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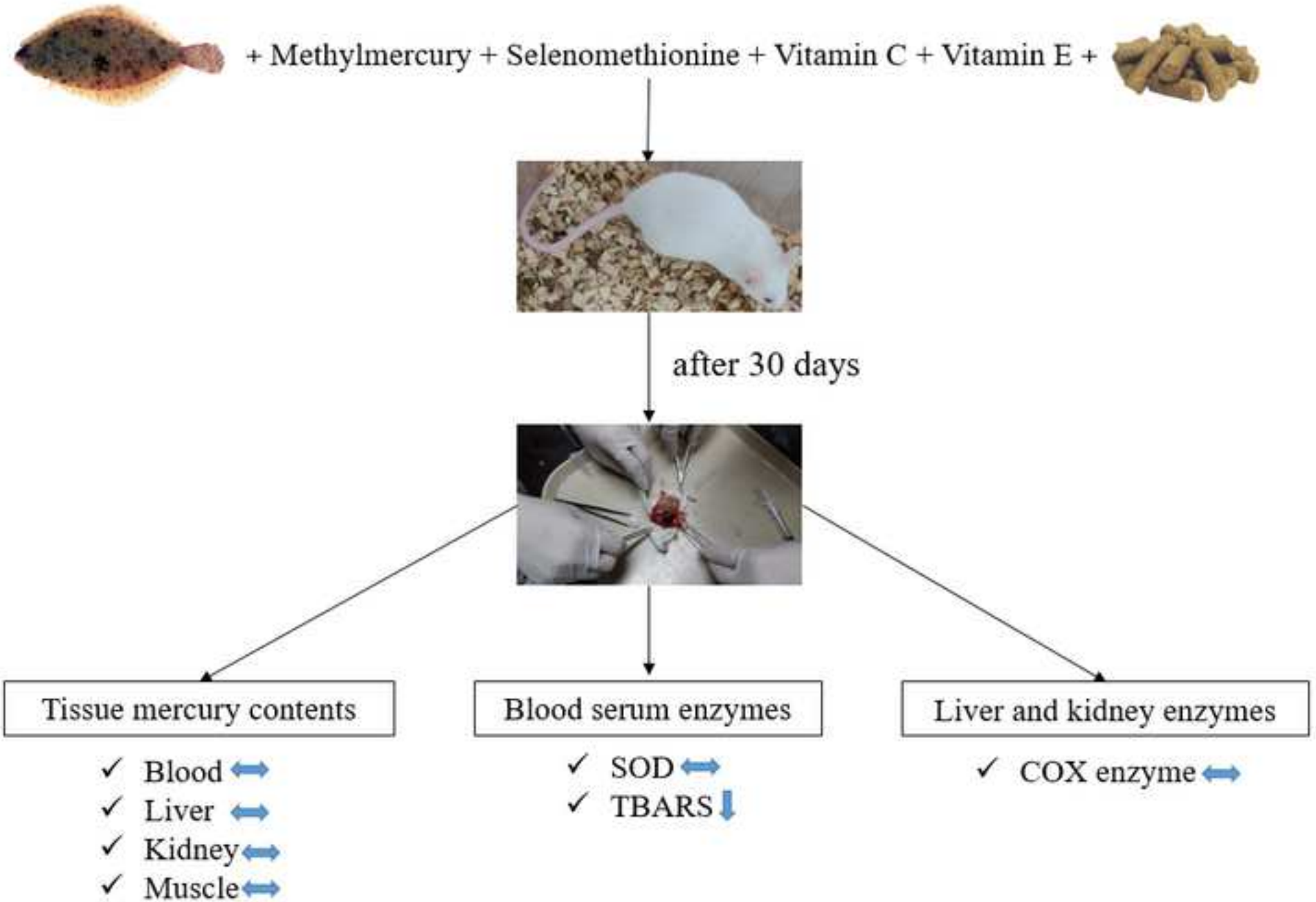
Graphical Abstract



Highlights

- Dietary Hg bioaccumulated, in decreasing order, kidney>liver>muscle>blood of mice
- Dietary Se, vitamin C and E had no effect on Hg bioaccumulation in tissues of mice
- The antioxidants partially reduced Hg toxicity based on oxidative stress in mice
- Dietary antioxidants prolonged the survivability in mice on induced Hg toxicity

CRediT authorship contribution statement

Mohammad Moniruzzaman: Investigation, Laboratory analyses, Data analysis, Writing - original draft. **Seunghan Lee:** Investigation, Laboratory analyses, Data analysis. **Youngjin Park:** Investigation. **Taesun Min:** Writing - review & editing. **Sungchul C. Bai:** Supervision, Funding acquisition, Resources and Approval of final draft.

Declaration of competing interest

The authors declare that they have no competing interests.

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Abstract

Mercury (Hg) in high exposures can be a potent life threatening heavy metal that bioaccumulate in aquatic food-chain mainly as organic methylmercury (MeHg). In this regard, fish and seafood consumptions could be the primary sources of MeHg exposure for human and fish-eating animals. The objective of the present study was to elucidate the effects of dietary supplementation of some antioxidants on induced mercury toxicity in mice model. In this study, a 30-day long investigation has been conducted to evaluate the dietary effect of selenium (Se) in combination with vitamin C and vitamin E on methylmercury induced toxicity in mice. Total 54 mice fed the diets with three levels of Hg (0, 50 or 500 $\mu g.kg^{-1}$) and two levels of Se in combination with vitamin C and E (Se: 0, 2 mg kg^{-1}; vitamin C: 0, 400 mg kg^{-1}; vitamin E: 0, 200 mg kg^{-1}) in triplicates. The results show that Hg accumulated in blood and different tissues such as muscle, liver and kidney tissues of mice on dose dependent manner. The bioaccumulation pattern of dietary Hg, in decreasing order, kidney > liver > muscle > blood. Superoxide dismutase levels in blood serum showed no significant differences in mice fed the diets. However, dietary antioxidants significantly reduced the levels of thiobarbituric acid reactive substances in mice fed the mercury containing diets. Cytochrome *c* oxidase enzyme activities showed no significant differences as the mercury level increases in liver and kidney tissues of mice. Kaplan-Meier curve showed a dose- and time- dependent survivability of mice. Cumulative survival rate of Hg intoxicated mice fed the antioxidant supplemented diets were increased during the experimental period. Overall, the results showed that dietary Se, vitamin C and vitamin E had no effect on reducing the mercury bioaccumulation in tissues but reduced the serum lipid peroxidation as well as prolonged the cumulative survival rate in terms of high Hg exposures in mice.

Keywords: methylmercury; antioxidants; tissue bioaccumulation; superoxide dismutase; reactive oxygen species; cytochrome *c* oxidase;

1. Introduction

Mercury is a xenobiotic, persistent and non-essential ubiquitously present heavy metal (Farina et al., 2013; Rand et al., 2016) which may be organic or inorganic in nature. Mercury is commonly available in the atmosphere in the form of elemental mercury (Hg^0), mercuric mercury (Hg^{2+}) and methylmercury (CH_3Hg^+) which is the most toxic form of Hg in high exposures and extensively investigated in recent years (Ordiano-Flores et al., 2012; Fernandes et al., 2020). Hg inputs to the environment is primarily involved with wet and dry environmental deposition of inorganic mercury (Hg^{2+}) that can be modified into volatile gaseous elementary mercury (Hg^0) or methylated to form the highly bioaccumulative methylmercury that biomagnifies in aquatic food chains and finally causes health hazards in human and fish-eating wildlife (Lavoie et al., 2018; Bourdineaud et al., 2019; Fernandes et al., 2020). For this reason, marine and freshwater fish are considered as the main sources of dietary methylmercury (MeHg) exposures (Ralston and Raymond, 2019). The organic MeHg has high neurotoxic effects to human especially during fetal development where MeHg is exposed in molar excess of selenium through fish and seafood consumptions (Ralston, et al., 2019; Kim et al., 2019) that are also good sources of quality protein and essential amino acids as well as omega-3 fatty acids (eicosapentaenoic acid or EPA and docosahexaenoic acid or DHA), minerals (iodine and selenium), and vitamins (NRC, 2011; Ralston et al., 2019). The MeHg absorption rate in an organism digestive tract is around 90-95% (Nielsen and Andersen, 1992) which potentially distributed in different organs and cause toxicity through the ingestion of polluted fish (Farina et al., 2011). The MeHg can accumulate by exceeding seven orders of magnitude from sub $ng.L^{-1}$ concentrations in water to more than 1 $mg.kg^{-1}$ in piscivorous fish which may cause serious health concerns in frequent seafood consumers (Hintelmann, 2010). In contrast, MeHg excretion from human body usually starts at the elimination rate (k_{el}) of near about 0.01 day^{-1} or 70 days of half-life [$t_{1/2}$]) and it is a potential determinant of the Hg

bioaccumulation resulted through fish consumption (Rand et al., 2016). Based on the Priority List of Hazardous Substances established by the Agency for Toxic Substances and Disease Registry (ATSDR, 2019), the toxic effects of heavy metals on human health status observed, in decreasing order, As > Pb > Hg > Cd > Cr > Co > Ni > Zn > Cu > Mn. Therefore, Hg is considered as the third most toxic heavy metal after arsenic (As) and lead (Pb), respectively, when it exceeds the normal or recommended level.

The U.S. Environmental Protection Agency (USEPA) recommended the reference dose for MeHg at 0.1 $\mu g.kg^{-1}$ $bw.day^{-1}$ for human. Methylmercury is known to create oxidative stress through the production of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and nitric oxide (NO) in animals, which have deleterious effects on organisms in terms of mitochondrial dysfunction to behavioral changes through the depletion of antioxidant enzyme activities by MeHg (Farina et al., 2011; Ishihara et al., 2016). High level of MeHg has the high affinity to the thiol or sulfhydryl (-SH) groups of proteins (Ajsuvakova et al., 2020). However, Hg has a lower affinity for thiol groups compared to Se containing groups (Spiller, 2017). It has been reported that Hg has ~1 million times higher affinity for Se than sulfur which is considered as the primary target of MeHg toxicity (Ralston and Raymond, 2019). Furthermore, Spiller et al. (2017) evidenced that the *in vivo* pathophysiological changes by Hg is dependent on the binding of Hg with Se but not with sulfur. Selenium has reciprocal effects on Hg^{2+} toxic behavior reported by Pariziek et al. (1971) who postulated that mortality of rats can be reduced by co-injection of Se and Hg. The bindings of Hg to Se compromised the biological functions and availability of Se (Folven et al., 2009). The high affinity of Hg for Se warrant it to specifically chelate Se at the active sites of important Se-containing enzymes (selenoenzymes) like glutathione peroxidase and thioredoxin reductase (Branco et al., 2014; Spiller, 2017). These selenoenzymes are continuously disrupted and reconstitute the insoluble Hg-Se bonds due to the excessive molar formation of Hg and Se (more than 1:1) which could

reduce the production of essential selenoproteins (selenocysteine) by depleting Se availability in the tissue level (Ralston, 2018) and finally the Hg-induced Se deficiency could not restore the cellular redox environment (Spiller, 2017). The impairment of selenoenzymes in the cellular redox environment causes proliferation of intracellular reactive oxygen species (ROS) which resulted the mitochondrial dysfunction, lipid peroxidation, protein impairment and apoptosis (Spiller, 2017). Nonetheless, selenoenzymes have potential to control reactive oxygen species (ROS) production and they can prevent as well as reverse the oxidative damage in various tissues especially liver and brain of organisms (Ralston et al., 2019; Zhang et al., 2020). Consumption of marine fish or seafood containing Se in molar excess of MeHg could prevent the disruption of selenoenzymes through alleviating Hg-exposure risks (Ralston et al., 2019) which is regarded as the positive Health-Benefit-Value (HBV) or they contain MeHg in excess of Se as regarded as the negative HBV for the consumers (Ralston et al., 2014; Ralston et al., 2019). However, the consequences are depend on the form of Hg and Se as well as the organ and amounts (Spiller, 2017). Furthermore, Prohaska and Ganther (1977) reported that dietary Se cannot decrease the tissue Hg levels in rat brain rather it increase the Hg uptake. Penglase et al. (2014) found that dietary Se had negative effect on reproductive performance of fish on higher levels of Hg in fish diets.

Mitochondria is known as the powerhouse of cell that brings energy to every cellular process. Mitochondria could produce high levels of ROS when exposed to MeHg. It has been reported that mitochondrial ROS are responsible for oxidative stress following cell death in organisms intoxicated by MeHg (Ishihara et al., 2016). In this case, both enzymatic and non-enzymatic defense systems are required to protect the organisms from deleterious effects caused by ROS (Halliwell and Gutteridge, 2015). There are two main categories in the defense systems such as: endogenous antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) (Halliwell and Gutteridge, 2015), and others are

134 exogenous antioxidants such as ascorbic acid (vitamin C), tocopherols (vitamin E), vitamin A,
135 carotenoids and some metals like selenium (Se) is essential for the function of antioxidant
136 enzymes (Halliwell and Gutteridge, 2015). The cytochrome *c* oxidase (COX) as the terminal
137 enzyme of mitochondria, is the terminal electron acceptor of the respiratory chain of
138 mitochondria which is responsible for the cellular respiration in most of the aerobic organisms
139 (Ostermeier et al., 1996) and in the final act it reduces dioxygen to water with four electron
140 from cytochrome *c* and four protons taken up from the mitochondrial matrix without formation
141 of ROS (Kadenbach et al., 2000). There are 13 different hetero-oligomeric sub-units of COX
142 enzymes localized to the inner mitochondrial membrane (Tsukihara et al., 1996; Zsengeller
143 and Rosen, 2016), of which, sub-units I, II, III and IV were widely described (Ostermeier et
144 al., 1996).

145 Vitamin C (L-ascorbic acid) have potential to maintain the normal health status in
146 organisms. Smirnoff (2018) reported that higher doses of vitamin C supplementation have
147 health benefits. Vitamin C is an important water-soluble antioxidant and a cofactor in metabolic
148 synthesis process (Smirnoff, 2018). Studies have shown that dietary vitamin C can reduce the
149 mercury toxicity in terms of reducing mercury concentrations in blood and tissues especially
150 liver and kidney tissues through the urinary excretion as well as enhance the immune responses
151 in organisms (Hounkpatin et al., 2012; Lee et al., 2016).

152 Vitamin E is a lipid soluble antioxidant, which plays a vital role to stabilize the tissue
153 membranes and reduces the ROS due to the oxidative stress (Agarwal et al., 2010). It can
154 reduce mercury and inhibits oxidative damage in the liver and other tissues caused by mercury
155 toxicity (Rana et al., 1996; Chapman and Chan, 2000; Rao et al., 2001). However, some
156 researchers reported that dietary vitamin E and Se can prevent the oxidative damage but cannot
157 reduce the Hg contents in tissues of animal (Ganther, 1978; Beyrouty and Chan, 2006; Folven
158 et al., 2009). Pillai and Gupta (2005) postulated that vitamin E can protect from mercury

toxicity due to its chelating efficacy which ultimately enhance the antioxidant defense system in living organisms. Furthermore, vitamin E together with vitamin C could reduce lipid peroxidation, ROS and increase immunity in male rats (Muthu and Krishnamoorthy, 2012), while the dietary Se and vitamin E were reported to show their synergistic protective effect against oxidative damage in animals (Marin-Guzman et al. 2000; Kolodziej and Jacyno, 2005).

Olive flounder is one of the most consumed and commercially important marine demersal fish species cultured in East-Asian countries like Republic of Korea, Japan and China (Raihan et al., 2020). As a demersal and predatory fish species, olive flounder fish has great importance in relation to the mercury contamination on public health. Moon et al. (2011) reported that organic and total Hg in seafood of Korean water reached 55.6 $\mu g.kg^{-1}$ and 100 $\mu g.kg^{-1}$, respectively which necessitates to investigate in terms of mercury contamination and its detoxification using animal models. Recently, a series of experiments have been conducted by our group where we documented that dietary antioxidants like selenium, vitamin C and E had protective effect against mercury-induced toxicity in marine fish like olive flounder (Lee et al., 2016; Park et al., 2017; Moniruzzaman et al., 2017a; Moniruzzaman et al., 2017b; Lee et al., 2017; Raihan et al., 2020). In this study, for the first time, we envisage to evaluate the effect of multi-antioxidants (Se, vitamin C and vitamin E) in mice fed with methylmercury (MeHg) toxified olive flounder fish muscle powder in terms of tissue mercury bioaccumulation, antioxidant enzyme activities and oxidative stress in mice model.

2. Materials and Methods

2.1. Ethics and animal welfare

The experiment was conducted under the guidelines of Animal Ethics Committee Regulations No. 554 issued by the Pukyong National University, Busan, Rep. of Korea. In this

experiment, we tried to reduce the number of animals and sacrificing stress as minimum as possible.

2.2. Chemicals and reagents

Methylmercury (II) chloride, Cl ~13% (CH_3HgCl) as a source of Hg, L-selenomethionine (97% pure), DL-α-tocopheryl acetate as a source of vitamin E were collected from Sigma-Aldrich, St. Louis, USA and L-ascorbyl-2-monophosphate (containing 35% ascorbic acid activity) as a source of vitamin C was purchased from Hoffman La Roche, Basel, Switzerland; diethyl ether, nitric acid (HNO_3) and hydrogen peroxide (H_2O_2) were procured from Merck, Darmstadt, Germany.

2.3. Acquiring mice

A total of 54 specific pathogen-free (SPF) and laboratory bred male albino mice (*Mus musculus*) of ICR (Institute of Cancer Research) strain (35~38g), were used for the experiment. Mice (10 weeks old) were maintained on standard chow or mouse feed (Purina Korea Inc., Seongnam-si, Gyeonggi-do, Korea) without antioxidants (Se, vitamin C and vitamin E) or mercury supplementations (Table 1), and the mice were supplied with normal water up to *ad libitum*. The animals and the standard chow were procured from Hyochang Science, Daegu, Korea. Mice were housed in 18 stainless steel cages (200×260×130 mm) each containing 3 mice reared during the experimental period. All mice were kept at a temperature of 23±2°C in animal house with air conditioner facilities and at a relative humidity of 58% and exposed to 12 h/12 h (light/dark) cycle for at least 7 days prior to the experiment as well as during the experimental period.

2.4. Preparation of mice diet

Six experimental diets supplemented with three levels of MeHg (0, 50 or 500 $\mu g.kg^{-1}$ and two levels of Se in constant combination with vitamin C and E (Se: 0, 2 $mg.kg^{-1}$; vitamin C: 0, 400 $mg.kg^{-1}$; vitamin E: 0, 200 $mg.kg^{-1}$) by 3×2 factorial design (Hg0C0E0Se0,

Hg0C400E200Se2, Hg50C0E0Se0, Hg50C400E200Se2, Hg500C0E0Se0, Hg500C400E200Se2 diets) were fed to the albino male ICR mice in triplicate number of cages (Table 1). Prior to the experiment, juvenile olive flounder (*Paralichthys olivaceus*) fish muscle powder was prepared with the inclusion of methylmercury (II) chloride (Sigma-Aldrich, St. Louis, MO, USA). The total Hg contents in fish muscle tissue was found to be 17.4 $\mu g.kg^{-1}$. For the experimental diets, dried and lyophilized olive flounder fish muscle powder was added in the diets with or without supplementation of methylmercury (MeHg) as the negative control (Hg0C0E0Se0 diet). In basal diets, when supplemented with selenium, vitamin C and E, an equivalent amount of cellulose were removed. The experimental pellet feeds were processed manually by proper mixing the required ingredients and pelleting using metal pellet machine. After pelleting, the actual levels of total Hg in the diets were found to be 50.7 $\mu g.kg^{-1}$ (in Hg0C0E0Se0 diet), 53.5 $\mu g.kg^{-1}$ (in Hg0C400E200Se2 diet), 111.8 $\mu g.kg^{-1}$ (in Hg50C0E0Se0 diet), 98.2 $\mu g.kg^{-1}$ (in Hg50C400E200Se2 diet), 523.6 $\mu g.kg^{-1}$ (in Hg500C0E0Se0 diet) and 529.6 $\mu g.kg^{-1}$ (in Hg500C400E200Se2 diet).

2.5. Sample collection and analyses

At the end of the 30-day feeding trial, all of the survived mice (n=32) were euthanized directly and excised humanely for the collection of blood, muscle, liver and kidney tissues. In brief, mice were euthanized with ether and blood was immediately collected by cardiac puncturing of 3 mice from each treatments to minimize the animal sufferings (1 mice from each replicate). Blood was divided in two separate parts, i.e. heparinized (for mercury determination) and non-heparinized (for serum biochemical assays, *viz.* superoxide dismutase and thiobarbituric acid reactive substances). Non-heparinized blood samples were centrifuged in order to separate blood serum at 5,000 ×g for 10 min at room temperature and stored at -80°C for later determination of blood biochemical parameters. Tissues from mice were dissected for the analyses of mercury contents in liver, kidney and muscle as well as the further

enzymatic analyses *viz.* cytochrome *c* oxidase (COX) enzyme activities in liver and kidney tissues. The analyses of protein contents in blood serum for superoxide dismutase and thiobarbituric acid reactive substances as well as tissue homogenates for COX activities in mice fed the experimental diets were done by previously described method (Bradford 1976) using the bovine serum albumin (BSA) as standard and optical density (OD) at 595 nm to get the absorbance of the samples.

2.6. Tissue mercury contents

For the analyses of Hg contents in the olive flounder fish muscle and the diets as well as blood and other tissues (muscle, liver and kidney) of mice, an argon gas assisted Inductively Coupled Plasma Mass Spectrometer, ICP-MS (Perkin-Elmer 3300, Waltham, MA, USA) was used previously described by Lee et al. (2016). Briefly, samples (0.2 g of each) were weighed and put into a Kjeldahl flask (250 mL) with the addition of 50 mL nitric acid (65% HNO3) to the flask. Then, a heating mantle was used to heat the sample with flask. An aliquot of 5 mL hydrogen per oxide (50% H_2O_2) was added to the sample to digest fully, and the digested sample was diluted with de-ionized water upto 100 mL. The Hg contents in the digested solution was determined by the method of EPA-6020-A by ICP-MS. For better comparison, we used a reference sample (DORM-2: 4.47±0.03 $\mu g.g^{-1}$ Dogfish liver; National Research Council, Ottawa, ON, Canada) and blanks (0.04±0.01 $\mu g.L^{-1}$) during the analyses. We found that Hg values were in accordance with the reference values (95% recovery level). The limit of detection (LOD) and the limit of quantification (LOQ) of the equipment were 0.001 $\mu g.L^{-1}$ and 0.47 $\mu g.kg^{-1}$, respectively. The concentrations of total mercury in blood, liver, kidney and muscle tissues of mice were expressed in $\mu g.g^{-1}$ on wet matter basis.

2.7. Serum superoxide dismutase (SOD) activity

Superoxide dismutase (SOD) activity was measured by the superoxide radical-dependent reaction inhibition rate of enzyme with WST-1 (water-soluble tetrazolium dye) substrate and

xanthine oxidase using the SOD Assay Kit (product number 19160, Sigma-Aldrich, St. Louis, MO, USA) following to the manufacturer's protocols. Each endpoint assay was monitored by absorbance at 450 nm (the absorbance wavelength for the colored product of WST-1 reaction with superoxide) after 20 min of reaction time at 37°C. The percent inhibition was normalized by mg protein and expressed as SOD unit per mg.

2.8. Serum thiobarbituric acid reactive substances (TBARS)

Thiobarbituric acid reactive substances (TBARS) is a well-established assay for the screening and monitoring of ROS-mediated lipid peroxidation (Buege and Aust, 1978). The degree of peroxidation of blood serum was assessed by measurement of pink color intensity by means of the reaction of malondialdehyde (MDA) and TBARS using a commercial kit (Oxiselect^{TM} TBARS Assay kit, Cell Biolabs, San Diego, CA, USA). Briefly, 100 mL of serum samples with a known MDA concentration were mixed with 100 mL SDS (sodium dodecyl sulfate) lysis buffer and 250 mL thiobarbituric acid solution and heated at 95 ^{0}C in a water bath for 1 h, and then the absorbance of centrifuged supernatant solution was measured at 532 nm. Results were expressed as µmole per ml per mg protein.

2.9. Tissue cytochrome c oxidase (COX) enzyme activity

The COX activities in the freshly prepared liver and kidney tissues of mice (~50 mg of each) were determined using the commercial kit (CYTOCOX1, Sigma-Aldrich, St. Louis, MO, USA). The assay in this kit is based on Lemberg method (1969) which is executed by COX oxidation to ferricytochrome *c* by cytochrome *c* oxidase in decreasing way of absorbance at 550 nm spectrophotometrically. The oxidation of cytochrome *c* by cytochrome *c* oxidase is a two-step procees with fast reaction followed by a slower reaction rate. Initially, cytochrome *c* oxidase was mixed with a 0.1-M dithiothreitol (DTT) solution to prepare its reduced form. The initial reaction rate of the assay was measured during the first 45 seconds of the reaction then the reduction was confirmed by the change of color from dark orange red to pale purple-red.

Changes in absorbance per min was measured and results were expressed as nmol per min per mg protein at pH 7.0 at 25 ^{0}C.

During the experimental period we recorded the number of mice died every day to establish the survivability curve at the end of the experiment.

2.10. Statistical analyses

Mice cage mean values (n=3) were used for statistical analysis. All data were subjected to two-way analysis of variance (ANOVA) test using SAS version 9.1 analytical software (SAS Institute, Cary, NC, USA) to test for the dietary treatments. Tukey's HSD (honestly significant difference) *post-hoc* test was used to compare means amongst treatments with significant effects. Data values are expressed as mean ± standard deviation of three replicates of each treatment groups. Treatment effects were considered with the significant level at $P < 0.05$. The normality and homogeneity of variance were confirmed by Shapiro–Wilk and O'Brien tests, respectively. We used GraphPad Prism 3 software (GraphPad Software, San Diego, CA, USA) based on Kaplan and Meier (1958) survival curves to determine the differences in survival rate among the treatment groups.

3. Results

3.1. Tissue mercury bioaccumulation

Total Hg bioaccumulations in blood and different tissues such as liver, kidney and muscle of male ICR mice fed the experimental diets for 30 days are shown in Table 2. The results show that dietary mercury deposited in blood and tissues on dose dependent manner. Mercury contents in blood, liver, kidney and muscle tissues of mice fed the Hg500C0E0Se0 and Hg500C400E200Se2 diets showed significantly higher values than the mice fed the Hg0C0E0Se0, Hg0C400E200Se2, Hg50C0E0Se0 and Hg50C400E200Se2 diets. Mice fed the Hg0C0E0Se0, Hg0C400E200Se2, Hg50C0E0Se0, Hg50C400E200Se2, Hg500C0E0Se0 and Hg500C400E200Se2 diets showed highest Hg contents in kidney tissues than the other tissues.

There were no significant differences in total Hg concentrations in the blood, liver, kidney and muscle tissues of mice fed the experimental diets in the respective treatment groups supplemented with or without antioxidants. Furthermore, the two-way ANOVA showed that dietary Hg had significant effect on tissue Hg bioaccumulations in male ICR mice. However, there were no significant effect of antioxidants as well as no interaction effect between Hg and antioxidants (Se, vitamin C and vitamin E) on reducing mercury contents in the tissue levels.

3.2. SOD enzyme activities

Serum antioxidants such as superoxide dismutase (SOD) activities in mice fed the experimental diets for 30 days is shown in Figure 1 (A). The result shows that there were no significant differences in terms of serum SOD activities in blood of mice fed the experimental diets. Furthermore, two-way ANOVA revealed that dietary Hg or antioxidants had no significant individual effects or interaction between Hg and antioxidants on SOD activities in mice fed the diets.

3.3. TBARS activities

Serum peroxidation such as thiobarbituric acid reactive substances (TBARS) activities in mice fed the experimental diets for 30 days is shown in Figure 1 (B). Serum TBARS activities in blood of mice fed the experimental diets revealed that the Hg50C400E200Se2 and Hg500C400E200Se2 diets had significantly lower TBARS activities than the Hg50C0E0Se0 and Hg500C0E0Se0 diets. The two-way ANOVA showed that both the Hg and antioxidants had significant effect on TBARS activity; however, no interaction effect was observed between dietary Hg and antioxidants.

3.4. Mitochondrial oxidative stress

The tissue specific mitochondrial oxidative stress in terms of cytochrome *c* oxidase (COX) enzyme activities in liver and kidney tissues in male ICR mice were shown in Figure 2 (A) and 2 (B), respectively. The COX enzyme activities in liver tissue of mice fed the Hg0C0E0Se0,

Hg0C400E200Se2, Hg50C0E0Se0 and Hg50C400E200Se2 diets were significantly higher than the Hg500C0E0Se0 and Hg500C400E200Se2 diets. However, there were no significant differences in liver COX activities in mice fed the Hg0C0E0Se0, Hg0C400E200Se2, Hg50C0E0Se0 and Hg50C400E200Se2 diets. Dietary Hg had significant effect on the treatment groups based on two-way ANOVA; however, there were no significant effects of antioxidants or interaction effect on the liver tissue of mice fed the experimental diets.

In case of kidney tissue, the COX enzyme activities in mice fed the Hg0C0E0Se0 and Hg0C400E200Se2 diets were significantly higher than the Hg50C0E0Se0, Hg50C400E200Se2, Hg500C0E0Se0 and Hg500C400E200Se2 diets. Moreover, mice fed the Hg50C0E0Se0 and Hg50C400E200Se2 diets showed significantly higher COX activities in kidney tissue than that of Hg500C0E0Se0 and Hg500C400E200Se2 diets. However, there were no significant differences between Hg0C0E0Se0 and Hg0C400E200Se2 diets, between Hg50C0E0Se0 and Hg50C400E200Se2 diets as well as between Hg500C0E0Se0 and Hg500C400E200Se2 diets in terms of COX activities of kidney tissue. The two-way ANOVA showed that dietary Hg had significant effect on COX enzyme activities; however, there were no significant effect of antioxidants or the interaction effect of dietary Hg and antioxidants related to COX enzyme activities in mice fed the experimental diets.

3.5. Survivability in mice

Cumulative survival rate of male ICR mice based on Kaplan-Meier survival curve during the 30 days of feeding trial has shown in Figure 3. The first mortality of mice was observed on day-6 in the cage of mice fed the Hg500C0E0Se0 diet and further became more evident on the following days in the cages of mice fed the Hg supplemented diets. The results showed that mice fed the Hg0C0E0Se0 and Hg0C400E200Se2 diets secured 100% survival rate than the other treatment groups. However, mice fed the Hg500C0E0Se0 diet showed highest mortality (80% of mortality) at the end of the experiment. At the end of 30 days of feeding trial, mice

357 fed the Hg0C0E0Se0 and Hg0C400E200Se2 diets had significantly higher cumulative survival
358 rates than those of mice fed the Hg50C0E0Se0, Hg50C400E200Se2, Hg500C0E0Se0 and
359 Hg500C400E200Se2 diets.
360 The two-way ANOVA showed that both the Hg and antioxidants had significant effects
361 based on K-M survival curve; however, no interaction effect was observed between dietary Hg
362 and antioxidants.
363 **4. Discussion**
364 In the present study, male ICR mice fed the diets containing organic mercury in the form of
365 methylmercury chloride (CH_3HgCl) added in the lyophilized olive flounder fish muscle with
366 or without supplementation of dietary antioxidants such as selenium (Se), vitamin C and
367 vitamin E in different doses were evaluated. In this experiment, the mercury and the multi-
368 antioxidant levels were selected based on the previous research findings in rodent and fish
369 models (Beyrouty and Chan, 2006; Bourdineaud et al., 2008; Bolkent et al., 2008; Folven et
370 al., 2009; Muthu and Krishnamoorthy, 2012; Park et al., 2016). The results revealed that dietary
371 organic mercury had profound effect on the ICR mice in terms of mercury toxicity based on
372 tissue mercury bioaccumulations as well as survivability of mice. Likewise, Beyrouty and Chan
373 (2006) reported the higher level of MeHg bioaccumulations in different tissues and higher
374 mortality of rats in a dietary experiment. Dietary analyses of the present study showed that Hg
375 contents in the fish muscle seemed to be very low compared to the basal or negative control
376 diet which may be attributed to the higher Hg contents in the standard chow from commercial
377 source. In consistent with the present study, Weiss et al. (2005) reported that a significant
378 amount of total Hg can be detected in the standard laboratory chow for animals which may
379 directly affect the results of the experimental findings. Furthermore, the researchers opined that
380 dietary ingredient like fish meal especially from tuna scraps could be the additional source of
381 Hg in the standard chow for rats which might be reflected in the present study by means of

382 elevated Hg levels in the experimental diets compared to the suppelemental levels. In this
383 study, we observed that the highest level of Hg bioaccumulated in tissues of mice, in decreasing
384 order, kidney > liver > muscle > blood. The results agree with our recent study in marine fish
385 model for organic mercury bioaccumulation (Raihan et al., 2020) as well as other researchers
386 who reported in mice fed the mercury contaminated fish flesh (Bourdineaud et al., 2008). In
387 the present study, dietary supplementation of multi-antioxidants could not reduce the Hg
388 concentrations in blood, liver, kidney and muscle tissues of mice may be due to the high levels
389 of Hg in the experimental mice feeds. In accordance of our findings, Ganther (1978) reported
390 that dietary antioxidants such as Se and vitamin E had no protective effect in terms of reducing
391 tissue mercury concentrations rather they can prevent the tissue oxidative damage in animals
392 which strongly supported the results of the present study. Moreover, Se could increase the
393 uptake of Hg and dietary Hg cannot be decreased by Se at the same time when Se is exerting a
394 protective effect in rat brain (Prohaska and Ganther, 1977). Likewise, Penglase et al. (2014)
395 postulated that dietary supplementation of Se could not enhance the reproductive performance
396 and it can negatively affect the fish reproductive potential as well as the effect on reproduction
397 that may enhance in the presence of elevated Hg. Beyrouty and Chan (2006) and Folven et al.
398 (2009) opined that there were no effects of dietary Se and vitamin E in reducing Hg
399 concentrations of brain, liver and kidney tissues of rodent model on induced mercury toxicity
400 which agrees with the results of the present study. However, in our previous findings, we
401 reported that dietary antioxidants had positive effects in reducing organic or inorganic mercury
402 contents in marine fish model (Lee et al., 2016; Park et al., 2016; Moniruzzaman et al., 2017
403 (a) (b); Lee et al., 2017). Furthermore, some researchers reported that dietary Se, vitamin C
404 and vitamin E could reduce the Hg burden in the tissue levels of rodent model (Rao et al., 2001;
405 Muthu and Krishnamoorthy, 2012; Glaser et al., 2013). Bolkent et al. (2008) shown that
406 antioxidants such as Se, vitamin C and Vitamin E partly prevent the toxicity of cadmium in

407 gastric fundus tissue of male Sprague-Dawley rats based on histopathological,
408 immunohistochemical and biochemical analyses.

409 Mercury is known to increase the cellular level of reactive oxygen species (ROS) and
410 induce oxidative stress which lead to the generation of superoxide radicals and enhancement
411 of lipid peroxidation levels (Agarwal et al., 2010). Redox homeostasis depends on the
412 antioxidant defense system that eliminate a wide range of oxidants, including ROS, lipid
413 peroxides, and metals (Oyewole and Birch-Machin, 2015). Antioxidants help to protect animal
414 from hazardous effect of ROS produced in the body. It have been reported that because of
415 having the antioxidant properties of Se, vitamin C and vitamin E, they can protect from the
416 ROS in animal due to the heavy metal toxicity (Ganther, 1978; Rao et al., 2001; NRC, 2005;
417 Bolkent et al., 2008; Agarwal et al., 2010; Glaser et al., 2013). In the present study, the results
418 showed that due to the supplementation of Se, vitamin C and vitamin E in the mice feed the
419 antioxidant enzyme such as serum superoxide dismutase (SOD) activity was unaltered even
420 though Hg was present in the corresponding diets. In agreement with our study, Agarwal et al.
421 (2010) reported that there was no significant difference in SOD levels of kidney tissue in terms
422 of vitamin E and Hg interaction in mice recieved gavage supplementation of vitamin E and Hg.

423 In the present study, we found that dietary Se, vitamin C and vitamin E had significant
424 effect on the reduction of lipid peroxidation in terms of serum TBARS levels. Likewise, we
425 reported previously that dietary Se, vitamin C and vitamin E could reduce the lipid peroxidation
426 in tissue levels of olive flounder fish (Park et al., 2016). Marin-Guzman et al. (2000) postulated
427 that Se and vitamin E both have protective role in peroxidative damage in animals and dietary
428 co-admisnistration of these antioxidants can act synergitiscally (Kolodziej and Jacyno, 2005).
429 The results of the present study revealed that dietary Hg and antioxidants both had significant
430 effect on serum TBARS activities. Furthermore, TBARS levels in the Hg containing diets were
431 significantly reduced by dietary antioxidants supplementations. In agreement with our study,

El-Demerdash (2001) postulated that oral doses of Se could reduce the Hg toxicity in terms of decreasing TBARS activities in brain and liver of male rats. However, in contrast to the present study, Perottoni et al. (2004) reported that Hg can increase the TBARS level in kidney but organoselenium compounds did not prevent the such effect in terms of Hg toxicity in male Wistar rats.

Mitochondria as the subcellular organelles are present in the cytoplasm of eukaryotic cells which play an important role in cellular energy generation by means of adenosine tri-phosphate (ATP) production. Albeit, mitochondria are the main source of ROS production (Oyewole and Birch-Machin, 2015). Mitochondrial ROS can be detrimental or beneficial based on the amount, duration, and location of their production (Onukwufor et al., 2019). In electron transport chain, SOD enzyme as the mitochondria-specific antioxidant can detoxify oxygen ion (O_2^-) by the formation of H_2O_2 which finally converted into water (H_2O). If the process hampered, then a highly reactive hydroxyl radical (OH) can form from the H_2O_2 which may react with the metal ions (Oyewole and Birch-Machin, 2015). Cytochrome c oxidase (COX) as the last enzyme of the mitochondrial respiratory chain, is the major oxygen consumer enzyme in the cell (Bourens et al., 2013). It has been reported that impaired COX could resulted the high deposition of ROS and oxidative stress in cell levels (Douiev et al., 2018). In the present study, we found that mice fed the Hg containing diets had profound effect on the liver and kidney tissues in terms of COX activities in male ICR mice. The concentrations of COX enzyme activities were significantly decreased in both tissues as the Hg contents increased in the diets. However, interestingly, we could not find any significant differences in individual group of diets supplemented with or without antioxidants. The possible mechanism may be due to the higher production of ROS by mitochondria in the respective tissue levels that of mice fed the Hg containing diets got oxidative stress during the experiemental period. In agreement with the present study, Bourdineaud et al. (2008) reported that Hg had significant deacreasing

457 effect on the COX activities in naïve male mice of the C57B1/6 Jico fed the mercury-
458 contaminated fish flesh for one month exposure period.

459 In the present study, male ICR mice fed the Hg contaminated fish with commercial
460 diet showed significantly higher mortality rate compared to the control diet which contained
461 baseline level of Hg at 50.7 $\mu g.kg^{-1}$ diet (no Hg and no antioxidant supplemented diet) as well
462 as the diet supplemented with antioxidants which contained baseline level of Hg at 53.5 $\mu g.kg^{-1}$
463 diet (no supplementation of Hg). The 100% cumulative survival rates were achieved in mice
464 fed the control and the diet supplemented with Se, vitamin C and vitamin E only. These may
465 attributed to the presence of selenium in fish meal of the diets as well as in the dietary inclusion
466 of marine fish, olive flounder fish muscle in the present study. However, a slight increase in
467 supplemention of Hg in the diet (Hg50C0E0Se0 diet that contained 111.8 $\mu g.kg^{-1}$ diet) caused
468 more than 40% of mice die might be due the presence of MeHg in molar excess of Se in the
469 diet which is in agreement with Ralston et al. (2019). Interestingly, the first mortality was
470 observed in mice fed the low Hg with antioxidant supplemented diet (Hg50C400E200Se2 diet)
471 on day 15; whereas, in case of high Hg with antioxidant supplemented diet
472 (Hg500C400E200Se2 diet) fed mice showed rapid onset of mortality (day 9). Here, in this
473 study, the results demostrated that antioxidant supplemented diets prolonged the survivability
474 of mice. Noteworthy, in the present study, we used selenomethionine as the source of biological
475 form of selenium in the diets which have high antagonistic behavior with organic MeHg
476 compared to inorganic selenium (Moniruzzaman et al., 2017b) that may enhanced the
477 survivability of mice due to the higher level of selenium supplementation in the diets (2 $mg.kg^{-1}$).
478 In agreement with our study, Beyrouty and Chan (2006) reported that co-consumption of
479 MeHg, Se and vitamin E can significantly increase the survivability of offspring of female
480 Sprague-Dawley rats. Likewise, in our previous experiment on marine fish model, we
481 demonstrated the synergistic positive effect of dietary vitamin E and selenomethionine on

induced Hg toxicity on the survival rate of the fish (Moniruzzaman et al., 2017b). However, some researchers reported a little or no death and higher concentrations of Hg in tissues of rodents supplied with high levels of Hg compared to the present study (Newland and Rasmussen, 2000; Bourdineaud et al., 2008; Fujimura et al., 2009; Ferrer et al., 2021). The results may be contradicted due to the use of different strain of rodent, different research methodology as well as different feeding regimen in the present study.

5. Conclusions

From the findings of the present study, taken together, it can be corroborated that dietary supplementation of antioxidants such as Se, vitamin C and vitamin E had no effect on Hg bioaccumulation in male ICR mice; however, antioxidants had partial effect in terms of reducing serum lipid peroxidation and pronounced effect on the cumulative survival rate in mice fed the Hg intoxicated diets. Importantly, the present study contradicted with our previous findings in marine fish model where we postulated that dietary supplementation of Se, vitamin C and vitamin E had protective role on Hg toxicity in olive flounder fish. Based on the present and the previous investigations (Lee et al., 2016; Park et al., 2017; Moniruzzaman et al., 2017a; Moniruzzaman et al., 2017b; Lee et al., 2017; Raihan et al., 2020), we may assume that marine fish like olive flounder had more effective metabolic system than male ICR mice in reducing Hg toxicity on the supplementation of dietary antioxidants. However, the present study further warranted the interaction effect of dietary Hg with different antioxidants based on molecular mechanisms.

CRediT authorship contribution statement

Mohammad Moniruzzaman: Investigation, Laboratory analyses, Data analysis, Writing - original draft. **Seunghan Lee:** Investigation, Laboratory analyses, Data analysis. **Youngjin Park:** Investigation. **Taesun Min:** Writing - review & editing. **Sungchul C. Bai:** Supervision, Funding acquisition, Resources and Approval of final draft.

Declaration of competing interest

The authors declare that they have no competing interests.

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712

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

Ingredients	Diets					
	Hg0C0E0Se0	Hg0C400E200Se2	Hg50C0E0Se0	Hg50C400E200Se2	Hg500C0E0Se0	Hg500C400E200Se2
Mouse[1] feed	86.2	86.2	86.2	86.2	86.2	86.2
Flounder[2] fish muscle powder (Hg 5 ppm)	0	0	1.0	1.0	10.0	10.0
Flounder[2] fish muscle powder (Hg 0 ppm)	10.0	10.0	9.0	9.0	0	0
Vitamin C[3] (50,000 ppm)	0	0.8	0	0.8	0	0.8
Vitamin E[4] (20,000 ppm)	0	1.0	0	1.0	0	1.0
Selenium[5] (100 ppm)	0	2.0	0	2.0	0	2.0
Cellulose	3.8	0	3.8	0	3.8	0

714

715 [1]Purina Korea Inc., Seongnam-si, Gyeonggi-do, Republic of Korea [2]Laboratory made dried
716 flounder muscle powder [3]Hoffman La Roche, Basel, Switzerland [4]Sigma Aldrich, St. Louis,
717 MO, USA [5]United States Biochemical, Cleveland, Ohio, USA.

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719

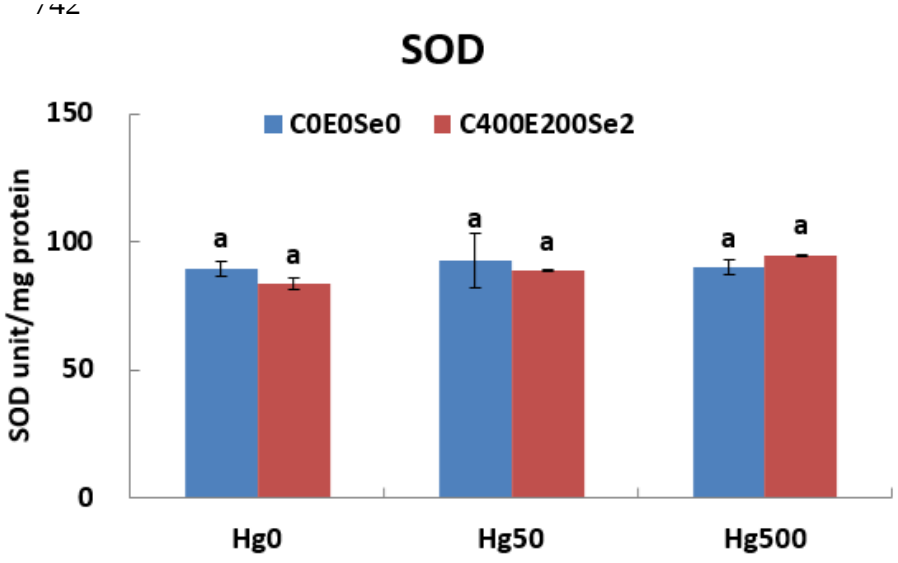
720

Table 2. Tissue total mercury contents (μg.g^{-1} of wet matter basis) in male ICR mice fed the experimental diets for 30 days.

Diets[1]	Muscle	Liver	Kidney	Blood
Individual treatment means				
Hg0C0E0Se0	0.0477±0.01^{c}	0.0739±0.01^{c}	0.3199±0.12^{c}	0.0071±0.00^{c}
Hg0C400E200Se2	0.0538±0.01^{c}	0.0736±0.01^{c}	0.4289±0.14^{c}	0.0076±0.00^{c}
Hg50C0E0Se0	0.2074±0.04^{b}	0.3560±0.08^{b}	2.6224±0.64^{b}	0.0469±0.02^{b}
Hg50C400E200Se2	0.1981±0.05^{b}	0.4165±0.12^{b}	3.3368±0.81^{b}	0.0633±0.01^{b}
Hg500C0E0Se0	0.4132±0.02^{a}	0.9226±0.07^{a}	5.7005±0.31^{a}	0.1453±0.02^{a}
Hg500C400E200Se2	0.4125±0.09^{a}	0.9861±0.03^{a}	5.7969±1.20^{a}	0.1493±0.03^{a}
Means of main effect				
Hg0	0.0507^{c}	0.0737^{c}	0.3744^{c}	0.0073^{c}
Hg50	0.2027^{b}	0.3862^{b}	2.9796^{b}	0.0550^{b}
Hg500	0.4128^{a}	0.9547^{a}	5.7484^{a}	0.1473^{a}
C0E0Se0	0.2227^{a}	0.4508^{a}	2.8809^{a}	0.0664^{a}
C400E200Se2	0.2214^{a}	0.4920^{a}	3.1875^{a}	0.0733^{a}
Two-way analysis of variance (ANOVA): *P*-value				
Hg effect	<0.0001	<0.0001	<0.0001	<0.0001
Antioxidants effect	0.9629	0.3372	0.4524	0.5483
Hg × Antioxidants	0.9729	0.7682	0.7613	0.8290

^{a,b,c} Values are means ± SD from triplicate groups of mice (n=3) where the values within a column without a common superscript differ ($P < 0.05$).

[1]Hg0C0E0Se0, Hg50C0E0Se0 and Hg500C0E0Se0 designate 0, 50, 500 μg.kg^{-1} Hg without multi-antioxidants supplements; Hg0C400E200Se2, Hg50C400E200Se2 and Hg500C400E200Se2 designate 0, 50, 500 μg.kg^{-1} Hg with selenium (2mg.kg^{-1}), vitamin C (400 mg.kg^{-1}) and vitamin E (200 mg.kg^{-1}) supplements.



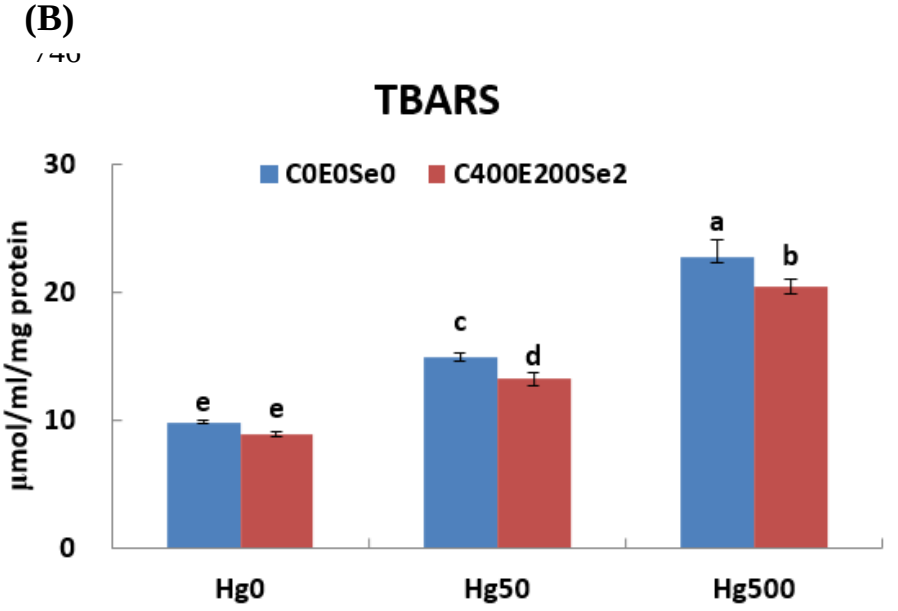
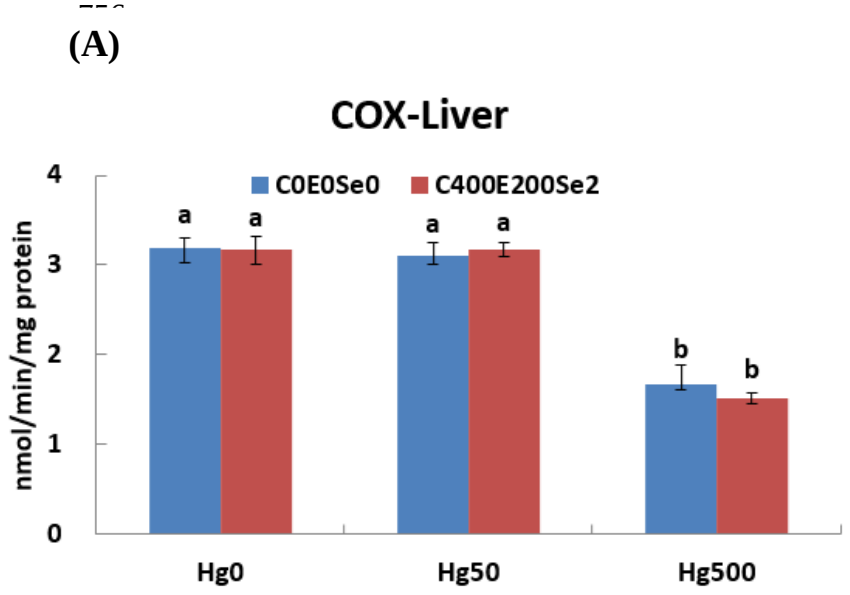
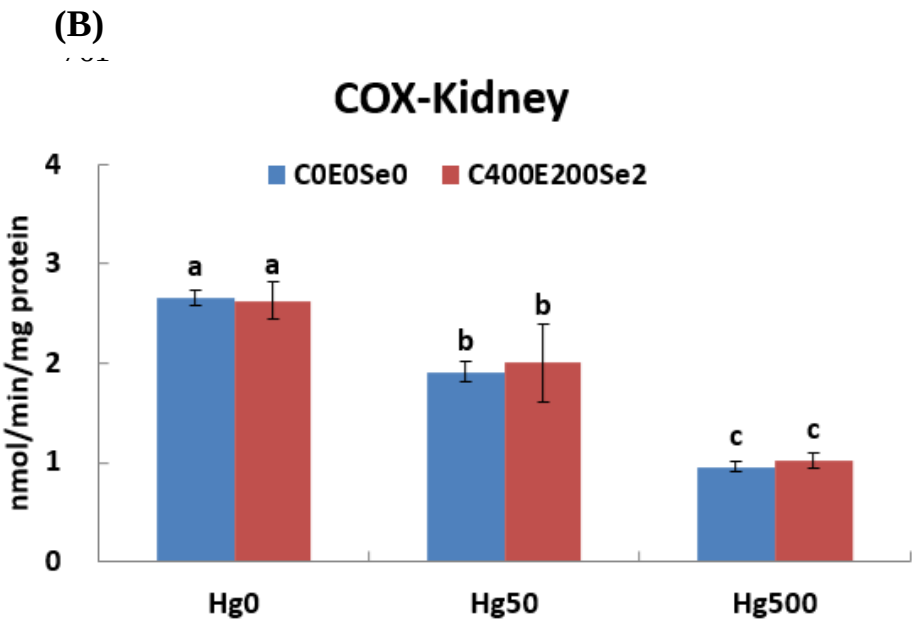


Figure 1. Blood serum superoxide dismutase (SOD) activities (A) and thiobarbituric acid reactive substances (TBARS) activities (B) in male ICR mice after 30 days. [a,b,c,d,e] Values are means ± SD from triplicate groups of mice (n=3) where different letters are

significantly different ($P < 0.05$). Effects of Hg and the combined effect of antioxidants (selenium, vitamin C and vitamin E) and their interaction effects were determined by 2-way ANOVA.



2-way ANOVA
Hg:
$P = 0.0001$
Antioxidants:
$P = 0.6861$
Interaction
$P = 0.5652$



2-way ANOVA
Hg:
$P = 0.0001$
Antioxidants:
$P = 0.6948$
Interaction
$P = 0.9016$

Figure 2. Cytochrome *c* oxidase (COX) enzyme activities in liver (A) and kidney (B) tissues in male ICR mice after 30 days.[a,b,c] Values are means ± SD from triplicate groups of mice (n=3) where different letters are significantly different ($P < 0.05$). Effects of Hg and the combined effect of antioxidants (selenium, vitamin C and vitamin E) and their interaction effects were determined by 2-way ANOVA.

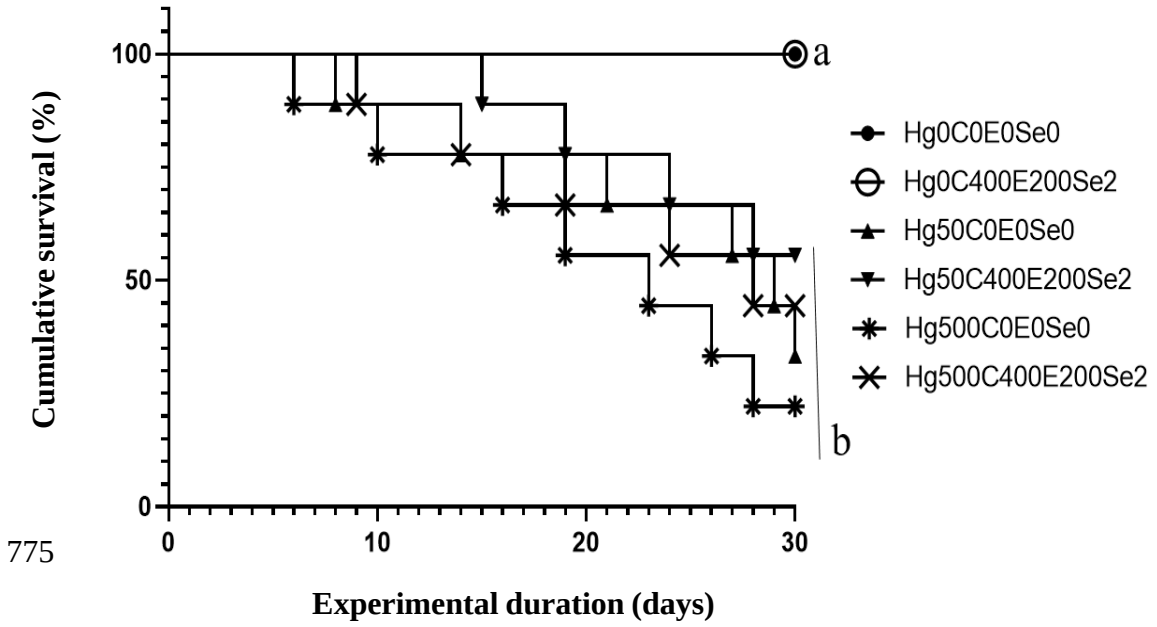


Figure 3. Cumulative survival rate (initial number of mice – final number of mice × 100/initial number of mice) of male ICR mice based on Kaplan-Meier (K-M) survival curve during the 30 days experimental period. Each value represents mean of 3 cages of each treatment (n=3). Different letters on survival data (a,b) are significantly ($P < 0.05$) different by Tukey's HSD test. Two-way ANOVA showed the following *P*-values: Hg, $P = 0.001$; antioxidants, $P = 0.039$; Interaction, $P = 0.300$.

Evaluation of dietary selenium, vitamin C and E as the multi-antioxidants on the methylmercury intoxicated mice based on mercury bioaccumulation, antioxidant enzyme activity, lipid peroxidation and mitochondrial oxidative stress

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Abstract

Mercury (Hg) in high exposures can be a potent life threatening heavy metal that bioaccumulate in aquatic food-chain mainly as organic methylmercury (MeHg). In this regard, fish and seafood consumptions could be the primary sources of MeHg exposure for human and fish-eating animals. The objective of the present study was to elucidate the effects of dietary supplementation of some antioxidants on induced mercury toxicity in mice model. In this study, a 30-day long investigation has been conducted to evaluate the dietary effect of selenium (Se) in combination with vitamin C and vitamin E on methylmercury induced toxicity in mice. Total 54 mice fed the diets with three levels of Hg (0, 50 or 500 $\mu g.kg^{-1}$) and two levels of Se in combination with vitamin C and E (Se: 0, 2 mg kg^{-1}; vitamin C: 0, 400 mg kg^{-1}; vitamin E: 0, 200 mg kg^{-1}) in triplicates. The results show that Hg accumulated in blood and different tissues such as muscle, liver and kidney tissues of mice on dose dependent manner. The bioaccumulation pattern of dietary Hg, in decreasing order, kidney > liver > muscle > blood. Superoxide dismutase levels in blood serum showed no significant differences in mice fed the diets. However, dietary antioxidants significantly reduced the levels of thiobarbituric acid reactive substances in mice fed the mercury containing diets. Cytochrome *c* oxidase enzyme activities showed no significant differences as the mercury level increases in liver and kidney tissues of mice. Kaplan-Meier curve showed a dose- and time- dependent survivability of mice. Cumulative survival rate of Hg intoxicated mice fed the antioxidant supplemented diets were increased during the experimental period. Overall, the results showed that dietary Se, vitamin C and vitamin E had no effect on reducing the mercury bioaccumulation in tissues but reduced the serum lipid peroxidation as well as prolonged the cumulative survival rate in terms of high Hg exposures in mice.

Keywords: methylmercury; antioxidants; tissue bioaccumulation; superoxide dismutase; reactive oxygen species; cytochrome *c* oxidase;

1. Introduction

Mercury is a xenobiotic, persistent and non-essential ubiquitously present heavy metal (Farina et al., 2013; Rand et al., 2016) which may be organic or inorganic in nature. Mercury is commonly available in the atmosphere in the form of elemental mercury (Hg^0), mercuric mercury (Hg^{2+}) and methylmercury (CH_3Hg^+) which is the most toxic form of Hg in high exposures and extensively investigated in recent years (Ordiano-Flores et al., 2012; Fernandes et al., 2020). Hg inputs to the environment is primarily involved with wet and dry environmental deposition of inorganic mercury (Hg^{2+}) that can be modified into volatile gaseous elementary mercury (Hg^0) or methylated to form the highly bioaccumulative methylmercury that biomagnifies in aquatic food chains and finally causes health hazards in human and fish-eating wildlife (Lavoie et al., 2018; Bourdineaud et al., 2019; Fernandes et al., 2020). For this reason, marine and freshwater fish are considered as the main sources of dietary methylmercury (MeHg) exposures (Ralston and Raymond, 2019). The organic MeHg has high neurotoxic effects to human especially during fetal development where MeHg is exposed in molar excess of selenium through fish and seafood consumptions (Ralston, et al., 2019; Kim et al., 2019) that are also good sources of quality protein and essential amino acids as well as omega-3 fatty acids (eicosapentaenoic acid or EPA and docosahexaenoic acid or DHA), minerals (iodine and selenium), and vitamins (NRC, 2011; Ralston et al., 2019). The MeHg absorption rate in an organism digestive tract is around 90-95% (Nielsen and Andersen, 1992) which potentially distributed in different organs and cause toxicity through the ingestion of polluted fish (Farina et al., 2011). The MeHg can accumulate by exceeding seven orders of magnitude from sub $ng.L^{-1}$ concentrations in water to more than 1 $mg.kg^{-1}$ in piscivorous fish which may cause serious health concerns in frequent seafood consumers (Hintelmann, 2010). In contrast, MeHg excretion from human body usually starts at the elimination rate (k_{el}) of near about 0.01 day^{-1} or 70 days of half-life [$t_{1/2}$]) and it is a potential determinant of the Hg

bioaccumulation resulted through fish consumption (Rand et al., 2016). Based on the Priority List of Hazardous Substances established by the Agency for Toxic Substances and Disease Registry (ATSDR, 2019), the toxic effects of heavy metals on human health status observed, in decreasing order, As > Pb > Hg > Cd > Cr > Co > Ni > Zn > Cu > Mn. Therefore, Hg is considered as the third most toxic heavy metal after arsenic (As) and lead (Pb), respectively, when it exceeds the normal or recommended level.

The U.S. Environmental Protection Agency (USEPA) recommended the reference dose for MeHg at 0.1 $\mu g.kg^{-1}$ $bw.day^{-1}$ for human. Methylmercury is known to create oxidative stress through the production of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and nitric oxide (NO) in animals, which have deleterious effects on organisms in terms of mitochondrial dysfunction to behavioral changes through the depletion of antioxidant enzyme activities by MeHg (Farina et al., 2011; Ishihara et al., 2016). High level of MeHg has the high affinity to the thiol or sulfhydryl (-SH) groups of proteins (Ajsuvakova et al., 2020). However, Hg has a lower affinity for thiol groups compared to Se containing groups (Spiller, 2017). It has been reported that Hg has ~1 million times higher affinity for Se than sulfur which is considered as the primary target of MeHg toxicity (Ralston and Raymond, 2019). Furthermore, Spiller et al. (2017) evidenced that the *in vivo* pathophysiological changes by Hg is dependent on the binding of Hg with Se but not with sulfur. Selenium has reciprocal effects on Hg^{2+} toxic behavior reported by Pariziek et al. (1971) who postulated that mortality of rats can be reduced by co-injection of Se and Hg. The bindings of Hg to Se compromised the biological functions and availability of Se (Folven et al., 2009). The high affinity of Hg for Se warrant it to specifically chelate Se at the active sites of important Se-containing enzymes (selenoenzymes) like glutathione peroxidase and thioredoxin reductase (Branco et al., 2014; Spiller, 2017). These selenoenzymes are continuously disrupted and reconstitute the insoluble Hg-Se bonds due to the excessive molar formation of Hg and Se (more than 1:1) which could

reduce the production of essential selenoproteins (selenocysteine) by depleting Se availability in the tissue level (Ralston, 2018) and finally the Hg-induced Se deficiency could not restore the cellular redox environment (Spiller, 2017). The impairment of selenoenzymes in the cellular redox environment causes proliferation of intracellular reactive oxygen species (ROS) which resulted the mitochondrial dysfunction, lipid peroxidation, protein impairment and apoptosis (Spiller, 2017). Nonetheless, selenoenzymes have potential to control reactive oxygen species (ROS) production and they can prevent as well as reverse the oxidative damage in various tissues especially liver and brain of organisms (Ralston et al., 2019; Zhang et al., 2020). Consumption of marine fish or seafood containing Se in molar excess of MeHg could prevent the disruption of selenoenzymes through alleviating Hg-exposure risks (Ralston et al., 2019) which is regarded as the positive Health-Benefit-Value (HBV) or they contain MeHg in excess of Se as regarded as the negative HBV for the consumers (Ralston et al., 2014; Ralston et al., 2019). However, the consequences are depend on the form of Hg and Se as well as the organ and amounts (Spiller, 2017). Furthermore, Prohaska and Ganther (1977) reported that dietary Se cannot decrease the tissue Hg levels in rat brain rather it increase the Hg uptake. Penglase et al. (2014) found that dietary Se had negative effect on reproductive performance of fish on higher levels of Hg in fish diets.

Mitochondria is known as the powerhouse of cell that brings energy to every cellular process. Mitochondria could produce high levels of ROS when exposed to MeHg. It has been reported that mitochondrial ROS are responsible for oxidative stress following cell death in organisms intoxicated by MeHg (Ishihara et al., 2016). In this case, both enzymatic and non-enzymatic defense systems are required to protect the organisms from deleterious effects caused by ROS (Halliwell and Gutteridge, 2015). There are two main categories in the defense systems such as: endogenous antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) (Halliwell and Gutteridge, 2015), and others are

134 exogenous antioxidants such as ascorbic acid (vitamin C), tocopherols (vitamin E), vitamin A,
135 carotenoids and some metals like selenium (Se) is essential for the function of antioxidant
136 enzymes (Halliwell and Gutteridge, 2015). The cytochrome *c* oxidase (COX) as the terminal
137 enzyme of mitochondria, is the terminal electron acceptor of the respiratory chain of
138 mitochondria which is responsible for the cellular respiration in most of the aerobic organisms
139 (Ostermeier et al., 1996) and in the final act it reduces dioxygen to water with four electron
140 from cytochrome *c* and four protons taken up from the mitochondrial matrix without formation
141 of ROS (Kadenbach et al., 2000). There are 13 different hetero-oligomeric sub-units of COX
142 enzymes localized to the inner mitochondrial membrane (Tsukihara et al., 1996; Zsengeller
143 and Rosen, 2016), of which, sub-units I, II, III and IV were widely described (Ostermeier et
144 al., 1996).

145 Vitamin C (L-ascorbic acid) have potential to maintain the normal health status in
146 organisms. Smirnoff (2018) reported that higher doses of vitamin C supplementation have
147 health benefits. Vitamin C is an important water-soluble antioxidant and a cofactor in metabolic
148 synthesis process (Smirnoff, 2018). Studies have shown that dietary vitamin C can reduce the
149 mercury toxicity in terms of reducing mercury concentrations in blood and tissues especially
150 liver and kidney tissues through the urinary excretion as well as enhance the immune responses
151 in organisms (Hounkpatin et al., 2012; Lee et al., 2016).

152 Vitamin E is a lipid soluble antioxidant, which plays a vital role to stabilize the tissue
153 membranes and reduces the ROS due to the oxidative stress (Agarwal et al., 2010). It can
154 reduce mercury and inhibits oxidative damage in the liver and other tissues caused by mercury
155 toxicity (Rana et al., 1996; Chapman and Chan, 2000; Rao et al., 2001). However, some
156 researchers reported that dietary vitamin E and Se can prevent the oxidative damage but cannot
157 reduce the Hg contents in tissues of animal (Ganther, 1978; Beyrouty and Chan, 2006; Folven
158 et al., 2009). Pillai and Gupta (2005) postulated that vitamin E can protect from mercury

toxicity due to its chelating efficacy which ultimately enhance the antioxidant defense system in living organisms. Furthermore, vitamin E together with vitamin C could reduce lipid peroxidation, ROS and increase immunity in male rats (Muthu and Krishnamoorthy, 2012), while the dietary Se and vitamin E were reported to show their synergistic protective effect against oxidative damage in animals (Marin-Guzman et al. 2000; Kolodziej and Jacyno, 2005).

Olive flounder is one of the most consumed and commercially important marine demersal fish species cultured in East-Asian countries like Republic of Korea, Japan and China (Raihan et al., 2020). As a demersal and predatory fish species, olive flounder fish has great importance in relation to the mercury contamination on public health. Moon et al. (2011) reported that organic and total Hg in seafood of Korean water reached 55.6 $\mu g.kg^{-1}$ and 100 $\mu g.kg^{-1}$, respectively which necessitates to investigate in terms of mercury contamination and its detoxification using animal models. Recently, a series of experiments have been conducted by our group where we documented that dietary antioxidants like selenium, vitamin C and E had protective effect against mercury-induced toxicity in marine fish like olive flounder (Lee et al., 2016; Park et al., 2017; Moniruzzaman et al., 2017a; Moniruzzaman et al., 2017b; Lee et al., 2017; Raihan et al., 2020). In this study, for the first time, we envisage to evaluate the effect of multi-antioxidants (Se, vitamin C and vitamin E) in mice fed with methylmercury (MeHg) toxified olive flounder fish muscle powder in terms of tissue mercury bioaccumulation, antioxidant enzyme activities and oxidative stress in mice model.

2. Materials and Methods

2.1. Ethics and animal welfare

The experiment was conducted under the guidelines of Animal Ethics Committee Regulations No. 554 issued by the Pukyong National University, Busan, Rep. of Korea. In this

experiment, we tried to reduce the number of animals and sacrificing stress as minimum as possible.

2.2. Chemicals and reagents

Methylmercury (II) chloride, Cl ~13% (CH_3HgCl) as a source of Hg, L-selenomethionine (97% pure), DL-α-tocopheryl acetate as a source of vitamin E were collected from Sigma-Aldrich, St. Louis, USA and L-ascorbyl-2-monophosphate (containing 35% ascorbic acid activity) as a source of vitamin C was purchased from Hoffman La Roche, Basel, Switzerland; diethyl ether, nitric acid (HNO_3) and hydrogen peroxide (H_2O_2) were procured from Merck, Darmstadt, Germany.

2.3. Acquiring mice

A total of 54 specific pathogen-free (SPF) and laboratory bred male albino mice (*Mus musculus*) of ICR (Institute of Cancer Research) strain (35~38g), were used for the experiment. Mice (10 weeks old) were maintained on standard chow or mouse feed (Purina Korea Inc., Seongnam-si, Gyeonggi-do, Korea) without antioxidants (Se, vitamin C and vitamin E) or mercury supplementations (Table 1), and the mice were supplied with normal water up to *ad libitum*. The animals and the standard chow were procured from Hyochang Science, Daegu, Korea. Mice were housed in 18 stainless steel cages (200×260×130 mm) each containing 3 mice reared during the experimental period. All mice were kept at a temperature of 23±2°C in animal house with air conditioner facilities and at a relative humidity of 58% and exposed to 12 h/12 h (light/dark) cycle for at least 7 days prior to the experiment as well as during the experimental period.

2.4. Preparation of mice diet

Six experimental diets supplemented with three levels of MeHg (0, 50 or 500 $\mu g.kg^{-1}$ and two levels of Se in constant combination with vitamin C and E (Se: 0, 2 $mg.kg^{-1}$; vitamin C: 0, 400 $mg.kg^{-1}$; vitamin E: 0, 200 $mg.kg^{-1}$) by 3×2 factorial design (Hg0C0E0Se0,

Hg0C400E200Se2, Hg50C0E0Se0, Hg50C400E200Se2, Hg500C0E0Se0, Hg500C400E200Se2 diets) were fed to the albino male ICR mice in triplicate number of cages (Table 1). Prior to the experiment, juvenile olive flounder (*Paralichthys olivaceus*) fish muscle powder was prepared with the inclusion of methylmercury (II) chloride (Sigma-Aldrich, St. Louis, MO, USA). The total Hg contents in fish muscle tissue was found to be 17.4 $\mu g.kg^{-1}$. For the experimental diets, dried and lyophilized olive flounder fish muscle powder was added in the diets with or without supplementation of methylmercury (MeHg) as the negative control (Hg0C0E0Se0 diet). In basal diets, when supplemented with selenium, vitamin C and E, an equivalent amount of cellulose were removed. The experimental pellet feeds were processed manually by proper mixing the required ingredients and pelleting using metal pellet machine. After pelleting, the actual levels of total Hg in the diets were found to be 50.7 $\mu g.kg^{-1}$ (in Hg0C0E0Se0 diet), 53.5 $\mu g.kg^{-1}$ (in Hg0C400E200Se2 diet), 111.8 $\mu g.kg^{-1}$ (in Hg50C0E0Se0 diet), 98.2 $\mu g.kg^{-1}$ (in Hg50C400E200Se2 diet), 523.6 $\mu g.kg^{-1}$ (in Hg500C0E0Se0 diet) and 529.6 $\mu g.kg^{-1}$ (in Hg500C400E200Se2 diet).

2.5. Sample collection and analyses

At the end of the 30-day feeding trial, all of the survived mice (n=32) were euthanized directly and excised humanely for the collection of blood, muscle, liver and kidney tissues. In brief, mice were euthanized with ether and blood was immediately collected by cardiac puncturing of 3 mice from each treatments to minimize the animal sufferings (1 mice from each replicate). Blood was divided in two separate parts, i.e. heparinized (for mercury determination) and non-heparinized (for serum biochemical assays, *viz.* superoxide dismutase and thiobarbituric acid reactive substances). Non-heparinized blood samples were centrifuged in order to separate blood serum at 5,000 ×g for 10 min at room temperature and stored at -80°C for later determination of blood biochemical parameters. Tissues from mice were dissected for the analyses of mercury contents in liver, kidney and muscle as well as the further

enzymatic analyses *viz.* cytochrome *c* oxidase (COX) enzyme activities in liver and kidney tissues. The analyses of protein contents in blood serum for superoxide dismutase and thiobarbituric acid reactive substances as well as tissue homogenates for COX activities in mice fed the experimental diets were done by previously described method (Bradford 1976) using the bovine serum albumin (BSA) as standard and optical density (OD) at 595 nm to get the absorbance of the samples.

2.6. Tissue mercury contents

For the analyses of Hg contents in the olive flounder fish muscle and the diets as well as blood and other tissues (muscle, liver and kidney) of mice, an argon gas assisted Inductively Coupled Plasma Mass Spectrometer, ICP-MS (Perkin-Elmer 3300, Waltham, MA, USA) was used previously described by Lee et al. (2016). Briefly, samples (0.2 g of each) were weighed and put into a Kjeldahl flask (250 mL) with the addition of 50 mL nitric acid (65% HNO3) to the flask. Then, a heating mantle was used to heat the sample with flask. An aliquot of 5 mL hydrogen per oxide (50% H_2O_2) was added to the sample to digest fully, and the digested sample was diluted with de-ionized water upto 100 mL. The Hg contents in the digested solution was determined by the method of EPA-6020-A by ICP-MS. For better comparison, we used a reference sample (DORM-2: 4.47±0.03 $\mu g.g^{-1}$ Dogfish liver; National Research Council, Ottawa, ON, Canada) and blanks (0.04±0.01 $\mu g.L^{-1}$) during the analyses. We found that Hg values were in accordance with the reference values (95% recovery level). The limit of detection (LOD) and the limit of quantification (LOQ) of the equipment were 0.001 $\mu g.L^{-1}$ and 0.47 $\mu g.kg^{-1}$, respectively. The concentrations of total mercury in blood, liver, kidney and muscle tissues of mice were expressed in $\mu g.g^{-1}$ on wet matter basis.

2.7. Serum superoxide dismutase (SOD) activity

Superoxide dismutase (SOD) activity was measured by the superoxide radical-dependent reaction inhibition rate of enzyme with WST-1 (water-soluble tetrazolium dye) substrate and

xanthine oxidase using the SOD Assay Kit (product number 19160, Sigma-Aldrich, St. Louis, MO, USA) following to the manufacturer's protocols. Each endpoint assay was monitored by absorbance at 450 nm (the absorbance wavelength for the colored product of WST-1 reaction with superoxide) after 20 min of reaction time at 37°C. The percent inhibition was normalized by mg protein and expressed as SOD unit per mg.

2.8. Serum thiobarbituric acid reactive substances (TBARS)

Thiobarbituric acid reactive substances (TBARS) is a well-established assay for the screening and monitoring of ROS-mediated lipid peroxidation (Buege and Aust, 1978). The degree of peroxidation of blood serum was assessed by measurement of pink color intensity by means of the reaction of malondialdehyde (MDA) and TBARS using a commercial kit (Oxiselect^{TM} TBARS Assay kit, Cell Biolabs, San Diego, CA, USA). Briefly, 100 mL of serum samples with a known MDA concentration were mixed with 100 mL SDS (sodium dodecyl sulfate) lysis buffer and 250 mL thiobarbituric acid solution and heated at 95 ^{0}C in a water bath for 1 h, and then the absorbance of centrifuged supernatant solution was measured at 532 nm. Results were expressed as µmole per ml per mg protein.

2.9. Tissue cytochrome c oxidase (COX) enzyme activity

The COX activities in the freshly prepared liver and kidney tissues of mice (~50 mg of each) were determined using the commercial kit (CYTOCOX1, Sigma-Aldrich, St. Louis, MO, USA). The assay in this kit is based on Lemberg method (1969) which is executed by COX oxidation to ferricytochrome *c* by cytochrome *c* oxidase in decreasing way of absorbance at 550 nm spectrophotometrically. The oxidation of cytochrome *c* by cytochrome *c* oxidase is a two-step procees with fast reaction followed by a slower reaction rate. Initially, cytochrome *c* oxidase was mixed with a 0.1-M dithiothreitol (DTT) solution to prepare its reduced form. The initial reaction rate of the assay was measured during the first 45 seconds of the reaction then the reduction was confirmed by the change of color from dark orange red to pale purple-red.

Changes in absorbance per min was measured and results were expressed as nmol per min per mg protein at pH 7.0 at 25 ^{0}C.

During the experimental period we recorded the number of mice died every day to establish the survivability curve at the end of the experiment.

2.10. Statistical analyses

Mice cage mean values (n=3) were used for statistical analysis. All data were subjected to two-way analysis of variance (ANOVA) test using SAS version 9.1 analytical software (SAS Institute, Cary, NC, USA) to test for the dietary treatments. Tukey's HSD (honestly significant difference) *post-hoc* test was used to compare means amongst treatments with significant effects. Data values are expressed as mean ± standard deviation of three replicates of each treatment groups. Treatment effects were considered with the significant level at $P < 0.05$. The normality and homogeneity of variance were confirmed by Shapiro–Wilk and O'Brien tests, respectively. We used GraphPad Prism 3 software (GraphPad Software, San Diego, CA, USA) based on Kaplan and Meier (1958) survival curves to determine the differences in survival rate among the treatment groups.

3. Results

3.1. Tissue mercury bioaccumulation

Total Hg bioaccumulations in blood and different tissues such as liver, kidney and muscle of male ICR mice fed the experimental diets for 30 days are shown in Table 2. The results show that dietary mercury deposited in blood and tissues on dose dependent manner. Mercury contents in blood, liver, kidney and muscle tissues of mice fed the Hg500C0E0Se0 and Hg500C400E200Se2 diets showed significantly higher values than the mice fed the Hg0C0E0Se0, Hg0C400E200Se2, Hg50C0E0Se0 and Hg50C400E200Se2 diets. Mice fed the Hg0C0E0Se0, Hg0C400E200Se2, Hg50C0E0Se0, Hg50C400E200Se2, Hg500C0E0Se0 and Hg500C400E200Se2 diets showed highest Hg contents in kidney tissues than the other tissues.

There were no significant differences in total Hg concentrations in the blood, liver, kidney and muscle tissues of mice fed the experimental diets in the respective treatment groups supplemented with or without antioxidants. Furthermore, the two-way ANOVA showed that dietary Hg had significant effect on tissue Hg bioaccumulations in male ICR mice. However, there were no significant effect of antioxidants as well as no interaction effect between Hg and antioxidants (Se, vitamin C and vitamin E) on reducing mercury contents in the tissue levels.

3.2. SOD enzyme activities

Serum antioxidants such as superoxide dismutase (SOD) activities in mice fed the experimental diets for 30 days is shown in Figure 1 (A). The result shows that there were no significant differences in terms of serum SOD activities in blood of mice fed the experimental diets. Furthermore, two-way ANOVA revealed that dietary Hg or antioxidants had no significant individual effects or interaction between Hg and antioxidants on SOD activities in mice fed the diets.

3.3. TBARS activities

Serum peroxidation such as thiobarbituric acid reactive substances (TBARS) activities in mice fed the experimental diets for 30 days is shown in Figure 1 (B). Serum TBARS activities in blood of mice fed the experimental diets revealed that the Hg50C400E200Se2 and Hg500C400E200Se2 diets had significantly lower TBARS activities than the Hg50C0E0Se0 and Hg500C0E0Se0 diets. The two-way ANOVA showed that both the Hg and antioxidants had significant effect on TBARS activity; however, no interaction effect was observed between dietary Hg and antioxidants.

3.4. Mitochondrial oxidative stress

The tissue specific mitochondrial oxidative stress in terms of cytochrome *c* oxidase (COX) enzyme activities in liver and kidney tissues in male ICR mice were shown in Figure 2 (A) and 2 (B), respectively. The COX enzyme activities in liver tissue of mice fed the Hg0C0E0Se0,

Hg0C400E200Se2, Hg50C0E0Se0 and Hg50C400E200Se2 diets were significantly higher than the Hg500C0E0Se0 and Hg500C400E200Se2 diets. However, there were no significant differences in liver COX activities in mice fed the Hg0C0E0Se0, Hg0C400E200Se2, Hg50C0E0Se0 and Hg50C400E200Se2 diets. Dietary Hg had significant effect on the treatment groups based on two-way ANOVA; however, there were no significant effects of antioxidants or interaction effect on the liver tissue of mice fed the experimental diets.

In case of kidney tissue, the COX enzyme activities in mice fed the Hg0C0E0Se0 and Hg0C400E200Se2 diets were significantly higher than the Hg50C0E0Se0, Hg50C400E200Se2, Hg500C0E0Se0 and Hg500C400E200Se2 diets. Moreover, mice fed the Hg50C0E0Se0 and Hg50C400E200Se2 diets showed significantly higher COX activities in kidney tissue than that of Hg500C0E0Se0 and Hg500C400E200Se2 diets. However, there were no significant differences between Hg0C0E0Se0 and Hg0C400E200Se2 diets, between Hg50C0E0Se0 and Hg50C400E200Se2 diets as well as between Hg500C0E0Se0 and Hg500C400E200Se2 diets in terms of COX activities of kidney tissue. The two-way ANOVA showed that dietary Hg had significant effect on COX enzyme activities; however, there were no significant effect of antioxidants or the interaction effect of dietary Hg and antioxidants related to COX enzyme activities in mice fed the experimental diets.

3.5. Survivability in mice

Cumulative survival rate of male ICR mice based on Kaplan-Meier survival curve during the 30 days of feeding trial has shown in Figure 3. The first mortality of mice was observed on day-6 in the cage of mice fed the Hg500C0E0Se0 diet and further became more evident on the following days in the cages of mice fed the Hg supplemented diets. The results showed that mice fed the Hg0C0E0Se0 and Hg0C400E200Se2 diets secured 100% survival rate than the other treatment groups. However, mice fed the Hg500C0E0Se0 diet showed highest mortality (80% of mortality) at the end of the experiment. At the end of 30 days of feeding trial, mice

357 fed the Hg0C0E0Se0 and Hg0C400E200Se2 diets had significantly higher cumulative survival
358 rates than those of mice fed the Hg50C0E0Se0, Hg50C400E200Se2, Hg500C0E0Se0 and
359 Hg500C400E200Se2 diets.
360 The two-way ANOVA showed that both the Hg and antioxidants had significant effects
361 based on K-M survival curve; however, no interaction effect was observed between dietary Hg
362 and antioxidants.
363 **4. Discussion**
364 In the present study, male ICR mice fed the diets containing organic mercury in the form of
365 methylmercury chloride (CH_3HgCl) added in the lyophilized olive flounder fish muscle with
366 or without supplementation of dietary antioxidants such as selenium (Se), vitamin C and
367 vitamin E in different doses were evaluated. In this experiment, the mercury and the multi-
368 antioxidant levels were selected based on the previous research findings in rodent and fish
369 models (Beyrouty and Chan, 2006; Bourdineaud et al., 2008; Bolkent et al., 2008; Folven et
370 al., 2009; Muthu and Krishnamoorthy, 2012; Park et al., 2016). The results revealed that dietary
371 organic mercury had profound effect on the ICR mice in terms of mercury toxicity based on
372 tissue mercury bioaccumulations as well as survivability of mice. Likewise, Beyrouty and Chan
373 (2006) reported the higher level of MeHg bioaccumulations in different tissues and higher
374 mortality of rats in a dietary experiment. Dietary analyses of the present study showed that Hg
375 contents in the fish muscle seemed to be very low compared to the basal or negative control
376 diet which may be attributed to the higher Hg contents in the standard chow from commercial
377 source. In consistent with the present study, Weiss et al. (2005) reported that a significant
378 amount of total Hg can be detected in the standard laboratory chow for animals which may
379 directly affect the results of the experimental findings. Furthermore, the researchers opined that
380 dietary ingredient like fish meal especially from tuna scraps could be the additional source of
381 Hg in the standard chow for rats which might be reflected in the present study by means of

382 elevated Hg levels in the experimental diets compared to the suppelemental levels. In this
383 study, we observed that the highest level of Hg bioaccumulated in tissues of mice, in decreasing
384 order, kidney > liver > muscle > blood. The results agree with our recent study in marine fish
385 model for organic mercury bioaccumulation (Raihan et al., 2020) as well as other researchers
386 who reported in mice fed the mercury contaminated fish flesh (Bourdineaud et al., 2008). In
387 the present study, dietary supplementation of multi-antioxidants could not reduce the Hg
388 concentrations in blood, liver, kidney and muscle tissues of mice may be due to the high levels
389 of Hg in the experimental mice feeds. In accordance of our findings, Ganther (1978) reported
390 that dietary antioxidants such as Se and vitamin E had no protective effect in terms of reducing
391 tissue mercury concentrations rather they can prevent the tissue oxidative damage in animals
392 which strongly supported the results of the present study. Moreover, Se could increase the
393 uptake of Hg and dietary Hg cannot be decreased by Se at the same time when Se is exerting a
394 protective effect in rat brain (Prohaska and Ganther, 1977). Likewise, Penglase et al. (2014)
395 postulated that dietary supplementation of Se could not enhance the reproductive performance
396 and it can negatively affect the fish reproductive potential as well as the effect on reproduction
397 that may enhance in the presence of elevated Hg. Beyrouty and Chan (2006) and Folven et al.
398 (2009) opined that there were no effects of dietary Se and vitamin E in reducing Hg
399 concentrations of brain, liver and kidney tissues of rodent model on induced mercury toxicity
400 which agrees with the results of the present study. However, in our previous findings, we
401 reported that dietary antioxidants had positive effects in reducing organic or inorganic mercury
402 contents in marine fish model (Lee et al., 2016; Park et al., 2016; Moniruzzaman et al., 2017
403 (a) (b); Lee et al., 2017). Furthermore, some researchers reported that dietary Se, vitamin C
404 and vitamin E could reduce the Hg burden in the tissue levels of rodent model (Rao et al., 2001;
405 Muthu and Krishnamoorthy, 2012; Glaser et al., 2013). Bolkent et al. (2008) shown that
406 antioxidants such as Se, vitamin C and Vitamin E partly prevent the toxicity of cadmium in

407 gastric fundus tissue of male Sprague-Dawley rats based on histopathological,
408 immunohistochemical and biochemical analyses.

409 Mercury is known to increase the cellular level of reactive oxygen species (ROS) and
410 induce oxidative stress which lead to the generation of superoxide radicals and enhancement
411 of lipid peroxidation levels (Agarwal et al., 2010). Redox homeostasis depends on the
412 antioxidant defense system that eliminate a wide range of oxidants, including ROS, lipid
413 peroxides, and metals (Oyewole and Birch-Machin, 2015). Antioxidants help to protect animal
414 from hazardous effect of ROS produced in the body. It have been reported that because of
415 having the antioxidant properties of Se, vitamin C and vitamin E, they can protect from the
416 ROS in animal due to the heavy metal toxicity (Ganther, 1978; Rao et al., 2001; NRC, 2005;
417 Bolkent et al., 2008; Agarwal et al., 2010; Glaser et al., 2013). In the present study, the results
418 showed that due to the supplementation of Se, vitamin C and vitamin E in the mice feed the
419 antioxidant enzyme such as serum superoxide dismutase (SOD) activity was unaltered even
420 though Hg was present in the corresponding diets. In agreement with our study, Agarwal et al.
421 (2010) reported that there was no significant difference in SOD levels of kidney tissue in terms
422 of vitamin E and Hg interaction in mice recieved gavage supplementation of vitamin E and Hg.

423 In the present study, we found that dietary Se, vitamin C and vitamin E had significant
424 effect on the reduction of lipid peroxidation in terms of serum TBARS levels. Likewise, we
425 reported previously that dietary Se, vitamin C and vitamin E could reduce the lipid peroxidation
426 in tissue levels of olive flounder fish (Park et al., 2016). Marin-Guzman et al. (2000) postulated
427 that Se and vitamin E both have protective role in peroxidative damage in animals and dietary
428 co-admisnistration of these antioxidants can act synergitiscally (Kolodziej and Jacyno, 2005).
429 The results of the present study revealed that dietary Hg and antioxidants both had significant
430 effect on serum TBARS activities. Furthermore, TBARS levels in the Hg containing diets were
431 significantly reduced by dietary antioxidants supplementations. In agreement with our study,

El-Demerdash (2001) postulated that oral doses of Se could reduce the Hg toxicity in terms of decreasing TBARS activities in brain and liver of male rats. However, in contrast to the present study, Perottoni et al. (2004) reported that Hg can increase the TBARS level in kidney but organoselenium compounds did not prevent the such effect in terms of Hg toxicity in male Wistar rats.

Mitochondria as the subcellular organelles are present in the cytoplasm of eukaryotic cells which play an important role in cellular energy generation by means of adenosine tri-phosphate (ATP) production. Albeit, mitochondria are the main source of ROS production (Oyewole and Birch-Machin, 2015). Mitochondrial ROS can be detrimental or beneficial based on the amount, duration, and location of their production (Onukwufor et al., 2019). In electron transport chain, SOD enzyme as the mitochondria-specific antioxidant can detoxify oxygen ion (O_2^-) by the formation of H_2O_2 which finally converted into water (H_2O). If the process hampered, then a highly reactive hydroxyl radical (OH) can form from the H_2O_2 which may react with the metal ions (Oyewole and Birch-Machin, 2015). Cytochrome c oxidase (COX) as the last enzyme of the mitochondrial respiratory chain, is the major oxygen consumer enzyme in the cell (Bourens et al., 2013). It has been reported that impaired COX could resulted the high deposition of ROS and oxidative stress in cell levels (Douiev et al., 2018). In the present study, we found that mice fed the Hg containing diets had profound effect on the liver and kidney tissues in terms of COX activities in male ICR mice. The concentrations of COX enzyme activities were significantly decreased in both tissues as the Hg contents increased in the diets. However, interestingly, we could not find any significant differences in individual group of diets supplemented with or without antioxidants. The possible mechanism may be due to the higher production of ROS by mitochondria in the respective tissue levels that of mice fed the Hg containing diets got oxidative stress during the experiemental period. In agreement with the present study, Bourdineaud et al. (2008) reported that Hg had significant deacreasing

457 effect on the COX activities in naïve male mice of the C57B1/6 Jico fed the mercury-
458 contaminated fish flesh for one month exposure period.

459 In the present study, male ICR mice fed the Hg contaminated fish with commercial
460 diet showed significantly higher mortality rate compared to the control diet which contained
461 baseline level of Hg at 50.7 μg.kg^{-1} diet (no Hg and no antioxidant supplemented diet) as well
462 as the diet supplemented with antioxidants which contained baseline level of Hg at 53.5 μg.kg^{-}
463 ^{1} diet (no supplementation of Hg). The 100% cumulative survival rates were achieved in mice
464 fed the control and the diet supplemented with Se, vitamin C and vitamin E only. These may
465 attributed to the presence of selenium in fish meal of the diets as well as in the dietary inclusion
466 of marine fish, olive flounder fish muscle in the present study. However, a slight increase in
467 supplemention of Hg in the diet (Hg50C0E0Se0 diet that contained 111.8 μg.kg^{-1} diet) caused
468 more than 40% of mice die might be due the presence of MeHg in molar excess of Se in the
469 diet which is in agreement with Ralston et al. (2019). Interestingly, the first mortality was
470 observed in mice fed the low Hg with antioxidant supplemented diet (Hg50C400E200Se2 diet)
471 on day 15; whereas, in case of high Hg with antioxidant supplemented diet
472 (Hg500C400E200Se2 diet) fed mice showed rapid onset of mortality (day 9). Here, in this
473 study, the results demostrated that antioxidant supplemented diets prolonged the survivability
474 of mice. Noteworthy, in the present study, we used selenomethionine as the source of biological
475 form of selenium in the diets which have high antagonistic behavior with organic MeHg
476 compared to inorganic selenium (Moniruzzaman et al., 2017b) that may enhanced the
477 survivability of mice due to the higher level of selenium supplementation in the diets (2 mg.kg^{-}
478 ^{1}). In agreement with our study, Beyrouty and Chan (2006) reported that co-consumption of
479 MeHg, Se and vitamin E can significantly increase the survivability of offspring of female
480 Sprague-Dawley rats. Likewise, in our previous experiment on marine fish model, we
481 demonstrated the synergistic positive effect of dietary vitamin E and selenomethionine on

induced Hg toxicity on the survival rate of the fish (Moniruzzaman et al., 2017b). However, some researchers reported a little or no death and higher concentrations of Hg in tissues of rodents supplied with high levels of Hg compared to the present study (Newland and Rasmussen, 2000; Bourdineaud et al., 2008; Fujimura et al., 2009; Ferrer et al., 2021). The results may be contradicted due to the use of different strain of rodent, different research methodology as well as different feeding regimen in the present study.

5. Conclusions

From the findings of the present study, taken together, it can be corroborated that dietary supplementation of antioxidants such as Se, vitamin C and vitamin E had no effect on Hg bioaccumulation in male ICR mice; however, antioxidants had partial effect in terms of reducing serum lipid peroxidation and pronounced effect on the cumulative survival rate in mice fed the Hg intoxicated diets. Importantly, the present study contradicted with our previous findings in marine fish model where we postulated that dietary supplementation of Se, vitamin C and vitamin E had protective role on Hg toxicity in olive flounder fish. Based on the present and the previous investigations (Lee et al., 2016; Park et al., 2017; Moniruzzaman et al., 2017a; Moniruzzaman et al., 2017b; Lee et al., 2017; Raihan et al., 2020), we may assume that marine fish like olive flounder had more effective metabolic system than male ICR mice in reducing Hg toxicity on the supplementation of dietary antioxidants. However, the present study further warranted the interaction effect of dietary Hg with different antioxidants based on molecular mechanisms.

CRediT authorship contribution statement

Mohammad Moniruzzaman: Investigation, Laboratory analyses, Data analysis, Writing - original draft. **Seunghan Lee:** Investigation, Laboratory analyses, Data analysis. **Youngjin Park:** Investigation. **Taesun Min:** Writing - review & editing. **Sungchul C. Bai:** Supervision, Funding acquisition, Resources and Approval of final draft.

Declaration of competing interest

The authors declare that they have no competing interests.

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

Ingredients	Diets					
	Hg0C0E0Se0	Hg0C400E200Se2	Hg50C0E0Se0	Hg50C400E200Se2	Hg500C0E0Se0	Hg500C400E200Se2
Mouse[1] feed	86.2	86.2	86.2	86.2	86.2	86.2
Flounder[2] fish muscle powder (Hg 5 ppm)	0	0	1.0	1.0	10.0	10.0
Flounder[2] fish muscle powder (Hg 0 ppm)	10.0	10.0	9.0	9.0	0	0
Vitamin C[3] (50,000 ppm)	0	0.8	0	0.8	0	0.8
Vitamin E[4] (20,000 ppm)	0	1.0	0	1.0	0	1.0
Selenium[5] (100 ppm)	0	2.0	0	2.0	0	2.0
Cellulose	3.8	0	3.8	0	3.8	0

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715 [1]Purina Korea Inc., Seongnam-si, Gyeonggi-do, Republic of Korea [2]Laboratory made dried
716 flounder muscle powder [3]Hoffman La Roche, Basel, Switzerland [4]Sigma Aldrich, St. Louis,
717 MO, USA [5]United States Biochemical, Cleveland, Ohio, USA.

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Table 2. Tissue total mercury contents (μg.g^{-1} of wet matter basis) in male ICR mice fed the experimental diets for 30 days.

Diets[1]	Muscle	Liver	Kidney	Blood
Individual treatment means				
Hg0C0E0Se0	0.0477±0.01^{c}	0.0739±0.01^{c}	0.3199±0.12^{c}	0.0071±0.00^{c}
Hg0C400E200Se2	0.0538±0.01^{c}	0.0736±0.01^{c}	0.4289±0.14^{c}	0.0076±0.00^{c}
Hg50C0E0Se0	0.2074±0.04^{b}	0.3560±0.08^{b}	2.6224±0.64^{b}	0.0469±0.02^{b}
Hg50C400E200Se2	0.1981±0.05^{b}	0.4165±0.12^{b}	3.3368±0.81^{b}	0.0633±0.01^{b}
Hg500C0E0Se0	0.4132±0.02^{a}	0.9226±0.07^{a}	5.7005±0.31^{a}	0.1453±0.02^{a}
Hg500C400E200Se2	0.4125±0.09^{a}	0.9861±0.03^{a}	5.7969±1.20^{a}	0.1493±0.03^{a}
Means of main effect				
Hg0	0.0507^{c}	0.0737^{c}	0.3744^{c}	0.0073^{c}
Hg50	0.2027^{b}	0.3862^{b}	2.9796^{b}	0.0550^{b}
Hg500	0.4128^{a}	0.9547^{a}	5.7484^{a}	0.1473^{a}
C0E0Se0	0.2227^{a}	0.4508^{a}	2.8809^{a}	0.0664^{a}
C400E200Se2	0.2214^{a}	0.4920^{a}	3.1875^{a}	0.0733^{a}
Two-way analysis of variance (ANOVA): *P*-value				
Hg effect	<0.0001	<0.0001	<0.0001	<0.0001
Antioxidants effect	0.9629	0.3372	0.4524	0.5483
Hg × Antioxidants	0.9729	0.7682	0.7613	0.8290

^{a,b,c} Values are means ± SD from triplicate groups of mice (n=3) where the values within a column without a common superscript differ ($P < 0.05$).

[1]Hg0C0E0Se0, Hg50C0E0Se0 and Hg500C0E0Se0 designate 0, 50, 500 μg.kg^{-1} Hg without multi-antioxidants supplements; Hg0C400E200Se2, Hg50C400E200Se2 and Hg500C400E200Se2 designate 0, 50, 500 μg.kg^{-1} Hg with selenium (2mg.kg^{-1}), vitamin C (400 mg.kg^{-1}) and vitamin E (200 mg.kg^{-1}) supplements.

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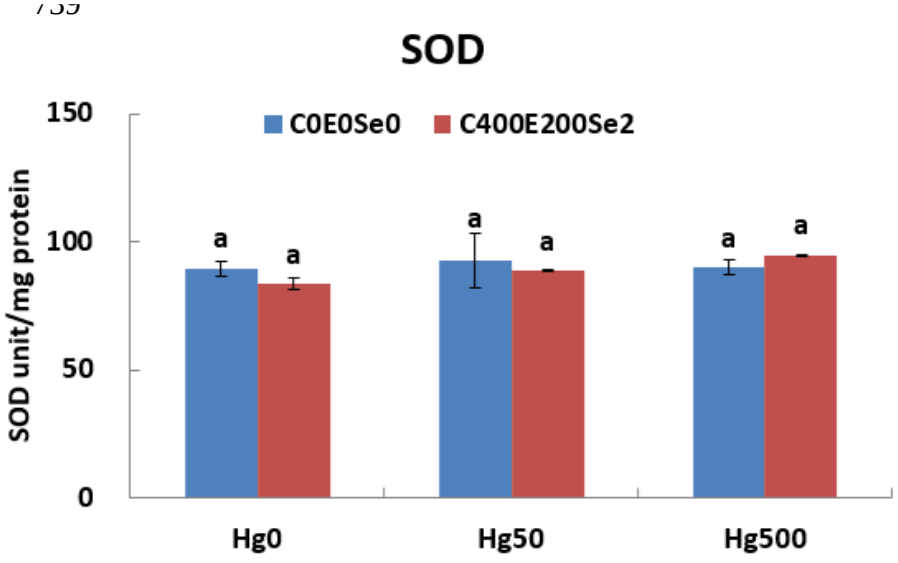
(A)



2-way ANOVA
Hg:
P = 0.2635
Antioxidants:
P = 0.5757
Interaction
P = 0.2900

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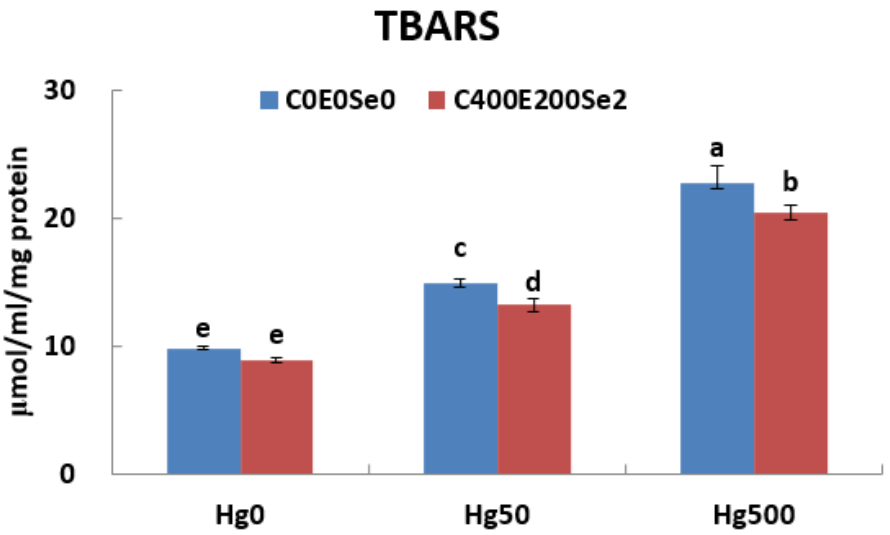
(B)



2-way ANOVA
Hg:
P = 0.0001
Antioxidants:
P = 0.0041
Interaction
P = 0.3751

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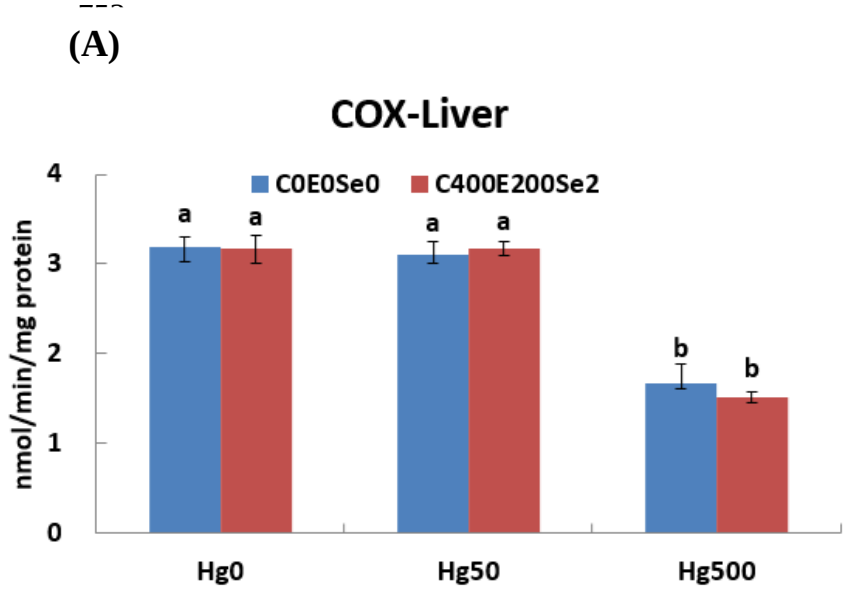
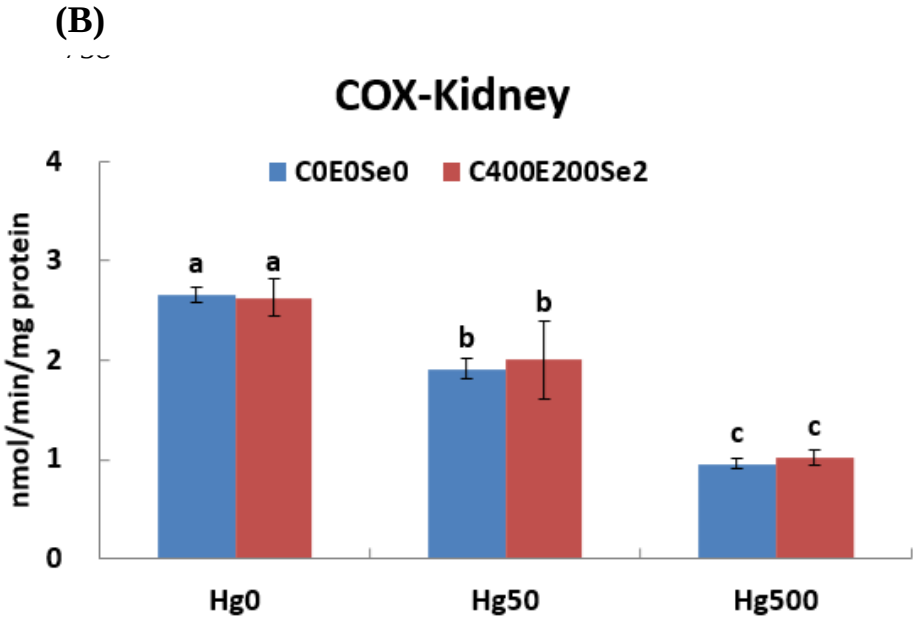
746 **Figure 1.** Blood serum superoxide dismutase (SOD) activities (A) and thiobarbituric acid
747 reactive substances (TBARS) activities (B) in male ICR mice after 30 days. [a,b,c,d,e] Values
748 are means ± SD from triplicate groups of mice (n=3) where different letters are
749 significantly different ($P < 0.05$). Effects of Hg and the combined effect of antioxidants
750 (selenium, vitamin C and vitamin E) and their interaction effects were determined by 2-
751 way ANOVA.



2-way ANOVA
Hg:
P = 0.0001
Antioxidants:
P = 0.6861
Interaction
P = 0.5652



2-way ANOVA
Hg:
P = 0.0001
Antioxidants:
P = 0.6948
Interaction
P = 0.9016

Figure 2. Cytochrome *c* oxidase (COX) enzyme activities in liver (A) and kidney (B) tissues in male ICR mice after 30 days.[a,b,c] Values are means ± SD from triplicate groups of mice (n=3) where different letters are significantly different ($P < 0.05$). Effects of Hg and the combined effect of antioxidants (selenium, vitamin C and vitamin E) and their interaction effects were determined by 2-way ANOVA.

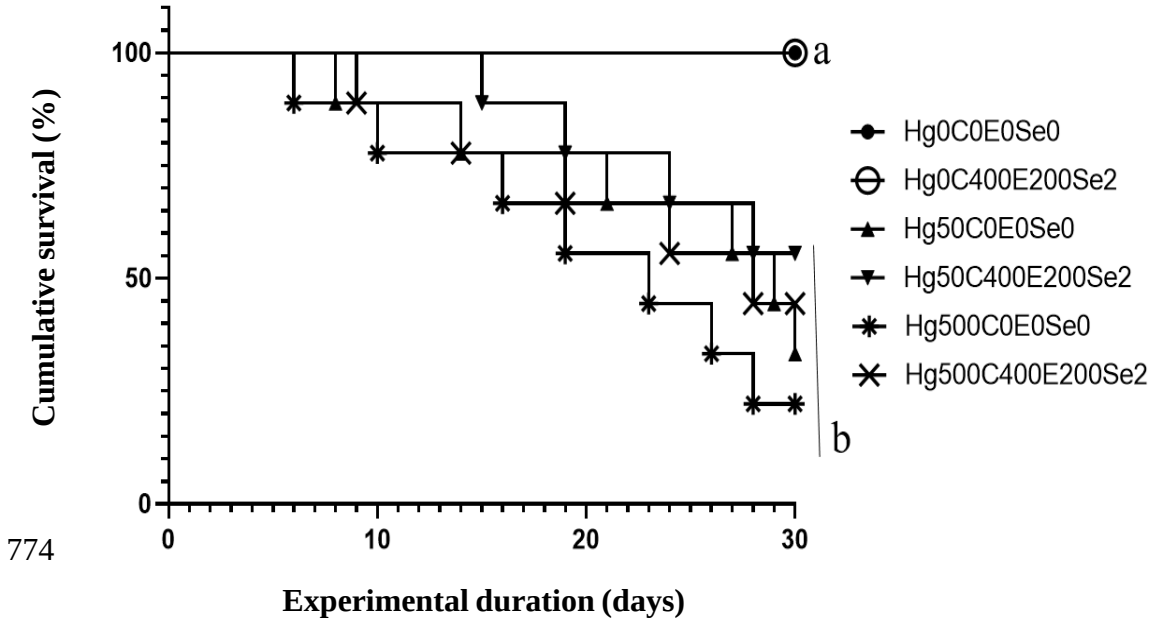


Figure 3. Cumulative survival rate (initial number of mice – final number of mice × 100/initial number of mice) of male ICR mice based on Kaplan-Meier (K-M) survival curve during the 30 days experimental period. Each value represents mean of 3 cages of each treatment (n=3). Different letters on survival data (a,b) are significantly ($P < 0.05$) different by Tukey's HSD test. Two-way ANOVA showed the following *P*-values: Hg, $P = 0.001$; antioxidants, $P = 0.039$; Interaction, $P = 0.300$.