Effect of *in vitro* exposure to cadmium and copper on sea bass blood cells

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ABSTRACT - Blood cells freshly collected from sea bass (*Dicentrarchus labrax*) were exposed *in vitro* to different concentrations of cadmium (Cd) and copper (Cu) at 10^{-7} M, 10^{-5} M, 10^{-3} M, and examined for neutral red retention capacity and for cell vitality with MTT assay. A relationship between heavy metal exposure and alteration in responses of blood cells in a dose-time-dependent was found. Our results showed that fish blood cells may constitute an interesting biological model for experimental and applied toxicology, especially in the case of environmental pollution.

Key words: Fish blood cells, Heavy metals, NR, MTT.

Introduction - Chronic contamination by heavy metals in marine habitats is a severe problem, especially because these pollutants can persist in the environment. It is possible to carry out numerous investigations on the effects of these metals on the biological functions of marine organism and, in particular, on the defence mechanism in fish. Exposure to environmental contaminants has been shown to alter fish immune responses including reactive oxygen intermediate (ROI) production during the phagocytosis (Elsasser *et al*., 1986; Anderson, 1994; Zelikoff *et al*., 1994, 1996; Roszell and Anderson, 1996). Thus, the immune system of fish can be used as a bioindicator of exposure to environmental pollutants. There are several assay methods for evaluating the cytotoxic effects of chemicals on cultured cells. One of these, the neutral red (NR) assay, is a quantitative colorimetric method (Borenfreud and Puerner, 1985). This assay is based on the uptake of neutral red dye which accumulates in the lysosomes. Lysosomes play an important role in the function of eukaryotic cells and, under certain conditions, many contaminants including heavy metals and organic xenobiotics are known to be sequestered in lysosomes (Moore, 1990). Living cells take up the neutral red, which is concentrated within the lysosomes. Another assay widely used is the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). This assay is based on the uptake and the reduction by mithocondrial succinic dehydrogenase of the soluble yellow MTT tetrazolium salt to an insoluble blue MTT formazan product. The formazan product is impermeable to the cell membranes and, therefore, it accumulates in healthy cells.

Material and methods - Sea bass (*Dicentrarchus labrax*) weighing 200–250 g were sampled at a commercial fish farm (Ecoittica, Marsala, TP) and anaesthetised in seawater with 0.05% MS222 (3-aminobenzoic acid ethyl ester) (Sigma-Aldrich Corp. St Louis, MO, U.S.A.). Blood was collected from heart into a sterile plastic syringe containing 0.2 ml of heparin. For experimental purpose, cells were cultured in 96-well plates. The optimum cell concentration to obtain a monolayer was 20 x 10^{-6}/ml in PBS (phosphate buffered saline) Ca^{2+} Mg^{2+} (200 µl/well). The cells were allowed to attach for 1 h before treatment with $CdCl_2$ (Cd) and $CuSO_4$ (Cu), then they were treated with cadmium and copper within a range of concentrations from 10^{-7} M, 10^{-5} M and 10^{-3} M in HBSS (Hanks Balanced Salt Solution) for 2 and 24 hours in humidified atmosphere containing 5% of

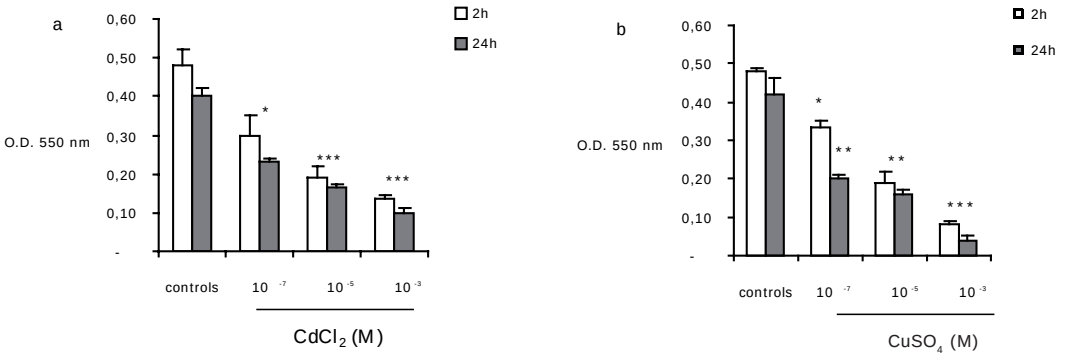
CO_2 at 18°C. After exposure of cell monolayer to cadmium and copper for 2 and 24 hours or HBSS for the control group, the cells were incubated with HBSS with 0.2% neutral red for 2 hours at 18°C, then the culture medium containing neutral red was aspirated, and each well was carefully rinsed twice with HBSS. Finally, the intracellular dye was extracted with 200 µl of a mixture of 100% ethanol and 99.9% acetic acid (1:1 v/v). The mixtures were mixed fully and evaluated at a 550 nm on a microplate reader Labsystems Uiniskan I. After exposure of cell monolayer to cadmium and copper for 2 and 24 hours or HBSS for the control group, the MTT assay was performed incubating cells for 3 h with 5 mg/ml of MTT, dissolved in L15 medium (Leibovitz medium). Following incubation, the microplates were centrifuged at 600g, the solution was aspirated and 100 µl of DMSO (dimethil sulfoxide) were added, gentle shaking for 10 minutes, then the complete dissolution was achieved. Aliquots of 100 µl of the resulting solutions were transferred in 96-well plates and absorbance was recorded at 550 nm using microplate reader Labsystems Uiniskan I. All the experiments were performed 3 times in triplicate and the data obtained were statistically analysed with the Student's t-test.

Results and conclusions - As shown in Figure 1, exposure of blood cells to both Cd and Cu resulted in the reduced capability of lysosomes to retain neutral red when compared with controls. A significant difference ($P<0.01$) of the lysosomal stability index with respect to controls was already present at the 2 higher concentrations for both Cd and Cu (10^{-5} M and 10^{-3} M) after 2h, but we observed major effects at 24h. The results showed that Cu is more toxic of Cd.

As shown in Figure 2, exposure of cells to both Cd and Cu resulted in a reduced capability of mitochondrial to effect enzymatic conversion of MTT when compared with controls. A significant difference ($P<0.01$) of mitochondrial activity index with respect to controls was already present at the 2 higher concentrations of both Cd and Cu (10^{-5} M and 10^{-3} M) after 2h and at 24h. In the latter case, we have observed major effects. The results showed that Cu is more toxic of Cd.

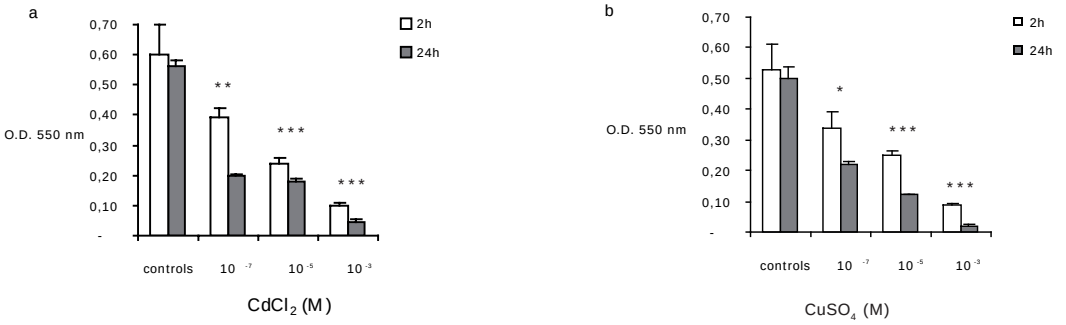
The results of the present study showed that exposure of the blood cells of *Dicentrarchus labrax* to cadmium and copper could influence their functional responses. The observed effects varied with the nature and concentration of these heavy metals, with copper being more toxic than cadmium. In conclusion, the biochemical variables we considered in this study could be useful for biomonitoring marine coastal environments.

Figure 1. Neutral red viability assays of blood cells treated with cadmium (a) and copper (b).
Values are the mean ±SD from 3 separate experiments. Significance levels compared to controls.



*$P<0.05$, **$P<0.01$, ***$P<0.001$.

Figure 2. MTT viability assays of blood cells treated with cadmium (a) and copper (b). Values are the mean ±SD from 3 separate experiments. Significance levels compared to controls.



**P<0.05, **P<0.01, ***P<0.001.*

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