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ORIGINAL PAPER

Effects of waterborne selenium exposure on the antioxidant and immunological activity in the goldfish, *Carassius auratus*

Young Jae Choi[1], Na Na Kim[1], Hyun Suk Shin[1], Mi Seon Park[2], Gyung-Suk Kil[3] & Cheol Young Choi[1]

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Abstract Selenium (Se) is an essential element that has relevant roles in growth, immunity, and the protection of cell membranes in organisms under normal conditions. The aim of this study was to test the antioxidant and immune functions of goldfish *Carassius auratus* after Se treatment. At the end of the experiment, levels of antioxidant enzymes (superoxide dismutase, catalase, and glutathione peroxidase) and immune functions (immunoglobulin M protein and plasma lysozyme, thyroxine, and triiodothyronine) were significantly higher in goldfish exposed to 1 and 2 mg/L of Se than those exposed to 0.5 and 3 mg/L doses. In addition, plasma glutathione, H_2O_2, and lipid peroxidation levels were significantly lower in the 1 and 2 mg/L treatment group than 0.5 and 3 mg/L treatment group. These results indicated that 1 and 2 mg/L of Se enhance antioxidant defense and immune system function under aquarium conditions.

Keywords Selenium, Antioxidant defense system, Immune activity, Thyroxine (T_4), Triiodothyronine (T_3)

Environmental changes such as temperature and salinity induce oxidative stress, which can generate reactive oxygen species (ROS) in teleost. ROS, including superoxide, hydrogen peroxide (H_2O_2), hydroxyl radicals, and singlet oxygen are produced naturally during oxidative metabolism[1,2]. However, overproduction of ROS in response to environmental stress can lead to increased lipid peroxidation (LPO) that may affect cell viability by causing membrane damage and enzyme inactivity. Subsequently, cell senescence and apoptosis as well as the oxidation of nucleic acids and proteins may be accelerated. The resultant DNA damage may provoke a variety of physiological disorders such as accelerated aging, and reduced disease resistance and reproductive ability[3,4]. Furthermore, ROS may seriously affect immune function by decreasing lysozyme activity[5].

Aerobic organisms have evolved complex antioxidant defense systems to protect themselves against ROS generating oxidative stress. Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and glutathione (GSH) have antioxidant functions in the livers and kidneys of marine organisms[6-9]. Phase 1 enzymes SOD and CAT directly scavenge ROS; SOD removes O_2^- through the process of dismutation to O_2 and H_2O_2 ($2O_2^- + H^+ \rightarrow H_2O_2 + O_2$) then the H_2O_2 produced by SOD is sequentially reduced to H_2O and O_2 by the oxidoreductase CAT. In the end CAT breaks down two molecules of H_2O_2 into two molecules of H_2O and O_2 ($2H_2O_2 \rightarrow 2H_2O + O_2$), thereby counteracting the toxicity of H_2O_2[10].

Selenium (Se) is an antioxidant substance and essential trace element required in the diet for the normal growth and physiological function of fish[11,12].

This element is an integral part of the functional units of all selenoenzymes known so far, themost prominent being GPX, iodothyronine deiodinase, thioredoxin reductase and selenophosphatase synthetase[13]. Selenoenzymes are part of the antioxidant defense system and are involved in thyroid hormone metabolism, in spermatogenesis, and probably in other processes unidentified to date. The activity of these enzymes

[1]Division of Marine Environment & BioScience, Korea Maritime and Ocean University, Busan 606-791, Korea
[2]Aquaculture Management Division, National Fisheries Research & Development Institute, Busan 619-705, Korea
[3]Division of Electrical and Electronic Engineering, Korea Maritime and Ocean University, Busan 606-791, Korea
Correspondence and requests for materials should be addressed to C. Y. Choi (choic@kmou.ac.kr)

depends on adequate Se intake, defining this trace element as an essential nutrient[13]. Therefore, Se is involved in many functions such as moderation of the immune system and prevention of cancer, acting directly as a support for the organismal health[14-16].

Bioaccumulation of Se is toxic at concentrations slightly above the homeostatic requirement[13,17], and a component of the enzyme glutathione peroxidase, which plays an important role in protecting cell membranes against oxidative damage[18-20]. This is especially important in intensive fish farming because fish often suffer from multiple microbial infections[21,22].

In addition, Se plays an important role in the control of thyroid hormone (TH) metabolism. The thyroid gland's major hormone product, thyroxine (T_4), is converted by 5′ (outer ring) deiodination to the more biologically active hormone, triiodothyronine (T_3) by type I and type II iodothyronine deiodinases (ID-I, ID-II). T_4 also can be converted to reverse triiodothyronine (3,5′,3′-triiodothyronine, γT3) by 5′ (inner ring) deiodination[23]. Therefore, Se may play a crucial role in the maintenance of normal thyroid physiology. The release of TH from thyroid follicles is regulated by the hypothalamus-pituitary-thyroid (HPT) axis[24,25]. According to a recent study performed in fish, Se processing could stimulate T_4 and T_3, in relation to the development and function of the lymphocytes[22].

Immunoglobulin M (IgM), which acts as an immune indicator is a major component of the teleost humeral immune system[26], and its secretion level is regulated by pituitary[27]. To date, Se toxicity has been mainly performed in fish related studies[28-30], while studies on the effects of Se as an antioxidant and immunity enhancer in fish remain very limited.

Most research on Se in animals is based on studies of inorganic forms of Se, principally sodium selenite; selenomethionine is the predominant organic form of Se in plants[31,32]. Currently, inorganic sodium selenite is the most common source of Se to supplement diets[33].

This study was conducted to confirm the optimal Se concentration range to maximize antioxidant capacity and immunity, and determine the exposure enhancement effect of Se on antioxidant reactions and immunity in goldfish *Carassius auratus*. We investigated the expression and activity changes of antioxidant enzymes (SOD, CAT, and GPX), IgM protein expression and GSH in goldfish treated with 0.5, 1, 2, and 3 mg/L of sodium selenite for 0 h, 6 h, 12 h, 24 h, 72 h, and 120 h. We also determined oxidative stress in goldfish by measuring changes in plasma H_2O_2 concentration and LPO levels, and assessed the effect of Se on immunity by measuring T_4, T_3, and lysozyme levels.

Time-course and dose-related effects of Se on antioxidant enzymes (SOD, CAT, and GPX)

We examined the expression and activities of antioxidant enzyme mRNAs and proteins during Se treat-

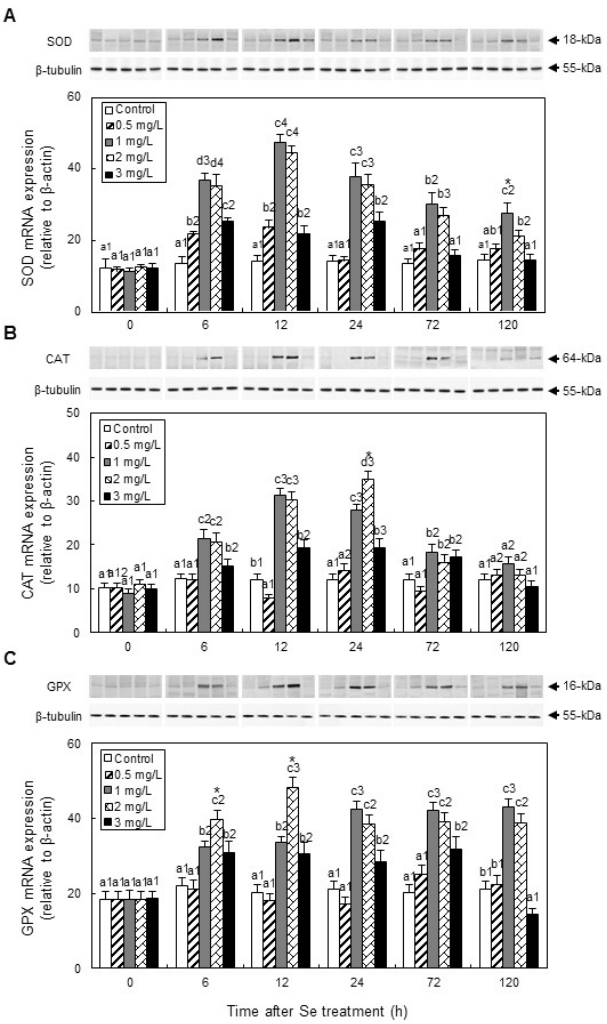

Figure 1. Changes of antioxidant enzymes [SOD (A), CAT (B), and GPX (C)] in the liver during Se treatment in the goldfish. Western blot of antioxidant enzymes (SOD, CAT, and GPX) protein expression in liver of goldfish after Se treatment; β-tubulin (55-kDa) was used as the internal control. Antioxidant enzymes [SOD (A), CAT (B), and GPX (C)] mRNA levels relative to β-actin mRNA levels in the liver of goldfish after Se treatment, based on quantitative real-time PCR. We reverse-transcribed 3 μg of total RNA prepared from liver and amplified the sample using gene-specific primers. Numbers indicate significant differences between different time points at the same Se concentration, and lower-case alphabet letters indicate significant differences between different Se concentrations at a single time point ($P<0.05$). The asterisks indicate significant differences between different Se concentration within the same time point (h) ($P<0.05$). All values are means ± SE ($n=5$).

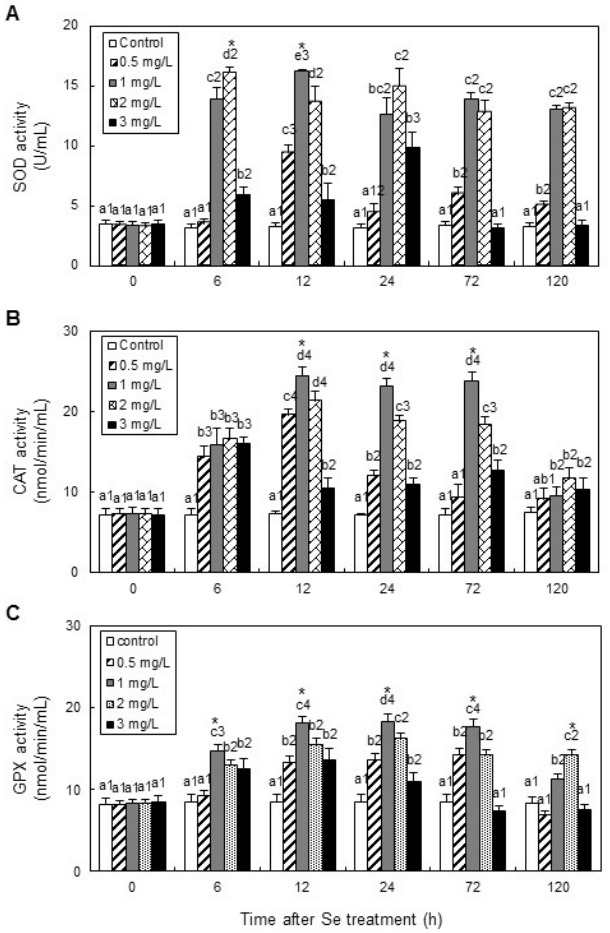

Figure 2. Changes in the activity of SOD (A), CAT (B), and GPX (C) in the liver during Se treatment of goldfish. Numbers indicate significant differences between different time points at the same Se concentration, and lower-case alphabet letters indicate significant differences between different Se concentrations at a single time point ($P<0.05$). The asterisks indicate significant differences between different Se concentration within the same time point (h) ($P<0.05$). All values are means ± SE (n=5).

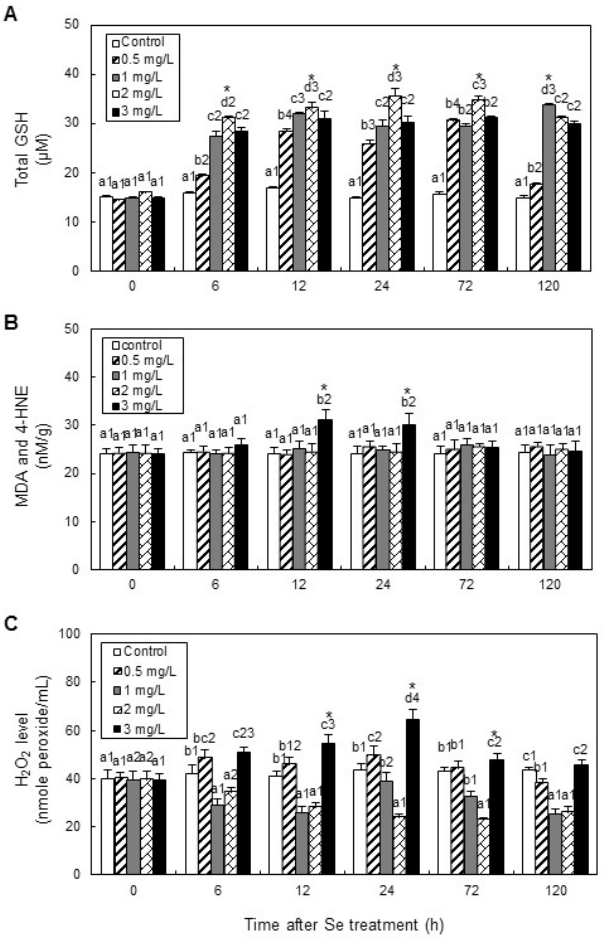

Figure 3. Change in the liver GSH activity (A), muscle LPO levels (B), and plasma H_2O_2 concentrations (C) during Se treatment of goldfish. Numbers indicate significant differences between different time points at the same Se concentration, and lower-case alphabet letters indicate significant differences between different Se concentrations at a single time point ($P<0.05$). The asterisks indicate significant differences between different Se concentration within the same time point (h) ($P<0.05$). All values are means ± SE (n=5).

ment by QPCR and western blot, respectively (Figures 1 and 2). Western blot analysis revealed a protein with antioxidant enzymes-specific immunoreactivity with a mass that corresponded to the predicted mass of goldfish antioxidant enzymes: SOD, 16-kDa; CAT, 64-kDa; GPX, 20-kDa (Figure 1). In addition, antioxidant enzyme mRNA expression in liver was significantly higher following 1 and 2 mg/L of Se after 12 h (approximately 2.03-, 3.43-, and 2.63-fold higher for SOD, CAT, and GPX, respectively) than 0.5 and 3 mg/L Se treatment (Figure 1). The protein expression patterns of SOD, CAT, and GPX resembled the mRNA expression patterns in goldfish liver. Similar to antioxidant enzymes mRNA expression, activity of antioxidant enzymes in liver was significantly higher following Se treatment at 1 and 2 mg/L than, 0.5 and 3 mg/L (Figure 2).

Time-course and dose-related effects of Se on GSH, LPO, and H_2O_2 levels

We observed the levels on liver GSH, plasma H_2O_2, and muscle LPO during Se treatment (Figure 3). At first, GSH levels in the liver significantly increased in all Se treatment groups (approximately 2.11-, 2.25-, 2.2-, and 2.08-fold higher at 0.5, 1, 2, and 3 mg/L Se, respectively) compared to control (Figure 3A). In contrast, LPO levels were significantly higher at 3 mg/L

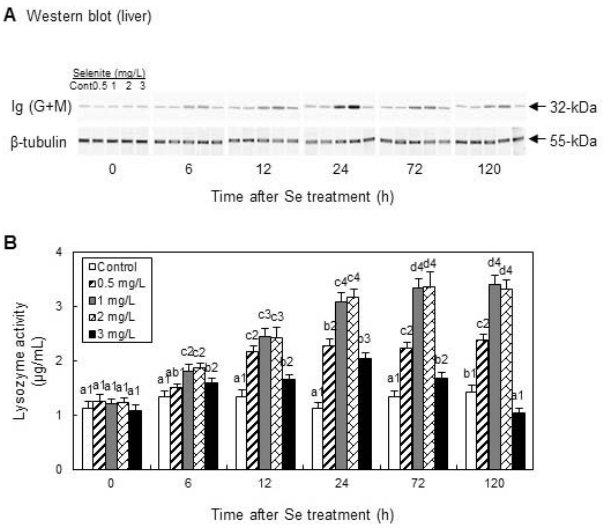

Figure 4. Changes in the IgM protein and plasma lysozyme levels during Se treatment in goldfish. (A) Expression levels of Ig (G+M) protein in the liver during Se treatment of goldfish; β-tubulin (55-kDa) was used as the internal control. (B) Changes in the plasma lysozyme during Se treatment of goldfish. Numbers indicate significant differences between different time points at the same Se concentration, and lower-case alphabet letters indicate significant differences between different Se concentrations at a single time point ($P<0.05$). The asterisks indicate significant differences between different Se concentration within the same time point (h) ($P<0.05$). All values are means ± SE (n=5).

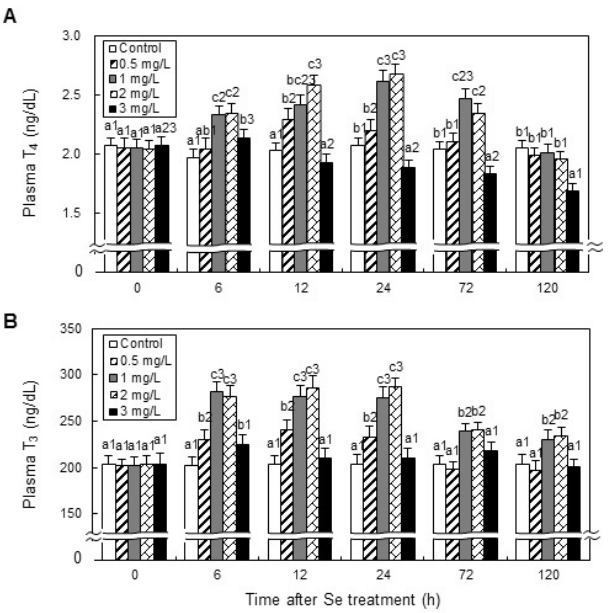

Figure 5. Change in plasma thyroxine (T_4, A) and triiodothyronine (T_3, B) during Se treatment of goldfish. Numbers indicate significant differences between different time points at the same Se concentration, and lower-case alphabet letters indicate significant differences between different Se concentrations at a single time point ($P<0.05$). The asterisks indicate significant differences between different Se concentration within the same time point (h) ($P<0.05$). All values are means ± SE (n=5).

Se (approximately 1.29-fold) than at other Se concentrations (Figure 3B). Furthermore, plasma H_2O_2 levels significantly increased over 120 h of Se treatment at 3 mg/L (approximately 1.62-fold than control) while H_2O_2 levels significantly decreased in groups treated with 1 and 2 mg/L Se (approximately 0.64- and 0.63-fold than control, respectively) (Figure 3C).

Time-course and dose-related effects of Se on IgM protein and lysozyme levels

To further elucidate the regulatory effects of Se, we measured IgM protein levels in liver tissue following Se treatment of goldfish. These proteins were detected in a size range corresponding to the predicted size for goldfish IgM (approximately 32-kDa). In all treatment groups, IgM protein expression levels significantly increased at 24 h and then decreased after treatment Se (Figure 4A).

While measuring immune system responses, we also quantified circulating levels of lysozyme in goldfish following treatment with Se (Figure 4B). Initial plasma lysozyme levels were 1.274 ± 0.132 μg/mL. In 0.5, 1, and 2 mg/L Se treatment groups, lysozyme levels significantly increased after Se treatment. In particular, lysozyme significantly increased 2.77- and 2.94-fold higher in the 1 and 2 mg/L Se treatment groups compared to 0.5 and 3 mg/L Se groups (1.209 ± 0.098 and 1.223 ± 0.081 μg/mL, respectively).

Time-course and dose-related effects of Se on plasma T_4 and T_3 Levels

A plate reader was used to observe plasma T_4 and T_3 levels during Se treatment (Figure 5). The plasma T_4 and T_3 levels were 2.07 ± 0.03 and 203.7 ± 7.8 ng/dL at start of the experiment, respectively. In groups treated with 0.5, 1, and 2 mg/L of Se, T_4 and T_3, levels significantly increased at 6 h then decreased. In particularly, T_4 and T_3 significantly increased and peaked at 24 h with 1 and 2 mg/L Se treatment (2.61 ± 0.04 and 2.61 ± 0.08 ng/dL on T_4; 275.3 ± 8.3 and 286.5 ± 9.3 ng/dL on T_3, respectively).

Discussion

To investigate the effects of waterborne Se on antioxidant enzymes expression, activities, and immunity, goldfish were treated with Se at 0.5, 1, 2, and 3 mg/L

for 0, 6, 12, 24, 72, and 120 h. Expression and activity changes of antioxidant enzymes (SOD, CAT, and GPX), and expression of immune system fusion proteins IgM and GSH were examined.

We investigated mRNA expression, activity, and protein expression changes of antioxidant enzymes (SOD, CAT, and GPX) in goldfish exposed to the four Se concentrations for up to 120 h. Antioxidant enzymes expression significantly increased at 6 h, peaked at 12 h, then decreased in all Se treatments. When comparing the effects of Se concentrations, we found that antioxidant enzymes expression was significantly higher in the liver of goldfish exposed to 1 and 2 mg/L of Se compared to 0.5 and 3 mg/L at 12 h (Figures 1 and 2). Results of this study were consistent with a previous study, which reported that oxidative stress was reduced in brown rat *Rattus norvegicus* fed a Se-added diet because Se is directly applied to the antioxidant defense system[34]. Monteiro *et al.*[35] also reported that the activity of antioxidant enzymes, such as SOD, CAT, and GST, and LPO increased in *Brycon cephalus* because of oxidative stress caused by a 4-day exposure to the toxic substance, Folisuper 600 BR. However, by supplying Se to *B. cephalus*, activity levels of SOD, CAT, GST, and LPO subsequently began to decrease, and the activities of GSH and GPX declined to normal values. Se may play an important role in balancing the oxidation/reduction state in the cell and maintaining antioxidant activity[35]. The results of this study, along with the study findings of Monteiro *et al.*[35] suggest that exposure to a Se environment *in vivo* increased the activity and expression of antioxidant enzymes in goldfish.

GSH levels in the liver were significantly higher following the 1 and 2 mg/L of Se treatment than control (Figure 3A); in contrast, the 1 and 2 mg/L Se treatment groups had significantly lower levels of GSH than other concentrations of Se (Figure 3). GSH, as a potent antioxidant is known to reduce LPO, reduce cell membrane damage[14,15], and protect tissues against ROS-induced oxidative stress[36]. In addition, Monteiro *et al.*[30] reported that Se has the ability to reduce ROS by maintaining the LPO level by increasing the utilization of GSH.

In this study, we speculated that GSH activity is increased by Se treatment, and LPO concentration is maintained, thereby reducing H_2O_2 levels (Figures 2 and 3). Specifically, Se plays an important antioxidant role since it is a GPX cofactor. GPX scavenges H_2O_2 and lipid hydroperoxides, using reducing equivalents from glutathione and protecting membrane lipids and macromolecules from oxidative damage[37]. So, we suggest that Se concentrations between 1 mg/L and 2 mg/L are optimal concentration intervals that can effectively suppress oxidative stress by increasing GSH, a powerful antioxidant.

In contrast, levels of H_2O_2 and LPO were observed to increase significantly at 3 mg/L of Se (Figure 3). In a previous study, rainbow trout *Oncorhynchus mykiss* were exposed to Se for 30 days. During this period, LPO levels were reduced at 2.5 mg/L of Se, but increased at the relatively high concentration of 3.6 mg/L[38]. Therefore, a concentration of 3.6 mg/L is excess concentrate and increases LPO by damaging the tissue confirm the results of the present study[38]. Weiller *et al.*[39] suggested that pro-oxidative reactions can be involved in the Se toxicity. When present in excessive amounts, selenium has been shown to interact with cellular sulphydryls, which leads to a depletion of intracellular glutathione and an increase in lipid peroxidation[40]. So, we observed that when a 3 mg/L Se treatment raised the level of H_2O_2 by damaging the tissue, LPO increased. In the goldfish, a Se concentrate of 3 mg/L is considered toxic and does not exert any antioxidant power.

To confirm the antioxidant effect of Se and its role in immunological processes, depending on the concentration by Se treatment, we investigated the expression of IgM protein, an immunoreactive substance that changes plasma parameters of the immune response lysozyme levels. After exposure to different Se concentrations, expression level of IgM and activity of plasma lysozyme was significantly higher with the passage of time (Figure 4). Se is an essential micronutrient, effectively used by the immune system of animals, which affects the immune process all cytoplasmic and cell membrane by reduction of antioxidant enzymes[41]. In addition, Arthur *et al.*[22] and Miller *et al.*[41] reported that the antioxidant effect of Se greatly enhances macrophage function and protects the neutrophil leukocytes from the active oxygen effects of the pathogen entering from the outside. A previous study reported that in juvenile Olive flounder *Paralichthys olivaceus* contained-Se concentration of 1.07 mg/kg feed increased the lysozyme activity and effective of immune activity[42]. Comparing the results of this study and previous research results, we suggest that while the immune system is stimulated with treatment of Se at adequate concentrations (1-2 mg/L), the expression level of IgM and activity of plasma lysozyme also increases in fish.

In addition, T_4 and T_3 are other immune parameters modulating the immune system by influence of growth, development, number of lymphocytes, and metabolism in fish[25,43]. Se is necessary in the process of converting the hormone T_4 into biologically active thyroid hormone T_3 (three to four times potent than T_4)[23]. In a previous study, Arthur *et al.*[22] reported that T_4 and T_3,

Table 1. Primers used for QPCR amplification.

Genes	Primer	DNA sequences
SOD (JQ776518)	Forward	5′-ACA ACC CTC ATA ATC AAA CTC A-3′
	Reverse	5′-GCA ACA CCA TCT TTA TCA GC-3′
CAT (JQ776513)	Forward	5′-ATC TTA CAG GAA ACA ACA CCC-3′
	Reverse	5′-CGA TTC AGG ACG CAA ACT-3′
GPX (JQ776515)	Forward	5′-CGG TGA ACA GGA ATG ACA-3′
	Reverse	5′-GTG AGG AAC CTT CTG CTG-3′
β-actin (AB039726)	Forward	5′-TTC CAG CCA TCC TTC CTA T-3′
	Reverse	5′-TAC CTC CAG ACA GCA CAG-3′

in relation to the development and function of the lymphocytes, decreased during stress, however, after Se processing, T_4 and T_3 increased again. In this study, we observed that in treatment groups of 1 and 2 mg/L Se, T_4 and T_3 levels significantly increased after treatment, however, at 3 mg/L Se, T_4 and T_3 significantly decreased (Figure 5). Based on these findings, we have considered that immunity of gold-fish increased at Se concentration intervals of 1 and 2 mg/L, however, in the 3 mg/L Se treated group, T_4 and T_3 levels and activity of lysozyme decreased (Figure 4A) therefore, immunity is degraded by Se concentrations of 3 mg/L.

In conclusion, we confirm that the concentrations of 1 and 2 mg/L Se effectively suppress the occurrence of oxidative stress in goldfish, and induce the antioxidant function and immune enhancement effect. However, we suggest that the high concentration of 3 mg/L Se or more not only increases H_2O_2, but also reduced the immune system.

Materials & Methods

Experimental fish

Goldfish (average length, 11.0 ± 0.2 cm; mass, 12.1 ± 0.6 g) were purchased from a commercial fish farm (Busan, Korea), and maintained in five 100 L circulation filter tanks prior to experiments in the laboratory. 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 activities and immunity, goldfish were treated with waterborne sodium selenite (Se, Na_2SeO_3; Sigma, St Louis, MO, USA) at 0.5, 1, 2, and 3 mg/L. Tissues (liver and muscle) were sampled from five different fish at each of the following 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. 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.

Quantitative PCR (QPCR)

QPCR was conducted to determine the relative mRNA expression of antioxidant enzymes (SOD, CAT, and GPX) using total RNA extracted from the liver of goldfish. Primers for QPCR were designed with reference to the known sequences of goldfish shown in Table 1. QPCR amplification was conducted using a BIO-RAD CFX96™ Real-Time System (Bio-Rad, Hercules, CA, USA) and the iQ™ SYBR Green Supermix (Bio-Rad) according to the manufacturer instructions. Each treatment group was treated three times to ensure consistency. As an internal control, β-actin was amplified for each sample, and all data were expressed as the change with respect to the corresponding β-actin calculated threshold cycle (Ct) levels. The calibrated ΔCt value (ΔΔCt) for each sample and internal control (β-actin) was calculated as $\Delta\Delta Ct = 2\text{^} - (\Delta Ct_{sample} - \Delta C_{tinternal\ control})$.

SOD, CAT, GPX, and GSH activity analysis

The liver tissues were homogenized in ice-cold 0.1 M PBS (pH 7.4) containing 1 mM EDTA. The homogenates were centrifuged at 10,000 × g for 15 min at 4°C. The supernatant was removed, and the remaining pellet was used for the analysis. SOD, CAT, and GPX activities were determined using commercial kits supplied by Cayman Chemical (Ann Arbor, MI, USA).

Western blot analysis

Total protein isolated from livers of goldfish during selenite treatment was extracted using protein extrac-

tion 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], sonicated, and quantified using the Bradford method (Bio-Rad). Total protein (30 μg) was loaded per lane onto a 4% acrylamide stacking gel and a 12% acrylamide resolving gel. The gels were immediately transferred to a 0.2-μm polyvinylidene diflouride membrane (Bio-Rad) 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 followed by washing in TBS. Membranes were incubated with SOD (1/2000 dilution; NBP1-47443, Novus Biologicals, USA), CAT (1/2000, sc-58332, Santa Cruz Biotechnology, USA), GPX (1/2000; CPBT-35941RH, Creative Diagnostics, USA), and Ig G+M (1/5000; C-57070, LSBio, Seattle, WA, USA) followed by horseradish peroxidase conjugated anti-rabbit IgG secondary antibody (GPX and IgM, 1/2,000; Bio-Rad) and anti-mouse IgG secondary antibody (SOD and CAT, 1/2,000; Bio-Rad) for 60 min. The internal control was β-tubulin (1/5000; ab6046, abcam, UK) followed by horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (1/5000; Bio-Rad) for 60 min. Bands were detected using WesternBright™ ECL (Advansta, Menlo Park, CA, USA) and exposure for 30 s with a Molecular Imager® ChemiDoc™ XRS+ Systems (Bio-Rad, Hercules, CA, USA). The membrane images were scanned by a high-resolution scanner and the band density estimated using a computer program (Image Lab™ Software, version 3.0, Bio-Rad).

LPO and H_2O_2 assay

LPO was quantified by measuring malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), the degradation products of polyunsaturated fatty acids hydroperoxides[44], and measured according to the manufacturer instructions (Lipid Hydroperoxide Assay Kit, Cayman Chemical). The absorbance was read at 500 nm using a plate reader.

H_2O_2 concentrations were measured using the modified methods of Nouroozzadeh *et al.*[45] and a Peroxid Detect kit (Sigma). Absorbance was read at 560 nm, and the concentration of H_2O_2 was interpolated from a standard curve.

Plasma parameter analysis

Plasma lysozyme (E17296Fh; Cusabio Biotech, Hubei, China) levels were analyzed using the enzyme-linked immunosorbent assay (ELISA) kit.

Additionally, plasma T_3 and T_4 levels were analyzed using enzyme immunoassay (EIA) with the T_3 and T_4 EIA Kit (Biosewoom, Seoul, Korea).

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 ($P<0.05$). The values were expressed as the mean ± SE of three independent measurements.

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