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ORIGINAL PAPER

Influence of quercetin on the physiological response to cadmium stress in olive flounder, *Paralichthys olivaceus*: effects on hematological and biochemical parameters

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Abstract The aim of the present study was to determine whether quercetin, an antioxidant and radical scavenger as natural flavonoid, would be able to offer any protection against cadmium (Cd) toxicity in olive flounder, *Paralichthys olivaceus* with emphasis on biochemical analysis. Fish were pre-treated with 0% (Diet 1), 0.25% (Diet 2) and 0.5% (Diet 3) quercetin for 30 and 60 day and after that, fish were post-exposed to 10 ppb Cd for 0, 6, 12, 24, and 48 hr. To understand the stress-resistance effect of quercetin, we measured the mRNA expression of metallothionein (MT), glucocorticoid receptor (GR), and level of acetylcholinesterase (AChE) in quercetin-treated flounder exposed to Cd. The MT and GR expression levels were lower in flounder fed Diets 2 and 3 than in those fed Diet 1, and AChE level was higher in flounder fed Diet 2 and 3 than in those fed Diet 1. Plasma cortisol increased in fish fed Diets 1, 2, and 3, but it was lower in fish fed Diets 2 and 3 than in those fed Diet 1. In addition, lipid peroxidation (LPO) levels lower than with Diet 1, which protected the cell membrane. We also investigated the effects of cortisol on stress resistance *in vitro*. Results showed that the MT and GR expression levels were lower in livers of flounder fed with Diets 2 and 3 than those fed with Diet 1, suggesting that quercetin reduced the stress induced by Cd. These results indicate that quercetin has a stress-resistance effect and acts to maintain physiological homeostasis.

Keywords Acetylcholinesterase activity, Glucocorticoid receptor, Metallothionein, Stress, Quercetin

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Cadmium (Cd) is a heavy metal that has natural and industrial sources. It is highly toxic to aquatic organisms, humans, animals, and plants, even at low doses[1]. Aquatic organisms accumulate Cd in the liver, kidney, and gills[2,3], which is deleterious to growth, reproduction, and osmoregulation[4]. Cd increases the formation of reactive oxygen species (ROS) and provokes oxidative stress in organisms[5]. Overproduction of ROS by Cd can increase lipid peroxidation (LPO), the oxidation of nucleic acid and proteins, and DNA damage.

Metallothionein (MT) synthesis is induced to protect cells from heavy metal toxicity. MTs are low-molecular weight (6.0-7.0 kDa), cysteine-rich proteins that bind Cd and detoxify heavy metals[6]. Also, MTs induced by a variety of environmental stressors also play roles in binding heavy metals[7]. Previous studies have reported that MT synthesis is induced by Cd exposure in the common carp, *Cyprinus carpio*[8]; yellow perch, *Perca fluviatilis*[9]; white sucker, *Catostomus commersonii*[10]; killifish, *Oryzias latipes*[11]; and pufferfish, *Takifugu obscures*[12].

Stress activates the hypothalamo-pituitary-interrenal axis, which causes the rapid release of corticotrophin-releasing hormone and cortisol into the blood[13-15]. Cor-

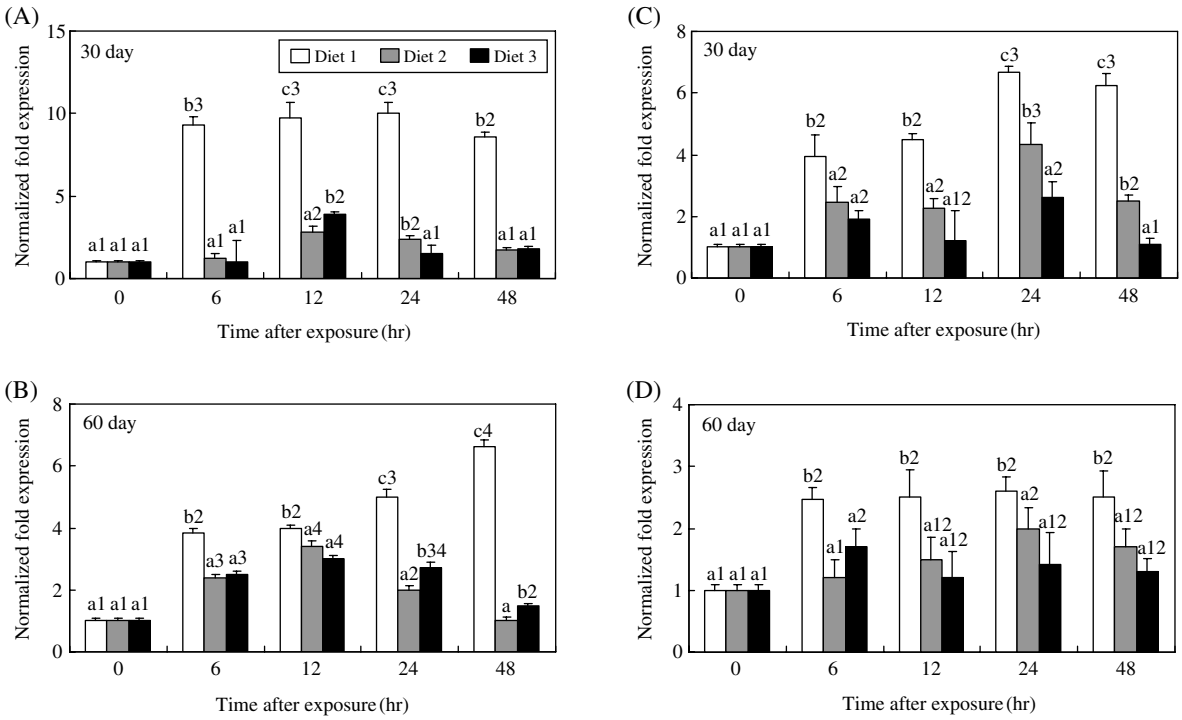

Figure 1. Expression of MT (A and B) and GR (C and D) mRNA, as measured by quantitative real-time PCR, in olive flounder fed a diet containing quercetin (Diet 2, 0.25%; Diet 3, 0.5%) or no quercetin (Diet 1, 0%) for 30 (A and C) or 60 day (B and D) and then exposed Cd (0, 6, 12, 24, and 48 hr). Total liver RNA (2.5 μg) was reverse-transcribed and amplified using gene-specific primers. Results are expressed as normalized fold expression with respect to β-actin in the same sample. Values with alphabets indicate significant differences between Diet 1, 2, and 3 within the same time after exposure. The numbers indicates significant differences from the control (within the same diet) ($P < 0.05$). All values are means ± SD ($n=5$).

tisol secreted into the plasma regulates a variety of physiological processes, which directly affect the cell by diffusion or binding the glucocorticoid receptor (GR), a specific receptor in the cell membrane, to regulate physiological activity[16]. Olsson[9] and Sathiyaa and Vijayan[17] reported that GR mRNA expression and plasma cortisol increases in rat, *Rattus norvegicus*[18], and tilapia, *Oreochromis niloticus*[19], exposed to Cd.

Furthermore, Cd acts on the central nervous system (CNS), causing behavioral and biochemical dysfunction[20], including changes in nucleic acid structure and the levels of various neurotransmitters[21]. Acetylcholine is a classical neurotransmitter that has multiple roles in the CNS. After its release, acetylcholine is rapidly removed from the synaptic cleft by acetylcholinesterase (AChE), which cleaves acetylcholine into choline and acetate[22]. However, Cd inhibits AChE activity, resulting in a negative effect on the nervous system[23].

Quercetin is a polyphenolic flavonoid compound that is almost ubiquitous in plants, protects organisms from stress[24,25], and is a powerful antioxidant[26]. Broccoli and apples contain 7-110 mg/kg, and onions contain 284-486 mg/kg[26]. Quercetin also plays a role in the excretion of heavy metal ions such as Cd[27].

No studies have examined the effects of quercetin on stress regulation in fish. Therefore, we investigated the effect of quercetin pretreatment on the toxicity induced by Cd in the olive flounder, *Paralichthys olivaceus*. We measured the expression of MT and GR mRNA in liver and change the activity of AChE in brain and LPO in liver to determine the stress by Cd exposure. We also measured changes in plasma cortisol following exposure to Cd to demonstrate stress response by toxicity, and MT and GR mRNA expression by primary cell culture of liver treated with cortisol as a general stress indicator to examine effect of cortisol on the changes of MT and GR mRNA expression related with stress response.

MT and GR mRNA expression

After 30 day of feeding with Diet 1, MT mRNA expression increased significantly at 6 hr, followed by a decrease at 48 hr, and GR mRNA expression increased gradually with changes in time after Cd exposure.

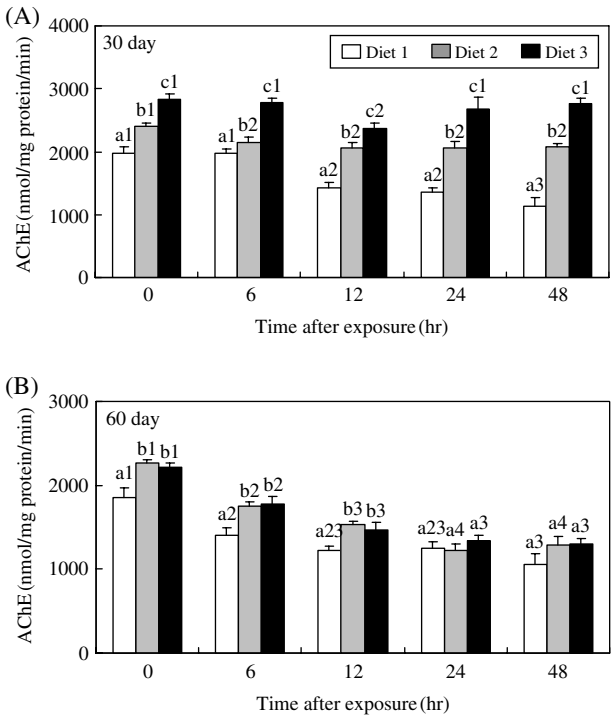

Figure 2. AChE activity in olive flounder fed diets containing quercetin (Diet 2, 0.25%; Diet 3, 0.5%) or no quercetin (Diet 1, 0%) for 30 (A) or 60 day (B) and then exposed Cd (0, 6, 12, 24, and 48 hr) using brain tissue. Values with alphabets indicate significant differences between Diet 1, 2, and 3 within the same time after exposure. The numbers indicates significant differences from the control (within the same diet) ($P<0.05$). All values are means ± SD ($n=5$).

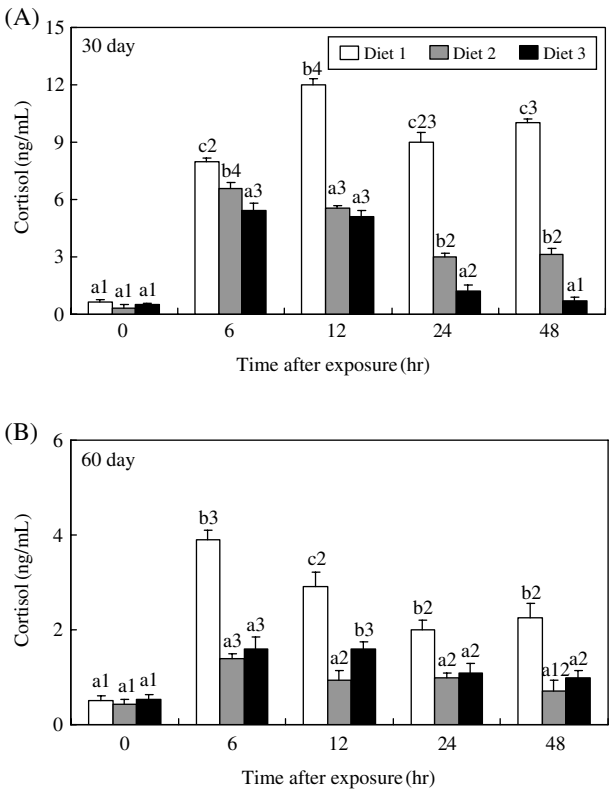

Figure 3. Plasma cortisol in olive flounder fed a diet containing quercetin (Diet 2, 0.25%; Diet 3, 0.5%) or no quercetin (Diet 1, 0%) for 30 (A) or 60 day (B) and then exposed Cd (0, 6, 12, 24, and 48 hr). Values with alphabets indicate significant differences between Diet 1, 2, and 3 within the same time after exposure. The numbers indicates significant differences from the control (within the same diet) ($P<0.05$). All values are means ± SD ($n=5$).

The MT and GR mRNA level in livers of fish fed Diets 2 and 3 was lower than that in fish fed Diet 1 (Figure 1A, C). After 60 day of feeding with Diet 1, the MT mRNA expression level gradually increased with changes in time after Cd exposure, and GR mRNA expression increased at 6 hr and was maintained until 48 hr in livers of fish fed Diet 1. The MT and GR mRNA levels in livers of fish fed Diets 2 and 3 were lower than that in those fed Diet 1 (Figure 1B, D).

AChE activity

To examine the effect of quercetin on stress in the flounder, Cd-inhibited AChE activity was measured in brains of flounder fed the three experimental diets (Figure 2). After 30 day of feeding with Diet 1, AChE activity gradually but significantly decreased with changes in time after Cd exposure. AChE activity in brains of fish fed Diets 2 and 3 decreased significantly, but remained higher than that in fish fed Diet 1 (Figure 2A). After 60 day of feeding with Diet 1, AChE activity decreased significantly at 6 hr. AChE activity in brains of fish fed Diets 2 and 3 decreased significantly, but remained higher than that in fish fed Diet 1 (Figure 2B).

Plasma cortisol

For both exposure periods of feeding with Diet 1, the plasma cortisol concentration increased significantly at 6 and 12 hr and decreased with time after Cd exposure. Although the plasma cortisol concentration increased significantly at 6 hr in fish fed Diets 2 and 3, they were lower than that in fish fed with Diet 1 for both exposure periods (Figure 3).

LPO assay

After feeding for 30 day, with Diet 1, the LPO level (expressed as MDA and 4-HNE compounds) increased significantly until 48 hr, and the LPO levels with

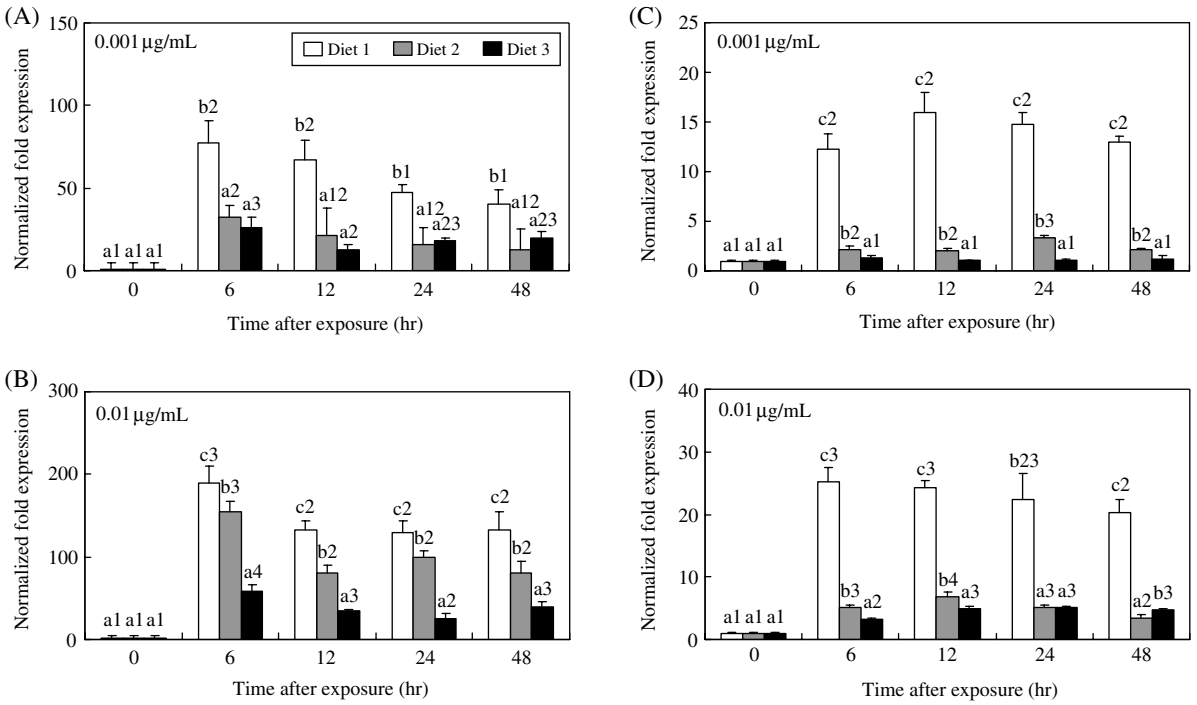

Figure 4. Expression of MT and GR, as measured by quantitative real-time PCR, in the livers of cortisol-treated olive flounder (*in vitro*) fed a diet containing quercetin (Diet 2, 0.25%; Diet 3, 0.5%) or no quercetin (Diet 1, 0%). Olive flounder livers were incubated for 0, 6, 12, 24, and 48 hr with medium alone (control), or with medium containing 0.001 or 0.01 μg/mL cortisol. Total liver RNA (2.5 μg) was reverse-transcribed and amplified using gene-specific primers. The results are expressed as the normalized fold-expression with respect to β-actin in the same sample. Values with alphabets indicate significant differences between Diet 1, 2, and 3 within the same time after exposure. The numbers indicates significant differences from the control (within the same diet) ($P<0.05$). All values are means ± SD ($n=5$).

Diets 2 and 3 were no significant differences with the initial levels, but they were lower than with Diet 1. After feeding for 60 day, with Diet 1, the LPO level increased significantly at 24 hr; with Diet 2, the LPO level increased significantly at 6 hr and was maintained until 48 hr; and with Diet 3, no significant difference was observed compared with the initial levels. The levels with Diets 2 and 3 were lower than with Diet 1 (Table 2).

Quantification of MT and GR mRNA expression by cortisol (*in vitro*)

To examine MT and GR mRNA expression by stress, the changes in MT and GR mRNA expression in cultured livers were compared among fish fed the diets (Figure 4). In the 0.001 μg/mL cortisol group, MT mRNA expression increased at 6 hr and then decreased until 48 hr, and GR mRNA expression increased at 6 hr and was maintained until 48 h in livers from fish fed Diets 1, 2, and 3, but MT and GR mRNA expression levels in livers from fish fed Diets 2 and 3 were lower than those in fish fed Diet 1. In the 0.01 μg/mL cortisol group, MT and GR mRNA expression increased at 6 hr and then decreased until 48 hr in livers from fish fed Diets 1, 2, and 3, but MT and GR mRNA expression levels in livers from fish fed Diets 2 and 3 were lower than those in fish fed Diet 1.

Discussion

We investigated the changes in liver MT and GR mRNA expression, brain AChE activity, LPO levels, and plasma cortisol to demonstrate the stress-resistance effects of quercetin on Cd-induced stress in olive flounder. Furthermore, we investigated the changes in MT and GR mRNA expression in livers treated with cortisol to explain the effect of quercetin on stress reduction.

To examine the stress-resistance effect of quercetin, we compared the changes in MT and GR mRNA expression when olive flounder were fed diets containing quercetin (Diet 1, 2, and 3) for 30 and 60 day and were exposed to Cd. MT and GR mRNA expression increased with time after exposure, but these expres-

sion levels in fish fed Diets 2 and 3 were lower than those in fish fed Diet 1. Previous studies have reported that Cd is a toxic material that induces MT[28,29] and that quercetin excretes heavy metals through chelation[30]. Furthermore, since MT is an antioxidant[31], an efficient scavenger of hydroxyl radicals[32], and also can functionally substitute for antioxidants in oxidative stress[33], the lower expression of MT mRNA in olive flounder fed Diets 2 and 3 than in those fed Diet 1 suggests that the antioxidant quercetin directly scavenged the ROS induced by Cd[27]. This oxidative stress caused by ROS generates LPO and damages cells[34]. In this study, the LPO levels with Diets 2 and 3 were significantly lower than with Diet 1. These results agree with Hiratsuka *et al.*[35], who reported that LPO levels were reduced in mice fed docosahexaenoic acid (DHA).

Plasma cortisol and GR mRNA expression were increased with stress[17]. Gupta *et al.*[36] reported that cortisol decreases in cows, *Bos taurus*, fed vitamin E and selenium, indicating that vitamin E reduces stress by decreasing cortisol concentration. Kawabata *et al.*[37] treated water immersion-restrain stress to rat fed quercetin, plasma cortisol level was lower than those of stressed rat fed no quercetin, this result indicated that quercetin is essential for attenuating hypothalamic-pituitary-adrenal axis activation, thus quercetin might lead to the down-regulation of secretions of cortisol into the plasma. Based on the results of previous studies, we suggest that quercetin reduced the stress of Cd by decreasing cortisol concentrations, which resulted in a decrease in GR mRNA expression.

Cd inhibits AChE activity, produces behavioral dysfunctions, and induces stress in fish[20,38]. The AChE activity in brains of olive flounder fed with Diets 1, 2, and 3 decreased with time after exposure, whereas the activity level in brains from fish fed Diets 2 and 3 was higher than that in fish fed Diet 1. AChE activity decreases in mosquito fish, *Gambusia affinis* brain exposed to ammonia, indicating that ammonia is an AChE inhibitor that inhibits neurotransmitters in fish[35]. More recently Mazzanti *et al.*[39] reported that AChE activity decreased in rat brain treated with the AChE inhibitor ethidium bromide, but that the activity level increased significantly in rats fed vitamin E, suggesting that vitamin E has a neuroprotective effect that inhibits repression of AChE activity by ethidium bromide[39]. Gelinas and Martinoli[40] reported that querce-

Table 1. Ingredients and nutrient composition of the experimental diets.

Ingredient (%)	Diet		
	Diet 1 (0%)	Diet 2 (0.25%)	Diet 3 (0.5%)
Fish meal[a]	45.00	45.00	45.00
Corn gluten meal	15.00	15.00	15.00
Wheat flour	**19.90**	**19.65**	**19.40**
Soybean meal	10.00	10.00	10.00
Fish oil-salmon	2.50	2.50	2.50
Squid meal	2.00	2.00	2.00
Krill meal	2.00	2.00	2.00
Lecithin	1.00	1.00	1.00
Mono-calcium	1.00	1.00	1.00
Choline	1.00	1.00	1.00
VITAMIX[b]	0.20	0.20	0.20
MINEMIX[c]	0.20	0.20	0.20
Vitamin C	0.20	0.20	0.20
Quercetin	**0.00**	**0.25**	**0.50**
Total	100	100	100

[a]Imported from Chile.

[b]Vitamin premix contained the following ingredients (g/kg mix): L-ascorbic acid, 121.2; DL-α-tocopheryl acetate, 18.8; thiamin hydrochloride, 2.7; riboflavin, 9.1; pyridoxine hydrochloride, 1.8; niacin, 36.4; Ca-D-pantothenate, 12.7; myo-inositol, 181.8; D-biotin, 0.27; folic acid, 0.68; p-aminobenzoic acid, 18.2; menadione, 1.8; retinyl acetate, 0.73; cholecalciferol, 0.003; cyanocobalamin, 0.003.

[c]Mineral premix contained the following ingredients (g/kg mix): $MgSO_4 \cdot 7H_2O$, 80.0; $NaH_2PO_4 \cdot 2H_2O$, 370.0; KCl, 130.0; Ferric citrate, 40.0; $ZnSO_4 \cdot 7H_2O$, 20.0; Ca-lactate, 356.5; CuCl, 0.2; $AlCl_3 \cdot 6H_2O$, 0.15; KI, 0.15; $Na_2Se_2O_3$, 0.01; $MnSO_4 \cdot H_2O$, 2.0; $CoCl_2 \cdot 6H_2O$, 1.0.

Table 2. MDA and 4-HNE concentrations in the livers of olive flounder fed for 30 or 60 day diets containing quercetin 0.25 or 0.5%, after 6, 12, 24, and 48 hr exposure to Cd.

	MDA and 4-HNE (nM/g)					
Feeding day	30 day			60 day		
Diet	Diet 1	Diet 2	Diet 3	Diet 1	Diet 2	Diet 3
0 h	77 ± 8.4^{a1}	70 ± 10.0^{a1}	69 ± 2.5^{a1}	72 ± 11.0^{b1}	46.5 ± 8.0^{a1}	41 ± 15.0^{a1}
6 h	105 ± 4.0^{b2}	80 ± 6.4^{a1}	76 ± 11.2^{a1}	94.5 ± 9.0^{c2}	65 ± 4.6^{b2}	45.5 ± 9.0^{a1}
12 h	129 ± 5.0^{b3}	90 ± 2.0^{a2}	82 ± 4.3^{a1}	96 ± 10.0^{b2}	62 ± 9.0^{a2}	60 ± 10.0^{a1}
24 h	130 ± 7.0^{b3}	69 ± 4.3^{a1}	71 ± 8.2^{a1}	117 ± 4.0^{b3}	65 ± 7.4^{a2}	55.5 ± 6.0^{a1}
48 h	127 ± 3.0^{b3}	72 ± 5.1^{a1}	70 ± 6.2^{a1}	110 ± 8.0^{c23}	66 ± 7.1^{b2}	30.5 ± 9.0^{a1}

Values with different letters indicate significant differences from controls for the same diet, and asterisks indicate significant differences between Diet 1 and Diets 2 and 3 ($P < 0.05$). All values are means ± SD ($n = 5$).

tin has also been associated with neural protection[41], repression of cell damage, and antioxidant and stress resistance. Therefore, we hypothesized that quercetin has neuroprotective capacity and indirectly inhibits repression of AChE activity caused by Cd[40,41].

Recent studies have reported that flavonoids, including quercetin, have a stress-resistance effect[24,25]. In this study, plasma cortisol concentrations increased with time after Cd exposure, but the levels in fish fed Diets 2 and 3 were significantly lower than those in fish fed Diet 1. Butterweck *et al.*[42] reported that cortisol concentrations decrease significantly in rats fed flavonoids including imipramine, hypericin, hyperoside, isoquercitrin, and miquelianin. Moreover, Zhou *et al.*[43] reported that plasma cortisol concentrations were lower in soft-shelled turtles, *Pelodiscus sinensis*, that were fed vitamin C than in those that were not, following an exposure to hydrochloric acid, suggesting that vitamin C protects organisms from stress. In combination, these results led us to consider that quercetin reduces the stress induced by Cd by decreasing plasma cortisol concentrations. We also compared changes in MT and GR mRNA expressions in cultured livers treated with two concentrations of cortisol. Results indicated that MT and GR mRNA expression increased with exposure to high concentration of cortisol, but livers from fish fed Diets 2 and 3 secreted significantly less cortisol than those in fish fed Diet 1, suggesting that MT and GR mRNA expression was decreased by quercetin uptake[24,25].

MT and GR mRNA expression levels in olive flounder exposed to Cd after being fed quercetin (Diets 2 and 3) were lower than those in fish fed Diet 1, suggesting that quercetin was involved in Cd excretion. Furthermore, the stress-resistance effects observed in fish fed Diets 2 and 3 were maintained for 30 and 60 day. And MT and GR mRNA expression and AChE activity levels in 60 day are basically lower than those of 30 day, these results indicated that fishes fed quercetin grow up than 30 day, it can be more resist against toxicity. Therefore additional molecular biological and physiological studies are necessary to examine the effect of quercetin on various stress factors.

Materials & Methods

Experimental fish

Olive flounder (n=800; length, 10±0.5 cm; weight, 19.9±1.3 g) were obtained from a commercial fish farm and permitted to acclimate to the experimental conditions for 2 weeks in nine 300-L circulation filter tanks in the laboratory. During the experiments, the water temperature and photoperiod were maintained at 20±1°C and 12-h light : 12-h dark, respectively. The fish were fed a commercial feed twice daily (09: 00 and 17:00). The flounder were randomly distributed among the nine 300-L flow-through tanks, divided into three experimental groups about 50, respectively, and then fed by diets (Diet 1, without quercetin pre-treated group; Diets 2 and 3, quercetin pre-treated groups) for 30 and 60 day.

Experimental diets

Fish meal, dehulled soybean, and corn gluten meal were used as protein sources in the diets, and wheat flour and salmon fish oil were used as the carbohydrate and lipid sources, respectively. The diet ingredients (Table 1) were mixed well with water at a 3 : 1 ratio of ingredients to water and then pelletized, dried at room temperature, and stored at −20°C until use. The crude protein and lipid contents ranged from 55.1 to 56.0% and 9.1 to 9.7%, respectively, and the estimated energy content was 4.1 kcal/g. The experimental diets also contained 0% (Diet 1), 0.25% (Diet 2), or 0.5% (Diet 3) quercetin at the expense of 0, 0.25, or 0.5% wheat flour, respectively (Table 1). The quercetin used in this study was extracted from discarded onion peels using a chromatographic column (Dongsun Industrial, Gyeonggi-do, Korea); the product was sterilized, and its quality was assured.

Cd exposure

The fish fed a diet containing quercetin (Diets 2 and 3) or no quercetin (Diet 1) for 30 or 60 day were exposed to Cd ($CdCl_2 \cdot 2.5H_2O$) (Kanto Chemical, Tokyo, Japan) dissolved in water to a Cd^{2+} concentration of 10 ppb in 50-L tanks for 0, 6, 12, 24, and 48 hr. Five fish from each group were selected randomly for blood and tissue sampling. The fish were anesthetized with 200 mg/L tricaine methanesulfonate (MS-222, Sigma, St. Louis, MO, USA) before blood collection. Blood was collected from the caudal vein using a 3-mL syringe coated with heparin. Plasma samples were separated by centrifugation (4°C, 10,000 rpm, 5 min), and stored at −80°C until analysis. Fish were euthanized by spinal transection to collect the liver tissues for MT and GR mRNA analysis and brain tissues for AChE measurement. The samples were immediately frozen in liquid nitrogen and stored at −80 °C until total RNA was extracted.

Quantitative PCR (QPCR)

QPCR was conducted to determine the relative expression of MT and GR mRNA in the total RNA extracted

from the liver. Primers for QPCR were designed with reference to the known sequences of olive flounder as follows (accession no. EF406132 [MT]; AB013444 [GR]; EU090804 [β-actin]): MT forward primer (5′-AGT GGA ACC TGC AAC TGC-3′), MT reverse primer (5′-ATG TCT TCC CTT TGC ACA CG-3′), GR forward primer (5′-TCT GTT TGG TGT GTT CGG ATG AAG C-3′), GR reverse primer (5′-ATC TGT GTT TTG TCG TGC TCT CCA TC-3′), β-actin forward primer (5′-AAA TGG GAA CCG CTG CCT C-3′), and β-actin reverse primer (5′-TTC CTT CTG CAT ACG GTC AG-3′). PCR amplification was conducted using a BIO-RAD iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) and iQ™ SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions. QPCR was performed as follows: denaturation at 95°C for 5 min, 40 cycles of denaturation at 95°C for 20 s, and annealing at 55°C for 20 s. The experiments were duplicated with β-actin as an internal control, and all data are expressed as change with respect to corresponding β-actin calculated threshold cycle (CT) levels.

AChE assay

Brain AChE levels were quantified with a fluorometric assay kit (A12217; Invitrogen, Carlsbad, CA, USA). In the assay, AChE hydrolyzes the acetylcholine substrate to choline, which is oxidized by choline oxidase to form H_2O_2. In the presence of horseradish peroxidase, the Amplex Red reagent (10-acetyl-3, 7-dihydroxyphenoxazine) reacts with H_2O_2, forming a fluorescent by-product. Samples and standards were diluted with assay buffer and pipetted into a 96-well microplate in duplicate. Horseradish peroxidase, choline oxidase, AChE, and the Amplex Red reagent were added. After 30 min incubation in the dark, the fluorescence was determined with a plate reader (FLUOstar, BMG LabTech, Offenburg, Germany) using an excitation wavelength of 540 nm and an emission wavelength of 590 nm. The acetylcholine values were calculated as nmol/mg protein.

Liver culture

After the livers were removed from five flounder, they were placed in a 24-well culture plate. The liver tissues were treated with 0.001 and 0.01 μg/mL cortisol (hydrocortisone 21-hemisuccinate; Sigma) in medium 199 (Invitrogen) according to the manufacturer's instructions and cultured for 0, 6, 12, 24, and 48 hr in an incubator at 28°C, 100% humidity, and 5% CO_2 in air. Following the incubation period, each sample was centrifuged (20°C, 10,000 rpm, 15 s), and the supernatant was removed and stored in individual microcentrifuge tubes at −80°C until assay.

LPO assay

LPO is quantified by measuring malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), the degradation products of polyunsaturated fatty acids (PUFAs) hydroperoxides[44]. The Cayman Chemical (Ann Arbor, MI, USA) lipid hydroperoxide assay kit was used to measure hydroperoxides directly, utilizing the redox reaction with ferrous ion. Hydroperoxides were extracted into chloroform and reacted with ferrous ions to produce ferric ions. The resulting ferric ions were detected using thiocyanate ion as the chromogen. The hydroperoxide concentration was determined based on the absorption at 500 nm.

Plasma cortisol analysis

Plasma cortisol was analyzed using a radioimmunoassay kit (Diagnostic Systems Laboratories, Atlanta, GA, USA).

Statistical analysis

All data were analyzed using the SPSS statistical package (version 10.0; SPSS Inc., USA). One way ANOVA followed by *post hoc* Duncan's multiple range test was used to compare the differences in the data ($P < 0.05$).

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