Environ Sci Pollut Res (2016) 23:12312–12322
DOI 10.1007/s11356-016-6445-3

RESEARCH ARTICLE

Cytotoxicity and alterations at transcriptional level caused by metals on fish erythrocytes in vitro

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Received: 19 December 2015 / Accepted: 8 March 2016 / Published online: 15 March 2016

Abstract The in vitro use of fish erythrocytes to test the toxicity of aquatic pollutants could be a valuable alternative to fish bioassays but has received little attention. In this study, erythrocytes from marine gilthead sea bream (*Sparus aurata* L.) and European sea bass (*Dicentrarchus labrax* L.) specimens were exposed for 24 h to Cd, Hg, Pb and As and the resulting cytotoxicity was evaluated. Exposure to metals produced a dose-dependent reduction in the viability, and mercury showed the highest toxicity followed by MeHg, Cd, As and Pb. Moreover, fish erythrocytes incubated with each one of the metals exhibited alteration in gene expression profile of metallothionein, superoxide dismutase, catalase, peroxiredoxin, glutathione reductase, heat shock proteins 70 and 90, Bcl2-associated X protein and calpain1 indicating cellular protection, stress and apoptosis death as well as oxidative stress. This study points to the benefits for evaluating the toxicological mechanisms of marine pollution using fish erythrocytes in vitro.

Responsible editor: Cinta Porte

Electronic supplementary material The online version of this article (doi:10.1007/s11356-016-6445-3) contains supplementary material, which is available to authorized users.

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Keywords Erythrocytes · Metals · Teleost fish · Flow cytometry · Oxidative stress · Cell death

Introduction

Anthropogenic actions have resulted in an increased flux of metallic substances into the aquatic environment (Yang and Rose 2003), a fact that has led to the investigation of the effect of metals and metalloids on the biological functions of marine organism such as fish. Among the adverse effects, metals can produce mortality or alterations in blood, metabolism, nutrition, reproduction, development and immunity (Bols et al. 2001; Di Giulio and Hinton 2008; Sweet and Zelikoff 2001). Some studies have shown that aquatic pollutants including metals alter fish haematological indices (haematocrit, red blood cell count per unit blood volume and haemoglobin (Hb) concentration), as well as blood/plasma ions, hormones, metabolites, proteins or enzymes (Schlenk et al. 2008). However, there are not many papers dealing with the toxicological effects on fish blood cells, namely erythrocytes.

In vitro toxicological tests are gaining traction as alternatives to in vivo tests because they are more cost- and time-effective and have fewer ethical issues. The fact that fish erythrocytes are nucleated and contain organelles that exhibit good resistance in primary cultures and are easily handled constitutes a very interesting cellular model for toxicological studies in vitro (Bogé and Roche 1996). Furthermore, experimental fish are not killed which turns them into a valuable alternative to fish bioassays and contributes to the three R's. In addition, erythrocytes are targets for metals and used to distribute them along the body. Owing to the high oxygen and iron concentrations in the cytoplasm, erythrocytes can continuously produce reactive oxygen species (ROS) as a result of haemoglobin oxidation to methaemoglobin (Çimen 2008;

Giulivi and Davies 2001) and therefore are exposed to potential oxidative stress. To maintain the ROS balance, as in mammals, fish erythrocytes are well protected by radical scavengers, including enzymatic and non-enzymatic systems. The enzymatic systems in erythrocytes consist of mitochondrial and cytosolic superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) and peroxiredoxins (Çimen 2008; Scott et al. 1989).

In vivo toxicological studies in fish erythrocytes have reported the effects of various chemicals in the morphology, haemolysis, nuclear deformation, amitosis (Bogé and Roche 1996; Witeska 2013) and genotoxic damage (Bagdonas and Vosylienė 2006; Monteiro et al. 2011). However, fewer studies have evaluated the toxicological role of metals on fish erythrocytes in vitro, which could provide basic information on the nature of the tested agents and/or the cellular response (Binelli et al. 2009). Thus, rainbow trout (*Oncorhynchus mykiss*) naïve erythrocytes exposed in vitro to cooper showed little effect on ROS production (Fedeli et al. 2010) whilst exposure to titanium dioxide nanoparticles produced cytotoxicity and DNA damage but not ROS production (Sekar and Falcioni 2014). In the case of a neotropical freshwater fish (*Prochilodus lineatus*), erythrocyte exposure to lead also confirmed genotoxic and cytotoxic effects (Monteiro et al. 2011). Nevertheless, very few papers have evaluated the specific cellular responses of marine fish erythrocytes against pollutants at protein or messenger RNA (mRNA) levels (Roche and Bogé 1993). To our knowledge, only one study (Fulladosa et al. 2006) has evaluated the metallothionein (MT) expression in vitro in fish erythrocytes, showing a similar trend to that recorded in different fish species and models (Carbonell et al. 1998; Morcillo et al. 2015a, b, 2016). MTs, together with heat shock proteins (HSPs), are important proteins involved in cellular protection and concretely in the protection against metals (Bourdineaud et al. 2006; Morimoto 2011). ROS induction by metals provokes DNA damage and apoptosis, but no studies have been found in the literature about the cell death mechanism after metal exposure in fish erythrocytes. In humans, it has been described a particular erythrocyte cell death mechanism called eryptosis that resembles to apoptosis of nucleated cells (Lang et al. 2006). Some differences between eryptosis and apoptosis reside in the fact that mammalian erythrocytes lack nuclei and mitochondria and that the molecular signalling pathways are not identical (Lang et al. 2012). Eryptosis occurs under natural conditions in erythrocytes, and eryptotic cells are engulfed and degraded by macrophages contributing to the animal haemostasis although it also occurs under pathophysiological situations. Among these situations, eryptosis has been demonstrated on human erythrocytes exposed to Cd, Hg, Pb, or As (Eisele et al. 2006; Kempe et al. 2005; Mahmud et al. 2009; Sopjani et al. 2008). In addition, a report has demonstrated the role of B cell lymphoma-2 (Bcl2) and Bcl-2 homologous antagonist killer (Bak) proteins in the human erythrocyte survival in vitro (Walsh et al. 2002), but no studies were found in fish erythrocytes.

Taking into consideration that pollutants affect fish haematological parameters and the scarce studies evaluating their effects on fish erythrocytes at molecular level, we aimed to evaluate the cytotoxicity of metals (Cd, Hg, Pb) and a metalloid (As) as well as the transcription pattern of genes related to cellular oxidative stress, protection and death after 24-h exposure in two teleost fish species: gilthead sea bream (*Sparus aurata* L.) and European sea bass (*Dicentrarchus labrax* L.), the most important Mediterranean cultured fish species.

Material and methods

Animals

Thirty specimens of 80–100-g body weight of the seawater teleost gilthead sea bream (*Sparus aurata* L.) and European sea bass (*D. labrax* L.) obtained from local fish farms were kept in seawater aquaria (250 l) in the Marine Fish Facilities at the University of Murcia (Spain). The water was maintained at 20 ± 2 °C, with a flow rate of 900 l/h, and 28 ‰ salinity. The photoperiod was 12-h light/12-h dark, and fish were fed with a commercial pellet diet (Skretting) at a rate of 2 % body weight/day. Fish were allowed to acclimatize for 15 days before the start of the experimental trial. All experimental protocols were approved by the Ethical Committee of the University of Murcia.

Erythrocyte isolation

Fish were taken from the aquaria and 200 μl of blood was immediately withdrawn into a heparinized syringe from the caudal vein and placed into 4 ml of phosphate-buffered saline (PBS, containing 0.35 % sodium chloride, to adjust the medium's osmolarity) with 10 mM glucose, and the fish were returned to the aquaria. Blood was layered over a 51 % Percoll density gradient (Pharmacia) and centrifuged (400×*g* for 30 min, 4 °C) to separate leucocytes and erythrocytes, which were located in the pellets, collected, washed twice with PBS, counted and adjusted to 5×10^8 cells/ml in PBS with 10 mM glucose.

Metal exposure

Different salts of the tested metals (Sigma-Aldrich) were used: cadmium chloride ($CdCl_2$), methylmercury (II) chloride [CH_3HgCl (MeHg)], mercury (II) chloride ($HgCl_2$), lead (II) nitrate ($Pb(NO_3)_2$) and trioxide arsenic (As_2O_3). Each salt was initially dissolved in PBS with 10 mM glucose, and dilutions for each concentration were daily prepared. Prior to carrying

out the assays, the osmolarity of these solutions was measured in an osmometer (Wescor) to avoid effects due to this parameter.

For each individual fish, 100 μl of blood erythrocytes was placed into separate 1.5 ml Eppendorf tubes in triplicate and exposed with 1 ml of PBS with 10 mM glucose (controls) or metal solutions, to make final concentrations of 10–100 μM for Cd, 5–75 μM for MeHg, 1–10 μM for Hg, 0.1–3 mM for Pb and 10–500 μM for As. Samples were gently shaken in an orbital agitator (24 h at 24 °C). Erythrocytes from six different and independent fish were used for cytotoxicity curves and from four for gene expression studies.

Cytotoxicity assays

Propidium iodide uptake

In order to determinate the viability of the sea bream and sea bass erythrocytes, we assessed the abundance of dead erythrocytes using a flow cytometry technique (Ormerod 1990). Following 24 h of metal exposure, 10 μl of each sample was transferred to 5-ml tubes (Becton Dickinson) containing 400 μl of PBS and 100 μl of propidium iodide (PI; 400 μg/ml; Sigma-Aldrich). All samples were analysed in a flow cytometer (Becton Dickinson) with an argon-ion laser adjusted to 488 nm. Analyses were performed on 10,000 cells, which were acquired at a rate of 300 cells/s. Data were collected in the form of two-parameter side scatter (SSC, granularity) and forward scatter (FSC, size), and green fluorescence (FL1) and red fluorescence (FL2) dot plots or histograms were constructed on a computerized system. With this method, dead (PI^+) and viable (PI^-) cells were discriminated and analysed.

Oxyhaemoglobin release

Erythrocyte exposure to metals results in cell death and liberation of their content being haemoglobin, in either oxyhaemoglobin (HbO_2) or deoxyhaemoglobin forms, the most abundant protein. Thus, the viability of the cells was determined by the haemolysis of erythrocytes and consequent liberation of the oxyhaemoglobin to the medium (De Kretser and Waldron 1963; Jan and Frantisek 2000; Martínez-López et al. 2005). After 24 h of exposure, the samples were centrifuged (10,000×*g*, 1 min) and 100 μl of the supernatant transferred to 96-flat-bottomed-well plates (Nunc) and the absorbance at 542 nm (the maximum absorbance for oxyhaemoglobin) analysed in a plate reader (BMG, Fluoro Star Galaxy). Positive (maximum haemolysis and absorbance) or negative (minimum haemolysis and absorbance) controls consisted on 100 μl of erythrocytes in 1 ml of sterile distilled water or in 1 ml PBS with 10 mM glucose, respectively.

Data analysis

For each method, cell viability data and the metal concentrations were represented and fitted with an exponential decay three-parameter curve [$f = y\ 0 + a \times exp\ (-bx)$]. Fitted curves always showed r^2 values higher than 0.96 which are therefore the only ones presented in the cytotoxicity curves. The concentration producing 50 % cell death (EC_{50}) was determined for all metals and assays (Table 1) using SigmaPlot software. According to the PI method, EC_0 (the minimum concentrations used and that failed to be cytotoxic) and EC_{50} were used in this study as the concentrations to measure gene expression.

Gene expression analysis by real-time PCR

After 24 h of erythrocyte exposure to the EC_0 or EC_{50} of the metals (see Table 1), samples were centrifuged, the supernatants were aspirated and TRIzol Reagent (Life Technologies) was added to the wells in order to extract the total RNA as indicated by the manufacturer. It was then quantified and the purity assessed by spectrophotometry; the 260:280 ratios were 1.8–2.0. The RNA was then treated with DNase I (Promega) to remove any genomic DNA contamination. Complementary DNA (cDNA) was synthesized from 1 μg of total RNA using

Table 1 Comparing the cytotoxicity of metals to fish erythrocytes from two species by two methods

Metal	Gilthead sea bream erythrocytes		European sea bass erythrocytes	
	PI uptake	Oxyhaemoglobin release	PI uptake	Oxyhaemoglobin release
Cd	22 ± 3.62^{aA}	23 ± 4.5^{aA}	80 ± 12.7^{aB}	71 ± 9.27^{aB}
MeHg	21 ± 1.06^{aA}	20 ± 0.71^{aA}	28 ± 0.76^{bAB}	29 ± 0.5^{bB}
Hg	2.4 ± 0.22^{bA}	2.7 ± 0.45^{bB}	3.6 ± 0.01^{cC}	$5.5 \pm 0.\ 3^{cD}$
Pb	523 ± 103^{cA}	374 ± 64^{cB}	$1{,}100 \pm 129^{dC}$	$1{,}000 \pm 201^{dC}$
As	134 ± 0.28^{dA}	129 ± 0.14^{dB}	$190 \pm 51\ ^{aC}$	189 ± 3^{eC}

Values of EC_{50} (μM; mean ± SEM; $n = 6$ independent fish) of fish erythrocytes after exposure to metals for 24 h. r^2 values of the fitted curves were higher than 0.96 in all cases. Statistically significant differences (ANOVA; $P \leq 0.05$) within each column (differences among metals) or row (differences between fish species and method for each metal) are denoted by different lowercase or capital letters, respectively

the SuperScript III reverse transcriptase (Life Technologies) with an oligo-dT18 primer.

The expression of genes involved in cellular oxidative stress [superoxide dismutase (*sod*), catalase (*cat*), glutathione reductase (*gr*) and peroxiredoxin 1 (*prx1*)], cellular protection [metallothionein-A (*mta*) and heat shock proteins 70 (*hsp70*) and 90 (*hsp90*)], cellular apoptosis [pro-apoptotic Bcl2-associated X gene (*bax*)] and eryptosis [calpain1 (*calp1*)] was evaluated by real-time PCR with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems). Reaction mixtures (containing 10 μl of 2× SYBR Green supermix, 5 μl of primers (0.6 mM each) and 5 μl of cDNA template) were incubated (10 min, 95 °C), followed by 40 cycles of 15 s at 95 °C, 1 min at 60 °C, and finally 15 s at 95 °C, 1 min at 60 °C and 15 s at 95 °C. For each mRNA, gene expression was corrected by the elongation factor 1α (*ef1a*) RNA content in each sample and calculated using the $2^{-\Delta\Delta}$Ct method (Livak and Schmittgen 2001). Negative controls had no amplification product, and control templates showed no primer–dimer formations. Gene names follow the accepted nomenclature for zebrafish (https://wiki.zfin.org/). The primers used in the present study are shown in Table 2. In all cases, each PCR was performed with triplicate samples from four specimens.

Statistical analysis

Data of the cytotoxic effects are presented for each metal and method using the fitted curves. Gene expression is expressed as fold change with respect to the control samples where values higher than 1 indicate up-regulation and lower than 1 down-regulation of each gene. EC_{50} values for each fish species and method were analysed by one-way analysis of variance (ANOVA; $P \leq 0.05$) to determine differences among metals, followed by a Student–Newman–Keuls (SNK) comparison mean test. Gene expression data were statistically analysed by two-way ANOVA ($P \leq 0.05$) to determine differences between control and metals and between the two fish species. Normality of the data was previously assessed using a Shapiro–Wilk test, and homogeneity of variance was also verified using the Levene test. A non-parametric Kruskal–Wallis test was used when data did not meet parametric assumptions. Statistical analyses were conducted using SPPS 20.0 software (SPSS).

Results

Cytotoxicity assays

After 24-h exposure to metals, the viability of gilthead sea bream and European sea bass erythrocytes declined in a dose-dependent manner compared to controls (Fig. 1). The data were used to fit curves in order to identify the EC_{50} (Fig. 1 and Table 1). According to the PI method, EC_{50} values for sea bream erythrocytes were of 22, 21, 2.4, 523 and 134 μM, and for sea bass 80, 28, 3.6, 1,100 and 190 μM for Cd, MeHg, Hg, Pb and As, respectively (Table 1). Hg was the most toxic metal for erythrocytes in both species followed by MeHg, Cd, As and Pb (Table 1). Cytotoxicity curves followed different shapes between fish species when the erythrocytes were exposed to Cd or MeHg. Thus, sea bream erythrocytes are more resistant to Cd, Pb and As whilst the susceptibility to MeHg and Hg was roughly the same. PI uptake and oxyhaemoglobin release techniques revealed almost identical cytotoxicity curves except for Hg-exposed sea bass erythrocytes (Fig. 1), which is also evidenced for the EC_{50} values (Table 1).

Metals generate oxidative stress in erythrocytes

Exposure of erythrocytes to the EC_{50} of the metals for 24 h provoked oxidative stress (Fig. 2 and Supplementary data Fig. S1). First, all the metals, except Cd in sea bass, significantly up-regulated the *sod* transcription in erythrocytes, but no differences were observed between species. Surprisingly, *cat* and *gr* gene expression was always down-regulated after EC_{50} metal exposure of sea bream erythrocytes, but statistically significant for Cd, MeHg and As. By contrast, they were always up-regulated in sea bass erythrocytes being only significant the *cat* gene for EC_{50} of As and *gr* for EC_{50} of MeHg, Hg and As. Significant effects between the two fish species were also observed. The *prx1* gene expression was significantly enhanced by EC_{50} of Cd, MeHg, Hg and Pb in sea bream erythrocytes while only Cd and As exposure induced its transcription, and Hg down-regulated, in sea bass erythrocytes (Fig. 2). As consequence, Hg and Pb effects were significantly different in the two fish species. Interestingly, exposure of erythrocytes to EC_0 provoked roughly the same profile at transcriptional level than the EC_{50} (Supplementary Fig. S1).

Cellular protection is differently altered in sea bream and sea bass erythrocytes

We evaluated the cellular protection to metals by the expression of the metallothionein-A gene, *mta* and heat shock proteins 70 and 90 (Fig. 3 and Supplementary Fig. S1). As expected, EC_{50} of Cd or MeHg exposure of erythrocytes from sea bream and Hg exposure of erythrocytes from both fish species significantly up-regulated *mta* transcription (Fig. 3). Surprisingly, exposure to EC_{50} of Pb or As did not alter *mta* mRNA abundance in the sea bream and sea bass erythrocytes. All the metals increased *hsp70* gene expression in sea bream erythrocytes; however, Hg and Pb significantly down-regulated it in sea bass erythrocytes although As up-regulated it. Thus, exposure to MeHg, Hg and Pb provoked significant differences between gene expression in

Table 2 Primers used for analysis of gene expression by real-time PCR

Gene name	Gene abbreviation	Fish specie	Acc. numbers	Sequence (5′-3′)
Elongation factor 1-alpha	*ef1a*	Sea bream	AF184170	CTGTCAAGGAAATCCGTCGT
				TGACCTGAGCGTTGAAGTTG
		Sea bass	AJ866727	CGTTGGCTTCAACATCAAGA
				GAAGTTGTCTGCTCCCTTGG
Cu/Zn superoxide dismutase	*sod*	Sea bream	AJ937872	CCATGGTAAGAATCATGGCGG
				CGTGGATCACCATGGTTCTG
		Sea bass	FJ860004	TGTTGGAGACCTGGGAGATG
				ATTGGGCCTGTGAGAGTGAG
Catalase	*cat*	Sea bream	FG264808	TTCCCGTCCTTCATTCACTC
				CTCCAGAAGTCCCACACCAT
		Sea bass	FJ860003	GAGGTTTGCCTGATGGCTAC
				TGCAGTAGAAACGCTCACA
Glutathione reductase	*gr*	Sea bream	AJ937873	CAAAGCGCAGTGTGATTGTGG
				CCACTCCGGAGTTTTGCATTTC
		Sea bass	FM020412	TGCACCAAAGAACTGCAGAA
				ACGAGTGTCACCTCCAGTCC
Peroxiredoxin 1	*prx1*	Sea bream	GQ252679	CTCCAAGCAATAATAAGCCCAAAG
				TCACTCTACAGACAACAGAACAC
		Sea bass	AM987213	CTGCCGAAGATTTCAGGAAGA
				CGCCGTGTGTCAGATACCAG
Metallothionein-A	*mta*	Sea bream	X97276	ACAAACTGCTCCTGCACCTC
				CAGCTAGTGTCGCACGTCTT
		Sea bass	AF199014	GCACCACCTGCAAGAAGACT
				AGCTGGTGTCGCACGTCT
Heat shock protein 70	*hsp70*	Sea bream	EU805481	AATGTTCTGCGCATCATCAA
				GCCTCCACCAAGATCAAAGA
		Sea bass	AY423555	CTGCTAAGAATGGCCTGGAG
				CTCGTTGCACTTGTCCAGAA
Heat shock protein 90	*hsp90*	Sea bream	DQ524994	GGAGCTGAACAAGACCAAGC
				AGGTGATCCTCCCAGTCGTT
		Sea bass	AY395632	CTCAGGGACAACTCCACCAT
				CTTTGTCGTTCTTGTCAGCA
Bcl-2 associated X protein	*bax*	Sea bream	AM963390	CAACAAGATGGCATCACACC
				TGAACCCGCTCGTATATGAAA
		Sea bass	FM011848	TGTCGACTCGTCATCAAAGC
				CACATGTTCCCGGAGGTAGT
Calpain1	*calp1*	Sea bream	KF444899	GTGGAGCTCCTCGCTGTATC
				GCTTGAACTGAGGGTTGAGC
		Sea bass	FJ821591	TGCAGAGGGGACTGAGTTCT
				CCGCCTGTAAAGTCCTCAAA

erythrocytes for the two fish tested species. In contrast, most of the metals down-regulated the transcription of *hsp90* but only Pb and As did to a significant extent in sea bream and sea bass erythrocytes, respectively (Fig. 3).

As above, exposure of sea bream or sea bass erythrocytes to EC_0 of metals induced a very similar transcriptomic profile as the EC_{50} (Supplementary Fig. S1).

Metals induce apoptosis and eryptosis cell death

The expression of typical markers for apoptosis (pro-apoptotic Bcl2-associated X gene, *bax*) and eryptosis (μ-calpain, *calp1*) was assayed in fish erythrocytes (Fig. 4 and Supplementary Fig. S1). Sea bream and sea bass erythrocytes exposed to EC_{50} of Hg, Pb and

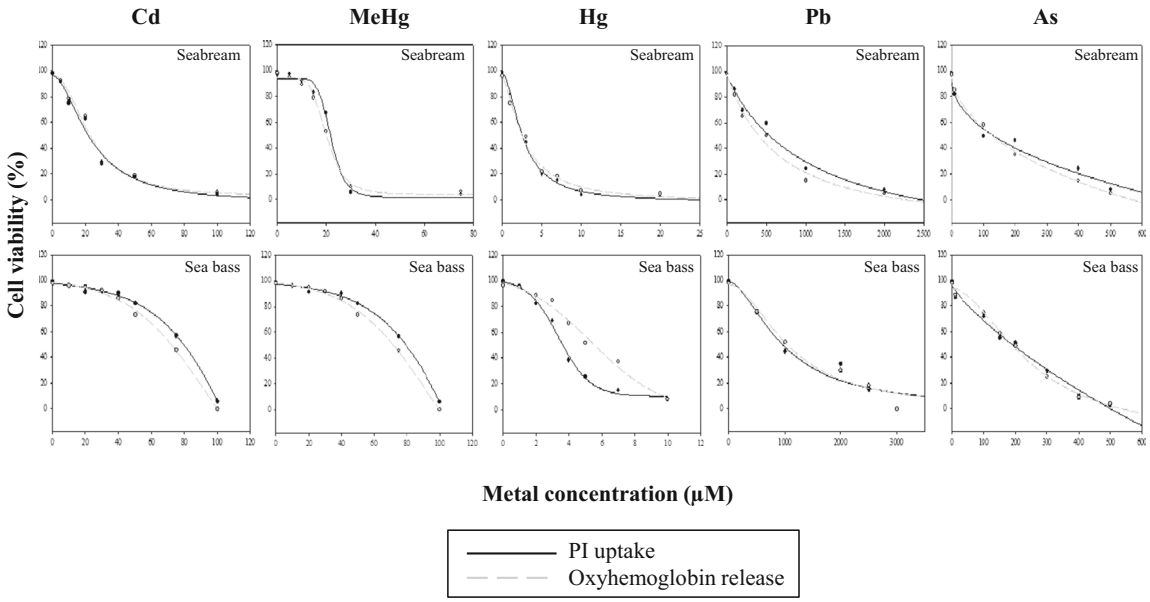

Fig. 1 Cytotoxicity curves of gilthead sea bream or European sea bass erythrocytes exposed to different metals for 24 h. *Lines* represent the fitted curve for each method: propidium iodide (PI) and oxyhaemoglobin release

As induced an up-regulation of *bax* transcripts, demonstrating the apoptosis cell death process. On the other hand, EC_{50} of Cd and MeHg did not alter it to a significant level. Surprisingly, *calp1* gene expression was greatly impaired by all EC_{50} of metals, except MeHg in sea bream erythrocytes; however, exposure to MeHg or Hg produced a general induction of *calp1* mRNA transcription in sea bass erythrocytes (Fig. 4), which could be related to the eryptosis process (Fig. 5) (Lang et al. 2006).

Finally, the effects provoked in fish erythrocytes at gene expression level by EC_0 and EC_{50} metals were comparable (Supplementary Fig. S1).

Discussion

Fish erythrocytes could be useful for toxicological studies of aquatic pollutants because they remain viable in primary cultures and are easy to obtain and manipulate. However, very few papers have evaluated the toxicological effects on fish erythrocytes and those available include morphological changes (Bogé and Roche 1996; Monteiro et al. 2011; Witeska 2004), genotoxic damage (Bagdonas and Vosylienė 2006; Mitchelmore and Chipman 1998) and metallothionein expression (Fulladosa et al. 2006).

Among the cytotoxicity tests used in vitro, flow cytometry and spectrophotometry methods were used to evaluate the cell

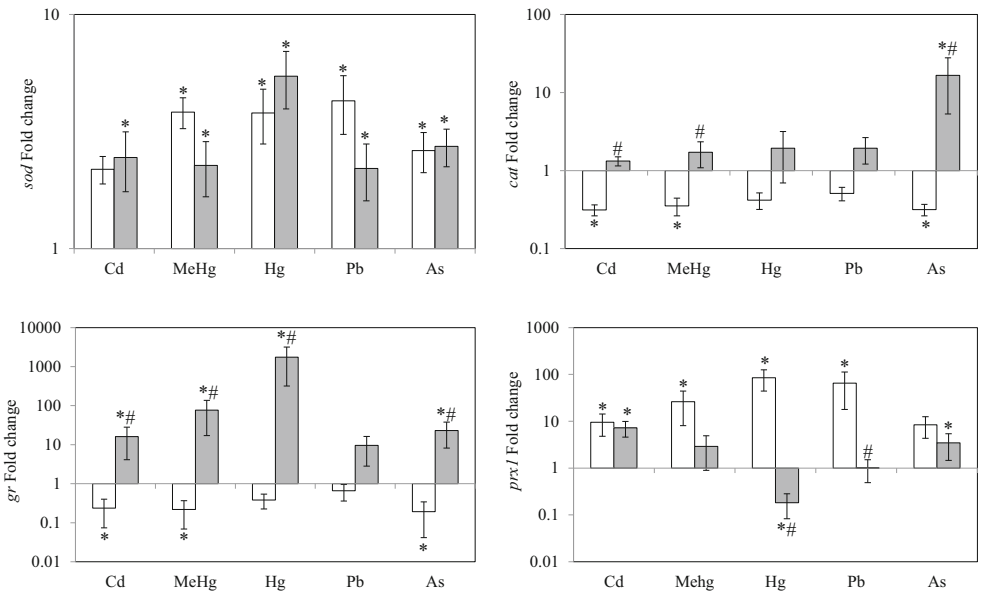

Fig. 2 Expression of genes related to oxidative stress (*sod*, *cat*, *gr* and *prx1*) in gilthead sea bream (*white bars*) or European sea bass (*grey bars*) erythrocytes exposed to the EC_{50} of each metal for 24 h. Data are expressed as fold change with respect to the control erythrocytes. *Bars* represent the mean ± SEM from four independent fish. Statistically significant differences ($P \leq 0.05$) between control and metal-exposed (*) and between the two species (#) are denoted

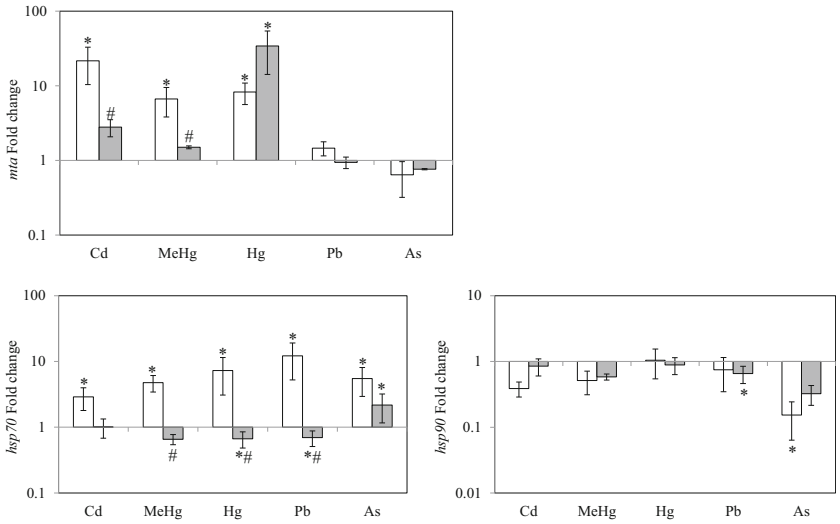

Fig. 3 Expression of gene related to cell protection (*mta*, *hsp70* and *hsp90*) in gilthead sea bream erythrocytes (*white bars*) or European sea bass (*grey bars*) erythrocytes after exposure to the EC_{50} of each metal for 24 h. Data are expressed as fold change with respect to the control erythrocytes. *Bars* represent the mean ± SEM from four independent fish. Statistically significant differences ($P \leq 0.05$) between control and metal-exposed (*) and between the two species (#) are denoted

death of fish erythrocytes. The cytotoxicity assays used revealed comparable profiles, showing a dose-dependent curve after 24-h exposure to the metals in erythrocytes from gilthead sea bream and European sea bass. The EC_{50} values showed that toxicity after 24-h exposure to metals was Hg > MeHg > Cd > As > Pb for erythrocytes from both species. However, sea bream erythrocytes were more sensitive to the metals than sea bass erythrocytes, showing lower EC_{50}. Moreover, we have shown for the first time that the oxyhaemoglobin release method could be used in fish ecotoxicological testing and is more sensible than the PI uptake. In addition, it has the advantage of using unsophisticated and affordable equipment and no need for reagents such as PI, which is carcinogenic. This method has been satisfactorily used in toxicological studies conducted in humans, rats and birds (De Kretser and Waldron 1963; Jan and Frantisek 2000; Martínez-López et al. 2005) and should be further explored in fish.

Cytotoxic effects of metals observed on fish erythrocytes were compared to the few studies available in the literature. For instance, sea bream erythrocytes exposed to 100 μM Hg for 1 h showed a 6.1 % of haemolysis (Gwozdzinski et al. 1992) while this dose for 24 h showed 100 % haemolysis, which could be attributed to a rapid and adverse effect of Hg upon membrane integrity. Human erythrocytes exposed to 10 mM As (III) for 5 h exhibited 0.7 % haemolysis (Shannon and Winski 1998). Thus, As seems to show a slower and lower toxicity mechanism than Hg, which is also observed in our results. Studies using bird erythrocytes reported a lower EC_{50} for Cd (0.027 mM) than for lead (1.84 mM) (Hernández-García et al. 2014), which also agrees with our data. Other studies in the RTG-2 fish cell line confirmed the highest toxicity of Hg compared to other metals (Maracine and Segner 1998), whilst in the case of the grass carp (*Ctenopharyngodon idella*) ZC-7901 cell line, the order was $Cd^{2+} > Hg^{2+} > Pb^{2+} > Cu^{2+} > Cr^{6+} > As^{5+}$ (Xiang et al. 2001). Moreover, in the rainbow trout RTL-W1 (derived from the liver) EC_{50} values for Cd are quite similar to our data (Dayeh et al. 2005). Differences in the fish environment (fresh or marine water), as well as the metal form, solubility, exposure time, interactions with culture medium components, etc., could be responsible for some of the differences found with those mentioned in the literature. In fact, it is known that culture medium composition or percentage of serum supplementation affects the metal cytotoxicity (Borenfreund and Puerner 1985; Segner 1998; Dayeh et al. 2005). For example, differences between culture medium could be behind the low

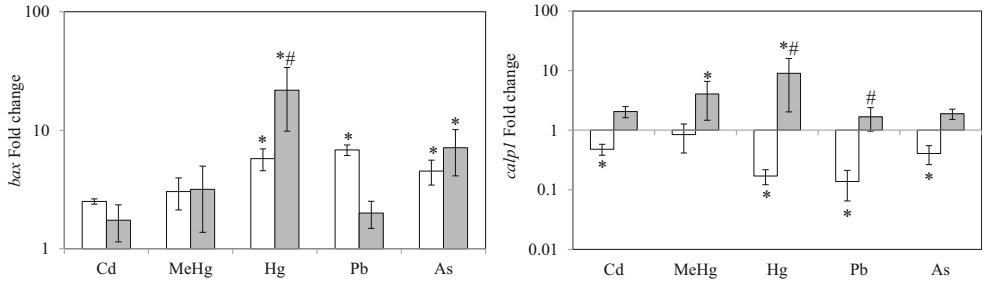

Fig. 4 Expression of genes related to cell death (*bax* and *calp1*) in gilthead sea bream (white bars) or European sea bass (*grey bars*) erythrocytes exposed to the EC_{50} of each metal for 24 h. Data are expressed as fold change with respect to the control erythrocytes. *Bars* represent the mean ± SEM from four independent fish. Statistically significant differences ($P \leq 0.05$) between control and metal-exposed (*) and between the two species (#) are denoted

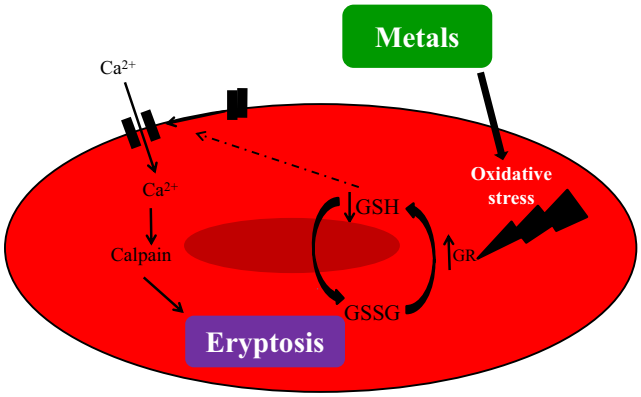

Fig. 5 Synopsis of the mechanisms and the signalling pathways involved in eryptosis. Modified from Lang et al. (2006). *GR*, glutathione reductase; *GSH*, glutathione; *GSSG*, glutathione disulfide

EC_{50} values observed in this study (PBS with 10 mM glucose) and the higher values observed in the SAF-1 cell line (derived from fins of gilthead sea bream and exposed in L-15 with 10 % serum) (Morcillo et al. 2016) that would be further investigated.

Very few studies concerned with the erythrocyte toxicology of metals at the gene level are found in the literature. Thus, we have evaluated the transcription of some important genes involved in oxidative stress, cell protection and death after 24 h of exposure to the EC_0 or EC_{50} of each metal. With respect to oxidative stress, we found that all the metals induced a significant up-regulation of *sod* gene expression in sea bream and sea bass erythrocytes. In sea bass, erythrocytes exposed to 10–100 μM Hg for 1 h the SOD enzyme activity increased in all doses (Gwozdzinski et al. 1992), which agrees with our results. However, the decrease in SOD activity of sea bass erythrocytes by Cu^{2+} and Zn^{2+} was unexpected (Roche and Bogé 1993), since these metallic ions are potent ROS activators. In vivo experiments with goldfish (*Carassius gibelio*) erythrocytes evaluating SOD activity showed a significant decrease after the first day of Cd exposure or an increase after 7 or 15 days (Zikić et al. 2001). This increased gene and protein SOD could be involved, as demonstrated in human erythrocytes, in the prevention of methaemoglobin formation (Dumaswala et al. 1999). In sea bream erythrocytes, both *cat* and *gr* transcriptions were significantly down-regulated, but in the case of sea bass, *cat* was up-regulated by As and *gr* by most metals. In sea bass, erythrocyte exposure to Cr or Zn increased CAT activity while GR activity was decreased by Cr, Cu and Zn exposure (Roche and Bogé 1993). These opposite effects between sea bream and sea bass erythrocytes were not observed in freshly isolated head-kidney leucocytes (Morcillo et al. 2015a, b). Contradictory results were also found in the literature. For example, CAT activity was increased or decreased after Cd exposure of fish erythrocytes (Firat and Kargin 2010; Kumar et al. 2009) and GR activity was reduced after Pb exposure in human erythrocytes in vitro or in vivo (Hunaiti et al. 1995; Hunaiti and Soud 2000). In rat erythrocytes exposed to As, the CAT activity remained unchanged (Dwivedi and Flora 2015). It was previously reported that reduced nicotinamide adenine dinucleotide-hydrogen (NADH) plays an important role in the activation of CAT from its inactivated form (Das et al. 2010) and insufficient supply of NADH during arsenic metabolism might decrease the activity of CAT (Kirkman et al. 1987). Special attention should be focussed on the increase (>1000-fold) of the *gr* transcription after Hg exposure in sea bass erythrocytes. Similarly, European sea bass erythrocytes exposed to low $HgCl_2$ concentrations for 1 h resulted in increased SOD, CAT, peroxidase and glutathione peroxidase activities, but not in the case of human red blood cells, suggesting the presence of ROS and its partial elimination (Gwozdzinski et al. 1992). Similarly, in vivo exposure to sublethal dosages of Hg resulted in increased metabolism of glutathione and its associated enzymes in an effort to control the ROS production (Elia et al. 2003). In contrast, inorganic mercury nitrate added in vitro to stroma-free rat erythrocyte haemolysates resulted in a clear inhibition of GR activity (Mykkanen and Ganther 1974). Thus, our data suggest that the overexpression of *gr* could imply that glutathione is in reduced (GSH) state and is ready to be oxidized by glutathione peroxidase and scavenge more ROS. The scavenging of H_2O_2 and peroxinitrites by Prx1 and Prx2 has been also demonstrated in erythrocytes (Dubuisson et al. 2004; Lee et al. 2003; Low et al. 2008; Neumann et al. 2003). In our study, *prx1* gene expression was intensely up-regulated by metals in sea bream erythrocytes; however, only Cd and As exposure induced *prx1* transcription in sea bass erythrocytes while Hg exposure down-regulated it. In another study, Cd, Hg forms and Pb exposure elicited the increase of *prx1* gene expression in the sea bream SAF-1 cell line but As decreased it (Morcillo et al. 2016). The antioxidant mechanisms (*cat* and *gr* gene expression) in sea bream erythrocytes were more inhibited than in sea bass erythrocytes, and this could explain higher sensitivity of sea bream erythrocytes to metal exposure.

As in mammals, metallothioneins and heat shock proteins are relevant in the cellular protection of fish erythrocytes (Currie and Tufts 1997; Ferencz and Hermesz 2015). Present results show an increase of the *mta* gene expression after Cd and Hg forms while Pb or As exposure did not alter it in sea bream or sea bass erythrocytes. The finding that Cd and Hg are transported in the blood bound to MTA cysteine residues (Goyer and Clarkson 1996; Zalups 2000) could partly explain our observations. In contrast, after 1- or 2-h exposure to sublethal concentrations of Cd and Pb in silver sea bream (*Sparus sarba*) in vitro, no overexpression of MTA was evidenced likely due to the short time exposure (Fulladosa et al. 2006). In common carp specimens exposed to Cd, the *mt* transcription was related with low adverse effects in the blood compared to those observed in skin (Ferencz and Hermesz 2015). In the case of *hsp* gene expression, mRNA coding for stress

proteins is actively produced in red blood cells of the brook trout (*Salvelinus fontinalis*) summited to a heat shock (Lund et al. 2003) while we found differences in the gene expression and fish species. This result suggests a possible influence of the metal concentration and exposure time in the stress protein expression. Fulladosa et al. (2006) found that the maximal overexpression of HSP70 occurred after 3-h exposure to 20 μM Cd but also that prolonged exposure reduced it. In addition, they showed that increasing concentrations of Cd failed to further increase the HSP70 overexpression, while in the case of Cr and Pb, this was reduced. Therefore, the relation between ROS production and the oxidative stress mechanisms and cell protection deserves further investigation in fish erythrocytes.

Finally, it is widely accepted that overproduction of ROS induced by metals provokes apoptotic cell death (Rana 2008). In the case of human erythrocytes, these ROS provoke a suicidal erythrocyte death named eryptosis (a type of apoptosis that takes place in erythrocytes) (Föller et al. 2008; Kempe et al. 2005; Lang and Lang 2015; Shin et al. 2007) that may involve stimulation of two proteases that play an essential role in apoptosis (caspases and calpain), with subsequent degradation of the cytoskeleton, but how erythrocyte cell death is regulated is still under debate. Walsh et al. (2002) have demonstrated the role of BAK and BCL2 proteins in the human erythrocyte survival in vitro, but no studies are found in fish erythrocytes in this respect. Fish erythrocytes possess nuclei and mitochondria, both absent in mature mammalian erythrocytes, and the last one are major players in the apoptosis cell death (Moyes et al. 2002). Thus, pro-apoptotic *bax* and μ-calpain (*calp1*) gene expression was assessed in sea bream and sea bass erythrocytes. As expected, a significant up-regulation of *bax* transcription after exposure to Hg, Pb and As in erythrocytes from both species suggested that these metals induced apoptosis cell death, which resulted positive to PI uptake and haemoglobin release after 24 h. However, no differences were observed after Cd or MeHg exposure in *bax* mRNA levels, so that this metals could not trigger apoptosis, or alter other genes involved in the regulation of apoptosis in erythrocytes from both species. For example, Cd and MeHg can provoke necrosis or necroptosis cell death in fish cell lines or leucocytes (Kim and Sharma 2004; Krumschnabel et al. 2005; Morcillo et al. 2015a; b; Rana 2008; Selvaraj et al. 2013). In fact, excessive oxidative stress can induce necrosis (Çimen 2008; Hong et al. 2009) and the conversion of apoptosis to necrosis in cultured cells (Higuchi and Yoshimoto 2002). Transcription of *calp1* was strongly down-regulated after metal exposure in sea bream erythrocytes in contrast to sea bass erythrocytes. A possible reason could be the fact that as a result of an oxidative stress situation, reduced glutathione (GSH) is depleted, which triggers activation of Ca^{2+}-permeable cation channels, provoking Ca^{2+} influx and activation of μ-calpain (Lang et al. 2006; Quintanar-Escorza et al. 2010). Some of the main molecules involved in the eryptosis process demonstrated in the present study are summarized in Fig. 5. In the case of sea bream erythrocytes, *gr* is down-regulated after metal exposure; thus, no GHS depletion and Ca^{2+} entry occurs, triggering an inactivation of μ-calpain, which is in accordance with the down-regulation of *calp1* in our study. Alternatively, an up-regulation of *gr* in sea bass erythrocytes could deplete GHS leading to the activation of *calp1*, at least at the gene level, which is in agreement with our results.

To conclude, the results of this study show that gilthead sea bream and European sea bass erythrocytes exposed in vitro for 24 h to Cd, MeHg, Hg, Pb and As suffered important toxicological effects. The cytotoxicity was in the order Hg > MeHg > Cd > As > Pb. In general, erythrocytes from both species exposed to either EC_0 or EC_{50} metals modulated oxidative mechanisms and cell protection and died by apoptosis. Furthermore, it is verified that the use of fish erythrocytes appears to be a useful tool to evaluate the toxicological impact of aquatic pollutants and should be further explored, as well as to study of the mechanisms affected by different contaminants.

Acknowledgments Financial support by grants AGL2011-30381-C03-01 and AGL2013-43588-P (*Ministerio de Economía y Competitividad* and FEDER) and 04538/GERM/06 (*Fundación Séneca de la Región de Murcia*, Spain) is gratefully acknowledged.

References

Bagdonas E, Vosylienė MZ (2006) A study of toxicity and genotoxicity of copper, zinc and their mixture to rainbow trout (*Oncorhynchus mykiss*). Biol 1:8–13

Binelli A, Cogni D, Parolini M, Riva C, Provini A (2009) Cytotoxic and genotoxic effects of in vitro exposure to triclosan and trimethoprim on zebra mussel (*Dreissena polymorpha*) hemocytes. Comp Biochem Physiol C 150:50–56

Bogé G, Roche H (1996) Cytotoxicity of phenolic compounds on *Dicentrarchus labrax* erythrocytes. Bull Environ Contam Toxicol 57:171–178

Bols NC, Brubacher JL, Ganassin RC, Lee LEJ (2001) Ecotoxicology and innate immunity in immunity in fish. Dev Comp Immunol 25: 853–873

Borenfreund E, Puerner JA (1985) Toxicity determined in vitro by morphological alterations and neutral red absorption. Toxicol Lett 24: 119–124

Bourdineaud JP, Baudrimont M, González P, Moreau JL (2006) Challenging the model for induction of metallothionein gene expression. Biochimie 88:1787–1792

Carbonell G, Martínez-Pereda J, Tarazona J (1998) Mobilization of essential metals during and after short-term lethal cadmium exposure in rainbow trout (*Oncorhynchus mykiss*). Ecotoxicol Environ Restor 1:85–91

Çimen MYB (2008) Free radical metabolism in human erythrocytes. Clin Chim Acta 390:1–11

Currie S, Tufts BL (1997) Synthesis of stress protein 70 (Hsp70) in rainbow trout *Oncorhynchus mykiss* red blood cells. J Exp Biol 200:607–614

Das AK, Bag S, Sahu R, Dua TK, Sinha MK, Gangopadhyay M, Zaman K, Dewanjee S (2010) Protective effect of *Corchorus olitorius* leaves on sodium arsenite-induced toxicity in experimental rats. Food Chem Toxicol 48:326–335

Dayeh VR, Lynn DH, Bols NC (2005) Cytotoxicity of metals common in mining effluent to rainbow trout cell lines and to the ciliated protozoan, *Tetrahymena thermophila*. Toxicol In Vitro 19:399–410

De Kretser AJ, Waldron HA (1963) The mechanical fragility of the red cell in patients with lead poisoning. Br J Ind Med 20:31–69

Di Giulio RT, Hinton DE (2008) The toxicology of fishes. CRC Press, Boca Raton

Dubuisson M, Vander-Stricht D, Clippe A, Etienne F, Nauser T, Kissner R, Koppenol WH, Rees JF, Knoops B (2004) Human peroxiredoxin 5 is a peroxynitrite reductase. FEBS Lett 571:161–165

Dumaswala UJ, Zhuo L, Jacobsen DW, Jain SK, Sukalski KA (1999) Protein and lipid oxidation of banked human erythrocytes: role of glutathione. Free Radic Biol Med 27:1041–1049

Dwivedi N, Flora SJS (2015) Sub-chronic exposure to arsenic and dichlorvos on erythrocyte antioxidant defense systems and lipid peroxidation in rats. J Environ Biol 36:383–391

Eisele K, Lang PA, Kempe DS, Klarl BA, Niemöller O, Wieder T, Huber SM, Duranton C, Lang F (2006) Stimulation of erythrocyte phosphatidylserine exposure by mercury ions. Toxicol Appl Pharmacol 210:116–122

Elia AC, Galarini R, Taticchi MI, Dorr AJM, Mantilacci L (2003) Antioxidant responses and bioaccumulation in *Ictalurus meias* under mercury exposure. Ecotoxicol Environ Saf 55:162–167

Fedeli D, Carloni M, Falcioni G (2010) Oxidative damage in trout erythrocyte in response to "in vitro" copper exposure. Mar Environ Res 69:172–177

Ferencz Á, Hermesz E (2015) Impact of acute Cd^{2+} exposure on the antioxidant defence systems in the skin and red blood cells of common carp (*Cyprinus carpio*). Environ Sci Pollut Res Int 22:6912–6919

Firat O, Kargin F (2010) Effects of zinc and cadmium on erythrocyte antioxidant systems of a freshwater fish *Oreochromis niloticus*. J Biochem Mol Toxicol 24:223–229

Föller M, Kasinathan RS, Koka S, Lang C, Shumilina E, Birnbaumer L, Lang F, Huber SM (2008) TRPC6 contributes to the Ca(2+) leak of human erythrocytes. Cell Physiol Biochem 21:183–192

Fulladosa E, Deane E, Ng AH, Woo NY, Murat JC, Villaescusa I (2006) Stress proteins induced by exposure to sublethal levels of heavy metals in sea bream (*Sparus sarba*) blood cells. Toxicol In Vitro 20:96–100

Giulivi C, Davies KJA (2001) Mechanism of the formation and proteolytic release of H2O2-induced dityrosine and tyrosine oxidation products in hemoglobin and red blood cells. J Biol Chem 276: 24129–24136

Goyer RA, Clarkson TW (1996) Toxic effects of metals. In: Klaassen, CD (ed) Casarett & Doull's toxicology: the basic science of poisons, 5th edn. New York, pp. 691–736

Gwozdzinski K, Roche H, Peres G (1992) The comparison of the effects of heavy metal ions on the antioxidant enzyme-activities in human and fish *Dicentrarchus labrax* erythrocytes. Comp Biochem Phsyiol 102:57–60

Hernández-García A, Romero D, Gómez-Ramírez P, María-Mojica P, Martínez-López E, García-Fernández AJ (2014) In vitro evaluation of cell death induced by cadmium, lead and their binary mixtures on erythrocytes of Common buzzard (*Buteo buteo*). Toxicol In Vitro 28:300–306

Higuchi Y, Yoshimoto T (2002) Arachidonic acid converts the glutathione depletion-induced apoptosis to necrosis by promoting lipid peroxidation and reducing caspase-3 activity in rat glioma cells. Arch Biochem Biophys 400:133–140

Hong JY, Lebofsky M, Farhood A, Jaeschke H (2009) Oxidant stress-induced liver injury in vivo: role of apoptosis, oncotic necrosis, and c-Jun NH2-terminal kinase activation. Am J Physiol Gastrointest Liver Physiol 296:572–581

Hunaiti AA, Soud M (2000) Effect of lead concentration on the level of glutathione, glutathione S-transferase, reductase and peroxidase in human blood. Sci Total Environ 248:45–50

Hunaiti A, Soud M, Khalil A (1995) Lead concentration and the level of glutathione, glutathione S-transferase, reductase and peroxidase in the blood of some occupational workers from Irbid City, Jordan. Sci Total Environ 170:95–100

Jan M, Frantisek N (2000) Cadmium-induced changes in cation-osmotic haemolysis in rats. Environ Toxicol Pharmacol 8:79–81

Kempe DS, Lang PA, Eisele K, Klarl BA, Wieder T, Huber SM, Duranton C, Lang F (2005) Stimulation of erythrocyte phosphatidylserine exposure by lead ions. Cell Physiol Biochem 18:151–154

Kim SH, Sharma RP (2004) Mercury-induced apoptosis and necrosis in murine macrophages: role of calcium-induced reactive oxygen species and p38 mitogen-activated protein kinase signaling. Toxicol Appl Pharmacol 196:47–57

Kirkman HN, Galiano S, Gaetani GF (1987) The function of catalase-bound NADPH. J Biol Chem 262:660–666

Krumschnabel G, Manzl C, Berger C, Hofer B (2005) Oxidative stress, mitochondrial permeability transition, and cell death in Cu-exposed trout hepatocytes. Toxicol Appl Pharmacol 209:62–73

Kumar P, Prasad Y, Patra AK, Ranjan R, Swarup D, Patra RC, Pal S (2009) Ascorbic acid, garlic extract and taurine alleviate cadmium-induced oxidative stress in freshwater catfish (*Clarias batrachus*). Sci Total Environ 407:5024–5030

Lang E, Lang F (2015) Triggers, inhibitors, mechanisms, and significance of eryptosis: the suicidal erythrocyte death. Biomed Res Int 2015:1–16

Lang KS, Lang PA, Bauer C, Duranton C, Wieder T, Huber SM, Lang F (2006) Mechanisms of suicidal erythrocyte death. Cell Physiol Biochem 8:1183–1192

Lang F, Lang E, Föller M (2012) Physiology and pathophysiology of eryptosis. Transfus Med Hemother 39:308–314

Lee TH, Kim SU, Yu SL, Kim SH, Park DS, Moon HB, Dho SH, Kwon KS, Kwon HJ, Han YH, Jeong S, Kang SW, Shin HS, Lee KK, Rhee SG, Yu DY (2003) Peroxiredoxin II is essential for sustaining life span of erythrocytes in mice. Blood 101:5033–5038

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) Method. Methods 25:402–408

Low FM, Hampton MB, Winterbourn CC (2008) Peroxiredoxin 2 and peroxide metabolism in the erythrocyte. Antioxid Redox Signal 10: 1621–1630

Lund S, Lund M, Tufts B (2003) Red blood cell Hsp70 mRNA and protein as bioindicators of temperature stress in the brook trout (*Salvelinus fontinalis*). Can J Fish Aquat Sci 60:460–470

Mahmud H, Föller M, Lang F (2009) Arsenic-induced suicidal erythrocyte death. Arch Toxicol 83:107–113

Maracine M, Segner H (1998) Cytotoxicity of metals in isolated fish cells: importance of the cellular glutathione status. Comp Biochem Physiol A 120:83–88

Martínez-López E, María-Mojica P, Martínez JE, Calvo JF, Romero D, García-Fernández AJ (2005) Cadmium in feathers of adults and blood of nestlings of three raptor species from a nonpolluted Mediterranean forest, southeastern Spain. Bull Environ Contam Toxicol 74:477–484

Mitchelmore CL, Chipman JK (1998) DNA strand breakage in aquatic organisms and the potential value of the comet assay in environmental monitoring. Mutat Res 399:135–147

Monteiro V, Cavalcante D, Vilela M, Sofia S, Martinez C (2011) In vivo and in vitro exposures for the evaluation of the genotoxic effects of lead on the Neotropical freshwater fish *Prochilodus lineatus*. Aquat Toxicol 104:291–298

Morcillo P, Cordero H, Meseguer J, Esteban MA, Cuesta A (2015a) Toxicological in vitro effects of heavy metals on gilthead seabream (*Sparus aurata* L.) head-kidney leucocytes. Toxicol In Vitro 30: 412–420

Morcillo P, Cordero H, Meseguer J, Esteban MA, Cuesta A (2015b) In vitro immunotoxicological effects of heavy metals on European sea bass (*Dicentrarchus labrax* L.) head-kidney leucocytes. Fish Shellfish Immunol 47:245–254

Morcillo P, Esteban MA, Cuesta A (2016) Heavy metals produce toxicity, oxidative stress and apoptosis in the marine teleost fish SAF-1 cell line. Chemosphere 144:225–233

Morimoto RI (2011) The heat shock response: systems biology of proteotoxic stress in aging and disease. Cold Spring Harb Symp Quant Biol 76:91–99

Moyes CD, Sharma ML, Lyons C, Leary SC, Leon M, Petrie A, Lund SG, Tufts BL (2002) Origins and consequences of mitochondrial decline in nucleated erythrocytes. Biochim Biophys Acta 1591: 11–20

Mykkanen HM, Ganther HE (1974) Effect of mercury on erythrocyte glutathione reductase activity. In vivo and in vitro studies. Bull Environ Contam Toxicol 12:10–16

Neumann CA, Krause DS, Carman CV, Das S, Dubey DP, Abraham JL, Bronson RT, Fujiwara Y, Orkin SH, Van Etten RA (2003) Essential role for the peroxiredoxin Prdx1 in erythrocyte antioxidant defence and tumour suppression. Nature 424:561–565

Ormerod MG (1990) Analysis of DNA. General methods. In: Ormerod MG (ed) Flow cytometry: a practical approach. Oxford University, Oxford, pp 69–87

Quintanar-Escorza MA, González-Martínez MT, del Pilar IO, Calderón-Salinas JV (2010) Oxidative damage increases intracellular free calcium [Ca2+]i concentration in human erythrocytes incubated with lead. Toxicol In Vitro 24:1338–1346.

Rana SVS (2008) Metals and apoptosis: recent developments. J Trace Elem Med Biol 22:262–284

Roche H, Bogé G (1993) Effects of Cu, Zn and Cr salts on antioxidant enzyme activities in vitro of red blood cells. Toxicol In Vitro 7:623–629

Schlenk D, Handy R, Steinert S (2008) Biomarkers. In: Di Giulio RT, Hinton DE (eds) The toxicology of fishes. CRC Press, Boca Raton, pp 684–713

Scott MD, Eaton JW, Kuypers FA, Chiu DT, Lubin BH (1989) Enhancement of erythrocyte superoxide dismutase activity: effects on cellular oxidant defense. Blood 74:2542–2549

Segner H (1998) Fish cell lines as a tool in aquatic toxicology. In: Braunbeck T, Hinton DE, Streit B (eds)Fish Ecotoxicology. Birkhäuser Basel, volume 86, pp 1–38

Sekar D, Falcioni M (2014) DNA damage and repair following in vitro exposure to two different forms of titanium dioxide nanoparticles on trout erythrocyte. Environ Toxicol 29:117–127

Selvaraj V, Armistead MY, Cohenford M, Murray E (2013) Arsenic trioxide (As(2)O(3)) induces apoptosis and necrosis mediated cell death through mitochondrial membrane potential damage and elevated production of reactive oxygen species in PLHC-1 fish cell line. Chemosphere 90:1201–1209

Shannon L, Winski DC (1998) Arsenate toxicity in human erythrocytes: characterization of morphologic changes and determination of the mechanism of damage. J Toxicol Environ Health A 53:345–355

Shin JH, Lim KM, Noh JY, Bae ON, Chung SM, Lee MY, Chung JH (2007) Lead-induced procoagulant activation of erythrocytes through phosphatidylserine exposure may lead to thrombotic diseases. Chem Res Toxicol 20:38–43

Sopjani M, Föller M, Lang F (2008) Gold stimulates Ca2+ entry into and subsequent suicidal death of erythrocytes. Toxicology 244:271–279

Sweet LI, Zelikoff JT (2001) Toxicology and immunotoxicology of mercury: a comparative review in fish and humans. J Toxicol Environ Health B 4:161–205

Walsh M, Lutz RJ, Cotter TG, O'Connor R (2002) Erythrocyte survival is promoted by plasma and suppressed by a Bak-derived BH3 peptide that interacts with membrane-associated Bcl-XL. Blood 99:3439–3448

Witeska M (2004) The effect of toxic chemicals on blood cell morphology in fish. Fresen Environ Bull 13:1379–1384

Witeska M (2013) Erythrocytes in teleost fishes: a review. Zool Ecol 23: 275–281

Xiang LX, Shao JZ, Meng Z (2001) Apoptosis induction in fish cells under stress of six heavy metal ions. Prog Biochem Biophys 28: 866–886

Yang H, Rose NL (2003) Distribution of mercury in six lake sediment cores across the UK. Sci Total Environ 304:391–404

Zalups RK (2000) Molecular interactions with mercury in the kidney. Pharmacol Rev 52:113–143

Zikić RV, Stajn AS, Pavlović SZ, Ognjanović BI, Saićić ZS (2001) Activities of superoxide dismutase and catalase in erythrocytes and plasma transaminases of goldfish (*Carassius auratus gibelio* Bloch.) exposed to cadmium. Physiol Res 50:105–111