Ecotoxicology and Environmental Safety 134 (2016) 280–285

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

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

# Effect of different metals on oxidative state and mitochondrial membrane potential in trout erythrocytes

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ARTICLE INFO

*Article history:*
Received 20 April 2016
Received in revised form
26 July 2016
Accepted 27 July 2016
Available online 25 August 2016

*Keywords:*
Trout erythrocytes
Metals
Flow cytometry
Mitochondrial membrane potential
Reactive oxygen species
Haemoglobin

ABSTRACT

Homeostasis of metal ions is critical for life and excessive exposure can promote cellular damage that could be due to oxidative damage. In this context we evaluated the effects of three different elements (copper, zinc and aluminum) on oxidative stress and mitochondrial functionality in nucleated trout erythrocytes (*Oncorhynchus mykiss*). Flowcytometric measurements using MitoProbe and DCFDA-$H_2$ as fluorescent probes, indicated that redox active copper was able to influence all the biological parameters considered while redox inert, zinc and aluminum, show no significant effects. Toxicity of Al and Zn represent a debated argument and their ability to interact with other endogenous metal ions/metal binding proteins could play a role modulating their cellular toxicity.



## 1. Introduction

Metals play important roles in various biological processes. Some of these, the so-called “trace elements”, are essential and thus required for the survival of all living organisms. They are present in all organisms, almost entirely as constituents of proteins acting as enzymes, transporters, reserve proteins and transcriptional factors (Hood and Skaar, 2012). However, all metals (essential and not) can cause toxic effects through excessive exposure mainly via induction of oxidative damage to cells and organs. Thus, the homeostasis of metal ions is critical for life and therefore, careful regulation of trace elements in order to reach the optimum concentration of each of them, is required. Organisms have evolved systems for maintaining trace elements homeostasis; these systems are composed primarily of transport proteins and storage proteins. Membrane transporter proteins play a role in the influx (in deficiency) and efflux (in excess) of the element (Colvin et al., 2000). In addition metallothioneins (MT), small cysteine-rich proteins, are involved in metal buffering (Isani and Carpenè, 2014). A role in metal-storage is played by ferritin, the main iron storage protein, that it is primarily present in the cytoplasm but it is also present in serum and mitochondria (Arosio and Levi, 2010; De Sole et al., 2013) . Breakdown of metal-ion homeostasis can lead to the metal binding to protein sites different to those designed for this purpose or replacement with other metals from their natural binding sites (Nelson, 1999).

In the last decades, the concentration of metals in the environment has increased. The highest exposure usually occurs at the work-place, even if, for example, significant aluminum exposure may take place due to its presence in certain foods (in particular in corn, yellow cheese, salt, spices, herbs) and beverages (such as tea) and drinking water (Majumdar et al., 2014).

In aquaculture systems, copper is widely used to control algal blooms and aquatic macrophyte infestations (Boyd and Massaut, 1999). Aluminum sulphate is instead used in lakes and ponds to reduce the development of phytoplankton and thus to improve water transparency (Wauer et al., 2004). Another metal that has a clear nutritional and biological role as cofactor in biological systems, similar to copper, is zinc. It is sure that all metals can cause diseases through excessive exposure (Jomova et al., 2011). The elements studied have different characteristics. Copper is an essential and redox active element that can easily cycle between the cuprous and cupric state generating ROS via Fenton reaction; zinc is essential but redox inactive although it could be involved in

*Abbreviations:* MMP, Mitochondrial membrane potential; ROS, Reactive oxygen species; Hb, Haemoglobin; Met-Hb, met-haemoglobin; MT, Metallothioneins; DilC1 (5), MitoProbe; DCFDA-$H_2$, 2′-7′-dichlorodihydrofluorescein diacetate; EDTA, Ethylenediaminetetraacetic acid; K-FC, Potassium ferricyanide; PMTs, Photomultiplier tubes

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http://dx.doi.org/10.1016/j.ecoenv.2016.07.040
0147-6513/

oxidative metabolism following its interaction with cysteine thiolates that confer an important role in redox signaling by ROS (Krezel et al., 2007; Maret and Li, 2009; Colvin et al., 2010; Maret, 2011a, 2011b, 2012) while aluminum is both non-essential and redox inactive.

The main aim of this work was to detect the ability of three different elements, copper, zinc and aluminum, to induce mitochondrial membrane depolarization, elevated production of reactive oxygen species (ROS) and thus reduced cell vitality in trout (*Oncorhynchusmykiss*) erythrocytes. These determinations could be useful to better understand the mechanism involved in oxidative toxicity due to excessive metal exposure and in particular to highlight the role of mitochondria in this process.

The toxicity of these three elements was followed in a biological system consisting of trout erythrocytes that possess nuclei, mitochondria and other organelles typical of somatic cells. Flow cytometry measurements were performed to evaluate the "*in vitro*" influence of the three elements on the intracellular production of ROS and mitochondrial membrane potential (MMP) using carboxy-$H_2$-DCFDA and MitoProbe respectively. In addition the oxidative state of haemoglobin was evaluated through meta-haemoglobin (met-Hb) formation, as another index of oxidative insult to cellular components.

## 2. Materials and methods

All reagents were of pure analytical grade. $CuSO_4{}^*5H_2O$, $AlCl_3{}^*6H_2O$, $ZnSO_4{}^*7H_2O$ from Sigma-Aldrich, Guava ViaCount Assay from Millipore, MitoProbe DilC1(5) and 2′-7′-dichlorodihydrofluoresceindiacetate (DCFDA-$H_2$) were purchased from Life Technologies.

### 2.1. Samples and treatments

Red blood cells were obtained from *Oncorhynchusmykiss* trout coming from the fish farm "Eredi Rossi Silvio" Sefro (Macerata), Italy. Blood was withdrawn by syringe from the lateral tail vein into an isotonic medium (0.1 M phosphate buffer, 0.1 M NaCl, 0.2% citrate, 1 mM EDTA at pH 7.8).

At least three trouts were collected for each experiment and the blood obtained from them was always pooled in order to minimize individual variability. EDTA was used as anticoagulant in virtue of its properties in preserving cellular morphology for flow cytometry. Nonetheless, considering potential chelating activity of EDTA, erythrocytes were washed twice with buffer without EDTA and resuspended in the same buffer for experimental purposes. Moreover, parallel experiments using heparin as anticoagulant were conducted incubating the cells with the higher concentrations of all of metals. No significant differences were observed in both, confirming that EDTA was efficiently removed during washes and its use did not bias the experimental setup.

The red blood cells were separated from the plasma and buffy coat by centrifugation for2 min at 300 *g* in 1 mL final volume and were washed 3 times with the same isotonic phosphate buffer without EDTA in order to prevent metal chelation. In fact in the concentration used at pH 7.8 chelating activity of citrate 0.2% might be considered marginal. To evaluate viability, intracellular reactive oxygen species and mitochondrial membrane potential, the washed cells were counted by using Guava ViaCount assay in flow cytometry, to determine both cell count and cell viability.

Subsequently, 1*106 viable cells for each condition were aliquoted in isotonic phosphate buffer at pH 6.3 and pH 7.8. For each evaluated endpoint, cells were incubated in the presence and in absence of the tested elements in the range of concentrations 1–300 μM for 30 min at 37 °C in the dark (in isotonic buffer pH 7.8 as well). Following incubation samples were washed with isotonic buffer pH 7.8 by centrifugation at 300 *g* for 2 min and resuspended in 300 μL of the same buffer.

The hemolyzate used to study the effects of metals on met-Hb formation was obtained by adding three volumes of distilled water to the washed packed cells and frozen at −20 °C for at least 30 min. Thawed hemolyzates were centrifuged at 15,000 *g* for 10 min at 4 °C to remove cellular debris.

### 2.2. Viability assay

Viability of erythrocytes was measured using Guava ViaCount kit. In brief, this kit contains two dyes: the nuclear dye binds DNA in all cells and is able to distinguish the cell from debris, while a second dye binds exclusively to DNA of dead cells since it is a membrane impermeable dye. Apoptotic cells have partially damaged cell membranes. This decrease in integrity produces an intermediate permeability associated with a weaker signal compared to dead cells. After incubation and wash, the cell suspensions were incubated with ViaCount reagent in 1:10 proportion for 5 min in the dark and analyzed in flow cytometry. Data are expressed as % of viable, apoptotic and dead cells ± DS.

### 2.3. Intracellular reactive oxygen species assay

Reactive oxygen species levels were assessed by means of 2′-7′-dichloro-dihydro-fluorescein-diacetate (DCFDA-$H_2$), a non-polar probe, which readily diffuses across cell membranes where it is hydrolyzed by intracellular esterases to the non-fluorescent polar derivative, $DCFH_2$. In the presence of ROS, $DCFH_2$ is oxidized to DCF which is highly fluorescent and whose emission maximum can be monitored at 520 nm.

Before incubation with isotonic buffer alone or with different salt concentrations, erythrocytes were incubated with DCFDA-$H_2$ (10 μM final concentration) at 37 °C for 45 min in the dark. Cells were then washed with isotonic medium by centrifugation at 300*g* for 2 minat 10 °C and resuspended in the same medium. Prior to flow cytometry analysis, fluorescence of the labeled cells was measured using Guava easyCyte (Millipore) at an excitation wavelength of 488 nm. Emission was recorded at 525/30 (Green), 583/26 (Yellow) and 690/50 (Red). Photomultiplier tubes were set at 49.4, 439 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 % of cells with high ROS production ± DS.

### 2.4. Mitochondrial membrane potential assay

Mitochondrial membrane potential (MMP) was evaluated using MitoProbe $DilC_1(5)$, a cationic cyanine dye that is able to accumulate in cells in response to membrane potential changes. The peaks of excitation and emission of this dye are 638 nm (red) and 658 nm (far red) respectively. After incubation with isotonic buffer alone or in association with different concentrations of metals, erythrocytes were incubated for 15 min at 37 °C in the dark with $DilC_1(5)$ (40 nM final concentration).

Subsequently, to remove excess $DilC_1(5)$, cells were washed in isotonic medium at pH 7.8 by centrifugation at 300*g* for 2 min at 10 °C and analyzed using Guava easyCyte equipped with a Kv-Av 633 nm laser. Photomultiplier tubes (PMTs) were set at 76.1 V and 58.7 V respectively. Mitochondrial depolarization was evaluated in terms of percentage of cells ± DS showing low fluorescence proportional to MMP, using the Guava InCyte software.