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Effects of arsenic (As) exposure on the antioxidant status of gills of the zebrafish *Danio rerio* (Cyprinidae)

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ABSTRACT

In fishes, arsenic (As) is absorbed via the gills and is capable of causing disturbance to the antioxidant system. The objective of present study was to evaluate antioxidant responses after As exposure in gills of zebrafish (*Danio rerio*, Cyprinidae). Fish were exposed for 48 h to three concentration of As, including the highest As concentration allowed by current Brazilian legislation (10 μg As/L). A control group was exposed to tap water (pH 8.0; 26 °C; 7.20 mg O_2/L). As exposure resulted in (1) an increase ($p<0.05$) of glutathione (GSH) levels after exposure to 10 and 100 μg As/L, (2) an increase of the glutamate cysteine ligase (GCL) activity in the same concentrations ($p<0.05$), (3) no significant differences in terms of glutathione reductase, glutathione-*S*-transferase and catalase activities; (4) a significantly lower ($p<0.05$) oxygen consumption after exposure to 100 μg As/L; (4) no differences in terms of oxygen reactive species generation and lipid peroxidation content ($p>0,05$). In the gills, only inorganic As was detected. Overall, it can be concluded that As affected the antioxidant responses increasing GCL activity and GSH levels, even at concentration considered safe by Brazilian legislation.

1. Introduction

Arsenic (As) is a pollutant widely distributed in nature and released into the environment through industrial processes and agricultural practices (Nandi et al., 2005). Different countries have established various As concentration limits for the protection of aquatic life (Mirlean et al., 2003). Brazilian legislation established that 10 μg/L of As is the maximum concentration considered to be safe for aquatic fauna (Conselho Nacional do Meio Ambiente, resolution 357, 17/03/2005; www.mma.gov.br/port/conama/res/res05/res35705.pdf). Arsenic can be found in both organic and inorganic compounds with variable oxidation states. In general, for inorganic forms, arsenite (As^{III}) is considered more toxic than arsenate (As^{V}) (Akter et al., 2005), while organic form seem to be less toxic (Kobayashi et al., 2005). In water, As is normally found in the pentavalent form (Caussy, 2003), but upon consumption by humans and most mammalian species its metabolic conversion includes reduction of arsenate to arsenite and ultimately methylated As species (Thomas et al., 2001). Methylated metabolites such as monomethylarsonic acid (MMA) and dimethylarsenic (DMA) are considered moderately toxic compared to inorganic As (iAs) and because of this, methylation of As appears to be primarily a detoxification mechanism (Thomas et al., 2004). However, recent studies suggest that As biomethylation is not always a detoxification event because methylated metabolites, such as DMA, can retain their toxicity and carcinogenic potential *in vitro* and *in vivo* (Sakurai et al., 2005).

Exposure to iAs can affect enzyme activities such as pyruvate dehydrogenase and α-ketoglutarate dehydrogenase (Ramanathan et al., 2003), cell signaling and DNA repair (Kitchin, 2001) and induce reactive oxygen species (ROS) generation in mammalians cells after very short times of exposure (Schuliga et al., 2002). To protect cells against some oxidative damage by ROS, a detoxification battery has evolved in aerobic organisms (Shila et al., 2005). This antioxidant defense system includes the tripeptide glutathione. Glutathione (GSH) and enzymes related to GSH comprise a system that maintains a reduced intracellular environment and acts as a primary defense against excessive generation of harmful ROS (Schuliga et al., 2002). Sakurai et al. (2005) showed that when cells (TRL 1215) were exposed to monomethylarsenic acid (MMA^{V}) at the mM range there was a

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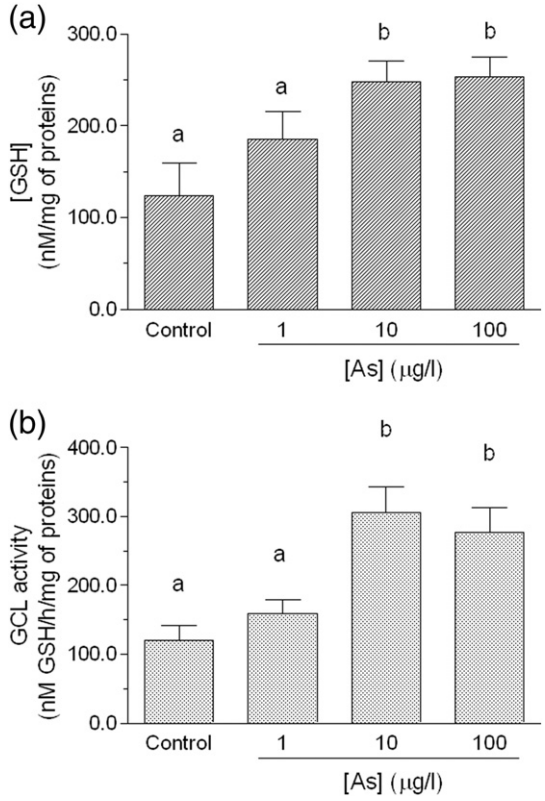

Fig. 1. (a) Glutathione (GSH) levels (nM/mg of protein). (b) Glutamate cysteine ligase (GCL) activity (nM GSH/h/mg of protein). Different letters indicate significant differences ($p<0.05$) between means of different treatments. Data are expressed as mean ± 1 standard error ($n=5$–6).

significant increase in cytolethality and enhanced the production of cellular ROS parallel with a depletion of cellular reserves of GSH. Moreover, some studies showed that GSH could be involved in the As biotransformation processes, serving as an electron donor for the reduction of arsenate to arsenite in aqueous solution and in erythrocytes (Thomas et al., 2001). Furthermore, specific glutathione-dependent methyltransferase catalyzes the methylation of iAs in mammals (Aposhian, 1997; Adair et al., 2005).

Most studies have evaluated As toxicity in mammalian cell lines. However, the study of As toxicity in aquatic animal species, including fishes, is limited (Wang et al., 2004). Previously, we showed that As affected antioxidant responses and evidence of oxidative stress in terms of lipid peroxidation in the estuarine polychaete *Laeonereis acuta* (Nereididae). Reduced catalase (CAT) activity, higher superoxide dismutase (SOD) activity and high lipid peroxidation (LPO) concentration were registered in worms exposed to the As concentration previously considered safe by Brazilian legislation (Conselho Nacional do Meio Ambiente, resolution 20, 30/07/1986; www.mma.gov.br/port/conama/res/res86/res2086.htm; 50 μg As/L).

In fishes, pollutants absorbed via gills can be metabolized before reaching the liver and, even with a relatively low metabolic capacity, the gills can contribute significantly to prevent accumulation of environmental pollutants in other organs. Gills thus fulfill a vital role in the overall protection against harmful substances by lowering the total uptake of toxic molecules by other organs (Mdgela et al., 2006).

Considering the facts cited above, the objective of the study was to evaluate antioxidant responses involving glutathione after As exposure in gills of the zebrafish *Danio rerio*. One of the concentrations assayed included the concentration actually considered safe for the preservation of aquatic fauna by current Brazilian Legislation (10 μg As/L) Fig. 1.

2. Materials and methods

2.1. Animals and exposure conditions

Organisms (~0.3 g) were maintained individually under laboratory condition in glass dishes (each dish with 200 mL of freshwater, pH 8.0 at 26 °C), under aeration (7.20 mg O2/L). Photoperiod was fixed at 12 L:12D. Fishes were exposed for 48 h to 1; 10; (maximum concentration allowed by Brazilian legislation, resolution 357, 17/03/2005), 100 μg As/L (As as KHAsO4, Sigma), plus a control group was also run in parallel, employing only water with the same characteristics cited above. Solutions were renewed once daily after exposure period, animals ($n=20$/group) were sacrificed and the gills were removed, homogenized and stored at −80 °C for further biochemical analyses. Reactive oxygen species concentration (ROS) was analyzed in fresh tissue.

The following variables were measured: total intracellular GSH content, enzymes activities (catalase, glutathione reductase, glutathione-S-transferase, and glutamate cysteine ligase), lipid peroxidation, reactive oxygen species levels and oxygen consumption. Total content of As and distribution of chemical forms were also analyzed in gills and water.

2.2. Enzymatic analysis

For enzymatic activities, gills and liver (weighing approximately 0.165 and 0.071 g, respectively) were homogenized (1:5 w/v) in buffer (100 mM Tris-HCL + 5 mM $MgCl_2$ + 2 mM EDTA, pH 7.75). Homogenates were then centrifuged at 10,000 × *g* for 20 min at 4 °C. The supernatant of each sample was employed for biochemical measurements.

Catalase (CAT) activity was analyzed following Beutler (1975), determining the initial of H_2O_2 (50 mM) decomposition at 240 nm. The results were expressed in CAT units, where one unit is the amount of enzyme that hydrolyzes 1 μmol of H_2O_2/min and per mg of protein, at 25 °C and pH 8.0.

Glutathione-*S*-transferase (GST) activity was determined by monitoring at 340 nm the formation of a conjugate between 1 mM GSH and 1 mM 1-chloro-2,4-dinitrobenzene (CDNB, Sigma) (Habig and Jakoby, 1981). The results were expressed in GST units, where 1 unit is defined as the amount of enzyme that conjugates 1 μmol of CDNB/min and per mg of protein, at 25 °C and pH 7.4.

Glutamate cysteine ligase (GCL) activity and baseline glutathione determination were analyzed following White et al. (2003). This method is based on the reaction of naphthalene dicarboxialdehyde (NDA) with glutathione (GSH) or γ-glutamylcysteine (γ-GC) to form cyclized products that are highly fluorescent. NDA-GSH fluorescence intensity was measured (472 nm ex/528 nm em) on a fluorescence microplate reader (Victor 2, Perkin Elmer). This assay has the advantage that baseline levels of GSH can also be measured in the same tissue sample. For GCL activity, a GCL reaction cocktail was prepared (400 mM Tris-HCl, 40 mM ATP, 20 mM glutamate, 2.0 mM EDTA, 20 mM sodium borate, 2 mM serine, 40 mM MgCl) just before the beginning of the assay to prevent ATP degradation. The samples were kept on ice until transferred to the reaction plate (25 °C) at 15 s time intervals. After 5 min of pre-incubation, the GCL reaction was initiated by adding 50 μL of cysteine (2 mM) to each GCL activity well (cysteine was not added to the GSH-baseline wells at this time). After this the plate was incubated for 30 min and the reaction stopped by adding 50 μL of 5-sulfosalicylic acid (SSA, 200 mM) and then 50 μL of 2 mM cysteine was added to the GSH-baseline wells. After protein precipitation, the plate was centrifuged for 5 min at 2,000 *g* and then, 20 μL aliquots of supernatant from each well of the reaction plate were transferred to a 96-well plate designed for fluorescence detection (Victor 2, Perkin Elmer).

Glutathione reductase (GR) activity was based on methods described previously by Nagalakshimi and Prasad (2001), using

sodium phosphate buffer (200 mM, pH 7.4), oxidized glutathione (GSSG, 1 mM) and NADPH (0.1 mM) and then monitoring the reduction in NADPH absorbance at 340 nm.

The total protein content was assayed using a commercial kit (Doles Ltda, Brazil) based on the Biuret method. Enzymatic determinations were performed at least in duplicate.

2.3. Reactive oxygen species (ROS) measurement

After sacrifice of experimental organisms, gills were removed and immediately homogenized (1:10 w/v) in cold buffer solution (0.32 M sucrose, 20 mM HEPES, 0.1 mM $MgCl_2$, 0.5 mM of PMSF, pH 7.4). The samples were centrifuged at 20,000 *g* for 20 min at 4 °C. The supernatants were used for determination of the ROS with 2',7'-dichlorofluorescein-diacetate (DCF-DA, Molecular Probes) that, in the presence of ROS, generates a fluorochrome measured by fluorescence at wavelengths of 488 nm and 525 nm for excitation and emission, respectively (Ferreira-Cravo et al., 2007). The readings were carried through in a fluorescence microplate reader (Perkin Elmer Victor 2 fluorescence, USA), in a medium containing 30 mM HEPES (pH 7.2), 200 mM KCl, 1 mM $MgCl_2$, 16 μM DCF-DA and 166 μg of proteins of tissue samples.

2.4. Oxygen consumption

Gills oxygen consumption was measured according to Geracitano et al. (2004). Oxygen concentration of the homogenization buffer (see Section 2) was measured at time zero and after 1 h using a portable oxymeter (Handlylab OX1/SET; SCOTT-GERÄTE, Hofheim, Germany). During the incubation period, dishes containing the gills were kept closed and at 20 °C. Oxygen consumption (OC) was calculated according to the following equation (based in Geracitano et al., 2004):

$$OC = \left[\left(O_i - O_f\right) * dv / 1{,}000\right] / ww$$

Where O_i and O_f represent the initial and final oxygen concentration (mg/L), respectively; dv is the buffer volume in the dish; 1,000 stands for the conversion of liter to milliliters; and ww is the wet mass (g) of the tissue tested. Oxygen consumption was expressed in mg O_2/g ww/h.

2.5. Thiobarbituric acid reactive substances (TBARS)

Tissue lipid peroxidation was measured by TBARS (Onkawa et al., 1979) with modifications. Gill homogenate was incubated with 8.1% (w/v) sodium dodecyl sulfate (SDS), 40% acetic acid (v/v), 0.2% BHT (dissolved in dimethyl sulfoxide) and 0.8% (w/v) of thiobarbituric acid (TBA) for 1 h in a water bath at 95 °C. Pink colour chromogen was extracted into pure n-butanol and read at 532 nm in a spectrophotometer. The values were expressed in μmol of malondialdehyde/mg of proteins.

2.6. As total content and speciation in gills

Chemical speciation of As was performed following a previously described and validated method (Fattorini and Regoli, 2004). Tissues were homogenized (1:10 w/v) in methanol (p.a.>99%, HPLC grade, Fluka) and As compounds were extracted using a microwave (Mars CEM, CEM Corporation) at 150 W and 55 °C for 15 min. After cooling the samples were centrifuged at 2,000 *g* for 15 min and supernatants were collected; these were concentrated by removing the solvent using a SpeedVac (RC1009; Jouan, Nantes, France) and finally recovered in 1 mL of methanol:water (70:30) solution. As species were separated by high performance liquid chromatography (HPLC), injecting 200 μL of the samples. Anionic forms were obtained with Supelcosil liquid chromatography-SAX1 column (25 cm, 4.6 mm ID, 5 μm, Supelco, Bellefonte, PA, USA) with 15 mM KH_2PO_4 (pH 6.1) as mobile phase at a flow rate of 1 mL/min; cationic exchange was performed with Supelcosil liquid chromatography-SCX column (25 cm, 4.6 mm ID, 5 μm, Supelco, Bellefonte, PA, USA) and 2.5 mM pyridine (pH 2.65) as mobile phase at a flow rate of 1 mL/min. Every 30 s from injection, 40 fractions were collected, added with 0.5 mL of nitric acid (p.a.>65%, Fluka), and analyzed for total As content by atomic absorption spectrometry as previously described. The same extraction and separation procedures were applied to standard reference material (SRM) DORM-2 (containing certified levels of TETRA and AsB; Table 1) and selected standards, As^V, DMA, TMA, AsB, as controls for accuracy, precision, and recovery. The limit of detection, analytical coefficient of variation (CV%) and the recovery of certified standard materials were analogous to those reported for the total As determination.

Table 1
Certified levels of total As, and As species in standard reference materials (NIST 2977 and DORM-2) and concentrations obtained in the present work.

		Certified value	Obtained value	Mean R%
NIST 2977	Total As	8.83 ± 0.91	8.72 ± 0.25	98.8%
DORM-2	Total As	18.0 ± 1.1	17.21 ± 0.21	95.6%
	AsB	16.4 ± 1.1	15.65 ± 0.87	95.4%
	TETRA	0.248 ± 0.054	0.239 ± 0.11	96.4%

Values are expressed as μg/g (dry weight) and were obtained analyzing 5 replicates. AsB: arsenobetaine, TETRA: tetramethylarsonium. Limit of detection: 5 ng of As.

Table 2
Operating conditions of the hydride generation atomic absorption spectrometer system.

Spectrometer conditions	Characteristics/conditions
Wavelength	193.7 nm
Bond width	0.5 nm
Lamp current	10 mA
Hydride generation	
Quartz cell	15 cm path length × 8 mm ID
Heating	Electrothermal
Temperature	925 °C
Nitrogen flow rate	1.5 L/min
Reductant concentration	1% (wt/vol) in 0.1 mol/L NaOH
Reductant flow rate	1.0 mL/min
Sample flow rate	8 mL/min
As(III) reduction milieu	
Sample (pH>7)	0.5 mol/L citric acid pH 2.0
Flow rate	1.0 mL/L
Total As (III; V) reduction milieu	6.0 mol/L HCl
Flow rate	1 ml/min

Limit of detection: 1.8 μg/L for As^{III} and 0.1 μg/L for total As (based on Bohrer et al., 2005).

2.7. As speciation in water

A SpectrAA 200 spectrometer (Varian, Australia), with a VGA 77 system (for hydride generation) was used. The operating conditions of the spectrometer used for both As species determination are shown in Table 2. A Berghof BSB 939-IR sub-boiling distillation apparatus (Eningen, Germany), a Phoenix AV 50 autoclave (São Paulo, Brazil), and a Digimed pH meter D-20 (São Paulo, Brazil) were used.

Arsenite was measured carrying out the reduction in citrate buffer pH 2.0. Arsenate was evaluated measuring the total As in solution by changing the citrate buffer to 6.0 mol/L.

HCl, and subtracting arsenite from the total As content. Sodium tetrahydroborate (III) in a concentration of 1% (w/v) was used as reducing agent in all determinations. Analytical curves were obtained with standards containing 5–40 μg/L As of each species (Bohrer et al., 2005).

2.8. Statistical analysis

Values of all determinations were expressed as mean ± 1 standard error (SE). Statistical analysis was performed through analysis of variance followed by Newman-Keuls test ($\alpha = 0.05$). Previously, the

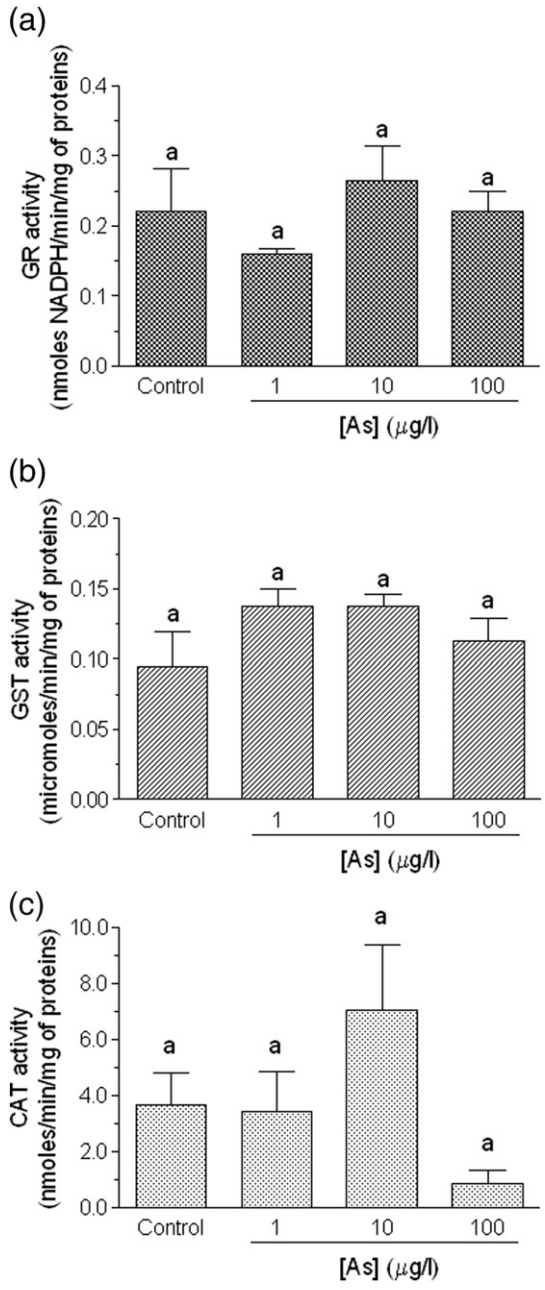

Fig. 2. (a) Glutathione reductase (GR) activity (nmoles NADPH/ min/ mg of protein). (b) Glutathione-S-transferase (GST) activity (µmoles/min/ mg of protein). (c) Catalase (CAT) activity (nmoles/min/mg of protein). Different letters indicate significant differences ($p<0.05$) between means of different treatments. Data are expressed as mean ± 1 standard error ($n=4$–6).

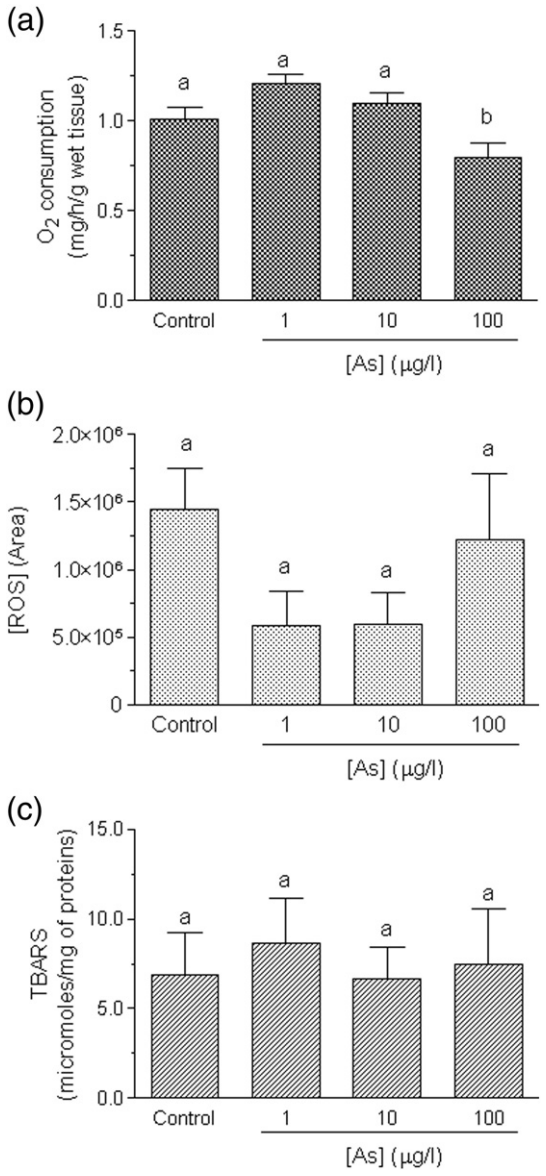

Fig. 3. (a) Oxygen consumption (mg/h/g wet tissue). (b) Reactive oxygen species (ROS) concentration (Area). (c) Lipid peroxides content expressed as thiobarbituric acid reactive substances (TBARS) (µmoles/mg of protein). Different letters indicate significant differences ($p<0.05$) between means of different treatments. Data are expressed as mean ± 1 standard error ($n=4$–6).

assumptions of normality and variance homogeneity were verified and mathematical transformation applied if at least one of them was violated (Zar, 1984).

3. Results

A significant ($p<0.05$) increase of GSH levels was observed in fishes exposed to 10 and 100 µg As/L in comparison to the control and 1 µgAs/L groups (Fig. 1a). Effects of As were also evident for GCL activity which was markedly increased ($p<0.05$) after exposure to 10 and 100 µg As/L (Fig. 1b). Concerning antioxidant enzymes, no variations ($p>0.05$) were observed in the antioxidant system GR, GST and CAT activities in fish exposed to various As concentrations (Fig. 2). A significant ($p<0.05$) decrease of oxygen consumption in gill tissues was registered after exposure to 100 µg As/L (Fig. 3a), while no differences ($p>0.05$) in terms of ROS and TBARS content (Fig. 3b and 3c) were observed in fish during various experimental conditions. Chemical speciation in water revealed that arsenite was the prevalent form (Table 3). The levels of As in gills of control fish and of organisms exposed to 1 and 10 µg of As/L were below the detection limit of the method (0.1 µg/g d.w.), while concentration measured in organisms exposed to 100 µg of As/L was 4.97 ± 0.78 µg/g (d.w.). The chemical

Table 3
Concentration of total inorganic As and As species in water where the fishes were exposed by 2 days to 1, 10 and 100 µg As/L.

Nominal concentration (µg/L)	As(III) (µg/L)	As(V) (µg/L)	Total As (µg/L)	As(III)/Total As (%)
Tap water	n.d	n.d	n.d	–
1.00	n.d	n.d	n.d	–
10.00	3.62 ± 0.67	2.50 ± 0.34	6.11 ± 1.02	59.24
100.00	69.86 ± 6.80	10.96 ± 7.50	80.83 ± 5.16	86.42

Table 4
Toxicological effects observed in zebrafish (*Danio rerio*) after arsenic exposure.

Concentration of As	Cell line/organ	Effect observed	References
22 mg/L	Liver cell lines	Induced apoptosis	Seok et al. (2007)
37 mg/L	Epithelial cells	Morphological aberrations	Wang et al. (2006)
1; 10; and 100 mg/L	Whole body	Uptake and accumulation of As	Liu et al. (2006)
50–117 mg/L	Thyroid	Hypothyroidism	Liu et al. (2008)
0.010 and 0.100 mg/L	Gills	Increase in GSH levels and GCL activity	Present study

speciation performed on organisms exposed to the highest As concentration, showed that the prevalent form of As was inorganic, while organic As compounds were not detected.

4. Discussion

Looking at Table 4, it is evident the scarce of data concerning antioxidant responses in zebrafish after As exposure. The studies listed in Table 4 considered accumulation, morphological or histological changes or even apoptosis induction. However, it seems that the present report is the first that considered toxicological responses in terms of one of the main antioxidants as GSH is, and the rate-limiting enzyme for its synthesis, GCL. Toxicological data for zebrafish are important, as this fish is broadly employed as animal model for toxicological studies together with at least other nine species (including turbot and trout, for example) (Carvan et al., 2007) and also as a biological model for several human diseases (Keller and Murtha, 2004).

In the present study, the role of GSH in antioxidant responses after As exposure was analyzed in zebrafish gills. In March of 2005, Brazilian legislation established a new maximum As concentration considered safe for aquatic fauna in 10 μg As/L (see the Introduction section). In this study, we observed that 10 μg As/L was sufficient to increase the GSH levels and GCL activity in gills of *D. rerio*. Similarly, Ochi (1997) observed elevated levels of intracellular GSH in mammalian keratinocytes exposed to iAs which were primarily attributed to GCL activity. Schuliga et al. (2002) showed that up-regulation of GCL activity by arsenite parallels a corresponding increase in the level of GCL mRNA and also showed that gene expression of GSH-related enzymes in human cells is increased under conditions of oxidative stress. GCL activity is the rate-limiting step in the *de novo* synthesis of GSH and its expression can be modulated by a number of different factors, including depleting agents, reactive oxygen and nitrogen species, cytokines, and hormones (White et al., 2003). It has been widely reported that iAs can be toxic in mammalian cells by induction of a severe burst of ROS production and that cellular GSH scavenges these ROS (Sakurai et al., 2005). Acute exposure to iAs is known to induce a cellular stress response in mammalian cells that involves increase in heat-shock proteins, heme-oxygenase, and GSH (Ochi, 1997).

The present results showed an increase in GSH levels, which can explain the absence of increased ROS concentration and of LPO levels in As exposed fishes. Other authors, in fact, have observed a significant increase in LPO content in the Indian catfish (*C. batrachus*) after exposure to low concentration of As(1–3 mM) (Battacharya and Battacharya, 2007). Interestingly, other authors as Bagnyukova et al. (2007) have observed augmented liver GSH levels after exposure of the goldfish to 200 μ of sodium arsenite. The same authors observed absence of arsenic effect of lipid peroxidation measured as thiobarbituric acid content and protein carbonyl groups, although higher levels of lipid hydroperoxides was registered in liver goldfish after 1 and 4 days of exposure.

In this study no significant changes were observed for glutathione reductase (GR), which has the critical role of reducing GSSG in order to maintain high levels of intracellular GSH. In fishes exposed to low concentration of As, a decrease in GR activity was reported (Battacharya and Battacharya, 2007), while Schuliga et al. (2002) showed an increase of GR activity in fibroblasts, but the mechanisms by which As regulates the activity of GR *in vivo* remain unclear. The increments in GSH levels and GCL activity observed in this study indicate that the GSH antioxidant response to the As pro-oxidant challenge is mediated through *de novo* synthesis of the thiol, while the reduction rate of GSSG remained unchanged. Note that, however, the As-induced antioxidant responses can be deleterious to the organisms at long term, taking into account that GSH synthesis divert ATP from other cellular purposes and that the alteration of the redox state, even when moved to a more reduced one, can alter cellular functions (Jones, 2006). Also, the lowering of oxygen consumption observed at the highest As concentration (100 μg/L) suggested a loss of aerobic metabolism which is another potential problem in ATP generation.

The enzyme GST catalyze conjugation of GSH to electrophilic substrates and, despite these enzymes are known to be involved in As metabolism and detoxification, our results did not reveal significant change in *Danio rerio*. In a previous study, GST activities were lowered in the polychaeta *Laeonereis acuta* after one-week exposure to 500 μg As/L (Ventura-Lima et al., 2007), while Sakurai et al. (2004) showed that human cells exposed to monomethylarsonic acid ($MMAs^V$) increased cellular GST activity, and addition of a specific GST inhibitor, significantly enhanced the cytolethality of both $MMAs^V$ and arsenite. Note that, in the context of present data, the fact that GST remained unaffected indicates that the tested concentration were not deleterious since some authors proposed that GST is involved in the detoxification of endogenous molecules such as 4-hydroxykenals (membrane peroxides) and base propenals which are products of the oxidative DNA degradation (Nagalakshimi and Prasad, 2001).

Also CAT activity was not affected in As exposed fishes, further supporting the efficacy of increased GCL and GSH in preventing additional oxidative perturbations. This is in contrast to observation in *C. batrachus* where an increase in CAT activity after As exposure was reported (Battacharya and Battacharya, 2007). These different responses in antioxidant system and oxidative damage observed in fishes and other organisms show clearly that As toxicity is dependent upon species and tissue analyzed. As metabolism and detoxification include absorption, distribution, biotransformation, and excretion factors (Schuliga et al., 2002) can also greatly vary in different tissues.

The As found in gills of *Danio rerio* was only inorganic, suggesting that this tissue is deficient in methyltransferases that are needed for As methylation. In gills of the fish *Mugil cephalus* the prevalent form of As was arsenobetaine (Maher et al., 1999), while Edmonds and Francesconi (1987) showed that iAs administered by food to the fish *Cnidoglanis macrocephalus* was accumulated only as TMAO. The predominance of certain compounds of As might thus be interpreted as a typical feature of each species. Fattorini and Regoli (2004) observed remarkable accumulation of As in the branchial crown of *Sabella spallanzanii* with dimethylarsinate (DMA) as the main As metabolite, while in another polychaete species, *Arenicola marina*, As is accumulated mostly in the inorganic forms (Geiszinger et al., 2002).

Considering the decrease of oxygen consumption in gills after exposure to 100 μg As/L, it is known that arsenite is an effective inhibitor of pyruvate dehydrogenase and α-ketoglutarate dehydrogenase enzymes. The decrease in activities of these two enzymes can inhibit the citric acid cycle and thereby decrease the generation of reducing equivalents such NADH and NADPH, impairing ATP production (Ramanathan et al., 2003; Tseng, 2004) and oxygen reduction to form water. Moreover, iAs can affect NADH dehydrogenase and cytochrome c oxidase. The significant decline in the activity of two enzymes would result in the inhibition of electron flow from NADPH to oxygen, augmenting the chance of ROS generation and lowering

oxygen consumption. Note that at long term, a reduction in ATP synthesis capability should impair GSH synthesis, favoring an oxidative stress scenario.

Inorganic As occurs in water mostly as arsenate, whereas arsenite may exist in anaerobic water or with low dissolved oxygen (Caussy, 2003). In this study, we showed that the predominant As form in water was arsenite, although under aerobic conditions, suggesting that the oxidation state of As observed in this study could be due mostly to microbial activity associated with fish. In aqueous environments, prokaryotes and eukaryotes reductively biomethylate inorganic As to dimethylarsenic (DMA) and monomethylarsonic (MMA).

As a general conclusion, it can be stated that As affected the antioxidant responses in zebrafish gills in terms of GSH concentration and GCL activity, although no oxidative damage was detected. It is likely that with longer exposure times, the decrease of oxygen consumption observed here could impair ATP production, affecting GSH synthesis and triggering oxidative damage.

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