Thesis for the Degree of Master of Science

Toxic effects of dietary mercury on the growth performance, hematological parameters, antioxidant and immune responses in starry flounder, *Platichthys stellatus*

by

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강도다리, *Platichthys stellatus*의 성장률, 혈액학적 성상, 항산화 및 면역반응에 미치는 수은의 독성 효과

Advisor: Prof. Ju-Chan Kang

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요약

수은 노출에 따른 생체축적, 혈액학적 성상, 항산화 및 면역반응의 변화를 확인하기 위해, 강도다리(평균 전장 14.2±0.4cm, 평균 체중 43.9±4.4g)를 먹이를 통해 수은 0, 4, 8, 12, 16 mg/kg의 농도로 4주간 노출시키고 이후 6주까지는 컨트롤 사료만을 급이 하여 회복을 관찰하였다. 수은의 조직 내 생체축적은 장, 간, 신장, 아가미, 근육을 관찰하였고 고농도 구간에서 유의적으로 축적되는 경향을 보였으며 장에서 가장 많이 축적되었다.

성장 요인으로는 BWG, FER을 측정하였으며 4주차 12mg/kg 이상의 구간에서는 2주차에 비해 유의성을 띄지 않았으나 회복기간에는 모든 구간에서 유의적으로 증가하였다. 또한 혈액학적 성상인 RBC count, Hematocrit, Hemoglobin은 12 Hg mg/kg 이상에서 유의적으로 감소하는 경향을 보였고 혈장무기성분인 Calcium과 Magnesium은 고농도 구간에서 감소하는 경향을 보였다. 혈장유기성분인 Glucose는 수은의 농도에 시간에 따라 증가하는 경향을 보였고 Total protein은 감소하는 경향을 보였으며 효소성분인 GOT와 GPT는 증가하는 경향을 보였다.

항산화효소인 SOD, CAT, GST는 간, 아가미, 신장을 분석하였으며 수은 함유 사료 급이에 따라 증가하는 경향을 보였다. 비효소 항산화제인 GSH도 간, 아가미, 신장을 분석하였으며 수은 함유 사료 급이에 따라 증가하는 경향을 보였다. 비특이적 면역인자인 Lysozyme activity은 신장, 혈장을 분석하였고 고농

도 구간에서 증가하는 경향을 보였다. .전체적으로 수은 노출이 강도다리의 여러 조직에 생체축적을 유발하였고 성장률을 감소시키고 혈액학적 성상도 변화시켰으며 산화스트레스로 인한 항산화효소, 면역학적 변화를 유발한 것으로 보이고 이후에도 계속 유해한 영향을 미친 것으로 보인다.

I. Introduction

Heavy metals are well known to be toxicity in aquatic animals. Mercury, one of the heavy metals, has become widely distributed through the biosphere due to the artificial activities caused by industrial processes and has increased steadily since industrialization began, and some mercury deposits originate from natural sources, but many are derived from artificial activity (Kim et al., 2012). Mercury has ability to biological binding with the food chain, mercury often poses an ecological hazard to aquatic life (Elia et al., 2003). When fish are exposed to mercury, cell homeostasis may be disrupted, DNA damage, and physiological activity may be adversely affected (Keum et al., 2008). In aquatic environments, mercury is found in elemental, organic or inorganic compounds. Organic mercury is the most toxic form, but inorganic mercury is the most emitted form of aquatic environment by industry, which has a greater impact on fish tissue (Monteiro et al., 2010).

The bioaccumulation of mercury has a negative effect on fish health such as fish's metabolic activity, reproduction, and growth. Mercury is accumulated in organs such as the intestine, gills, and liver, and it affects various defense mechanisms causing various stress and deterioration of physiological activity (CHI et al., 2007). Due to stress, the osmotic pressure of fish changes, the concentration

of energy sources such as glucose and fatty acids changes, and the activity of various enzymes increases or decreases and immunity decreases (Hwang et al., 2016).

When mercury enters the body of fish, gill or liver damage can occur, which can lead to abnormalities in hematological parameters. Typically, red blood cell counts, hematocrit, and hemoglobin levels are affected, and in the liver detoxification process, the organic components glucose and total protein levels may be affected (Witeska, 2005). In addition, the levels of inorganic components such as calcium and magnesium may also be affected, causing problems with ion control functions in the blood (Sawsan et al., 2017). When liver is damaged, the enzymes GOT and GPT that affect metabolic activity, which can also be an important indicator of mercury toxicity (Kim et al., 2019).

Heavy metals, such as mercury, cause oxidative stress on fish to generate free radicals (ROS). Free radicals are oxygen compounds produced during metabolism of cells in the body, when excessively generated, they attack normal cells and tissues. (Monteiro et al., 2013). Fish have a defense mechanism to prevent damage from oxidative stress, such as superoxide dismutase (SOD) and catalase (CAT). Superoxide dismutase (SOD) converts superoxide to hydrogen peroxide(H_2O_2) or general oxygen(O_2), while catalase (CAT) decomposes hydrogen peroxide into water and oxygen (Žikić et al, 1996). And other antioxidant enzymes include GST (Glutathione S-transferase), GR (Glutathione reductase), and GPx (Glutathione

peroxidase). GSH (Glutathione) turns into GSSG(Glutathione disulfide), which breaks the disulfide bonds of surrounding proteins and reduces them to cysteine. It is GPx that oxidizes GSH to GSSG, and GR that returns GSSG to GSH (Martínez-Álvarez et al., 2005).

Lysozyme is one of the important immune elements in fish and is active against both Gram-positive and Gram-negative bacteria and performs immune functions (Dalmo et al., 1997). Lysozyme is mainly found in neutrophils, monocytes, and small amounts of macrophages, and it acts to break down the β(1 → 4) bond between N-acetylmuramic acid and N-acetylglucosamine in the cell wall of Gram-positive bacteria, and also assists phagocytosis (Saurabh & Sahoo, 2008). Heavy metals, such as mercury, enter the fish's body, inhibiting the immune system. At this time, lysozyme is activated and serves to prevent it (Kong et al., 2012). Therefore, the activity of lysozyme is an important factor in immunologically examining the effects of toxic substances on fish.

Starry flounder, *Platichthys stellatus* has recently been increasing its aquaculture production in Korea due to its characteristics such as disease resistance and euryhaline. Fish are highly exposed to toxic substances through food intake and gills, especially through food intake. It can also accumulate in humans through the food chain. Therefore, the purpose of this study was to investigate the bioaccumulation, hematological parameters, antioxidants and immune responses of *P.stellatus* by exposure to mercury through food.

II. Materials and methods

1. Experimental fish and culture conditions

Starry flounder, *Platichthys stellatus* were obtained from a local farm in Gijang, Korea. Before the experiment, it was acclimated for one week under the experimental conditions, and feeds were 1% of body weight twice a day and changed water daily. The seawater components used in the experiment are expressed in Table 1. After acclimation, the selected fishes were raised in five 250L circular tanks and 15 in each section. Feeds were provided twice a day for one week up to 1% of the weight of feed prepared for each concentration until 4 weeks, and mercury-free feed was paid until 6 weeks. The mercury concentrations were 0, 4, 8, 12, 16 mg/kg.

Table 1. The chemical components of seawater and experimental condition used in the experiments.

Item	Value
Temperature (℃)	13 ± 1.0
pH	8.11 ± 0.4
Salinity (‰)	32.75 ± 0.6
Dissolved Oxygen (mg/L)	7.21 ± 0.31
Chemical Oxygen Demand (mg/L)	1.19 ± 0.85
Ammonia (μg/L)	11.4 ± 1.0
Nitrite (μg/L)	1.2 ± 0.5
Nitrate (μg/L)	11.21 ± 1.04

2. Feed ingredients and diets formulation

Formulation of the diets in shown in Table 2. Mercury was obtained from Sigma Chemical Co., Ltd. All diets contained white fish meal 55%, casein 15%, dextrin 20%, fish oil 3%, soybean oil 3%, carboxymethylcellulose 1%, vitamin premix 0.5%, mineral premix 0.5%, coline salt 0.5%. Mercury premix was made up of 1 g mercury with 99 g cellulose. Five diets were formulated with supplementation of different dietary mercury concentrations of 0, 4, 8, 12, and 16 mg/kg diet. All ingredients were blended thoroughly. At last, water was added into the mixture to produce stiff dough. Then the dough was pelleted by experimental feed mill, and dried for 24 h at room temperature. After processing, all the diets were packed and kept -20 ℃ until use.

Table. 2. Formulation of the experimental diet (% dry matter)

Ingerdient	concentration (%)				
	0	4	8	12	16
White fish meal[1]	55	55	55	55	55
Casein[2]	15	15	15	15	15
Dextrin[3]	20	20	20	20	20
Fish oil[4]	3	3	3	3	3
Soybean oil[5]	3	3	3	3	3
Carboxymethylcellulose[6]	1	1	1	1	1
α-Cellulose[7]	1.5	1.496	1.492	1.488	1.484
Vitamin premix[8]	0.5	0.5	0.5	0.5	0.5
Mineral premix[9]	0.5	0.5	0.5	0.5	0.5
Coline salt[10]	0.5	0.5	0.5	0.5	0.5
Mercury[11]	0	0.004	0.008	0.012	0.016
Total	100	100	100	100	100

1. Dajeon Co., Ltd., Pusan, Korea
2. The Feed Co., Ltd., Pusan, Korea
3. TS CO,. Ltd., Incheon, Korea
4. Sigma Chemical Co., St. Louis, MO
5, 6, 7. Sigma, USA
8. dl-calcium pantothenate, 368; Choline chloride, 10; Inositol, 400; Menadione, 1800; Nicotivamide, 1030; Pyridoxine·HCl, 88; Riboflavin, 380; Thiamine mononitrate, 115; dl-a-tocopherol acetate, 210; Retinyl acetate, 38; Biotin, 10; Folic acid, 20; Cyanocobalamin, 1.3; Cholecalcifero, 13.2
9. Ferrous Fumarate, 12.5; Dried Ferrous sulfate, 20; Manganese Sulfate, 11.25; Dried Cupric Sulfate. 1.25; Cobaltous sulfate, 0.75, Zinc sulfate, 13.75; Calcium iodate, 0.75, Magnesium Sulfate, 80.2; Aluminum Hydroxide, 0.75
10. Kofavet Co., Ltd., Ulsan, Korea
11. 10.000 mg Hg/kg diet

3. Bioaccumulation

The intestine, liver, kidney, gill and muscle tissues of the experimental fish *P. stellatus* were sampled and freeze-dried. After that, it was crushed and 0.1 g of each sample was used for analysis. Measurement was carried out with a mercury analyzer (MA-3000, Nippon Instruments Corporation, Tokyo, Japan) using atomic absorption spectrometry. Accuracy was verified using TORT-3 (NRC-Canada) as the CRM (Certified Reference Materials), and the mercury content in the organ was expressed as μg/g dry wt.

4. Growth Performance

Body weight gain (BWG) and feed efficiency ratio (FER) of fish were measured at 2, 4 and 6 weeks, respectively. Calculation methods are as follows.

BWG(%) = 100 * (Final weight - Initial weight) / (Initial weight)

FER(%) = 100 * (Increase in biomass of fish) / (Feed intake)

5. Hematological analysis

Blood was collected from the caudal vein of fish using a disposable 1ml syringe, and RBC (red blood cell) count, Hb (hemoglobin), and Ht (hematocrit) were immediately analyzed. The RBC count was calculated by diluting 400 times with Hendrick's solution, counting with an optical microscope using a hemocytometer (Improved Neubauer, Germany), and multiplying the dilution factor. The Hb concentration was measured by the Cyan-methemoglobin method using a clinical kit (Asan Pharm. Co., Ltd.). The Ht value was measured by putting blood into a Micro-hematocrit capillary tube and centrifuging at 12,000 rpm for 5 minutes at 4 ℃ with Micro-hematocrit centrifugation (Model; 01501, HAWKSLEY AND SONS Ltd, England). And it was measured through a reading plate (Micro-Hematocrit reader, HAWKSLEY AND SONS Ltd., England).

The blood samples were centrifuged at 3000g, 4 ℃ for 5 minutes to separate the plasma. The plasma samples were used to analyze inorganic components, organic components, and enzyme components. Calcium and magnesium as inorganic components were analyzed by OCPC (o-cresolphthalein-complexon) method and Xylidyl blue method, respectively. Glucose and total protein as organic components were analyzed by GOD/POD method and Biuret method, respectively. Glutamic oxalate transaminase (GOT) and glutamic pyruvate transaminase (GPT) analysis for enzyme activity were both analyzed by the Reitman Frankel method.

6. Antioxidant system analysis

The liver, gill and kidney were collected for analysis, and the tissues were washed with a washing buffer (0.1 M KCl, pH 7.4). After washing, the tissues were put in a ratio of 1:10 to the weight of the homogenizing buffer (0.1 M KCl, pH 7.4), and then homogenized using a Teflon-glass homogenizer (099CK4424, Glass-Col, Germany). The homogeneous solution was centrifuged for 30 minutes at 4 ℃ and 10000 g, and the supernatants were separated and stored at -80 ℃. To measure the protein content, it was measured using a Bio-rad protein assay kit (Bio-rad laboratories GmbH, Munich, Germany) using the Bradford (1976) method.

6-1. Superoxide dismutase activity (SOD)

Superoxide dismutase activity (SOD) was analyzed using the SOD Assay Kit-WST (Dojindo Molecular Technologies, Inc.). The solutions used in the experiment are WST-1 working soultion, enzyme working solution, sample solution, and dilution buffer. There were added to each sample according to the manual. Thereafter, these were incubated at 37 ℃ for 20 minutes and absorbance was measured at 450 nm using a spectrophotometer. One unit of SOD activity was the amount of enzyme at the point where 50% of the reduction reaction between WST-1 and superoxide anion was inhibited. SOD activity

was expressed as unit/mg protein.

WST - 1 = 2 - (4-iodophenyl) - 3 - (4-nitrophenyl) - 5 - (2,4-disulfo-phenyl) - 2H - tetrazolium, monosodium salt

6-2. Catalase activity (CAT)

Catalase activity(CAT) was analyzed using the OxiSelect^{TM} CAT Assay Kit (Cell Biolabs. Inc.). The quinonimine dye coupling product, which is related to the amount of hydrogen peroxide remaining in the reaction mixture, was measured for absorbance at 520 nm using a spectrophotometer. One unit of CAT was expressed as the amount of enzyme that decomposes 1 μm of hydrogen peroxide per minute at 25 ℃, and CAT activity was expressed as unit/mg protein.

6-3. Glutathione (GSH)

Glutathione (GSH) was analyzed using the method of Beutler et al. (1963). After adding 20 µl of sample to 180 µL of D.W, 300 µL of precipitating solution (0.167 g metaphosphoric acid, 0.02 g EDTA and 3 g NaCl in 10 mL distilled water) was mixed with the diluted supernatant sample. Then, $NaHPO_4$ solution and 0.5 mL DTNB (5,5`-dithiobis-2-nitrobenzoic acid) were sequentially added to 40 µL of this mixture, and the absorbance was measured at 412 nm using a

spectrophotometer. GSH level was expressed as nmol GSH/mg protein.

6-4. Glutatione-S-transferase (GST)

Glutathione-S-transferase (GST) activity was analyzed using the method of modified Habig et al. (1974). 100 μl 0.2 M phosphate buffer (pH 6.5), 20 μl 10 mM GSH (Sigma-Aldrich, Inc.), 20 μl 10 mM 1-chloro-2, -dinitrobenzene, and CDNB (Sigma-Aldrich, Inc.) were sequentially mixed with a 20μl sample. The mixture was measured for 5 minutes at 30 second intervals at an absorbance of 340 nm using a spectrophotometer, and the section with the largest change was used. GST activity was expressed as nmol/min/mg protein.

7. Lysozyme activity

Lysozyme activity was analyzed using the Lysozyme Detection Kit (Sigma, USA). This assay to detect lysozyme activity uses *Micrococcus lysodeikticus* cells as the substrate. The plasma samples and *Micrococcus lysodeikticus* cells were mixed and then mixed with 66 mM potassium phosphate buffer (pH 6.24). And the resulting mixture was measured for absorbance at 450 nm using a spectrophotometer, and the result value was expressed in μg/ml using a standard curve.

8. Statistical analysis

Statistical significance of the experimental analysis results was indicated using the SPSS/PC+ statistical package (SPSS Inc., Chicago, IL, USA). One-way ANOVA and Duncan's multiple range test were used to identify significantly different values between groups. The significance level was determined at $P < 0.05$.

III. Results

1. Bioaccumulation

Mercury accumulations in intestine, liver, kidney, gill and muscle of starry flounder fed the varying concentration of mercury diet are shown in Fig. 1 ~ Fig 5. The largest accumulation of mercury was in intestine. Bioaccumulation of intestine was a significant increase at over 8 mg Hg/kg at all weeks. In the kidney and liver, mercury accumulation significantly increased over 4mg Hg/kg at all weeks. Bioaccumulation of gill was significant increased at over 4 mg Hg/kg at 2, 4 weeks and over 8 mg Hg/kg at 6 week. The mercury accumulation in the muscle was significantly increased at 16 mg Hg/kg at all weeks.

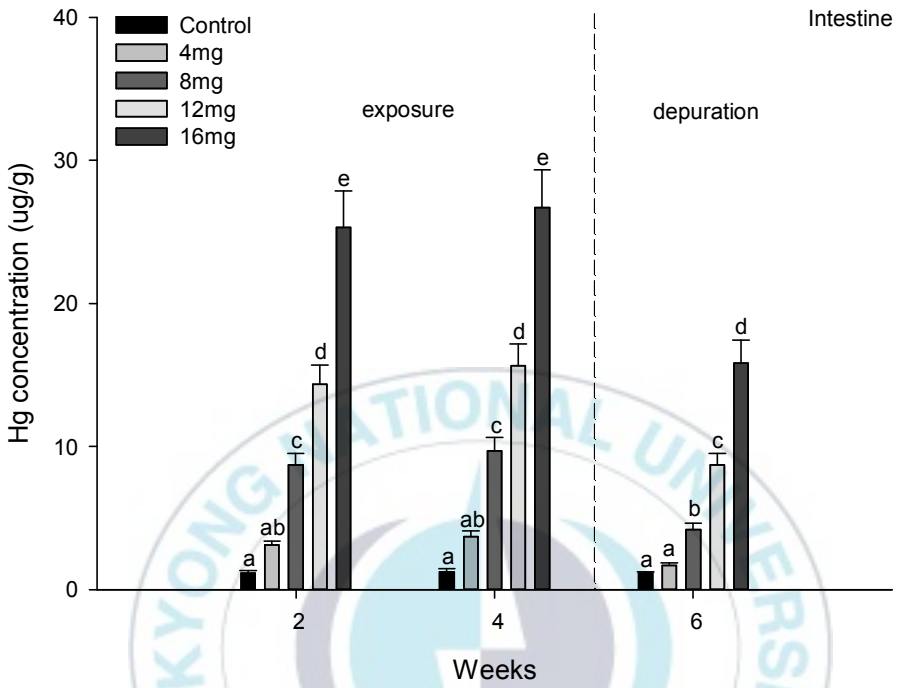

Fig. 1. Mercury accumulation in intestine of starry flounder, *Plyatichthys stellatus* fed diets containing different levels of mercury for 2, 4 and 6 weeks. Values are mean ± S.D. Vertical bar denotes a standard error. Values with a different superscript are significantly different from others at 2, 4 and 6 weeks ($P < 0.05$) as determined by Duncan's multiple range test.

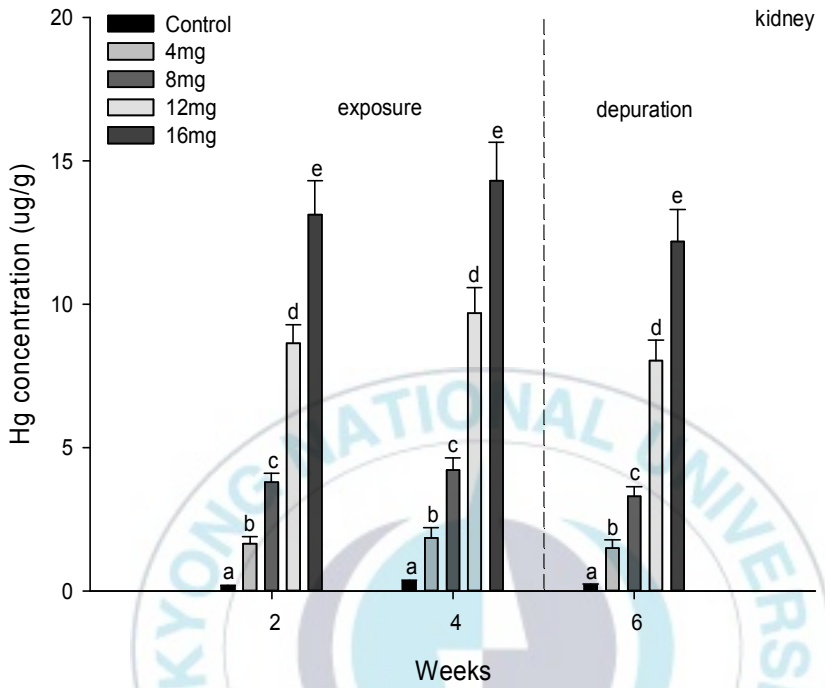

Fig. 2. Mercury accumulation in kidney of starry flounder, *Plyatichthys stellatus* fed diets containing different levels of mercury for 2, 4 and 6 weeks. Values are mean ± S.D. Vertical bar denotes a standard error. Values with a different superscript are significantly different from others at 2, 4 and 6 weeks ($P < 0.05$) as determined by Duncan's multiple range test.

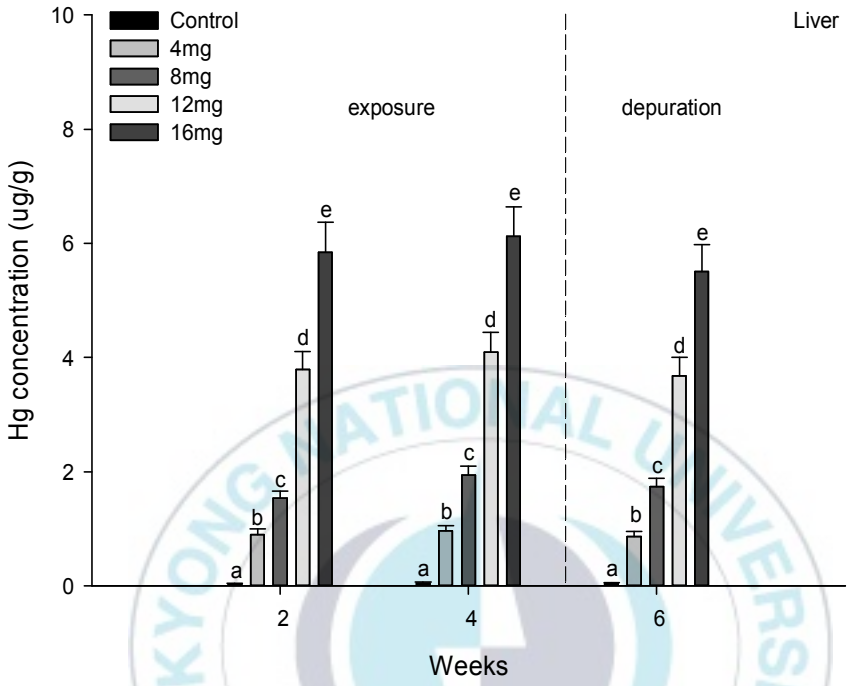

Fig. 3. Mercury accumulation in liver of starry flounder, *Plyatichthys stellatus* fed diets containing different levels of mercury for 2, 4 and 6 weeks. Values are mean ± S.D. Vertical bar denotes a standard error. Values with a different superscript are significantly different from others at 2, 4 and 6 weeks ($P < 0.05$) as determined by Duncan's multiple range test.

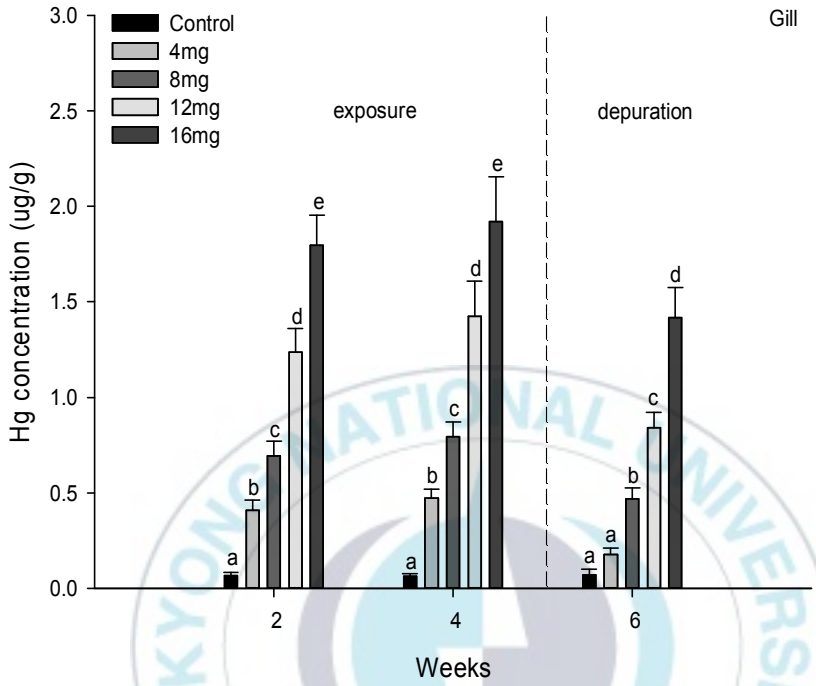

Fig. 4. Mercury accumulation in gill of starry flounder, *Plyatichthys stellatus* fed diets containing different levels of mercury for 2, 4 and 6 weeks. Values are mean ± S.D. Vertical bar denotes a standard error. Values with a different superscript are significantly different from others at 2, 4 and 6 weeks ($P < 0.05$) as determined by Duncan's multiple range test.

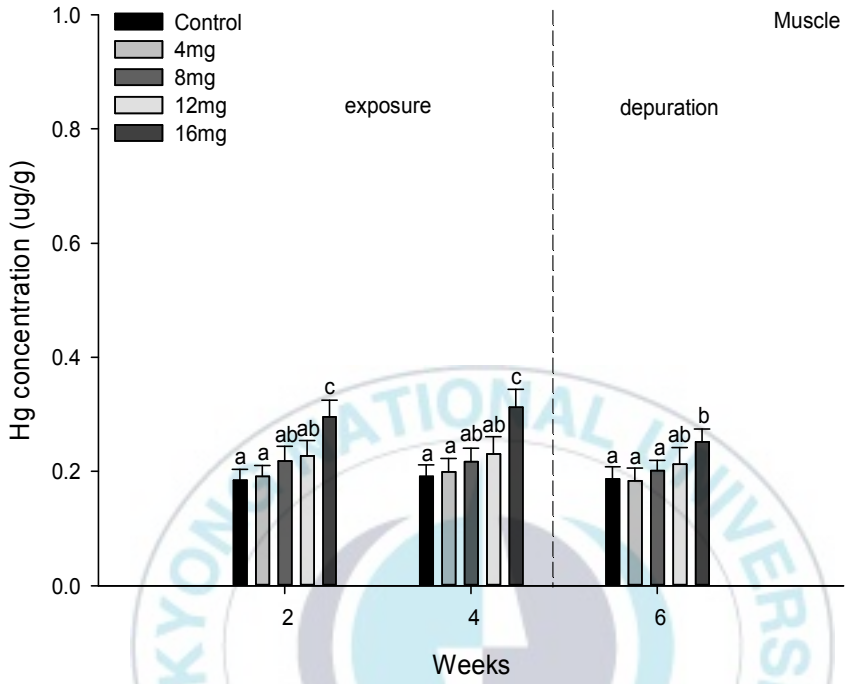

Fig. 5. Mercury accumulation in muscle of starry flounder, *Plyatichthys stellatus* fed diets containing different levels of mercury for 2, 4 and 6 weeks. Values are mean ± S.D. Vertical bar denotes a standard error. Values with a different superscript are significantly different from others at 2, 4 and 6 weeks ($P < 0.05$) as determined by Duncan's multiple range test.

2. Growth performance

The growth performance of Starry flounder according to mercury feed is shown in Fig. 6 ~ 8. Mercury dietary had negative effect on growth performance such as BWG and FER.. BWG and FER was significantly increased at control, 4, and 8 mg Hg/kg at 2 and 4 weeks, and significantly increased at all sections during the depuration period.

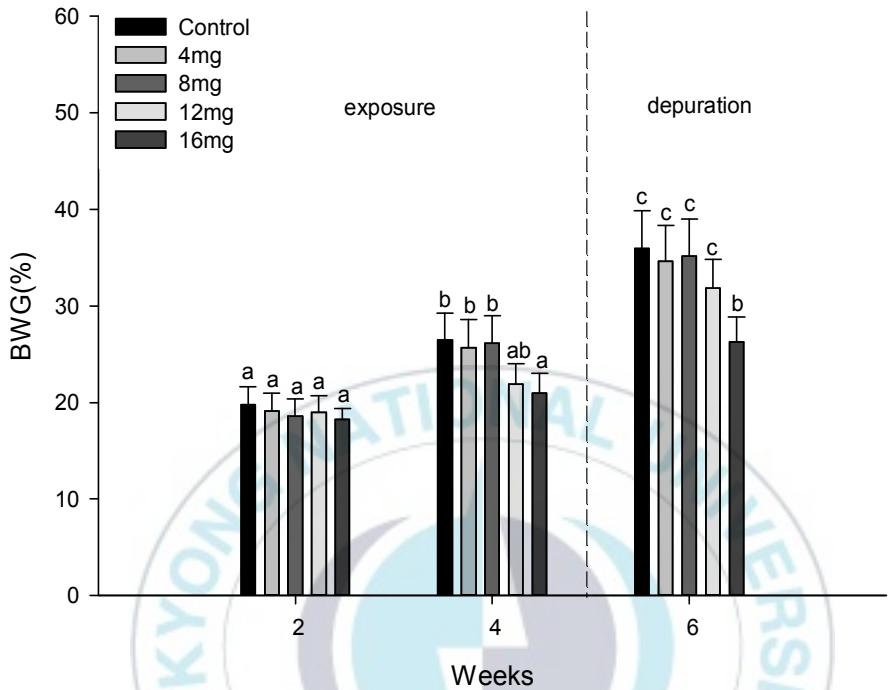

Fig. 6. BWG of starry flounder, *Plyatichthys stellatus* fed diets containing different levels of mercury for 2, 4 and 6 weeks. Values are mean ± S.D. Vertical bar denotes a standard error. Values with a different superscript are significantly different from others at 2, 4 and 6 weeks ($P < 0.05$) as determined by Duncan's multiple range test.

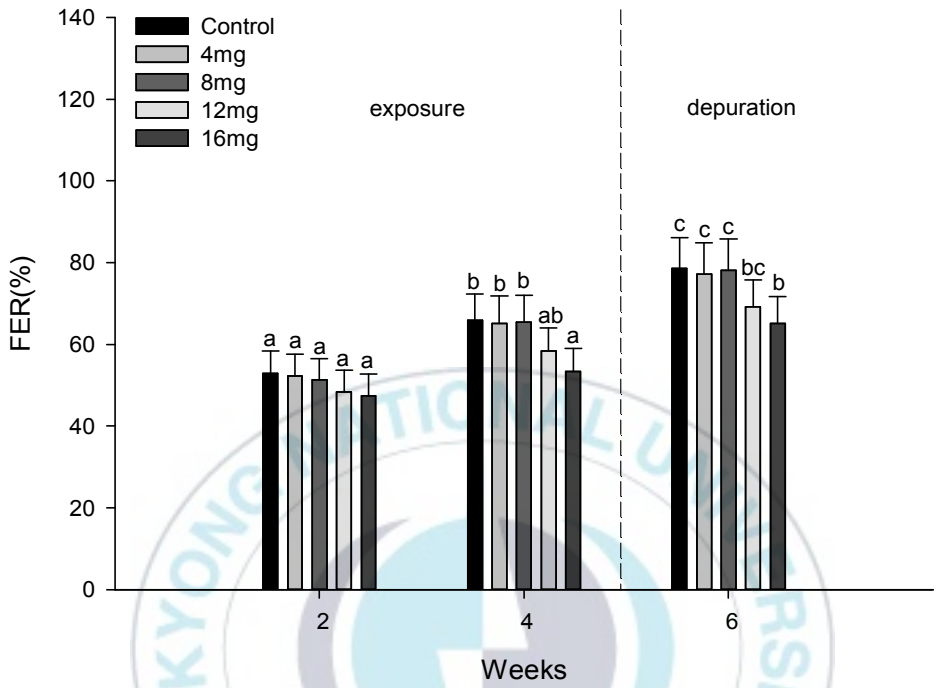

Fig. 7. FER of starry flounder, *Plyatichthys stellatus* fed diets containing different levels of mercury for 2, 4 and 6 weeks. Values are mean ± S.D. Vertical bar denotes a standard error. Values with a different superscript are significantly different from others at 2, 4 and 6 weeks ($P < 0.05$) as determined by Duncan's multiple range test.

3. Hematological parameters

The RBC count, Ht value and Hb concentration of starry flounder fed the varying concentration of mercury diet are shown in Table 3. As a result of the RBC count experiment, it was significantly decreased at over 12 mg Hg/kg in 2, 4 weeks and 16 mg Hg/kg during the depuration period. Ht value was significantly decreased at 16 mg Hg/kg at 2, 4 and 6 weeks, and Hb concentration was also equal to Ht value.

The plasma parameters of starry flounder were influenced by the mercury diet and are shown in Table 4, 5 and 6. Calcium and magnesium did not show any significance. Glucose was significantly increased at over 12 mg Hg/kg at 2 week and over 8 mg Hg/kg at 4 week. During the depuration period, it was significantly increased 16 mg Hg/kg. Total protein was significantly decreased at over 12 mg Hg/kg at 2 week and over 8 mg Hg/kg at 4 week. During the depuration period, it was significantly decreased at over 12 mg Hg/kg. Both GOT and GPT were significantly increased at over 12 mg Hg/kg at 2, 4 and 6 weeks.

Table 3. RBC count, Hematocrit, Hemoglobin in starry flounder, *Platichthys stellatus* fed diet containing different levels of inorganic mercury for 2, 4 and 6 weeks [1]

Parameters	Period (weeks)	Inorganic mercury concentration (mg/kg)				
		0	4	8	12	16
RBC count ($\times 10^4 mm^3$)	2	301.58±12.75^{a}	299.76±12.19^{a}	296.92±11.96^{a}	273.49±12.43^{b}	250.18±11.12^{c}
	4	299.55±12.70^{a}	298.31±12.71^{a}	295.68±11.64^{a}	271.64±11.81^{b}	248.64±10.89^{c}
	6	300.86±11.97^{a}	299.25±12.74^{a}	295.75±11.59^{a}	279.21±11.16^{ab}	272.43±10.09^{b}
Hematocrit (%)	2	29.75±2.44^{a}	28.59±2.40^{ab}	26.44±2.31^{ab}	21.49±2.44^{c}	21.07±1.92^{c}
	4	29.47±2.41^{a}	28.19±2.93^{ab}	26.88±2.21^{ab}	21.39±2.16^{c}	20.69±1.80^{c}
	6	29.64±2.47^{a}	28.51±2.33^{ab}	27.29±2.30^{ab}	25.44±2.23^{ab}	24.76±1.88^{bc}
Hemoglobin (g/dL)	2	6.44±0.54^{a}	6.29±0.53^{a}	5.74±0.51^{ab}	5.22±0.44^{bc}	5.09±0.49^{bc}
	4	6.31±0.53^{a}	6.04±0.52^{ab}	5.70±0.52^{ab}	5.08±0.47^{bc}	4.70±0.48^{c}
	6	6.19±0.56^{a}	6.27±0.49^{a}	5.89±0.50^{ab}	5.72±0.43^{ab}	5.13±0.47^{bc}

Values are mean±S.D. Values with a different letter are significantly different from others at 2, 4 weeks and at 6 weeks ($P<0.05$) as determined by Duncan's multiple range test.

Tabel 4. Change of Calcium, Magnesium in starry flounder, *Platichthys stellatus* fed diet containing different levels of inorganic mercury for 2, 4 and 6 weeks [1]

Parameters	Period (weeks)	Inorganic mercury concentration (mg/kg)				
		0	4	8	12	16
Calcium (mg/dL)	2	11.24±0.96^{a}	11.12±0.92^{a}	10.62±0.93^{ab}	9.75±0.88^{ab}	7.84±0.85^{c}
	4	11.31±0.95^{a}	11.01±0.93^{a}	9.94±0.98^{ab}	9.17±0.89^{bc}	7.72±0.86^{c}
	6	11.21±0.94^{a}	11.09±0.90^{a}	10.31±0.92^{ab}	9.57±0.89^{ab}	9.26±0.82^{bc}
Magnesium (mg/dL)	2	4.14±0.20^{a}	4.09±0.17^{ab}	3.85±0.19^{ab}	3.26±0.21^{c}	2.97±0.13^{c}
	4	4.15±0.21^{a}	4.02±0.24^{ab}	3.79±0.25^{ab}	3.15±0.21^{c}	2.92±0.11^{c}
	6	4.16±0.22^{a}	4.08±0.21^{ab}	3.91±0.26^{ab}	3.75±0.15^{b}	3.25±0.13^{c}

Values are mean±S.D. Values with a different letter are significantly different from others at 2, 4 weeks and at 6 weeks ($P<0.05$) as determined by Duncan's multiple range test.

Table 5. Change of glucose, total protein in starry flounder, *Platichthys stellatus* fed diet containing different levels of inorganic mercury for 2, 4 and 6 weeks [1]

Parameters	Period (weeks)	Inorganic mercury concentration (mg/kg)				
		0	4	8	12	16
Glucose (mg/dL)	2	59.28±5.10^{a}	62.65±5.50^{ab}	68.17±5.74^{ab}	71.99±5.82^{bc}	79.55±5.16^{c}
	4	60.49±4.62^{a}	64.55±5.36^{ab}	72.55±6.65^{bc}	77.32±5.62^{c}	81.96±6.51^{c}
	6	60.32±5.69^{a}	63.95±5.51^{ab}	65.48±5.97^{ab}	65.99±5.84^{ab}	73.66±6.25^{bc}
total protein (g/dL)	2	3.34±0.17^{a}	3.38±0.26^{a}	3.16±0.19^{a}	2.55±0.20^{c}	2.43±0.23^{c}
	4	3.27±0.20^{a}	3.18±0.22^{a}	2.72±0.17^{bc}	2.46±0.21^{c}	2.34±0.19^{c}
	6	3.25±0.18^{a}	3.27±0.23^{a}	3.06±0.24^{ab}	2.70±0.22^{bc}	2.64±0.16^{c}

Values are mean±S.D. Values with a different letter are significantly different from others at 2, 4 weeks and at 6 weeks ($P<0.05$) as determined by Duncan's multiple range test.

Table 6. Change of GOT, GPT in starry flounder, *Platichthys stellatus* fed diet containing different levels of inorganic mercury for 2, 4 and 6 weeks [1]

Parameters	Period (weeks)	Inorganic mercury concentration (mg/kg)				
		0	4	8	12	16
GOT (Karmen/mL)	2	23.87±2.90^{a}	24.08±2.89^{a}	26.57±2.02^{a}	32.57±2.11^{bc}	34.06±2.94^{c}
	4	23.67±2.08^{a}	24.35±2.95^{a}	28.63±2.80^{ab}	32.63±2.92^{bc}	34.83±2.89^{c}
	6	23.95±2.61^{a}	23.87±2.11^{a}	26.46±2.90^{a}	31.76±2.94^{bc}	32.14±2.42^{bc}
GPT (Karmen/mL)	2	15.60±0.97^{a}	15.92±0.82^{a}	16.66±0.88^{ab}	17.60±0.99^{b}	19.78±0.56^{c}
	4	15.70±0.91^{a}	16.05±0.67^{a}	16.85±0.73^{ab}	17.91±0.83^{b}	19.84±0.68^{c}
	6	15.88±0.82^{a}	16.11±0.65^{a}	16.46±0.64^{ab}	17.65±0.79^{b}	19.57±0.85^{c}

Values are mean±S.D. Values with a different letter are significantly different from others at 2, 4 weeks and at 6 weeks ($P<0.05$) as determined by Duncan's multiple range test.

4. Antioxidant responses

4-1. Superoxide dismutase (SOD)

SOD activity of liver, gill and kidney in starry flounder fed mercury diet were shown in Fig. 9 ~ 11. SOD activity in the liver, kidney showed a tendency to significantly increased at over 12 mg Hg/kg at 2, 4 and 6 weeks. In the gill, SOD activity showed a tendency to significantly increased at over 12 mg Hg/kg at 2, 4 weeks, but no significance was observed during the depuration period.

4-2. Catalase (CAT)

CAT activity of liver, gill and kidney in starry flounder fed mercury diet were demonstrated in Fig. 12 ~ 14. In the liver and kidney, CAT activity showed a tendency to significantly increased at over 12 mg Hg/kg at 2, 4 and 6 weeks. CAT activity of gill was observed to increase significantly at over 12 mg Hg/kg at 2, 4 weeks. On the other hand, no significance was observed during the depuration period.

4-3. Glutathione (GSH)

GSH levels of liver, gill and kidney in starry flounder fed mercury diet were shown in Fig. 18 ~ 20. GSH levels of liver was significantly increased at over 12 mg Hg/kg at 2, 4 weeks. However, during the depuration period, it increased significantly only at 16 mg Hg/kg. GSH levels of gill was significantly increased at over 12 mg Hg/kg at 2, 4 weeks. Whereas, it was no significance during the depuration period. In the kidney, a significant increase was found at over 12 mg Hg/kg at 2, 4 and 6 weeks.

4-4. Glutathione-S-transferase (GST)

GST activity of liver, gill and kidney in starry flounder fed mercury diet were shown in Fig. 15 ~ 17. In the liver, GST activity was significantly increased at over 12 mg Hg/kg at 2, 4 weeks, and during the recovery period, it increased significantly only at 16 mg Hg/kg. GST activity of gill was significantly increased at over 12 mg Hg/kg at 2 week and over 8 mg Hg/kg at 4 week, but during the depuration period, it was significantly increased only at 16 mg Hg/kg. GST activity of kidney was significantly increased at over 12 mg Hg/kg at 2, 4 and 6 weeks.

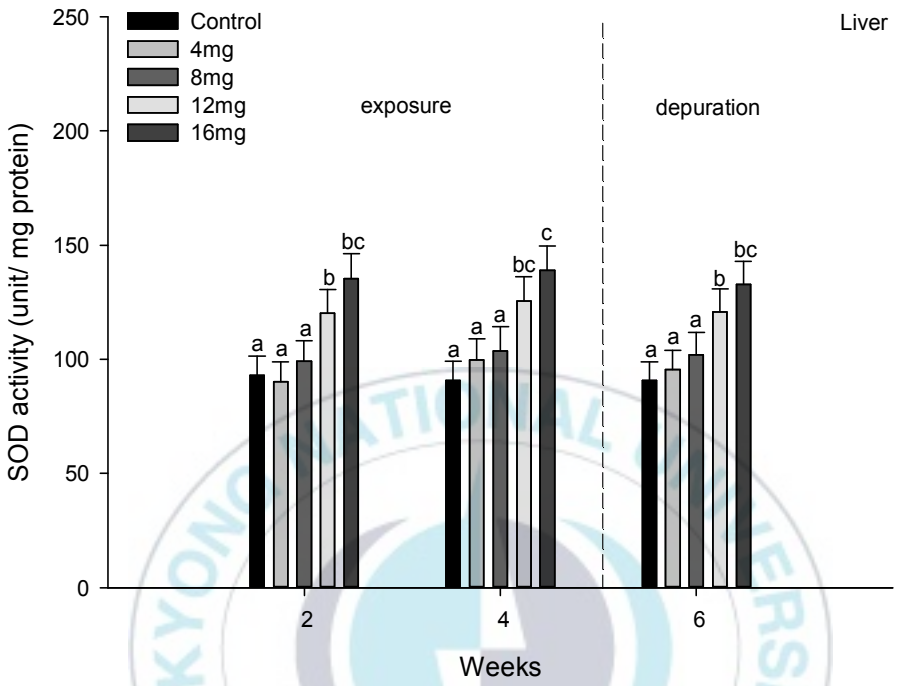

Fig. 9. SOD activity in liver of starry flounder, *Plyatichthys stellatus* fed diets containing different levels of mercury for 2, 4 and 6 weeks. Values are mean ± S.D. Vertical bar denotes a standard error. Values with a different superscript are significantly different from others at 2, 4 and 6 weeks ($P<0.05$) as determined by Duncan's multiple range test.

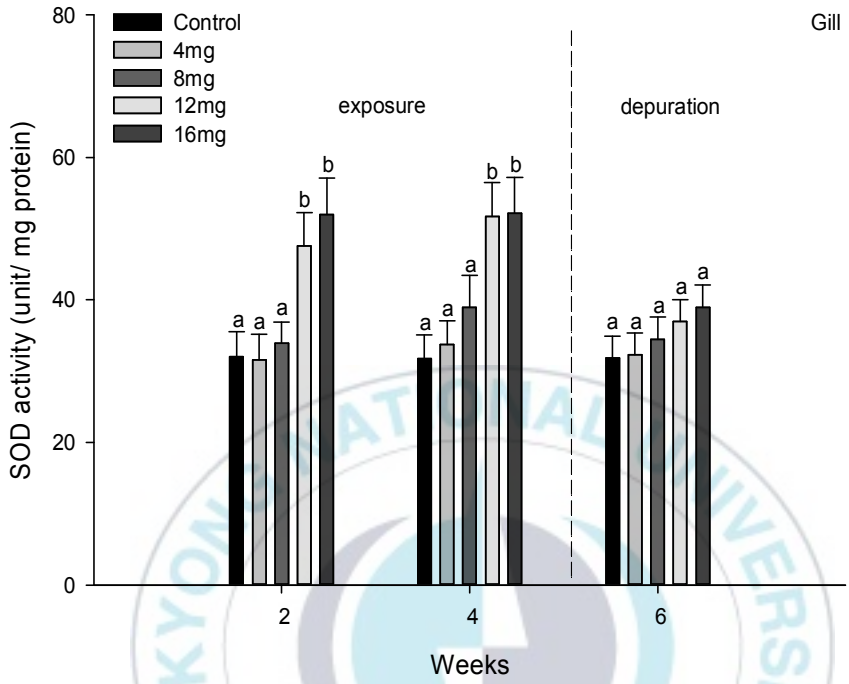

Fig. 10. SOD activity in gill of starry flounder, *Plyatichthys stellatus* fed diets containing different levels of mercury for 2, 4 and 6 weeks. Values are mean ± S.D. Vertical bar denotes a standard error. Values with a different superscript are significantly different from others at 2, 4 and 6 weeks ($P<0.05$) as determined by Duncan's multiple range test.

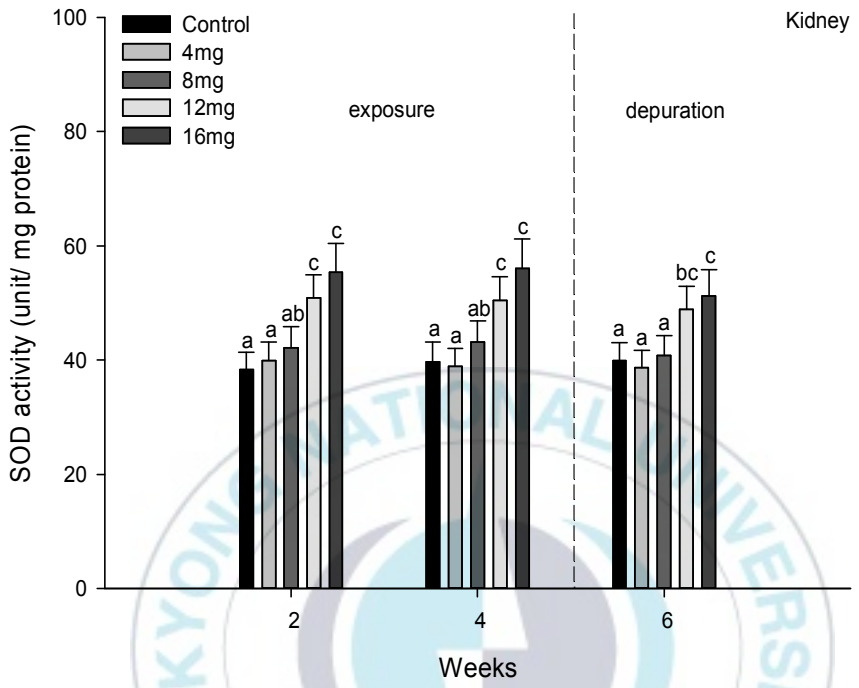

Fig. 11. SOD activity in kidney of starry flounder, *Plyatichthys stellatus* fed diets containing different levels of mercury for 2, 4 and 6 weeks. Values are mean ± S.D. Vertical bar denotes a standard error. Values with a different superscript are significantly different from others at 2, 4 and 6 weeks ($P<0.05$) as determined by Duncan's multiple range test.

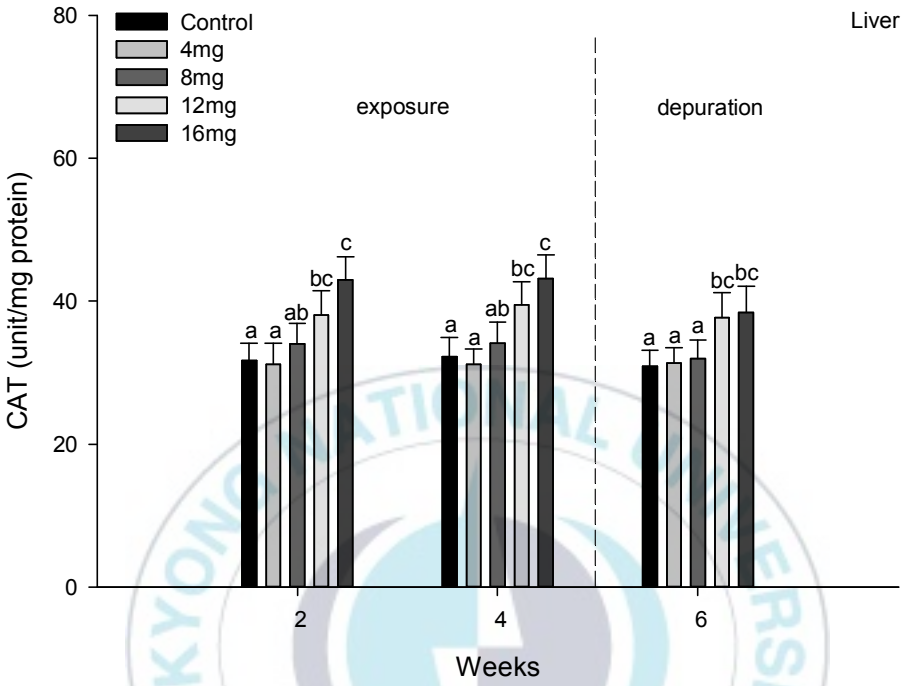

Fig. 12. CAT activity in liver of starry flounder, *Plyatichthys stellatus* fed diets containing different levels of mercury for 2, 4 and 6 weeks. Values are mean ± S.D. Vertical bar denotes a standard error. Values with a different superscript are significantly different from others at 2, 4 and 6 weeks ($P<0.05$) as determined by Duncan's multiple range test.

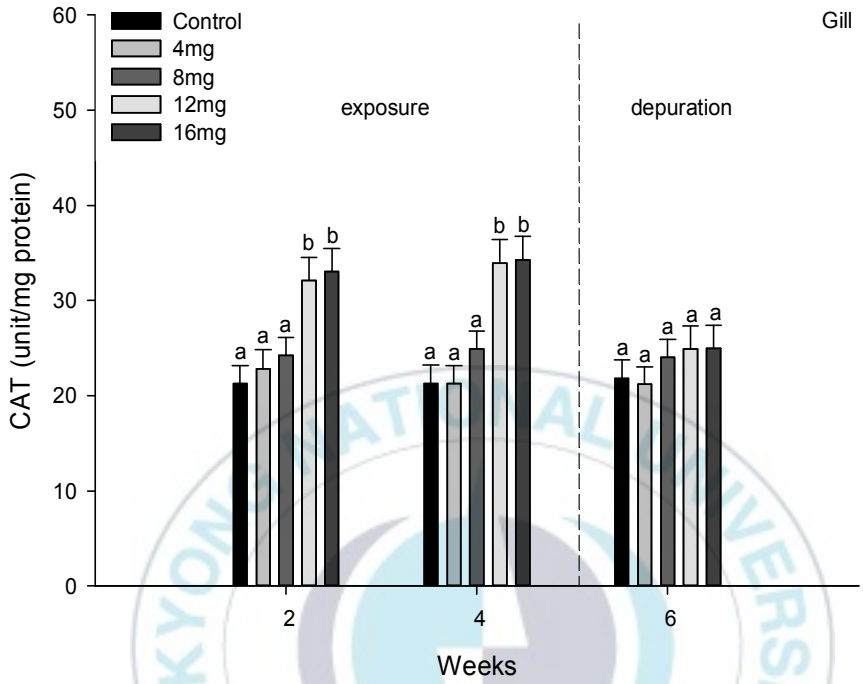

Fig. 13. CAT activity in gill of starry flounder, *Plyatichthys stellatus* fed diets containing different levels of mercury for 2, 4 and 6 weeks. Values are mean ± S.D. Vertical bar denotes a standard error. Values with a different superscript are significantly different from others at 2, 4 and 6 weeks ($P<0.05$) as determined by Duncan's multiple range test.

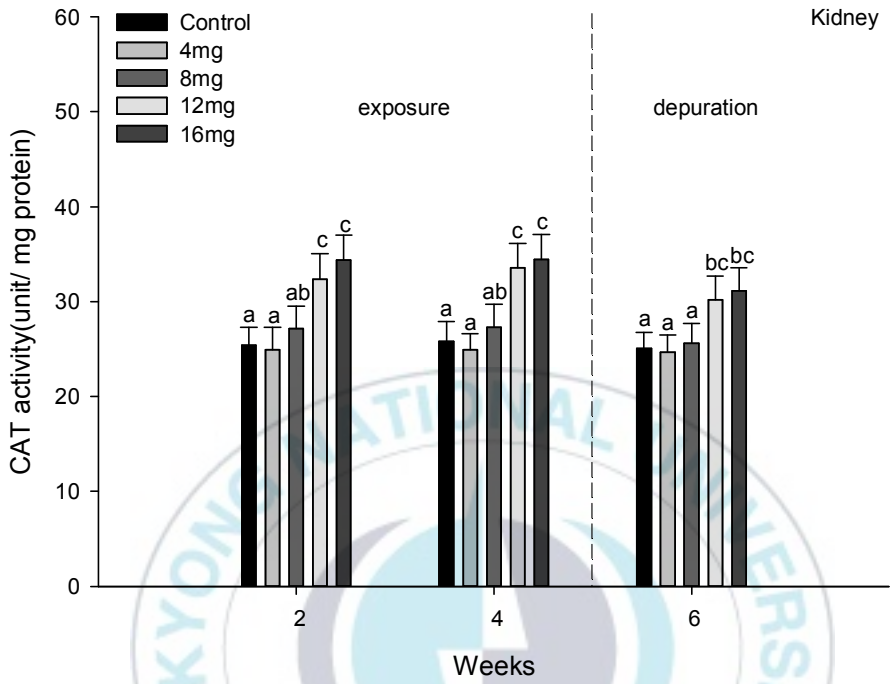

Fig. 14. CAT activity in kidney of starry flounder, *Plyatichthys stellatus* fed diets containing different levels of mercury for 2, 4 and 6 weeks. Values are mean ± S.D. Vertical bar denotes a standard error. Values with a different superscript are significantly different from others at 2, 4 and 6 weeks ($P<0.05$) as determined by Duncan's multiple range test.

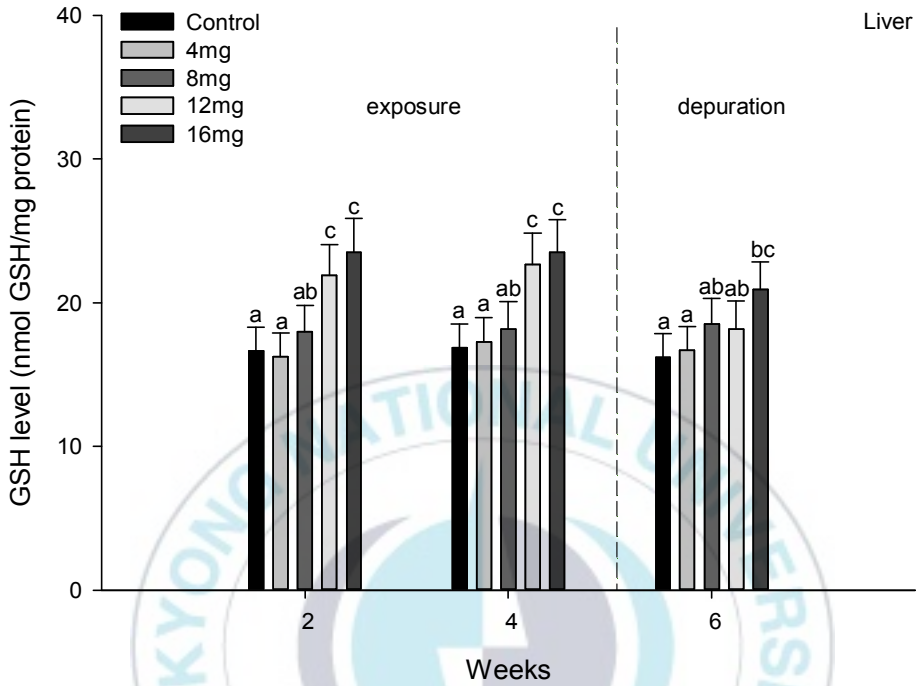

Fig. 15. GSH level in liver of starry flounder, *Plyatichthys stellatus* fed diets containing different levels of AMP for 2, 4 and 6 weeks. Values are mean ± S.D. Vertical bar denotes a standard error. Values with a different superscript are significantly different from others at 2, 4 and 6 weeks ($P<0.05$) as determined by Duncan's multiple range test.

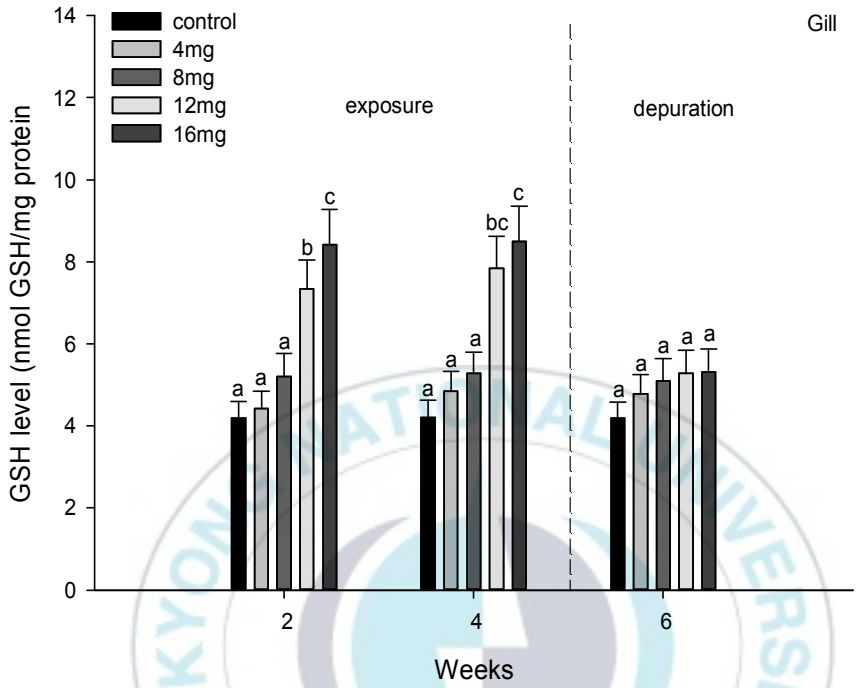

Fig. 16. GSH level in gill of starry flounder, *Plyatichthys stellatus* fed diets containing different levels of AMP for 2, 4 and 6 weeks. Values are mean ± S.D. Vertical bar denotes a standard error. Values with a different superscript are significantly different from others at 2, 4 and 6 weeks ($P<0.05$) as determined by Duncan's multiple range test.

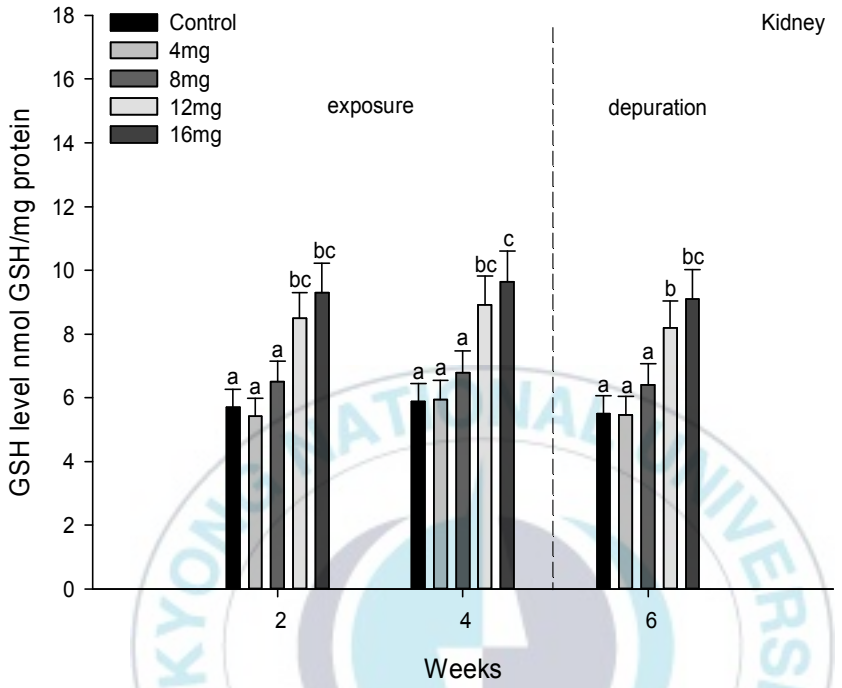

Fig. 17. GSH level in kidney of starry flounder, *Plyatichthys stellatus* fed diets containing different levels of AMP for 2, 4 and 6 weeks. Values are mean ± S.D. Vertical bar denotes a standard error. Values with a different superscript are significantly different from others at 2, 4 and 6 weeks ($P<0.05$) as determined by Duncan's multiple range test.

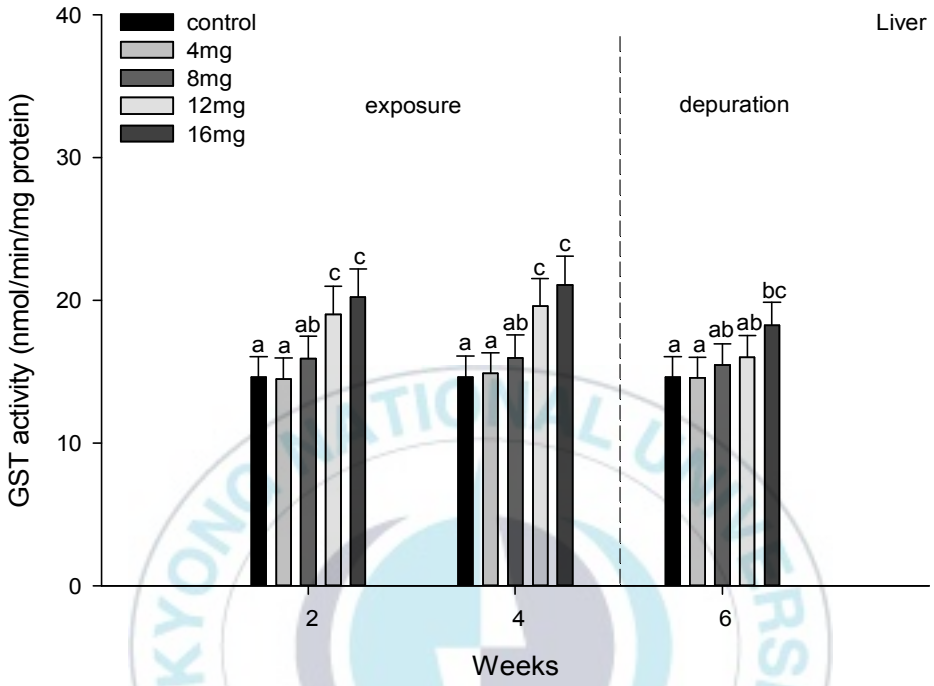

Fig. 18. GST activity in liver of starry flounder, *Plyatichthys stellatus* fed diets containing different levels of mercury for 2, 4 and 6 weeks. Values are mean ± S.D. Vertical bar denotes a standard error. Values with a different superscript are significantly different from others at 2, 4 and 6 weeks ($P<0.05$) as determined by Duncan's multiple range test.

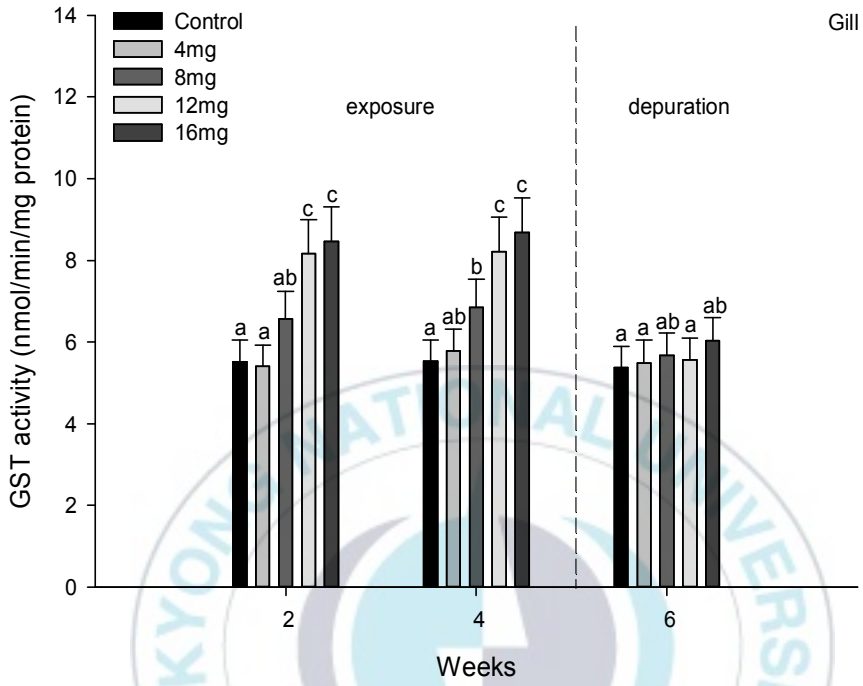

Fig. 19. GST activity in liver of starry flounder, *Plyatichthys stellatus* fed diets containing different levels of mercury for 2, 4 and 6 weeks. Values are mean ± S.D. Vertical bar denotes a standard error. Values with a different superscript are significantly different from others at 2, 4 and 6 weeks ($P<0.05$) as determined by Duncan's multiple range test.

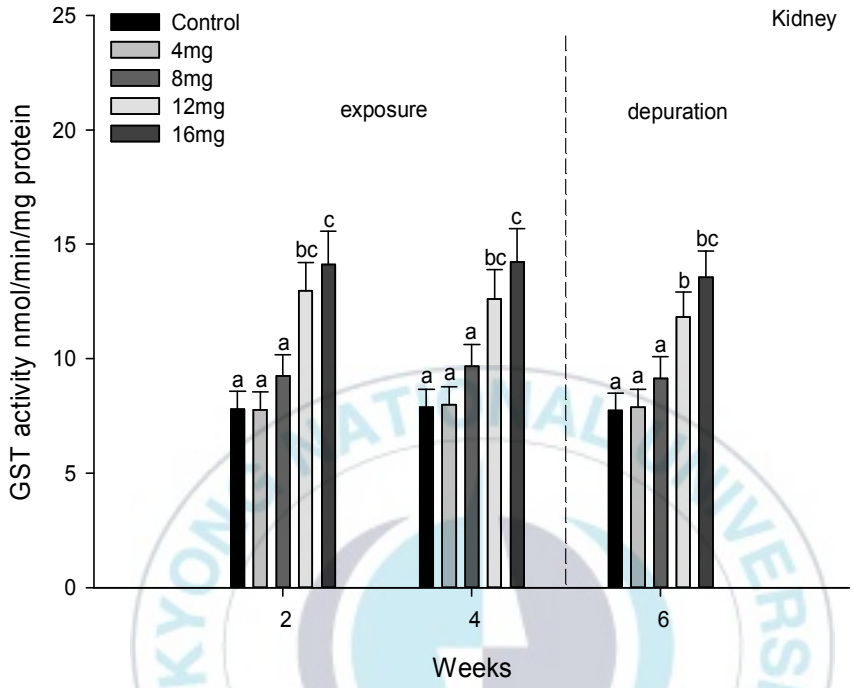

Fig. 20. GST activity in kidney of starry flounder, *Plyatichthys stellatus* fed diets containing different levels of mercury for 2, 4 and 6 weeks. Values are mean ± S.D. Vertical bar denotes a standard error. Values with a different superscript are significantly different from others at 2, 4 and 6 weeks ($P<0.05$) as determined by Duncan's multiple range test.

5. Lysozyme activity

Lysozyme activity of kidney and plasma in starry flounder fed mercury diet were shown in Fig. 21, 22. Lysozyme activity of plasma and kidney was significantly decreased at over 12 mg Hg/kg at 2, 4 weeks, and During the depuration period, it was no significant difference in plasma, but in the kidney, it was significantly decreased at over 12mg Hg/kg.

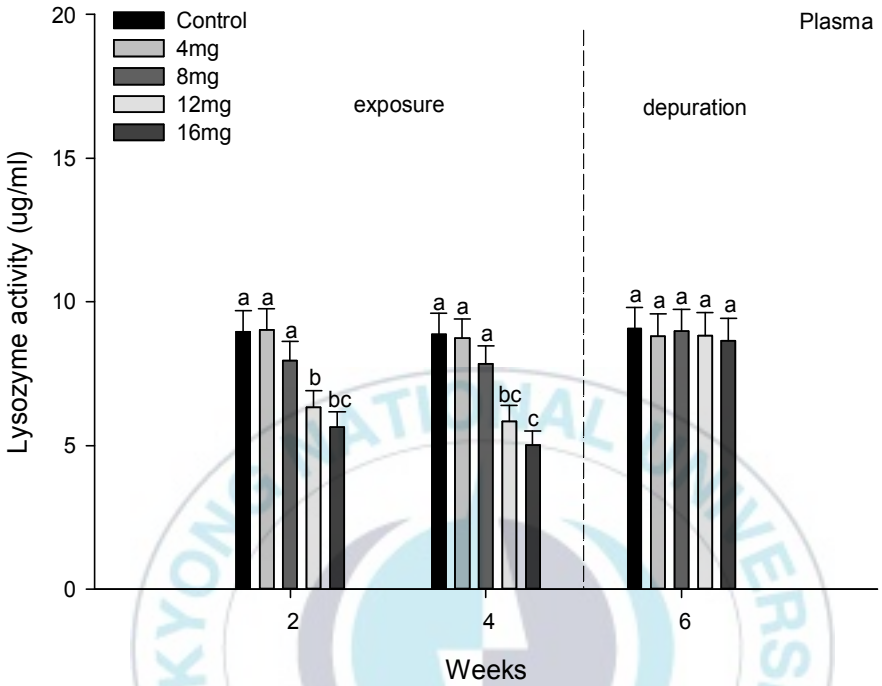

Fig. 21. Lysozyme activity in plasma of starry flounder, *Platichthys stellatus* exposed to the different concentration of dietary mercury for 6 weeks. Value with different superscript are significantly different in 2, 4 and 6 weeks ($P < 0.05$) as determined by Duncan's multiple range test.

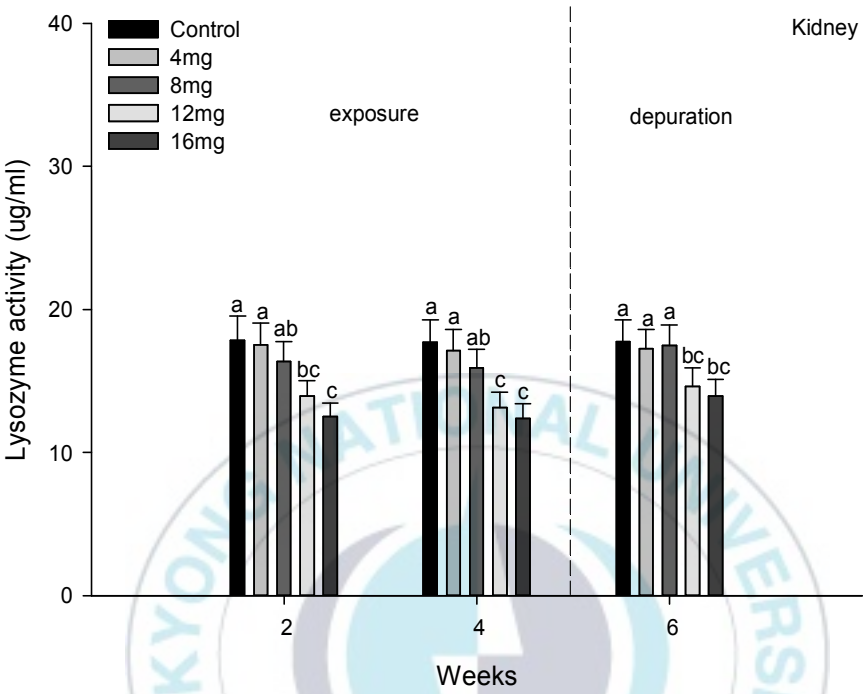

Fig. 22. Lysozyme activity in kidney of starry flounder, *Platichthys stellatus* exposed to the different concentration of dietary mercury for 6 weeks. Value with different superscript are significantly different in 2, 4 and 6 weeks ($P < 0.05$) as determined by Duncan's multiple range test.

IV. Discussion

Mercury is a toxic pollutant that raises concerns around the world, and the accumulation of mercury in the aquatic food chain can be a major problem in that it can affect humans through seafood consumption (Wang, 2012). Mercury has a more negative impact on fish growth and other areas than any other heavy metal (Wang et al., 2009). The accumulation of metals in fish is related to various factors, such as temperature and age, in addition to exposure concentration and time, and is related to absorption and removal rates in tissues (Heath, 1995).

Fish absorb several trace elements through their food and gills, and heavy metals are also absorbed through this pathway (Ciardullo et al., 2008). In this study, accumulation of heavy metals was observed in the starry flounder in the intestine, kidney, liver, gill and muscle, and the organ with the highest accumulation was the intestine. Kim et al. (2006) reported that the highest accumulation was observed in the intestines of rockfish *Sebastes schlegeli* exposed to heavy metal. In addition, significant accumulation was observed in the kidney. The kidney involved in the storage of metals, so large amounts of heavy metals accumulate (Palaniappan & Karthikeyan., 2009). Next, the liver showed the third highest accumulation. The liver is an organ primarily used to analyze damage caused by toxic substances

(Mourgaud et al., 2002). It has been reported that high accumulation was observed in the liver of zebrafish exposed to mercury (Amlund et al., 2015). Next, the accumulation of mercury was observed in gills. The gill of fish is tissue that play an important role in ion control, gas exchange and waste discharge (Kim et al., 2006) and greatly effects the accumulation of toxic substances (Kim & Kang, 2014). Mercury contained in food is absorbed into the blood by the intestine and then accumulate as it enter the epithelium of the gill (Ciardullo et al., 2008). A trace amount of mercury was also observed in the muscle. Because the muscle of fish is mainly consumed by humans, the accumulation of heavy metals in the muscle is important. In this study, it was no significant accumulation except for 16 mg Hg/kg at all weeks. Cinier et al. (1999) reported that a large amount of heavy metals were accumulated in the kidney and liver of fish, thus relatively little was accumulated in the muscle. In addition, other studies reported that it was little accumulation in the muscle of zebrafish exposed to dietary mercury (Amlund et al., 2015).

Fish generally remove heavy metals from the body through gill, mucus, bile, and urine. During the depuration period, slight recovery was observed in the intestine and gill, but there were no significant changes in the kidney, liver, and muscle. Heavy metals such as mercury have a long half-life and take a long time to be removed once accumulated (Sallsten et al., 1994). Trudel & Rasmussen. (1997) reported that the time it takes for mercury to be removed varies depending on the concentration of mercury, body size of fish, and

temperature.

Observation of the growth rate of fish is an indicator of toxicity assessment that is widely used in toxicity studies. Usually fish grows through nutrient intake and absorption. However, heavy metals can interfere with metabolic and biological activities (Quig, 1998; MacRury et al. 2002). In our study, BWG (Body weight gain) and FER (Feed efficiency ratio) were measured to observe the change in growth rate, and it was observed that the higher the mercury content, the more inhibited the growth. Berntssen et al. (2004) reported a significant reduction in the growth rate of Atlantic salmon exposed to dietary mercury above 10 mg Hg/kg. Other studies have also reported that the growth rate of fish varies depending on the concentration of mercury (Wang et al., 2009).

Hematological parameters are also widely used as indicators of toxicity assessment. This is because changes in hematological parameters may indicate the presence of stressors caused by toxic substances (Gil Barcellos et al., 2004). The influence of heavy metals causes swelling of red blood cells in fish, and the modified red blood cells cannot function properly and causes changes in hemoglobin and hematocrit levels (Maheswaran et al., 2008). Ishikawa et al (2007) reported that the hematological parameters of Nile Tilápia, *Oreochromis niloticus* significantly decreased with mercury exposure. In our study, RBC count, hemoglobin and hematocrit levels, which are the hematological parameters of starry flounder exposed to mercury, all significantly decreased. This is probably due to the effect of

mercury, causing damage to gills or red blood cells in the starry flounder, resulting in anemia.

Calcium and magnesium, which are inorganic components in plasma, are also widely used as toxicity evaluation indicators, and these are involved in ion control for maintaining homeostasis (Rogers et al., 2003). Calcium and magnesium levels can be affected by oxidative stress, and symptoms of magnesium deficiency in particular lead to growth retardation, spinal abnormalities and lipid peroxidation (Sales et al., 2014). MacDonald et al. (2002) reported that heavy metals affect the ion-exchange process of calcium and magnesium, which lowers the level. In this study, calcium and magnesium levels were significantly decreased. The reason is that heavy metals caused problems in the ion control function. (CHI et al., 2007). Organic components in plasma include glucose and total protein. In this study, the glucose level of starry flounder significantly increased according to the mercury exposure, and the total protein level significantly increased at all weeks. When heavy metals enter the body, stress can occur, and glucose can increase due to gluconeogenesis to provide energy for increased metabolic demand (Kavitha et al., 2010). Therefore, it can be seen that mercury generated stress in the body of starry flounder, and the effect of overcoming stress was achieved based on glucose changes. The change of total protein in plasma is an important parameter in assessing health and metabolism caused by toxic stress (Lavanya et al., 2011). Decreased plasma protein as a result of heavy metal accumulation can cause protein synthesis

disorders (Han et al., 2019). GOT and GPT values were confirmed as indicators of liver damage due to oxidative stress. These two enzymes are liver enzymes, and when the liver is toxic due to oxidative stress, its excretion increases and its concentration in the blood increases (Narra, 2017). In this study, when mercury was exposed to starry flounder, the values of GOT and GPT were significantly increased as the duration and concentration increased. Mercury accumulated in the liver caused toxicity, so it can be seen that the secretion of GOT and GPT were increased in the liver.

As shown in several studies, heavy metals such as mercury are known to affect many cells in the body by promoting the production of ROS (Martínez-Álvarez et al., 2005; Monteiro et al., 2010). Monteiro et al., (2013) reported that mercury exposure caused an imbalance between ROS production and antioxidant defense, resulting in oxidative stress. Fish have antioxidant enzymes, such as SOD and CAT, a means of defense to prevent excessive production of ROS (Kim et al., 2020). SOD catalyzes the reaction of converting superoxide radical (O^{2-}) to hydrogen peroxide (H_2O_2), and hydrogen peroxide (H_2O_2) is converted to water by CAT (Yu & Kang, 2020). Zhang et al. (2007) reported that SOD increased in proportion to the concentration and time of heavy metals. Hansen et al. (2007) reported an increase in CAT in the gills of *Salmo trutta* exposed to heavy metals in water, and Wei Huang et al. (2010) also reported that an increase in CAT was observed in Japanese flounder embryos and larvae exposed to mercury. GST is an enzyme that catalyzes the

bond between GSH and electrophilic xenobiotics and GSH, a non-enzymatic antioxidant, acts as a cofactor for glutathione peroxidase, which directly removes ROS or converts hydrogen peroxide into water and oxygen (Srikanth et al., 2013). GST and GSH are important in preventing oxidative damage and play a role in maintaining the redox balance of cells (Oliva et al., 2012). Marí & Cederbaum. (2001) reported that GST activity may be increased by oxidative stress, which is a defense mechanism to prevent cell damage. And Atli & Canli. (2008) reported that the GSH level of *Oreochromis niloticus* exposed to lead was significantly increased, and the reason was the defense system to protect against oxidative stress. Starry flounder increased GST and GSH acitivity when exposed to heavy metals, and this increase was due to a defense mechanism to maintain homeostasis (Park et al., 2018). Studies have shown that *Mugil cephalus* exposed to toxic substances recovered their antioxidant response after the depuration period (Ferreira et al. 2007). Boudjema et al. (2014) also reported the recovery of *Perna perna* exposed to Cd, Pb and Cu. In this study, SOD, CAT, GST and GSH were analyzed to observe these changes. Concentrations of SOD, CAT, GST, and GSH all increased significantly during the exposure period, and no significant SOD and CAT in the gills during the depurtation period. In addition, GST activity and GSH levels of gills and kidneys also did not show significance.

Lysozyme in the fish is an immune substance that can show the effect of toxic substances on non-specific immunity of fish, and it is

most measured in plasma. Lysozyme destroys the peptidoglycan layer on the cell walls of Gram-positive and some Gram-negative bacteria (Saurabh and Sahoo., 2008). Sanchez-Dardon et al. (1999) reported that the lysozyme activity of rainbow trout exposed to cadmium and zinc was significantly reduced. However, other studies have shown that the lysozyme activity of Starry Flounder exposed to lead was increased (Park et al., 2018), and the lysozyme of olive flounder affected by the toxic effect of water nitrate exposure was increased (Kim et al., 2020). Different results of lysozyme activity may be due to reasons such as fish species, exposed substances, and differences in analysis time. In this study, the lysozyme activity of Starry flounder exposed to mercury decreased and recovered to the initial level during the depuration period. Therefore, Starry flounder exposed to mercury decreased the ability to synthesize lysozyme.

In conclusion, this experiment proves that dietary Hg can exert negative effects on the growth performance (BWG and FER), hematological parameters, antioxidant responses (SDO, CAT, GST and GSH) in starry flounder. Moreover, lysozyme activity also tended to decrease due to stimulation of non-specific immunity. Considering the results of this study, exposure to mercury above 12 mg/kg appears to have a negative effect on starry flounder and accumulated mercury appears to have a negative effect during the depuration period.

V. References

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