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Histopathological Study: The Effect of Ascorbic Acid on Cadmium Exposure in Fish (*Puntius altus*)

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Abstract: The effects of cadmium and ascorbic acid on the red-tailed tinfoil barb (*Puntius altus*) were compared using light microscopic study for the period 24, 48, 72 and 96 h. In the gills, edema, lamellar cell hyperplasia, epithelial lifting and aneurysm were observed. In the liver, there were blood congestion in sinusoids, vacuolation of hepatocytes, hemosiderin accumulation, apoptosis and nuclear pyknosis. In the kidney, glomerulus atrophy and apoptotic cells were seen. In tubular cells, there were hydropic swelling, hyaline casts, apotosis and necrosis. Their changes occurred predominantly in the 48 h treatment. In the combination of Cd and ascorbic acid treated group, they showed similar alterations as those observed in the Cd treated alone group but they were less severe. The findings of this study can be used as guidelines for developing programs to help the fish, which are cultured near the cadmium contaminated areas.

Key words: Red-tailed tinfoil barb, *Puntius altus*, ascorbic acid, cadmium, histopathology

Introduction

Not only does enviroumental pollution caused a decrease in water quality, but subsequently affects all living organisms in that system. It is therefore, necessary to not only identify and manage these pollution sources, but also to 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 Cd as a coloring agent, a stabilizer and in alloy mixtures.

Cd 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 Cd 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, but its role in modulating cytogenetic damage in any fish has few reported (Guha and Khuda-Bukhsh, 2002).

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

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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 (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 expanding and, for aquatic systems, fish have become indicators for the evaluation of the effects of noxious compounds. Red-tailed tinfoil barb (*Puntius altus*) is a commercialized freshwater fish that is not only widely known around the world, but it is also one of the most popular fish in Thailand. To the best of our knowledge no study has been done so far on the efficacy of AA against the Cd 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.

Materials and Methods

Animals

Red-tailed tinfoil barb (*P. altus*) 27.61±5.17 g in body weight and 13.50±0.77 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, 1998). Conductivity was measured with Hanna instrnments Model 3 DiST WP (Hanna Instrnments Inc., USA). The pH was measured with a Cyberscan 510 (Eutech Instrnments 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 = 29.0±1.0°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 37% protein commercial fish food (Charoen Pokphand Group, Bangkok, Thailand). The quantity of food was 2% of the initial body weight per day.

Experimental Design

Fish were exposed to 10 mg L^{-1} Cd. The dose chosen was 50% of the 96 h LC50 value from the acute toxicity test, which was 20.12±0.61 mg L^{-1}. Fish (n = 32) were randomly assigned to four equally sized groups as follows: (1) distilled water; (2) group II, 10 mg L^{-1} $CdCl_2.H_2O$ (Sigma, Germany, CAS No.10108-64-2); (3) 10 mg L^{-1} $CdCl_2.H_2O$ plus 500 mg kg^{-1} BW AA (Sigma, Germany, CAS No.50-81-7) and (4) 500 mg kg^{-1} BW AA. 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 earlier.

At different times (24, 48, 72 and 96 h), 2 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. The organs (gills, liver and kidney) were removed and prepared for histopathological studies.

Specimen Preparation for Light Microscopic Study

The procedures for light microscopy were performed following by the protocol routinely practice in the Department of Pathobiology, Faculty of Science, Mahidol University. The tissues 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).

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. Ten slides were observed from each organ and treatment.

Results

Gills

Control

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 time periods sampled (Fig. 1A and B). 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. Each gill consisted of a primary filament and secondary lamellae. The primary filament had two rows of secondary lamellae that run perpendicular to each filament. The primary lamellar epithelium was one or two cell layers thick. Chloride cells were identified as large epithelial cells with light cytoplasm, usually present at the base of secondary lamellae. 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).

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. 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. At 48 h, the gills showed severe edema of epithelial cells with some lifting, hypertrophy and hyperplasia of secondary lamellar epithelium (Fig. 1C). At 72 and 96 h, the gills of many fish showed moderate hyperplasia of secondary lamellae (Fig. 1E). In the Cd plus AA treated group, they showed similar alterations as those observed after Cd alone treatment but they were less severe (Fig. 1D and F). The semiquantitative scoring of gill lesion is shown in Table 1.

Liver

Control

The liver histology of the control and AA treated groups revealed the typical parenchymatous appearance. At the light microscopic level, the liver was divided into irregularly shaped lobules separated by the hepatopancreas and bile duct. The liver was made up of hepatocytes that were polygonal cells with a central spherical nucleus and a densely stained nucleolus. Venous blood entered the liver caudally from the intestine via the hepatic portal veins and branched into capillaries known as sinusoids. Sinusoids were lined with reticulo-endothelial cells which were in turn surrounded by hepatocytes (Fig. 2A).

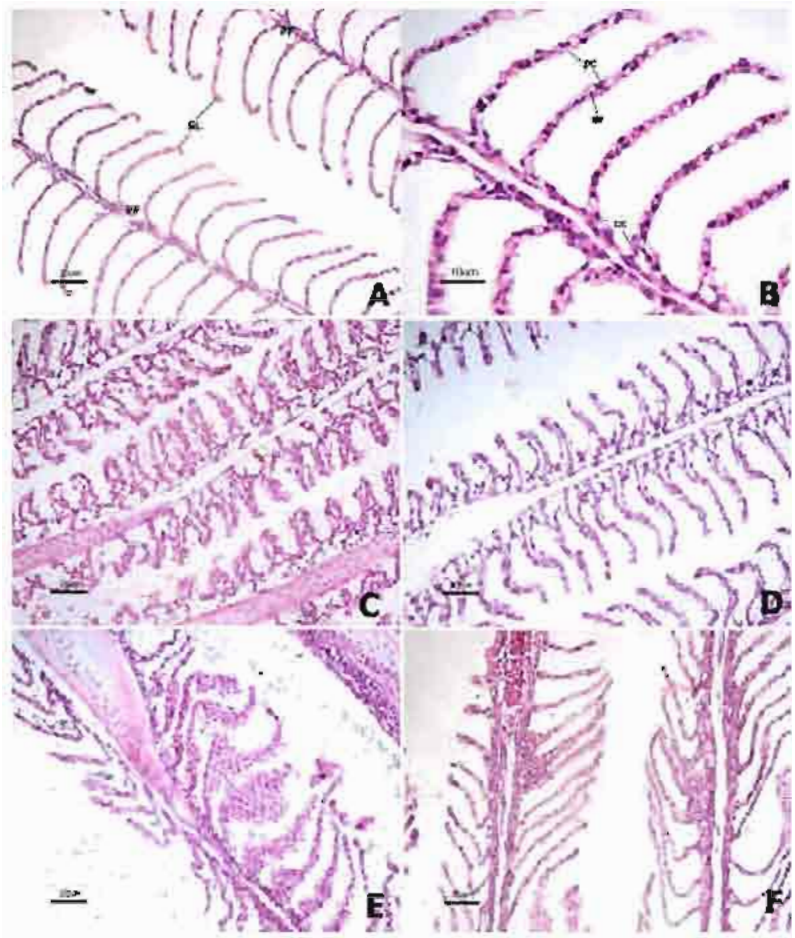

Fig. 1: Light micrographs of gills of *Puntius altus* in each treatment. (A) Control group showing Primary Filament (PF), Secondary Lamellae (SL). (B) High maginification showing erythrocytes (er) within capillary lumen delimited by pillar cells (pc) and chloride cells (cc) at the base of secondary filament. (C) Cd exposure at 48 h, gills showed sever edema of epithelial cells. (D) Cd plus AA expusure at 48 h, gills showed mild to moderate edema of epithelial cells. (E) Cd exposure at 72 h, gills showed moderate hyperplasia of epithelial cells. (F) Cd plus AA expusure at 72 h, gills showed mild hyperplasia of epithelial cells

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

	Cont group				Cd group				VitC group				Cd+VitC group			
Lesion	24	48	72	96	24	48	72	96	24	48	72	96	24	48	72	96
Edema	-	-	-	-	-	+++	++	++	-	-	-	-	-	++	+	+
Aneurism	-	-	-	-	-	++	+	+	-	-	-	-	-	+	-	-
Hyperplasia	-	-	-	-	-	+++	++	++	-	-	-	-	-	++	+	+

[a]Score value (-) none, (+) mild, (++) moderate, (+++) severe occurrence

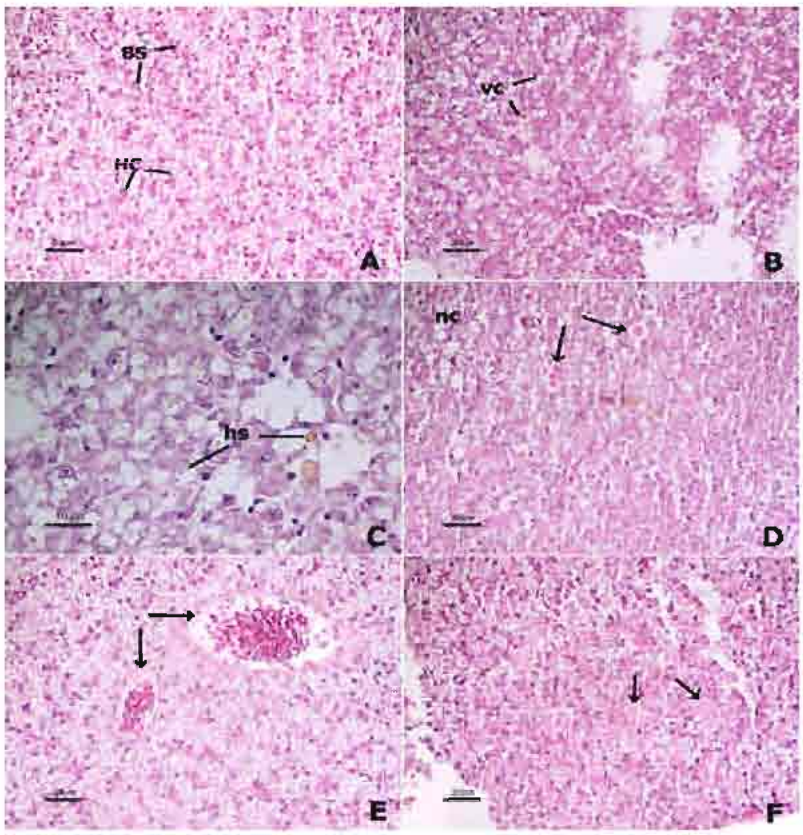

Fig. 2: Light micrographs of liver of *Puntius altus* in each treatment. (A) Control group showing normal hepatocytes (HC) and Blood Sinusoids (BS). (B-C) Cd exposure at 24 h, accumulation of vacuoles (vc) and hemosiderin (hs) were seen in many hepatocytes. (D) Cd exposure at 48 h showing many apoptotic cells (arrows) and necrosis (nc). (E) Cd exposure at 72 h showing dilation of sinusoid with blood congestion (arrows). (F) Cd exposure at 96 h, hepatocytes showing hydropic swelling and hypertrophy (arrows)

Treated Groups

At 24 h Cd treated group, the hepatocytes began to swell and vacuolated were observed (Fig. 2B). The hemosiderin pigments were observed in all time intervals (Fig. 2C). At 48 h, the hepatocytes were still swelling and exhibiting darkly stained specks of necrotic nuclei and several apoptotic cells (Fig. 2D). At 72 and 96 h, the hepatocytes showed moderate congestion (Fig. 2E) and exhibited increased size and pyknotic nuclei in many areas (Fig. 2F). In the Cd plus AA treated group, they showed similar alterations as those observed after Cd treatment but they were less severe. The semiquantitative scoring of liver lesion is shown in Table 2.

Kidney

Control

No recognizable changes were observed in the kidney of the control and AA treated groups (Fig. 3A). At the light microscopic level, the kidney was composed of numerous renal corpuscles with

Table 2 Semiquatitative scoring of liver lesion in red-tailed tinfoil barb (*P altus*) exposed to different time and treatments[a]

	Cont group				Cd group				VitC group				Cd+VitC group			
Lesion	24	48	72	96	24	48	72	96	24	48	72	96	24	48	72	96
Vacuolation	-	-	-	-	+	++	+	+					+	+	-	-
Blood congestion	-	-	-	-	+	+	++	++	-	-	-	-	-	-	+	-
Necrosis	-	-	-	-	-	++	+	+	-	-	-	-	-	+	+	-
Apotosis	-	-	-	-	-	+++	-	-	-	-	-	-	-	-	-	-
Hemosidenn	-	-	-	-	+	+	+	+	-	-	-	-	+	+	+	+
Hypertrophy	-	-	-	-	-	-	++	++	-	-	-	-	-	-	-	-

[a] Score value (-) none, (+) mild, (++) moderate, (+++) severe occurrence

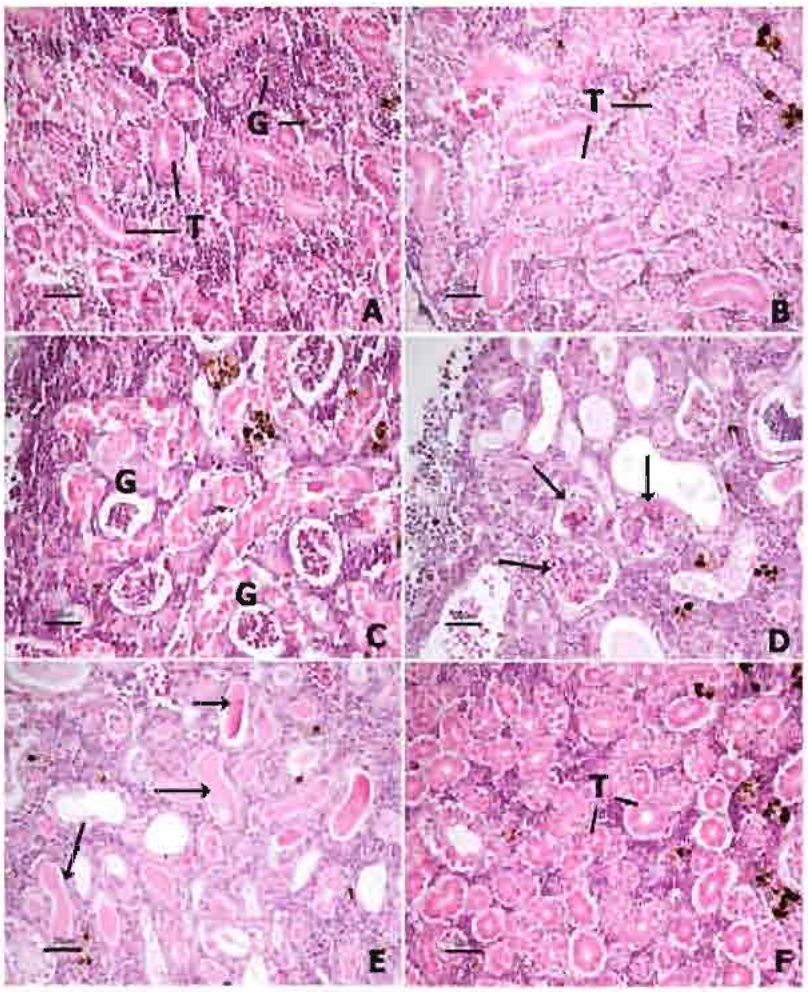

Fig. 3: Light micrographs of kidney of *Puntius altus* in each treatment. (A) Control group showing normal glomerulus (G) and proximal tubules (T). (B) Cd exposure at 24 h showing hydropic swelling of tubules (T) (C-E) Cd exposure at 48 h showing atrophy of glomeruli (G), tubular necrosis and apoptotic cells (arrows in D) and (hyaline casts (arrows in E). (F) Cd exposure at 96 h showing normal appearance of renal tubules (T)

Table 3: Semiquatitative scoring of kidney lesion in red-tailed tinfoil barb (*P. altus*) exposed to different time and treatments[a]

	Cont group				Cd group				VitC group				Cd+VitC group			
Lesion	24	48	72	96	24	48	72	96	24	48	72	96	24	48	72	96
Glomerulus atrophy	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
Tubular cells-swelling	-	-	-	-	+	+	-	-	-	-	-	-	+	+	-	-
hemosiderin	-	-	-	-	+	+	+	+	-	-	-	-	+	+	+	+
apoptosis	-	-	-	-	-	+++	-	-	-	-	-	-	-	-	-	-
necrosis	-	-	-	-	+	+++	+	+	-	-	-	-	+	+	+	+
hyaline casts	-	-	-	-	-	+++	-	-	-	-	-	-	-	+	-	-

[a] Score value: (-) none, (+) mild, (++) moderate, (+++) severe occurrence

developed glomeruli and a system of tubules. The proximal tubule was covered by cuboidal or low columnar epithelial cells with round basal nuclei and brush border (Fig. 3A)

Treated Groups

At the first 24 h Cd treated group, the epithelial cells of the proximal tubule were swollen (Fig. 3B). The hemosiderin pigments were observed in all time intervals. At 48 h, some glomeruli were collapsed or atrophy and tubular necrosis (Fig. 3C); there were also observed apoptotic cells and necrosis in some areas (Fig. 3D). Some of the proximal tubular epithelial cells still swollen and they were exhibited hyaline casts in their lumen (Fig. 3E). Their changes occurred predominantly in the 48 h treatment. At 72 and 96 h, the epithelial cells of many tubules had shown fewer damages, their morphology similar to those of the control (Fig. 3F). In the Cd plus AA treated group, they showed similar alterations as those observed after Cd treatment but they were less severe. The semiquantitative scoring of kidney lesion is shown in Table 3.

Discussion

Pollution by heavy metals is an important problem due to their stable and persistent existence in the envirounent. It is well known that heavy metals interferes the regular chromosome segregation during cell division mainly by inhibition of polymerization of actin tubules, an essential structure of the mitotic spindle (Miura and Imura, 1987). It was suggested that the mechanism of Cd is mainly conditioned by single strand breaks in DNA through the direct cadmium-DNA interactions as well as by the action of incision nucleases and/or DNA-glycosylase during DNA repair (Privezentsev *et al*., 1996). Correspondingly, most of the toxic chemicals have been known to form reactive oxygen species as well as electrophilic free-radical metabolites that interact with DNA to cause disruptive changes. It has been suggested that during the heavy metal exposure, electrophilic ions and radicals were produced, interacting with nucleophilic sites in DNA and leading to breaks and other related damage in the latter.

Further, it would be revealed from the results of this study that Cd produced histopathological alterlations. Interestingly the injection of ascorbic acid appeared to minimize 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. In

the present study the gills of red-tailed tinfoil barb treated with Cd showed thickening of the primary lamellar epithelium, edema, lifting and hyperplasia of secondary lamellae. 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 gases and 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 *Poronotus triacanthus* exposed to copper (Jiraungkoorskul *et al*., 2005) and *Oreochromis niloticus* exposed to herbicide (Jiraungkoorskul *et al*., 2002, 2003).

In the fish liver, as known to be one of the major organs that accumulates cadmium. It not only acts as a storage organ, but is also the primary site for detoxification mechanisms (Olsson *et al*., 1996). The histopathological alterations in the liver observed in the present study were sinusoid dilation with blood congestion, vacuolation, hemosiderin accumulation, apoptosis and cell necrosis. The vacuolation, or fatty change, was characteristic of many exposed livers. The large vacuole in the cell forces the nuclei to the periphery of the hepatocyte and this fatty change is usually accompanied by nuclear atrophy. The vacuolation of hepatocytes might indicate an imbalance between the rate of synthesis of substances in the parenchymal cells and the rate of their release into the systemic circulation (Gingerich, 1982). This vacuolation of hepatocytes was observed in the Cd treated alone and the combination of Cd and AA treated fish. Congestion of blood sinusoids occurred in many of the Cd treated fish, especially the portal veins. A definite higher level of congestion was observed in the 72-96 h exposure time when compare to that of the control group. Toxic of Cd can result in the injured cells dying by apoptosis or necrosis. Cells normally undergo apoptosis in response to mildly adverse conditions, while exposure to severe conditions will result in necrosis. Apoptosis is an active cellular death process characterized by distinctive morphological changes that include condensation of nuclear chromatin, cell shrinkage, nuclear disintegration, plasma membrane blabbing and formation of membrane-bound apoptotic bodies (Risso-de Faverney *et al*., 2006). According to the semiquantitative scoring, it showed an increase as the short-term exposure period, 48 h, but was mostly decrease in fish exposed over the long-term exposure period.

In the fish kidney, it is one of the main targets for cadmium accumulation. The histopathological alterations occurred in the kidney in this study were glomeruli atrophy, tubular swelling with hyaline casts in the lumen. Tubular degeneration and necrosis were also observed. The accumulation of hyaline casts in tubular cells of this study might be the result of a glomerular alteration or an increased permeability of the glomerular filter (Bucher and Hofer, 1993) Similar results were reported in *P. conchonius* exposed to $CdCl_2$ (Gill *et al*., 1989).

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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References

American Public Health Association (APHA). 1998. Standard Methods for the Examination of Water and Wastewater. 20th Edn., The American Public Health Association, American Water Works Association and Water Pollution Control Federation, Washington DC.

Bucher, F. and R. Hofer, 1993. The effects of domestic sewage on three organs (gills, kidney, liver) of brown trout (*Salmo trutta*). Water Res., 27: 255-261.

Gebhart, E., H. Wagner, K. Grziwok and H. Behnsen, 1985. The action of anticlastogens in human lymphocyte cultures and their modification by rat liver S9 II. Studies with vitamin C and E. Mutat. Res., 149: 83-94.

Gill, T.S., J.S. Pant and H. Tewari, 1989. Cadmium nephropathy in a freshwater fish, *Puntius conchonius* hamilton. Ecotoxicol. Environ. Safe., 18: 165-172.

Gingerich, W.H., 1982. Hepatic Toxicology of Fishes. In: Weber, L.J., (Edn.), Aquatic Toxicology. Raven Press: New York. pp: 55-105.

Goering, P.L., M.P. Waalkers and C.D. Klaassen, 1995. Toxicology of Cadmium. In: Goyer, R.A. and M.G. Cherian (Eds.), Toxicology of Metals: Biochemical Aspects. Springer :Berlin, pp: 189-214.

Goncharova, R.I. and T.D. Kuzhir, 1989. A comparative study of the antimutagenic effects of antioxidants on chemical mutagenesis in *Drosophila melanogaster*. Mutat. Res., 214: 257-265.

Guha, B. and A.R. Khuda-Bukhsh, 2002. Efficacy of vitamin C (L-ascorbic acid) in reducing genotoxicity in fish (*Oreochromis mossambicus*) induced by ethyl methanesulphonate. Chemosphere, 47: 49-56.

Humason, G.L., 1972. Animal Tissue Techniques. 3rd Edn., W.H. Freeman and Company: San Francisco.

Jiraungkoorskul, W., E.S. Upatham, M. Kruatrachue, S. Sahaphong, S., S. Vichasri- Grams and P. Pokethitiyook, 2002. Histopathological effects of Roundup, a glyphosate herbicide, to Nile tilapia (*Oreochromis niloticus*). Sci. Asia, 28: 121-127.

Jiraungkoorskul, W., E.S. Upatham, M. Kruatrachue, M.S. Sahaphong, S. Vichasri- Grams and P. Pokethitiyook, 2003. Biochemical and histopathological effects of glyphosate herbicide on Nile tilapia (*Oreochromis niloticus*). Environ. Toxicol., 18: 260-267.

Jiraungkoorskul, W., S. Sahaphong and N. Kangwanrangsan, 2006. Toxicity of copper in butterfish (*Poronotus triacanthus*): Tissues accumulation and ultrastructural changes. Environ. Toxicol., (In Press).

Liehr, J.G., D. Roy and A. Gladek, 1989. Mechanism of inhibition of estrogen-induced renal carcinogenesis in male Syrian hamster by vitamin C. Carcinogenesis, 10: 1983-1988.

Miura, K. and N. Imura, 1987. Mechanism of methyl mercury toxicity. CRC Crit. Rev. Toxicol., 18: 161-187.

Olsson, P.E., A. Larsson and C. Haux, 1996. Influence of seasonal changes in water temperature on cadmium inducibility of hepatic and renal metallothionein in rainbow trout. Mar. Environ. Res., 42: 41-44.

Pauling, L., J.C. Nixon, F. Stiff, R. Marcuson and W.B. Dunham *et al*., 1985. Effect of dietary ascorbic acid on the incidence of mammary tumors in RIII mice. Proc. Natl. Acad. Sci. USA., 82: 5185-5189.

Privezentsev, K.V., N.P. Sirota and A.I. Gaziev, 1996. The genotoxic effects of cadmium studied *in vivo*. Tsitol. Genet., 30: 45-51.

Risso-de Faverney, C., A. Devaux, M. Lafaurie, J.P. Girard, B. Bailly and R. Rahmani, 2001. Cadmium induces apoptosis and genotoxicity in rainbow trout hepatocytes through generation of reactive oxygen species. Aquatic Toxicol., 53: 65-76.

Sato, K., E. Niki and H. Shimasaki, 1990. Free radical-mediated chain oxidation of low density lipoprotein and its synergistic inhibition by vitamin E and vitamin C. Arch. Biochem. Biophy., 279: 402-405.

Shamberger, R.J., 1984. Genetic toxicology of ascorbic acid. Mutat. Res., 133: 135-159.

Van der Oost, R., J. Beyer and N.P.E. Vermeulen, 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: A review. Environ. Toxicol. Pharmacol., 13: 57-149.

Verlhac, V. and J. Gabaudan, 1994. Influence of vitamin C on the immune system of salmonids. Aquacult. Fish Manag., 25: 21-36.

WHO, 1992. Enviroumental Health Criteria No 134, Cadmium. International Programme on Chemical Safety (IPCS), World Health Organisation, Geneva.