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**BIOCHEMICAL RESPONSES TO CADMIUM EXPOSURE IN *ONCORHYNCHUS MYKISS* ERYTHROCYTES**

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**ABSTRACT**

Cd is known for its carcinogenic effects, however its mechanism of toxicity is debated and in particular in this context its ability to promote oxidative stress. In fact, although it is considered a redox-inactive metal, in high concentration Cd was shown to promote indirectly oxidative stress. In this study we investigated metal accumulation in *ex vivo* exposed trout (*Oncorhynchus mykiss*) erythrocytes and its dose-dependent effect in terms of RBC viability, cytosolic and mitochondrial ROS levels as well as its effects on mitochondrial membrane depolarization, haemoglobin stability and precipitation.

In the concentration range used, Cd did not affect cell viability. However metal accumulation was associated to an increase of all oxidative indexes evaluated excluding mitochondrial superoxide anion production that, on the contrary, was significantly decreased, probably due to a lowered respiration rate associated to interference of Cd with complex I, II and III, as suggested by the observed Cd-dependent mitochondrial membrane depolarisation. On the other hand hemoglobin destabilisation seems to be the major trigger of oxidative stress in this cell type.

**Keywords:** Cadmium, flow-cytometry, haemoglobin, oxidative stress, mitochondria, trout erythrocytes.

**Abbreviations**: Cd, Cadmium; MT, metallothionein; ROS, Reactive Oxygen Species; met-Hb, ferric haemoglobin; DCFDA-H2, dichlorodihydrofluoresceindiacetate; RBC, Red Blood Cells, MMP, Mitochondrial Membrane Potential.

1. **INTRODUCTION**

Cd is generally considered a non-essential trace element ubiquitously present in the natural environment. In the last decades, the intensification of anthropogenic environmental pressure led to a rise of the concentration of this metal in waters, sediments, soil and biota, generating concern about this increasing trend (Crea et al., 2013). The average Cd content in the world's oceans has variously been reported as low as <5 ng/L (WHO, 1992) and 5-20 ng/L (OECD 1994, Jensen and Bro-Rasmussen, 1992) to as high as 110 ng/L (CRC, 1996), 1 00 ng/L (Cook and Morrow 1995) and 10 to 100 ng/L (Elinder, 1985). Weathering and erosion are estimated to contribute 15000 tonnes of Cd annually to the global aquatic environment, while atmospheric fall-out (of anthropogenic and natural emissions) is estimated to contribute between 900 and 3600 tonnes (UNEP, 2008). Cd has been investigated mainly for its toxicity in several species belonging to different taxa. Most research was focused on its adverse effects on humans and model animals (Waalkes, 2003, Madejczyk et al., 2015) and it has been ranked seventh in the Priority List of Hazardous Substances by the Agency for Toxic Substances and Disease Registry (2015) (https://www.atsdr.cdc.gov/spl/). The toxicity of Cd is mostly linked to cancerogenesis through mechanisms acting at different molecular levels (Chen et al., 2016), including the inhibition of DNA repair (Whiteside et al., 2010) and modulation of apoptosis (Liu et al., 2009). Moreover Cd, a redox-inactive metal, can indirectly induce oxidative stress binding to sulfhydryl groups of proteins, resulting in depletion of glutathione (Koedrith and Seo, 2011, Valko et al., 2016). Cd is also one of the stronger inducer of metallothionein (MT) which in turn can act as an antioxidant (Ruttkay-Nedecky et al., 2013). However, it cannot be excluded that, in certain circumstances, Cd could be considered an essential trace element for some organisms; indeed, according to Lane and Morel (2000), Cd at low concentrations can be beneficial for the marine diatom *Thalassosira weissflogii* and can act as an algal nutrient under Zn limitation.

In aquatic animals, the puzzling effects of Cd have been investigated *in vivo* in different fish species (Isani et al., 2009, Cirillo et al., 2012), and *in vitro* both in isolated organs (Carpené and George, 1981) and in cell cultures (Wright et al., 2000, Zhu and Chan, 2012). Novel perspectives can be open considering fish erythrocytes as a new model to study *ex vivo* the effects of Cd exposure at a cellular level. These cells possess the complete cellular machinery with functional ribosomes (Tiano et al., 2004) and mitochondria (Tiano et al., 2000, Tiano et al., 2001), thus allowing protein synthesis and full cellular activity. In addition, at the level of the gills, erythrocytes are separated by only two cell layers from the external environment and for these reasons the effects of metals in *ex vivo* exposure is closely related to the effects *in vivo*.

In this context we propose to use trout (*Oncorhynchus mykiss*) erythrocytes as a model to investigate the effects of Cd exposure on metal accumulation, its ability to promotes Reactive Oxygen Species (ROS) formation and to induce oxidative damage by interfering with cellular components. Since the erythrocytes maintain their vitality only for few hours during *ex-vivo* metal exposure condition, the experiments were designed in order to acutely expose cells to relatively high Cd concentrations. In the exposed erythrocytes we measured Cd accumulation, viability and intracellular ROS, mitochondrial superoxide anion, ferric haemoglobin (met-Hb) production, mitochondrial membrane depolarization and finally haemoglobin precipitation.

1. **MATERIALS AND METHODS**

**2.1 Chemicals**

All reagents were of pure analytical grade. NaCl, citrate, EDTA, CdCl2, K3[Fe(CN)6] were from Sigma Aldrich, Guava ViaCount Assay from Millipore, MitoProbeDilC1(5), 2’-7’-dichlorodihydrofluoresceindiacetate (DCFDA-H2) and MitoSOX™ Red reagentwere purchased from Life Technologies.

**2.2 Experimental animalsand erythrocyte preparation**

Red Blood Cells (RBC) were obtained from rainbow trouts *(Oncorhynchus mykiss) (*mean weight 350 ± 50 g, length 25 ± 5 cm) from the fish farm “Eredi Rossi Silvio” Sefro (Macerata), Italy*.*

For each experiment 4 mL blood were withdrawn by a syringe from the caudal vein of at least 3 trouts into an isotonic medium consisting of 0.1 M phosphate buffer (0.1 M NaCl, 0.2% citrate, 1 mM EDTA) at pH 7.8. Blood was always pooled to minimize individual variability and, in order to prevent metal chelation, RBC, obtained by centrifugation at 300 *g* for 2 minutes in 1 mL final volume, were washed with the same isotonic medium but without EDTA; this medium was also used to resuspend the erythrocytes and dilute Cd solution. Samples were stored on ice for subsequent analyses*.*

**2.3 Erythrocytes exposure to Cd**

Three different Cd exposure protocols were applied to study its accumulation and effects on both oxidative and energetic biomarkers and on haemoglobin stability during *ex vivo* erythrocyte incubation.

1. Cd accumulation was determined in erythrocyte suspensions exposed for 4 hours to 0 (control), 9, 90, 235 μM CdCl2 (n=3); in parallel, part of the erythrocyte suspensions was washed with the isotonic medium and analyzed for Cd as well. At the end of the experiments all erythrocyte suspensions were centrifuged for 10 min at 300 *g* and the erythrocytes pellet was immediately frozen and kept at -20°C for Cd analysis.
2. Cd effects on oxidative and mitochondrial biomarkers were evaluated on viable cells after cell count and viability determination carried out using Guava ViaCount assay in flow cytometry. In particular 1\*10^6 viable cells for each condition were aliquoted in isotonic phosphate buffer atpH 7.8. For each evaluated endpoint, cells were incubated in the presence and in absence of 25-75-150-300 μM of CdCl2for 30 min at 37°C in the dark (in isotonic buffer pH 7.8 as well). Following incubation, samples were washed with isotonic buffer at pH 7.8 by centrifugation at 300 *g* for 2 min and resuspended in 300 μL of the same buffer.
3. Haemoglobin stability was evaluated in both whole, cells and hemolysates. In particular met-Hb formation was evaluated in whole RBC while precipitation was measured in hemolysates (obtained by adding three volumes of distilled water to the washed packed cells and, after freezing at - 20°C for at least 30 min, thawed hemolysates were centrifuged at 15000 *g* for 10 min at 4°C to remove cellular debris).

**2.4 Cadmium accumulation assay**

To avoid contamination, polyethylene disposables were thoroughly washed with HCl 1 N under a fume hood and disposable gloves were worn during the procedure. All reagents, including distilled water, were from Merck, Darmstadt (Germany); acids were of Suprapur grade. Samples (200±20 mg of erythrocytes) were placed in individual acid-washed Teflon jars and were digested in 1–2 mL 65% HNO3 and 0.25–0.5 mL 30% H2O2 in a microwave oven (Milestone, Model mls 1200). Cooled samples were transferred into polyethylene volumetric flasks; the acid solutions were made up to a final volume of 5–10 mL with analytical grade distilled water and directly aspirated into the flame of an atomic absorption spectrophotometer (Instrumentation Laboratory, Model IL11, equipped with a deuterium lamp background correction, USA). Two blanks were digested simultaneously during each run. The detection limit foranalytes was established by analysis of 10 blank solutions. The detection limits was 4 ng Cd/mL. The trace metal standard was run every 20 samples. The accuracy of the method was evaluated by calibration versus an international standard (CRM 278: lyophilized mussels, Community Bureau of Reference-BCR,Brussels); the concentration values obtained with the method used in this study fell within the confidence interval given by the Community Bureau of Reference.Data are expressed as μg of Cd on g of erythrocytes ± SEM.

**2.5 Viability assay**

Viability of trout erythrocytes was measured in flow cytometry by using Guava ViaCount kit that permit to discriminate viable cells from apoptotic and dead ones (Silvestri et al.,2016). The kit exploits the different membrane permeability of two DNA binding fluorochromes. Trout erythrocytes are able to undergo programmed cell death through mitochondrial involvement as we previously reported (Tiano et al., 2003). Cells incubated with cadmium/pH 7.8 isotonic buffer and washed were incubated with ViaCount reagent for 5 minutes in the dark and then analyzed. Data are expressed as % of viable, apoptotic and dead cells ± SEM.

**2.6 Intracellular Reactive Oxygen Species assay**

Intracellular ROS levels were quantified by flow cytometry by means 2’-7’-dichloro-dihydro-fluoresceindiacetate (DCFDA-H2), a non-polar probe that, after going through cell membranes, it is hydrolyzed by intracellular esterases in DCFH2. In its reduced form, this probe is not fluorescent but in presence of intracellular ROS, it is oxidized to DCF fluorescent and whose emission maximum can be monitored at 520 nm (Silvestri et al., 2016). Before incubation with isotonic buffer alone or with different cadmium concentrations, erythrocytes were incubated with DCFDA-H2 (10 μM final concentration) at 37°C for 45 min in the dark. Subsequently cells were washed with isotonic medium by centrifugation at 300 *g* for 2 min at 10°C and resuspended inthe same medium. Prior to flow cytometry analysis, fluorescence of the labelled cells was measured using Guava easyCyte (Millipore) at an excitation wavelength of 488 nm. Emission was recorded at 525/30 nm (Green), 583/26 nm (Yellow) and 690/50 nm (Red). Photomultipliers were set at 5.19, 279 and 41.5 respectively. Counter staining was performed with ViaCount in order to quantify ROS levels only in viable cells and 5000 events from each sample were measured. Results were analyzed using the Guava InCyte software and expressed as percentage of cells with low, mid and high ROS production ± SEM. Regions of low, mid and high ROS were arbitrarily set using as reference population a negative control (cells incubated at 37°C for 45 min at pH of 7.8) and a positive control (cells incubated at 37°C for 45 min at pH of 6.3). In this last condition methaemoglobin formation and inactivation of primary detoxification systems are promoted (Falcioni et al., 1987)

**2.7 Mitochondrial Superoxide Anion production assay**

Superoxide anion levels produced by mitochondria were quantified in flow cytometry using a derivate of dihydroethidium, known as MitoSOX™ Red reagent, that permeates live cells where it selectively targets mitochondria. After oxidation by superoxide, resulting in hydroxylation at the position 2, it exhibits a fluorescence excitation peak at ~400 nm that is absent in the excitation spectrum of the ethidium oxidation product generated by ROS other than superoxide. Erythrocytes washed following incubation with cadmium or isotonic buffer alone, were incubated with MitoSOX™ Red reagent (5 μM final concentration) at 37°C for 20 min in the dark. Successively, in order to remove excess MitoSOX™ Red reagent, cells were washed in isotonic medium at pH 7.8 by centrifugation at 300 *g* for 2 min at 10°C and resuspended in the same medium. After that, cells were analyzed using Guava easyCyte equipped with a blu laser at 588nm. Emission was recorded at 583/26nm (Yellow). Photomultiplier was set at 39.7 V. Results were analyzed using the Guava InCyte software and data are expressed as percentage of cells with high and low superoxide anion ± SEM.

**2.8 Mitochondrial Membrane Potential assay**

Mitochondrial Membrane Potential (MMP) was evaluated using MitoProbeDilC1(5) (Silvestri et al. 2016). After incubation with isotonic buffer alone or in association with different concentrations of metal, erythrocytes were incubated for 20min at 37°C in the dark with MitoProbeDilC1(5) (40 nM final concentration). Subsequently, RBC were washed and analyzed using Guava easyCyte equipped with a red 633 nm laser. Emission was recorded at far red emission 658 nm. Photomultiplier was set at 86.7V. Mitochondrial depolarization was evaluated in terms of percentage of cells ± SEM showing low fluorescence proportional to MMP, using the Guava InCyte software.

**2.9 Ferric-haemoglobin formation assay**

Met-Hb was quantified after incubation of washed erythrocyte suspended in isotonic buffer pH 7.8 for 30 min at 37°C in the presence or in absence of 75-150-300µM of CdCl2. Ferric-haemoglobin formation was evaluated by using a microplate reader (Synergy HT, Biotek). The ratio between the absorbance at 405 nm and that at 413 nm of each hemolyzate solution was calculated according to the method previously reported (Orlando et al. 2014). Data were normalized considering met-Hb amount at time 0 in control samples (pH 7.8) as 0%, while complete oxidation of Hb (100%) was obtained by the addition of potassium ferricyanide, K3[Fe(CN)6], (molar ratio 1:1 per eme) to the oxygenated derivative. Data are expressed as percentage of met-Hb formation ± SEM.

**2.10 Haemoglobin precipitation assay**

The effect of cadmium on the haemoglobin precipitation was monitored spectrophotometrically for 6 hours at 700 nm during incubation of a standardised hemolysates containing1mg/mL Hb in presence of 75-150-300 µM of cadmium chloride or phosphate buffer pH 7.8 at 37°C.

**2.11 Statistical analysis**

Data analyzed were from 3 independent experiments each conducted in triplicate (technical

replicates). In each experiment blood was pooled from 3 animals. Differences in means were calculated using One-way ANOVA and Post-hoc analysis of differences between samples was calculated using Tukey's Honestly Significant Difference (HSD) method. p values ≤0.05 are considered statistically significant, p values ≤0.01 and ≤0.001 are considered highly significant. Data are reported as mean of three experiments (n=3) ±SEM. To analyse Cd erythrocyte accumulation, correlation and regression analysis were performed between the different Cd concentrations in exposure medium and the metal concentrations in the erythrocytes.

**RESULTS**

**3.1 Cadmium accumulation**

Exposure of trout erythrocytes to different Cd concentrations for four hours resulted in a significant (r=0.977, p<0.01) dose-dependent metal accumulation. Cd reached a maximum of 16.8 µg/g after four hours at 235 µM (Fig. 1A). Differently, in a parallel incubation performed with erythrocytes washed at the end of the exposure, Cd reached the maximum already at 90 µM without a further increase at 235 µM Cd (Fig. 1B). Nevertheless, also in this case the accumulation pattern was significantly (r=0.78, p<0.05) dose-dependent.



**Fig. 1.** **Cadmium accumulation.** Dose-dependent cadmium accumulation after 4 hours of erythrocytes exposure with 0 (control), 9-90-235 µM of CdCl2 without (A) and after (B) washing with isotonic buffer. Values (n=3) are reported as mean±SEM.

**3.2 Viability**

Incubation of trout erythrocytes suspended only in isotonic medium at pH 7.8 for 30 min at 37°C showed an high level of viable cells (78.4%±4.1) and low amount of apoptotic, with partially compromised membranes, (12.5%±4.0) and dead cells (8.8%±1.5). Figures 2A, B and C show that Cd concentrations used in this study did not cause any significant variation in terms of viable/apoptotic/dead cells with respect to unexposed control.



**Fig. 2.** **Viability.** Percentage of viable (A), apoptotic (B) and dead (C) erythrocytes incubated for 30 min at 37 °C in isotonic buffer at pH 7.8 alone (control) or in presence of 25-75-150-300 µM of CdCl2. Data are reported as mean of three experiments (n=3) ±SEM.

**3.3 Intracellular levels of Reactive Oxygen Species**

Intracellular ROS levels were quantified after pre-incubation with DCFDA-H2 as reporter fluorochrome. According to arbitrarily defined gates it was possible to discriminate a percentage of cells showing different levels of fluorescence corresponding to high, mid and low intracellular ROS production. In the negative control, after 30 min incubation at 37°C, 86%±0.27 of cells showed a low ROS content (Fig 3A), while in the presence of Cd at each tested concentration a significantly higher percentage of ROS-producing cells was observed, and proportion of ROS positive cells increased in a dose-dependent manner (Fig. 3B, C). In particular the lowest Cd concentrations (25 and 75 µM) produced a significant decrease of cells with low cytosolic ROS content (-14%, p=0.0180 and -33%, p<0.0001, respectively) and concomitantly a significant increase of cells at with mid ROS content (+13%, p=0.0388 and +31%, p=0.0002) compared to unexposed control. Moreover, cells characterised by high ROS production did not increase significantly.

Conversely, exposure to higher concentrations of Cd (150 and 300 µM) produced a marked oxidative effect, characterized by a decrease of low ROS-producing cells compared to unexposed controls (-51%, p<0.0001 and -63%, p<0.0001, respectively) paralleled by an increase of cells with both mid (+46%, p<0.0001 and +53%, p<0.0001) and high ROS content (+4%, p=0.0335 and +9%, p=0.0003).



**Fig. 3.** **Intracellular** **ROS production.** Percentage of cells producing low (A), mid (B) and high (C) levels of ROS after incubation for 30 min at 37 °C with isotonic buffer at pH 7.8 alone (control) or in presence of 25-75-150-300 µM of CdCl2.\* p≤0.05, \*\* p≤0.01,\*\*\* p≤0.001 vs ctr (a), 25 µM (b), 75 µM (c), 150 µM (d). Data are reported as mean of three experiments (n=3) ±SEM.

**3.4 Mitochondrial superoxide anion production**

Mitochondrial superoxide anion production was quantified after pre-incubation with MitoSOX Red reagent, a probe sensitive to superoxide anion that localizes in the mitochondria permitting to discriminate cells producing a high levels of superoxide anion. As shown in figures 4A and B, exposure of the RBC suspension to the lowest Cd concentrations for 30’ at 37°C did not result in significant changes in mitochondrial superoxide anion production compared to unexposed control. On the contrary, exposure to higher Cd concentrations unexpectedly produced a significant reduction in MitoSOX positive cells (-5%, p=0.0486 and -7%, p=0.0057) with elevated superoxide anion level compared to unexposed controls (Fig. 4B) and a concomitant increase of cells with low superoxide anion production (Fig. 4A).



**Fig. 4.** **Mitochondrial** **Superoxide Anion production.** Percentage of cells producing low (A) and high (B) mitochondrial superoxide anion after incubation for 30 min at 37 °C with isotonic buffer at pH 7.8 alone (control) or in presence of 25-75-150-300 µM of CdCl2. \* p≤0.05, \*\* p≤0.01 vs ctr (a), 25 µM (b), 75 µM (c). Data are reported as mean of three experiments (n=3) ±SEM.

**3.5 Mitochondrial membrane depolarization**

The effect of Cd on the mitochondrial membrane potential of trout erythrocytes was detected by MitoprobeDilC1(5) using flow cytometry. Cd produced a depolarizing effect on these cells (Fig.5) both in the low range (+25%, p=0.0164 and +26%, p=0.0135 for 25 and 75µM of Cd) and high range (+36%, p=0.0009 and +35%, p=0.0011 for 150 and 300 µM, respectively) compared to unexposed control.



Fig. 5. Mitochondrial membrane depolarization. Percentage of cells with mitochondrial membrane depolarized after incubation for 30 min at 37 °C with isotonic buffer at pH 7.8 alone (control) or in presence of 25-75-150-300 µM of CdCl2. \*p≤0.05, \*\* p≤0.01,\*\*\* p≤0.001 vs ctr (a). Data are reported as mean of three experiments (n=3) ±SEM.

**3.6 Haemoglobin stability and precipitation**

The effect of Cd on the conversion of oxy-Hb in the ferric form in whole erythrocytes is reported in figure 6A. Using the negative control as a reference for 0% of met-haemoglobin, Cd exposure significantly increased the percentage of met-Hb formation after 30 min of incubation at 37°C in a dose-dependent manner, at concentrations higher than 25 µM (2.25%±0.63, p=0.0296; 2.75%±0.85, p=0.0096; 3.25%±1.03, p=0.0033, 75-150-300 µM respectively). The effect of Cd on the stability of oxy-Hb has been also studied using hemolysates instead of whole erythrocytes. In this case we monitored Hb precipitation for 6 hours by following the increase of the absorbance at 700 nm. In figure 6B is reported the effect of different Cd concentrations on the time course of the precipitation and it is evident the accelerating effect of cadmium on this process.



Fig. 6. Met-Hb formation and Hb precipitation. Percentage of met-Hb formation in erythrocyte suspension after incubation for 30 min at 37 °C with isotonic buffer at pH 7.8 alone (control) or in presence of 75-150-300 µM of CdCl2 (A) and percentage of haemoglobin precipitated in hemolyzate during 6 hours of incubation at 37 °C with isotonic buffer at pH 7.8 alone (control) or in presence of 75-150-300 µM of CdCl2 (B). \* p≤0.05, \*\* p≤0.01 vs ctr (a). Data are reported as mean of three experiments (n=3) ±SEM.

1. **DISCUSSION**

In contrast to mammalian erythrocytes, fish have metabolically active nucleated erythrocytes containing the complete cellular machinery with functional ribosomes and mitochondria (Stier et al., 2013). Indeed these cells can be considered an interesting *ex vivo* model for translational studies for mammals. In this scenario, the first step of our study was aimed at investigating Cd accumulation in erythrocytes exposed to different metal concentrations at 37°C. Cd accumulation was dose-dependent, also at high metal concentrations in the exposure medium. It is noteworthy that at 9 μM Cd, the erythrocyte metal concentration (3.5 μg/g) is higher than in the external medium (1 μg/ml). Cell washing influenced the metal accumulation pattern only at the highest Cd concentration, suggesting that non-specific extracellular Cd ligands are weakly saturated and Cd can be easily removed, while a relevant percentage of the metal is strictly bound to the membranes or internalized and presumably chelated by MT. Several researches were performed on a variety of non-specific Cd transporters, including Zn and Ca transporters and divalent metal transporter 1 (Yang and Shu, 2015). To our knowledge, no Cd-specific membrane transporter has been identified so far. Lou et al., (1991) hypothesized that membrane anion exchangers provided a major transport mechanism for Cd uptake in the human red cells and it was shown that 1 mM Cd strongly interacted with human erythrocyte membrane inducing evident cellular shape changes (Suwalsky et al., 2004). Experimental evidence of the ability of Cd to cross the cell membrane is supported by the Cd accumulation in nucleated erythrocytes of the arcid clam *Scapharca inaequivalvis* experimentally exposed to Cd for 6 weeks (de Vooys et al., 1991) and by a recent *in vivo* study reporting that environmentally relevant concentrations of CdCl2 were genotoxic and cytotoxic to the erythrocytes of the fish *Labeo rohita* (Jindal and Verma, 2015).

Oxidative stress, defined as an altered “Steady State” of ROS, has been associated to the toxicity of excessive metal exposure. Redox active metals as Cu or Fe are able to generate directly ROS via Fenton reaction and this depends on their capacity to easily cycle between two valences (Cu+/Cu2+ and Fe2+/Fe3+). For Cd, a no-redox-active element, the direct ROS production is not possible also if toxicological exposure to Cd has been shown to induce oxidative stress conditions. Mitochondria play an important role in the induction of oxidative stress. It has been widely reported that mitochondrion via respiratory chain is a source of superoxide anion. Despite the suggestion that mitochondria-derived oxidative stress impacts many disease states and aging, the molecular mechanism of mitochondrial superoxide production is still vigorously debated. One of the latest discussions involves the recent observation about the association of respiratory complexes in the form of super-complexes which control ROS generation by the respiratory chain (Genova and Lenaz, 2015).

The data reported in this study are clearly indicating that, in our experimental conditions (30 min of incubation at 37°C and pH 7.8), the presence of Cd in the suspension medium, although at elevated concentration, does not influence in a significant manner the viability of the cells. The increased levels of ROS reported in figure 3 is therefore not sufficient to damage plasma membrane in a significant manner and this could be also due to the efficiency of the antioxidant repair systems which are particularly active in the erythrocytes.

To justify the effect of Cd on the mitochondrial superoxide formation reported in figure 4, we hypothesised that the reduced levels of superoxide anion observed in presence of elevated Cd concentration were due to a lowered respiration rate associated to interference of Cd with complex I, II and III (Adiele et al., 2012). In fact, in such condition, although mitochondria resulted significantly depolarised, as reported in figure 5, and hence more prone to proton leakage from the respiratory chain, in percentage this might still explain a lower formation of superoxide anion. Thus, reduced respiration with increased amount of Cd could be associated with lower mitochondrial superoxide production. On the other hand, the same high Cd concentration could induce met-Hb formation and consequent superoxide anion production. Therefore the effect of Cd on the Hb oxidation could be a major contributor to superoxide anion production in our experimental setting (Fig. 6), although other sources (i.e. xanthine/xanthine oxidase or other oxidases) could be involved as well.

It is important to point out that oxidative stress could be generated also by lowering the efficiency of antioxidant defence systems. It is well known that the conversion of oxy-Hb into met-Hb associates to superoxide formation and thereby its derivatives (H2O2 and hydroxyl radicals). Inactivation of glutathione peroxidase, a key enzyme that metabolizes either H2O2 and lipid peroxides, following met-Hb formation has been previously reported (Falcioni et al., 1987).

In conclusion, our results highlight the role of the interaction between Cd and haemoglobin on the mechanism of Cd toxicity. A reduced oxygen transport capacity due to formation of oxidative haemoglobin intermediate species and superoxide production associated to this oxidation could be, at least in part, the cause of Cd toxicity. In fact, teleosts present multiple haemoglobin components and this relates to the fact that haemoglobins have to provide oxygen for different purposes, namely the metabolic demands and the operation of the swim bladder. These haemoglobins are prone to oxidation, either as purified proteins or in the whole cell. A marked difference has been observed in the autoxidation of the two major components HbI and HbIV (Fedeli et al, 2001), directly related to the different pH dependence of their oxygen affinity. A higher oxidation rate of HbIV is in part due to its fractional saturation. It is known (Brunori, 1975) that at constant pO2 (=air) and at pH lower than 7.5, HbIV is only partially saturated with O2 and thus more easily oxidizable with respect to the fully oxygenated derivative.

In relation to an increased susceptibility to oxidation it is possible that HbIV plays a particular role in Cd toxicity in this cellular model; however further investigations are necessary to better investigate these aspects and to understand the molecular mechanism involved in Cd cellular transport and toxicity.

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