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RESEARCH PAPER

Synergistic Effects of Dietary Vitamin C and Selenium on Induced Methylmercury Toxicity in Juvenile Olive Flounder *Paralichthys olivaceus*

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Abstract

The synergistic effects of dietary vitamin C and selenium (Se) on induced methylmercury (MeHg) toxicity in juvenile olive flounder *Paralichthys olivaceus* were examined in this study. Nine diets containing 3 different vitamin C levels (0, 200 and 400 mg kg^{-1} diet in the form of L-ascorbyl-2-monophosphate), 3 different Se levels (0, 2 and 4 mg kg^{-1} diet in the form of selenomethionine) at a constant level of MeHg (20 mg kg^{-1} diet in the form of MeHg) were formulated and fed to triplicate groups of juvenile olive flounder with mean weight of 2.00±0.04 g (mean± SD) in semi recirculation system using 3^2 factorial design. Growth performance and tissue Hg burden were determined after 8 weeks of feeding. Fish fed diets containing 400 mg kg^{-1} vitamin C together with 2 and 4 mg kg^{-1} Se ($C_{400}Se_2$ and $C_{400}Se_4$) showed significantly ($P<0.05$) higher weight gain (WG), specific growth rate (SGR) and feed efficiency (FE). Whereas fish which were under the $C_{400}Se_4$ diet exhibited significantly ($P<0.05$) higher and protein efficiency ratio (PER) than other feeding groups. Tissue Hg burden in muscle, liver and kidney showed a tendency of increasing with decreasing the levels of vitamin C and Se. However, significantly low tissue Hg burden was observed from fish fed diets containing 400 mg kg^{-1} vitamin C together with 2 and 4 mg kg^{-1} Se ($C_{400}Se_2$ and $C_{400}Se_4$). The results suggested that tissue Hg burden could be reduced and MeHg mediated growth problems could be ameliorated by supplementing dietary vitamin C and Se in juvenile olive flounder.

Keywords: Mercury, bioaccumulation, tissue burden, growth.

Introduction

Fish is a good source of two long chain omega 3 polyunsaturated fatty acids (n-3 lcPUFAs) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Fish consumption is also known to reduce the risk of death due to coronary heart disease by 36% (Mozaffarian & Rimm, 2006). Nevertheless, organic compounds such as polychlorinated biphenyls (PCBs), dioxins and insecticides together with inorganic chemicals such as arsenic, cadmium, lead, mercury, copper, zinc and iron can contaminate fish and seafood in general (FAO, 2005). As a result, concerns have been raised in recent years about contaminants found in fish originated from environmental pollution.

Among all of these contaminants, mercury (Hg) is the major contaminant and ubiquitous environmental toxin (Moniruzzaman *et al*., 2015) which is appearing to be a threat to human health. In fact, both inorganic and organic Hg may be found in fish (Lee *et al*., 2016). However, methylmercury (MeHg) is the predominant form of Hg in fish (Hoffman, Rattner, Burton, & Cairns, 2002). Its chemical properties allow it to rapidly diffuse and tightly bind to proteins in aquatic biota, including the proteins in muscle tissue of fish. This leads to bioaccumulation in the fish, with the Hg level increasing with age of the fish. In turn, biomagnification along the food chain leads to higher Hg levels in piscivorous fish that are higher in the food chain. The ability of MeHg to biomagnify in aquatic food chains was responsible for past epidemics in human populations and is a continuing concern for both human and environmental health (Ausili *et al.*, 2008; Driscoll, Mason, Chan, Jacob, & Pirrone, 2013).

Commercial fish diets are composed of various ingredients and Hg contamination in commercial fish feeds is mainly due to the high metal levels in the raw materials (Wang, Onsanit, & Dang, 2012). For this reason dietary exposure is one of the main routes of Hg contamination in fish (Choi & Cech, 1998). As the result, Hg has been regarded as undesirable substance in animal feed (EFSA, 2008). Fish consumption is one of the main paths through which human exposure to MeHg occurs (Passos, Mergler, Lemire, Fillion, & Guimaraes, 2007). Seafood contamination by Hg is a

public health concern particularly in countries with high rate of fish consumption such as Korea. Daily seafood consumption in Korea has reached 50.6 g, which accounted for 3.8% of the total food ingested (Moon, Kim, Choi, Yu, & Choi, 2009; Choi, Moon, & Choi, 2012). Consequently, blood Hg level in a representative sample taken from Korean adult population found to be associated with fish consumption (Kim & Lee, 2010). Moon *et al*. (2011) suggested that implementation of systematic monitoring programs for seafood contaminations by Hg are necessary in Korea. The various toxic effects induced by Hg in biological systems are often due to alterations in the antioxidant defense system (Sheweita, 1998; Berntssen, Waagbø, Toften, & Lundebye, 2003; Alves, Rosa, & Santana, 2007; Berg, Puntervoll, Valdersnes, & Goksøyr, 2010).

Vitamin C is a potent antioxidant and enzyme cofactor which can protect indispensable molecules in the body, such as proteins, lipids, carbohydrates, and nucleic acids (DNA and RNA), from damage by free radicals and reactive oxygen species (ROS) that are generated during normal metabolism, by active immune cells, and through exposure to toxins (Combs Jr., 2012). Vitamin C also participates in redox recycling of other important antioxidants; for example, vitamin C is known to regenerate vitamin E from its oxidized form (Bruno *et al*., 2006).

Selenium (Se) as an essential element required in small amounts to maintain good health, plays a role in antioxidant defenses and is a cofactor for the antioxidant enzyme glutathione peroxidase. Selenium interacts with the accumulation and toxicity of Hg in aquatic organisms in various ways. Because of its biological importance and nutraceutical component Se has attracted various researchers. It is involved in numerous biological functions including, preventing oxidative damage, maintaining homeostasis of thyroid hormone, enhancing immune functions (Hoffmann & Berry, 2008). Fish and crayfish treated with Se showed decreased Hg content, and fish in Se contaminated areas have been shown to contain reduced amounts of Hg (Southworth, Peterson, & Turner, 1994; Southworth, Peterson, & Ryon, 2000).

Various antioxidants including vitamin C, vitamin E, and Se are known to decrease Hg toxicity in Japanese quail (Kung, Soares, & Haltman, 1987) and in various other organisms (Chapman & Chan, 2000). Vijayalakshmi, Bapu and Sood (1992) and Bapu, Vijayalakshmi and Sood (1994) also examined the effects of vitamin C treatment after subcutaneous injections of methylmercuric chloride (MeHgCl) for 7 days in mice and found improvements in recoveries of enzymes activities. Chen *et al*. (2006) demonstrated that selenoproteins help eliminate ROS induced changes by metals because of their antioxidant properties. In this study, we evaluated effects of dietary vitamin C and Se levels on induced Hg accumulation in juvenile olive flounder *Paralichthys olivaceus*, a most commercially important marine aquaculture fish species in Republic of Korea (Lee *et al*., 2016).

Materials and Methods

Experimental Diets

Composition of the semi-purified basal diet is shown in Table 1. Nine diets contain three different vitamin C levels (0, 200 and 400 mg kg^{-1} diet in the form of L-ascorbyl-2-monophosphate) and three different Se levels (0, 2 and 4 mg kg^{-1} diet in the form of selenomethionine) with similar Hg toxicity levels (20 mg Hg kg^{-1} diet in the form of MeHg) were formulated. The 20 mg MeHg level kg^{-1} diet was chosen based on previous study from our lab. In diets

Table 1. Composition of the experimental diets (% dry matter basis)

Ingredients	Diets								
	C_0Se_0	C_0Se_2	C_0Se_4	$C_{200}Se_0$	$C_{200}Se_2$	$C_{200}Se_4$	$C_{400}Se_0$	$C_{400}Se_2$	$C_{400}Se_4$
Casein[1]	32	32	32	32	32	32	32	32	32
Defatted fish meal[2]	25	25	25	25	25	25	25	25	25
Wheat flour[3]	18	18	18	18	18	18	18	18	18
Corn starch[3]	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Fish oil[4]	9.6	9.6	9.6	9.6	9.6	9.6	9.6	9.6	9.6
Vitamin premix (C free)[5]	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Minerals premix (Se free)[6]	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Hg-premix	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Cellulose	6.0	5.0	4.0	4.0	3.0	2.0	2.0	1.0	0.0
Vitamin C premix	0.0	0.0	0.0	2.0	2.0	2.0	4.0	4.0	4.0
Se-premix	0.0	1.0	2.0	0.0	1.0	2.0	0.0	1.0	2.0

[1] United States Biochemical (Cleveland, OH) 44122.
[2] Suhyup Feed Co. Ltd.
[3] Young Nam Flour Mills Co., Pusan, Korea.
[4] E-Wha oil Co., Ltd., Buasn Korea.
[5] Contains (as mg kg^{-1} diet): dl-calcium pantothenate, 150; choline bitartrate, 3,000; inositol, 150; menadione, 6; niacin, 150; pyridoxine·HCl, 15; riboflavin, 30; thiamine mononitrate, 15; retinyl acetate, 6; biotin, 1.5; folic acid, 5.4; B12, 0.06; cholecalciferol, 2.4
[6] Contains (as mg kg^{-1} diet): Al, 1.2; Ca, 5000; Cl, 100; Cu, 5.1; Co, 9.9; Na, 1280; Mg, 520; P, 5000; K, 4300; Zn, 27; Fe, 40; I, 4.6; Mn, 9.1.

supplemented with a MeHg and ascorbic acid sources, an equivalent amount of cellulose was removed. In a 3^2 factorial design 9 experimental diets (C_0Se_0, C_0Se_2, C_0Se_4, $C_{200}Se_0$, $C_{200}Se_2$, $C_{200}Se_4$, $C_{400}Se_0$, $C_{400}Se_2$ and $C_{400}Se_4$) were formulated to be isonitrogenous and isoenergetic, containing 50% crude protein (CP) and 16.7 kJ available energy g^{-1} diet (16.7, 16.7 and 37.7 kJ g^{-1} for protein, carbohydrate and lipid respectively). Vitamin free casein was used as the main protein sources. All the ingredients were mixed completely and then pelleted by using 1-mm- and 2-mm-diameter dies (Bai & Lee, 1998). After processing, all the diets were packed into small bags and kept at -20°C until use.

Experimental Fish and Feeding Trials

Juvenile olive flounder, *Paralichthys olivaceus* were obtained from Tong-Yeong, Korea. Prior to the start of feeding trial, fish were fed the basal diet for 10 days to adjust to the semi-purified diet and to deplete possible body reserves of vitamin C. The feeding trial was conducted in a semi-circulated system with 30 L aquariums receiving filtered sea water at a rate of 2 L min^{-1}. Supplemental aeration was provided to maintain dissolved oxygen near saturation and water temperature was kept at 20±1°C. Experimental fish averaging 2.00±0.04 g (mean±SD) were randomly distributed into each aquarium as a group of 20 fish. Each diet was fed to triplicate groups to satiation level three times a day at a feeding rate of 2.0 to 3.5% of wet body weight. Total fish weight in each aquarium was determined every three weeks and the amount of diet fed to fish was adjusted accordingly. Aquariums were kept clean during the experiment time to minimize algae and fungal growth which could provide a source of vitamin C to cultured fish.

Sample Collection and Analysis

Weight gain (WG), specific growth rate (SGR), feed efficiency (FE), protein efficiency ratio (PER), and survival rate were measured and calculated after weight checking. After the final weighing, 3 fish was randomly removed from each aquarium for analysis.

Vitamin C Analysis

Ascorbic acid concentration was determined by High Performance Liquid Chromatography (HPLC; Dionex Softron, Sunnyvale, CA, USA). The ultraviolet detector was set at 254 nm and the mobile phase was 0.05 M KH_2PO_4 with flow rate of 1.0 mL min^{-1}. Weighed samples were homogenized in 10% cold metaphosphoric acid. Homogenates were centrifuged at 3000×g for 20 minutes and supernatants were analyzed after filtered through a 0.45 μm pore size syringe filter.

Selenium Analysis

Diet and tissue Se concentrations were assessed by the digestion of samples in nitric acid. Weighed samples were put into a 250 mL Kjeldahl flask, and 50 mL of HNO_3 was added to the flask. Then, the flask was heated in a heating mantle until the sample was fully digested. Approximately 5 mL of H_2O_2 was added to make sure that the sample was totally digested, and the digested sample was diluted with H_2O. The concentration of Se in the diluted digest solution was determined using a Perkin-Elmer 3300 Inductively Coupled Plasma Mass Spectrometer (ICP-MS, Perkin-Elmer, Waltham, MA, USA).

Mercury Analysis

Over 90% of Hg present in fish is MeHg. For this reason, direct total concentration of Hg was measured instead of MeHg (Bloom, 1992; Amlund, Lundebye, & Berntssen, 2007). Hg analyzer (DMA-80, Milestone, Inc., Shelton, CT) was used to determine tissue Hg concentration following the method similar to the one used in Lee *et al.* (2011). A certified reference material (DORM-2 dogfish liver, National Research Council, Canada) was used simultaneously during the analyses.

Statistical Analysis

Data were analyzed by two-way ANOVA to test for the effect of dietary treatments using SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). Least Significant Difference (LSD) was used to compare means when significant difference between treatments was observed and P-values of 0.05 or less ($P \geq 0$) were considered to be statistically significant.

Results

Growth Performance

Growth performance of fish is summarized in Table 2. Fish fed 400 mg kg^{-1} vitamin C comprising diets ($C_{400}Se_0$, $C_{400}Se_2$ and $C_{400}Se_4$) showed significantly ($P<0.05$) higher WG at all Se levels, than other feeding groups. Even though no significant difference was observed between feeding groups which were under 200 mg kg^{-1} vitamin C containing diets at all Se levels, all of them showed significantly ($P<0.05$) higher WG than those groups which were not supplemented with vitamin C. Specific growth rate of fish which supplemented 400 mg kg^{-1} vitamin C with 2 and 4 mg kg^{-1} Se ($C_{400}Se_2$ and $C_{400}Se_4$) appeared to be significantly ($P<0.05$) higher than the other feeding groups. In general, feeding groups which were under 400 mg kg^{-1} vitamin C categories ($C_{400}Se_0$, $C_{400}Se_2$ and $C_{400}Se_4$) exhibited significantly higher SGR than groups which were given 0 and 200 mg kg^{-1} vitamin C at all Se levels. No significant

Table 2. Growth performance of juvenile olive flounder fed the experimental diet for 8 weeks [1]

Diets	WG (%)[2]	SGR (% day^{-1})[3]	FE (%)[4]	PER[5]
C_0Se_0	123^{f}	1.66^{e}	39.0^{e}	0.76^{e}
C_0Se_2	127^{f}	2.16^{f}	64.0^{d}	1.27^{d}
C_0Se_4	214de	2.38^{d}	64.2^{d}	1.26^{d}
$C_{200}Se_0$	166^{cd}	2.04^{e}	43.6^{e}	0.86^{e}
$C_{200}Se_2$	242^{cd}	2.56^{cd}	67.0^{d}	1.30^{d}
$C_{200}Se_4$	267^{bcd}	2.71^{c}	78.0^{c}	1.53^{c}
$C_{400}Se_0$	310^{abc}	2.94^{b}	91.6^{b}	1.81^{b}
$C_{400}Se_2$	334^{ab}	3.05^{ab}	93.6^{ab}	1.83^{b}
$C_{400}Se_4$	374^{a}	3.24^{a}	102^{a}	2.06^{a}
Pooled SEM[6]	16.1	0.09	3.95	0.08
Two-way ANOVA				
Vitamin C	0.0001	0.0001	0.0001	0.0001
Selenium	0.0001	0.0001	0.0001	0.0001
Vitamin C × Selenium	0.0100	0.0001	0.0064	0.0086

[1] Values are means from groups (n=3) of fish where the values in each row with different superscripts are significantly different (P<0.05).
[2] Weight gain (%) = (final weight - initial weight) × 100 / initial weight
[3] Specific growth rate (%) = 100 × (Ln final wt. - Ln initial wt.) / days
[4] Feed efficiency (%) = (wet weight gain / dry feed intake) × 100
[5] Protein efficiency ratio = (wet weight gain / protein intake)
[6] Pooled standard error of means: SD/√n

difference in SGR observed between fish fed 200 mg kg^{-1} vitamin C at 2 and 4 mg kg^{-1} Se levels ($C_{200}Se_2$ and $C_{200}Se_4$). Similarly, significantly (P<0.05) higher FE observed in ($C_{400}Se_4$) feeding group. However, no significant differences were observed between fish fed C_0Se_2, $C_{200}Se_2$, or C_0Se_4 and between fish fed $C_{200}Se_0$ or C_0Se_0 diets. Protein efficiency ratio followed quite similar pattern like FE where, fish fed $C_{400}Se_4$ diet exhibited significantly (P<0.05) higher PER than other feeding groups.

Tissues Mercury Burden

Muscle of fish fed diets containing 400 mg kg^{-1} vitamin C along with Se ($C_{400}Se_2$ and $C_{400}Se_4$) showed significantly (P<0.05) lower Hg burden than other feeding groups. Whereas the control group (C_0Se_0) deposited significantly (P<0.05) higher Hg in their muscle (Table 3). Tissue Hg burden showed a tendency of increasing with decreasing levels of vitamin C and Se. Similarly, liver Hg burden appeared to be significantly lower for fish which fed 400 mg kg^{-1} vitamin C diets at 2 and 4 mg kg^{-1} Se levels. However, no significant difference in liver Hg burden was observed between fish fed C_0Se_0, C_0Se_2 or C_0Se_4 diets and between fish fed C_0Se_2, C_0Se_4 or $C_{200}Se_0$ diets. Kidney Hg deposition followed similar pattern like muscle where fish fed $C_{400}Se_2$ and $C_{400}Se_4$ diets accumulated significantly lower Hg than other feeding groups.

Discussion

In the present study, olive flounder fed MeHg containing diet (C_0Se_0) demonstrated clear growth depression possibly due to the higher accumulation of Hg in fish. Similar results have been found from the one of our recent study dealt with the effects of dietary vitamin C on inorganic mercury ($HgCl_2$) toxicity in juvenile olive flounder (Lee *et al*., 2016). Moreover, in the present study, the poor growth performance observed in olive flounder fed diets which do not contain either vitamin C and/or Se might have occurred due to decreased enzyme activity, altered structural functionality and transport process problems caused by Hg accumulation (Zalups & Lash, 1994). However, when the diets were supplemented with higher level of Se (C_0Se_4), significant growth improvement was observed in fish which may be due to the antioxidative nature of selenium. Interestingly, the diet supplied with higher level of vitamin C ($C_{400}Se_0$) showed more pronounced effect in terms of improvement of WG, SGR, FE and PER in fish compared with the high level of Se (C_0Se_4) containing diet. The results suggest that vitamin C might have more protective effect than Se in terms of growth improvement in fish. In addition, when the diets were supplied with higher level of vitamin C (400 mg/kg diet) with irrespective level of Se (2 or 4 mg/kg diet) showed significant growth improvement in fish compared to rest of the diets. Lee *et al*. (2016) reported that dietary vitamin C (100 or 200 mg kg^{-1} diet) could significantly improve the growth performance of olive flounder on inorganic mercury ($HgCl_2$) induced toxicity which is in agreement of the present study. In this study, the two-way ANOVA also demonstrated that there is a significant interactive effect between dietary vitamin C and Se on growth performance in terms of WG, SGR, FE and PER of fish on induced methylmercury toxicity. These results suggested that dietary vitamin C and Se had synergistic effects on reduction of methylmercury toxicity in this fish species.

In this study, dietary methylmercury

Table 3. Total tissue mercury concentrations (µg g^{-1} of wet matter basis) juvenile olive flounder fed the experimental diets for 8 weeks [1]

Diets	Muscle	Liver	Kidney
C_0Se_0	15.2^{a}	18.4^{a}	23.7^{a}
C_0Se_2	13.6^{b}	17.6^{ab}	21.3^{b}
C_0Se_4	12.1^{c}	17.6^{ab}	18.2^{c}
$C_{200}Se_0$	12.0^{c}	16.6^{b}	18.5^{c}
$C_{200}Se_2$	10.7^{d}	15.2^{c}	16.6^{d}
$C_{200}Se_4$	9.7^{et}	13.2^{d}	14.8^{e}
$C_{400}Se_0$	10.1^{de}	13.0^{d}	15.0^{e}
$C_{400}Se_2$	9.0^{g}	11.6^{e}	12.5^{t}
$C_{400}Se_4$	9.3^{tg}	11.4^{e}	12.1^{t}
Pooled SEM [2]	0.37	0.49	0.69
Two-way ANOVA			
Vitamin C	0.0001	0.0001	0.0001
Selenium	0.0001	0.0001	0.0001
Vitamin C × Selenium	0.0031	0.0154	0.0128

[1] Values are means from groups (n=3) of fish where the values in each row with different superscripts are significantly different ($P<0.05$).
[2] Pooled standard error of means: SD/√n

accumulated in the tissues, in increasing order, muscle<liver<kidney. The highest deposition of Hg was found in kidney tissue and the lowest in muscle tissue of fish which is in agreement with Lee *et al.* (2016). The results of the present study suggest that accumulation of organic mercury (methylmercury) is higher than inorganic mercury accumulation in juvenile olive flounder which was reported by Lee *et al.* (2016). Our results also showed that higher amounts of Hg accumulated in muscle tissue of fish compared to that of reported by Lee *et al.* (2016). It has been reported that a substantial amount of organic mercury can accumulated in fish muscle (NRC, 2005). In this study, the amount of Hg deposited in fish muscle is very close to the Hg contents in liver and kidney. However, as the dietary vitamin C and Se levels increases in the diets, Hg contents in the muscle, liver and kidney tissues of fish also decreases. Significantly lowest amount of Hg was found in fish fed the higher level of vitamin C with lower/higher levels of Se in diets which supports the growth performance data of the present study. In present study, both of the dietary vitamin C and Se have shown their strong effects in reducing mercury contents in tissue levels of fish. In addition, dietary vitamin C and Se exhibited their interaction effect in reducing Hg contents in muscle, liver and kidney tissue of fish. However, Lee *et al.* (2016) found interaction effect between dietary vitamin C and Hg in kidney tissue only on induced $HgCl_2$ toxicity in olive flounder. Therefore, in the present study, the results clearly demonstrated the synergistic effects of dietary vitamin C and Se on induced MeHg toxicity in terms of tissue mercury reduction.

In the present study, the better growth performance and lower tissue Hg burden observed in fish fed diets supplemented with dietary vitamin C (200 and 400 mg kg^{-1}) together with Se (2 and 4 mg kg^{-1}) might, presumably, attribute to the protective role of vitamin C and Se against MeHg induced immune suppression and their ability to maintain/or elevate the activities of several key antioxidants. Consequently, effective disposal of Hg from SH groups by vitamin C along with its ability to inhibit, minimize and remove free radicals might have reduced tissue Hg burden and helped to improve the poor growth performance observed in this study which is in agreement with Durak, Kalender, Uzun, Demır and Kalender (2010).

Conclusion

In this study, the results showed that the supplemental Se might have ensured adequate levels of Se and replaced the amount of Se lost to Hg sequestration. This in turn helped the normal selenoprotein synthesis to continue. On the other hand, vitamin C likely inhibited free radical formation and lipid peroxidation that could have been caused by MeHg. Therefore, we may conclude that poor growth performance resulted from induced MeHg could be enhanced and Hg burden on muscle, liver and kidney could be reduced by supplementing dietary vitamin C (400 mg kg^{-1} diet) together with Se (2 or 4 mg kg^{-1} diet) in juvenile olive flounder.

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