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Behavioral and neurotoxic effects of arsenic exposure in zebrafish (*Danio rerio*, Teleostei: Cyprinidae)

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ABSTRACT

This study investigated the passive avoidance conditioning in zebrafish (*Danio rerio*). An instrument was developed for measuring escape responses triggered by a conditioned stimulus. This system allowed quantification of latency of crossing from a light to a dark zone. Zebrafish were trained to swim from an illuminated to a dark compartment, where they received a body shock (training session). The proposed methodology was efficient for evaluation of working, short, and long-term memory formation of an aquatic animal model. The possibility of employing memory measurements in toxicity tests, in order to obtain an ecologically meaningful biomarker response, was also analyzed. In this experiment, immediately after the training session, fish were exposed to three arsenic (As^V) concentrations. After the test session, the brain was removed for biochemical analyses. A control group was kept in tap water. After exposure, animals were submitted to a one-trial inhibitory avoidance test for measurement of long-term memory (LTM). Results from behavioral and biochemical analyses showed that the three As^V concentrations impaired LTM ($p<0.05$) and increased protein oxidation, which suggests an amnesic and pro-oxidant effect of As^V. Evaluation of behavior parameters in aquatic models is an important complement in studies concerning the environmental impact of chemical substances.

1. Introduction

Learning and memory formation comprise a complex mechanism that encloses the archive and the recovery of experiences, being therefore associated with learning, which is defined as the ability to alter behavior through experiences that have been stored in memory (Clark et al., 2000). Three types of memories are commonly acknowledged: working, short, and long-term memory. One-trial inhibitory avoidance (IA) is commonly employed as a conditioned fear paradigm, known since Pavlov. In passive avoidance conditioning, animals learn to associate the conditioned stimulus (CS) with unconditioned stimulus (US), and through CS–US association formation, the conditioned stimulus (CS) generates various conditioned responses (CRs) that share similar characteristics with innate fear response (Rescorla, 1968; Kim and Jung, 2006). Latency time is a good indicator of learning. There are several reasons for using this particular task, but the most important is that it is acquired in seconds, so the LTM initiation time and the formation and duration of STM can be determined quite accurately (Izquierdo et al., 2000; Kim and Jung, 2006). Therefore, inhibitory avoidance is used to evaluate behavioral responses in several animal models like crabs, rats, mice and fish (Denti et al., 1988; Izquierdo and Medina, 1995).

Zebrafish, *Danio rerio* (Cyprinidae), have been extensively used in many research areas. In the late 1970s, investigators began to employ this species in neuroscience, studying the startle response and Mauthner cells (Serra et al., 1999). Zebrafish are also being developed as sensitive models for studying the neurobehavioral impact of neurotoxicants, as well as pharmacological agents (Carvan III, 2003; Parng et al., 2007).

Arsenic (As) is considered as an environmental pollutant. It is widely distributed in nature and released into the environment through industrial processes and agricultural practices (Nandi et al., 2005). Different countries have established various arsenic concentration limits for the protection of aquatic life (Mirlean et al., 2003). Brazilian legislation determines that 10 μg of arsenic (As)/L is the maximum concentration considered safe for aquatic fauna (CONAMA, 2005). The effects of arsenic on nervous system function have received considerably less attention than its association with cancer, genotoxicity, and cellular disruption (Rodríguez et al., 2003).

Arsenic can be found in both organic and inorganic compounds with variable oxidation states. In general, for inorganic forms, arsenite

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[As (III)] is more toxic than arsenate [As (V)] (Akter et al., 2005). In water, arsenic is normally encountered in the pentavalent form (Caussy et al., 2003). Several data are available concerning the toxic effects of As on whole brain (Tripathi et al., 1997) of rats and mice. Toxic manifestations of arsenic are considered as being primarily caused by the imbalance between pro-oxidant and antioxidant homeostasis, and also by a high affinity of arsenic for thiol groups in functional proteins (Aposhian and Aposhian, 1989). Free radicals have previously been shown to be capable of damaging many cellular components such as DNA (Liu et al., 1996), proteins (Liu et al., 1996) and lipids (Alper et al., 1999). Oxidative modification of proteins *in vivo* may affect a variety of cellular functions involving these molecules, such as signal transduction mechanisms, transport systems, receptors and enzymes activity (Samuel et al., 2005).

The oxidative damage to proteins is reflected in the increase of protein carbonyl (PCO) levels (Butterfield et al., 1998), and the decrease of protein thiols (PSH) (Dubey et al., 1996). Reaction of free radicals, such as hydroxyl radical ($HO^{\bullet}$) or superoxide anion ($O_2^{\bullet -}$), with side chains of lysine, arginine, proline, threonine and glutamic acid residues of proteins, leads to the formation of carbonyl derivatives (Stadtman and Berlett, 1997). Furthermore, aldehydes, such as 4-hydroxy-2-nonenal or malondialdehyde, produced during lipid peroxidation can be incorporated into proteins by reaction with either the ε-amino moiety of lysine or the sulfhydryl group of cysteine residues, forming carbonyl derivatives (Uchida and Stadtman, 1993). Carbonyl groups can also be introduced into proteins by glycation and glycoxidation reactions (Baynes, 1991). Therefore, protein carbonyl groups become reasonable markers for free radical-induced protein oxidation.

The aim of this work was to develop and validate an apparatus for evaluation of conditioned fear in zebrafish, as well as investigate the effects of an important pollutant such as arsenic on behavioral and oxidative stress parameters.

2. Materials and methods

2.1. Subjects and chemicals

Zebrafish of both sexes and mean weight of approximately 0.3 g were obtained from commercial suppliers at the city of Rio Grande, RS, Brazil. Animals were maintained individually under laboratory condition in glass dishes (each dish with 200 mL of freshwater) at 20 ± 1 °C, 12/12 light/dark (0700–1900 h light) cycle, in a continuously filtered and aerated 30-liter aquarium, for 7 days prior to experiments. Arsenic was used under the form of KH_2AsO_4 (Sigma-Aldrich). For method validation, nicotinic receptor agonist (nicotine — Sigma-Aldrich) and antagonist (mecamylamine hydrochloride — Sigma-Aldrich) were used.

2.2. Apparatus description

The apparatus, under a patent protocol in INPI (Instituto Nacional de Propriedade Industrial) under number 0000220704494890 in 07/09/2007, consisted of a PVC pipe, (33.0 × 7.3 × 8.0 cm), divided into a dark zone (13.0 cm) and a bright zone (20.0 cm). The internal diameter was 7.3 cm, and the water column height was 2.6 cm. In the dark zone, two metallic plates with 10.5 cm length were fixed in parallel bars (vertically), equidistant in 4.0 cm. This capacitor was fed continuously by a 6-volt source on to an interrupting key that allows or impedes pulse generation, both being controlled by a voltmeter and an amperimeter, respectively. The pipe presents one transversal cut (see Fig. 1) for fixing the two zones: the bright zone, which allows entrance of light and the dark zone, which should theoretically be preferred by the fish [46]. Since the objective of the test was to evaluate fear conditioning behavior, a mild electrical shock (5 mA; 1.5 V; unconditioned stimulus — US) was applied once the fish entered the dark zone. The capacitor (flow intensifier) and the chain were tested observing if the physical integrity of the fishes was not affected. Through a few tests, it was verified that the chain value was in the order of 5 mA. The instrument uses direct current (DC), guaranteeing the constant and commanded flow of electrons, always in one direction. Current was generated by four 1.5 V alkaline batteries, connected in series (Fig. 1).

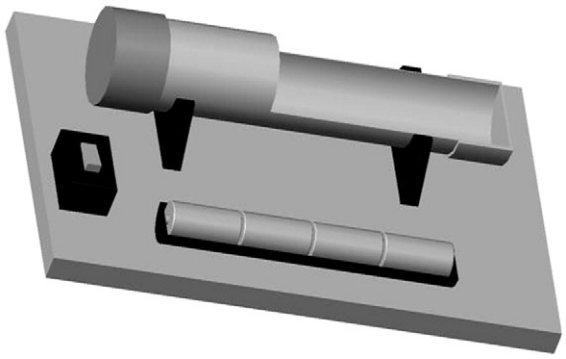

Fig. 1. Schematic drawing of one-trial inhibitory avoidance apparatus for zebrafish *D. rerio*.

2.3. Behavior experimental procedure

Fish were placed inside the apparatus, at the illuminated zone. As observed by Serra et al. (1999), they preferred the dark compartment, probably seeking protection. Latency of entrance in the dark side was measured with a chronometer and immediately after an electric shock was applied. After the shock, the zebrafish returned to the bright zone, learning that a specific region of the instrument is associated with the shock. Working memory (WM) was measured 10 s after the training session. Short (STM) and long-term (LTM) memories were determined 90 min and 96 h after the training session, respectively. Latency of entry in the dark zone was measured in order to evaluate WM, STM and LTM.

For test validation, animals were exposed to nicotine (50 and 100 mg/L) (Levin and Chen, 2004) and a non-selective antagonist, mecamylamine hydrochloride (200 mg/L) (Levin et al., 2007), following the same experimental protocol as described above.

In the assay conducted to test the device applicability, fish were maintained in tap water (20 °C, pH 7.1, 7.20 mg O_2/L) with arsenic (As) immediately after training, for 96 h. The assayed As concentrations were: 1, 10 (maximum concentration allowed by Conselho Nacional do Meio Ambiente, resolution 357, 17/03/2005; www.mma.gov.br/port/conama/res/res05/res35705.pdf) and 100 μg As/L. Also, a control group was run in parallel ($n = 20$/group), employing only tap water with the same characteristics mentioned above. Solutions were renewed every day. After 96 h, the inhibitory avoidance test was carried out, and the subjects were placed again in the apparatus. Latency of entrance in the dark zone was measured, and a ceiling of 120 s of permanence in the light zone was established. After session test, animals were euthanized and the brain were removed, homogenized and stored at −80 °C for further biochemical analyses.

2.4. Biochemical procedures

2.4.1. Protein oxidation

Whole brains were homogenised (1:6) in a cold buffer solution (0.32 M sucrose, 20 mM HEPES, 0.1 mM $MgCl_2$, 0.5 mM PMSF, pH 7.4). Samples were centrifuged at 20,000 *g*, 4 °C, for 20 min. Supernatant was kept and total protein content determined by the biuret assay (Doles).

Quantification of oxidised proteins was performed using a Western blot protocol. Samples were derivatized by a 30-min incubation at room

temperature with dinitrofenil hydrazine (DNPH, Sigma-Aldrich), which reacts with the carbonyl groups of oxidised proteins, resulting in the formation of dinitrophenyl hydrazone (DNP) (Levine et al., 1990). After electrophoresis separation in precast polyacrylamide gradient gel (NuPAGE® 4–12% Bis–Tris Gel, Invitrogen Corporation), samples were blotted onto a PVDF membrane (Invitrogen) and incubated with primary (for the DNP–protein complex) and secondary rabbit antibodies (Invitrogen). Finally, the membrane was scanned and analyzed using the free software Scion Image. For analysis, proteins were divided into three groups, including proteins of low (MW<86.0 kDa), medium, (86.01<MW<131.00 kDa) and high molecular weight (MW> 131.01 kDa). The number of oxidised proteins in each category was registered.

2.4.2. DNA damage

DNA damage was evaluated through the K^+/SDS test, which detects covalent protein–DNA crosslinks. In the nucleus and close to the DNA, many protein associations are involved in gene expression regulation and chromatin structure. Several agents promote the covalent bond between proteins and DNA, leading to the generation of adducts called DNAPCs (Barker et al., 2005). The employed assay is based on the capacity of sodium dodecyl sulphate (SDS) to selectively bind proteins. Addition of potassium promotes precipitation of a complex formed by K^+/SDS, protein and DNA. Afterwards, samples were incubated with proteinase K, promoting protein release from the complex. Quantification of DNAPCs was accomplished by estimating the DNA/protein ratio, with regard to total DNA. DNA quantification was carried out through the fluorescence generated by Syber Gold dye (Molecular Probes), employing wavelengths of 485 and 535 nm for excitation and emission, respectively. Reading was carried through a fluorescence microplate reader (Victor 2, Perkin Elmer).

2.4.3. Total antioxidant capacity

Quantification of total antioxidant capacity was performed by means of reactive oxygen species (ROS) concentration (Regoli and Winston, 1999), using 2′,7′-dichlorofluorescin diacetate (H_2DCF-DA, Molecular Probes). Fluorescence intensity was determined using a fluorometer (Victor 2, Perkin Elmer), with excitation and emission wavelengths of 485 and 520 nm, respectively. Background fluorescence was determined before the addition of H_2DCF-DA. Total antioxidant capacity was measured against peroxyl radicals, generated by thermolysis of 2,2′-azobis (2 methylpropionamidine) dihydrochloride (ABAP; 20 mM; Sigma-Aldrich) at 37 °C. Therefore, ROS concentration was determined in samples with and without ABAP, in order to analyze the difference in fluorescence. Comparison of fluorescence emitted with and without ABAP in each sample permitted evaluating if arsenic exposure modified the capacity to intercept and/or to degrade peroxyl radicals. Protein concentration of samples was fixed at 3.32 mg/mL.

2.5. Statistical analysis

Results of the inhibitory avoidance task were analyzed through the Kruskall–Walis and Mann–Whitney *U* tests, and expressed as median and interquartile intervals. Biochemical data were computed as mean ± 1 standard error (SE). Statistical analysis was performed through analysis of variance followed by Newman–Keuls test ($\alpha = 0.05$). Assumptions of normality and variance homogeneity were verified previously and mathematical transformations applied if any were violated (Zar, 1984).

3. Results

3.1. Behavioural results

Results are expressed as median and interquartile time range (seconds). Animals trained in the inhibitory avoidance task were once more placed in the device 10 s, 90 min and 96 h after the training session, in order to measure WM, STM and LTM, respectively. In all cases, significant difference ($p<0.05$) was verified between the training and retention sessions. The registered latency times were 3.9 s (2.8–5.3) for the training session; 78.2 s (48.3–118.5 s) for WM; 77.1 s (37.5–102.3 s) for STM; and 104.1 s (72.0–114.7 s) for LTM (Fig. 2a).

The mecamylamine hydrochloride and nicotine experiment showed the efficiency of the apparatus for long-term memory measurement. Nicotine in both doses [50 mg/L — 76.22 (44.16–92.17) and 100 mg/L — 74.05 (57.04–96.70)] and mecamylamine hydrochloride [200 mg/L — 5.83 (2.81–11.00)] significantly differed from the control group [32.69 (14.16–88.22)], ($p<0.05$) (Fig. 2b).

Exposure to As demonstrated that fish submitted to the three concentrations presented lower latency for entrance in the dark zone

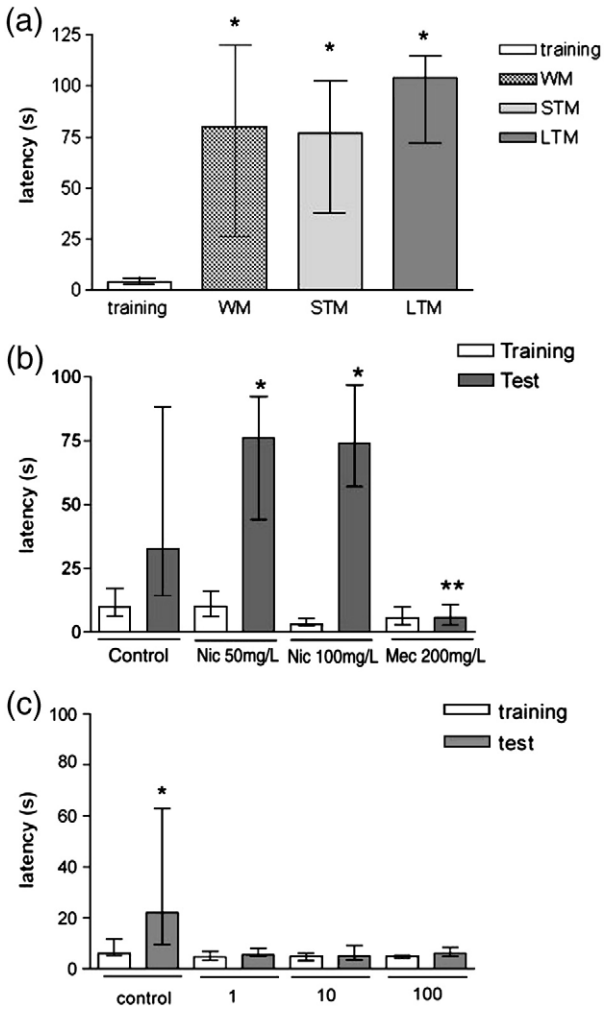

Fig. 2. (a) Effect of body shock exposure in zebrafish *D. rerio* in one-trial inhibitory avoidance. Working memory (WM) was measured 10 s after the training session. Short (STM) and long-term (LTM) memory were measured 90 min and 96 h after the training session, respectively. Test session latencies are expressed as medians (in seconds) and vertical lines represent interquartile ranges. Asterisks indicate significant differences from the training session ($p<0.05$). (b) Effect of the agonist and antagonist of memory. Zebrafish *D. rerio* were exposed to nicotine (agonist) and mecamylamine hydrochloride (antagonist), and long-term memory (LTM) was estimated. Test session latencies are expressed as medians (interquartile ranges), in seconds. Asterisks indicate significant differences from the training session ($p<0.05$) in relation to the control group, and two asterisks indicate significant differences between the control group and the nicotine group (50 and 100 mg/L). (c) Effect of arsenic exposure on zebrafish *D. rerio* memory. Retention test latency was measured 96 h after training, in order to estimate long-term memory (LTM). Ordinates express median (interquartile range) test session latency in seconds. Asterisk indicates significant difference between training and test sessions ($p<0.05$).

when compared to the control group [22.2 (9.6–62.8)], indicating that arsenic impaired LTM at the concentrations of 1 μg/L [5.6 s (4.9–8.0 s)], 10 μg/L [5.2 s (3.5–9.2 s)] and 100 μg/L [6.2 s (4.8–8.4 s)] ($p<0.05$) (Fig. 2c).

3.2. Biochemical results

Fish exposed to 10 and 100 μg As/L presented a higher number of oxidised proteins of an intermediate molecular weight (MW) range ($86.01<MW<131.00$), when compared to the control group ($p<0.05$; Fig. 3a). There were no differences ($p>0.05$) in antioxidant capacity against peroxyl radicals after arsenic exposure (Fig. 3b). Results obtained for DNA damage in terms of DNA and protein covalent binding (protein–DNA crosslink; DNAPC) showed no effect ($p>0.05$) of arsenic exposure (Fig. 3c).

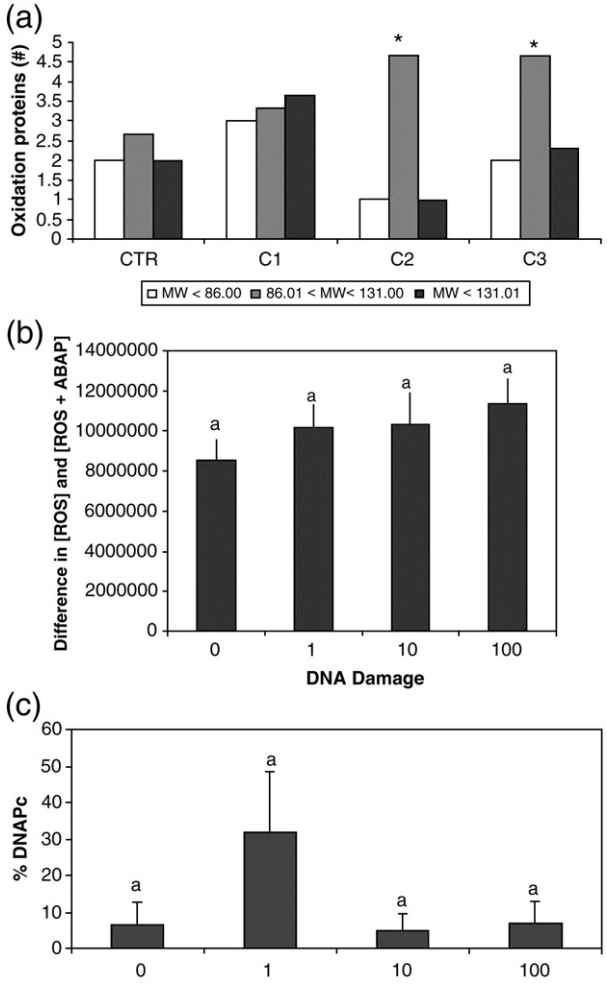

Fig. 3. (a) Protein oxidation measured through Western blot. Data are expressed in total number of oxidised proteins. The performed treatments were CTR = 0, C1 = 1, C2 = 10 and C3 = 100 μg As/L; asterisk indicates statistical difference ($p<0.05$) in the number of oxidised proteins of arsenic-exposed groups in regard to the control. (b) Total antioxidant capacity after zebrafish *D. rerio* exposure to arsenic (0, 1, 10 and 100 μg As/L). Total antioxidant capacity was measured against peroxyl radicals generated by thermolysis of 2,2′-azobis (2 methylpropionamidine) dihydrochloride (ABAP) at 37 °C. Comparison of fluorescence emitted with and without ABAP in each sample permitted evaluating if arsenic exposure modified the capacity to intercept and/or to degrade peroxyl radicals. Protein concentrations of samples were fixed at 3.32 mg/mL. Equal letters indicate absence of statistical differences ($p>0.05$). ROS, reactive oxygen species. (c) DNA damage in terms of adduct formation of DNA and protein covalent binding (protein–DNA crosslinks, DNAPC), after zebrafish *D. rerio* exposure to arsenic during 96 h, at concentrations 0, 1, 10 and 100 μg As/L. Values are expressed in percentage of formed DNAPC in relation to free DNA. Equal letters indicate absence of statistical differences ($p>0.05$).

4. Discussion

Learning and memory have been studied in many animal models, such as fruit flies, mice, rats and others (Goodwin et al., 1997; Izquierdo et al., 1998b; Barros et al., 2002; Crow, 2004). Zebrafish behavioral assays are becoming increasingly versatile and utilized in studies of genetic and neural underpinnings of cognitive functions (Darlang and Dowling, 2001; Xu et al., 2007).

In the first experiment, for validation of the inhibitory avoidance apparatus, zebrafish showed increases in avoidance response (Fig. 2a), suggesting the establishment of an association between CS (dark zone) with the US (shock). In tasks that are acquired in seconds, such as one-trial inhibitory avoidance, evidence indicates that WM lasts for a few seconds and is usually measured as immediate memory (Goldman-Rakic, 1991; Izquierdo et al., 1998a,b). WM should not be considered equivalent to STM, since it belongs to an entirely separate memory system with a different subset of underlying brain areas, at least in rats (Izquierdo et al., 1998a,b; Barros et al., 2002; Izquierdo et al., 2006). WM lasts only few seconds or minutes and no biochemical traces are formed (Goldman-Rakic, 1991). STM processes and holds information for 1–6 h while LTM is being consolidated (Izquierdo and McGaugh, 2000). LTM becomes established over a period of up to 6 h through a series of biochemical cascades in several brain areas (Izquierdo et al., 1998b; Izquierdo and McGaugh, 2000). Recent evidence indicates that WM, STM and LTM are in essence separate and parallel processes (Izquierdo et al., 1998b), but also suggests that they are linked at some levels (Izquierdo et al., 1998a,b).

Standardized validated tests of learning and memory are essential for determining the neural bases for cognition function (Levin and Chen, 2004). Nicotine enhancers and nAChRr antagonists, such as mecamylamine, hinder memory (Levin et al., 1993, 1996; Felix and Levin, 1997). The effect of nicotine on improving memory function is a common finding across a wide variety of species, including rats, mice, monkeys and humans (Levin and Simon, 1998; Levin et al., 2003).

In the present work, nicotine and mecamylamine were used to validate the technique. Nicotinic acetylcholine receptors (nAChRs) have been shown to be important for maintaining optimal memory performance, and their malfunction is implicated on a variety of CNS disorders including nicotine addiction, neurodegeneration and emotional disorders (Picciotto et al., 2000; Ankarberg et al., 2001).

Experiments performed in the second phase of this work aimed to analyze toxicological effects in the zebrafish *D. rerio* after exposure to an arsenic concentration considered safe by Brazilian legislation. The results showed that As^V impaired LTM, even at the concentration considered safe for aquatic fauna (10 μg As/L). Previous studies indicate that arsenic can be toxic in fishes causing disturbance to the antioxidant system (Ventura-Lima et al., 2009) and by affecting the neurotransmitter system, and these alterations may be reflected in behavioral tests (Rodriguez et al., 1998).

Arsenate acts in the cholinergic systems (Rodríguez et al., 2003), through interactions with the sulphydryl groups involved in mechanisms of high-affinity uptake of choline and with the disulfide group of acetylcholinesterases (AChE) (Trevor et al., 1978). Glutamic acid decarboxylase (GAD), a marker of GABAergic system activity, was measured and found to be decreased in certain regions of the brain, while glutamate (Glu) content was increased in animals treated with arsenic (Nagaraja and Desiraju, 1993). Glutamate excess can be excitotoxic, leading to neuronal death (Tekkok et al., 2007). It is well established that cholinergic, gabaergic and glutamatergic systems are involved in memory consolidation and retrieval (Barros et al., 2001, 2003, 2005; Izquierdo et al., 2006). The occurrence of neuronal loss in the neurotransmission system may cause cognitive deficits.

Arsenic also acts as an oxidative stress generator. In recent years, evidences have been accumulated to show that reactive oxygen species are involved in arsenic toxicity, leading to oxidative damage of DNA, proteins and lipids (Bau et al., 2002; Shi et al., 2004; Wang et al.,

2007). In the present work, both arsenic doses increased protein oxidation in animals, in an intermediate molecular weight range. The presence of carbonylated proteins in tissue samples has become a widely accepted biomarker of oxidative stress (Samuel et al., 2005). An increase in carbonyl proteins under oxidizing conditions could create a high percentage of dysfunctional proteins that may be a major contributor to cell damage and death due to oxidative stress. Its is well known that proteins are susceptible to damage by ROS *in vitro* and *in vivo*, and protein modification may lead to a structural alteration and functional inactivation of many enzyme proteins. Enzyme inactivation is correlated with the formation of carbonyl groups (Halliwel and Gutteridge, 1999), these oxidative modifications are essential for enzyme activity.

In this study, DNA damage in terms of DNAPCs was not verified. Previous results suggest that arsenic only induces DNA damage at high concentrations (Bau et al., 2002). However, the work of Bau et al. (2002) indicates that different cell types have different sensitivity to arsenic. A possible explanation for such result is that the brain can be less sensible to damage at the DNA level, in the arsenic concentrations utilized in our experiment. Measurement of central nervous system competence to scavenger peroxyl radicals showed absence of antioxidant response after arsenic exposure. The lack of antioxidant capacity responses against peroxyl radicals was concomitant with the onset of protein oxidation, suggesting that a lack of compensatory response lead to an oxidative stress scenario. The inability of brain tissue to augment its antioxidant competence after pro-oxidants exposure was previously reported in *in vivo* studies with rats (Maidana et al., 2006; Barros et al., 2007).

As a general conclusion, results suggest that the one-trial inhibitory avoidance apparatus developed by our laboratory successfully evaluates learning and memory parameters and arsenic-caused deleterious effects in long-term memory in fish exposed for 96 h to three arsenic concentrations, including the highest concentration considered safe for preservation of aquatic fauna by Brazilian legislation (10 μg As/L). At this level a higher number of oxidised proteins was detected, in the range of 86.01–131.0 kDa.

Fish life is intimately associated with numerous inter-specific (e.g. predation) and intra-specific (both reproductive and non-reproductive) interactions, which invariably depend on the performance of appropriate behaviors. In that manner, behavioral interactions associated with predator avoidance, reproductive, and social behaviors, form an important part of a successful, adapted life strategy. Alterations of normal behavioral patterns caused by exposure to pollutants therefore pose serious risks to the success of fish populations. Many contaminants disrupt normal fish behavior after exposures much less severe than those causing significant mortality; in this manner, behavioral indicators of toxicity are ideal for assessing sub-lethal impacts of pollutants. The results obtained in this study are of extreme importance when considering that that amnesia may affect behaviour, ecology and preservation of the animal species.

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