Ecotoxicology and Environmental Safety 102 (2014) 113–120

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

In vitro effect of cadmium and copper on separated blood leukocytes of *Dicentrarchus labrax*

Mirella Vazzana [a], Monica Celi [a], Cecilia Tramati [b], Vincenzo Ferrantelli [c], Vincenzo Arizza [a,*], Nicolò Parrinello [a]

[a] Dipartimento STEBICEF, Università degli Studi di Palermo,Via Archirafi 18, Palermo, Italy
[b] Dipartimento DISTEM, Università degli Studi di Palermo, Via Archirafi 18, Palermo, Italy
[c] Istituto Zooprofilattico della Sicilia "A. Mirri", Palermo, Italy

ARTICLE INFO

Article history:
Received 26 August 2013
Received in revised form
10 January 2014
Accepted 13 January 2014

Keywords:
Heavy metal
Leukocytes
NR
MTT
Hsp70 gene
Real-time-PCR

ABSTRACT

The immunotoxic effects of heavy metals on blood leukocytes of sea bass (*Dicentrarchus labrax*) were examined. The cells, separated by a discontinuous Percoll-gradients, were exposed *in vitro* to various sublethal concentrations of cadmium and copper (10^{-7} M, 10^{-5} M, and 10^{-3} M) and their immunotoxic effect was then evaluated by measuring neutral red uptake, MTT assay, DNA fragmentation and Hsp70 gene expression. First of all, we demonstrated that the cells treated *in vitro* could incorporate Cd and Cu. A relationship between heavy metal exposure and dose-time-dependent alterations in responses of leukocytes from blood was found for both metals, but copper was more immunotoxic than cadmium in all assays performed. A significant reduction in the cells' ability to uptake neutral red and viability by MTT assay was recorded, indicating that both cadmium and copper could change the membrane permeability, inducing cellular apoptosis when the concentration of metals reached 10^{-3} M. The apoptotic effect may also explain the high level of cytotoxicity found when the leukocytes were exposed to higher concentration of metals. These results demonstrated that toxic effect of copper and cadmium affect on the mechanisms of cell-mediated immunity reducing the immune defences of the organism.

1. Introduction

In recent years a great deal of effort has been directed towards elucidating the possible correlation between environmental pollutants and stress-related disease conditions in animals. Industrial development has caused an increase of metal emissions and aquatic environments have been continuously contaminated by so-called heavy metals. They usually occur in a highly soluble form when present in seawater, and their uptake and accumulation are therefore usually proportional to the concentration. Among these metals, copper (Cu) and cadmium (Cd) are of particular importance for their harmful effects on fish. Chronic contamination in the marine environment produces severe problems, especially because these pollutants persist in the environment, and fish show the ability to absorb and accumulate – through the gill surface and gut – higher levels of heavy metals in their tissue than the level of toxic concentration in their environment (Alyakoob et al., 1994), with a consequent negative influence on their homeostatic mechanisms.

Although copper is essential for cellular metabolism, it becomes extremely toxic at high concentration (Carvalho and Fernandes, 2006). Many papers describe its toxic effect on DNA and the damage to and inhibition of relative repair mechanisms (Emmanouil et al., 2007; Joseph, 2009; Pruski and Dixon, 2002), and the general reduction of protein synthesis coupled with an increase in stress proteins (Qian et al., 2012; Said Ali et al., 2010). Cadmium, whose effects in sublethal concentrations on fish metabolism are well known, has been shown to alter the structure and to cause morpho-pathological changes of varying severity in various organs (Atif et al., 2005; Thophon et al., 2003).

The exposure of fish to sublethal doses of Cd and Cu altered the macrophage functions such as phagocytic activity and production of free radicals (Elsasser et al., 1986; Zelikoff, 1993), respiratory burst (Sanchez-Dardon et al., 1999) and the blastogenic and antibody-production responses of lymphocytes (Anderson et al., 1989; O'Neill, 1981; Thuvander, 1989). The effects of metal also affect the levels of circulating leukocytes, thus altering the blood cell ratios (Dick and Dixon, 1985; Murad and Houston, 1988; Nussey et al., 1995). Since leukocytes, through their elevated phagocytic activity, are important to protect the body against bacterial infection, the alteration of their ratio could have deleterious effects on both specific and cellular immune responses.

* Corresponding author.
E-mail address: vincenzo.arizza@unipa.it (V. Arizza).

http://dx.doi.org/10.1016/j.ecoenv.2014.01.018
0147-6513

In previous papers, *Dicentrarchus labrax* was used as model organism to investigate the immunotoxic effects of xenobiotics. When a mixture of cadmium and benzo(a)pyrene was injected in the fish body, it reduced phagocytosis both in spleen and kidney macrophages (Lemairegony et al., 1995). Copper and cadmium were shown to have an *in vivo* inhibitory toxic effect on the phagocytosis and on the production of reactive oxygen intermediates from macrophages from the anterior kidney of sea bass (Bennani et al., 1996).

Previously we observed that all the blood cells of *D. labrax* exposed to cadmium and copper were affected in their functional responses, including the reduced capability of lysosomes to retain neutral red and the reduced capability of mitochondria to effect enzymatic conversion of MTT (Celi et al., 2008; Vazzana et al., 2009). The aim of this study was to evaluate the impact of cadmium chloride ($CdCl_2$) and copper sulfate ($CuSO_4$) on leukocytes isolated from *D. labrax* blood cells at different concentrations after 2 and 24 h of exposure. We measured metal accumulation in the cells by mass spectrometer and the varying effects of toxicity by neutral red uptake and MTT assays. Moreover, we evaluated DNA integrity and Hsp70 gene expression levels by RT-PCR analysis.

2. Materials and methods

2.1. Procedures related to fish and bleeding

A total of 32 sea bass (*D. labrax*) weighing 200–250 g were obtained from a commercial fish farm (Ecoittica, TP) were utilized for all the experiments. The fish were acclimated in the department aquaria at constant environmental conditions for at least 2 weeks, and fed with commercial pellets (Biomar for sea bass).

Before sampling, the fish were anesthetized with 0.05 percent MS222 (3-aminobenzoic acid ethyl ester) (Sigma-Aldrich Corp. St Louis, MO, U.S.A.) in seawater.

Blood was collected from the heart into a sterile plastic syringe containing 0.2 ml of heparin and diluted with three shares of cold medium (Leibovitz L15 medium, 100 units penicillin ml^{-1}, 100 units streptomycin/ml and 10 units heparin/ml). Culture medium components were from Gibco Carlsbad, CA, U.S.A. The medium osmolarity was measured by an osmometer (Röebling) and adjusted to that of fish serum (370 ± 20 mOsm/kg).

All experiments were performed in full compliance with the national rules and guidelines (D. Lgs 116/92 and subsequent amendments) and international European Commission Recommendation guidelines for the accommodation and care of animals used for experimental and other scientific purposes (2007/526/EC).

2.2. Density gradient separation of the cells

Blood samples of 32 individuals were diluted 1:3 with Hanks balanced salt solution (HBSS: NaCl 190 mM, KCl 5.36 mM, glucose 5.54 mM, KH_2PO_4 0.44 mM, Na_2HPO_4 0.56 mM; pH 7.6, 370 mOsm/kg) and to separate leukocytes, the total blood cell suspension was layered on a 34–46 percent discontinuous Percoll/density gradient as previously described (Vazzana et al., 2003). After centrifugation at $400 \times g$ for 25 min at 4 °C, a cellular band, enriched in leukocytes, was visible at the interface of the two Percoll gradients and after collection, the cells were washed twice with HBSS. The cellular debris was located at the top of the gradient and the erythrocyte pellet was packed at the bottom. The leukocyte band was counted in a haemocytometer and resuspended at 2×10^6 cells/ml in HBSS. Cell viability was determined using a Trypan blue (0.01 percent in HBSS) exclusion test.

2.3. Copper and cadmium exposure

For experimental purpose, enriched leukocytes from 32 fish were suspended in HBSS at a concentration of 2×10^7/ml and aliquots of 200 μl, per well in double, were seeded in sterile PVC flat bottom 96-well plates. The cells were allowed for 1 h to adhere to the bottom of the wells to form a monolayer, to which were then added 200 μl of $CdCl_2$ or $CuSO_4$ solution at a concentration of 10^{-7} M, 10^{-5} M and 10^{-3} M or HBSS as control. The cell cultures were incubated in a humidified atmosphere containing 5 percent of CO_2 at 18 °C and sampled at 2 and 24 h.

2.4. Determination of heavy metals in the leukocytes

After exposure to the metals, the cells-5×10^6 from nine specimens for each metal standardized concentrations – were mineralized with 5 ml of HNO_3 Suprapur (67–70 percent), 1 ml of H_2O_2 Suprapur (30 percent) and 4 ml of MilliQ water in an automatic microwave digestion system (MARS® CEM) using for sample digestion the CEM XP1500-Plus high-pressure digestion vessels.

The presence of heavy metals in the leukocytes was evaluated with a spectrometer ICP–OES Varian Vista MPX (Inductively Couplet Plasma-Optical Emission Spectrometer) in according to manufacture instruction. The specific wavelengths (λ) used for each element were Cd: 214.439 nm and Cu: 324.754 nm.

Only reagents of Suprapur quality and MilliQ water were used during the laboratory procedures. Results were given in μg/ml per 1000 cells

2.5. Neutral red uptake assay

Neutral red uptake assay (NRU) is used to measure cell viability (Borenfreund and Puerner, 1985). It has been used as an indicator of cytotoxicity in cultures of primary hepatocytes (Fautz et al., 1991) and other cell lines (Morgan et al., 1991). Living cells take up the neutral red, which is concentrated within the lysosomes of cells. Chemicals causing membrane damage inhibit the accumulation of this dye. The amount of dye released from the cells is proportional to their vitality. The NRU evaluation was performed, with slight modification, as previously described (Arizza et al., 2013). The cell monolayers from nine fishes, after treatment with metals, were incubated with 200 μl of 0.2 percent neutral red in HBSS for 2 h at 18 °C in double. To minimize crystal formation during the neutral red uptake assay, the dye solution was stirred overnight at 37 °C and filtered (0.2 μm) before use. After the incubation period, the supernatant of cell monolayers containing the neutral red dye was discharged, and each well was carefully rinsed twice with 200 μl of fresh HBSS. Finally, the neutral red adsorbed by the leukocytes was extracted treating the cellular monolayers with 200 μl of ethanol and acetic acid (1:1 v/v). After mixing weakly, the absorbance of the extracted dye was read on a Labsystems Uiniskan I microplate reader equipped with a 540 nm filter. Results from triplicate samples were recorded as the average number of optical density units after subtracting the blanks and as NRU per number of cells.

2.6. MTT reduction assay

The MTT (3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay is a sensitive and quantitative colorimetric assay that measures the viability of cells based on the cellular uptake of MTT and its subsequent reduction by mitochondrial succinyl dehydrogenase in living cells to convert the yellow substrate into a dark blue formazan product. The original protocol of Mosmann (1983) was optimized for the cells used in these experiments. Briefly, at the end of the incubation time, cells from nine fishes were incubated for 3 h with 20 μl of MTT (5 mg/ml) in HBSS in double. After incubation the microplates were centrifuged at 500 rpm for 5 min at 4 °C and the supernatant of the cell monolayer was aspirated. To extract MTT from the intact cells, 100 μl of dimethyl sulfoxide (DMSO) were added and gentle shaking was performed for 10 min. When complete dissolution was achieved aliquots (100 μl) of the resulting solutions were transferred into 96-well plates and absorbance was recorded at 550 nm using a Labsystems Uiniskan I microplate reader.

2.7. Hsp70 gene expression

2.7.1. Total RNA extraction and cDNA synthesis

Total RNA was isolated from the leukocytes, held in RNA later, from every individual by using a RNAqueousTM-Midi Kit purification system (Ambion), and was then reverse-transcribed by the Kit Ready to GoTM T-primed first-strand using random primers (Amersham-Pharmacia Biotech, USA).

2.7.2. mRNA quantification by real-time quantitative PCR

Changes in the gene expression of heat shock proteins were quantified using fluorescence real-time quantitative PCR. Analyses were performed in triplicate using a quantitative thermal cycler. Cellular expression of the Hsp70 genes was studied by Real-time PCR using the Taqman method. Primers and hybridization probes were designed using Primer Express software V.0 and synthesized commercially (Applied Biosystems, Foster City, USA). Real-time PCR analysis was performed using the Applied Biosystems 7500 Realtime PCR System, as described previously (Celi et al., 2012). Tissue expression was performed using Hsp70 probe (ACCATGAAGTCCACTGTG), Hsp70 forward (5'-TGGCCTGGAGTCATATGCTTT-3') and reverse (5'-TCTTACCGGCAAGCCTTTCA-3') primers, for housekeeping actin gene probe (ACCACAGCCGAGACG) forward (5'-CAGAGCGTGGCTACTCCTTCA-3') and reverse (5'-TCCTTGATGTCACGCACGAT-3') primers (*D. labrax* actin Accession number AY148350). For each time point/treatment five individuals were used and each cDNA sample was run in triplicate together with negative controls. The amplification efficiencies of the target and reference genes were approximately equal, thereby validating the $\Delta\Delta Ct$ calculation. The amount of Hsp70 transcript from tissue was normalized to actin in order to compensate for variations in input RNA amounts. Relative Hsp70 expression was determined by dividing the normalized value of the target gene in the tissue by the normalized value obtained from untreated tissue.

2.8. DNA apoptotic fragment

DNA was extracted from the cells of five fishes by using the Sigma-Aldrich geneluite mammalian genomic miniprep kit based on a silica-based membrane, specially selected for genomic DNA purification, in a convenient spin column format according to the manufacturer's instructions.

Briefly, a suspension of 5×10^6 cells in 200 µl of HBSS was prepare and to lyse the cells 20 µl of proteinase K, 20 µl of RNase and 200 µl of lysis buffer (supplied by the kit) were added. After incubation at 70 °C for 10 min, 200 µl of 100 percent ethanol were added to the suspension and layered at the top of the column (supplied by the kit), then centrifuged at 6500 × g (8000 rpm) for 1 min to remove the medium. The column was washed two times with washing buffer (supplied by the kit) at 12,000 × g for 3 min. To elute the DNA, the column was treated with eluting buffer (supplied by the kit), incubated for 5 min at 20 °C and then centrifuged at 6500 × *g*. Each eluted sample was analyzed through standard 1 percent agarose gel electrophoresis and visualized with ethidium bromide (5 µg ml^{-1}) reaction. The DNA fragmentation was analyzed after electrophoresis at 200-BP intervals to check for a "ladder" pattern due to apoptosis. Cellular treatments with 100 mM of 2-deoxy-D-ribose (dRib) were used as a positive control (Malagoli et al., 2005).

2.9. Statistical analysis

All experiments were performed three times utilizing for each trial three fish for a total of 27 specimens. For the experiment of Real-time PCR analysis were used a sample size of five fish. Significant differences between values of different incubation groups and the reference control groups were determined with the Statistical analysis by one-way ANOVA with Tukey's post-test.

3. Results

3.1. Leukocytes enrichment

After density gradient centrifugation, a band enriched mainly with leukocytes was evident at the interface of two gradient steps. It was composed by lymphocytes (~59.3 percent), monocytes-macrophages (~28.7 percent), neutrophils (~7.1 percent) and eosinophils (~4.9 percent), thus confirming the results obtained by Vazzana et al. (2003). The viability test, performed on leukocytes after each treatment, showed vitality values greater than 95 percent as determined by trypan blue.

3.2. Heavy metal analysis

After exposure to metals, the cells were examined to evaluate the incorporation of Cd and Cu. The evaluation, performed using a spectrometer ICP–OES, showed that metals were adsorbed by the leukocytes in a dose- and time-dependent manner. Indeed a significant presence of metals was found only after 2 h of treatment at a concentration of 10^{-5} M for cadmium (12.6 ng/ml) and for copper (9.3 ng/ml). The effects were significantly more evident for the higher concentration and exposure time, reaching respectively the values of 386 ng/ml for Cd and 456 ng/ml for Cu (Table 1). The untreated cells (controls) showed values below of the minimum detectable concentrations of the instrument.

3.3. NRU assay

Exposure of leukocytes for different times and at different concentrations of the metals produced a general decrease in the incorporation of neutral red. As shown in Fig. 1a, treatments for 2 h with Cd at a concentration of 10^{-5} M significantly reduced ($p < 0.01$) the capability to retain neutral red, and decreased the control value from 0.22 O.D. to 0.15 O.D. While longer treatment (24 h) significantly decreased ($p < 0.01$) the control value of 0.67 O.D. to 0.18 O.D. at 10^{-7} M.

In Fig. 1b are shown the modulating effects of Cu on NRU. In general, Cu appeared to have a greater toxic effect than Cd. In fact it showed a significant decrease in retention of neutral red for both

Table 1
Cellular concentration of Cd and Cu in separated blood leukocytes of *D. labrax* after 2 h and 24 h incubation time.

M	2 h	24 h
$CdCl_2$ cellular absorption (ng/ml)		
10^{-7}	0.1 ± 0.09	0.1 ± 0.05
10^{-5}	12.6 ± 0.9	24.4 ± 1.2[a]
10^{-3}	262.0 ± 23.1[b]	386.0 ± 12.5[c, d]
$CuSO_4$ cellular absorption (ng/ml)		
10^{-7}	0.3 ± 0.01	0.3 ± 0.09
10^{-5}	9.3 ± 0.7	11.7 ± 0.8[a]
10^{-3}	371.0 ± 5.2[e]	456.0 ± 9.6[f, d]

The values are expressed as ng/ml evaluated by a spectrometer ICP–OES. The data are the means of three distinct measures from three separated cell treatments ± standard deviation. Statistical analysis by one-way ANOVA with Tukey's post-test.

[a] $p < 0.05$,
[b] $p < 0.001$ significance degree between control and $CdCl_2$ concentration at 2 h.
[c] $p < 0.01$ significance degree between control and $CdCl_2$ concentration at 24 h.
[d] $p < 0.01$ significance degree between 2 h and 24 h incubation time.
[e] $p < 0.001$ significance degree between control and $CuSO_4$ concentration at 2 h.
[f] $p < 0.01$ significance degree between control and $CuSO_4$ concentration at 24 h.

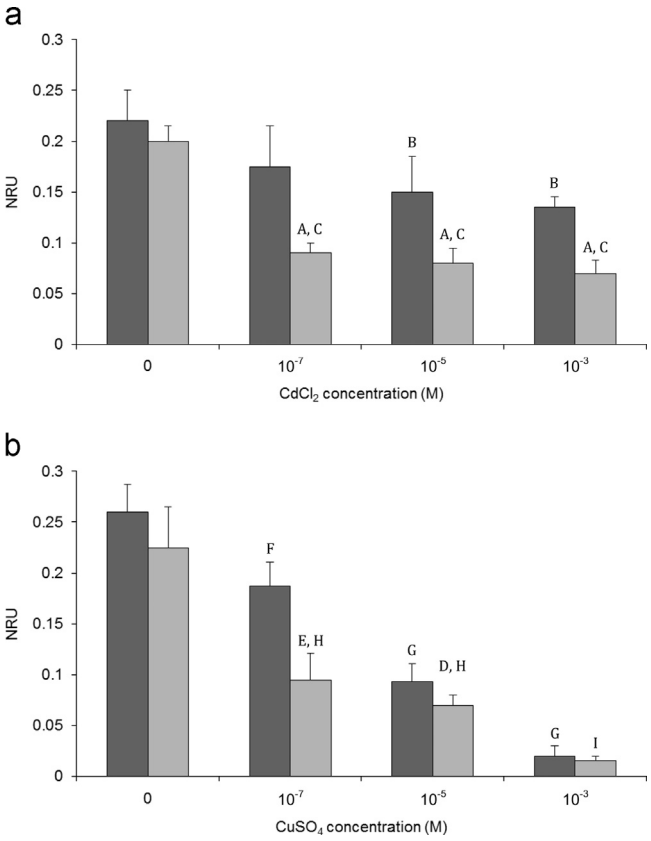

Fig. 1. Neutral red retention activity of separated blood leukocytes of *D. labrax* exposed to $CdCl_2$ (A) and CuSO4 (B) after 2 h (■) and 24 h (■) incubation time. The values are expressed as the optical density of Neutral red retention at 550 nm. Data are the means of three distinct experiments ± standard deviation. Statistical analysis by one-way ANOVA with Tukey's post-test. $A = p < 0.05$ significance degree between 2 h and 24 h exposure; $B = p < 0.01$ significance degree between control and $CdCl_2$ concentration at 2 h. $C = p < 0.01$ significance degree between control and $CdCl_2$ concentration at 24 h; $D = p < 0.05$ and $E = p < 0.001$ significance degree between 2 h and 24 h exposure to $CuSO_4$; $F = p < 0.05$ and $G = p < 0.001$ significance degree between control and $CuSO_4$ concentration at 2 h; and $H = p < 0.01$ and $I = p < 0.001$ significance degree between control and $CuSO_4$ concentration at 24 h.

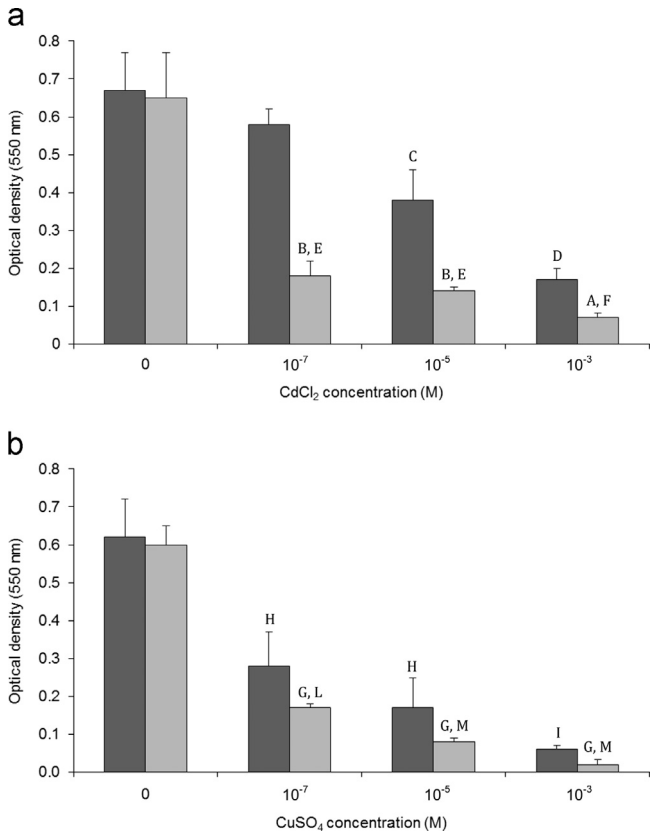

Fig. 2. Cytotoxic effects of $CdCl_2$ (A) and $CuSO_4$ (B) on separated blood leukocytes of *D. labrax* after 2 h (■) and 24 h (■) incubation time. The values are expressed as the optical density of MTT at 550 nm. Data are the means of three distinct experiments ± standard deviation. Statistical analysis by oneway ANOVA with Tukey's post-test. $A = p < 0.05$ and $B = p < 0.01$ significance degree between 2 h and 24 h incubation time; $C = p < 0.01$ and $D = p < 0.001$ significance degree between control and $CdCl_2$ concentration at 2 h; $E = p < 0.01$and $F = p < 0.001$ significance degree between control and $CdCl_2$ concentration at 24 h; $G = p < 0.05$ significance degree between 2 h and 24 h incubation time for CuSO4; $H = p < 0.01$ and $I = p < 0.001$ significance degree between control and $CuSO_4$ concentration at 2 h; and $L = p < 0.01$ and $M = p < 0.001$ significance degree between control and $CuSO_4$ concentration at 24 h.

exposure times ($p < 0.05$ for 2 h and $p < 0.001$ for 24 h) at the lowest concentration of the metal (10^{-7} M). Indeed, leukocytes exposed for 2 and 24 h at a concentration of 10^{-7} M of Cu decreased the uptake of neutral red respectively to 1.187 and 0.095.

3.4. MTT reduction assay

The cytotoxic activity of the metals on leukocytes is shown in Fig. 2. The cells treated with Cd and Cu resulted in the reduced capability of mitochondria to effect enzymatic conversion of MTT when compared with controls. Cells exposed for 2 h to Cd showed a significant difference ($p < 0.01$) of MTT activity index with respect to controls only at a concentration of 10^{-5} in which the control index value was reduced from 0.67 to 0.38 (Fig. 2a). After an exposure of 24 h, the cells showed a strong reduction of MTT index ($p < 0.001$) already at the lower concentration of metal (10^{-7} M), reducing the index value of the control from 0.65 to 0.18 (Fig. 2a).

The effects of treatment with Cu are similar to those observed for Cd, but the Cu seems to be more toxic than the Cd because 2 h of treatment at the concentration of 10^{-7} M was already able to decrease the control value of MTT index significantly ($p < 0.01$), from 0.62 to 0.28.

3.5. Induction of apoptosis by cadmium and copper

After treatment with the metals, at the two concentrations and for the two established times, the genomic DNA was extracted from the cells and agarose gel electrophoresis was performed. Non-random DNA fragmentation has been regarded as one of the typical end points of apoptosis (Khodarev et al., 1998). Fig. 3 shows that DNA fragmentation induced by cadmium and copper was time- and dose-dependent. DNA fragmentation appeared only after 24 h of treatment with the highest concentration (10^{-3} M) of two metals. No results were evident after 2 h of incubation (data not shown).

3.6. Real-time PCR analysis

The expression of Hsp70 mRNA from cells treated with Cd and Cu was analyzed through real-time PCR. Fig. 4a indicates that the cells, after an exposure with Cd, at a concentration of 10^{-5} M and after 24 h, show a significant increase in the transcription of mRNA specific for Hsp70 (2.47, $p < 0.05$) compared to the control. The value of transcription, however, at higher concentrations (10^{-3} M) for both 2 and 24 h treatments matched the control values (1.35 and 0.85 respectively) (Fig. 4a).

Fig. 3b illustrates that treatment with the Cu causes an increase in transcription of mRNA specific to Hsp70 for all concentrations used, and already at a concentration of 10^{-5} M, after 2 h of treatment, there was a significant increase (2.8 $p < 0.01$) compared to untreated samples (1.1). At higher concentrations (10^{-3} M) there was an almost 3-fold increase (2.8) compared to the control (Fig. 3b). After 24 h treatment it was observed that there was a significant increase of the transcription of mRNA specific for Hsp70 compared to untreated cells both at the lowest concentration ($p < 0.01$, 10^{-7} M) and at the intermediate concentration ($p < 0.001$, 10^{-5} M). At the highest concentration (10^{-3} M) the values reached those of the control.

4. Discussion

Heavy metals, particularly cadmium and copper, have been identified as the major source of aquatic pollution and have been detected in alarming quantities in many water bodies, particularly at or near industrial localities where effluents are routinely discharged. Although there have been marked advances in our understanding of how organic toxic agents can affect living organisms, the mechanisms by which toxic metals such as Cd or Cu produce their biochemical effects are still largely unknown (Arizza et al., 2009; Prozialeck, 2000; Prozialeck et al., 2003). Prior to this study, the toxic effects of Cd in fish had been studied in the rainbow trout *Oncorhynchus mykiss*, specifically for its role in toxicopathological symptoms and bioaccumulation (Battaglini et al., 1993; Kamunde and MacPhail, 2011; Schwartz et al., 2004), and these authors advocated the need to use different fish models for testing metal toxicity. Some studies documented the accumulation of cadmium and copper in the gills, kidneys and liver and the pathological changes of varying severity in the above mentioned organs in sea bass. (Cattani et al., 1996; Giari et al., 2007; Romeo et al., 2000; Viarengo et al., 1997). In this work, we show preliminarily that the cultures of isolated blood leukocytes of *D. labrax* were able to incorporate Cd and Cu after treatment, thus confirming that these metals could cross membranes (for a review see Foulkes (2000)), and accumulate in the cells. In fact, after 24 h of treatment the concentration was significantly higher. The consequences of the incorporation of cadmium and copper were subsequently evaluated utilizing the NRU and MTT *in vitro* cytotoxicity assays on separated blood leukocytes. Leukocytes are cells

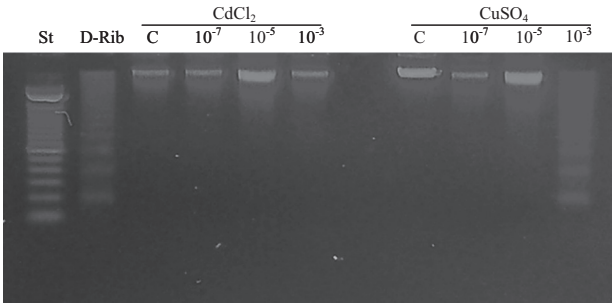

Fig. 3. Agarose gel electrophoresis of genomic DNA from *D. labrax* enriched leukocytes. The cells were *in vitro* treated for 24 h with 10^{-7} M, 10^{-5} M, and 10^{-3} M solutions of $CdCl_2$ or $CuSO_4$. The positive control was performed treating the genomic DNA with 100 mM of 2-deoxy-D-ribosio (D-Rib). St = 100 bp DNA ladder marker.

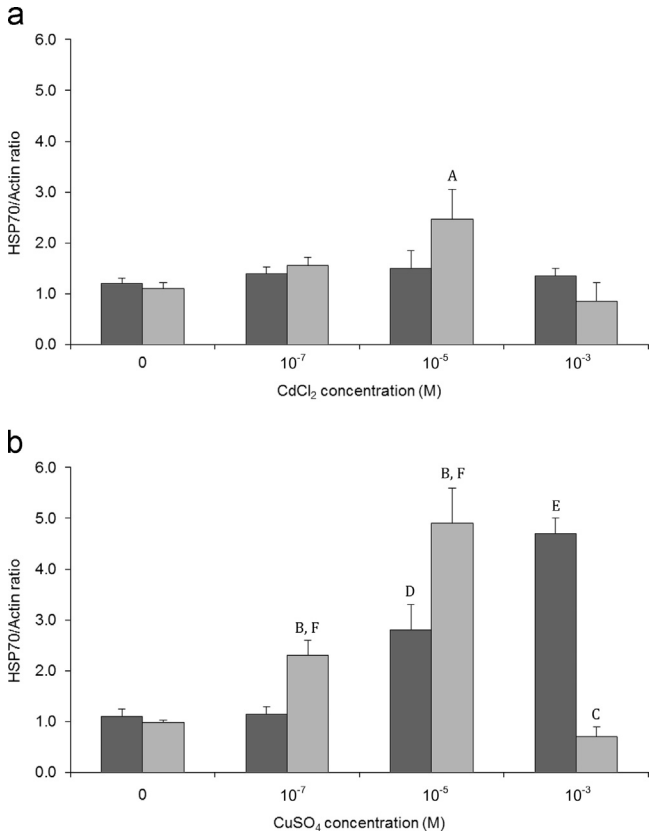

Fig. 4. Expression level of Hsp70 on separated blood leukocytes of *D. labrax* after 2 h (■) and 24 h (■) incubation time with $CdCl_2$ (A) and $CuSO_4$ (B). The IDV values were used to express the ratio of Hsp70 mRNA to actin after background correction. Data are the means of three distinct experiments ± standard deviation. Statistical analysis by one-way ANOVA with Tukey's post-test. $A = p < 0.05$ significance degree between control and $CdCl_2$ concentration at 24 h; $B = p < 0.05$ and $C = p < 0.001$ significance degree between 2 h and 24 h incubation time for $CuSO_4$; $D = p < 0.01$ and $E = p < 0.001$ significance degree between control and $CuSO_4$ concentration at 2 h; and $F = p < 0.01$ significance degree between control and $CuSO_4$ concentration at 24 h.

responsible for a variety of responses of immunity such as phagocytosis, antigen presentation, production of cytokines and the release of anti-microbial and anti-tumor agents. The *in vivo* modulation of the activity of these cells by environmental factors could have serious consequences on the functioning of the immune system and the organism's resistance to diseases.

Current standard approaches to gauge the degree of cell damage include assays that measure various aspects of cell viability, such as metabolic activity and plasma membrane integrity. The MTT reduction assay, which determines cell metabolic activity, is among the most commonly used end points. This method measures the reduction of MTT salt to a colored insoluble formazan in active mitochondria in viable cells and also, in certain cases, outside the mitochondria (Berridge et al., 2005; Liu et al., 1997). The neutral red uptake (NRU) assay, which is also widely used in biomedical applications, measures the uptake of neutral red dye by viable cells with intact plasma membrane, and its concentration in lysosomes (Repetto et al., 2008).

Our results indicate that the treatment of leukocytes with metals produce a decrease in the uptake of neutral red that is probably caused by a general reduction in lipid membrane stability, which usually precedes cell death (Romeo et al., 2000). The cytotoxic effects of cadmium and copper are reflected in the alteration of the lysosomal membrane, probably due to the high levels of metals that may accumulate in lysosomes rather than being eliminated from the body (Moore, 1990; Regoli, 1992). It is likely that Cd and Cu lead to the inhibition of Mg^{2+}-ATPase, a proton pump of lysosomal membranes that maintains the internal environment of lysosomes acid (Lowe et al., 1992). Dysfunction of this ATPase allows the free passage of lysosomal contents, including NRU, into the cytosol (Lowe et al., 1995). In our NRU tests, leukocytes treated with cadmium showed a lower capacity to retain neutral red in the lysosomes. The effects were more pronounced at higher concentrations and for a greater exposure time. However, the effect of cadmium was lower compared with that of copper. Indeed cells exposed to Cu showed significant reductions in NRU already at 2 h of treatment and at lower concentrations of the metal (10^{-5} M).

To validate the cytotoxicity results obtained by the NRU assay, the cytotoxic sensitivities of the *D. labrax* leukocyte to the heavy metals were also evaluated using the MTT test, which is known to be more sensitive in detecting early toxicity (Fotakis and Timbrell, 2006) than NRU assays; it is an index of cellular metabolic activity, mainly based on the enzymatic conversion of MTT in the mitochondria. The tetrazole ring of MTT can be reduced by mitochondrial dehydrogenases in formazan if the cells are metabolically active. The data obtained on leukocytes of *D. labrax* show that both 2 and 24 h after the treatment with both heavy metals ($CuSO_4$ and $CdCl_2$) there was a constant time-dose dependent decrease of cell viability compared to the control. These data are in agreement with previous work, in particular with a study on six cell lines of fish, in which a time-dose dependent decrease in cell viability following treatment with of $CdCl_2$ and $CuSO_4$ (Tan et al., 2008) was evidenced. The toxicity measured by the MTT was consistent with that obtained by NRU, highlighting the greater impact of copper compared to that of cadmium. Our results seem to be consistent with data present in the literature. In fact similar studies have shown that injecting cadmium or copper into the peritoneal cavity of *D. labrax* led to a decrease in the stability of the lysosomal membrane of cells of the pronephros, and the impact of copper was greater than that of cadmium (Romeo et al., 2000). The toxic effects of the metals can be produced by an increase of the membrane fluidity leading to a higher rate of fusion of the lysosomes (Moore and Viarengo, 1987). In the digestive gland of mussels submitted to xenobiotic exposure, Moore (1988) reported that enlarged and lipid-enriched lysosomes frequently showed reduced membrane stability, which is indicative of their increased fragility. Metal toxicity may also be exerted through lipid peroxidation, considered as a first step of cellular membrane damage by xenobiotics (Viarengo, 1989). Romeo et al. (2000) showed that lipid peroxidation measured *in vitro* in the kidney extracts of *D. labrax* was shown to be higher in the presence of copper than in that of cadmium. Moreover, in the common carp *Cyprinus carpio morpha* exposed to copper sulfate for 24 h, Radi and Matkovics (1988) found significant increases in the lipid peroxidation measured in the liver, gill and white muscle.

The cytotoxicity caused by heavy metals may be due to apoptosis. Several reports have shown that cadmium induces apoptosis in various tissues and cells, both *in vivo* and *in vitro* (Hamada et al., 1997). E.g., cadmium-induced apoptosis was reported in rat testes (Xu et al., 1996), mouse liver (Habeebu et al., 1998), rat lung epithelial cells (Hart et al., 1999), CL-3 human lung carcinoma cells (Chuang et al., 2000), HeLa human cervix carcinoma cells (Szuster-Ciesielska et al., 2000) and Rat-1 fibroblast cells (Kim et al., 2000). Copper, for its part, can induce an up-regulation of apoptosis and related genes in zebrafish (Luzio et al., 2013) or induces the apoptotic cell death in the copepod *Tigriopus japonicus* (Rhee et al., 2013). Thus in order to assess the phenomenon of apoptosis we examined the integrity of the DNA of leukocytes. During apoptosis, apoptotic cells activate distinct proteases (so-called caspases), and there is also chromatin condensation followed by activation or newly synthesized distinct calcium-dependent nuclease that cuts double-strand DNA at the linker regions between the nucleosomes, resulting in apoptosis-specific DNA fragmentation. The result is electrophoretic separation of apoptotic DNA fragments (multiples of 180–200 bp) corresponding to a typical ladder pattern (Arends et al., 1990). This whole process is triggered by a variety of agents, including calcium ionophores, radiation, heat shock, cytokines, serum deprivation or oxidative stress.

The DNA fragmentation was analyzed using agarose gel electrophoresis 1 percent to demonstrate a “ladder” pattern at ~200-bp intervals. The DNA extracted from *D. labrax* leukocytes was perfectly intact at the lower concentrations examined for both treatments with $CuSO_4$ and $CdCl_2$, both at 2 and 24 h. At the highest concentration (10^{-3} M) after 24 h of treatment, a phenomenon detectable with apoptotic DNA fragmentation every 180 bp typical of such an event (Weber and Janz, 2001) was evident. DNA fragmentation which forms a “smear” on agarose gels disclosing necrosis was never found. The cytotoxic effects of Cd are related to its accumulation in the nucleus by specific metallothioneins and by a strong inhibition effect on superoxide dismutase, peroxidase and catalase, which are major scavenger enzymes of intracellular superoxides. Oxidative stress has been shown to be one of the predominant factors related to apoptosis through peroxinitrite formation (Lin et al., 1997; Stoian et al., 1996; Wolfe et al., 1994). Copper may induce apoptosis through pore formation in the mitochondrial membrane. This would give rise to increased amounts of mitochondrial protein. Among these proteins, there is the EndoG, a mitochondrion-specific nuclease that translocates to the nucleus during cell cycle arrest and apoptosis. Once released from mitochondria, EndoG cleaves chromatin DNA into nucleosomal fragments (independently of caspases); thus, EndoG represents a novel caspase independent apoptotic pathway (Huang et al., 2006; Mitra et al., 2012).

The exposure of organisms to xenobiotics such as heavy metals creates a stress condition that might interfere with the maintenance of homeostasis. Cells respond to stress conditions with a variety of gene families and biochemical pathways that protect and repair them when faced with environmental hazards. Among them there are the heat shock proteins (HSPs): a large group of proteins with a molecular size ranging from 10 kDa to 170 kDa. They play a predominant role in chaperoning the folding and/or degradation of proteins (Young et al., 2004) and are highly conserved through evolution, and Hsp70 and Hsp90 have been found in all organisms examined (Kiang and Tsokos, 1998). In particular, Hsp70 is the most ubiquitous and universal chaperone family member involving the unfolding/refolding of newly synthesized and damaged proteins as well as the sequestering and breaking down of proteins that are damaged beyond repair (Mayer and Bukau, 2005). Over-expression of several chaperone proteins can facilitate the repair of misfolded proteins or the elimination of aggregated proteins by the ubiquitin–proteasome system (Esser et al., 2004). The increased expression of HSPs in response to Cd and Cu were demonstrated in tissues and cells of many organisms and, among the aquatic invertebrates, mainly in sponges, mollusks, arthropods, echinoderms and fish (Cebrian et al., 2006; Clark and Peck, 2009; Dimitriadis et al., 2012; Lauritano et al., 2012; Matranga et al., 2012).

The cell is provided with protection mechanisms that are able, to some extent, to block the adverse effects of heavy metals. Among these there are two classes of anti-stress proteins: the metallothioneins (MTs) and heat shock proteins (HSPs). The MTs are the first defense mechanism against the toxicity of heavy metals, but when metal concentrations reaches high levels and produce protein denaturation, HSPs act to repair the damage (Giudice et al., 1999). To evaluate the expression of Hsp70 after metal treatment, we analyzed the expression of specific mRNA by RT-PCR. The data obtained indicate that separated leukocytes treated with $CdCl_2$ at lower concentrations did not show significant differences between concentrations and treatment time compared to the controls. Only at a 10^{-5} M concentration was there a significant increase, evident only after 24 h treatment (Fig. 3a). Instead, the cells treated for 2 h with $CuSO_4$ significantly increased the transcription of mRNA for Hsp70. Our experiments show that at 10^{-5} M, $CuSO_4$ after 24 h of treatment significantly increased mRNA expression. At a concentration of 10^{-3} M, the transcript reaches lower levels compared to control values, and this decrease could coincide with a possible apoptotic phenomenon. This finding is in agreement with Steiner et al. (1998), who showed that the Hsp70 mRNA transcription increased significantly after stress, and they considered that expression a good indicator of stress. The different behavior of the two metals could be due to a higher resistance of cells to Cd than Cu (Matozzo et al., 2001; Murata et al., 1999; Romeo et al., 2000), rapidly activating several genes involved in defense mechanisms such as MTs. At higher concentrations of Cd (10^{-3} M), the MTs are not able to chelate all Cd ions and, due to protein denaturation, they may activate the transcription of HSPs.

5. Conclusions

The results of the present study shows that exposure of the leukocytes from the blood of *D. labrax* to Cu and Cd may influence the functional responses of cells.

The integrity of the immune system is fundamental for the preservation of homeostasis and the defense against fish pathogens (parasites, bacteria, and viruses). Leukocytes are part of the immune system and are involved in a variety of immunity responses such as phagocytosis, antigen presentation, production of cytokines and the release of anti-microbial and anti-cancer agents. Therefore, the complex network of immune cells regulated by a variety of multistep control processes of cellular interactions could be impaired in one or more steps by pollutants. In fact, the alteration of activity of these cells caused by environmental factors could have serious consequences for the functioning of the organism's immune system and resistance to disease. Therefore, the study of the biochemical parameters that here we have considered could be useful for biomonitoring marine coastal environments.

Funding

Funds (“ex. 60 percent”) were provided by Italian Minister of University, Research and Public Instruction (MIUR) to M. Vazzana.

Acknowledgments

We are gratefully to Dr. Benedetto Savona for the help in statistical analysis.

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