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## Efficacy of Ascorbic Acid Reducing Waterborne Copper Toxicity in Butterfish (*Poronotus triacanthus*)

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**Abstract:** This study was investigated the effects of copper (Cu) and Ascorbic Acid (AA) on butterfish (*Poronotus triacanthus*), using the micronucleus (MN) and Nuclear Abnormality (NA) tests for the period of 7, 14 and 21 days. NA shapes in erythrocytes were scored into blebbed nuclei (BL), lobed nuclei (LB), notched nuclei (NT) and binuclei (BN). It was observed that, fish showed significant sensitivity to the different treatments. In general, the highest value of both MN and NA cells were significantly increased in the Cu treated group followed by the combination of Cu and AA treated group. These values revealed the highest number after 21 days treatment in all cases. The frequencies of each NA shape in erythrocytes of all treatments were observed in the following NT>LB>BN>BL. Present results demonstrated the efficacy of ascorbic acid in reducing genotoxicity in fish induced by waterborne copper.

**Key words:** Butterfish, *Poronotus triacanthus*, ascorbic acid, copper, micronucleus test, nuclear abnormalities test

### INTRODUCTION

Heavy metals are toxicants commonly found in mining, industrial, agricultural. In high concentrations these metals could have a negative effect not only on aquatic systems, but also on fish populations. It is therefore, important to determine the effect of these heavy metals on the fish at an early stage, before the fish show any sign of being exposed to the pollutants and before any bodily functions are affected. It is known that polluted water, especially heavy metal pollution like copper (Cu), can cause physiological and biochemical alterations in fish (WHO, 1998). Cu is used to control fungal diseases in vineyard plants in France, South Africa (Schlotfeldt, 1992) and orange orchard in Thailand. High concentrations of Cu were detected in some aquatic ecosystems collecting vineyard runoff water. These high as 0.6 mg L<sup>-1</sup> Cu were detected in orange orchard runoff in Pre province, Thailand (Duangduen *et al.*, 1999). The toxic effect of Cu is related to its capacity for catalyzing oxidative reactions, leading to the production of reactive oxygen species. These highly reactive compounds may also induce tissue alterations and physiological derangement in fish (Varanka *et al.*, 2001).

Ascorbic Acid (AA) is an essential vitamin for normal growth and physiological functions in animals including fish. Most teleosts are unable to synthesize AA due to the lack of L-gulonolactone oxidase (EC 1.1.3.8)

(Fracalossi *et al.*, 2001). Therefore, an exogenous source of AA is required in fish diets. It functions as a general water-soluble redox reagent, on collagen formation, iron metabolism and the response to stress (Vijayavel *et al.*, 2006). It has also been reported to have anticarcinogenic (Guha and Khuda-Bukhsh, 2002), anticlastogenic (Nefic, 2001) and even antimutagenic (Aly and Souria, 2002) 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).

It has been suggested that a variety of biomarkers and bioassays in the laboratory and field studies be used in determining the effects of genotoxic pollution. These include the presence of DNA adducts, chromosomal aberrations, DNA strand breaks and measurement of micronuclei frequencies. Among the currently available test systems, the micronucleus assay is the most widely applied method due to its simplicity, reliability, sensitivity and proven suitability for fish species. Although originally developed for its application in mouse, it was subsequently modified by Hoofman and de Raat (1982) for the application in the laboratory to fish. The micronucleus test is a measure of subcellular process such as induced chromosomal breaks (clastogenesis) or cell spindle malfunction (aneuploidy) (Heddle *et al.*, 1991). Otherwise, in most cases the method of exposure has been intraperitoneal injection of the pollutant assayed, for example *Puntius altus*, to cadmium (Jiraungkoorskul *et al.*,

2006, 2007). However, in field situations pollutants are dissolved in the water; therefore, the direct application of experimental results to freshwater ecosystems is questionable.

Several species of freshwater fish have been reported to be good targets for biomonitoring of rivers and lakes using the micronucleus test as a genotoxicity indicator: rainbow trout, *Oncorhynchus mykiss* (De Flora *et al.*, 1993), brown trout, *Salmo trutta* (Sanchez-Galan *et al.*, 1999). None of these species is appropriate to biomonitor Southeast Asia freshwater ecosystems because they are not native to them. Butterfish (*P. triacanthus*), is a commercialized freshwater fish that is one of the most popular fish in Thailand. It can provide a good model to study responses and possible adaptations of local fish populations to aquatic pollutants. In the present study, we aimed to evaluate the efficacy of ascorbic acid reducing sublethal toxicity of waterborne copper in butterfish (*P. triacanthus*). We also investigated the new parameter in erythrocytes as the incidence of abnormal shape nuclei.

## MATERIALS AND METHODS

**Experimental fish:** This study was performed at the Department of Pathobiology, Faculty of Science, Mahidol University, Bangkok, Thailand, in 2006. Butterfish (*P. triacanthus*), 14.80±3.96 g in body weight and 9.26±0.74 cm in total length, were purchased from a commercial hatchery in Thailand. Tap water was filtered with activated charcoal 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, Rhode Island). The pH was measured with a Cyberscan 510 (Eutech Instruments, Illinois) and the temperature was measured with a glass mercury thermometer. A 16 h light and 8 h dark photo-period was maintained.

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<sup>-1</sup> (as CaCO<sub>3</sub>), alkalinity = 75-80 mg L<sup>-1</sup> and conductivity = 185-210 µmhos cm<sup>-1</sup>. Chlorine residual and ammonia were below detection limits. Fish were fed twice a day with commercial fish food contains 28% protein, 3% fat and 4% fiber (Charoen Pokphand Group, Bangkok, Thailand). The quantity of food was 2% of the initial body weight per day.

**Acute toxicity test:** The acute toxicity tests were performed according to the US EPA procedure for the static non-renewal technique (US EPA, 2002) as described in the previous study (Jiraungkoorskul *et al.*, 2002). Fish were not fed 48 h before starting and 96 h during the experiment. Preliminary screening was carried out to determine the appropriate concentration range for testing the chemical. The test consisted of a control and at least five concentrations of Cu, three replicates per group, with ten fish in each replicate. At the beginning of the tests and after every 24 h, the symptoms and the number of dead fish were recorded. The results of the median lethality concentration (LC<sub>50</sub>) at 24, 48, 72 and 96 h were computed using the SPSS probit analysis computer program (Finney, 1971).

**Diet preparation:** All diets were prepared with commercially fish food that was used to feed the fish during acclimation. Ascorbic acid (Sigma, Germany) supplemented diets were prepared by mixing 500 mg kg<sup>-1</sup> 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 pellets 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.

**Sublethal toxicity test:** Fish were exposed to 26 mg L<sup>-1</sup> CuSO<sub>4</sub>·5H<sub>2</sub>O (Sigma, Germany). The dose chosen was 25% of the 96 h LC<sub>50</sub> value from the acute toxicity test, which was 104.78 mg L<sup>-1</sup>. Fish (n = 60) were randomly assigned to four equally sized groups as follows: Gr.1: normal diet and water; Gr. 2: normal diet and waterborne Cu; Gr.3: AA diet and waterborne Cu and Gr.4: AA diet and water. The fish were kept in the glass flow-through aquaria (50×50×120 cm<sup>3</sup>) with continuous aeration were filled with 200 L of dechlorinated tap water whose physicochemical characteristics were the same as those described previously.

After 7, 14 and 21 days, 5 fish of each group were anesthetized with 0.2 g L<sup>-1</sup> MS-222 (tricaine methan sulphonate, Sigma, Germany), weighed and measured. Peripheral blood samples were drawn from the caudal vessel and placed in tube containing EDTA for MN and NA analysis.

**Micronuclei (MN) and Nuclear Abnormality (NA) analysis:** Blood was smeared on clean grease free microscope slides, air dried for 12 h and then fixed in

absolute ethanol for 20 min. Each slide was stained with 5% Giemsa solution for 30 min. Three slides were prepared and each slide 1000 cells were scored under 1000× magnification using a Nikon E600 light microscope and photographed using a Nikon DXM 1200 digital camera (Tokyo, Japan). Slides were scored by a single observer using blind review. Frequencies of micronuclei (MN) and Nuclear Abnormality (NA) cells were expressed per 1000 cells (%).

**Micronuclei and nuclear abnormality cells scoring:** Only the cells clearly isolated from the surrounding cells were scored. The criteria for the identification of MN were earlier described: (a) MN must be smaller than one-third of the main nuclei, (b) MN must be clearly separated from the main nuclei, (c) MN must be on the same plane of focus and have the same color (Fenech *et al.*, 2003). Nuclear abnormality shapes were scored into one of the following categories: blebbed nuclei (BL), lobed nuclei (LB), notched nuclei (NT) and binuclei (BN) (Carrasco *et al.*, 1990). The result was expressed as the mean value (%) of the sum for all the individual abnormality observed.

**Statistical analysis:** All data were expressed as means±SD. A two-way analysis of variance was used to determine the significance of micronuclei and nuclear abnormalities test. The Least-Significant Difference (LSD) was used for determination of significant differences at  $p < 0.05$ .

## RESULTS

**Acute toxicity tests:** From the probit transformed responses curve of Butterfish exposed to 24, 48, 72 and 96 h of Cu, the values of  $LC_{50}$  were log of 2.11, 2.10, 2.03 and 2.02 or 129.72, 127.12, 108.36 and 104.78  $mg L^{-1}$ , respectively (Fig. 1).

**General growth parameters:** Mortality did not occur during the experiments and the control fish did not show any gross or behavioral changes. Otherwise, the treated fish were less active than the controls and a few of the treated fish rested on the bottom of the aquaria. The swimming became slower and there was reduction in their rate of feeding. Overall, the exposure to copper caused only a minor reduction in mean growth rate. These differences were not statistically significant.

**Micronuclei (MN) and Nuclear Abnormality (NA) analysis:** Normal erythrocyte, approximately diameter 7  $\mu m$ , was contained mainly elliptical nuclei (Fig. 2). The small non-refractile circular or ovoid particle lying in the cytoplasm and resembling a nucleus with respect to

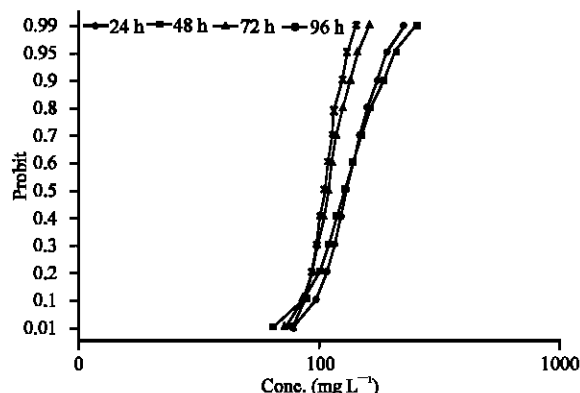


Fig. 1: The probit transformed responses of *P. triacanthus* exposed to  $LC_{50}$  value of Copper

staining properties was considered as micronuclei. The size of the micronuclei varied to some extent (between  $1/25^{th}$  and  $1/5^{th}$  that of nuclear size) but the number was always one. The position of the micronuclei in the cytoplasm also varied, some located very near to the nucleus or some located vary far even at the periphery of the cell. Some of the nuclei clearly deviated from their normal shape and were either blebbed (BL), lobed (LB), notched (NT) and binucleated cells (BN). All abnormalities of nuclei were scored. In briefly, cells with two nuclei were considered as binucleates. The two nuclei should be approximately equal size, staining pattern and staining intensity, within the same cytoplasmic boundary. Blebbed nuclei presented a relatively small evagination of the nuclear membrane, which contained euchromatin. Evaginations larger than the blebbed nuclei, which could have several lobes, were classified as lobed nuclei. Nuclei with depth into a nucleus were recorded as notched nuclei.

The frequencies of MN observed in various treated and control fish have been summarized in Table 1. It is evident that MN was not significantly induced in the control group at 7, 14 and 21 days. Otherwise, the differences were statistically significant in treated group when compared with those of the control group at all the time intervals. The frequencies of MN were significant highest number in fish treated with Cu alone. However, when both Cu and AA were conjointly administered, the MN appeared to be reduced to some extent. The frequencies of NA observed in various treated and control fish have been summarized in Table 2. Similarly to MN, the frequencies of NA were also significant greater number in fish treated with Cu. It was observed that the frequency of each nuclear abnormality shapes in erythrocytes were found in the following order:  $NT > LB > BN > BL$ .

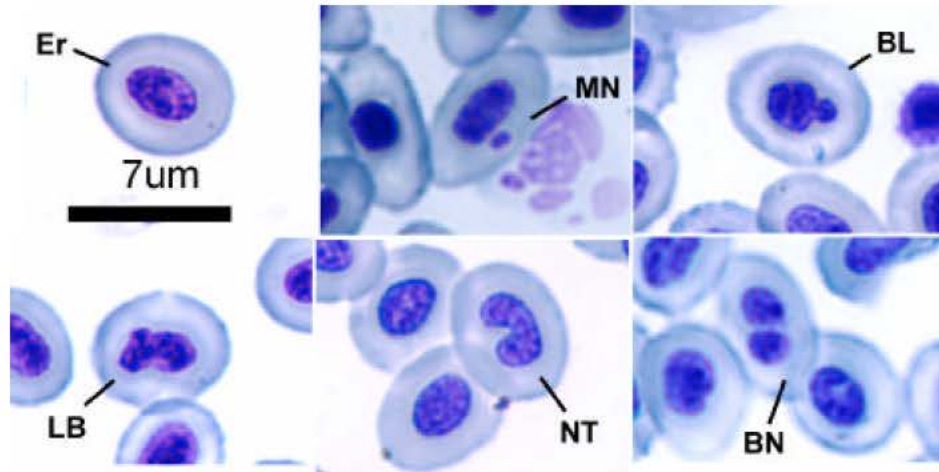


Fig. 2: Photomicrographs of erythrocytes with normal nucleus (Er); micronuclei (MN) and nuclear abnormalities: blebbed nuclei (BL); lobed nuclei (LB); notched nuclei (NT); binuclei (BN) of *P. triacanthus*

Table 1: Comparison of the frequencies (%) of micronuclei and total nuclear abnormalities in erythrocytes *P. triacanthus* after different treatments and times (mean±SD)

Erythrocytes	Time (days)	Cont.	Cu	Mix	AA
Micronuclei	7	0.133±0.058	0.600±0.100*	0.433±0.153*	0.133±0.058
	14	0.167±0.058	0.967±0.115*	0.633±0.058*	0.167±0.058
	21	0.167±0.058	1.067±0.058*	0.667±0.058*	0.200±0.100
Total NA	7	0.467±0.058	0.833±0.058*	0.633±0.058	0.467±0.058
	14	0.500±0.100	0.933±0.058*	0.533±0.058	0.567±0.058
	21	0.533±0.058	0.967±0.153*	0.633±0.058	0.433±0.058

Cont. = Control group, Cu = Copper group, Mix = Copper plus ascorbic acid, AA = Ascorbic acid, \* Mean difference was significant when compared the control group (p•0.05)

Table 2: Comparison of the frequencies (%) of nuclear abnormalities cells in erythrocytes of *P. triacanthus* after different treatments and times (mean±S.D.)

Nuclear abnormalities	Time (days)	Cont.	Cu	Mix	AA
Blebbed nuclei	7	0.033±0.058	0.067±0.058	0.067±0.058	0.033±0.058
	14	0.033±0.058	0.133±0.058	0.033±0.058	0.133±0.058
	21	0.033±0.058	0.067±0.058	0.067±0.058	0.033±0.058
Lobed nuclei	7	0.067±0.058	0.267±0.058*	0.200±0.000	0.133±0.058
	14	0.133±0.058	0.267±0.058*	0.167±0.058	0.133±0.058
	21	0.133±0.058	0.200±0.100	0.167±0.058	0.133±0.058
Notched nuclei	7	0.2467±0.058	0.367±0.115	0.233±0.058	0.233±0.058
	14	0.267±0.058	0.333±0.058	0.200±0.100	0.233±0.058
	21	0.267±0.058	0.600±0.173*	0.267±0.058	0.133±0.058
Binuclei	7	0.100±0.000	0.133±0.058	0.133±0.058	0.067±0.058
	14	0.067±0.058	0.200±0.000	0.133±0.058	0.067±0.058
	21	0.133±0.058	0.167±0.058	0.133±0.058	0.133±0.058

Cont. = Control group, Cu = Copper group, Mix = Copper plus ascorbic acid, AA = Ascorbic acid, \* Mean difference was significant when compared the control group (p•0.05)

## DISCUSSION

MN is chromatin masses with the appearance of small nuclei that arise from chromosome fragments or intact whole chromosomes lagging behind in the anaphase stage of cell division. Their presence in cells is a reflection of structural and/or numerical chromosomal aberrations arising during mitosis (Heddle *et al.*, 1991). MN and NA tests in fish are generally performed in enucleated peripheral blood erythrocytes mainly due to its technical feasibility. There are some assays in fish aimed

to evaluate the differential sensitivity of diverse species to different chemicals (Ayllon and Gardía-Vazquez, 2000). But in most cases the method of exposure has been intraperitoneal injection, for the example, cadmium significantly induced micronuclear expression in red-tailed tinfoil barb, *P. altus*, (Jiraungkoorskul *et al.*, 2006, 2007). Otherwise, in the present study, we investigated the genotoxicity of copper sulfate in fish using immersion protocol.

Pollution by heavy metals is an important problem due to their stable and persistent existence in the

environment. 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). The biochemical effects of Cu can be related to binding to various electron donors, sulfhydryl groups in particular and possibly represents the major toxic action of metal. These bonds induce changes in protein structure and enzymatic function. Another effect seems to affect nucleic acids; it has led to speculations about the possible induction of chromosomal aberrations like lead (Wozniak and Blasiak, 2003). Correspondingly, most of the toxic chemicals that produce genotoxic effects 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 are produced, interacting with nucleophilic sites in DNA and leading to breaks and other related damage in the latter.

This result revealed that Cu produced genotoxic effects. Interestingly ascorbic acid supplemented diet appeared to minimize the genotoxic effects of Cu. The exact mechanisms are still unknown. However, it is known that ascorbic acid has marked nucleophilic properties it might intercept reactive electrophilic metabolites produced by Cu, 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 Cu 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 Cu induced genotoxicity could actually be accomplished through one or many of these inhibition mechanisms. Blanco and Meade (1980) indicated that dietary supply of AA not only helps in promoting growth but also protects the fish from the toxicities of many chemicals. In the present study, there was a general tendency of occurrence of notched and lobed types in higher frequencies. An analysis revealed spontaneous frequencies of nuclear abnormalities in erythrocytes were found in the following order: NT>LB>BN>BL. Thus, our results seem to be in agreement with previous studies (Jiraungkoorskul *et al.*, 2007; Cavas and Ergene-Gozukara, 2003).

In conclusion, Present data indicated that the efficacy of ascorbic acid in reducing genotoxicity in fish associated with sublethal concentration of waterborne

copper. The micronuclei and abnormal shape nuclei in erythrocytes fish are the valid technique for monitoring genotoxic effects induced by heavy metals.

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