Thesis for the Degree of Master of Science

Change of hematological parameters, antioxidant and immune responses by ascorbic acid dietary in starry flounder, *Platichthys stellatus*

by

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The Graduate School

Pukyong National University

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Ascorbic acid 급이에 따른 강도다리의 혈액학적성상, 항산화 및 면역반응의 변화

Advisor: Prof. Ju-Chan Kang

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요 약

L-ascorbic acid(AA)는 수생 생물에게 생리적 항상성 유지와 성장에 필요한 영양소이다. 또한 산화스트레스에 의한 세포손상을 보호하는 항산화제 역할을 한다. AA는 빛과 열에 약해 사료제조 및 보관 과정에서 많은 손실이 일어나므로 빛과 열에 내성이 있는 것으로 알려진 L-ascorbic acid derivatives인 L-ascorbyl-2-monophate(AMP)를 사용했다. 강도다리(*Platichthys stellatus*)에 급이한 사료의 AMP는 50, 100, 200, 400, 600, 800 mg/kg으로 설정했으며 총 4주간 실험을 했다.

성장 요인인 BWG, FER와 SGR은 AMP 400 mg/kg에서 유의적인 증가가 나타났으며 긍정적인 영향을 미쳤다. 또한 혈액학적성상인 RBC counts와 Hematocrit과 Hemoglobin 모두 AMP를 급이했을 때 유의적으로 증가했다. 혈청성분인 Calcium과 Magnesium은 AMP 600 mg/kg 이상 급이에 따라 증가했으며 Glucose, Total protein, GOT, GPT 모두 AMP가 증가함에 따라 유의적인 감소가 나타났다.

항산화 효소인 SOD, CAT, GST는 간, 아가미, 신장, 비장 조직을 분석하였으며 AMP 급이함에 따라 감소하는 경향이 나타났다. 비효소 항산화제인 GSH도 AMP 급이에 따라 감소하는 경향이 나타났다. 비특이적 면역인자인 Lysozyme activity와 Phagocytosis는 AMP 급이에 따라 증가하는 경향이 나타났다. 전체적으로 AMP가 성장인자, 혈액학적인자 및 면역인자에 긍정적인 영향을 미친 것으로 보인다.

Ⅰ. Introduction

L-ascorbic acid(AA) is a important nutrient in aquatic animals. Ascorbic acid is well known to improve growth, antioxidant defence, immunity and maintain physiological homeostasis. In the case of antioxidant mechanism, the ascorbic acid deactivates a free radical by giving it an electrons. A abundant studies reported that high concentrations of L-ascorbic acid supplementation was resulted in improve immune response in fish such as turbot(Roberts et al., 1995), japanese seabass(Ai et al., 2004), large yellow croaker(Ai et al., 2006) and juvenile grouper(Lin et al., 2005). Aquatic animals can not synthesize AA since they don't have a lot of the enzyme L-gulonolactone oxidase that is essential to change L-gulonic acid to AA(Sato et al., 1976). For this reason, AA supplementation is necessary for fish and deficiency symptoms occur when AA is insufficient in the body. Deficiency symptoms indicate reduced growth rate, external and internal hemorrhage, deformed spinal columns, erosion of fins and dark skin color. This deficiency symptoms was reported in salmonids(Halver et al., 1969), ictalurids(Wilson and Poe, 1973; Lim and Lovell, 1978), cyprinids (Dabrowski et al., 1988).

AA is unstable due to heat and light, resulting in a loss of AA activity during processing and storage(Lovell et al., 1978). Numerous studies reported that L-ascorbic acid derivatives was

able to replace unstable L-ascorbic acid. L-ascorbic acid derivatives with phosphate and sulfate parts at the unstable C-2 position in the lactone ring have resistance to oxidation(Tolbert et al., 1975). For instance, Mg-L-ascorbyl-2-phosphate used as a vitamin source for Kumar shrimp, Penaeus japonicus(Kunihiko Shigueno et al., 1988) and L-ascorbyl-2-sulfate was reported to have vitamin C activity in channel catfish(Brandt et al., 1985).

Starry flounder, *Platichthys stellatus* is cold-water fish and euryhaline fish. Starry flounder is suitable for aquaculture considering Korean seawater, which has a long period of low temperature below 15℃ and flow in freshwater frequently. It also succeeded in mass seedling production in 2004 and now it is one of the representative aquaculture of Korea. Many studies of AA supplementation of other species such as olive flounder(Wang et al., 2002) and cobia(Zhou et al., 2012) have been under way, but the relationship of AA supplementation related to starry flounder culture has not been studied yet sufficiently. Therefore, this sturdy was to evaluate growth, hematological, antioxidant effects of AA supplementation in starry fish, *Platichthys stellatus*.

Ⅱ. Materials and methods

1. Experimental diet

Composition of the experimental basal diet is shown in Table 1. The AMP ratio in diet were set at 0, 50, 100, 200, 400, 600, 800 mg/kg. As increasing the amount of AMP, α-Cellulose was reduced at the same rate. Ascorbic acid in white fish meal was removed before making diet. All ingredients were mixed using hobart-type mixer, then squid liver oil and water were added. All ingredients were extruded as pellet type and dried. After this processing, all diets were packed into vacuum bag and kept at -20 ℃ until using.

2. Experimental fish and feeding trial

Starry flounder, *Platichthys stellatus* were obtained from Hwanam fish hatchery in Gijang, Korea. Prior to the experiment, starry flounder were acclimatized to laboratory condition for 1 weeks. After acclimation, 15 fishes (body length, 19.87±0.21 cm; body weight, 139.47±1.41 g) randomly selected for each sections were experimentally sorted into 150 L cylindrical water tank. Each water tank was provided with a constant flow of water. Range of water temperature was 12.0±1.0 ℃ and photoperiod was 12h light : 12h

dark (06:00 to 18:00) during the experimental period. Considering a nocturnal, fish of all section were fed the same amount at 20:00 which was 2 % of body weight for 4 weeks. Allowing for growth rate for experiment period, the amount of feed was recalculated every two weeks.

3. Growth performance

7 fish in each tank were selected randomly every two weeks. After selecting, fish were weighed and measured the length, body weight gain (BWG), feed efficiency ratio (FER), specific growth rate (SGR). BWG, FER, SGR was calculated by the following method.

BWG (%) = 100 * (Final weight-Initial weight) / (Initial weight)
SGR (%) = 100 * (In(Final weight)-In(Initial weight)) / (Duration)
FER (%) = 100 * (Increase in biomass of fish) / (Feed intake)

4. Sample collection and hematological analysis

Blood samples of each fish were collected by puncture of the caudal vein using heparinized disposable syringes and analyzed RBC(Red blood cell count), hematocrit(Ht), hemoglobin(Hb). centrifuged at 3000 g for 5 min at 4 ℃. RBC counts were counted by using an optical microscope with hemo-cytometer(Improved

Neubauer, Germany) after diluted 400 times by Hendrick's diluting solution. The Ht value was determined by Micro-hematocrit reader (HAWKSLEY AND SONS Ltd., England) after blood samples put in Micro-hematocrit capillary tubes and centrifuged at 12000 rpm for 5 min using the Micro-hematocrit centrifuge(Model; 01501, HAWKSLEY AND SONS Ltd., England). The Hb concentration was determined by a clinical kit(Asan Pharm. Co., Ltd) according to the Cyan-methemoglobin method.

The blood samples were centrifuged at 3000 g for 5 min at 4 ℃ to separate plasma from blood samples. The plasma samples were analyzed for serum parameters and enzyme activity. Glucose, total protein were measured by a clinical kit(Asan Pharm. Co., Ltd). Glucose was analyzed by GOD/POD method, total protein was Biuret method. Calcium and magnesium were measured by a clinical kit (Asan Pharm. Co., Ltd.). Calcium was analyzed by OCPC method and magnesium was Xylidyle blue-I method. Glutamic oxalate transaminase(GOT) and glutamic pyruvate transminase(GPT) were measured as serum enzyme activities. GOT and GPT were measured by a clinical kit(Asan Pharm., Co., Ltd) according to Reitman Frankel method.

5. Antioxidant response analysis

The liver, gill, kidney and spleen tissues of the starry flounder were used to analyze antioxidant response. Each tissues were

excised and homogenized by 10 volumes of 0.1 M PBS buffer(pH 7.4). The homogenate was centrifuged at 10,000 g for 30 min at 4 ℃. The supernatants were acquired after centrifuging process. Quantitative test of protein was measured by Ellman's method (1961).

5-1. Superoxide dismutase activity (SOD)

Superoxide dismutase activity (SOD) was measured by using SOD Assay Kit(Dojindo Molecular Technologies, Inc.). Simply, the WST working solution, Enzyme working solution and Dilution buffer were added in the sample of the 96 well plate according to manual. After that, incubate the plate at 37 ℃ for 20 min and read the absorbance at 450 nm by a spectrophotometer.

5-2. Catalase activity (CAT)

Catalase activity (CAT) was measured by using CAT Assay Kit (CELL BIOLABS, INC.). Simply, add 20 μL of the samples and 50 μL of the Hydrogen Peroxide Working Solution(12mM) to a 96 well plate. Mix thoroughly and incubate at room temperature for exactly 1 min. After that, stop the reaction by adding 50 μL of the Catalase Quencher into each well and mix thoroughly. Transfer 5 μL of each reaction well to a fresh well and add 250 μL of the Chromogenic Working Solution to each well. Finally, incubate the plate at room temperature for 40-60 min with vigorous mixing and

read the plate absorbance at 520 nm.

5-3. Glutathione (GSH)

Glutathion (GSH) was measured by the method of Beutler (1984). Simply, 20 μL samples, 180 μL D·W and 300 μL Precipitation solution were added in E-tube. 40 μL solution in E-tube, 160 μL Phosphate solution and 20 μL DTNB solution was added in 96 well plate. After protecting from light for 3 min, read the 96 well plate absorbance at 412 nm by a spectrophotometer.

5-4. Glutathione-S-transferase (GST)

Glutathione-S-transferase (GST) activity was measured by the method of Habig et al., (1974). Simply, 100 μL Phosphate Buffered Saline(0.2M), 20 μL GSH(10mM), 40 μL D·W, 20 μL samples and 20 μL CDNB(10mM) were added in 96well plate in order. 96 well plate was determinated by a spectrophotometer in absorbance at 340 nm at 30 sec intervals for 5 min.

6. Immune response analysis

6-1. Lysozyme activity

Lysozyme activity in serum was measured by using Lysozyme Detection Kit(Sigma). Simply, add 800 μL *Micrococcus lysodeikticus*

cell suspension 30 μL Reaction buffer and 30 μL serum in the plate at 25 ℃. After this process. read the plate absorbance at 450 nm per minute for 5 min.

6-2. Phagocytosis

Phagocytosis was measured by using Phagocytosis Assay Kit (CELL BIOLABS, INC.). Briefly, add 200 μL of cold 1 X PBS to well and remove the PBS solution by centrifugation. Add 100 μL Fixation Solution and incubate for 5 min at room temperature. After that, remove the Fixation Solution by centrifugation at 300 g for 5 min. Add 100 μL prediluted 1 X Blocking Solution to well and Incubate for 30 min at room temperature and remove the Blocking Solution by centrifugation at 300 g for 5 min. Add 100 μL prediluted 1 X Permeabilization Solution to well and incubate for 5 min at room temperature and remove the Permeabilization Solution by centrifugation 300 g for 5 min. Add 100 μL Substrate and incubate for 10 min at room temperature. Finally, add 100 μL Stop Solution and read the absorbance at 450 nm.

7. Statistical analysis

The SPSS/PC+ statistical package(SPSS Inc, Chicago, IL, USA) were used to perform statistical analysis. Significantly different values between groups were identified by using one-way ANOVA

and Duncan's multiple range test. The significance level was determined at $P<0.05$.

Table 1. Composition of the experimental basal diet

Ingredient	L-ascorbyl-2-monophosphate concentration (%)						
	AMP_0	AMP_{50}	AMP_{100}	AMP_{200}	AMP_{400}	AMP_{600}	AMP_{800}
White fish meal[1]	62	62	62	62	62	62	62
Casein[2]	10	10	10	10	10	10	10
Dextrin[3]	19.65	19.65	19.65	19.65	19.65	19.65	19.65
Fish oil[4]	2	2	2	2	2	2	2
Squid liver oil[5]	2	2	2	2	2	2	2
Carboxymethylcellulose[6]	1	1	1	1	1	1	1
α-Cellulose[7]	0.85	0.8	0.74	0.64	0.42	0.21	0
Vitamin Premix (AA-free)[8]	1	1	1	1	1	1	1
Mineral Premix[9]	1	1	1	1	1	1	1
Coline salt[10]	0.5	0.5	0.5	0.5	0.5	0.5	0.5
AMP[11]	0	0.05	0.11	0.21	0.43	0.64	0.85
Total	100	100	100	100	100	100	100

1. White fish meal, Dajeon Co., Ltd., Pusan, Korea
2. Casein, The Feed Co., Ltd., Pusan, Korea
3. Dextrin, TS Co., Ltd., Incheon, Korea
4. Fish oil, Sigma Chemical Co., St. Louis, MO
5. Squid liver oil, Sigma, USA
6. Carboxymethylcellulose, Sigma, USA
7. α-Cellulose, Sigma, USA
8. Vitamin Premix (AA-free) (mg/kg diet): dl-calcium pantothenate, 368; Choline chloride, 10; Inositol, 400; Menadione, 1800; Nicotinamide, 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. Mineral Premix (g/kg): 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. Coline salt, Kofavet Co., Ltd., Ulsan, Korea
11. L-ascorbyl-2-monophosphate (mg/kg) (Woosung Co. Ltd., Seoul, Korea, containing 35% ascorbic acid activity)

Ⅲ. Results

1. Growth factors

Growth factors of the starry flounder fed diets containing AMP are shown in Figure 1 ~ Figure 4. Body weight gain(BWG) of fish fed diet containing over AMP 400 mg/kg at 2 weeks and over AMP 200 mg/kg at 4 weeks was significantly higher than fish fed other diets. Feed efficiency ratio(FER) of fish fed diet containing AMP 800 mg/kg at 2 weeks and over AMP 600 mg/kg 4 weeks was significantly higher than fish fed other diets. It was specific growth rate(SGR) of fish fed diet containing AMP 800 mg/kg at 2 weeks and over AMP 400 mg/kg at 4 weeks was significantly higher than fish fed other diets.

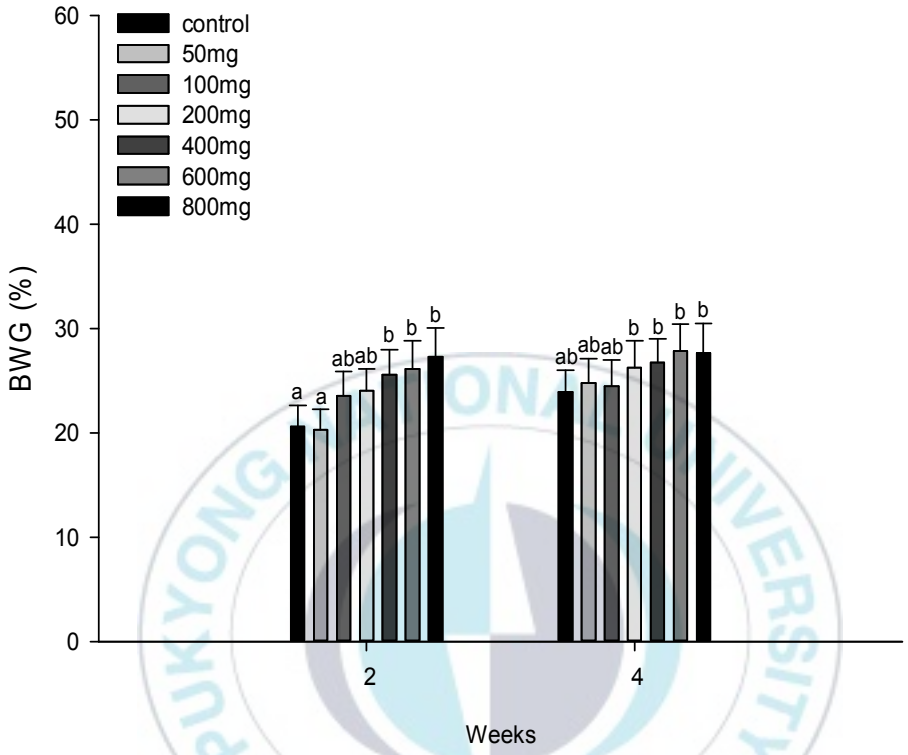

Figure 1. BWG of starry flounder, *Platichthys stellatus* fed diet containing different levels of AMP for 2, 4 weeks. Values are mean±S.D. Values with a different letter are significantly different from others at 2 weeks and at 4 weeks ($P<0.05$) as determined by Duncan's multiple range test.

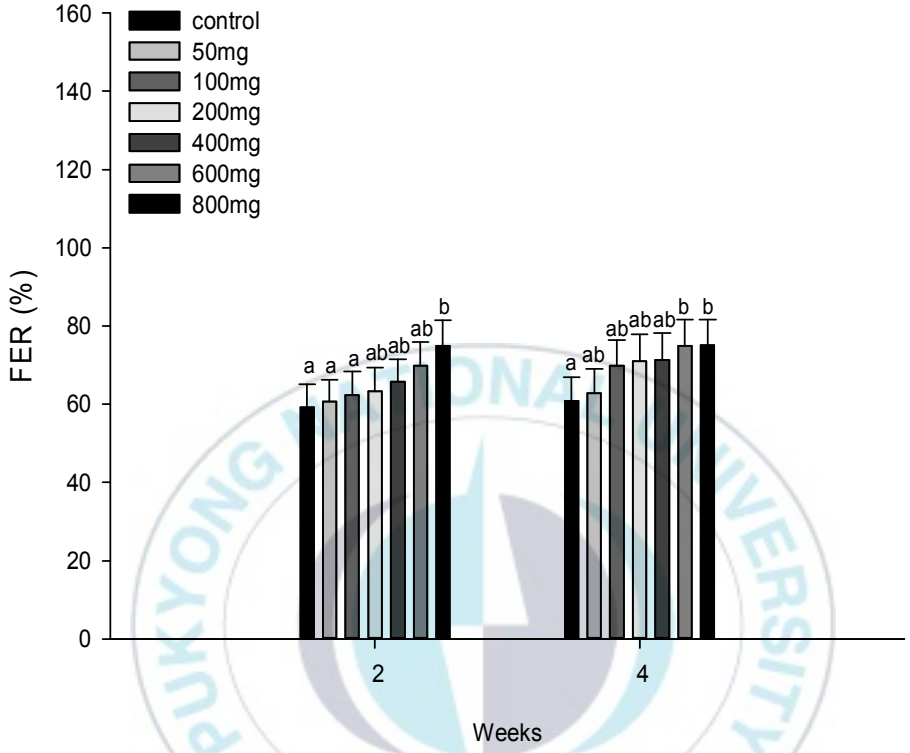

Figure 2. FER of starry flounder, *Platichthys stellatus* fed diet containing different levels of AMP for 2, 4 weeks. Values are mean±S.D. Values with a different letter are significantly different from others at 2 weeks and at 4 weeks ($P<0.05$) as determined by Duncan's multiple range test.

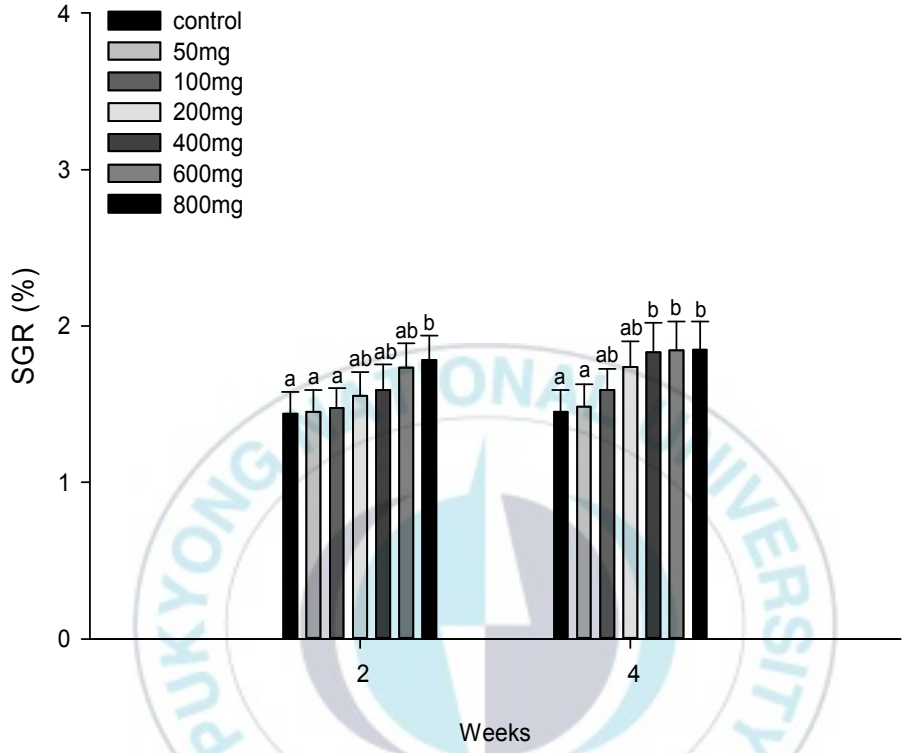

Figure 3. SGR of starry flounder, *Platichthys stellatus* fed diet containing different levels of AMP for 2, 4 weeks. Values are mean±S.D. Values with a different letter are significantly different from others at 2 weeks and at 4 weeks ($P<0.05$) as determined by Duncan's multiple range test.

2. Hematological parameters

Hematological parameters of the starry flounder fed diets containing AMP are shown in Table 2. RBC count of fish fed diet including AMP 800 mg/kg at 2 weeks and over AMP 200 mg/kg at 4 weeks were notably higher than fish fed other diets. Hematocrit values of fish deit containing fed AMP 800 mg/kg at 2, 4 weeks were notably higher than other fish fed AMP diet. Hemoglobin of fish was significantly increased from over AMP 600 mg/kg in both 2 and 4 weeks.

The change of serum parameters in the serum of the starry flounder fed AMP diet are shown in Table 3. Calcium and magnesium was considerable affected in over AMP 600 mg/kg at 2 and over AMP 400 mg/kg at 4 weeks. Glucose was lowest in AMP 800 mg/kg at 2 weeks and over AMP 600 mg/kg at 4 weeks were significantly lower than other fish fed other diets. Total protein was significantly decreased at over AMP 100 mg/kg in both 2 and 4 weeks. GOT of fish fed diet including over AMP 100 mg/kg at 2, 4 weeks were significantly lower than fish fed other diets. GPT of fish fed diet containing over AMP 100 mg/kg at 2, 4 weeks were considerable lower than fish fed other diets.

Table 2. The change of hematological parameters in starry flounder, *Platichthys stellatus* fed diet containing different levels of AMP for 2, 4 weeks [1]

Parameters	Period (weeks)	L-ascorbyl-2-monophosphate concentration (mg/kg)						
		AMP_0	AMP_{50}	AMP_{100}	AMP_{200}	AMP_{400}	AMP_{600}	AMP_{800}
RBC count (X $10^4 mm^3$)	2	255.16±14.48^a	256.67±17.82^a	258.86±14.43^a	261.14±13.82^a	277.43±16.26^{ab}	274.86±16.25^{ab}	294.29±10.95^b
	4	262.86±16.37^a	278±16.96^{ab}	274.57±16.33^{ab}	293.14±14.21^b	301.14±12.95^b	296±16.58^b	303.43±12.61^b
Hematocrit (%)	2	28.43±2.76^a	28.50±2.88^a	29.71±2.46^a	32.29±2.39^{ab}	30.89±2.60^{ab}	31.60±2.76^{ab}	35.33±2.19^b
	4	28.14±2.24^a	28.89±2.15^a	29.54±2.57^a	32.79±3.18^{ab}	32.45±2.45^{ab}	33.20±2.51^{ab}	35.71±2.80^b
Hemoglobin (g/dL)	2	5.86±0.36^a	6.13±0.54^a	5.84±0.59^a	6.67±0.54^{ab}	6.77±0.52^{ab}	7.23±0.58^{bc}	7.83±0.29^c
	4	5.85±0.41^a	5.89±0.51^a	6.43±0.49^{ab}	6.69±0.59^{ab}	6.74±0.55^{ab}	7.30±0.57^{bc}	8.07±0.38^c

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

Table 3. The change of serum parameters in starry flounder, *Platichthys stellatus* fed diet containing different levels of AMP for 2, 4 weeks [1]

Parameters	Period (weeks)	L-ascorbyl-2-monophosphate concentration (mg/kg)						
		AMP_0	AMP_{50}	AMP_{100}	AMP_{200}	AMP_{400}	AMP_{600}	AMP_{800}
Calcium (mg/dL)	2	9.03±0.81^{a}	8.80±0.54^{a}	9.05±0.55^{a}	9.69±0.45^{ab}	9.80±0.43^{ab}	10.87±0.51^{c}	11.12±0.47^{c}
	4	9.42±0.66^{a}	8.79±0.46^{a}	9.63±0.44^{a}	9.80±0.77^{ab}	10.73±0.71^{bc}	11.50±0.49^{c}	11.54±0.60^{c}
Magnesium (mg/dL)	2	3.48±0.27^{a}	3.73±0.35^{a}	3.89±0.32^{ab}	4.04±0.29^{ab}	3.80±0.41^{ab}	4.37±0.40^{bc}	4.70±0.37^{c}
	4	3.47±0.31^{a}	3.82±0.38^{ab}	4.02±0.20^{ab}	3.94±0.16^{ab}	4.39±0.23^{bc}	4.95±0.37^{c}	4.95±0.45^{c}
Glucose (mg/dL)	2	97.10±4.93^{a}	92.98±5.38^{ab}	92.70±6.60^{ab}	91.37±5.68^{ab}	89.26±7.18^{ab}	90.41±4.44^{ab}	86.34±4.36^{b}
	4	99.41±4.38^{a}	98.01±5.22^{a}	91.64±7.34^{ab}	89.27±5.59^{ab}	89.87±4.62^{ab}	86.22±3.82^{b}	85.53±4.75^{b}
Total protein (g/dL)	2	3.09±0.25^{a}	2.90±0.19^{ab}	2.64±0.20^{bc}	2.61±0.20^{bc}	2.30±0.17^{c}	2.29±0.22^{c}	2.29±0.13^{c}
	4	3.13±0.21^{a}	2.94±0.19^{ab}	2.60±0.22^{bc}	2.59±0.13^{bc}	2.61±0.17^{bc}	2.29±0.21^{c}	2.27±0.20^{c}
GOT (Karmen/ml)	2	23.32±1.44^{a}	21.90±1.76^{a}	18.95±1.73^{b}	17.76±0.81^{bc}	16.85±1.67^{bc}	17.04±1.63^{bc}	15.87±1.16^{c}
	4	22.72±1.37^{a}	22.18±1.87^{a}	18.74±1.68^{b}	17.43±0.82^{bc}	16.46±1.29^{bc}	16.96±1.39^{bc}	15.29±0.78^{c}
GPT (Karmen/ml)	2	15.49±0.88^{a}	15.37±0.73^{a}	12.85±0.92^{b}	11.98±0.96^{b}	12.57±0.53^{b}	9.69±0.85^{c}	9.53±0.84^{c}
	4	15.51±0.90^{a}	15.50±0.71^{a}	12.26±0.84^{b}	13.01±0.52^{b}	11.82±0.96^{b}	10.04±0.93^{c}	8.89±0.70^{c}

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

3. Antioxidant responses

3-1. Superoxide dismutase activity (SOD)

SOD activity of the starry flounder fed diets containing AMP are shown in Figure 5 ~ Figure 8. In the liver, SOD activity of fish fed diet including over AMP 400 mg/kg at 2, 4 weeks were significantly lower than fish fed other diets. In the gill, SOD activity of fish fed diet including over AMP 100 mg/kg at 2, 4 weeks were significantly lower than fish fed other diets. In the kidney and spleen, SOD activity of fish fed diet including over AMP 100 mg/kg at 2, 4 weeks were significantly lower than other dietary.

3-2. Catalase activity (CAT)

CAT activity of the starry flounder fed diets containing AMP are shown in Figure 9 ~ Figure 12. In the liver, CAT activity of fish fed diet including over AMP 400 mg/kg at 2 weeks and over AMP 200 mg/kg at 4 weeks were significantly increased. In the gill, CAT activity of fish fed diet including over AMP 400 mg/kg at 2 weeks and over AMP 200 mg/kg at 4 weeks also were significantly increased. In the kidney, CAT activity of fish fed over AMP 100 mg/kg at 2, 4 weeks were significantly decreased. In the spleen, over AMP 600 mg/kg at 2, 4 weeks were also significantly decreased.

3-3. Glutathione (GSH)

GSH levels of starry flounder fed diets containing AMP are shown in Figure 13 ~ Figure 16. In the liver, GSH level of fish fed diet including over AMP 200 mg/kg at 2 weeks and over AMP 100 mg/kg at 4 weeks were decreased substantially. In the gill, GSH level of fish fed diet including over AMP 200 mg/kg at 2, 4 weeks were significantly decreased. In the kidney, over AMP 100 mg/kg at 2, 4 weeks were significantly decreased. In the spleen, over AMP 600 mg/kg at 2, 4 weeks were significantly decreased.

3-4. Glutathione-S-transferase (GST)

GST activity of the starry flounder fed diets containing AMP are shown in Figure 17 ~ Figure 20. In the liver, GST activity of fish fed diet including over AMP 100 mg/kg at 2 weeks and over AMP 50 mg/kg at 4 weeks were significantly decreased. In the gill, GST activity of fish fed diet over AMP 400 mg/kg at 2 weeks and over AMP 100 mg/kg at 4 weeks were substantially decreased. In the kidney, over AMP 200 mg/kg at 2 weeks and over AMP 400 mg/kg at 4 weeks were significantly decreased. In the spleen, over AMP 200 mg/kg at 2, 4 weeks were significantly decreased.

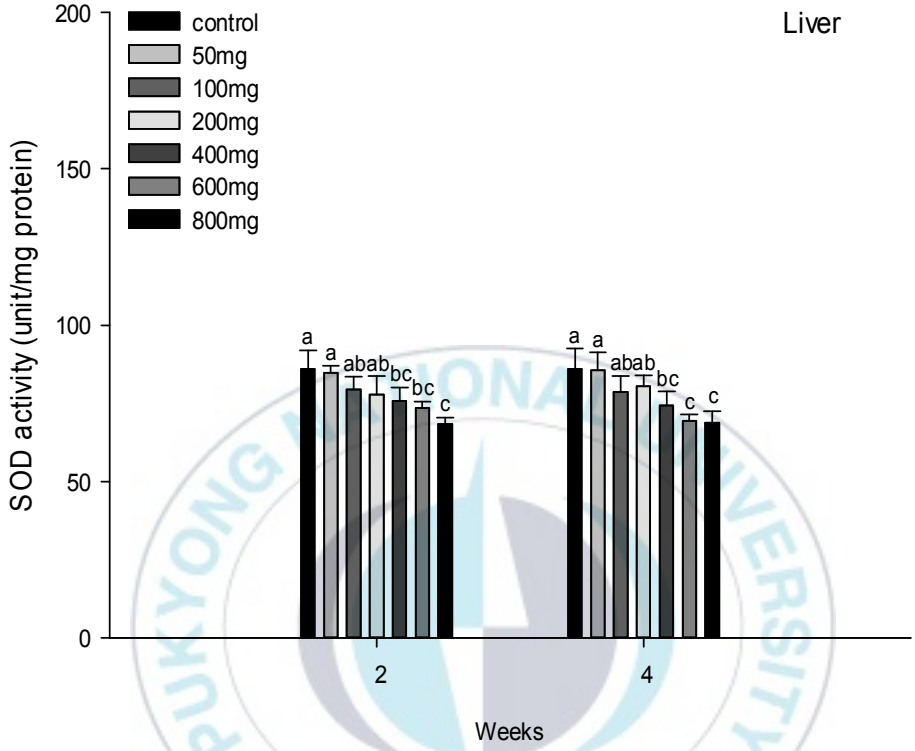

Figure 5. SOD activity of starry flounder, *Platichthys stellatus* fed diet containing different levels of AMP for 2, 4 weeks. Values are mean±S.D. Values with a different letter are significantly different from others at 2 weeks and at 4 weeks ($P<0.05$) as determined by Duncan's multiple range test.

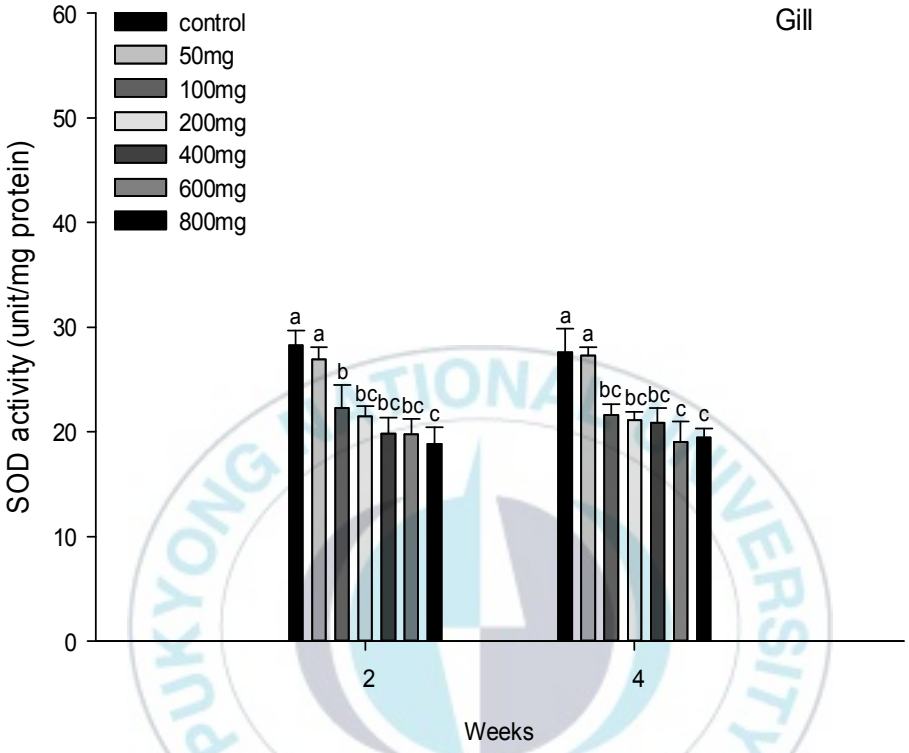

Figure 6. SOD activity of starry flounder, *Platichthys stellatus* fed diet containing different levels of AMP for 2, 4 weeks. Values are mean±S.D. Values with a different letter are significantly different from others at 2 weeks and at 4 weeks ($P<0.05$) as determined by Duncan's multiple range test.

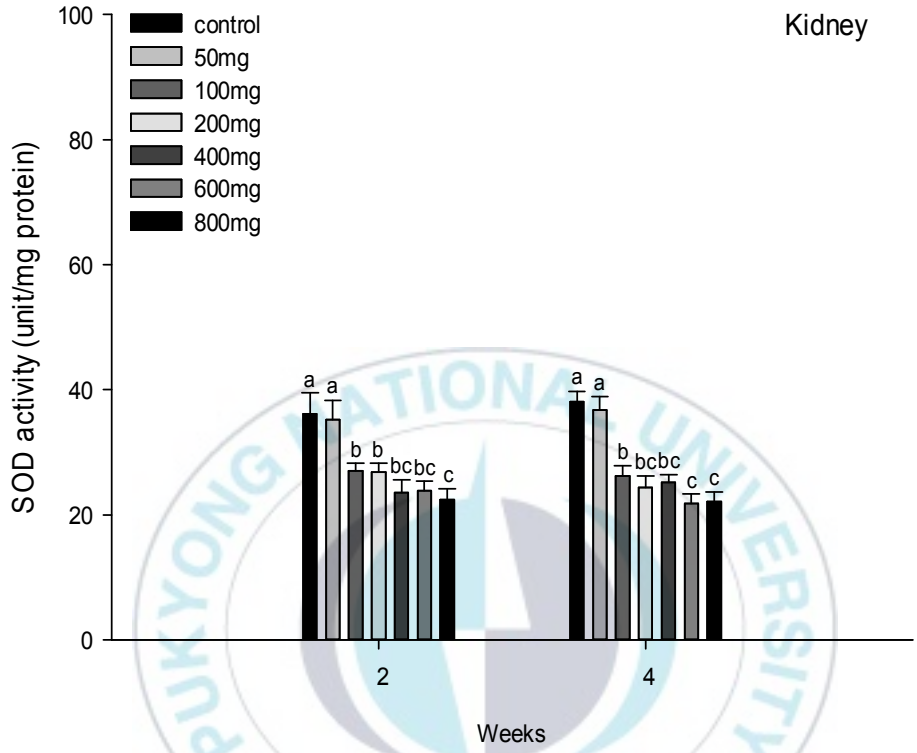

Figure 7. SOD activity of starry flounder, *Platichthys stellatus* fed diet containing different levels of AMP for 2, 4 weeks. Values are mean±S.D. Values with a different letter are significantly different from others at 2 weeks and at 4 weeks ($P<0.05$) as determined by Duncan's multiple range test.

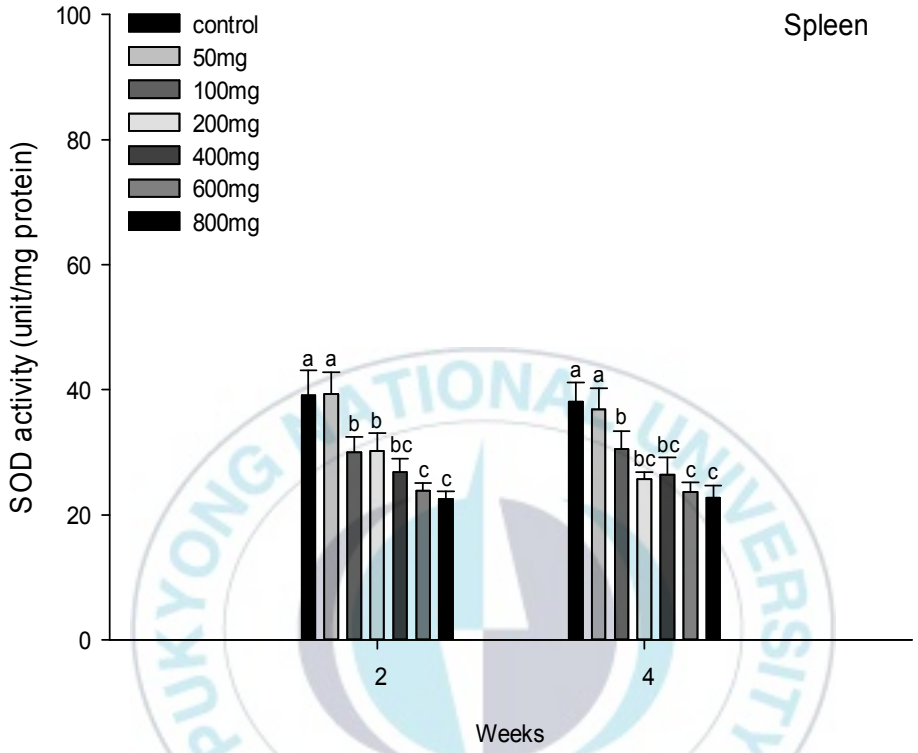

Figure 8. SOD activity of starry flounder, *Platichthys stellatus* fed diet containing different levels of AMP for 2, 4 weeks. Values are mean±S.D. Values with a different letter are significantly different from others at 2 weeks and at 4 weeks ($P<0.05$) as determined by Duncan's multiple range test.

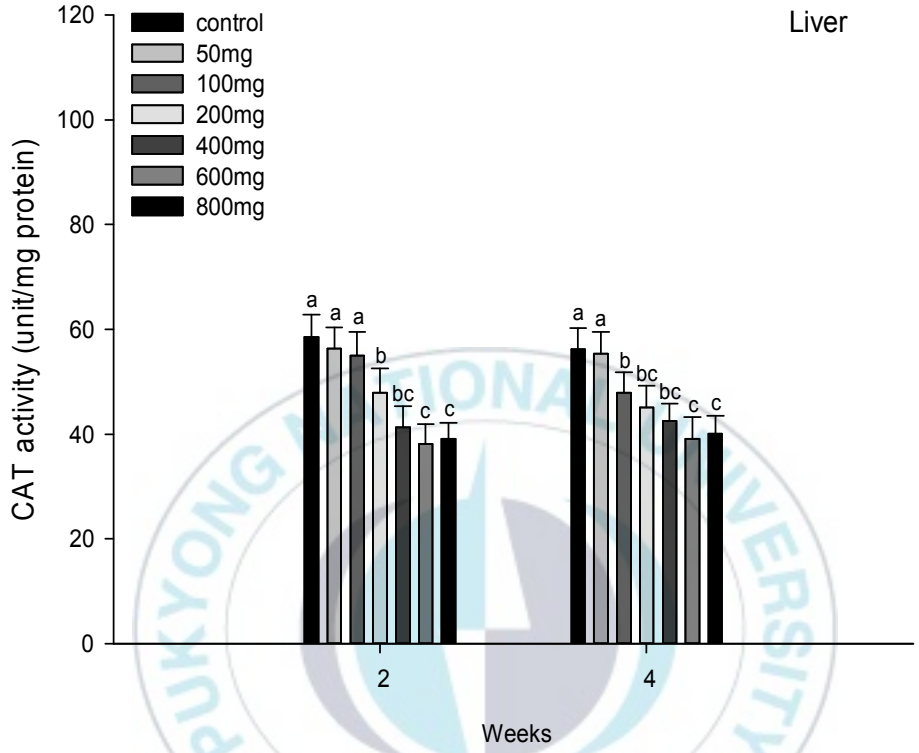

Figure 9. CAT activity of starry flounder, *Platichthys stellatus* fed diet containing different levels of AMP for 2, 4 weeks. Values are mean±S.D. Values with a different letter are significantly different from others at 2 weeks and at 4 weeks ($P<0.05$) as determined by Duncan's multiple range test.

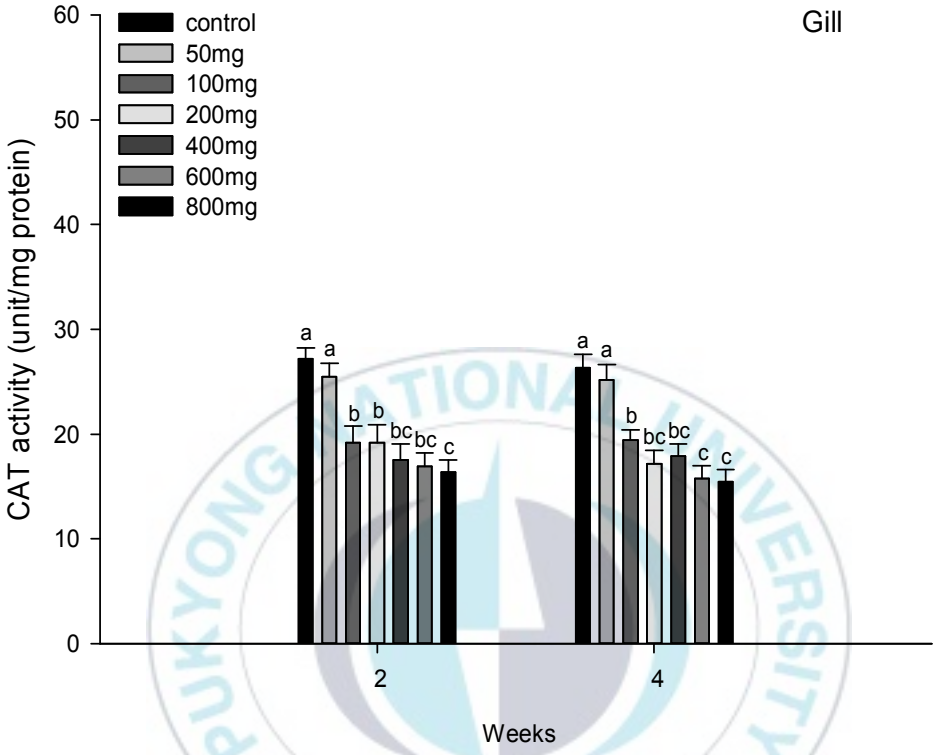

Figure 10. CAT activity of starry flounder, *Platichthys stellatus* fed diet containing different levels of AMP for 2, 4 weeks. Values are mean±S.D. Values with a different letter are significantly different from others at 2 weeks and at 4 weeks ($P<0.05$) as determined by Duncan's multiple range test.

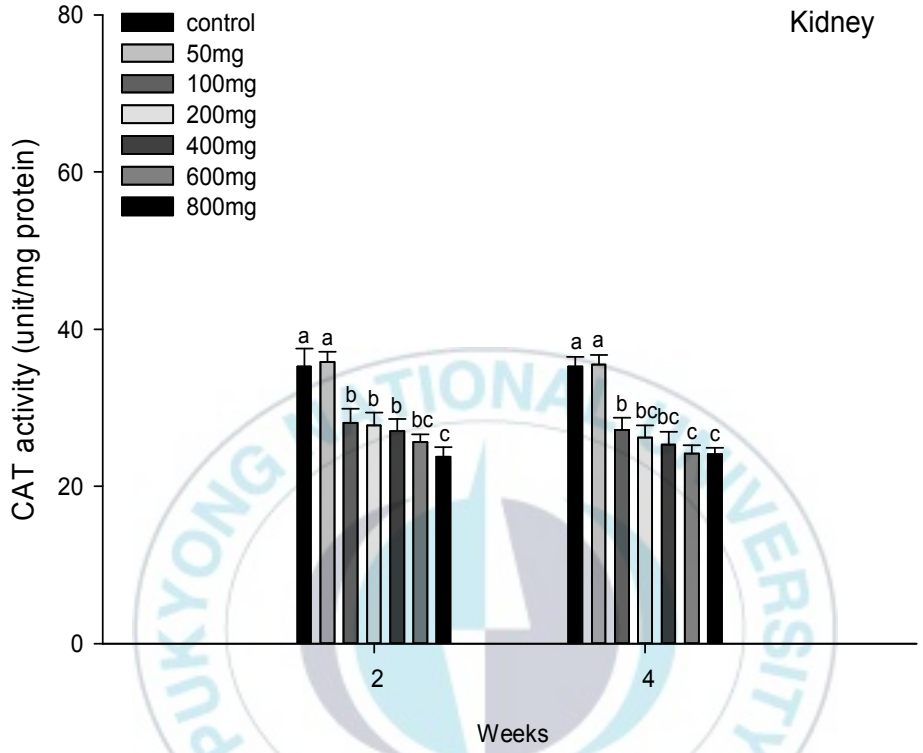

Figure 11. CAT activity of starry flounder, *Platichthys stellatus* fed diet containing different levels of AMP for 2, 4 weeks. Values are mean±S.D. Values with a different letter are significantly different from others at 2 weeks and at 4 weeks ($P<0.05$) as determined by Duncan's multiple range test.

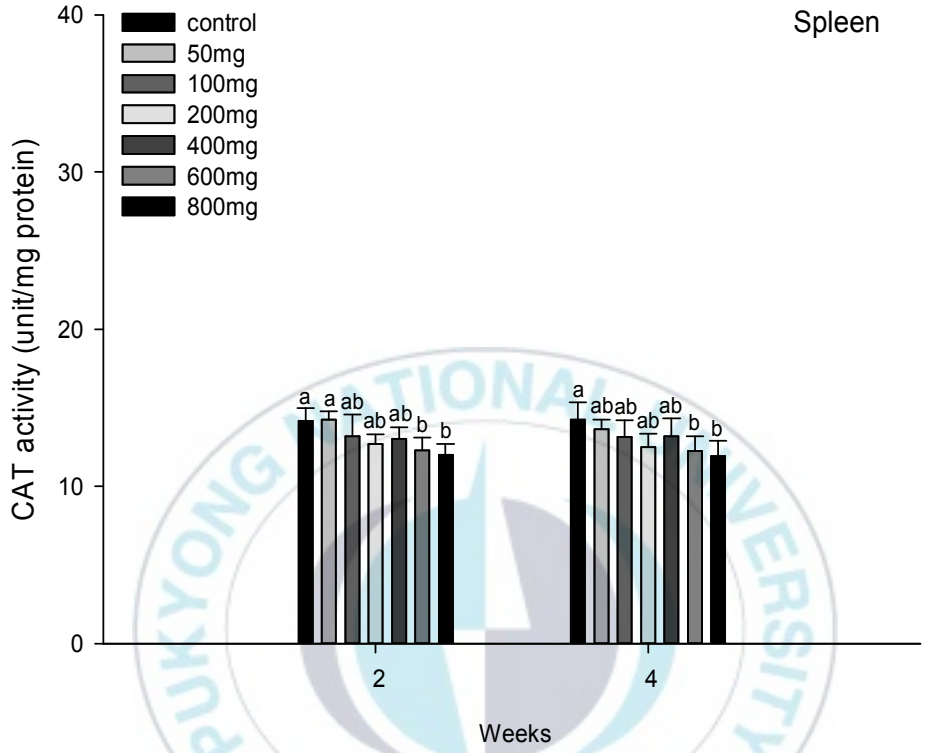

Figure 12. CAT activity of starry flounder, *Platichthys stellatus* fed diet containing different levels of AMP for 2, 4 weeks. Values are mean±S.D. Values with a different letter are significantly different from others at 2 weeks and at 4 weeks ($P<0.05$) as determined by Duncan's multiple range test.

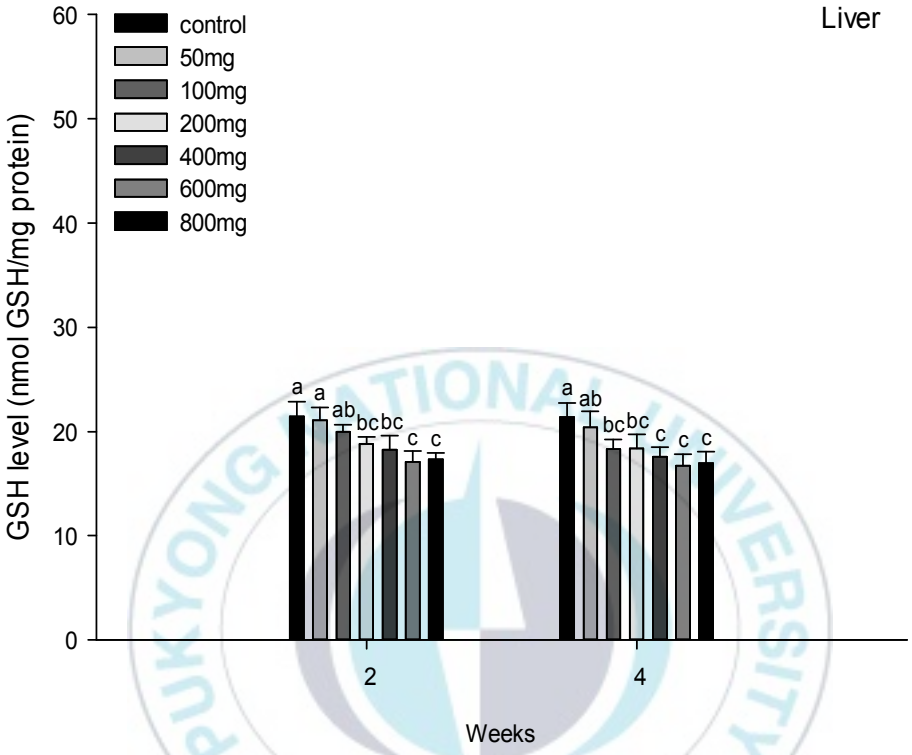

Figure 13. GSH level of starry flounder, *Platichthys stellatus* fed diet containing different levels of AMP for 2, 4 weeks. Values are mean±S.D. Values with a different letter are significantly different from others at 2 weeks and at 4 weeks ($P<0.05$) as determined by Duncan's multiple range test.

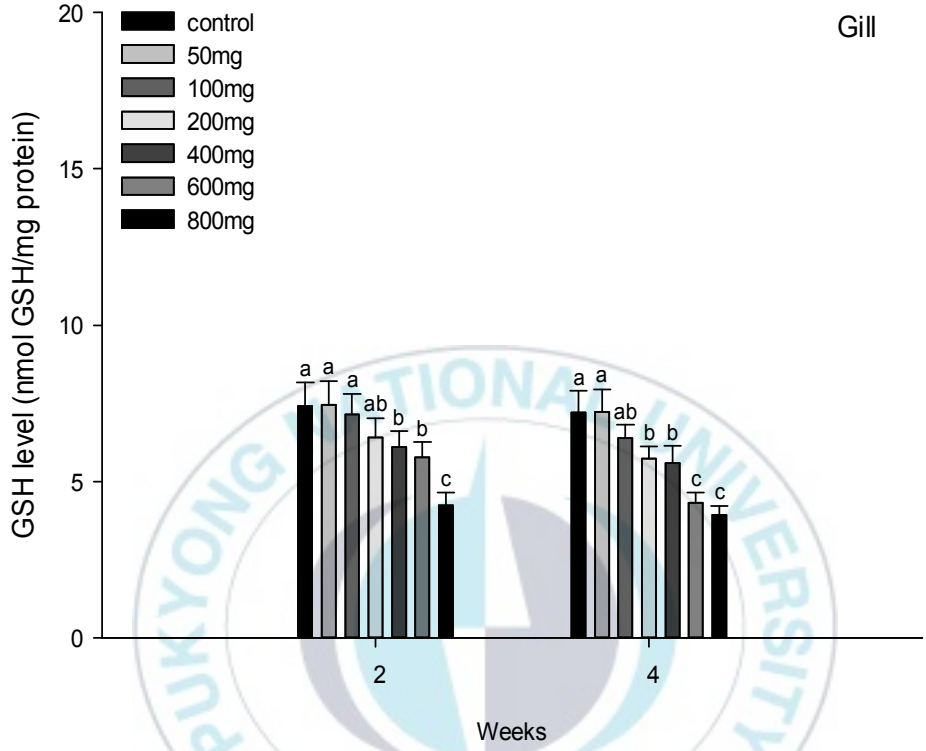

Figure 14. GSH level of starry flounder, *Platichthys stellatus* fed diet containing different levels of AMP for 2, 4 weeks. Values are mean±S.D. Values with a different letter are significantly different from others at 2 weeks and at 4 weeks ($P<0.05$) as determined by Duncan's multiple range test.

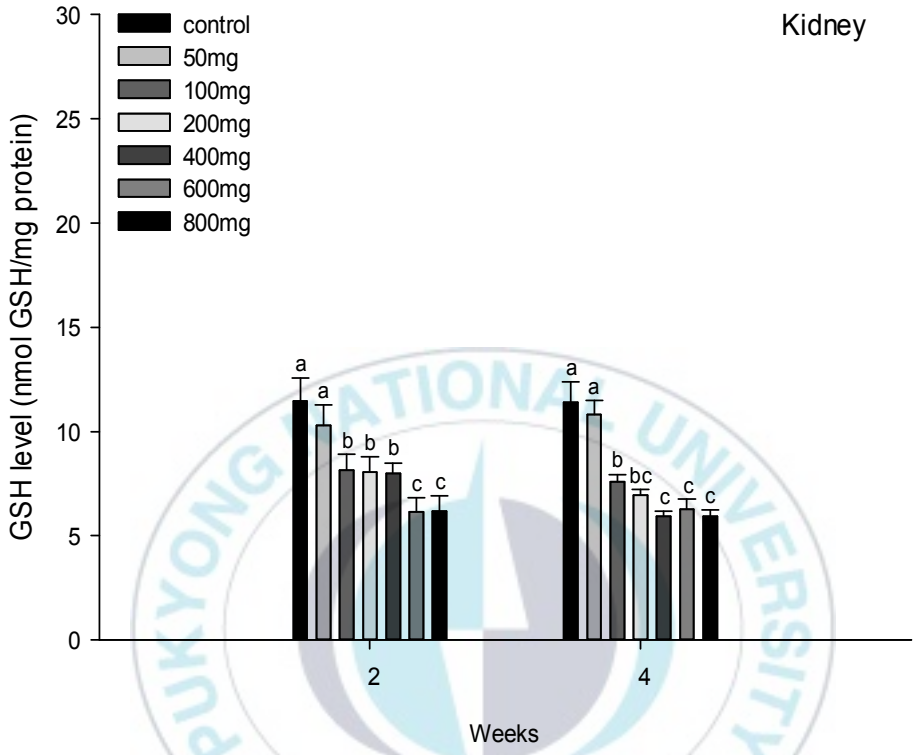

Figure 15. GSH level of starry flounder, *Platichthys stellatus* fed diet containing different levels of AMP for 2, 4 weeks. Values are mean±S.D. Values with a different letter are significantly different from others at 2 weeks and at 4 weeks ($P<0.05$) as determined by Duncan's multiple range test.

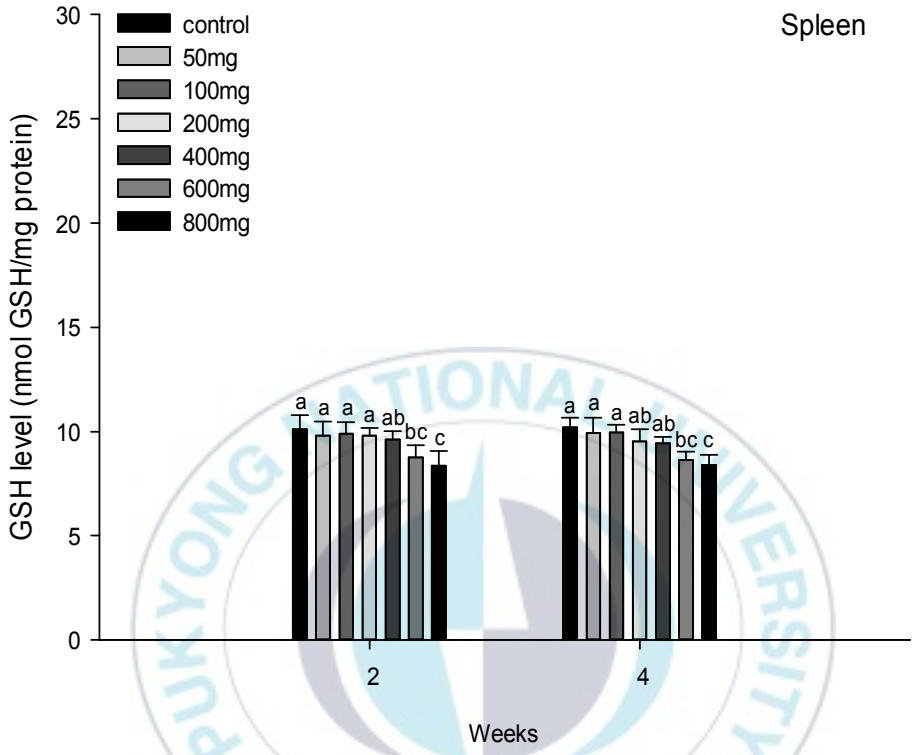

Figure 16. GSH level of starry flounder, *Platichthys stellatus* fed diet containing different levels of AMP for 2, 4 weeks. Values are mean±S.D. Values with a different letter are significantly different from others at 2 weeks and at 4 weeks ($P<0.05$) as determined by Duncan's multiple range test.

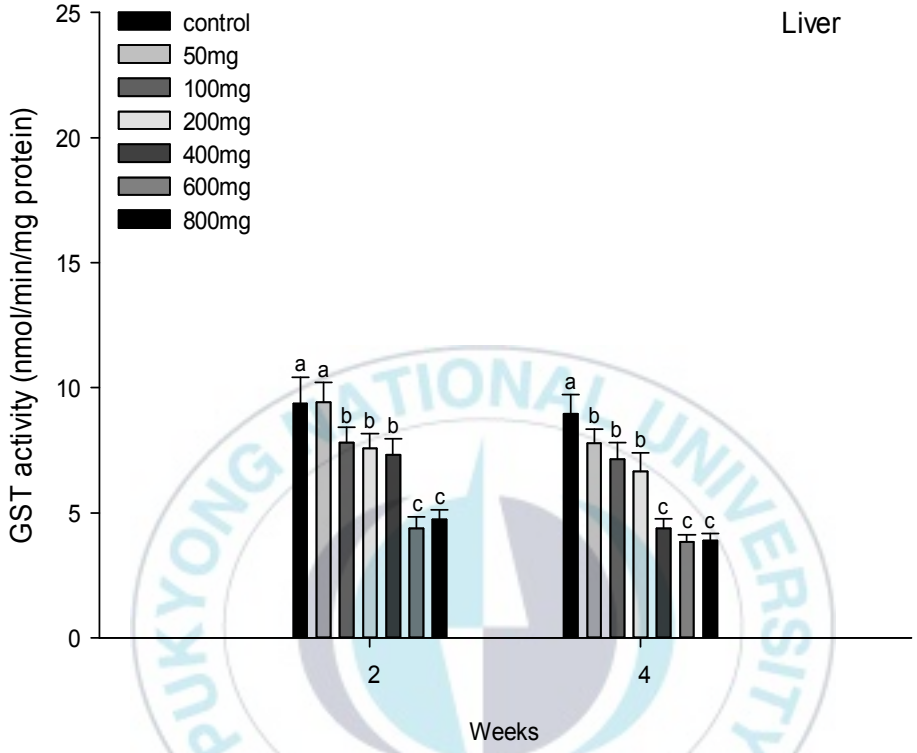

Figure 17. GST activity of starry flounder, *Platichthys stellatus* fed diet containing different levels of AMP for 2, 4 weeks. Values are mean±S.D. Values with a different letter are significantly different from others at 2 weeks and at 4 weeks ($P<0.05$) as determined by Duncan's multiple range test.

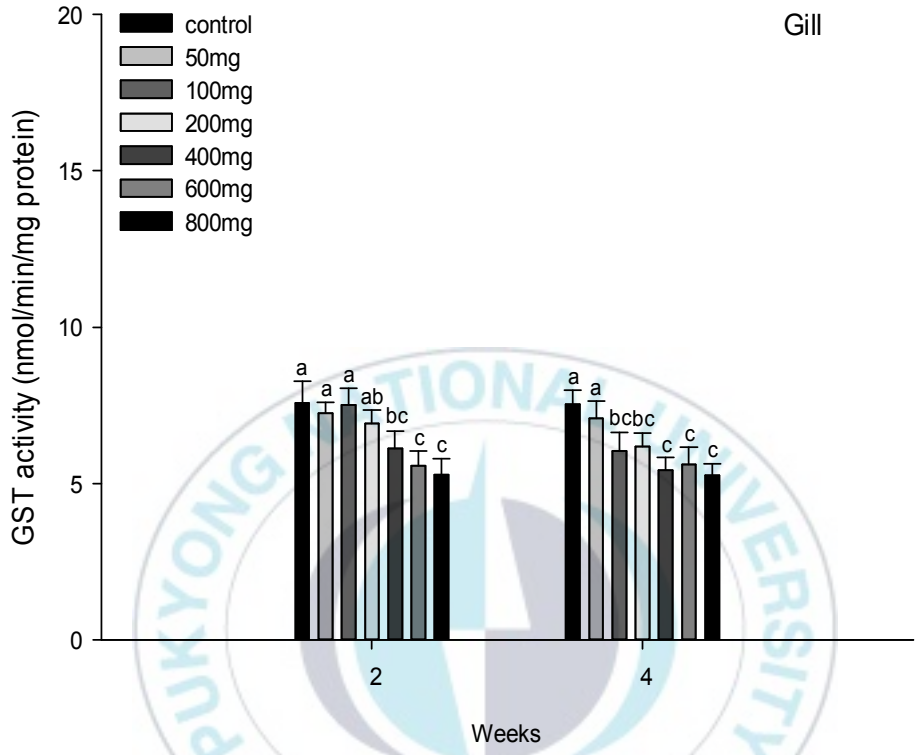

Figure 18. GST activity of starry flounder, *Platichthys stellatus* fed diet containing different levels of AMP for 2, 4 weeks. Values are mean±S.D. Values with a different letter are significantly different from others at 2 weeks and at 4 weeks ($P<0.05$) as determined by Duncan's multiple range test.

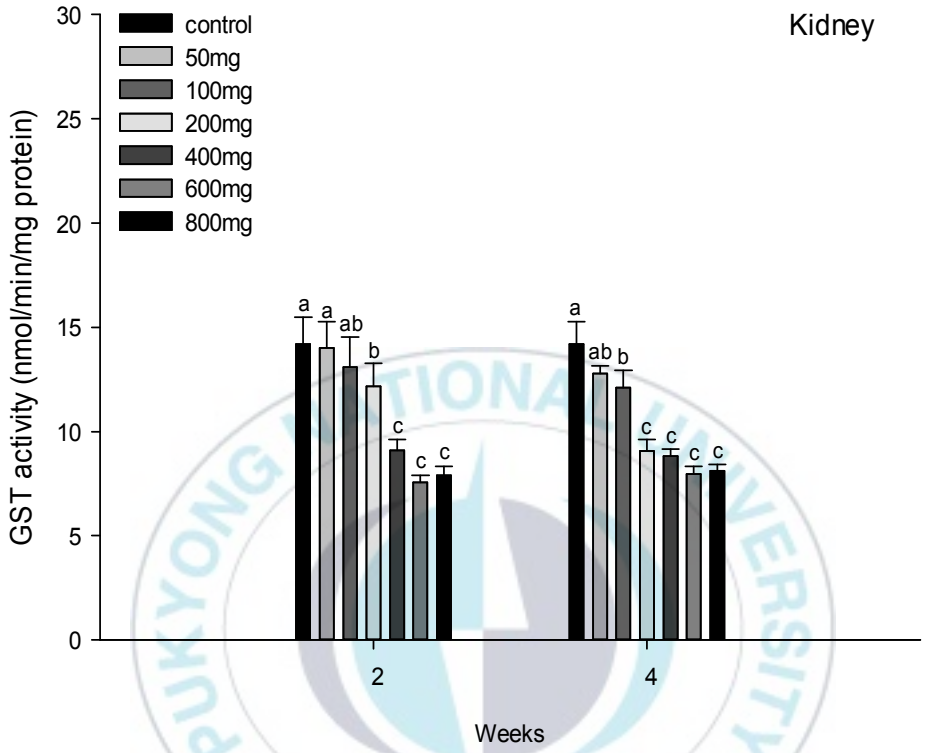

Figure 19. GST activity of starry flounder, *Platichthys stellatus* fed diet containing different levels of AMP for 2, 4 weeks. Values are mean±S.D. Values with a different letter are significantly different from others at 2 weeks and at 4 weeks ($P<0.05$) as determined by Duncan's multiple range test.

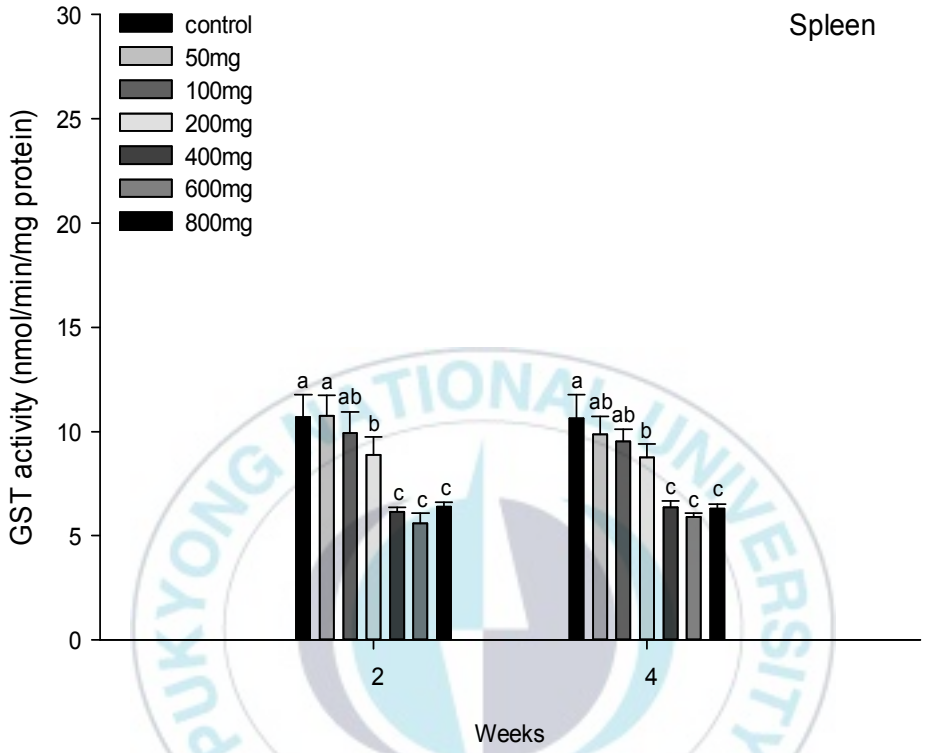

Figure 20. GST activity of starry flounder, *Platichthys stellatus* fed diet containing different levels of AMP for 2, 4 weeks. Values are mean±S.D. Values with a different letter are significantly different from others at 2 weeks and at 4 weeks ($P<0.05$) as determined by Duncan's multiple range test.

4. Immune responses

4-1. Lysozyme activity

Lysozyme activity of the starry flounder fed diets containing AMP are shown in Figure 21. Fish fed over AMP 400 mg/kg dietary was significantly increased at 2 weeks and over AMP 200 mg/kg dietary at 4 weeks.

4-2. Phagocytosis

Phagocytosis of the starry flounder fed diets containing AMP are shown in Figure 22. Fish fed over AMP 600 mg/kg dietary was significantly increased at 2 weeks and over AMP 400 mg/kg dietary at 4 weeks.

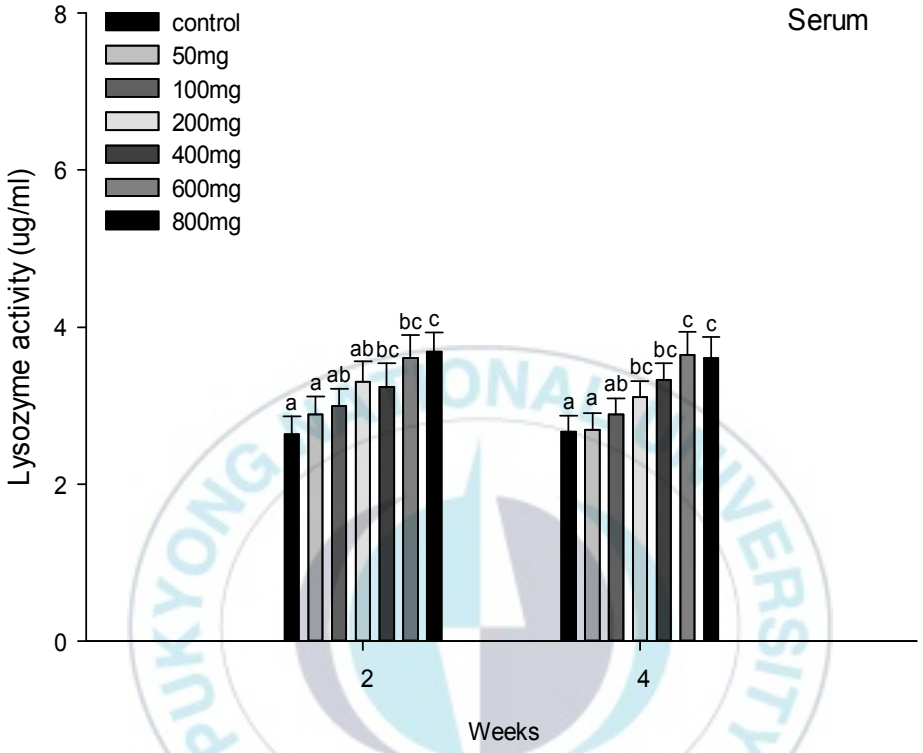

Figure 21. Lysozyme activity of starry flounder, *Platichthys stellatus* fed diet containing different levels of AMP for 2, 4 weeks. Values are mean±S.D. Values with a different letter are significantly different from others at 2 weeks and at 4 weeks ($P<0.05$) as determined by Duncan's multiple range test.

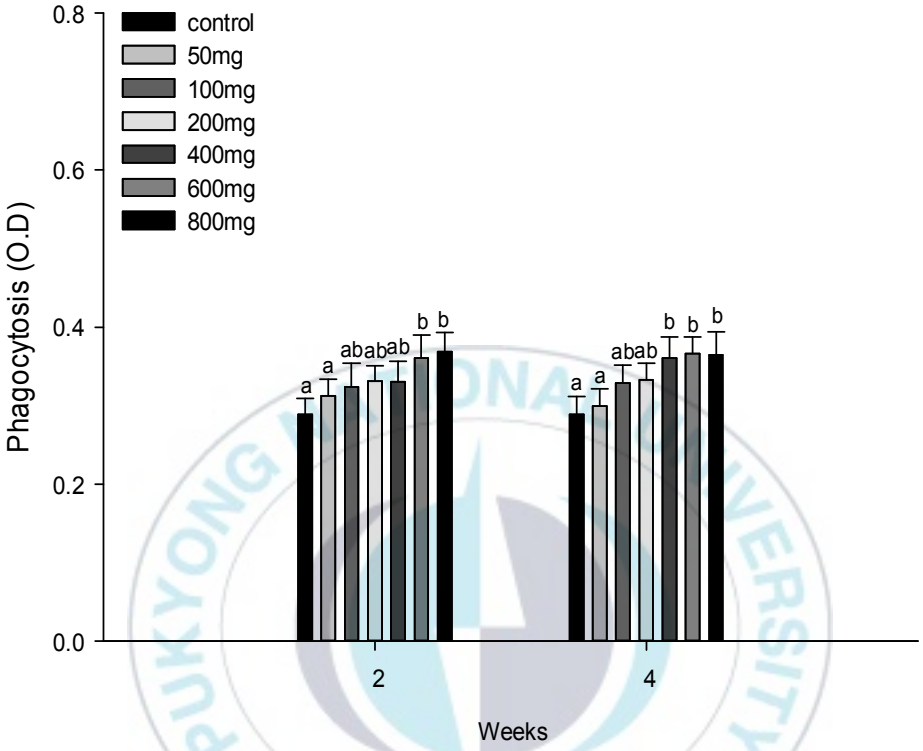

Figure 22. Phagocytosis of starry flounder, *Platichthys stellatus* fed diet containing different levels of AMP for 2, 4 weeks. Values are mean±S.D. Values with a different letter are significantly different from others at 2 weeks and at 4 weeks ($P<0.05$) as determined by Duncan's multiple range test.

Ⅳ. Discussion

Ascorbic acid(AA) is a water-soluble vitamin that is not accumulated in the body but is discharged as urine. Therefore, ascorbic acid is that continuous feeding by diet is required. AA is easily inactivated due to oxidizing during processing and storage. Numerous studies reported that L-ascorbic acid derivatives was able to replace unstable L-ascorbic acid. Matusiewicz and Dabrowski(1995) reported that L-ascorbyl-2-polyphosphate(ASPP) was hydrolyzed by the reaction of intestinal enzyme alkaline phosphatase in rainbow trout. Shiau and Hsu(1995) reported that L-ascorbyl-2-sulfate was able to have antiscorbutic activity in tilapia, *oreochromis niloticus*. These studies may interpret that ascorbic acid phosphate such as L-ascorbyl-2-monophosphate(AMP) is used by diet.

In the deficiency of AA in diet, the japanese eel, *Anguilla japonica* decreased growth rate for 10 weeks and showed hemorrhage after 14 weeks(Arai et al., 1972). The channel catfish, *Ictalurus punctatus* showed erosion of fin, external and internal hemorrhage and dark skin color between 8 weeks and 12 weeks(Lim C et al., 1978). The juvenile tilapia, *Oreochromis niloticus* also showed hemorrhage, caudal fin erosion, exophthalmia after 8 weeks(Soliman et al., 1986). The juvenile Korean rockfish, *Sebastes schlegeli* showed deficiency symptoms such as fin

hemorrhage, scoliosis and exophthalmia after 12 weeks(Lee et al., 1998). In this study, on other hand, no deficiency symptoms such as hemorrhage, scoliosis, dysplasia, abnormality of eye, gill and fins were found in all AMP concentrations for 4 weeks. No deficiency symptoms in the control and low levels of AMP supplementation were depending on species, fish size, experimental environment and duration of experiment. It was confirmed that AMP diet containing high levels concentration was a positive factor for growth, hematological and antioxidants.

AA has a positive effect on the growth factors. The requirement of AA depend on species and is related to growth(Dabrowski et al., 1991). When *cyprinus carpio* larva fed diet containing AA 90 mg/kg, growth rate was significantly increased, the minimum requirement of AA was 45 mg/kg(Gouillou-Coustans et al., 1998). The minimum AA requirement for parrot fish was 118 mg/kg and the growth rate was significantly increased at AA doses above 1869 mg/kg(Wang et al., 2003). In addition, fingerlings of *O. spilurus* showed a significant increase in growth rate when AA was fed at 100 mg/kg(Al-Amoudi et al., 1992). A significant increase in the growth rate of a walking catfish, *clarias batrachus* was AA 500 mg/kg for 4 weeks(Kumari and Sahoo., 2005) and the indian major carp, c*irrhina mrigala* was AA 650~800 mg/kg(Mahajan and Agrawal., 1980). A significant difference growth rate in cobia, r*achycentron canadum linneaus* was showed at AA 44.7 mg/kg(Xiao et al., 2010). Thus the difference in AA requirement for growth rate

varies depending on the size, species and experimental fish. In our experiment, BWG, SGR and FER of starry flounder fed AMP-free were low, but BWG, SGR and FER of experimental fish were increased proportionally with increasing AMP concentration.

Hematological parameters are good indicator of the status of fish such as stress, metabolic disorders, reproductive dysfunction and disease(Fazio et al., 2012), and indicate changes in various blood characteristics depending on the concentration of AA(Sandnes et al., 1990). AA acts as an antioxidant to protect fish tissues from oxidative damage in the body and improves antioxidant activity of erythrocyte membrane(Pearce et al., 2003). Therefore RBC count, hematocrit and hemoglobin on red blood cells are good indicators of oxidative status. A significant increase in RBC counts was observed in pirarucu, *arapaima gigas* fed AA 800 mg/kg and 1200 mg/kg(Andrade et al., 2007). Affonso et al (2007) also reported that Ht, Hb, RBC, MCV, MCHC of *brycon amazonicus* fed ascorbic acid was significantly increased. Increasing hematological factors suggest that it improve the ability of oxygen supply. When *arapaima gigas* was fed L-ascrobyl-2-polyphosphate(APP), RBC count, hematocrit and hemoglobin were significantly increased in APP 500 mg/kg(Menezes et al., 2006). In our experiment, RBC count of starry flounder in which AMP 800 mg/kg was fed showed a significant increase at 2 weeks and over AMP 200 mg/kg at 4 weeks. Hematocrit increased significantly at AMP 800 mg/kg at 2, 4 weeks and hemoglobin increased at AMP 600 mg/kg at 2, 4 weeks.

As a result, AMP seemed to positive effect on the hematological parameters.

Calcium and magnesium can be affected by changes in osmotic pressure(Wook et al., 2001). Commonly, when fish exposed environment stress such as the heavy metal, calcium and magnesium levels were decreased. *O. niloticus* exposed the Cu or Pb was significantly decreased in calcium and magnesium levels(Canli et al., 2015). In our experiment, there was significant difference in AMP 600 mg/kg at 2 weeks and AMP 400 mg/kg at 4 weeks.

Several studies have shown that glucose levels are closely correlated with fish stress and indicate respiratory and nutritional status(Saha et al., 2008). In stressed fish, glucose in the blood was increased, but was decreased in the fish fed AA 3 mg/kg(Ortuño et al., 2002). Catfish exposed to synthetic pyridrine pesticide, Deltamethrin(0.005 mg/L) for 24 hours, showed an increase in glucose level in the blood, whereas the glucose level of catfish fed AA 100 mg/kg for 60 days decreased under the same conditions(Datta et al., 2003). This is due to the increase of plasma AA levels and the decrease of glucose by AA-mediated insulin action, thereby increasing non-oxidative glucose metabolism (Paolisso et al., 1994). In our experiment, glucose level was significantly decreased in AMP 800 mg/kg at 2 weeks and over AMP 600 mg/kg at 4 weeks.

Total protein is an indicator of the nonspecific immune response

of fish fed diets containing AA because it increases the protein activity of the complement system, a nonspecific immune system(Lin and Shiau., 2005). Respiratory burst activity and alternative complement activity of juvenile grouper, *Epinephelus malabaricus* fed AA 288 mg/kg were higher than those fed AA deficient or AA 77 mg/kg(Lin and Shiau., 2005). In addition, total protein of indian major carp fed APP 100 mg/kg was significantly increased(Nayak et al., 2007). Considering our experiment, total protein of starry flounder fed AMP 100 mg/kg at 2, 4 weeks were significantly decreased. As AMP increases, further research on total protein reduction in our experiment is needed.

GOT and GPT are indicators of liver damage. GOT values were significantly decreased in japanese flounder, *Paralichthys olivaceus* fed AA 500 mg/kg or more(Gao et al., 2014). Japanese eel, *Anguilla japonica* was also significantly decreased at AA 762 mg/kg(Ren et al., 2007). However, it has been reported that excess of AA supplement may result in lipid peroxidation in fish tissues resulting in increased GOT values(Gao et al., 2014). GOT and GPT values of labeo rohita fed AA 1000 mg/kg were the highest, and the lowest values were obtained in the control section(Tewary et al., 2008). Kim et al (2017) reported that *S. schlegelii* fed the dietary AsA appeared a considerable decrease in GOT and GPT levels. In our experiment, GOT and GPT values significantly decreased at over AMP 100 mg/kg in 2, 4 weeks. Generally, the feeding AMP dietary has influenced positive effect on serum parameters excepted total

protein.

When a fish eats food, it forms free radicals in the process of creating energy in the mitochondrial membrane of cells. The process of making free radicals into water and oxygen is called antioxidant. Antioxidant enzymes play important a role in maintaining cell homeostasis(Doyotte et al., 1997). Free radical is an oxidized stress material that travels through the body. In addition, free radical is unstable since it does not form a pair, and it takes electrons from neighboring cells. The main role of vitamin c is to neutralize free radicals and can prevent both inside and outside the cell from free radical since vitamin c is water soluble. The free radicals seek to an electron for restoration of stability. Since ascorbic acid is an source of electrons, it can provide an electron to free radicals such as hydroxyl and superoxide ions and neutralize reactivity(Bindhumol et al., 2003).

Free radicals do not only have harmful effects. An appropriate amount of free radicals is used to remove pathogens and foreign substances and ROS act as a signaling material to disease(Ray et al., 2012). Therefore, normal cells maintain adequate amounts of antioxidant and free radicals for keeping homeostasis. The adaptive mechanism of oxidative stress is to scope with stressors to maintain a normal state. However, severe or prolonged stress damages the physiological adaptive mechanism. For this reason, oxidative stress occurs when there are more free radicals or less antioxidant than antioxidant(Panieri and Santoro., 2016).

Superoxide dismutase(SOD) is an enzyme that catalyzes the disproportionate reaction that converts reactive oxygen species (ROS) into oxygen and hydrogen peroxide(H_2O_2). It is known that cells exposed to oxidative stress are antioxidant defense mechanisms. Since the free radical anions of ROS have detrimental effects on cells, SOD plays an important role in defending cells from toxicity by converting superoxide ions into oxygen and hydrogen peroxide. The liver SOD activity of tiger puffer, *Takifugu rubripes* increased as AMP concentration increases, but dietary of AMP 700 mg/kg decreased at SOD activity(Eo et al., 2008). When wuchang bream, *Megalobrama amblycephala* was under heat stress of 34℃, the SOD activity was significantly decreased. However, the SOD activities of fish fed vitamin c were significantly higher than that of control groups(Ming et al., 2012). In other hand, SOD activity of *Bufo raddei* exposed the heavy metals was increased to adjust SOD activity and protect SOD levels against a polluted environment(Zhang et at., 2007). The SOD activity increases in *S. schlegelii* was occurred by the dietary lead exposure due to defence of oxidative damage(Kim et al., 2017). Hussain(2008) also reported that the brain SOD activity of rat was simultaneously increased due to the generation of ROS. In our experiment, the liver SOD activity of starry flounder at 2, 4 weeks were significantly decreased at AMP 400 mg/kg or more. The gill SOD activity of starry flounder were significantly decreased at AMP 100 mg/kg or more at 2, 4 weeks. The kidney and spleen SOD activity of fish fed over AMP

100 mg/kg were significantly decreased at 2, 4 weeks. It seems that ascorbic acid plays a role in providing electrons to free radicals and results that the SOD level in each tissue seem to maintain homeostasis of normal tissue.

Catalase(CAT) is a peroxisomal haem protein as antioxidant enzyme that catalyses hydrogen peroxide(H_2O_2) into water(H_20) and oxygen(O_2). CAT activity of *channa punctatus* exposed to delta-methrin was significantly decreased(Sayeed et al., 2003). CAT activity of *Terapon jarbua* also decreased when fish was exposed to copper(Vijayavel et al., 2006). In other hand, The liver CAT activity of *O. niloticus* was significantly increased by exposing chlorpyrifos. The liver CAT activity of fish exposed with chlorpyrifos and vitamin c was lower than exposed chlorpyrifos alone(O¨zkan et al., 2012). Hussain(2008) also reported that the increase CAT activity of rat brain also was due to the generation of ROS. In our experiment, CAT activities in liver were decreased at over AMP 200 mg/kg at 2 weeks and over AMP 100mg/kg at 4 weeks. In the gill and kidney, over AMP 100 mg/kg were significantly decreased at 2, 4 weeks. In the spleen, over AMP 600 mg/kg were also decreased at 2, 4 weeks. CAT, which plays a role in removing H_2O_2 produced by SOD, is thought to have reduced the CAT activity in response to less H_2O_2 due to AMP diet.

Reduced glutathione(GSH) is an non enzyme antioxidant as ascorbic acid that converts hydrogen peroxide(H_2O_2) into water(H_20) and oxygen(O_2), turning into the oxidized form of glutathione

disulfide(GSSG). When exposed to oxidative stress, glutathione reductase(GR) enzymes are increased to maintain high GSH content in cells and glutathione peroxidase(GPx) enzymes work by using GSH. The GSH levels of african cat fish, *clarias gariepinus* was significantly increased when fish was exposed in heavy metals. This reason is that an adaptive and protective role against oxidative stress by heavy metals(Farombi et al., 2007). In our experiment's results, it was that GSH levels of the liver significantly decreased at over AMP 200 mg/kg at 2 weeks and over AMP 100 mg/kg at 4 weeks. In the case of the gill, GSH levels significantly decreased at over AMP 400 mg/kg at 2 weeks and over AMP 200 mg/kg 4 weeks. In the kidney, over AMP 100 mg/kg at 2, 4 weeks were significantly decreased and in the spleen, over AMP 600 mg/kg at 2, 4 weeks. As a AMP increases, GSH levels tended to decrease. This is due to the lower amount of H_2O_2 than fish fed less AMP.

Glutathione-S-transferase(GST) is enzymes that catalyze reduced glutathione to form glutathione complex through thioether linkages (Baysoy et al., 2012). In vivo, it is involved in the detoxification of various cytotoxic substances, cell defense from oxidative damage. GST activity according to the metal exposure are conformed by GSH depletion because GSH is essential for functioning of GST(Elia et al., 2003). The hydroxyl radical(OH•) is reactive to a lipid to yield the lipid alkyl radical(L•) and L• combines with O_2 to form the lipid peroxyl radical(LOO•) which yield the lipid hydroperoxides(LOOH) and GST act as remove directly LOOH(Meda et al., 2019). Vitamin

E(α-tocopherol) also act as a radical scavenging antioxidant and LOO• is scavenged by α-tocopherol(Etsuo Niki., 2014). After that, α-tocopherol becomes α-tocopheroxyl radical and α-tocopheroxyl radical is regenerated to α-tocopherol by AA(Rock et al., 1996). Although AA does not directly remove LOO•, it interacts with α-tocopherol to reduce LOO•. For this reason, when fish fed AMP, the production of LOOH has decreased, which seems to have reduced GST activity. In our experiment, The liver GST activity of fish fed diet including over AMP 100 mg/kg at 2 weeks and over AMP 50 mg/kg at 4 weeks were significantly decreased. The gill GST activity of fish fed diet over AMP 400 mg/kg and over AMP 100 mg/kg were substantially decreased. The kidney GST activity of over AMP 200 mg/kg at 2 weeks and over AMP 100 mg/kg at 4 weeks was significantly decreased. The spleen GST activity of AMP 200 mg/kg at 2, 4 weeks was significantly decreased.

Lysozyme activity is one of the non-specific immune system. Ai et al., (2004) reported that lysozyme activity of japanese seabass, *Lateolabrax japonicus* was significantly elevated by increasing ascorbic acid dietary doses. The lysozyme activity of Juvenile grouper, *Epinephelus malabaricus* also was higher in over AA 76 mg/kg than other AA dietary(Lin and Shiau., 2005). Tiger puffer, *Takifugu rubripes* of lysozyme activity was significantly increased at AMP 80 mg/kg and 160 mg/kg(Eo et al., 2008). Yellow croaker, *Pseudosciaena crocea* of lysozyme activity was significantly increased at 500 mg/kg(Ai et al., 2006). In our study, the serum

lysozyme activity were significantly increased at over AMP 400 mg/kg at 2 weeks and over AMP 200 mg/kg at 4 weeks.

Fish seem to depend on non-specific immune system for disease and ascorbic acid affects the positive effect of fish immune system(Roberts et al., 1995). Phagocytosis is also the non-specific immune system factor and a primitive defence mechanism of both vertebrates and invertebrates (Harford et al., 2006). Fish has several types of phagocytic leucocytes in blood, a variety of tissue locations(Secomebes., 1994). Tewary et al., (2008) reported that vitamin C enhanced phagocytic activity. In our study, phagocytosis were significantly increased at over AMP 600 mg/kg at 2 weeks and AMP 400 mg/kg at 4 weeks. This suggests that AMP dietary improve the non-specific immune of starry flounder.

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