A THESIS

FOR THE DEGREE OF MASTER OF SCIENCE

Effect of dietary ascorbic acid on growth and non-specific immune responses of tiger puffer, *Takifugu rubripes*

Jinee Eo

Department of Marine Life Science

GRADUATE SCHOOL

CHEJU NATIONAL UNIVERSITY

2009. 2.

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Jinee Eo

(Supervised by Professor Kyeong-Jun Lee)

A thesis submitted in partial fulfillment of the requirement for the degree of Master of Science

2009. 2.

This thesis has been examined and approved.

.....

Kwang-Sik Choi, Prof. of Marine Life Science

.....

Joon Bum Jeong, Full-time lecturer of Marine Life Science

.....

Kyeong-Jun Lee, Assistant Prof. of Marine Life Science

2009. 02

Date

Department of Marine Life Science

GRADUATE SCHOOL

CHEJU NATIONAL UNIVERSITY

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국문초록

Vitamin C(ascorbic acid, AA)는 어류를 포함하여 정상적인 대사 작용에 필수적이다. Vitamin C는 항산화 작용, collagen 합성, carnitine, norepinephrine, neuroendocrine peptides 생합성에 중요한 역할을 한다. 그러나 대부분의 경골어류는 vitamin C를 합성하는 L-gulonolatone oxidase의 효소가 없기 때문에 vitamin C를 합성할 수 없다. 따라서 효소가 없는 동물은 vitamin C를 자체 생성하지 못하므로 식품을 통해 공급 받아야 한다. 자주복은 횟감으로 선호도가 좋아 우리나라 최근 새로운 양식 어종으로 각광 받는 어종이다. 이 연구는 자주복에 있어서 vitamin C의 요구량이 아직까지 밝혀진 바가 없으므로, 자주복에 있어서 사료 내 vitamin C의 적정 요구량과 간 내 vitamin C 축적함량, collagen 축적함량 그리고 면역에 미치는 영향을 조사하고자 수행되었다.

Casein과 gelatin을 기초로하는 사료에 L-ascorbyl-2-monophosphate의 함량을 0, 40, 80, 160 그리고 700 mg/kg으로 첨가하여 (AMP0, AMP40, AMP80, AMP160 그리고 AMP700) 실험이 수행되었다. 10주간의 실험결과 vitamin C가 결핍된 AMP0 실험구에서 vitamin C가 첨가된 실험구에 비하여 유의적으로 낮은 성장을 보였다. 또한 AMP0 실험구에서 유의적으로 낮은 hematocrit, condition factor, HSI를 보였다. 대식세포 활성 분석결과 AMP0 실험구가 유의적으로 낮았으며 lysozyme activity 분석결과 AMP80, AMP160 실험구에서 AMP0 실험구에 비하여 유의적으로 높았다. 간 내 superoxide dismutase 함량은 사료 내 vitamin C 첨가 농도와 유의적으로 비례하였으며 myeloperoxidase activity 분석결과 AMP0 실험구가 다른 실험구에 비하여 유의적으로 낮았다. 간에서의 vitamin C 축적농도는 사료 내 vitamin C 첨가 농도와 유의적으로 비례하였고 bone collagen 함량 역시 vitamin C 첨가 농도와 비례하는 경향을 보였으나 유의적인 차이는 나타나지 않았다(Part I).

10주간의 이전 실험을 통해서(Part I) 자주복에 있어서 AA는 정상적인 성장과 생리적용, 면역기능에 있어서 필수적임을 알 수 있었으나 척추측만, 척추전만, 껍질이 벗겨지는 현상, 콜라겐 합성 등 명확한 vitamin C 결핍증상을 관찰 할 수 없었다. 따라서 두번째 실험은 33주의 실험을 통하여 명확한 vitamin C의 결핍증을 알아보기 위하여 수행되었다.

10주 후, 고기를 선별하여 150 L 사육수조에 재배치 하였다. Casein과 gelatin을 기초로 하는 사료에 L-ascorbyl-2-monophosphate의 함량을 0, 80, 700 mg/kg으로 첨가하여 (AMP0, AMP80, 그리고 AMP700) 실험이 수행되었다. 27주 후 AMP0 실험구에서 50%의 어류가 빈혈증상을 보였으며 30주 후에는 대부분의 실험어류에서 빈혈증상이 관찰되었다. 33주 후에는 20%의 어류의 담즙이 검게 되는 현상을 관찰하였으며 10% 의 어류가 폐사하였다. 31주 후부터는 실험어류의 껍질이 벗겨지는 현상이 관찰되었다. 간에서의 vitamin C 축적농도는 사료 내 vitamin C 첨가 농도와 유의적으로 비례하였고 bone collagen 함량 역시 vitamin C 첨가 농도와 유의적으로 비례하였다(Part II).

위 결과들을 종합해 볼 때, 자주복에 있어 사료 내 vitamin C의 적정 함량은 성장에 기초하여 27ppm, 면역 작용에 있어서는 82ppm이상의 vitamin C가 첨가되어야 한다고 판단된다. 그러나 사료 내 더 높은 함량의 vitamin C 첨가는 (> 160 mg/kg) 불필요한 것으로 판단된다.

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REFERENCE REVIEW

Vitamin C (ascorbic acid, AA) is an essential vitamin for normal physiological functions in animals including fish [1,2]. AA is a strong antioxidant and essential for collagen synthesis, helps to maintain various enzymes in their required reduced form, and participates in the biosynthesis of carnitine, norepinephrine and certain neuroendocrine peptides [3]. However, most teleosts cannot synthesize AA due to the lack of L-gulonolactone oxidase (EC 1.1.3.8) which is the key enzyme for AA synthesis [4]. Thus, an exogenous source of AA is required in fish diets. Many studies reported the positive effects of AA on growth performances of fish [5–10]. On the other hand, there were some contradictory reports that dietary AA did not improve the growth performance of seabream [3] and yellow croaker [11].

AA is also known to be beneficial for immune responses in fish, even though its exact mechanism has not been demonstrated. A number of studies reported the improved immune responses and disease resistance in many fish species by feeding higher level of dietary AA than required. Increased immunity was demonstrated by the increased immunological parameters, such as lysozyme, complement activities, phagocytic activity, and respiratory burst [9–12].

AA is very unstable in heat and light. Phosphate derivatives of AA, which is more stable and bioavailable forms of AA, are currently available and shown to have antiscorbutic activity in channel catfish [5], tilapia [13], rainbow trout [14], yellowtail [15], olive flounder [16] and parrot fish [17].

No information on AA is available in tiger puffer *Takifugu rubripes* which is one of the emerging and important aquaculture species. The species is known to have a high market value in China, Japan, and Korea. Therefore, this study was conducted to determine the essentiality and requirements of AA in diets for the tiger puffer based on growth performances, liver AA and bone collagen concentrations, and non-specific immune responses of juvenile tiger puffer.

AA deficiency symptoms such as spinal deformation, impaired collagen formation, internal hemorrhage and retarded growth [28, 34, 35] have been reported. Duncan and Lovell [47] found hematological abnormalities in channel catfish fed diets without AA. Lack of AA in diet reduced efficiency of intestinal iron absorption in rainbow trout [48]. Ai et al. [10] reported that Japanese seabass fed AA free diets for 8 weeks exhibited scoliosis, lordosis and caudal fin erosion. Wang et al. [16] reported that olive flounder fed without AA supplementation exhibited deficiency signs such as scoliosis and lordosis after 12 weeks.

In the previous study (Part I) we found that tiger puffer needs adequate exogenous AA to maintain normal growth, physiological functions and immune response during 10 weeks feeding trial. However, none of the signs of vitamin C deficiency including scoliosis, lordosis, skin erosion and impaired collagen formation were observed during the feeding period. Therefore, the second study was conducted to examine the effect of vitamin C deficiency on tiger puffer for 33 weeks of feeding trial.

Part Ⅰ

Effect of dietary ascorbic acid on growth and non-specific immune responses of tiger puffer, *Takifugu rubripes*

Part 1

Effect of dietary ascorbic acid on growth and non-specific immune responses of tiger puffer, *Takifugu rubripes*

ABSTRACT

We report nutritional physiology and non-specific immune responses of ascorbic acid (AA) in puffer fish for the first time. This study aimed to examine the essentiality and requirements of AA in diets for the tiger puffer, *Takifugu rubripes* based on growth performances, liver AA and bone collagen concentration, and non-specific immune responses. Five casein-gelatin based semi-purified diets were formulated to contain five graded levels of L-ascorbyl-2-monophosphate at 0, 40, 80, 160 and 700 mg/kg (designated as AMP0, AMP40, AMP80, AMP160 and AMP700, respectively) and fed to triplicate groups of fish. After 10 weeks of feeding trial, growth performances of fish (initial body weight, 35 g) fed the AMP0 were significantly lower compared to that of fish fed diets supplemented with AMP. The fish fed the AMP0 diet also exhibited significantly lower hematocrit, condition factor and hepatosomatic index compared to the fish fed diets supplemented with AMP. Phagocytic acitivity (NBT assay) was significantly lower in fish fed the AMP0 diet than in fish fed the AMP containing diets. Plasma lysozyme activity of fish fed the AMP 80 and AMP 160 was significantly higher than that of fish fed the AMP0. Dietary supplementation of AMP significantly increased the liver superoxide dismutase in the fish. Myeloperoxidase activity of fish fed the AMP0 was significantly lower compared to that of fish fed the AMP containing diets. Bone collagen level tended to increase numerically and total AA concentration in liver of fish was significantly increased in a dose dependent manner by the supplementation of AMP. Therefore, tiger puffer requires exogenous ascorbic acid and the optimum dietary level could be 29 mg AA/kg diet for normal growth and physiology. Dietary AA concentration over 82 mg/kg could be required to enhance non-specific immune responses of the fish. However, it does not seem that the fish needs an overdose of dietary AA (> 160 mg/kg) for a better non-specific immune responses.

MATERIALS AND METHODS

Experimental diets

Five casein-gelatin based semipurified diets were formulated (Table 1) to contain five different levels of L-ascorbyl-2-monophosphate (0, 40, 80, 160, and 700 mg AA equivalent/kg, designated as AMP0, AMP40, AMP80, AMP160 and AMP700, respectively) at the expense of cellulose. L-ascorbyl-2-monophosphate was claimed to have 35% ascorbic acid activity by Woo-sung feed Co., Ltd. (Daejeon, Korea). Ethanol-extracted fish meal was employed in the diets as an attractant to enhance palatability in semi-purified diets [18]. Fish meal was extracted twice using 70% aqueous ethanol solution for 48 h, and then the extracted fish meal was completely dried using an electric fan at room temperature. AA concentration in the test diets was measured according to Dabrowski and Hinterleitner [19]. The analyzed AA concentrations of the test diets were 0, 35.8, 88.6, 157 and 688 mg/kg diet for AMP0, 40, 80, 160, and 700, respectively (Table 2).

The experimental diets were prepared by thoroughly mixing ingredients with oil and 35% distilled cold water in a mixer (NVM-14-2P, Korea). The wet dough was pelleted by a chopper machine (SMC-12, Kuposlice, Busan, Korea) at 4 mm of diameter. Then, the diets were freeze-dried for 24 h, crushed into desirable particle sizes (1 – 3.0 mm) and stored at – 45 ℃ until use.

Fish and feeding trial

Juvenile tiger puffer were transported from a private hatchery (Sa-Jo Fisheries Co.) in Jeju Island to Marine and Environmental Research Institute, Cheju National University, Jeju, Korea. All the transported fish were fed a commercial diet for 1 month to be acclimated in the experimental facilities and conditions. After the acclimation, the fish (initial body weight 35.0±0.04g) were randomly assigned to eighteen 150 L polyvinyl conical tanks (triplicate groups per dietary treatment) at a density of 15 fish/tank. The feeding trial was conducted for 10 weeks in a flow through system supplied with sand filtered seawater. Aeration was also provided to maintain enough dissolved oxygen levels. The photoperiod was scheduled by 11:13 h (light/dark) by fluorescent light. Water temperature ranged from 15 to 21 ℃

according to the seasonal change. Salinity of the water was maintained at 32-34 ppt, dissolved oxygen was ranged from 7.80 to 8.05 mg/L, and pH was 8.02±0.01. The experimental diets were fed to the fish at a feeding rate of 1-2 % body weight twice daily (9:00 and 17:00 h). After feeding, uneaten feeds were removed by siphoning to calculate feed utilization. Inside of the tanks were routinely cleaned by a sponge to prevent the growth of microflora. The growth of fish was measured every three weeks and feeding rate was adjusted accordingly. Feeding was stopped 24 h prior to weighing.

Table 1-1. Composition of the basal diet (%DM).

Ingredients	%
Casein (vitamin free) [1]	28.0
Gelatin[1]	7.0
Fish meal (defatted) [2]	15.0
Dextrin[1]	21.0
Starch	10.0
Squid liver oil	14.0
Vitamin Mix.(vitamin C free) [3]	2.0
Mineral Mix.[4]	2.0
Cellulose	1.0

[1] United States Biochemical (USB), Cleveland, OH, USA.
[2] White fishmeal, Rukoss, Russia. Fish meal were extracted by 70% aqueous ethanol (water : ethanol = 3 : 7) for 48h.
[3] Vitamin premix (g/kg of mixture): retinyl acetate, 1.0; cholecalciferol,0.05; menadione, 0.2; thiamine hydrochloride, 4.0; riboflavin, 4.4; d-pantothenic acid hemicalcium, 14.5; pyridoxine hydrochloride, 4.0; cyanocobalamin, 0.01; niacinamide, 30.0; folic acid, 0.48; d-biotin, 0.2; *myo*-inositol, 40.0; α-tocopherol, 10.0.
[4] Mineral mixture was based on the composition of Lee et al., 2003[45].

Table 1-2. Analyzed ascorbic acid concentrations and proximate composition of the experimental diets (% DM).

Diets	Added AA levels (mg kg^{-1})	Analyzed AA levels (mg kg^{-1})	Dry matter (%)	Protein (% DM)	Lipid (% DM)	Ash (% DM)
AMP0	0	0	92.4	42.3	13.5	3.1
AMP40	40	35.8	92.7	42.4	13.9	3.1
AMP80	80	88.6	89.4	42.2	13.9	3.2
AMP160	160	157	89.6	42.5	13.7	3.1
AMP700	700	688	89.5	42.5	13.8	3.1

Values are means from duplicate sample of experimental diets.

Sample collection and analyses

At the end of the 10 weeks of feeding trial, all fish were weighed and counted for the calculations of growth performances, feed utilizations and condition factor. Four fish per tank (12 fish per dietary treatment) were randomly collected and anaesthetized with 2-phenoxyethanol (100 ppm). Blood was taken from the caudal vein for the determination of hematocrit, hemoglobin, respiratory burst and lysozyme and myeloperoxidase activities.

After fish were bled for the hematological analyses and non-specific immune responses, liver was removed on an icy-plastic board, weighed for calculating hepatosomatic index (HSI) and stored at -70°C for further vitamin C analysis. Total ascorbic acid (TAA) and dehydroascorbic acid (DHAA) concentrations in liver were measured using the 2,4-dinitrophenylhydrazine (DNPH) colorimetric method described by Dabrowski and Hinterleitner [19]. Sample was homogenized in 5% trichloroacetic acid (Sigma, USA) solution containing 250 mM $HClO_4$ (Sigma, USA) and 0.08% EDTA (Sigma, USA). The homogenate was centrifuged 15,000 rpm for 30 min at 4°C. Twenty five micro liter of 0.2% dichorophenolindophenol (Sigma, USA) was added into supernatants (250 μl) and incubated at room temperature for 20 min. Then 250 μl of 2% thiourea (Sigma, USA) in 5% meta-phosphoric acid (MPA; Sigma, USA) and 250 μl of 2% DNPH (Sigma, USA) were added to the mixture and incubated at 60°C for 3 hours. The absorbance of the mixture was measured at 524 nm after adding 500 μl of ice cold 18M sulfuric acid (Fluka, Germany). For DHAA, 250 μl of tri-distilled water was added instead of MPA. L-ascorbic acid (Sigma, USA) was used to develop a standard curve.

Vertebral columns were analyzed for bone collagen content using the method of Wilson and Poe [1] with some modifications by Ai et al. [11]. Approximate compositions of the experimental diets were analyzed by the method of AOAC [20].

Monitoring of non-specific immune responses

The oxidative radical production by phagocytes during respiratory burst was measured by the nitro-blue-tetrazolium (NBT; Sigma, USA) assay described by Anderson and Siwicki [21] with some modifications by Kumari and Sahoo [22]. Briefly, blood and 0.2% NBT were mixed in equal proportion (1:1), incubated for 30 min at room temperature, then 50 μl was taken out and dispensed into glass tubes. Then, 1 ml of dimethylformamide (Sigma, USA) was added and centrifuged at 2000 x g for 5 min. Finally, the optical density of supernatant was measured at 540 nm. Dimethylformamide was used as the blank.

Plasma lysozyme activity was determined by a turbidometric assay utilizing lyophilized

Micrococcus lysodeikticus cells (Sigma, USA). Its original method [23] was slightly modified by Swain et al. [24]. Briefly, *M. lysodeikticus* at a concentration of 0.2 mg/ml (in 0.02 M sodium citrate buffer) was added to plasma samples at 10:1 ratio, and the OD of the mixture was immediately read at 450 nm. After incubating for 1 h at 24℃, the final OD was read. Lyophilized hen egg white lysozyme (HEWL; Sigma, USA) was used for a standard curve. Plasma activity was expressed as μg/ml equivalent of HEWL activity.

Liver Superoxide dismutase activity was determined by the superoxide dismutase assay kit (Cayman, Ann Arbor, USA).

Myeloperoxidase activity was measured according to Quade and Roth [25] with a slight modification by Kumari and Sahoo [22]. Briefly, serum (20 μl) was diluted with HBSS (Hans balance solution without Ca^{2+} or Mg^{2+}, Sigma, USA) in 96-well plates. Then, 35 μl of 20 mM 3.3', 5, 5'-tetramethylbenzidine hydrochloride (Sigma, USA) and 5 mM H_2O_2 were added. The color change reaction was stopped after 2 min by adding 35 μl of 4 M sulfuric acid. Finally, OD was read at 450 nm.

Statistical analysis

All experimental diets were assigned by a completely randomized design. Data were subjected to one-way ANOVA in SPSS version 11.0 (SPSS Inc., Chicago, IL, USA). The significant differences ($P < 0.05$) between group means were compared using Duncan's multiple range test [26]. Dietary ascorbic acid requirement for the fish was estimated by broken-line regression method based on growth performance and non-specific immune response data. Data are presented as mean±SD. Percentage data were arcsine transformed before analysis.

RESULTS

Growth performances

Weight gain and specific growth rate of fish fed AMP supplemented diets were significantly ($P<0.05$) higher than those of fish fed the control diet (no AMP) deficient in AA. Dietary AMP over 88 mg AA/kg diet significantly improved the feed efficiency and protein efficiency ratio of the fish whereas a lower dietary AMP concentration (~36 mg/kg diet) did not. The survival of fish fed diets the AMP0 and AMP40 were significantly lower compared to that of fish fed diets AMP80 and AMP160.

Table 1-3. Weight gain (WG), specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency ratio (PER) and survival of tiger puffer fed graded levels of AA for 10 weeks.

Diets	AMP0	AMP40	AMP80	AMP160	AMP700
WG [1]	34.27 ± 1.66^{a}	42.39 ± 2.66^{cd}	45.62 ± 2.92^{d}	38.31 ± 0.85^{b}	38.73 ± 1.75^{bc}
SGR[2]	0.81 ± 0.03^{a}	0.94 ± 0.04^{bc}	0.99 ± 0.04^{c}	0.88 ± 0.02^{b}	0.89 ± 0.03^{b}
FCR[3]	2.1 ± 0.21^{a}	1.9 ± 0.10^{ab}	1.5 ± 0.10^{c}	1.7 ± 0.13^{bc}	1.8 ± 0.07^{ab}
PER[4]	1.1 ± 0.11^{a}	1.2 ± 0.06^{ab}	1.6 ± 0.10^{c}	1.4 ± 0.11^{bc}	1.3 ± 0.05^{ab}
Survival	73 ± 6.67^{a}	71 ± 7.70^{a}	90 ± 4.71^{b}	90 ± 4.71^{b}	80 ± 0^{ab}

Data were presented as mean±SD ($n = 3$). Value in the same row having different superscripts is significantly different ($P<0.05$).

[1] WG (g): (final mean body weight (g)-initial mean body weight (g)

[2] SGR (%): 100×(ln final mean body weight-ln initial mean body weight)/days

[3] FCR: dry feed fed/ wet weight gain

[4] PER (%): wet weight gain (g)/total protein given (g)

Hematological parameters, hepatosomatic index and condition factor

Hemoglobin was not significantly different among all treatments. However, the hematocrit of fish fed the AMP0 diet was significantly lower than that of fish fed the diets containing AMP except for the AMP160. Interestingly, hepatosomatic index (HSI) of the fish fed the AMP0 was significantly lower than that of fish fed diets supplemented with AMP (Table 1-4). Fish fed the AMP0 diet exhibited significantly lower condition factor compared to the fish fed the AMP80 and AMP160.

Table 1-4. Hemoglobin, hematocrit, HSI and CF of tiger puffer fed graded levels of AA for 10 weeks.

Diets	AMP0	AMP40	AMP80	AMP160	AMP700
Hemoglobin (g/dl)	6.00±0.94	6.90±0.80	7.03±0.80	6.30±0.15	6.28±0.94
Hematocrit (%)	20.3±0.88^{a}	23.7±2.31^{b}	23.4±0.38^{b}	22.3±0.33^{ab}	22.8±1.35^{b}
HSI[1]	7.98±0.19^{a}	9.61±0.80^{b}	10.17±1.38^{b}	9.49±0.42^{b}	9.87±0.28^{b}
CF[2]	3.31±0.26^{a}	3.69±0.22^{ab}	3.91±0.22^{b}	3.73±0.20^{b}	3.69±0.15^{b}

Data were presented as mean±SD (n = 3). Value in the same row having different superscripts is significantly different ($P<0.05$).
[1] HSI: [liver weight (g)/body weight (g)] ×100
[2] CF: [fish weight (g)/fish length (cm^3)] ×100

Monitoring of non-specific immune responses

Non-specific immune responses such as nitro-blue-tetrazolium (NBT), lysozyme, liver superoxide dismutase (SOD) and myeloperoxidase (MPO) activities are provided in Figure 1-1. NBT activity was significantly lower in fish fed the AMP0 diet deficient in AA than in fish fed the AMP supplemented diets. Plasma lysozyme activity of fish fed the AMP 80 and AMP 160 was significantly higher than that of fish fed the AMP0. Dietary supplementation of AMP significantly increased the liver SOD in the fish. MPO of fish fed AMP0 was significantly lower compared to that of fish fed diets containing AMP. Beneficial effects on the non-specific immune responses of the tiger puffer were not clearly observed in the dietary AA level over 160 mg/kg during the 10 weeks of feeding trial.

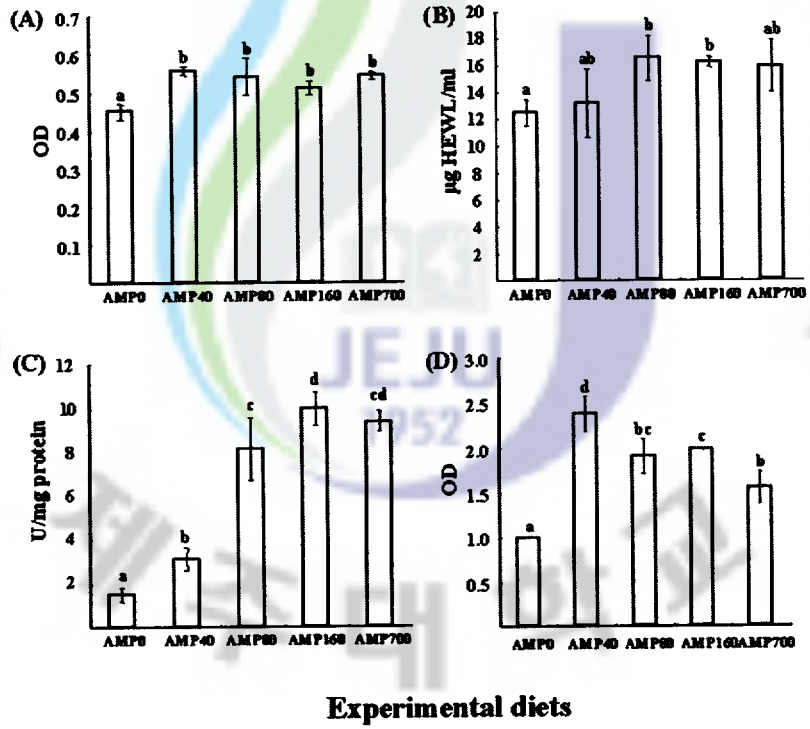

Fig. 1-1. Non-specific immune responses of tiger puffer fed graded levels of L-ascorbyl-2-monophosphate for 10 weeks: Nitro-blue-tetrazolium (NBT) assay (A), serum lysozyme activity (B), liver superoxide dismutase (SOD) activity (C) and myeloperoxidase (MPO) activity (D)

Bone collagen and liver ascorbic acid concentrations

Bone collagen tended to increase as dietary AMP increased, although there were no significant differences among all the dietary treatments (Fig. 1-2). Total AA concentration in liver of fish was significantly increased in a dose-dependent manner by the supplementation of dietary AMP. The ratio of dehydroascorbic acid to total AA concentrations in the liver of the fish was decreased by higher supplementation of the AMP over 89 mg/kg (diet AMP 80) compared to that of fish fed diets deficient or lower in AMP (~36 mg/kg).

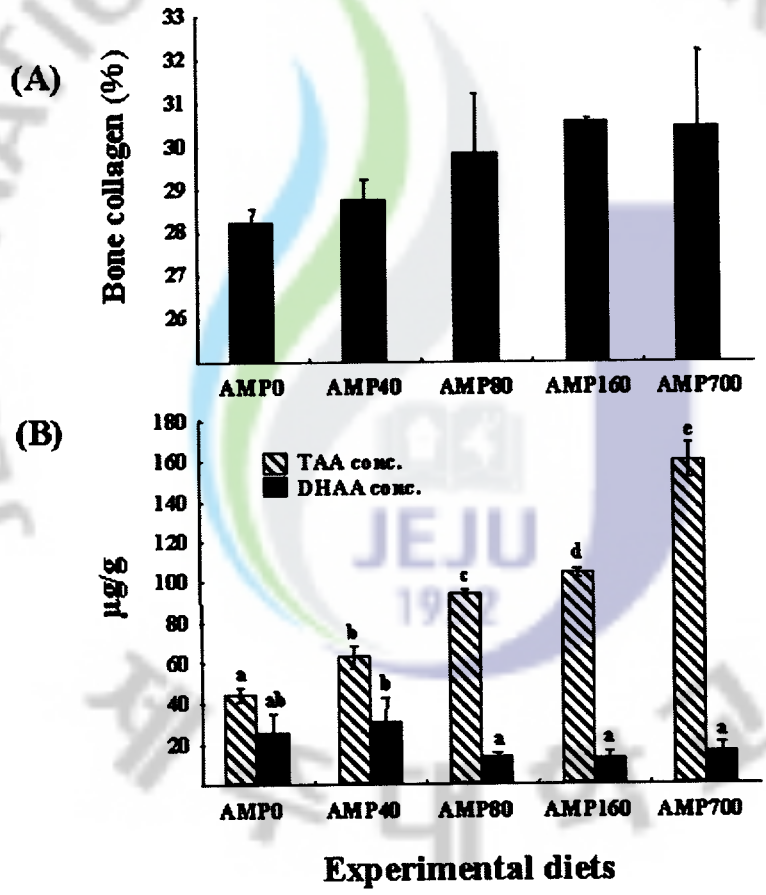

Fig. 1-2. Bone collagen (A) and total and dehydroascorbic acid (DHAA) concentrations in the liver (B) of tiger puffer fed graded levels of L-ascorbyl-2-monophosphate for 10 weeks.

DISCUSSION

Phosphate derivatives of AA is hydrolyzed in fish by intestinal alkaline phosphatase [27] and is as effective as AA when supplemented in diets on AA equivalent basis [17]. In the present study AA activity was well proved when supplemented in diets as the type of AMP (L-ascorbyl-2-monophosphate). The results in growth performances in the present study (Table1-3) indicate that the tiger puffer needs adequate exogenous AA to maintain normal growth and physiological functions, which agree with most previous studies on other fish species [13,17,28]. And, the estimated minimum dietary vitamin C level for the maximum growth performance of tiger puffer was approximately 36 mg AA /kg diet. The present result is the first in tiger puffer in terms of AA nutrition, to our knowledge, and is very significant in feed formulation for the species. The tiger puffer grew well during the feeding trial and the growth performance in this study was comparable and/or higher than that in other studies with puffer fish [29–31]. Kikuchi et al. [29] observed that the weight gain of tiger puffer (180 g) was 33 - 100 % when fed with a commercial feed for 12 weeks. River puffer (18 g) grew by 100 % body weight when fed with a commercial feed for 120 days [30] and tiger puffer (30 g) grew by 6 – 33 % when fed with a semipurified diet for 3 weeks [31]. The fish tended to bite each other and most of the fish after 10 weeks of the feeding trial did have injured caudal fin, which is a common symptom by the specific behavior of puffer fish. It seemed that the mortality (Table 1-3) was caused by biting behavior of the fish.

The required dietary AA level for tiger puffer in the present study was higher than that for other fish species. Dietary AA requirements based on growth performances ranged from 10 to 25 mg AA/kg in channel catfish [32], rainbow trout [33] and hybrid tilapia [13]. However, the required dietary AA for tiger puffer was lower than that for tilapia (100-200 mg/kg)[28], parrot fish (118mg/kg)[17] and olive flounder (93mg/kg)[16]. The differences are probably due to differences in fish species, feeding behavior, forms of AA, feed compositions, rearing conditions and nutritional status of fish.

Many studies reported that the signs of vitamin C deficiency as spinal deformation, impaired collagen formation, internal hemorrhage and retarded growth [28, 34, 35]. In the present study, however, no signs of clinical deficiency were observed during the experimental period. This result was similar to previous studies in gilthead seabream [3] and yellow croakers [11]. Dabrowski [36] reported that the deficiency of AA is affected by fish size, feeding duration and previous nutrition status. In the present study the fish size was relatively larger compared to many other studies on the requirement of vitamin C [10, 13, 35]. Ai et al. [11] reported that there was no AA in muscle of yellow croaker (initial body

weight, 17.82 g) fed AA free diet, however, AA was still detected in its liver (16.6 μg/g wet weight). This is similar to the results of Ai et al. [10] that also detected no AA in muscle of seabass, but detected in its liver tissue (16.5 μg/g wet weight) after feeding AA free diet for 10 weeks. These results confirmed that large fish depleted AA relatively slower. It suggests that longer experimental duration and smaller fish should be adapted to further evaluation of AA functions in terms of clinical deficiency signs for tiger puffer.

In the present study, HSI and condition factor were positively correlated to dietary AMP supplementation (Table 4). This result was similar to previous studies on olive flounder, [16] and Korean rockfish [37]. However, the HSI value of tiger puffer (8.0-10.2) was much higher than previous studies (0.7-3.8) on other fish species [3,16,17,37]. It is well known that the tiger puffer has a larger liver compared to the other fish species. Ghosh et al. [38] reported that the HSI in different puffer fish ranged from 3.1 to 15.0 depending on the season and HSI was the highest in spottyback puffer (15.0%) followed by green rough-backed puffer (7.9%), and smoothback puffer (9.1%) in the monsoon (July–October). The large liver of the tiger puffer could explain its AA concentration (~40 μg /g) in the control groups (AMP0 diet) and no clinical deficiency signs in the present study of 10 weeks.

In fish, non-specific immune system is more important for disease resistance than specific immune system [39]. The microbicidal activity is led by a production of reactive oxygen species due to an abrupt rise in oxygen consumption of organisms. In the present study mean phagocytes activated with NBT were significantly increased by the supplementation of dietary AMP (Fig.1-1). Ortuno et al. [9] reported that enhanced immune response was observed in gilthead seabream by the dietary intake of AA after 8weeks. Lysozyme is released by leukocytes and plays an important role in antibiotic activity. Lysozyme activity is positively correlated with supplementation of AA [10.11]. In the present study such a correlation was also observed indicating that proper level of dietary AA could improve the immunity of tiger puffer. The superoxide dismutase (SOD), an antioxidant enzyme, removes damaging reactive oxygen species by catalyzing dismutation of two superoxide radicals to hydrogen peroxide and oxygen [40]. The SOD activity of tiger puffer in this study was increased with increased AMP in a dose dependent manner suggesting that SOD activity can be used as a good parameter for the immune response to dietary AA in the fish. However, megadose (~700 mg/kg) of dietary AA did not improve the immunity of the fish compared to optimal levels (80-160 mg/kg) in this study.

Myeloperoxidase (MPO), an important enzyme having microbicidal activity, utilizes one of the oxidative radical (H2O2) to produce hypochlorous acid [41], which is potent in killing pathogens. This process is believed to be important in killing microbes. In the present

study MPO activity of fish fed AMP-supplemented diets was significantly higher than that of fish fed the control diet (Fig.1-1). Kumari and Sahoo [42] also determined the MPO activity in Asian catfish and reported an improved immune response by dietary intake of AA for 8 weeks. The findings with respect to the immune parameters in this study suggest that the optimal supplementation of dietary AMP could enhance non-specific immune responses of tiger puffer. In general, AA is known to enhance immune responses as a free radical scavenger. The reason for the enhanced non-specific immune system of the tiger puffer in this study might be attributed to the mechanism by protecting cells from auto-oxidation and maintaining their integrity for an optimal function of the immune system, even though its exact mechanism has not been verified yet in the fish.

Lim and Lovell [2] found that bone collagen was below 29.5% for the impaired collagen formation in channel catfish and suggested that it could be a criterion for scurvy. In the present study, bone collagen tended to increase from 28.2% to 30.5% as the dietary AMP increased, although there was no significant difference among all the treatments (Fig.1-2). The value was higher than yellow croaker as16.5-20.2% [11] and lower than Japanese seabass and yellow sea bream [43] as 40.7% and 40.1%, respectively.

Total and reduced AA concentrations in liver of tiger puffer were increased with increasing dietary AMP in a dose dependent manner in the present study (Fig. 1-2). The significant increase in the ratio of reduced AA to total AA in this study indicates significantly enhanced tissue antioxidant properties that might be an explanation for the improved immunity [44]. The liver AA concentration in this study was similar to that of other studies with other fish species that were ranged from 17 to 164 μg/g wet tissue [10,13,16]. In the present study higher concentration of AA was detected in the liver of the fish fed the diet containing higher levels of AMP. This indicates that AMP is hydrolyzed and converted to AA in the fish body and stored into liver suggesting that AMP could be used as dietary AA source for tiger puffer.

In conclusions, dietary ascorbic acid requirement for the normal growth and the increased non-specific immune system of the fish was estimated by broken-line regression method based on growth performance and non-specific immune responses; Tiger puffer requires exogenous ascorbic acid and the optimum dietary level is 29 mg AA/kg diet for normal growth and physiology. Dietary AA concentration over 82 mg/kg could be required to enhance non-specific immune responses of the fish. However, it does not seem that this species needs an overdose of dietary AA (> 160 mg/kg diet) for better non-specific immune responses.

Part Ⅱ

Vitamin C deficiency in the tiger puffer, *Takifugu rubripes*

Part Ⅱ

Vitamin C deficiency in the tiger puffer, *Takifugu rubripes*

MATERUALS AND METHODS

Experimental diets

Three casein-gelatin based semipurified diets were formulated (Table 2-1) to contain three different levels of L-ascorbyl-2-monophosphate 0, 80 and 700 mg AA equivalent/kg, (designated as AMP0, AMP80 and AMP700, respectively) at the expense of cellulose. Ethanol-extracted fish meal was employed in the diets as an attractant to enhance palatability in semi-purified diets [18]. The analyzed AA concentrations of the test diets were 0, 88.6 and 688 mg/kg diet for AMP0, 80 and 700, respectively.

The experimental diets were prepared by thoroughly mixing ingredients with oil and 35% distilled cold water in a mixer (NVM-14-2P, Korea). The wet dough was pelleted by a chopper machine (SMC-12, Kuposlice, Busan, Korea) at 4 mm of diameter. Then, the diets were freeze-dried for 24 h, crushed into desirable particle sizes (1 – 3.0 mm) and stored at – 45 ℃ until use.

Fish and feeding trial

Juvenile tiger puffer were transported from a private hatchery (Sa-Jo Fisheries Co.) in Jeju Island to Marine and Environmental Research Institute, Cheju National University, Jeju, Korea. All the transported fish were fed a commercial diet for 1 month to be acclimated in the experimental facilities and conditions. After acclimated, we conducted the effect of dietary AA on growth and immune responses of tiger puffer for 10 weeks. After 10 weeks, we selected fish and randomly assigned to six 150 L polyvinyl conical tanks (duplicates groups per dietary treatment) at a density of 10 fish/tank. The feeding trial was conducted for 33 weeks in a flow through system supplied with sand filtered seawater. Aeration was also provided to maintain enough dissolved oxygen levels. The photoperiod was scheduled by 11:13 h (light/dark) by fluorescent light. Water temperature ranged from 14 to 22 ℃ according to the seasonal change. Salinity of the water was maintained at 32-34 ppt,

dissolved oxygen was ranged from 7.80 to 8.05 mg/L, and pH was 8.02±0.01. The experimental diets were fed to the fish by satiation twice daily (9:00 and 17:00 h). Inside of the tanks was routinely cleaned by a sponge to prevent the growth of microflora.

Table 2-1. Composition of the basal diet (%DM)

Ingredients	%
Casein (vitamin free) [1]	28.0
Gelatin[1]	7.0
Fish meal (defatted) [2]	15.0
Dextrin[1]	21.0
Starch	10.0
Squid liver oil	14.0
Vitamin Mix.(vitamin C free) [3]	2.0
Mineral Mix.[4]	2.0
Cellulose	1.0

[1] United States Biochemical (USB), Cleveland, OH, USA.
[2] White fishmeal, Rukoss, Russia. Fish meal were extracted by 70% aqueous ethanol (water : ethanol = 3 : 7) for 48h.
[3] Vitamin premix (g/kg of mixture): retinyl acetate, 1.0; cholecalciferol,0.05; menadione, 0.2; thiamine hydrochloride, 4.0; riboflavin, 4.4; d-pantothenic acid hemicalcium, 14.5; pyridoxine hydrochloride, 4.0; cyanocobalamin, 0.01; niacinamide, 30.0; folic acid, 0.48; d-biotin, 0.2; *myo*-inositol, 40.0; α-tocopherol, 10.0.
[4] Mineral mixture was based on the composition of Lee et al., 2003[45].

Sample collection and analysis

At the end of 33 weeks of feeding trial, five fish per each tank (10 fish per treatment) were randomly collected and anaesthetized with 2-phenoxyethanol (100 ppm). Total ascorbic acid (TAA) and dehydroascorbic acid (DHAA) concentrations in liver were measured using the 2,4-dinitrophenylhydrazine (DNPH) colorimetric method described by Dabrowski and Hinterleitner [19]. Vertebral columns were analyzed for bone collagen content using the method of Wilson and Poe [1] with some modifications by Ai et al. [11]. Approximate compositions of the experimental diets were analyzed by the method of AOAC [20].

Statistical analysis

All experimental diets were assigned by a completely randomized design. Data were subjected to one-way ANOVA in SPSS version 11.0 (SPSS Inc., Chicago, IL, USA). The significant differences between group means were compared using Duncan's multiple tests ($P<0.05$). Data are presented as mean±SD. Percentage data were arcsine transformed before analysis.

RESULTS

Vitamin C deficiency signs

AA deficiency signs of tiger puffer after 33 week feeding trial are given in Table 2-2. After 27 weeks, 50% fish fed the AA free diet exhibited anemia symptom and over the 30 weeks, most fish were observed have anemia symptom. By the end of the 33 weeks, 20% of fish showed blacken bile and 10% fish fed the AA-free diet were dead. After 31 weeks, fish fed AA free diet had skin erosion symptom. However, no gross deficiency sighs were observed in any fish fed the diets supplemented with AA.

Table 2-2. Vitamin C deficiency symptoms of fish fed vitamin C free diet for 33 weeks of tiger puffer.

	Anemia	Skin erosion	Blacken bile	Death
Fish (%)	85	25	20	10

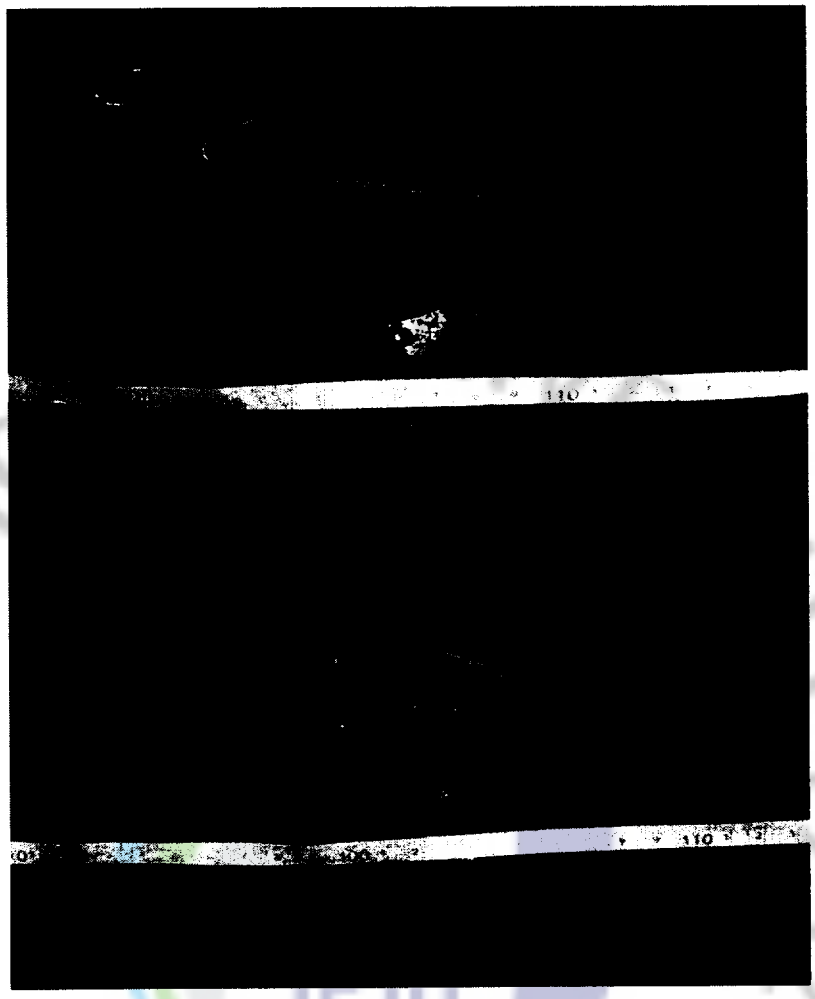

Figure 2-1. Deficiency symptom (B, skin erosion) of fish fed AMP0 diet of tiger puffer for 33 weeks.

Bone collagen concentrations

Bone collagen concentration of tiger puffer fed the diets containing different vitamin contents is presented in Fig. 2-2. The bone collagen concentration was significantly increased with the increment of dietary L-ascorbic-2-monophosphate (AMP) and ranged from 27.2 to 31.5% for the fish.

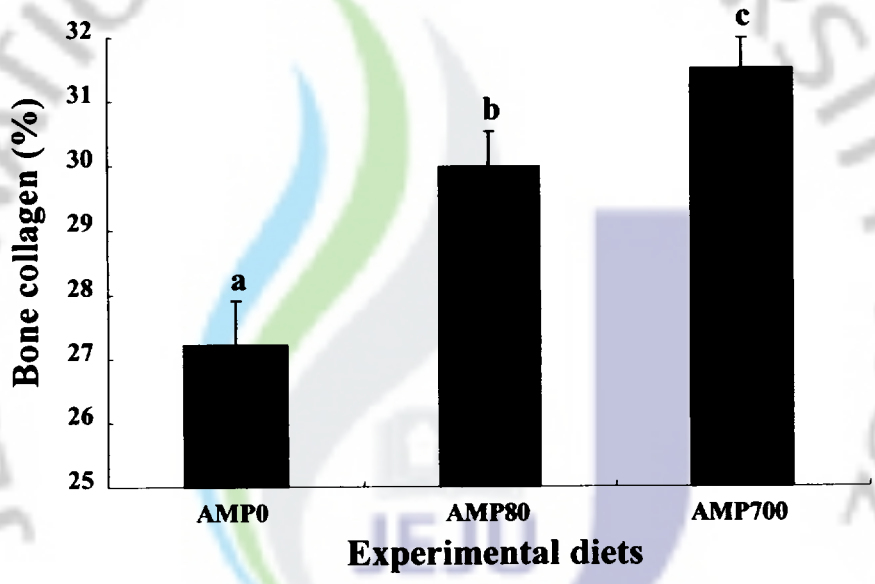

Fig. 2-2. Bone collagen concentrations of tiger puffer fed graded levels of L-ascorbyl-2-monophosphate for 33 weeks.

Liver ascorbic acid concentrations

Total AA concentration in liver of tiger puffer is presented in Fig. 2-3. Total AA concentration in liver of fish was significantly increased in a dose-dependent manner by the supplementation of dietary AMP.

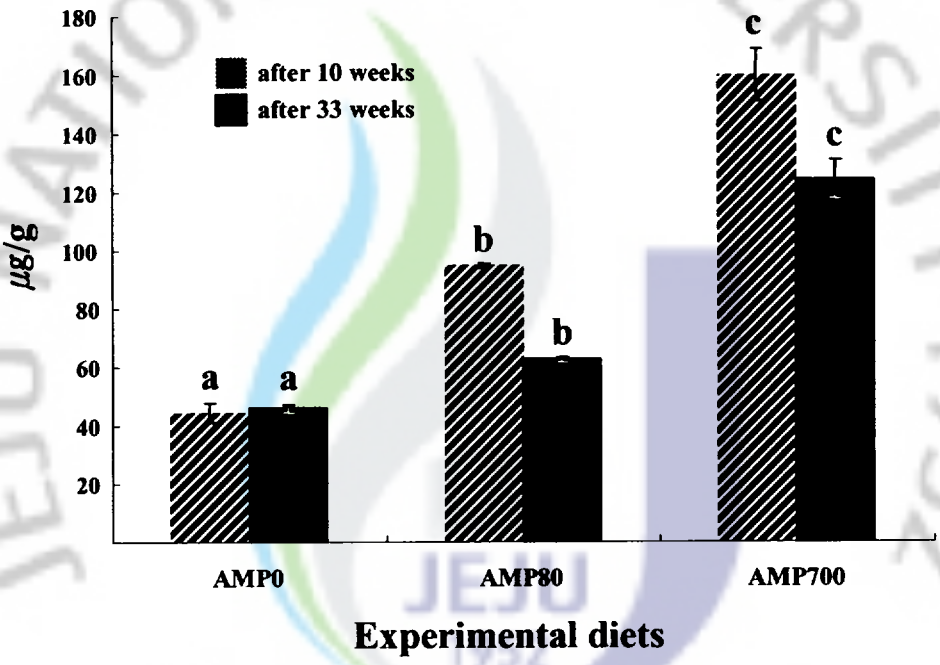

Fig. 2-3. Total ascorbic acid concentrations in the liver of tiger puffer fed graded levels of L-ascorbyl-2-monophosphate for 10 weeks and 33 weeks.

DICCUSSION

Teleosts require dietary source of ascorbic acid for their normal growth and physiological functions, but quantitative needs vary among species. Several deficiency signs of AA including retarded growth, lowered stress resistance, and reduced immune responses have been reported [46]. In the present study, tiger puffer fed an AA-free diet over 27 wk were shown to have anemia symptom. Also, deficiency signs such as lethargy and erratic swimming were observed during the experimental period. In the previous study, after 10 weeks of feeding trial, hematocrit of fish fed AMP0 diet was significantly lower compared to that of fish fed diets supplemented with AMP. Duncan and Lovell [46] reported hematological abnormalities in channel catfish fed AA free diets. Deficient fish had reduced hematocrit and erythrocyte and leukocyte counts, and had abnormally large erythrocytes. Fish fed a diet with a marginal level of AA (20 mg/kg) also had greater numbers of other abnormalities such as binuclear erythrocytes and condensation of the nucleus.

In the present study, 10% mortality was observed in fish fed the AA-free diet. Many studies were reported that high mortality occurred in the AA deficient fish with the development of advanced deficiency signs [10, 11, 17, 39, 49]. Ai et al. [11] reported that 17% mortality was observed in Japanese seabass fed the AA free diet after 8 week of feeding trial. Wang et al. [17] reported that all fish fed the AA free diet dead after 7 weeks. Gouillou-Coustans et al. [49] interpreted that a severe impairment of tyrosine metabolism in turbot fed an AA-free diet can lead to renal granulomatous disease which can cause high mortalities. This may explain the mortalities in tiger puffer fed AA free diet in the present study.

In the present study fish fed diets without AA supplementation observed the skin erosion (Fig.2-1.). This was the first report for the deficiency signs of tiger puffer, to our knowledge.

AA functions as a regulator of the catabolism of cholesterol to bile acids in the

guinea pig and has been demonstrated to be an important factor in lipid regulation of the guinea pig, rabbit and rat [50]. In the present study, fish fed diets without AA supplementation exhibited a darken bile color.

In the previous study, no signs of clinical deficiency were observed during 10 weeks. However, after 33 weeks long feeding period, fish fed diets without supplementation observed clinical deficiency sign. These results confirmed that large fish depleted AA relatively slower.

Lim and Lovell [2] reported that bone collagen below 29.5% indicated impaired collagen formation and resulted in scurvy in channel catfish. In our previous study, bone collagen was not significantly different among all treatments for 10 weeks [51]. However, in the present study, after 33 weeks of long term feeding period, dietary supplementation of AMP significantly increased the bone collagen concentration in the fish (Fig.2-2.). Bone collagen concentration of tiger puffer fed the experimental diets for 33 weeks ranged from 27.2 to 31.5%. Li and Lovell [52] speculated that AA affects the collagen-like regions of the C1 complex of complement through its role in proline hydroxylation. Reduced bone collagen was observed in fish fed the AA free diet for 33weeks whereas it was not clear after 10 weeks. This result indicated that AA affects the collagen formation during long term feeding trial of tiger puffer. Many study reported that the minimum requirement to maintain normal collagen formation has been estimated in the range of 10–20 mg AA/kg in juveniles rainbow trout [33], hybrid tilapia [53] channel catfish [32] and Asian seabass (Boonyaratpalin et al., 1992).

In the present study, total AA concentration in liver of fish was significantly increased in a dose-dependent manner by the supplementation of dietary AMP (Fig.2-3.). The liver AA concentration in this study was similar to that of previous study and other studies with other fish species that were ranged from 17 to 164 μg/g wet tissue [10, 16 53] In the present study higher concentration of AA was detected in the liver of the fish fed the diet containing higher levels of AMP. This indicates that AMP is hydrolyzed and converted to AA in the fish body

and stored into liver suggesting that AMP could be used as dietary AA source for tiger puffer.

In the present study the total AA concentration in liver of fish fed AA free diet for 33 weeks was less than 44 μg/g tissue and lower than that in our previous study. Kikuchi et al. [54] reported that optimal temperature is around 25℃ for tiger puffer. However, in the present study the lowest water temperature was 14℃ for long feeding period. The present findings suggest that total ascorbic acid concentration in liver below 44 μg/g tissue might be a sensitive indication of AA deficiency in tiger puffer. Hilton et al. [48] showed that rainbow trout fed an AA-free diet for 16 weeks exhibited overt anorexia, lethargy and lying prostrate on the bottom, and the liver AA concentration was below 20 μg/g. Meanwhile, Lim and Lovell [2] reported that a liver concentration of AA in cannel catfish less than 30 μg/g tissue indicated a dietary deficiency of AA. Wang et al. [16] reported that olive flounder fed without AA supplementation exhibited deficiency signs and the liver AA concentration was below 28 μg/g.

In conclusion, the present results indicate that tiger puffer require dietary vitamin C for their normal growth performance and prevention of clinical deficiency signs. High mortality, anemia, skin pigmentation, liver vitamin C concentration, and decrement of bone collagen could be sensitive indicators for vitamin C deficiency symptoms in tiger puffer.

SUMMARY

Vitamin C (ascorbic acid, AA) is an essential vitamin for normal physiological functions in animals including fish. AA is a strong antioxidant and essential for collagen synthesis, helps to maintain various enzymes in their required reduced form, and participates in the biosynthesis of carnitine, norepinephrine and certain neuroendocrine peptides. However, most teleosts cannot synthesize AA due to the lack of L-gulonolactone oxidase (EC 1.1.3.8) which is the key enzyme for AA synthesis. Thus, an exogenous source of AA is required in fish diets. No information on AA is available in tiger puffer *Takifugu rubripes* which is one of the emerging and important aquaculture species. Therefore, the purposes of this study was conducted to determine the essentiality and requirements of AA in diets for the tiger puffer based on growth performances, liver AA and bone collagen concentrations, and non-specific immune responses of juvenile tiger puffer.

Five casein-gelatin based semi-purified diets were formulated to contain five graded levels of L-ascorbyl-2-monophosphate (AMP) at 0, 40, 80, 160 and 700 mg/kg (designated as AMP0, AMP40, AMP80, AMP160 and AMP700, respectively) and fed to triplicate groups of fish. After 10 weeks of feeding trial, growth performances of fish (initial body weight, 35 g) fed the AMP0 were significantly lower compared to that of fish fed diets supplemented with AMP. The fish fed the AMP0 diet also exhibited significantly lower hematocrit, condition factor and hepatosomatic index compared to the fish fed diets supplemented with AMP. Phagocytic activity (NBT assay) was significantly lower in fish fed the AMP0 diet than in fish fed the AMP containing diets. Plasma lysozyme activity of fish fed the AMP80 and AMP160 was significantly higher than that of fish fed the AMP0. Dietary supplementation of AMP significantly increased the liver superoxide dismutase in the fish. Myeloperoxidase activity of fish fed the AMP0 was significantly lower compared to that of fish fed the AMP containing diets. Bone collagen level tended to increase numerically and total AA concentration in liver of fish was significantly increased in a dose dependent manner by the supplementation of AMP (Part I).

In the previous study (Part I) we found that tiger puffer needs adequate exogenous AA to maintain normal growth, physiological functions and immune response during 10 weeks feeding trial. However, none of the signs of vitamin C deficiency including scoliosis, lordosis, skin erosion and impaired collagen formation were observed during the feeding period. Therefore, the second study was conducted to examine the effect of vitamin C deficiency on tiger puffer for 33 weeks of feeding trial.

After 10 weeks, we selected fish and randomly assigned to six 150 L polyvinyl

conical tanks. Three casein-gelatin based semi-purified diets were formulated to contain three graded levels of AMP at 0, 80 and 700 mg/kg (designated as AMP0, AMP80 and AMP700, respectively) and fed to duplicates groups of fish. After 27 weeks, 50% fish fed the AA free diet exhibited anemia symptom and over the 30 weeks, most fish were observed to have anemia symptom. By the end of the 33 weeks, 20% of fish showed blacken bile and 10% fish fed the AA-free diet were dead. After 31 weeks, fish fed AA free diet had skin erosion symptom. The bone collagen concentration and total AA concentration in liver of fish were significantly increased in a dose-dependent manner by the supplementation of dietary AMP (Part II).

Therefore, this study concluded that tiger puffer requires exogenous ascorbic acid and the optimum dietary level could be 29 mg AA/kg diet for normal growth and physiology. Dietary AA concentration over 82 mg/kg could be required to enhance non-specific immune responses of the fish. However, it does not seem that the fish needs an overdose of dietary AA (> 160 mg/kg) for a better non-specific immune responses.

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