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Preexposure to Copper Modulates Nonenzymatic Antioxidants in Liver of *Channa punctata* (Bloch) Exposed to the Herbicide Paraquat

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Herbicide contamination of surface waters derived from agricultural practices is a problem of worldwide importance. Several scientific investigators have examined the use of herbicides in terrestrial ecosystems and the toxicity of herbicides to animals (Szarek et al. 2000). However, less is known about their toxicity to life in aquatic ecosystems, including fish. The presence of herbicides in water is a consequence of weed control in terrestrial ecosystems and water reservoirs. Paraquat (methyl viologen) is an effective quaternary bipyridyl herbicide with a broad spectrum of activity, ranging from crop desiccation to weed control, that has become a ubiquitous contaminant of the environment (Tortorelli et al. 1990). Its rate of application for aquatic weed control ranges from 0.1 to 2 parts per million (Calderbank 1972). It is also used in orchards and vineyards. Among other things it passes via rain into natural waters where it is accumulated in different organisms living in water, especially in fish, thus making them vulnerable to several discernible effects (Gabryelak and Klekot 1985). Toxic effects of paraquat have been found in some aquatic non-target organisms. It produces gill, kidney and liver alterations in fish (Di Marzio and Tortorelli 1994). It is also known to modify enzymatic activities in fish and affects cardiac contraction and operculative ventilation. These effects may alter early development of the organism (Tortorelli et al. 1990).

Pesticide-induced oxidative stress has been also a focus of toxicological research for the last decade as a possible mode of toxicity. Role of antioxidants in protection against toxicity of environmental chemicals and their use as surrogate biomarkers of aquatic pollution are being studied in aquatic animals (Lackner 1998). Our studies have shown that non-enzymatic antioxidants of fish may be useful biomarkers of exposure to aquatic pollutants (Ahmad et al. 2000; Ali et al. 2004).

Copper is an essential trace element for all biota. Essentiality of copper arises from its specific incorporation into a variety of enzymes, which play important roles in physiological processes (e.g. enzymes involved in cellular respiration, free radical defense, neurotransmitter function, connective tissue biosynthesis and other functions), as well as into some structural proteins (WHO 1998). Copper is reported to have a protective role against toxicity of a variety of chemicals mainly

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in mammals (Lauren and McDonald 1987). Furthermore, in our previous study a substantial amount of copper present in paper mill effluent was found to provide protection to liver from peroxidative damage induced by paper and pulp mill effluent. The observed protection was mainly due to the induction of copper-metallothionein (Cu-MT) and other antioxidants (Ahmad et al. 2000). We have also reported copper induces an array of non-enzymatic antioxidants that may be beneficial to fish in oxidative stress resulting from chemical pollutants (Parvez et al. 2003). The present study was undertaken to investigate whether copper pre-exposure on the herbicide paraquat modulated non-enzymatic antioxidants in the freshwater fish *Channa punctata* (Bloch).

MATERIALS AND METHODS

The investigations were carried out in a freshwater fish, *Channa punctata* (Bloch), procured from pollution-free water bodies (fish hatcheries, which were free from any kind of pollutant exposure). The morphometric parameters of fish included weight (40 ± 3 g) and length (15 ± 2.5 cm). Fish were maintained following standard fish maintenance procedure (APHA 1998; Parvez and Raisuddin 2005), in glass aquaria (1m x 0.75m x 0.75m) containing 160-liter dechlorinated water, with n=4 fish in each exposure chamber. Fish were acclimatized for 15 days before use. Aquarium water was kept aerated and its temperature was maintained at ambient laboratory temperature (27 ± 2°C). Fish were transferred to a fresh volume of water every 24 h to minimize contamination from metabolic wastes.

One group of fish (n=16) was exposed to paraquat (1 mg/L) for 24 h duration. Another group with n=16, served as the control while the third group with n=32 were exposed to 10 ppb (1 μg/L) of cupric chloride for 4 weeks. After 4 weeks of exposure, half of the copper-acclimatized fish were exposed to paraquat (1 mg/L) for 24 h. There were four replications within each treatment with (n=4) fish in each exposure chamber (capacity of 160-liter) for all the exposure conditions. The biological load of 1 g tissue/L of solution was maintained in all conditions. The concentration and duration of copper exposure were based on information in previously reported studies (Ahmad et al. 2000; Parvez et al. 2003). Discernible toxic effects are reported to appear at the concentration and duration of exposure of paraquat used (Matkovics et al. 1987). Six samples from each treatment group were taken for this study as other exposed fish that survived were used to collect information not presented in this paper. The exposure was planned in such a way that all the fishes were sacrificed on the same day. After decapitation, the liver tissues were cleaned, thoroughly minced and homogenized in chilled phosphate buffer (0.1M, pH 7.4) containing KCl (1.17%) to prepare post-mitochondrial supernatant (PMS) using the method of Parvez and Raisuddin (2005). The homogenate was filtered through a muslin cloth and centrifuged at 800 *g* for 5 min at 4°C to separate the nuclear debris. The supernatant was centrifuged in a refrigerated centrifuge at 10,500 *g* for 30 min at 4°C to obtain the PMS, which was used for estimation of various biochemical parameters.

Reduced glutathione (GSH) was determined in PMS by the method of Ahmad et al. (2000). The values are expressed as μM/g of wet tissue. Total (T-SH), protein bound (P-SH) & non-protein bound thiol (NP-SH) groups in the PMS were determined using the method of Parvez et al. (2003). The values are expressed as μM/g of wet tissue. Protein carbonyl content was assayed by the procedure of Levine et al. (1990) as modified by Parvez and Raisuddin (2005). The results were expressed as nanomoles of DNPH incorporated/mg protein using a $\in$ of 21,000 M^{-1} cm^{-1}. Ascorbic acid (AsA) content was estimated by the method of Sayeed et al. (2003). Absorbance was taken at 540 nm and the results were expressed as mg of AsA/g tissue. Protein content in various samples was estimated by the method of Lowry et al. (1951) using Folin's reagent and bovine serum albumin (BSA) as the standard. The statistical analysis of data was done using analysis of variance (ANOVA). The significance of results was ascertained at $P < 0.05$.

RESULTS AND DISCUSSION

Paraquat (1mg/L) caused a significant ($P < 0.001$) reduction in liver GSH levels when compared with control values (Table 1). The levels of T-SH and P-SH also increased significantly ($P < 0.05$) in liver of paraquat-only fish when compared with control group values (Table 1). The level of NP-SH decreased significantly ($P < 0.001$) in liver when compared to the values for control fish (Table 1).

Table 1. Effect of copper pre-exposure and paraquat-only exposure on the thiol-profile in livers of *Channa punctata* (Bloch.)

Parameter (Units)	Groups			
	Control	*Paraquat*	*Cu pre-exposed*	*Cu + Paraquat*
GSH (nmol/g tissue)	0.99 ± 0.08	0.71 ± 0.09[b]	1.08 ± 0.09	0.88 ± 0.07*
T-SH (μmol/g tissue)	231.42 ± 18.6	272.81 ± 19.2[a]	240.29 ± 20.1	249.12 ± 15.2*
NP-SH (μmol/g tissue)	1.29 ± 0.24	1.05 ± 0.34[b]	1.35 ± 0.16	1.19 ± 0.21*
P-SH (μmol/g tissue)	230.11 ± 17.9	270.92 ± 18.4[a]	242.29 ± 18.5	246.97 ± 14.5*

Values are expressed as mean ± S.E. (n=6); the significance level observed are [a]$P < 0.05$ and [b]$P < 0.001$ when compared with control group values; and *$P < 0.05$ when compared with paraquat-only exposed group values.

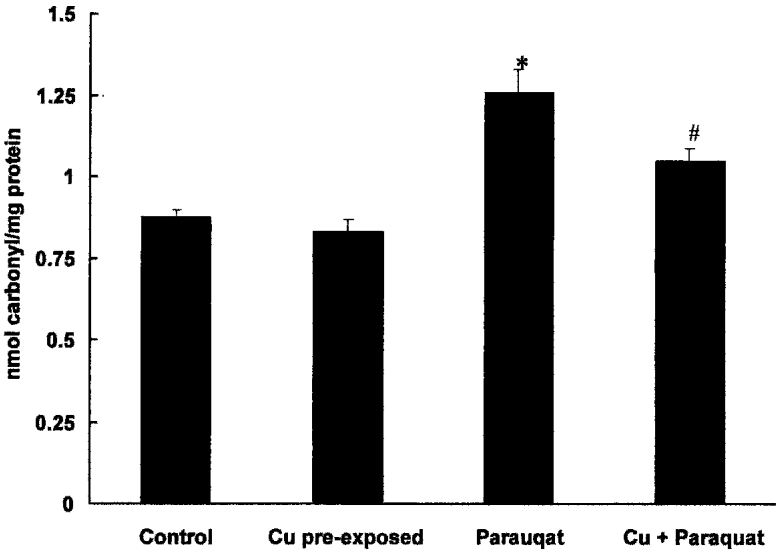

Figure 1. Protein carbonyl levels in the livers of different groups of fish. The values are expressed as means ± SE (n=6). Protein carbonyl level is expressed as mg/g of tissue. The significance levels observed are $^{*}P < 0.05$ when compared with control group values and $^{\#}P < 0.05$ when compared with paraquat-only group values

Copper pretreatment for 4 weeks showed no significant effect on any of the thiol profile parameters in liver tissue of the fishes. A significant increase ($P < 0.05$) in GSH level in liver of the Cu-acclimatized paraquat-exposed group was observed when compared with the paraquat-only group. T-SH and P-SH levels of the Cu-acclimatized, paraquat-only group were significantly lowered ($P < 0.05$) in liver (Table 1) when compared with the paraquat-exposed group, while NP-SH levels showed a significant ($P < 0.05$) increase in the liver of the Cu-acclimatized, paraquat-exposed group when compared with the paraquat-only group (Table 1). Glutathione is a ubiquitous thiol-containing tripeptide that is involved in numerous processes that are essential for normal biological function, such as DNA and protein synthesis (Meister and Anderson 1983). It scavenges reactive oxygen species directly or in a reaction catalysed by glutathione peroxidase (GPx) through the oxidation of two molecules of GSH to a molecule of glutathione disulphide (GSSG). GSH comprises of the bulk of non-protein thiol content of cells. The similarity of trends in GSH and NP-SH levels further strengthens our findings. Protective and adaptive roles of GSH against oxidative stress-induced toxicity are well established in aquatic animals (Ahmad et al. 2000). The decrease in GSH level in paraquat-only fish could be attributed to an adaptive response of fish either due to induction of MT or ceruloplasmin-like oxyradical scavengers. The central thiol status is of great importance in coordinating the antioxidant defense network (Dafre et al. 2004). Its estimation has been used as a biomarker of exposure in the aquatic environment (Sayeed et

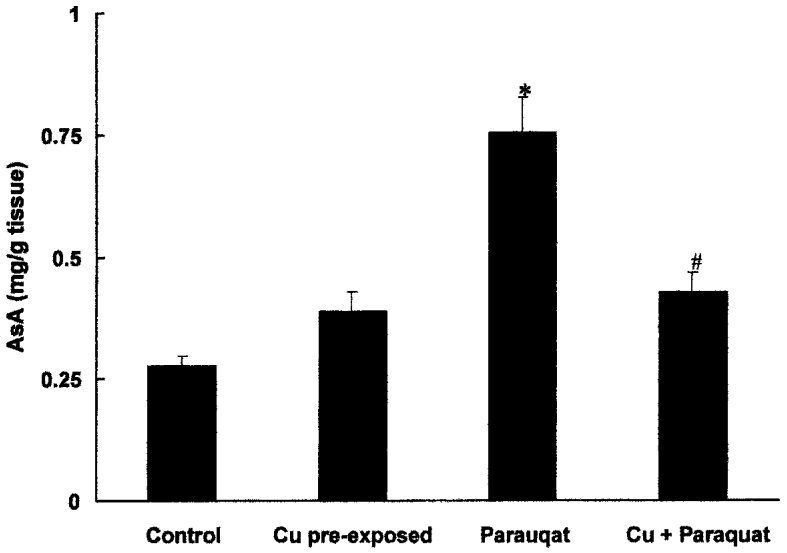

Figure 2. AsA levels in liver of different groups of fish. The values are expressed as means ± SE (n=6). AsA is expressed as mg/g of tissue. The significance level observed are $^{*}P < 0.001$ when compared with control group values and $^{\#}P < 0.05$ when compared with paraquat exposed group values.

al. 2003). Pre-exposure to copper restored the thiol status in fish exposed to paraquat. Copper is a micronutrient for aquatic life present in all natural waters and sediment. This metal is essential to the health of virtually all-living organisms. Natural flux of copper concentrations has lead to adaptation by many organisms, which has influenced aquatic species composition, diversity and abundance of the populations. Invertebrates and fish possess elaborate homeostatic mechanisms that can maintain internal concentrations of this metal within narrow limits while external concentrations fluctuate over several orders of magnitude. Fish utilize proteins called metallothionein that are involved with copper transport and metabolism. We have previously reported that copper pre-exposure increases the serum ceruloplasmin activity in fish (Parvez et al. 2003). This could explain the restoration of the thiol profile in fish pre-exposed to copper. The levels of protein carbonyl increased significantly ($p < 0.05$) in liver of paraquat exposed ($p < 0.05$) fish when compared with control fish (Fig 1). Copper pre-exposure significantly reduced the levels of protein carbonyl levels when compared with paraquat-only fish (Fig 1). It has been established in mammalian systems including humans, that direct damage to proteins or chemical modification of amino acids in proteins during oxidative stress can give rise to protein carbonyls (Zusterzeel et al. 2001). It has been suggested that induction of protein carbonyl may serve as a surrogate biomarker for general oxidative stress. Recently, protein carbonyl has been used as a biomarker of pesticide-induced oxidative stress in freshwater fish (Parvez and Raisuddin 2005). Protein oxidation can lead to loss of critical sulfhydryl groups in addition

to modification of amino acids leading to the formation of carbonyl and other oxidized moieties. High oxygen tension in many areas of the circulation favors reactive oxygen species formation and membrane proteins are cross-linked. Oxidative modification leads to proteolytic degradation, which may affect structure, function and integrity (Carney et al. 1991). Pre-exposure to copper significantly reduced the AsA levels in paraquat-only fish, by inhibiting the oxidation of proteins by reactive oxygen species. The AsA level increased significantly ($p < 0.001$) in livers of paraquat-only fish when compared with control fish (Fig 2). In fishes which were copper pre-exposed and subsequently paraquat exposed the AsA levels were significantly ($p < 0.05$) reduced when compared with paraquat-only fish (Fig 2). Ascorbic acid is an important cellular metabolite with various functions, one of them being potent antioxidant activity (Fraga et al. 1991). Elevated ascorbic acid levels have been reported in the livers of fish exposed to paper and pulp mill effluents (Andersson et al. 1988) and also to pesticides like deltamethrin (Sayeed et al. 2003). Copper pre-exposure helped in reaching normalized AsA in fishes exposed to paraquat suggesting protection against toxicity of pesticides. Copper pre-exposure modulates the non-enzymatic antioxidants in the liver of the fish *Channa punctata* and these findings can be employed as surrogate biomarkers in biomonitoring programs.

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