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Reactive oxygen species generation and expression of DNA repair-related genes after copper exposure in zebrafish (*Danio rerio*) ZFL cells

Juliana Zomer Sandrini, Adalto Bianchini, Gilma Santos Trindade, Luiz Eduardo Maia Nery, Luis Fernando Fernandes Marins*

Programa de Pós-Graduação em Ciências Fisiológicas - Fisiologia Animal Comparada, Instituto de Ciências Biológicas, Universidade Federal do Rio Grande, Av. Itália km 8, Campus Carreiros, 96200-970, Rio Grande, RS, Brazil

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ABSTRACT

Copper is an essential metal to aquatic animals, but it can be toxic when in elevated concentrations in water. The objective of the present study was to analyze copper effects in zebrafish hepatocytes (ZFL cell-line). The number of viable cells and copper accumulation were determined in hepatocytes exposed *in vitro* to different copper concentrations (5–30 mg Cu/L). Intracellular reactive oxygen species (ROS) formation, total antioxidant capacity against peroxyl radicals, and expression of genes related do DNA repair system were also measured in hepatocytes exposed to 5 and 20 mg Cu/L. After 24 h of exposure, hepatocytes showed an exponential kinetics of copper accumulation. Copper exposure (24 and 48 h) significantly reduced hepatocyte number in all concentrations tested, except at the lowest one (5 mg Cu/L). Exposure to 20 mg Cu/L for 6, 12 and 24 h significantly increased intracellular ROS formation. However, no significant change in total antioxidant capacity was observed. After 12 and 24 h of exposure to 20 mg Cu/L, a significant decrease in expression of *p53* and *CDKI* genes was observed. Conversely, expression of *Gadd45α*, *CyclinG1* and *Bax* genes was significantly induced after 24 h of exposure to 20 mg Cu/L. In hepatocytes exposed to 5 mg Cu/L, any significant alteration in expression of these genes was observed. In a broad view, most of genes encoding for DNA repair proteins were inhibited after copper exposure, especially in hepatocytes exposed to 20 mg Cu/L. Taken all together, results obtained suggest that the increased intracellular ROS formation induced by copper exposure would be responsible for the alteration in gene expression pattern observed.

1. Introduction

Sources of copper present in aquatic environments could be natural or anthropogenic, such as mine run-off or agricultural leaching. Copper is a transition metal having differential effects in aquatic animals, depending on its concentration in water. It is an essential micronutrient, being part of the structure of several enzymes and other proteins. However, it could be toxic at elevated concentrations (Griffitt et al., 2007; Paris-Palacios and Biangianti-Risbourg, 2006). On top of that, copper is a redox-active metal, existing in two major oxidation states, Cu^{1+} and Cu^{2+}. Redox cycling of copper between Cu^{1+} and Cu^{2+} catalyzes the intracellular production of hydroxyl radicals (Stohs and Bagchi, 1995; Verhaegh et al., 1997). In fact, several studies have attributed the toxic effects of copper, at least in part, to its ability in inducing reactive oxygen species (ROS) formation (Bopp et al., 2008; Pourahmad et al., 2003).

ROS generation induced by copper ions could lead to an oxidative stress situation, which in turns would cause oxidative damage to cellular macromolecules like proteins, lipids and DNA. Damage to DNA molecule might cause mutations and it is suggested that these kind of damage play a major role in the development of chronic diseases such as cancer (Powell et al., 2005). In turn, cells have evolved a complex mechanism, known as DNA repair system, to reduce the yield of mutations and chromosomal aberrations. This complex cellular system acts at three levels: (a) arresting the cell cycle to allow time for DNA repair; (b) triggering the signal transduction events to activate the repair components; and (c) directly reversing, excising or tolerating DNA damage via constitutive and induced activities (Begley and Samson, 2004). If DNA damage is not repaired, cells undergo complex enzymatic reactions that might lead to apoptosis, necrosis or other forms of cell death (Nyberg et al., 2002; Su, 2006). The tumor suppressor protein p53 has a central role in cellular stress responses. It elicits its normal functions mainly by acting as a transcription factor, regulating the transcription of genes involved in cell cycle arrest (e.g. *p21*, *Gadd45α*, *CyclinG1*), DNA repair (e.g. *XPC*, *DDB2*) and apoptosis (e.g. *Bcl-2*, *Bax*) (Ford, 2005; Kohn, 1999).

* Corresponding author.
E-mail address: lfmarins@pq.cnpq.br (L.F.F. Marins).

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Given that several different types of lesions could be produced in the DNA molecule, different pathways for DNA repair are known, each one being specific for a type of lesion (Hoeijmakers, 2001). Several components of the DNA repair system for these pathways are described in literature. In this context, the major DNA repair mechanisms are the following: (1) base excision repair (BER), involving proteins like Ogg1 and Apex1, (2) nucleotide excision repair (NER), involving proteins like XPC; (3) non-homologous end-joining (NHEJ), involving proteins like Ku80; (4) and homologous recombination (HR), involving proteins like Nbs1 and Rad51 (Hoeijmakers, 2001).

The zebrafish *Danio rerio* has been used as a model in several toxicological studies (Amanuma et al., 2000; Craig et al., 2007; Pomati et al., 2007; Seok et al., 2006). These studies have demonstrated that many physiological mechanisms between zebrafish and mammals are highly conserved. Recently, Hernández and Allende (2008) published a review on the use of zebrafish as a model for studies concerning copper metabolism in vertebrates. Regarding some components of the DNA repair system, Langheinrich et al. (2002) described the conservation of the Mdm2/p53/p21 system in zebrafish.

In light of the above, the main objective of the present study was to analyze the physiological effects of copper exposure on zebrafish hepatocytes (ZFL cell-line). Cells were exposed to different copper concentrations and the following endpoints were analyzed: number of viable cells, copper accumulation, ROS production, total antioxidant capacity, and expression of some genes related to DNA repair system.

2. Material and methods

2.1. Cell-line and culture conditions

Hepatocyte-like cells (lineage ZFL) from zebrafish were obtained from the American Type Culture Collection (ATCC® number CRL-2643™). Cells were grown at 28 °C in disposable plastic flasks containing RPMI 1640 (Gibco) medium supplemented with sodium bicarbonate (0.2 g/L), L-glutamine (0.3 g/L), Hepes (25 mM), β-mercaptoethanol (5×10^{-5} M), fetal bovine serum (10%; Gibco) and 1% of antibiotics and antimycotic (penicillin [100 U/mL], streptomycin [100 μg/mL], amphotericin B [0.25 μg/mL]; Gibco).

2.2. Copper exposure

Cells (10^6 cells per well) were transferred to 24-well culture plates and allowed to attach for 48 h at 28 °C. After this period, the culture medium was removed and the attached monolayer of cells was washed with Ca^{2+}- and Mg^{2+}-free phosphate buffer saline (PBS). Copper exposure was performed adding 1 mL of fresh culture medium (without β-mercaptoethanol) containing different copper concentrations (0, 5, 10, 20 and 30 mg Cu/L, as $CuCl_2$) in each well. The experimental medium was prepared at least 3 h prior to cell exposure to allow copper to equilibrate with the culture medium. During exposure, cells were kept at 28 °C in dark. After different times of exposure, the experimental medium was removed, the monolayer of cells was washed with fresh PBS, and trypsin–EDTA (1.25 g/L trypsin and 0.2 g/L EDTA) was added. After trypsinization, the same volume of culture medium was added. Number of viable cells was measured using the trypan blue exclusion assay, as previously described (Sandrini et al., 2009).

Copper concentration in experimental medium was measured using non-filtered and filtered (0.45 μm mesh filter) samples (10 mL) using an atomic absorption spectrophotometer (GBC, AAS 932 Avanta Plus, IL, USA), as previously described (Pinho et al., 2007).

2.3. Cell copper accumulation

ZFL cells were exposed (24 h) to different copper concentrations (0, 5, 10, 20 and 30 mg Cu/L, as $CuCl_2$), as described above. After trypsinization, cells (four groups of 6×10^6 cells per group) were centrifuged (2 min) at 1500 rpm. Pellet was resuspended in PBS containing 12 mM EDTA PBS and centrifuged. The new pellet was dried (60 °C) and completely digested in 50 μL of HNO_3 (Suprapur; Merck) for 24 h. Copper concentration in digested samples was measured by AAS, as described above. Results were expressed as ng Cu/10^6 cells.

2.4. Intracellular reactive oxygen species (ROS) formation

ZFL cells were exposed to three different copper concentrations (0, 5 and 20 mg Cu/L) for 6, 12 and 24 h. These concentrations were selected based on cell viability results obtained (see Section 3). After copper exposure, cells (eight groups of 10^6 cells per group) were trypsinized and washed two times with PBS, as described above. These procedures permit that the residual copper, which could interfere with the H_2DCF-DA assay, could be reduced. Cells were incubated at 28 °C (30 min) in PBS containing the fluorogenic compound 2′,7′-dichlorofluorescein diacetate (H_2DCF-DA; Molecular Probes) at a final concentration of 40 μM, as described by Viarengo et al. (1999). The acetate groups of H_2DCF-DA are cleaved by intracellular esterases and the non-fluorescent compound H_2DCF generated is oxidized by ROS present in samples to the fluorescent compound, DCF. After incubation with H_2DCF-DA, two cell washes with PBS were performed and cells were resuspended in 1 mL of fresh PBS. Aliquots of 160 μL of each sample (containing approximately 10^4 cells) were placed into a white 96-well microplate and fluorescence intensity (excitation = 485 nm; emission = 520 nm) was determined (fluorometer Victor 2, Perkin Elmer). Total fluorescence production was calculated by integration of fluorescence units (FU) over the measurement period, after adjusting FU data to a second order polynomial function. The results were normalized by the number of viable cells present in the aliquot. These normalized results were then divided by the values for the untreated control group to express enhanced ROS formation as "relative ROS production".

2.5. Measurement of total antioxidant capacity against reactive oxygen and nitrogen species (RONS)

ZFL cells were exposed (24 h) to three different copper concentrations (0, 5 and 20 mg Cu/L). Each treatment was composed of four samples (10^6 cells in each sample). After cell trypsinization and washing with PBS, as described above, cells were centrifuged and the resulting pellet was resuspended in 150 μL of PBS. Total protein content in samples was measured using the Quant-iT™ Protein Assay Kit (Invitrogen). The measurement of total antioxidant capacity was performed according to the method described by Amado et al. (2007). Samples (11 μg of proteins) were incubated with H_2DCF-DA at a final concentration of 40 μM, as described above (see Section 2.5). Incubations were performed, in either the presence or the absence of 2,2′-azobis(2methylpropionamidine)dihydrochloride (ABAP) at 20 μM. Peroxyl radicals were generated by thermal (35 °C) decomposition of ABAP. Total fluorescence production (RONS) was calculated by integration of fluorescence units (FU) over the measurement period, after adjusting FU data to a second order polynomial function. Total antioxidant capacity against peroxyl radicals (ACAP) was estimated as the difference in RONS area with and without ABAP, with low ACAP values indicating high antioxidant competence. These results were then normalized by the values for the untreated control group to express the total antioxidant capacity relative to control.

Table 1
Gene-specific primers used for RT-PCR analysis, NCBI accession numbers for sequences used in primer design and PCR specific conditions used for each pair of primers (for more detail see Section 2.6).

Gene	Mechanism	NCBI accession number	PCR specific conditions		Forward primer (5′–3′)	Reverse primer (5′–3′)
			Extension time (s)	Cycles		
EF1α	Translation	NM131263	30	21	ACATCAAGAAGATCGGCTACAAC	GACCCACAGGTACAGTTCCAATA
Apex1	DNA repair (BER)	BC055545	30	30	GAGTTTCCTGATTTCTTCCTGGT	CATGTAGGTCCAGAAGGTGTAGG
Bax	Apoptosis	BC055592	40	29	GAGCTGCACTTCTCAACAACTTT	CTGGTTGAAATAGCCTTGATGAC
Bcl-2	Apoptosis	BC133848	40	31	TTGTGGAGAAATACCTCAAGCAT	GAGTCTCTCTGCTGACCGTACAT
CyclinG1	Cell cycle arrest	BC052125	40	27	TCTCTCCTTGACTCGATTCTTTG	AATATTCAACCAGGCACTTAGCA
Gadd45α	Cell cycle arrest	NM001002216	30	35	TTGAAGAACCGTGTGGAGATAAC	TGTTCACTCGCAAGATATTGATG
Ku80	DNA repair (NHEJ)	AY877316	45	34	TGGAGGAGATTGAGAGAGAACTG	GTTTCATCATCATCGTTCAGACA
Nbs1	DNA repair (HR)	AY858826	60	29	GTCACCCTGTCTCAAGATATTCG	GAGGACATCACTGTTTCTTGGAC
Ogg1	DNA repair (BER)	XM001332667	30	36	ACAGAAAGGAAAAGTGGAAGAGG	TGAGAAAAGACACTCCACAGGAT
CDKI	Cell cycle arrest	CU062631	30	31	GATCCTACGTTCACTCGGTAATG	TTGGCTTGGTAGAAATCTGTGAT
p53	Cell cycle arrest	U60804	30	29	CTATAAGAAGTCCGAGCATGTGG	GGTTTTGGTCTCTTGGTCTTTCT
Rad51	DNA repair (HR)	BC062849	45	30	GATCCTGACAGAAGCTGCTAAAA	CACCTCTGCCTTTCCTAAGGTAT
XPC	DNA repair (NER)	NM00104521	30	33	TATGGAGACCAAAAAGAACCTCA	CAGGTTTCGACTCAAGTGCTAGT

2.6. Gene expression analysis

ZFL cells were exposed to three different copper concentrations (0, 5 and 20 mg Cu/L) as described above. Gene expression analysis was performed by semiquantitative reverse transcription–polymerase chain reaction (PCR), as described by Chelly et al. (1988). After 6, 12 and 24 h of copper exposure, cells from three different culture dishes were pooled and employed for total RNA isolation using TRIzol reagent (Invitrogen), following the manufacturer's recommendations. For each experimental group, three or four samples were used for total RNA isolation. The total RNA extracted was quantified using Qubit™ Quantitation Kit (Invitrogen). First-strand cDNA was synthesized from 1 μg of total RNA according to the protocol of the 3′-RACE system (Rapid Amplification of cDNA Ends; Invitrogen) using the adapter primer provided with the kit. First-strand cDNA was amplified by PCR using gene-specific primers (Table 1), which were designed from sequences available in the DDBJ/GenBankTM/EBI Data Bank, using the Primer3 online software (Rozen and Skaletsky, 2000). PCR was carried out employing the *Platinum* Taq DNA Polymerase (Invitrogen) according to the following protocol: initial denaturation at 94 °C for 2 min, followed by variable number of cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30–60 s (Table 1), then a final extension period of 10 min at 72 °C. The elongation factor 1α (*EF1α*) gene was used as an internal control for data normalization. The PCR products were photodocumented after electrophoresis on 1% agarose gels and analyzed employing the software *1DScan* (Scanalitica, USA). For each gene, the densitometry value calculated by the software was divided by the value for *EF1α* gene for each sample. These results were then divided by the values for the untreated control group to express relative gene expression as "fold induction".

2.7. Statistical analysis

All experiments were done at least in triplicate. Data were expressed as mean (±standard error) and subjected to analysis of variance (ANOVA) followed by the Tukey's test. ANOVA assumptions (normality and homogeneity of variances) were previously checked. The significance level adopted was 95% ($\alpha = 0.05$). The relative data obtained in the experiments of ROS production, total antioxidant capacity and gene expression were all analyzed after arcsin$\sqrt{}$ transformation.

3. Results

3.1. Copper concentrations in the experimental media and cell copper accumulation

Nominal copper concentrations used to perform experiments on ZFL cells were 0 (control), 5, 10, 20 and 30 mg Cu/L, as $CuCl_2$. Total measured copper concentration in the control medium was below the AAS detection limit (<0.01 mg Cu/L). Total measured copper concentrations in experimental media deviated 0.7–9.6% from the desired nominal concentrations. All virtually copper present in the experimental media was in the dissolved phase (Table 2).

ZFL cells significantly accumulated copper after 24 h of exposure (Table 2). As expected, this accumulation was dependent on copper concentration and followed an exponential kinetics ($R^2 = 0.976$).

3.2. Effects of copper exposure on viable cells number

The number of viable cells in the groups exposed to copper for 24 and 48 h significantly reduced when compared to that of ZFL cells kept under control conditions, i.e. non-copper addition to the culture medium. Copper effect on the number of viable cells was concentration- and time-dependent. All copper concentrations tested significantly reduced the number of viable cells, except the lowest one, i.e. 5 mg Cu/L (Fig. 1). Based on these results, two copper concentrations were selected for the experiments on ROS formation, total antioxidant capacity, and gene expression. Concentrations selected were 5 and 20 mg Cu/L. The first was selected for not inducing any significant decrease in the number of cells. The second one was selected because it was the lowest concentration

Table 2
Copper concentrations (mg Cu/L) in experimental media and copper accumulation (ng Cu/10^6 cells) in zebrafish ZFL cells exposed to different metal concentrations.

Nominal copper	Total measured copper	Dissolved copper	Copper accumulation in ZFL cells
0	<0.01	<0.01	0.52 ± 0.15^{a}
5	4.549 ± 0.43	4.369 ± 0.156	$0.85 \pm 0.10^{a,b}$
10	9.035 ± 1.61	9.323 ± 1.03	1.64 ± 0.25^{b}
20	19.850 ± 0.96	20.023 ± 0.89	5.62 ± 0.16^{c}
30	27.942 ± 0.42	29.513 ± 0.36	12.39 ± 1.51^{d}

Data are expressed as mean ± SE ($n = 3–8$). Equal letters indicate absence of significant difference ($p > 0.05$) between mean values.

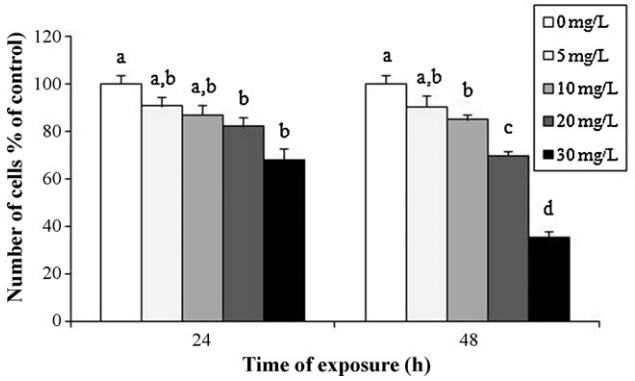

Fig. 1. Number of viable cells after 24 and 48 h of exposure to different copper concentrations (0, 5, 10, 20, 30 mg Cu/L) using the Trypan blue staining. Values are expressed as percentage of viable cells in the control treatment (no addition of copper in the experimental medium). Data are expressed as mean ± SE (n = 3–8). Equal letters indicate absence of significant difference (p > 0.05) between mean values at the same exposure time.

tested that induced a significant effect on cell viability after 24 h of exposure.

3.3. ROS formation and total antioxidant capacity

Copper effect on intracellular ROS generation was dependent on the concentration tested (Fig. 2). When compared to the control cells, those exposed for 6 h to 5 mg Cu/L showed significantly lower ROS formation (Fig. 2a). Conversely, those exposed for 6, 12 and 24 h to 20 mg Cu/L showed increased levels of ROS generation (Fig. 2b). No significant change in total antioxidant capacity against RONS was observed in cells exposed for 24 h to 5 or 20 mg Cu/L (Fig. 3).

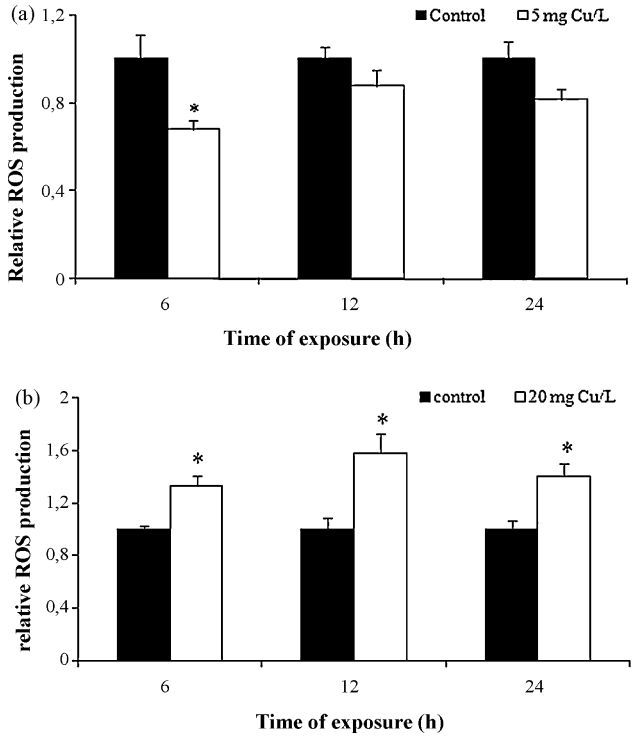

Fig. 2. Intracellular reactive oxygen species (ROS) formation in zebrafish ZFL cells exposed to 5 mg Cu/L (a) and 20 mg Cu/L (b). Values are relative to the control group and expressed as mean ± SE (n = 8). Significant difference (p < 0.05) between control and copper exposed group at the same time of exposure is indicated by an asterisk.

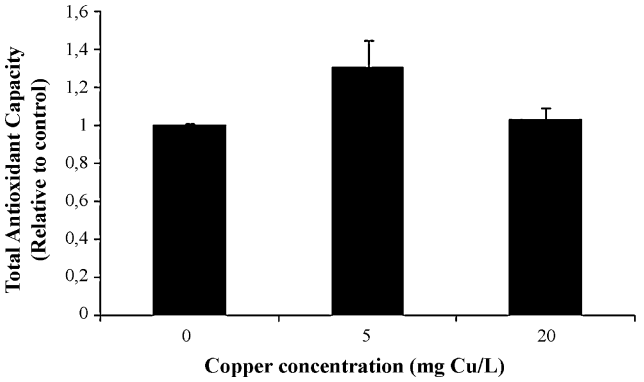

Fig. 3. Total antioxidant capacity against peroxyl radicals in zebrafish ZFL cells exposed to 5 and 20 mg Cu/L. Values are relative to control group and expressed as mean ± SE (n = 4). No significant difference (p > 0.05) was observed between treatments.

3.4. Gene expression

Genes analyzed in the present study are related to DNA repair system and could be grouped according to their function: cell cycle arrest (*p53*, *CDKI*, *Gadd45α* and *CyclinG1*; Fig. 4), apoptosis (*Bcl-2* and *Bax*, Fig. 5) and DNA repair (*Apex1*, *Ogg1*, *Rad51*, *Nbs1*, *XPC* and *Ku80*; Fig. 6).

In a broad view, copper effects on gene expression were more evident in ZFL cells exposed to 20 mg Cu/L. A significantly lower *p53* and *CDKI* (cyclin-dependent kinase inhibitor p21-like) expression

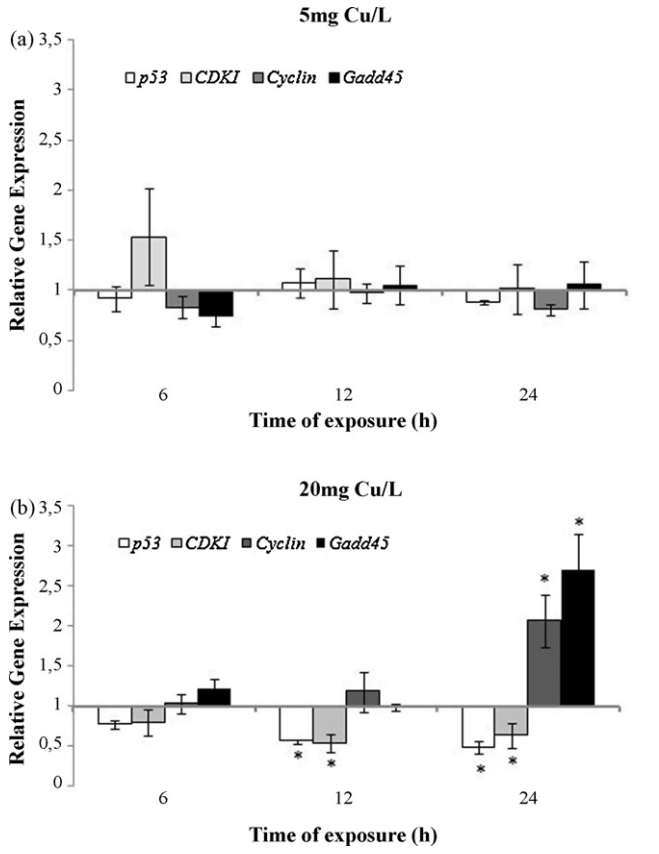

Fig. 4. Expression of genes involved in cell cycle arrest (*p53*, *CDKI*, *Gadd45α* and *CyclinG1*) in zebrafish ZFL cells exposed to 5 mg Cu/L (a) and 20 mg Cu/L (b). Values are relative to control group and expressed as mean ± SE (n = 4). Significant difference (p < 0.05) between control and cells exposed for the same time to copper is indicated by an asterisk.

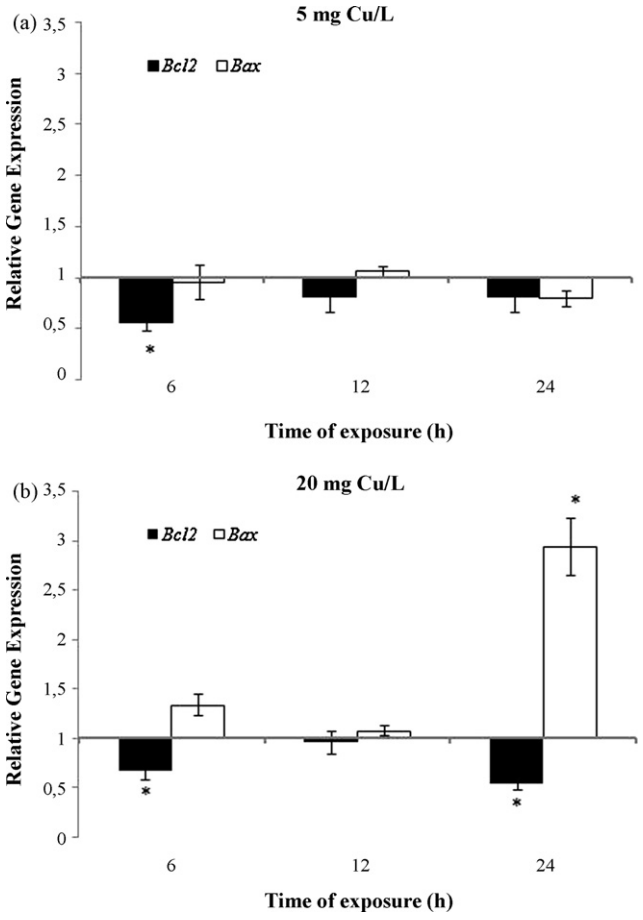

Fig. 5. Expression of genes involved in cell apoptosis (*Bcl-2* and *Bax*) in zebrafish ZFL cells exposed to 5 mg Cu/L (a) and 20 mg Cu/L (b). Values are relative to control group and expressed as mean ± SE ($n = 4$). Significant difference ($p < 0.05$) between control and cells exposed for the same time to copper is indicated by an asterisk.

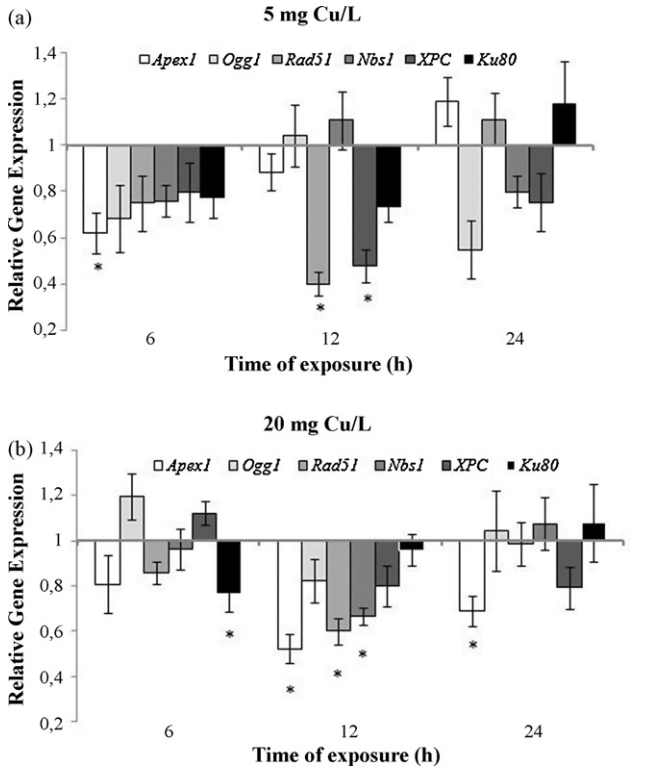

Fig. 6. Expression of genes involved in DNA repair (*Rad51*, *XPC*, *Ku80*, *Nbs1*, *Apex1* and *Ogg1*) in zebrafish ZFL cells exposed to 5 mg Cu/L (a) and 20 mg Cu/L (b). Values are relative to control group and expressed as mean ± SE ($n = 4$). Significant difference ($p < 0.05$) between control and cells exposed for the same time to copper is indicated by an asterisk.

was observed in cells exposed to copper for 12 and 24 h than in control ones (Fig. 4b). Moreover, cells exposed to 20 mg Cu/L showed lower levels of *Apex1*, *Rad51*, *Ku80*, *Nbs1* and *Bcl-2* expression after different times of copper exposure (Fig. 5b and Fig. 6b). *Gadd45α*, *CyclinG1* and *Bax* expressions were increased after 24 h of exposure to 20 mg Cu/L (Figs. 4 and 5b).

In ZFL cells exposed to 5 mg Cu/L, expression of genes related to cell cycle arrest was not significantly altered by copper exposure (Fig. 4a). However, a significant decrease in *Apex1*, *Rad51*, *XPC* and *Bcl-2* expression was observed when compared to that measured in control cells (Fig. 5a and Fig. 6a).

4. Discussion

In the present study, copper effects on zebrafish ZFL cells were investigated. Several studies have reported the effects of metal exposure in zebrafish (Craig et al., 2007; Gonzalez et al., 2006; Griffitt et al., 2007; Paris-Palacios and Biangianti-Risbourg, 2006). Regarding copper, little is known about its effects on the expression of genes related to DNA repair system. In the present study, copper is shown to accumulate in zebrafish cells, inducing an increased intracellular ROS formation without any significant change in total antioxidant capacity. Furthermore, copper changed the expression level of several genes involved in DNA repair system. These effects resulted in a reduced number of viable cells.

The use of *in vitro* systems in toxicological studies is rapidly growing in the recent years. ZFL cell-line was isolated in 1992 and was already used in toxicological studies (Miranda et al., 1993; Seok et al., 2007). Cheuk et al. (2008) and Chan et al. (2006) reported data describing the effects of ZFL cell-line exposure to metals, including copper. According to Chan et al. (2006), the lethal copper concentration for 50% of cells exposed for 24 h (24 h LC50) is 308.1 μM, which corresponds to 19.6 mg Cu/L. This copper concentration is within the range used in the present study. According to these authors, ZFL cell-line is much more resistant to copper than the zebrafish embryo-larvae (24 h LC50 = 5.16 μM = 0.33 mg Cu/L). It is important to note that the cell-line used in the present study has a hepatic origin, being liver known as an important site of metal detoxification. The presence of a well-developed detoxifying system in this organ could explain the high tolerance of ZFL cells to copper. According to Cheuk et al. (2008), ZFL cell-line possesses a higher tolerance to several metals when compared to SJD cells (ATCC® number CRL-2296), a cell-line derived from zebrafish caudal fin. However, Dayeh et al. (2005) using two different rainbow trout cell-lines (derived from gill and liver) did not show differences in copper sensitivity between these two trout cell lineages. According to this study, the media used for the exposure has a great influence on copper toxicity, since many of its constituents could bind metals, reducing their availability, and some components could act as antioxidants, like the amino acid cysteine.

Toxic effects of copper are generally attributed to its high affinity for thiol groups and to its capacity in participating in redox reactions to form reactive oxygen species. In fact, several authors reported an increased ROS formation after copper exposure in different aquatic species (Ferreira-Cravo et al., 2008; Pourahmad et al., 2003). In the present study, exposure for 6, 12 and 24 h to 20 mg Cu/L significantly increased intracellular ROS generation in ZFL cells. Despite this increase in ROS formation, copper exposure did not significantly alter the total antioxidant capacity of cells against peroxyl radicals. This was not expected since copper is known to reduce the

intracellular levels of glutathione (Canesi et al., 1999). This result could be explained by a possible induction of some components of the antioxidant defense system. This statement is based on the fact that exposure for 24 h to 20 mg Cu/L induced a higher expression of some genes related to the antioxidant defense system, including the glutathione synthesizing enzymes, in ZFL cell-line (data not published, manuscript in preparation).

The increased intracellular ROS formation without a concomitant increase in antioxidant capacity could be leading to an oxidative stress situation in ZFL cells exposed to 20 mg Cu/L, which could generate DNA damage. In this context, results from the present study show significant effects of copper exposure on the expression of some genes related to the DNA repair system. Several studies reported an induced expression of these genes after exposure to different genotoxic compounds (Gonzalez et al., 2005; Green et al., 2007; Weber et al., 2007; Zegura et al., 2008). However, in the present study some unexpected results were found. For example, expressions of *p53* and *CDKI* genes were reduced after 12 and 24 h exposure to 20 mg Cu/L. This finding seems to be contradictory with the fact that the expressions of these two genes were induced after ZFL cell-line exposure to UVB exposure (Sandrini et al., 2009). However, some authors described the inactivation of p53 protein after metal exposure (Méplan et al., 2000; Tassabehji et al., 2005). According to Hainaut and Milner (1993) redox conditions in cells influence the conformational folding of p53 protein, through oxidation/reduction of specific thiol groups in the DNA-binding domain of this protein. Moreover, Verhaegh et al. (1997) described an inhibition of p53 protein through direct interaction with copper. Therefore, the decrease in expression of *CDKI* and *p53* genes observed in the present study could be explained by a possible inhibition of p53 protein after copper exposure. This could be possible if we consider that *p53* and *CDKI* genes are both regulated by p53 protein. Actually, p53 DNA-binding sites were shown to be localized in the promoter region of both *p53* and *p21* genes in humans (Halazonetis and Bartek, 2006; Wang and El-Deiry, 2006).

In the present study, expression of genes like *Gadd45α*, *CyclinG1* and *Bax* was increased after 24 h of exposure to 20 mg Cu/L. These results seem to argue against a possible p53 inactivation given that these genes are known targets of p53 (Grace and Blakely, 2007; Hollander et al., 1993; Kimura et al., 2001). However, some evidences have emerged in the literature indicating a possible activation of these genes through p53-independent pathways (Hartman and Ford, 2003; Zhao et al., 2003).

In the present study, it was observed an increased *Bax* expression and a decreased *Bcl-2* expression after copper exposure. These results could indicate a possible increase in the apoptosis pathway. According to a review paper by Roos and Kaina (2006), there are some pathways for apoptosis activation that are p53 independent. According to these authors, they could involve NF-κB and the p53 homologs p63 and p73. The p53 homolog p73 is known to activate some p53 target genes, like *Bax*. In this context, results obtained in the present study open a field of investigation concerning the possible activation of these genes independently of p53 in zebrafish.

According to Powell et al. (2005), the expression of genes involved in base excision repair, like *Ogg1* and *Apex1*, is sensitive *in vivo* biomarkers for oxidative DNA damage. Several studies have reported increased levels of some genes involved in DNA repair system after exposure to different genotoxic stress in zebrafish (Gonzalez et al., 2005; Pomati et al., 2007). When ZFL cells were exposed to UVB (23.3 mJ/cm^2), which could cause direct DNA damage, expression of many DNA repair genes was induced up to 24 h of exposure (Sandrini et al., 2009). Contrary to this, in the present study the copper treatment decreased the expression of many DNA repair genes. These differential gene responses observed in the two studies could be due to different mechanisms involved in DNA damage by UVB and copper. UV-B is known to act mostly through direct interaction with the DNA molecule, leading to the formation of photoproducts that could cause distortion in double helix structure (Cadet et al., 2005; Häder and Sinha, 2005). On the other hand, copper acts mainly through generation of ROS, which in turn could damage cellular macromolecules like DNA (Stohs and Bagchi, 1995; Verhaegh et al., 1997). Therefore, copper is not directly inducing DNA damage as UVB. Thus, it is possible that copper takes longer to induce DNA damage than UVB. In the present study, the expression of genes related to DNA repair system was analyzed up to 24 h of copper exposure. It could be possible that this exposure time was not long enough to generate DNA damage levels capable of inducing expression of DNA repair-related genes, as observed after UVB exposure (Sandrini et al., 2009). In this context, it was possible that ROS generation induced by copper exposure had affected mainly cytosolic macromolecules like proteins and lipids. This idea is consistent with the fact that nuclear DNA seems to be less susceptible to oxidative modifications than lipids and proteins (Trachootham et al., 2008).

Taking all results together, the major copper effects were already observed in ZFL cells exposed to the highest metal concentration (20 mg Cu/L) after only 24 h of exposure. On the other hand, the lowest copper concentration tested (5 mg Cu/L) only induced slight changes in few endpoints analyzed, without any significant impact on cell viability, even after 48 h of exposure. However, longer periods of exposure to 5 mg Cu/L could probably also induce significant changes in the endpoints analyzed. Further studies are necessary to verify this hypothesis.

In conclusion, results reported in the present study show some important copper effects in ZFL cell-line, mainly in cells exposed to high copper concentration. Copper exposure induced an increased intracellular ROS generation without influence on the total cellular antioxidant capacity. Regarding gene expression, data obtained suggest a possible p53 inactivation due to a direct copper interaction with the protein or an indirect effect through ROS oxidation of the thiol residues, which could explain the reduction in *p53* and *CDKI* gene expression. The data also suggest a possible involvement of others pathways independent of p53 for activation of genes like *Gadd45α*, *CyclinG1* and *Bax*. Finally, copper exposure did not induce expression of DNA repair genes, which could indicate that copper toxicity would be related to damage to biomolecules other than DNA. In fact, our findings point to interesting questions on the mechanisms activated by copper exposure in ZFL cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2009.02.016.

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