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The Effect of Ascorbic Acid on Cadmium Exposure in the Gills of *Puntius altus*

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Abstract: The effects of cadmium and ascorbic acid on the gills of Red-tailed tinfoil barb (*Puntius altus*) were compared using light and scanning electron microscopic study for the period 24, 48, 72 and 96 h. The main alterations in the cadmium treated group were edema, filament cell proliferation, lamellar cell hyperplasia, lamellar fusion, epithelial lifting and aneurysm. These changes occurred predominantly in the 96 h treatment. In the combination of cadmium and ascorbic acid treated group, they showed similar alterations as those observed in the cadmium treated alone group but they were less severe.

Key words: Red-tailed tinfoil barb *Puntius altus*, ascorbic acid cadmium, light microscope scanning electron microscope

INTRODUCTION

Not only does enviroumental pollution caused a decrease in water qnality, but subsequently affects all living organisms in that system. Therefore, not only identify and manage these pollution sources, but also monitor their effects on the health of aquatic ecosystem. Cadmium (Cd) is an extremely toxic element of continuing concern because its environmental levels have risen steadily (WHO, 1992; Goering *et al*., 1995). They are being used in a wide variety of industrial processes in Thailand, for example, the use of cadmium as a coloring agent, a stabilizer and in alloy mixtures. Cadmium has been shown to alter the structure of fish and to cause histopathological changes of varying severity in various fish organs. The highest levels of cadmium have been detected in the kidney and liver (Olsson *et al*., 1996).

Ascorbic acid (AA), commonly known as vitamin C, is essential for many aquatic animal species, as they cannot synthesize this micronutrient and depend on an exogenous supply. The function of AA is a strong reducing agent in many tissues and is therefore involved in several physiological processes including growth, reproduction, immunity and the response to stress and infectious agents (Verlhac and Gabaudan, 1994). It has also been reported to have anticarcinogenic (Pauling *et al*., 1985), anticlastogenic (Gebhart *et al*., 1985) and even antimutagenic (Shamberger, 1984) roles in a variety of test systems.

Many different types of biomarkers are in use nowadays, ranging from biochemical and cellular biomarkers to physiological indicators and ecosystem monitors. They have to be more sensitive, less variable and often easier to measure. When the concentration of pollutants is high enough, changes occur within an entire organ or specific parts of it. Changes that alter the cells and tissues of an organism and don't result in death can be viewed under the light microscope or electron microscope. Histopathology has received increasing interest as an endpoint because histopathological changes are often the result of the integration of a large number of interactive physiological processes

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(van der Oost *et al*., 2003). Ultrastructure of tissues and organs is altered when levels of the contaminant are still at low levels, therefore histopathological assays provide a valuable screening method before sever damage occurs.

The application of environmental toxicology studies among non-mammalian vertebrates is rapidly expandingand, for aquatic systems, fish have become indicators for the evaluation of the effects of noxious compounds. Red-tailed tinfoil barb (*Puntius altus*) is one of the popular commercialized freshwater fish in Thailand. To the best of our knowledge no study has been done so far on the efficacy of AA against the cadmium exposure in fish. Therefore, we aimed to evaluate the effect of ascorbic acid associated with acute exposed to cadmium in red-tailed tinfoil barb (*P. altus*) using light microscopy and scanning electron microscopy.

MATERIALS AND METHODS

Animals

Red-tailed tinfoil barb (*P. altus*) 14.77±2.15 g in body weight and 9.02±0.61 cm in total length, were purchased from a commercial hatchery in Bangkok, Thailand. Tap water was filtered with activated charcoal (Aquapur, thysen, FRG) to eliminate chemical contamination. The physicochemical characteristics of water were measured daily, according to the experimental procedures described in Standard Methods for the Examination of Water and Wastewater (APHA, 2005). Conductivity was measured with Hanna instruments Model 3 DiST WP (Hanna Instruments Inc., USA). The pH was measured with a Cyberscan 510 (Eutech Instruments Inc., USA) and the temperature was measured with a glass mercury thermometer. A 16:8 h light-dark cycle was maintained throughout.

Acclimatization to laboratory conditions for 7 days was done using dechlorinated tap water that had the following physicochemical characteristics: temperature = 27.0±1.5°C, pH = 6.5-6.8, total hardness = 65-75 mg L^{-1} (as $CaCO_3$), alkalinity = 75-80 mg L^{-1} and conductivity = 185-210 μmhos cm^{-1}. Chlorine residual and ammonia were below detection limits. Fish were fed twice a day with commercial fish food contains 28% protein, 3% fatand 4% fiber (Charoen Pokphand Group, Bangkok, Thailand). The quantity of food was 2% of the initial body weight per day.

Diet Preparation

All diets were prepared with commercially fish food that was used to feed the fish during acclimation. Ascorbic acid (Sigma, Germany, CAS No.50-81-7) supplemented diets were prepared by mixing 500 mg kg^{-1} BW ascorbic acid with commercial fish food. The fish food was ground in a blender, followed by hydration with approximately 75% v/w deionized water and added to the food paste. The resulting paste was mixed well and put it into a pasta maker then broke the food paste into small pallets by hands or cutter. It was air dried in 60°C 3 h hot air oven (Zohouri *et al*., 2001). The control diet was prepared by the same method but with the addition of deionized water only.

Experimental Design

Fish were exposed to 40 mg L^{-1} $CdCl_2.H_2O$ (Sigma, Germany, CAS No.10108-64-2). The dose chosen was 25% of the 96-h LC_{50} value from the acute toxicity test, which was 166.22 mg L^{-1} (Jiraungkoorskul *et al*., 2007). Fish (n = 32) were randomly assigned to four equally sized groups as follows: (1) Control group (normal diet and water); (2) Cadmium exposure group (normal diet and 40 mg L^{-1} waterborne cadmium); (3) Mix exposure group (Ascorbic acid supplemented diets and 40 mg L^{-1} waterborne cadmium) and (4) Ascorbic acid exposure group (Ascorbic acid supplemented diets and normal water). The fish were kept in the glass flow-through aquaria (50×50×120 cm) with continuous aeration were filled with 200 L of dechlorinated tap water whose physicochemical characteristics were the same as those described previously.

At different times (24, 48, 72and 96 h), 5 fish of each group were anesthetized with 0.2 g L^{-1} MS-222 (tricaine methan sulphonate, Sigma, Germany, CAS No.886-86-2), weighed and measured. Gills were removed and prepared for histopathological studies.

Specimen Preparation for Light Microscopic Study

Gills were fixed in the 10% buffered formaldehyde for 24 h, dehydrate through a graded series of ethanol and clear with xylene solutions. They were embedded in a block using melted paraffin at the embedding station (Axel Johnson Lab System, USA). The paraffin blocks were sectioned at 4-5 μm thickness using a rotary microtome (HistoSTAT, Reichert, USA)and stained with hematoxylin and eosin. The tissue glass slides were examined for abnormalities by a Nikon E600 light microscope and photographed by a Nikon DXM 1200 digital camera (Tokyo, Japan) (Humason, 1972).

Specimen Preparation for Scanning Electron Study

Gills were fixed in 2.5% glutaraldehyde-phosphate buffer (0.1 mol L^{-1}, pH 7.4) at 4°C for 24 h and post-fixed in 1% osmium tetroxide for 1 h. They were dehydrated through a graded series of ethanol, dried in a Hitachi HCP-2 critical point dryer machine using liquid carbon dioxide as a transitional medium. After drying, they were mounted on aluminium stubs and coated with platinum and paladium in an ion-sputtering apparatus, Hitachi E-102, at 10-15 mA for 6 min. They were examined and photographed in a Hitachi scanning electron microscope S-2500 (Hitachi High-Technologies Co., Hitachi-Naka City, Japan), operating at 15 kV (Humason, 1972).

Semiquantitative Scoring

Histopathological alteration was assessed using a score ranging from - to + + + depending on the degree and extent of the alteration: (-) none, (+) mild occurrence, (+ +) moderate occurrence, (+ + +) severe occurrence. A total of 20 slides were observed from each treatment.

RESULTS

Control Group

No recognizable changes were observed in the gills of the control and AA treated groups throughout the course of this experiment; therefore, the following description was representative of all times sampled. Investigation of the gill structure showed significant differences between control and treated groups. Briefly, there were four-gill arches on each side of the buccal cavity, which were termed from lateral to medial as first, second, third and fourth. Each gill arch was semilunar in shape consisting of numerous primary filaments, which had a cartilaginous central structure and highly vascularized. The primary filament had two rows of secondary lamellae that run perpendicular to each filament (Fig. 1A). Each secondary lamella was made up of two sheets of epithelium delimited by many pillar cells, which were contractile and separated the capillary channels. One to two erythrocytes were usually recognized within each capillary lumen (Fig. 1B). These secondary lamellae, together with the central vascular spaces, form the gaseous exchange barrier, or respiratory barrier. The interlamellar space contained a multilayered epithelium composed of numerous cells and supported by a visible basal membrane that separated the cells from the vascular and cartilaginous structures of the primary filament and secondary lamellae. The cell types involved were epithelial cells, chloride cells, mucousand pavement cells. Chloride cells were mainly located on the trailing edge of the filamental epithelium and at the bases of lamellae. The cells displayed a characteristic apical surface morphology of microvilli. Mucus cells and pavement cells were also present in the epithelium of the filament and at the base of lamellae, but they lacked the light cytoplasm and were smaller than chloride cells. The gill filaments were covered with squamous pavement cells showing characteristic concentric patterns of microridges (Fig. 2A).

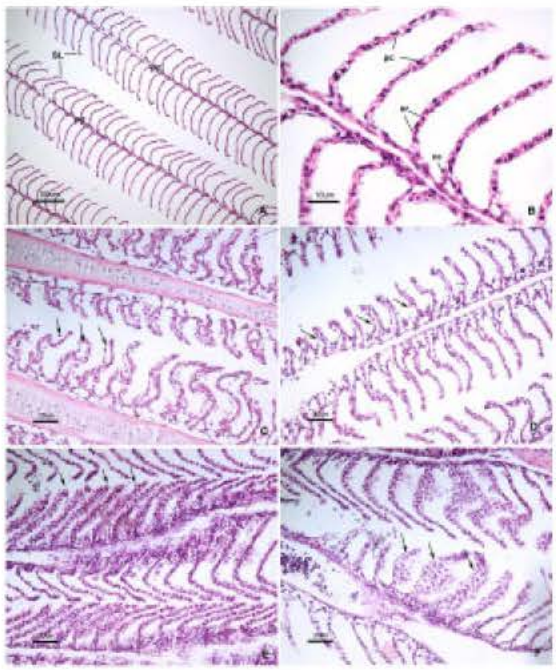

Fig. 1: Light micrographs of gills of *P. altus* in each treatment. (A) Control group showing normal appearance of primary filament (PF)and secondary lamellae (SL). (B) High magnification showing erythrocytes (er) within capillary lumen delimited by pillar cells (pc)and chloride cells (cc) at the base of secondary filament. (C) Cd exposure at 48 h, showing severe edema of epithelial cells (arrows). (D) Cd plus AA exposure at 48 h, showing mild to moderate edema of epithelial cells (arrows). (E) Cd exposure at 72 h, showing moderate hyperplasia of epithelial cells (arrows). (F) Cd plus AA exposure at 72 h, showing mild hyperplasia of epithelial cells (arrows).

Treated Groups

Light microscopic study of the gills of red-tailed tinfoil barb exposed to Cd for 24, 48, 72 and 96 h showed several pathological changes and their frequencies increased with increasing time. SEM study in each group and each time were shown in Fig. 2-5. At the first 24 h, filament cell proliferation was quantified by the height of the filament epithelium. The thickening of the primary lamellar epithelium appeared regular, similar to that of the control. No lesions were observed in the epithelium of the interlamellar space. No recognizable changes were observed in SEM examination (Fig. 2D). At 48 h, lesions tended to appear in the secondary lamellae, where mild edema was observed with some lifting, hypertrophy and hyperplasia of secondary lamellar epithelium and chloride cells. Small areas of necrosis had begun to appear (Fig. 1C). The deformation of primary branch was major defect in SEM examination. It was also found the existence of a mild intercellular edema of the secondary lamellae. The distal extremities of secondary lamellae were bent (Fig. 3D). At 72 and 96 h, the gills of many fish showed moderate to severe hypertrophy and hyperplasia of chloride cells and mucous cells at the base of the gill filaments and secondary lamellae. Extensive aneurism with some ruptures in many secondary lamellae and the breakdown of pillar cell system were seen (Fig. 1E). In addition, SEM examinations showing the micro-ridges on the primary branches were either edema or completely wiped out. The ruptures of the outer tissues were continuously observed entirely (Fig. 5D). In the Cd plus AA treated group, they showed similar alterations as those observed after Cd treatment but they were less severe (Fig. 1-5). The semi-quantitative scoring of gill lesion is shown in Table 1.

Fig. 2: Scanning electron micrograph of gills of *P. altus* after 24 h exposure showing gill filament (GF), secondary lamellae (SL) in control (A); ascorbic acid (B); mix (C) and cadmium (D) exposure group

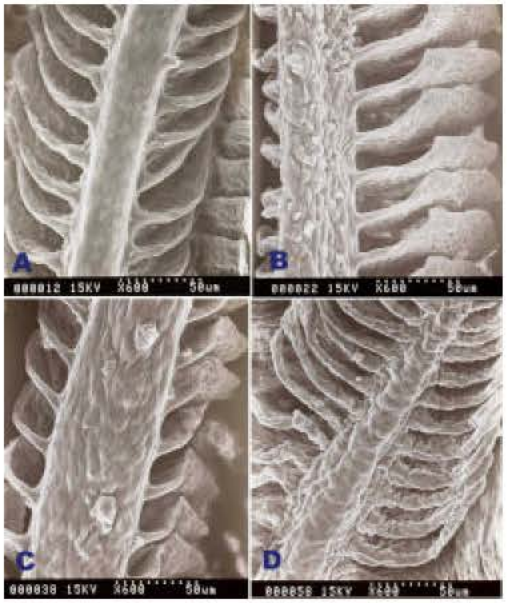

Fig. 3: Scanning electron micrograph of gills of *P. altus* after 48 h exposure in control (A) and showing the distal extremities of secondary lamellae were bent in treatment group: ascorbic acid (B); mix (C) and cadmium (D) exposure group

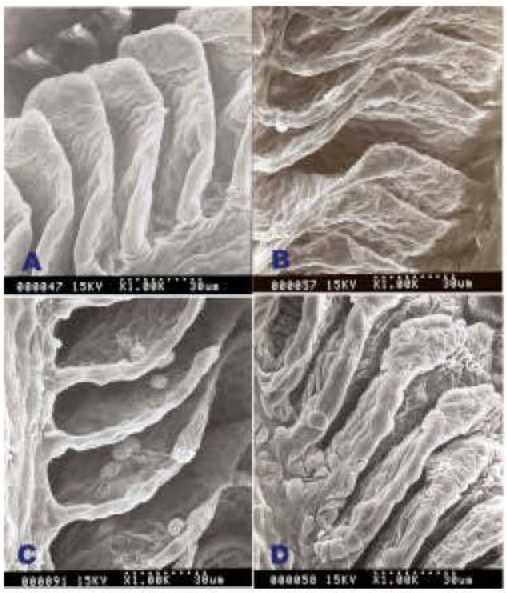

Fig. 4: Scanning electron micrograph of gills of *P. altus* after 72 h exposure in control (A) and showing the distal extremities of secondary lamellae were bent in treatment group: ascorbic acid (B); mix (C) and cadmium (D) treatment group

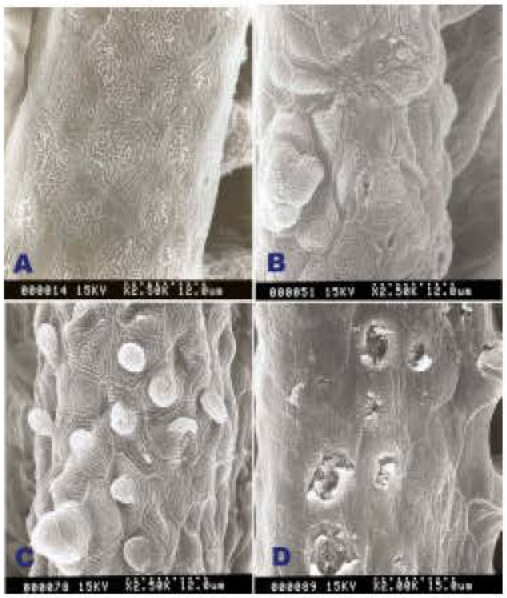

Fig. 5: Scanning electron micrograph of gills of *P. altus* after 72 h exposure in control (A) and showing the micro-ridges on the primary branches were either edema or completely wiped out in treatment group: ascorbic acid (B); mix (C) and cadmium (D) treatment group

Table 1: Semi-quantitative scoring of gill lesion in red-tailed tinfoil barb (*P. altus*) exposed to different time and treatments[a]

	Cont gr.				Cd gr.				Cd+AA gr.				AA gr.			
Lesion	24	48	72	96	24	48	72	96	24	48	72	96	24	48	72	96
LM: Edema	-	-	-	-	-	+++	++	++	-	++	+	+	-	-	-	-
Aneurism	-	-	-	-	-	++	+	+	-	+	-	-	-	-	-	-
Hyperplasia	-	-	-	-	-	+++	++	++	-	++	+	+	-	-	-	-
SEM: Aneurism	-	-	-	-	-	++	+	+	-	+	-	-	-	-	-	-
Hyperplasia	-	-	-	-	-	+++	++	++	-	++	+	+	-	-	-	-

[a]Score value: (-) none, (+) mild, (+ +) moderate, (+ + +) severe occurrence. LM = Light, microscopy, SEM = Scanning electron microscopy

DISCUSSION

Several studies have been described the changes of the gills upon Cd exposure. The data were mainly focused on gill epithelial similarly in this study i.e., gill cells degeneration, uplifting of epithelial, necrosis, lesions and inflammatory infiltration (Randi *et al*., 1996; Wong and Wong, 2000). The nature of the stimulus that triggers the morphological transformation of the gill during Cd exposure is unknown. Interestingly the injection of ascorbic acid appeared to modulate the effects of Cd at all time intervals. The exact mechanism which ascorbic acid minimizes the effect of Cd is not known. However, it is known that ascorbic acid has marked nucleophilic properties it might intercept reactive electrophilic metabolites produced by Cd, thereby preventing their attack on nucleophilic sites on DNA and hence blocking adduct formation (Liehr *et al*., 1989). Otherwise ascorbic acid is an anti-oxidant, which might inhibit the oxidative metabolism of Cd and thus could prevent the production of mutagenic electrophilic metabolites (Goncharova and Kuzhir, 1989). Also as part of a redox buffer system ascorbic acid can scavenge harmful free radical metabolites or reactive oxygen species (Sato *et al*., 1990). Thus, the general protective effect of ascorbic acid observed against Cd induced toxicity could actually be accomplished through one or many of these inhibition mechanisms.

In the fish gills, serve as a major organ for osmotic and ion regulation and respiration. Because of the highly vascular structure of the gill epithelium, it is a primary target for waterborne toxicants. The first sign of lesions in the present study included edema of epithelial cells. The secondary lamellae showed capillary congestion or aneurism, similar to those reported in *Gnathonemus petersii* exposed to Cd (Alazemi *et al*., 1996). The lamellar aneurism resulted from the collapse of the pillar cell system and the breakdown of vascular integrity with a release of large quantities of blood that push the lamellar epithelium outward. Otherwise, thickening of the primary lamellar epithelium, hypertrophy and hyperplasia of epithelial and chloride cells, lifting and fusion of secondary lamellae were also observed. The distal extremities of secondary lamellae were bent, with a reduction of the interlamellar space. Several studies pointed out that chloride cell hyperplasia occurred in response to the need to eject the Cd^{2+} absorbed by the gills (Oransaye and Brafield, 1984; Gill *et al*., 1988).

All lesions in the present study may impair respiratory function. The respiratory diffusion distance is the distance separating blood lacunae in the lamellae from the external medium. Lifting of epithelium or hyperplasia of epithelium results in an increase of the diffusion distance, thus affecting the exchange of gasesand the fusion of lamellae caused a decrease in the total respiratory area of the gills, resulting in a decreased oxygen-uptake for total metabolic activities. Similar results were reported in *P. altus* exposed to cadmium (Jiraungkoorskul *et al*., 2006a), *Poronotus triacanthus* exposed to copper (Jiraungkoorskul *et al*., 2006b) and *Oreochromis niloticus* exposed to herbicide (Jiraungkoorskul *et al*., 2002, 2003). Variations in the epithelial surface of gills show important physiological adaptations, related to the area available for increased gaseous exchange. The previous studies found that Cd was taken up across the epithelial layer of fish gills via calcium channels (Farag *et al*., 1994; Wicklund-Glynn *et al*., 1994). Calcium was known to exert considerable control

over the permeability of the gills and displacement of calcium could stimulate ion loss and water uptake. Cd affected calcium balance and induced damage in gill structure of zebra fish, rainbow trout and tilapia (Karlsson-Norrgren *et al*., 1985; Pratap and Wendelaar Bonga, 1993).

In conclusion, the results presented in this study show that the efficacy of ascorbic acid in reducing histopathological alterations in fish associated with acute exposed to cadmium.

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