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Immunological Response of the Freshwater Fish *Colossoma macropomum* as a Biomarker of Copper Exposure

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Because of their aquatic environment, fishes are an excellent model for studying the immunotoxic effects of water and sediment-borne pollutants. Many piscine diseases are related to poor environmental quality. Various environmental pollutants have immunotoxic potential, and many related diseases have an immunological component. Moreover, there is concern about the health status of aquatic ecosystems in relation to pollution, and many fishes are useful as sentinel species for detecting these effects via development of such biomarkers, including various immunological markers, which are necessary for effective monitoring of environmental pollutants and their effects.

Although copper is an essential nutrient, high concentration in water can exert an immunosuppresive effect in fishes and lead to increased incidence of infectious diseases (Carballo *et al.*, 1995). In Venezuelan rivers, copper concentration has been increased reaching legal limited stablished (García *et al.*, 2004; Vaquero *et al.*, 2004). This investigation describes the sensitivity of non-specific cellular immunological responses in *Colossoma macropomum* as biomarker of effects of copper exposure. Because this fish is distributed throughout South America, and laboratory culture and manipulation are relatively easy it is a good candidate sentinel species in immunotoxicity studies (Goulding and Carvalho, 1982).

MATERIALS AND METHODS

Juveniles of *C. macropomum* (31.43-48.41g) supplyed by National Institute of Agropecuary Research (Tucupita, Venezuela), were maintained at 23±1 °C in aquariums with continuosly aereated and flowing dechlorinated tap water (pH 7.2- 7.8) at least 15 days prior to the experiments. Fish were fed *ad libitum.* Before copper exposure, total and differential count of leukocytes and phagocytosis by polymorphonuclear cells were determined in 24 fishes. Then, two groups of 6 fishes (each group with its respective replicate) were exposed to 0.2 mgCu L^{-1} ($CuCl_2.5H_2O$, Merck) during 21 days in a 16L glass aquarium, using a semi-static test system. The 96 hours LC_{50} of copper, previously calculated, for this size and weight was 8.35 $mgCuL^{-1}$. Each aquarium was continuously aerated and the same physical and chemical characteristics of water as those in laboratory acclimation were maintained. Copper concentration in the

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water was measured each two days using an atomic absorption spectrophotometer. Eighty percent of metal aquarium water was renewed all days. Before renewal of water, fish were fed and the remaining food was removed. Control fishes were maintained under the same conditions in water devoid of detectable copper. Total and differencial cell counts were analysed at 7, 15 and 21 days of copper exposure. Bacterial killing was assayed at 21 d in 6 Cu-exposed and 6 control fishes.

Blood samples were taken from anaesthetized fishes (0.01% benzocaine) by caudal puncture with heparinized 3 ml syringes with 21 gauge needles. Blood smears were fixed with methanol and stained with Giemsa dye. Total cell count was determined by quantification in a Neubauer chamber (Blanhall and Daisley, 1973) Differential leukocyte counts were made by differentiation of 200 leukocytes in each slide (Dick and Dixon, 1985). Phagocytic cells were obtained from 0.5 ml of heparinized fish blood with 1.5 ml cold (4°C) isotonic ammonium chloride, mixed well and held for 30 min to permit erythrocyte haemolysis. Cell suspensions were then centrifuged at 160 X g for 10 min and the red haemolysate discarded. The white cell pellet was gently resuspended in 1 ml of Hanks' balanced salts solution (HBSS). The cells were centrifuged twice at 55 X g for 10 and 5 min, respectively, and the supernatant was carefully discarded. The resulting phagocytic cells (PC) pellet was gently resuspended in 0.5 ml HBSS and held at 4°C until required (about 5 minute). Cell viability was determined by staining with 0.4% Trypan blue (Sigma Chemical Co) in HBBS solution and reported as the percentage of live cells at counting. Dead or damaged cells appeared blue, intact cells were transparent.

The method of Eggletton *et al.* (1989) was used to determine the opsonisation, phagocytosis and bacterial killing. Briefly, bacteria *Escherichia coli* (ATCC) were grown overnight in Brain Heart Infusion Broth (Merck) washed twice and resuspended in HBSS. The number of bacteria in the suspension was estimated at 620 nm. After appropriate dilutions the bacteria were opsonised by adding fish serum to a final concentration of 10% (v/v) and incubed for 30 min at 37°C. A phagocytic cell suspension (PCS) of 200 µl was added to an equal volume of opsonised bacteria to give bacteria to PCS ratio of approximately 10:1. The bacteria/PCS mixture was rotated for 1 h at 37 °C, a 100 µl sample removed, and a slide prepared to assess phagocytosis. To estimate bacterial killing, a 50 µl sample of the bacteria/PCS suspension was removed immediately upon mixing, added to 4.95 ml of distilled water for 5 min, shaken vigorously, diluted and plated out for duplicate viable counts. This step was repeated after 45 min. Bacterial killing was expressed as the percent reduction of viable count after 45 min. All experiments were performed in triplicate.

Blood parameter mean values in experimental fish were statistically analyzed by simple ANOVA, and the SNK test with 95% confidence limit was applied to compare the means whenever the data were significant. Phagocytic response and bacterial killing were analysed by the t-test.with 95% confidence. All statistics analysis were made with the program Statgraphics Plus Version 5.1 (Addlink Software Scientific, S. L.).

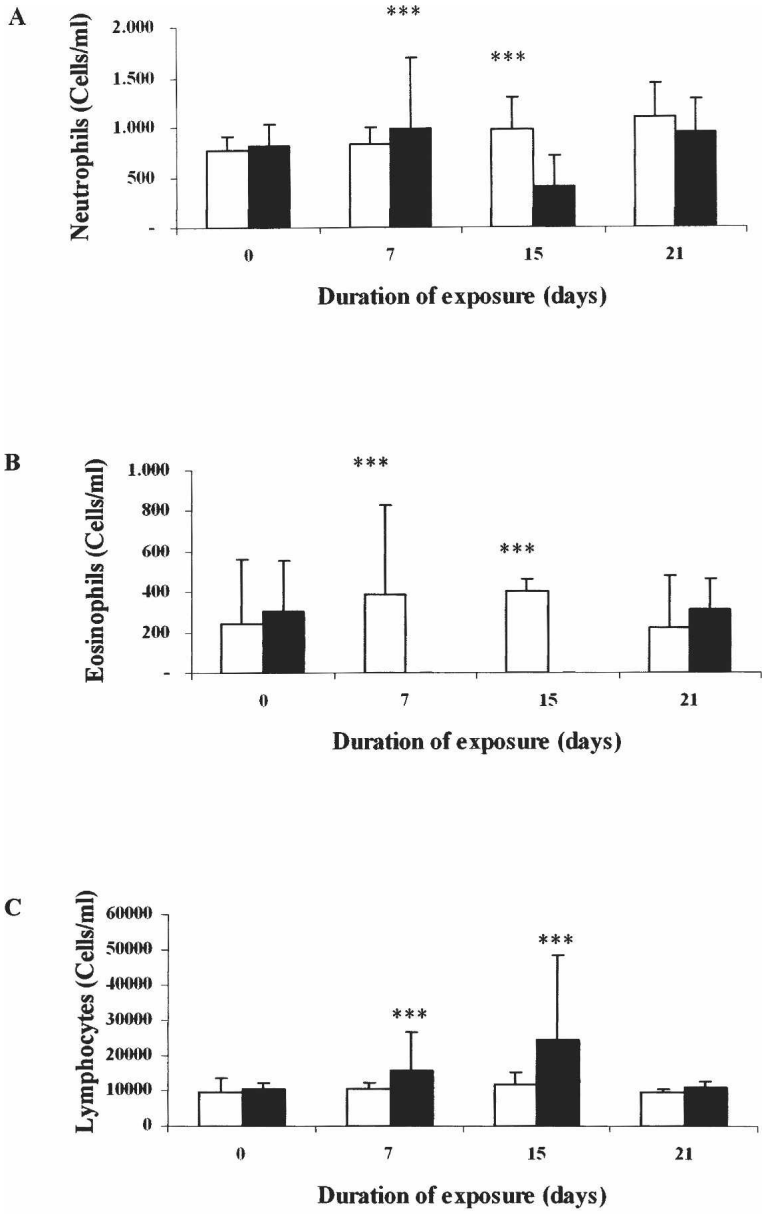
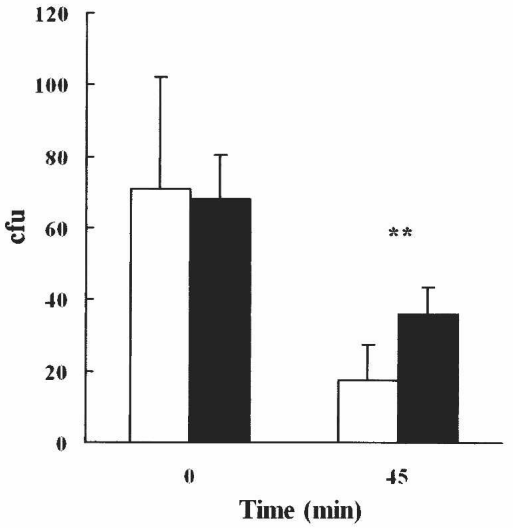


Figure 1. Changes in the percentage of differential leukocyte counts of *C. macropomum* blood after exposure to copper. Control (open bars) and copper exposed fish (solid bars). The vertical rectangles and the bars denote mean values and standard deviation, *** significant to $P<0.001$.

A



B

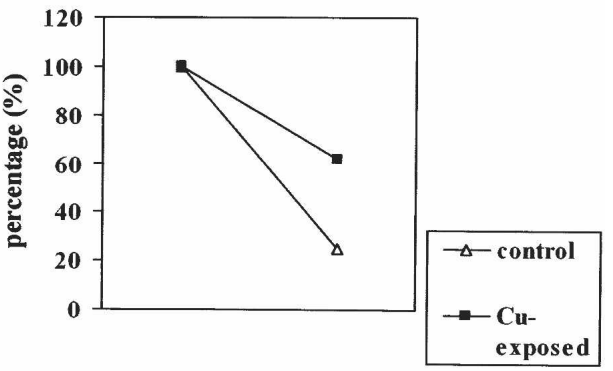


Figure 2. A. Bacterial killing induced by phagocytic cells (PC) of *C. macropomum* exposed to copper during 21 days. Control (open bars) and copper exposed (solid bars). The vertical rectangles and the bars denote mean values and standard deviation. Cfu: colony forming units. B. Percentage of reduction to colony forming units by PC of *C. macropomum*, ** significant to $P<0.01$.

RESULTS AND DISCUSSION

The copper increased the total count leukocytes of *C. macropomum* at 7d (16510 ± 11790 cell/ml; control= 13000 ± 3763 cell/ml) similar to reported by Dick and Dixon, (2000) in *Salmo gardneri* Cu-exposure. However, at 15-21d Cu exposure, leukocyte count reached similar values that control organism (Cu-exposure: 25000 ± 24228 cell/ml; 12000 ± 2198 cell/ml, respectively; control: 10833 ± 1402 cell/ml). The high variability showed by Cu-exposed fishes maybe result of intraspecific difference since these were wild-caught fish increased samples sizes wolud be necessary to evaluate this problem.

On the other hand, we observed increase total count of lymphocytes and neutrophils in short time Cu-exposure (7 days) but not at 15-21d Cu-exposure (Figure 1A y B). A decrease in lymphocyte with increase in neutrophils is the pattern observed in copper exposed fish for long and short periods (Mazon *et al.*, 2002), as well as to other metals (Khangarot *et al.*, 2000; Witeska and Kosciut, 2003); its pattern was not totally showen in *C. macropomum* exposed to copper. Eosinophils were betwen 0 to 305 cell/ml in both groups; however, in Cu-exposed fish 7-15d these cells were few (< 100 cells/ml, Figure 1B). Cell viability was 95 ± 2% in both groups. The phagocytic response was significantlly higher for Cu-exposed fish compared to controls; (Cu exposure= 19±3%; control= 11±5 %; t-test = 3.61; $P < 0.01$). However, bactericidal activity by phagocytic cells was significantly lower (t-test = 3.66 $P < 0.01$) as evidenced by the increase in the percent of reduction the colonies forming units (cfu) arising from bac/PCS of exposed organisms (Figure 2A y B). This indicates that the chronic copper exposure affected immunological function of phagocytic cells of *C. macropomum*, inducing defects in intracellular killing of ingested microorganisms. This defect could be related with copper inactivation of enzyme involved in the 'respiratory burst' (Elaser *et al.*, 1986). It is known that other organisms, including human with normal endocytosis and defective killing of polymorphonuclear cells tend to develop chronic granulomatous responses (Hagey, 2002). Evidence suggests that fish from contaminant rich environments develop granulomas in their tissues and also develop increased sizes and numbers of melanomacrophage aggregates in spleens or other organs (Kane *et al.*, 2000). Suppression of bactericidal activity of phagocytic cells could be considered as a biomarker of chronic copper effects on the immune system in fish and should be included, with other parameters, for immunotoxicity studies in aquatic organisms living in environments contaminaated with metals and other toxic chemicals. However more it is work needed to describe these effects on respiratory burst activity.

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