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Effects of vitamin C supplementation on infectivity of *Opisthorchis viverrini* metacercaria in silver barb (*Barbonymus gonionotus*)

Naiyana Senasri[1], Smarn Tesana[2], Bundit Yuangsoi[1] and Sutee Wongmaneeprateep[1*]

[1]Department of Fisheries, Faculty of Agriculture, Khon Kaen University, Khon Kaen 40002, **Thailand**
[2]Food-Borne Parasite Research Group, Department of Parasitology, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, **Thailand**

***Correspondence:** sutee_8888@hotmail.com, suthwo@kku.ac.th **Accepted:** 09 June 2019 **Published online:** 20 June. 2019

The present study was carried out to evaluate effects of dietary vitamin C levels on infectivity of *Opisthorchis viverrini* metacercaria in silver barb (*Barbonymus gonionotus*). The Experiment I was conducted under laboratory conditions to examine susceptibility of different ages of *B. gonionotus* to infection with *O. viverrini* cercaria. Five groups (2, 5, 15, 30, and 50-day-old fish) exposed to 50 *O. viverrini* cercariae per fish for 24 h. The 100% of *O. viverrini* infection rate was achieved in all age-groups of fish. However, the highest intensity of the infection (12.90±0.52 metacercariae/fish) and the highest metacercarial recovery (25.80%) were observed in the group of age 50-day-old fish. For the Experiment II, 50-day-old *B. gonionotus* was conducted under laboratory conditions to investigate effects of different dietary vitamin C levels on disease resistance against *O. viverrini*. Five levels of vitamin C (0 as a control group, 500, 1,000, 1,500 and 2,000 mg per kg of diet) were used to prepare diets. Fish from each group were randomly sampled after 0, 3, 7 and 14 days post-feeding (30 fish at each sampling time) in order to be exposed to 50 *O. viverrini* cercariae per fish for 24 h. Challenged fish in a group fed diet supplemented with 2,000 mg/kg vitamin C for 14 days showed no infection and were *O. viverrini*-negative by histopathological examination. In contrast, 100% *O. viverrini* infection rate occurred all sampling time of the control group and 0 day sampling time of fish fed the dietary vitamin C supplementation. In addition, fish fed the diet supplemented with 2,000 mg/kg vitamin C for 14 days had 1.6 folds higher levels of SOD activity than those of the control group at the same sampling time. This study clearly indicated that *B. gonionotus* fed the diet supplemented with 2,000 mg vitamin C/kg of diet for 14 days were able to resist *O. viverrini* infection.

Keywords: vitamin C, *Opisthorchis viverrini*, metacercaria, silver barb

INTRODUCTION

Opisthorchis viverrini, a liver fluke, is an important public health problem and a major cause of cholangiocarcinoma in the Greater Mekong Subregion including Lao PDR, Cambodia, Vietnam and Thailand (Jongsuksuntigul and Imsomboon, 2003; Sayasone et al., 2007; Dang et al., 2008; IARC, 2011; Donthaisong et al., 2014). Over 10 million people are estimated to be infected by a liver fluke, especially Northeast Thailand is a hotspot for *O. viverrini* high transmission (Sripa and Echaubard, 2017). The *O. viverrini* infection in humans caused by consumption of local/traditional dishes, such as Koi pla, Pla-som, Jom Planoi and Pla-ra, which are favorite meals of rural people. Those dishes are generally made from fermented, raw and partially cooked cyprinid

fish which is containing *O. viverrini* metacercariae (Harinasuta and Harinasuta, 1984; Grundy-Warr et al., 2012). The life cycle of *O. viverrini* is complex; for example, the bithynia snails and the cyprinid fish, are considered as the first and second intermediate hosts, respectively; and cats, dogs, and humans become as the final hosts (Sithithaworn and Haswell-Elkins, 2003; Upatham and Viyanant, 2003). The infection rate of *O.viverrini* in cyprinid fish shows high variation (Rim et al., 2008; Manivong et al., 2009; Touch et al., 2013). Likewise, the susceptibility to infection depends on several factors including physiological, mechanical, nutritional, immunological defenses and survival after infection.

Barbonymus gonionotus (Silver barb) is an economically important fish species of freshwater aquaculture, commonly consumed in Thailand, Lao PDR and Cambodia. The naturally infection rate of *O. viverrini* in *B. gonionotus* was low (0.95%) (Sukontason et al., 1999); however, the infection rate in laboratory conditions was high (100%) (Donthaisong et al., 2014). Previously published studies on infectivity of *O. viverrini* metacercariae in *B. gonionotus* reported that the old age-groups (e.g. 45-49, 50-54, 55-59 and 60-64 days) of *B. gonionotus* had high infection rates of *O. viverrini* and found that fish immune responses related to damage of cyst walls of metacercariae resulting in failing in development into adult metacercariae (Donthaisong et al., 2014; 2016).

Vitamin C, L-ascorbic acid (AA), does not only influences growth performance, reproduction, response to stressful factors, feed utilization and wound healing but also has an ability to be an immunomodulatory vitamin promoting the immune system in fish, especially non-specific immunity. In fish, the dietary vitamin C supplementation can boost immunity by enhancing several non-specific immune parameters, such as respiratory burst (also called oxidative burst) (Verlhac et al., 1998; Ortuño et al., 2001; Kumari and Sahoo, 2005; Lin and Shiau, 2005; Tewary and Patra, 2008; Leal et al., 2017), phagocytic activity (Tewary and Patra, 2008), lysozyme activity and bactericidal activity (Lin and Shiau, 2005; Ren et al., 2007; Verlhac et al., 1998; Dawood et al., 2016). Respiratory burst activity of phagocytic cells of the non-specific immune response in fish produces many kinds of chemicals to eliminate parasites or antigens. Superoxide dismutase (SOD), one of the main antioxidant enzymes, catalyzes the dismutation of the superoxide anion into molecular oxygen and hydrogen peroxide (H_2O_2) (Fridovich, 1989). SOD involves in defense defense and/or destruction of parasites in fish and hosts through toxic compounds, namely hypochlorous acid (HOCl). Therefore, the infectivity of fish or hosts is related to the immunity levels including levels of SOD expression. However, information on the vitamin C to protect or resist the *O. viverrini* infection in *B. gonionotus* is not available.

The objectives of the present study were to investigate the susceptibility of different ages of *B. gonionotus* to infection with *O. viverrini* cercaria, and the effective levels of dietary vitamin C supplementation on disease resistance of *B. gonionotus* against *O. viverrini* infection.

MATERIALS AND METHODS

The experimental protocols and animals were reviewed and approved by the Institutional Animal Care and Use Committee of Khon Kaen University, based on the Ethic of Animal Experimentation of National Council Research of Thailand (record No. IACUC-KKU-14/61 and reference No. 0514.1.75/7).

Experimental animals

Freshwater snails, *Bithynia siamensis goniomphalos*, were collected by a scoop net and by hand from the agricultural areas where endemic infection of *Opisthorchis viverrini* is present in Khon Kaen Province, Thailand. The snails were transported to the laboratory of Food-Borne Parasite Research Group, Department of Parasitology, Faculty of Medicine, Khon Kaen University. Snails were identified by using morphological characteristics according to Pace (1973) and Brandt (1974). Newly shed cercariae were used in this study. Briefly, snails were placed individually in plastic cups (diameter = 3 cm and height = 2.5 cm) contained 10 ml dechlorinated tap-water. The *O. viverrini* cercariae were released from snails into the water by using cercarial shedding method (induced by exposure to electric light (40 W) for 2-3 h). The morphology of cercariae were identified under a compound microscope based on Wykoff et al. (1965), Schell (1970) and Frandsen and Christensen (1984). The cercariae indentification was confirmed by polymerase chain reaction (PCR) analysis using species-specific primers, OV-6F (5'-CTG AAT CTC TCG TTT GTT CA-3') and OV-6R (5'-GTT CCA GGT GAG TCT CTC TA-3') (Wongratanacheewin et al., 2001).

Parasite-free *B. gonionotus* fry and fingerlings were obtained from the hatchery of Department of

Fisheries, Faculty of Agriculture, Khon Kaen University. This experiment was conducted in the laboratory of Food-Borne Parasite Research Group. Fish were acclimatized in a 500-L fiberglass tank contained dechlorinated tap-water and equipped aeration. After yolk-sac completely disappeared, fish (4-15 days old) were fed 4 times daily to satiation with a commercial powdered feed (KT020, Krungthai Food Public Company Limited, Thailand). For 16-50 days old, fish were fed 3 times daily to satiation with a commercial pelleted feed (Hi-grade 9961, Charoen Pokphand Foods Public Company Limited, Thailand) before being transferred for experiments. The water temperature was maintained within a range from 28 to 30°C and pH values were within a range from 7.5 to 8.0 with over 5 mg/L of dissolved oxygen. Levels of nitrite and unionized ammonia nitrogens were monitored throughout the experiment to ensure that the concentrations did not exceed 0.1 and 0.25 mg/l, respectively. Prior to the experiments, fish were randomly sampled and verified to be *O. viverrini*-free by PCR assay.

Experiment I: Susceptibility of different ages of *B. gonionotus* to infection with cercarial *O. viverrini*.

A total of 150 *O. viverrini*-free fish were transferred into fifteen 32-L aquaria (10 fish/aquarium) contained dechlorinated tap-water and equipped aeration, and reared under laboratory conditions. The experiment designedly consisted of five treatments with three replicates. Fish were divided into five groups according to the morphology reported by Termvidchakorn and Hortle (2013) and Basak et al. (2014) into 5 age-groups of 2, 5, 15, 30 and 50 days after hatching and were exposed to 50 *O. viverrini* cercariae per fish under the conditions of 100 ml dechlorinated tap-water in plastic cups (diameter = 14 cm in and height = 5 cm) for the groups of age 2, 5 and 15 days of fish; and 500 ml dechlorinated tap-water in plastic cups (diameter = 24 cm and height = 10 cm) for the groups of age 30 and 50 days of fish at room temperature (25±3°C) as described by Donthaisong et al. (2014). After 24 h of exposure, all fish were euthanized by immersion in an ice water bath at 4°C to examine *O. viverrini* infection by hand-compressed between two slides. The number of *O. viverrini* metacercariae in fish was counted under a light microscope (40 magnification), and recorded as the infectivity of *O. viverrini* metacercariae in fish.

Experiment II: Effects of dietary vitamin C supplementation on *O. viverrini* infection in *B. gonionotus* fingerlings.

Based on the result of the susceptibility of different ages of *B. gonionotus* to infection with *O. viverrini* cercaria in Experiment I. A total of one thousand two hundred *O. viverrini*-free fish (50-day-old fish and average body length was 2.54±0.05 cm) were randomly distributed into forty 32-L aquaria (30 fish/aquarium). Five treatments (Five levels of dietary vitamin C supplementation were 0, 500, 1,000, 1,500 and 2,000 mg per kg of diet) were used. Fish in Treatment 1 were fed a commercial diet (Hi-grade, Charoen Pokphand Foods Public Company, Thailand) used as a control group (without supplementary vitamin C). Fish in the other four groups were fed with commercial diet containing graded levels of vitamin C (98% L-ascorbic acid, Aq & G Tech Co., Ltd.) as 500, 1,000, 1,500 and 2,000 mg per kg of diet, respectively. The feed used in present study contained 40% crude protein and 8% lipid. Fish from each treatment group were randomly sampled after 0, 3, 7 and 14 days post-feeding (30 fish at each sampling time) in order to be exposed to 50 *O. viverrini* cercariae per fish under the conditions of 500 ml dechlorinated tap-water in plastic cups (diameter = 24 cm and height = 10 cm) at room temperature (25±3°C) for 24 h. After exposure to *O. viverrini* cercariae, all fish were examined for *O. viverrini* infection using the same procedure as Experiment I. The number of *O. viverrini* metacercariae was counted, and recorded infectivity of *O. viverrini* metacercariae in the fish under a light microscope (40 magnification) to calculate infection rate of fish, intensity of infection and percentages of metacercarial recovery. The other 30 fish from each treatment group were randomly sampled to evaluate the superoxide dismutase (SOD) activity, and histopathological examination.

Superoxide dismutase (SOD) activity

At the end of *O. viverrini*-immersion challenge for 24 h in Experiment II, ten *B. gonionotus* fingerlings were randomly selected from each dietary vitamin C group. In brief, an individual fish was explored and rinsed with phosphate buffer saline (PBS). The whole body was homogenized with 3-5 ml of cold 0.1M Tris/HCl, pH 7.4 containing 0.5% Triton X-100, 5mM 2-mercapto ethanol (β-ME), 0.1 mg/ml phenylmethylsulfonyl fluoride (PMSF). Homogenized tissue was centrifuged at 14,000*g* for 5 min at 4°C. The supernatant was collected to be measured the

superoxide dismutase (SOD) activity using a Superoxide Dismutase Activity Assay kit (Colorimetric) (ab65354; Abcam). The SOD activity was measured at 450 nm using microplate reader (Sunrise-Basic Tecan, Grodig, Austria). A unit of the enzyme is generally defined as the amount of enzyme that inhibits the reaction by 50%. The activity was expressed as units per gram fresh weight (U/g FW).

Histopathology

Challenged fish (10 fish in each group) in Experiment II were euthanized by immersion in an ice water bath at 4°C. The scarified fish were preserved in 10% neutral buffered formalin for 24 h and decalcified by decalcification solution (0.5 M $AlCl_3.6H_2O$, 2.6 M HCl, 1.325 M formic acid) for 4 to 5 h. Specimens were processed for histopathological examination as previously described in Humason (1979) and stained with hematoxylin and eosin (H&E). Histopathological changes were examined under a compound microscope. Infections were considered positive when samples showed histopathological features including fibrous tissue formation or encapsulated metacercaria of *O. viverrini*.

RESULTS

Cercariae released from *Bithynia siamensis goniomphalos* by using cercarial shedding method in this study were *O. viverrini* identified by morphological classification. In Figure 1, *O. viverrini* have a tobacco-pipe form when briefly hanged head down or laid on the bottom, a pair of eye-spots and long tail with fin on both lateral sides (Wykoff et al., 1965; Arunsan et al., 2014; Donthaisong et al., 2014). Besides, they were confirmed by PCR with *O. viverrini*-specific according to Wongratanacheewin et al., (2001). The result showed *O. viverrini*-specific amplified DNA band of 330 base pairs (bp) (Figure 2).

Figure 1. **The feature of *O. viverrini* cercaria collected from the snail (bar = 100 µm).**

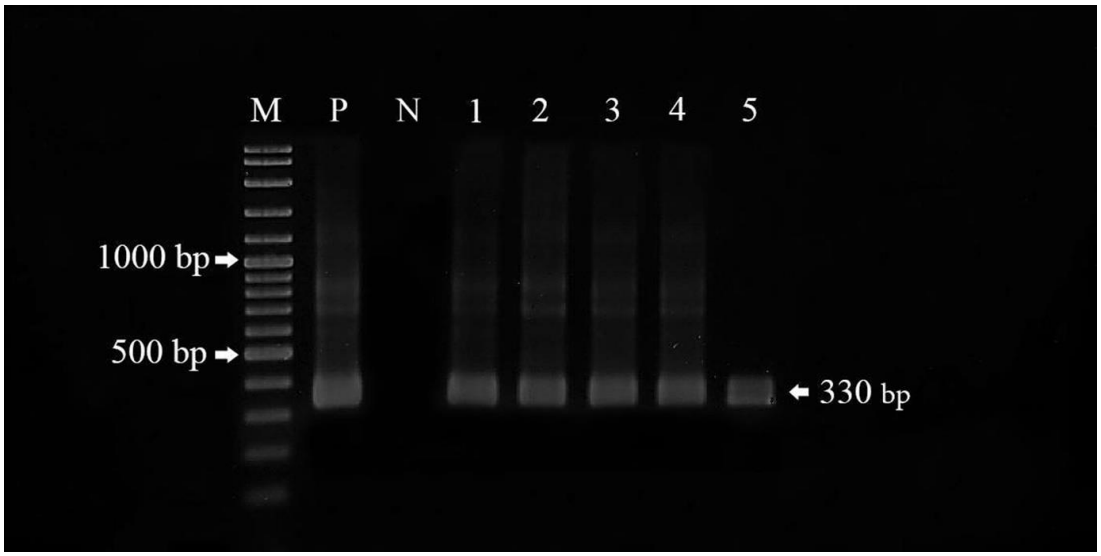


Figure 2. Cercarial *O. viverrini* were confirmed by using specific polymerase chain reaction (PCR) primers of producing 330 bp. M: 100 bp DNA ladder; Lane P: positive control (adult of *O. viverrini*); Lane N: negative control; and Lanes 1-5: PCR amplified products with DNA template sample extracted from cercariae released from snail into the water by using cercarial shedding method.

Experiment I: Susceptibility of different ages of *B. gonionotus* to infection with cercarial *O. viverrini*.

Percentage of infected fish, intensity of infection and percentage of metacercarial recovery are shown in Table 1. All fish in five age-groups (2, 5, 15, 30 and 50-day-old fish) were *O. viverrini* infection, representing infection rate of 100% after exposed to *O. viverrini* cercariae for 24 h. The intensity of the infection (average number of metacercariae per fish) in groups of 2, 5 and 15-day-old fish in range of 2.2±0.26 to 3.66±1.58 metacercariae/fish which increased to 9.60±1.37 metacercariae/fish in age-group of 30-day old fish. The highest intensity of the infection was 12.90±0.52 metacercariae/fish observed in age-group of 50-day old fish. The percentage of metacercarial recovery was found in range from 4.40 to 25.80% (approximately 66 to 387 metacecariae of 1,500 cercariae infection dose). The lowest level of metacercarial recovery (4.40%) was found in 5-day age group of fish. On the other hand, the highest level of metacercarial recovery was 25.80% which was found in 50-day age group of fish.

Experiment II: Effects of dietary vitamin C supplementation on *O. viverrini* infection in *B. gonionotus* fingerlings.

After 24 h of the exposure to *O. viverrini* cercariae, experimental fish were observed in percentage of infected fish, intensity of infection and percentage of metacercarial recovery (Table 2 to 4). The infection of *O. viverrini* was found in all groups of fish fed the experimental diets for 0, 3, 7 and 14 days except in the group of fish fed 2,000 mg/kg vitamin C for 14 days that showed no infection (Table 2). The percentage of infected fish in control group showed 100% in all sampling time (0, 3, 7 and 14 days). Nevertheless, the other groups of fish fed the dietary vitamin C supplementation showed 100% of infected fish in only 0 day of sampling time (Table 2). The intensity of the infection in Table 3 ranged from 0 to 1.30 metacercariae/fish in groups of fish fed the diets supplemented with vitamin C for 3, 7 and 14 days. For the control group, the intensity of the infection ranged from 7.13 to 7.33 metacercariae/fish, and in groups of fish fed the experimental diets for 0 day ranged from 7.26 to 7.56 metacercariae/fish. The percentage of metacercarial recovery (Table 4) in groups of fish fed the experimental diets for 0, 3, 7 and 14 days varied from 0 to 15%.

Table 1. Infectivity of *O. viverrini* metacercariae in each age-group of *B. gonionotus*. Data are expressed as mean ± SD (n=30).

Age of fish (days)	Percentage of infected fish (%)	Intensity of the infection (metacercariae/fish)	Percentage of metacercarial recovery (%)
2	100	3.66±1.58	7.33
5	100	2.20±0.26	4.40
15	100	3.03±0.47	6.06
30	100	9.60±1.37	19.20
50	100	12.90±0.52	25.80

Table 2. Percentage of *O. viverrini* infection in *B. gonionotus* (n = 30) fed the diets supplemented with different levels of vitamin C.

Number of day of vitamin C administration	Level of vitamin C (mg/kg)				
	0	500	1,000	1,500	2,000
0	100.00%	100.00%	100.00%	100.00%	100.00%
3	100.00%	63.33%	23.33%	10.00%	6.67%
7	100.00%	26.67%	13.33%	13.33%	3.33%
14	100.00%	23.33%	13.33%	10.00%	0.00%

Table 3. The intensity of *O. viverrini* infection (metacercariae/fish) in *B. gonionotus* (n = 30) fed the diets supplemented with different levels of vitamin C. Data are expressed as mean ± SD.

Number of day of vitamin C administration	Level of vitamin C (mg/kg)				
	0	500	1,000	1,500	2,000
0	7.33±0.49	7.30±0.30	7.56±0.80	7.26±0.05	7.36±0.25
3	7.16±0.80	1.30±0.20	0.26±0.15	0.13±0.05	0.06±0.05
7	7.13±0.87	0.33±0.20	0.13±0.11	0.13±0.11	0.03±0.05
14	7.23±0.75	0.26±0.15	0.13±0.11	0.10±0.00	0.00±0.00

Table 4. Percentage of metacercarial recovery infected in *B. gonionotus* (n = 30) fed the diets supplemented with different levels of vitamin C.

Number of day of vitamin C administration	Level of vitamin C (mg/kg)				
	0	500	1,000	1,500	2,000
0	14.67%	14.60%	15.13%	14.53%	14.73%
3	14.33%	2.60%	0.53%	0.27%	0.13%
7	14.47%	0.67%	0.27%	0.27%	0.07%
14	14.47%	0.53%	0.27%	0.20%	0.00%

The lowest level of metacercarial recovery (0%) was found in the group of fish fed 2,000 mg/kg vitamin C for 14 days. The highest level of metacercarial recovery was observed in fish fed 1,000 mg/kg vitamin C for 0 day.

Fish fed with a commercial diet without supplementary vitamin C (control group) had the lowest levels of superoxide dismutase (SOD) activity throughout the experiment. In contrast, fish fed diet supplemented with 2,000 mg/kg vitamin C for 14 days showed the highest level of SOD activity. Moreover, fish fed diet supplemented with 2,000 mg/kg vitamin C showed higher level of SOD activity than that of the groups fed diets supplemented with 500, 1000, and 1,500 mg/kg vitamin C after 3, 7 and 14 days of administration of vitamin C (Figure 3). In addition, fish fed the diet supplemented with 2,000 mg/kg vitamin C for 14 days (1,375±161.2 U/g FW) had 1.6 folds higher levels of SOD activity than those of fish fed only with a commercial diet without supplementary vitamin C (870±115.7 U/g FW) at the same sampling time. Therefore, differences of SOD defense response of fish were influenced by

the supplement of vitamin C in diet.

Histological section of infected fish from the groups fed the diets supplemented with 500, 1,000, 1,500 and 2,000 mg/kg vitamin C for 0, 3, 7 and 14 days after infection with *O. viverrini* cercariae and control group were *O.viverrini*-positive except fish from a group fed diet supplemented with 2,000 mg/kg vitamin C for 14 days was *O.viverrini*-negative (no infection). *O. viverrini*-infected fish exhibited the chronic inflammatory reactions including infiltration, and fibrous tissue formation. However, fibrous tissues observed in the groups of infected fish fed the diets supplemented with vitamin C were enlarged and formed thickly around metacercarial cysts compared to that in the control group (Figure 4). In addition, *O. viverrini* metacercariae in the groups of infected fish fed the diets supplemented with vitamin C for 14 days could not continue to mature or develop throughout metacercariae infective stage.

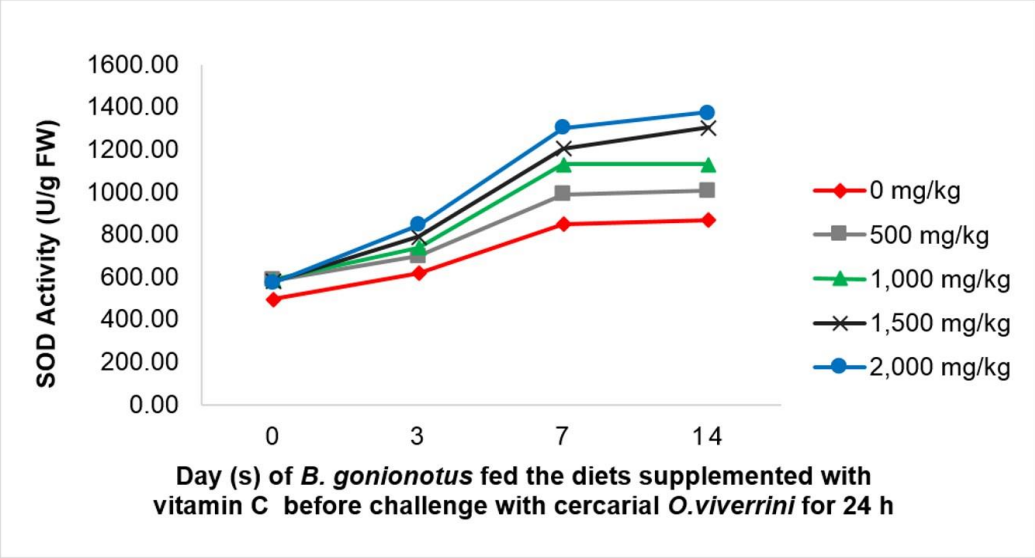
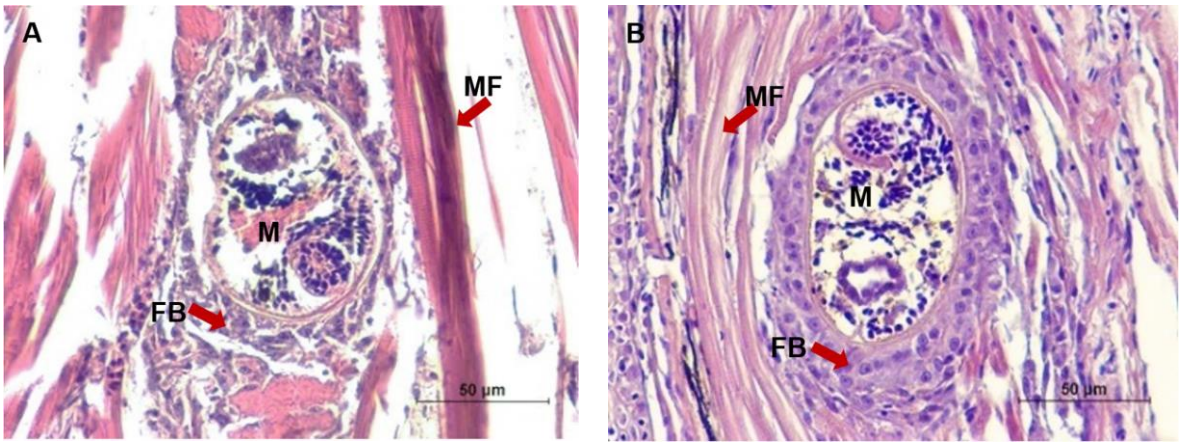


Figure 3. Superoxide dismutase of *B. gonionotus* fed the diets supplemented with different levels of vitamin C for 0, 3, 7 and 14 days before challenge with cercarial *O. viverrini* for 24 h.



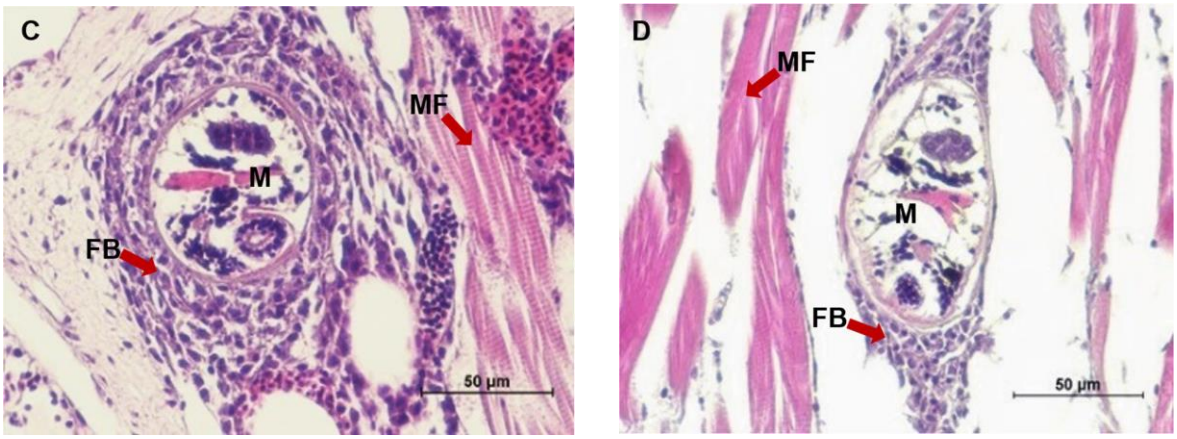


Figure 4. Photomicrographs of tissues from *B. gonionotus* fed the diets supplemented with (A) 500, (B) 1,000 and (C) 1,500 mg vitamin C/kg of diet for 14 days before challenge with cercarial *O. viverrini* for 24 h and (D) control group showing chronic inflammation, hyperplasia and infiltration (H&E, bar = 50 µm). M: metacercariae, FB: fibrous tissue, MF: striated muscle of fish.

DISCUSSION

In the present study, the susceptibility of *B. gonionotus* to *O. viverrini* cercarial infection did not only increase with increasing age of fish, but also *O. viverrini* metacercaial recovery increased with increasing age of fish, especially fish in age-groups of 30 and 50 days old was high (19.20 to 25.80%). Similar to the finding of Donthaisong et al. (2014), the difference in susceptibility caused by different stage of fish might be related to the quantity or composition of biochemical compounds secreted by fish that might change when fish increase in size and age (Haas, 1992). In fact, the surface area of fish host for *O. viverrini* cercarial attachment increased in older age-group (Donthaisong et al., 2014). In addition, the result of the present study indicates that the increased intensity of *O. viverrini* cercarial infection was influenced by the age and stage of *B. gonionotus* due to increasing area of attachment for *O. viverrini* cercariae increases the chance of infection in older age-group. Similar results were revealed by Phan et al. (2010), it was reported no infections of fish-borne zoonotic trematodes (FZTs) in fish fry from hatcheries. However, in nurseries, increasing prevalence of infected fish was 14% in one-week-old juveniles, 49% in four-week-old juveniles; and up to 58% in overwintered juveniles in the ponds. Boerlage et al., (2011, 2012) reported that small sized (1 g) common carp (*Cyprinus carpio*) had the high percentages of infected fish, metacercariae burdens, and attack rates of parapleurolophocercous cercariae (*Haplorchis* and *Centrocestus* sp., family Heterophyidae). In the present study, fish in age-groups of 2, 10 and 15 days old had low intensity of the infection and percentage of metacercarial recovery compared to that of 30 and 50 days old. This could be due to the feeding behavior of fish larvae after yolk-sac almost completely disappeared starts ingesting zooplanktons; and the size and appearance of *O. viverrini* cercariae are similar to zooplanktons which might have been eaten and also ingested by fish larvae. However, *O. viverrini* infection was related to the behavior (e.g. feeding, moving and swimming) and morphological changes (e.g. attachment area for cercariae) of fish in each age-group (Haas, 1992; Donthaisong et al., 2014). This result is similar to the report of Donthaisong et al. (2014), they observed the relationship between behavior of fish and intensity of *O. viverrini* infection, percentage of metacercarial recovery and percentage of infected fish post-exposed to *O. viverrini* cercariae. The result showed no infection in the groups of 9-10 days old; further, low intensity of *O. viverrini* infection, percentage of metacercarial recovery and percentage of infected fish were found in the groups of 1-2, 3-4, 5-6 and 7-8 days old as the fish actively search for food. In contrast, 100% infected fish was observed in age-groups of 30-34, 45-49, 50-54, 55-59 and 60-64. As a result, 50-day-old fish would be therefore selected as experimental animal in Experiment II.

In experiment II, the effects of dietary vitamin C supplementation on disease resistance against *O. viverrini* infection in 50-day-old *B. gonionotus* were investiagted. The results showed that the infection rate of *O. viverrini* in groups of *B. gonionotus* fed the diets supplemented with

vitamin C for 3, 7 and 14 days was decreased when compared to control group. The lowest infection rate was observed in fish fed the diet supplemented with 2,000 mg/kg vitamin C (0 to 6.67%) in all sampling times, especially fish in a group fed diet supplemented with 2,000 mg/kg vitamin C for 14 days showed no infection. As far as we know, this result is the first report to demonstrate the effects on dietary vitamin C supplementation on disease resistance against *O. viverrini* of *B. gonionotus*. Our finding showed that *B. gonionotus* fed the diets supplemented with vitamin C resulted in increased resistance against *O. viverrini*. In fish, previous studies suggested that dietary vitamin C in fish improves their natural resistance to infections which increase modulate the non-specific immune response (by enhancing phagocytosis-mediated leucocyte functions including respiratory burst activity and superoxide dismutase activity, bactericidal activity, alternative complement activity, etc.), and also increase survivability against pathogenic agents (Lin and Shiau, 2005; Nayak et al., 2007; Tewary and Patra, 2008; Ming et al., 2012; Guardiola et al., 2014; Roosta et al., 2014; Shahkar et al., 2015; Khan et al., 2017; Leal et al., 2017; Hossain et al., 2018).

The levels of metacercarial recovery in fish fed the diet supplemented with 2,000 mg/kg vitamin C for 3, 7 and 14 days were very low (0 to 0.13%) compared to the other groups. In 14 days of dietary vitamin C supplementation, 0% of metacercarial recovery was found in a group fed diet supplemented with 2,000 mg/kg vitamin C, and intensity of metacercarial infection was 0 metacercariae/fish. The results suggested that fish immunity involves in the infection process of *O. viverrini* cercariae to the fish. It is in agreement with previous research findings indicate that beneficial effect of dietary supplementation with vitamin C does not only influences growth and feed utilization in fish (Dupree, 1966; Halver et al., 1969; Lovell, 1973; Durve and Lovell, 1982; Mazik et al., 1987; Landolt, 1989; Navarre et al., 1989; Hardie et al., 1991; Waagbø et al., 1993; Rougier et al., 1994; Roberts et al., 1995; Verlhac et al., 1998; Adel and Khara, 2016) but also has ability to stimulate the non-specific immune response and disease resistance of a variety of cultured fish species, such as Bagrid catfish, *Mystus gulio* (Anbarasu and Chandran, 2001), Gilthead seabream, *Sparus aurata* (Ortuño et al., 2003), Asian catfish, *Clarias batrachus* (Kumari and Sahoo, 2005), Rohu, *Labeo rohita* (Tewary and Patra, 2008), Nile tilapia, *Oreochromis niloticus* (Ibrahem et al., 2010; Barros et al., 2014), Cobia, *Rachycentron canadum* (Zhou et al, 2012), Groper, *Polyprion oxygeneios* (Salinas et al., 2012), Wuchang bream, *Megalobrama amblycephala* (Wan et al., 2014; Liu et al., 2016), Largemouth bass, *Micropterus salamoides* (Chen et al., 2015), Japanese eel, *Anguilla japonica* (Shahkar et al., 2015), Red sea bream, *Pagrus major* (Dawood et al., 2016; Dawood et al., 2017; Hossain et al., 2018), Mahseer fish, *Tor putitora* (Khan et al., 2017).

Recent study was reported by Donthaisong et al. (2016) found that *B. gonionotus* administered with immunosuppressant (prednisolone) prior to infection with *O. viverrini* cercariae had higher metacercarial recovery than that of a control group without the immunosuppressant administration. On the other hand, the administration of prednisolone after the *O. viverrini* infection allowed metacercariae to develop to the infective stage, and also found that immunosuppression decreases superoxide dismutase (SOD) activity and fibrous tissue in infected fish; moreover, this circumstance promoted *O. viverrini* metacercariae to develop to the infective stage. Our study showed that SOD activity was enhanced by dietary vitamin C supplementation and the intake period of vitamin C. The SOD activity of the groups fed the diets supplemented with vitamin C were increased compared to the control group. Additionally, the levels of SOD activity increased with increasing period of vitamin C intake. Therefore, findings of the present study clearly demonstrate that vitamin C can improve the SOD activity of *B. gonionotus* resulting in stimulating the immune response of fish against *O. viverrini* infection. The enhancement of SOD induced by dietary vitamin C has been reported in several fish species (Kumari and Sahoo, 2005; Zhou et al., 2012; Chen et al., 2015; Shahkar et al., 2015; Dawood et al., 2016; Dawood et al., 2017).

The histological studies confirm that both of the dietary vitamin C supplementation and the intake period of vitamin C increase formation of fibrous tissue of infected fish. After 24 h exposure to *O. viverrini* cercariae, the group of infected fish in control group and the groups of infected fish fed the diet supplemented with 0 mg/kg vitamin C had less formation of fibrous tissue, and encysted metacercariae were resistant to the effects of immune response of fish and were not damaged (*O. viverrini* metacercariae become fully infective stage) compared to in groups fed the diets supplemented with vitamin C for 3, 7 and 14 days,

except in a group of fish fed the diet supplemented with 2,000 mg/kg vitamin C for 14 days which could resist *O. viverrini* infection. The metacercarial cysts in the groups of infected fish fed the diets supplemented with vitamin C for 3, 7 and 14 days were surrounded by a thick fibrous capsule produced by the host (*B. gonionotus*). Later, the metacercarial cysts were damaged in the groups of fish fed the diets supplemented with vitamin C for 14 days. According to Mumford et al. (2007), the defense mechanisms of immune response include chronic inflammation, hyperplasia and infiltration consisting mixture of macrophages, lymphocytes, plasma cells, fibroblasts, and sometimes neutrophils surround the metacercarial cysts. The SOD defense response to the *O. viverrini* infection was similar to the report by Donthaisong et al. (2016) which revealed that the SOD activity and fibrous tissue formation are enhanced by parasite exposure. The elevation of the SOD and fibrous tissue formation could interfere with the meatacercarial development of *O. viverrini*.

CONCLUSION

This study obviously indicated that *B. gonionotus* fed the diets supplemented with vitamin C for 14 days were able to reduce *O. viverrini* infection, and improve the non-specific immune response as well as SOD activity and formation of fibrous tissue, especially *B. gonionotus* fed the diet supplemented with 2,000 mg vitamin C/kg of diet for 14 days were able to resist *O. viverrini* infection. Results of the present study can be applied for preventing *O. viverrini* infection in *B. gonionotus* reared in nursing pond by feeding the diet supplemented with 2,000 mg/kg vitamin C for 14 days before stocking the fish in the culture ponds.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

NS and SW designed and performed the experiments. ST and BY carried out the experiments, NS and SW also wrote the manuscript. All authors read and approved the final version.

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