed homoscedastic (equal variance) t-test ($P < 0.05$). The values were expressed as the mean ± SE of three independent measurements.

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