Environmental Toxicology and Chemistry

Environmental Toxicology

ACUTE TOXICITY OF ARSENIC AND OXIDATIVE STRESS RESPONSES IN THE EMBRYONIC DEVELOPMENT OF THE COMMON SOUTH AMERICAN TOAD *RHINELLA ARENARUM*

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Environ Toxicol Chem., **Accepted Article** • DOI: 10.1002/etc.2856

Environmental Toxicology **Environmental Toxicology and Chemistry**
DOI 10.1002/etc.2856

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Running title: Effects of arsenic in toad embryos

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Submitted 21 May 2014; Returned for Revision 10 October 2014; Accepted 12 December 2014

Abstract: Arsenic, a natural element of ecological relevance, is found in natural water sources throughout Argentina in concentrations between 0.01 and 15 mg/L. The autochthonous toad *Rhinella arenarum* was selected to study the acute toxicity of As and the biochemical responses elicited by the exposure to As in water during its embryonic development. The LC50 value averaged 24.3 mg/L As and remained constant along the embryonic development. However, As toxicity drastically decreased when embryos were exposed from "heartbeat"-stage on (4 d of development), suggesting the onset of detoxification mechanisms. Given the environmental concentrations of As in Argentina, there is a probability of exceeding lethal levels at 1 percent of sites. Arsenic at sublethal concentrations caused a significant decrease in the total antioxidant potential, but generated an increase in endogenous GSH content and GST activity. This protective response might prevent a deeper decline in the antioxidant system and further oxidative damage. Alternatively, it might be linked to As conjugation with GSH for its excretion. The authors conclude that toad embryos are more sensitive to As during early developmental stages and that relatively high concentrations of this toxic element are required to elicit mortality, but oxidative stress may be an adverse effect at sublethal concentrations.

Keywords: Amphibians, Aquatic toxicology, Biomarkers, Glutathione S-transferase, Antioxidant potential

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INTRODUCTION

Arsenic (As) is considered a toxic element, widely distributed in the environment. It is mobilized by water and it can be released from natural sources by weathering of rocks, wind-blown dirt and volcanic activity, or from human activities such as mining, industries, and application of fertilizers and pesticides, followed by atmospheric deposition [1,2]. Arsenic can be found as both inorganic and organic compounds, with variable oxidation states and differential toxicity. Inorganic As is the predominant form in surface and underground water reservoirs [3]. Argentina was the first country in Latin America where As occurrence in groundwater was reported. In natural water sources throughout this country, concentrations of total As between 0.01 and 15 mg/L were found. The clinical manifestations in individuals who consume water from these highly As-contaminated sites were described a long time ago, including cardiovascular and cutaneous manifestations of this disease and collectively named as "Chronic Endemic Regional Hydroarsenicism" [4].

One of the most important causes of As toxicity is an imbalance between pro-oxidant and antioxidant homeostasis that results in oxidative stress [5]. Besides, As can react with protein and non protein thiol groups leading to the alteration of signaling pathways, structural modifications and enzyme inactivation [6], that may stimulate reactive oxygen species (ROS) production [5]. Oxidative stress is recognized as a possible mechanism implicated in both arsenic-induced apoptosis and carcinogenicity [7,8]. It has been demonstrated that the resistance to As toxicity in mammalian cells is correlated with higher levels of reduced glutathione (GSH) and GSH-related enzymes [5,6].

Arsenic is an element of ecological significance due to its toxicity, persistence and bioaccumulation [2,9]. The presence of As in water is a potential threat to aquatic ecosystems. It is accepted that amphibians are good indicators of overall environmental quality at freshwater sites because they live in both aquatic and terrestrial environments during larval and adult stages [10]. They are particularly sensitive to chemical contamination, due in part to their permeable skin and to their susceptibility to stress during metamorphosis. Despite the fact that there are well-established amphibian models such as *Xenopus laevis* for toxicological studies, it is also highly relevant the use of native species to assess ecotoxicological impact in a local context [11]. *Rhinella arenarum* is an

autochthonous toad of Argentina which inhabits the template and warm areas of this country, from Northern Patagonia (parallel 40°S) to Puna (parallel 23°S), including the agricultural productive area of the valley of Río Negro and Neuquén. This species spends its embryonic and larval stages in water bodies of the region. Accordingly, water quality greatly influences the growth and development of this species [12]. It has been shown that *R. arenarum* has a sensitivity towards diverse contaminants similar to other anurans [12–14]. Field studies have shown bioaccumulation of As both in tadpoles and adult amphibians at contaminated sites, as well as in laboratory-exposed tadpoles [2,10]. Arsenic ecotoxicity in amphibians has been a matter of controversy, as they appear to be able to develop and survive in highly contaminated water, in spite of the fact that inorganic As has long been considered toxic to frogs [10]. Nevertheless, the study of As toxicity is particularly limited in amphibians [15]. Considering these facts, our aim in the present study was to assess the acute toxicity of As in water during the embryonic development of *R. arenarum* and to determine the biochemical responses elicited by the exposure to sublethal and lethal As concentrations.

MATERIALS AND METHODS

Chemicals

Sodium (meta)arsenite ($NaAsO_2$) was purchased from Anedra (purity >99.95%). GSH, bovine serum albumin (BSA), 1,chloro,2,4-dinitrobenzene (CDNB), 5,5-dithio-bis-nitrobenzoate (DTNB) and luminol sodium salt were purchased from Sigma Co. Trolox, sodium hydroxide (NaOH) and 2,2'Azobis(2-methylpropionamidine) dihydrochloride (AAPH) were purchased from Aldrich Chemical Co. All the other reagents used were also of analytical grade.

Toad embryo development and assessment of arsenic toxicity

Adult *R. arenarum* were collected in Los Barreales Lake (S38.45344 W68.72918), a pristine environment, during the breeding season (spring and early summer). Animals used in this study were treated with regard for the alleviation of suffering according to recommended standards [16]. Upon collection, adults were transported to an outdoor terrarium. Ovulation was induced by intraperitoneal injection of 2500 international units (IU) of human chorionic gonadotrophin (ELEA Laboratory, Buenos Aires, Argentina). Freshly extruded eggs were fertilized *in vitro* with a testicular homogenate. To assess arsenic toxicity, thirty minutes after fertilization, groups of fifty

embryos were transferred to glass receptacles containing either amphibian Ringer's solution (0.65 g/L NaCl; 0.01 g/L KCl; 0.02 g/L $CaCl_2$) alone (control group) or with different concentrations of As solution, keeping a ratio of 1 embryo per mL of solution [17]. In order to determine the lethal concentration fifty (LC50), solutions of 0, 10, 20, 25, 30, 40 and 50 mg/L As were prepared. Three independent experiments were performed and the treatments were carried out in duplicate. The solutions were renewed every 48 h until embryos reached the end of the embryonic development at the stage of "complete operculum" (CO, 7 d of development). Also, groups of 50 embryos were exposed to 0, 20 and 50 mg/L As from "heartbeat" (HB) stage (4 d post-fertilization, when heartbeat can be visually detected) until one day after the end of embryonic development (CO stage + 1), in the same set of experiments. The embryos were maintained at 18–20°C in a 12:12 h light: dark photoperiod, without feeding. Embryonic stages were assessed according to Del Conte and Sirlin [18]. Embryo morphology and mortality were evaluated with the aid of a stereoscopic microscope. Mortality was determined at HB, operculum at right (OR, 6 d, when the right gill is covered by the operculum) and CO stages by the absence of blood circulation in the gills and caudal fin, and the absence of heartbeat. Dead embryos were removed and were not included in any morphological or biochemical analysis.

Biochemical analysis

Sampling and homogenization. At the end of the continuous exposure when the CO stage was reached, samples consisting of 20 embryos were taken. Embryos were thoroughly washed with cold Ringer's solution, briefly cooled on ice and homogenized in 1mL of 143 mM potassium phosphate buffer pH 7.5 with 6.3 mM EDTA. The homogenates were divided into aliquots either for immediate GSH and total reactive antioxidant potential (TRAP) determinations, or centrifuged at 10,000 *x g* for 30 min at 4°C; the resulting supernatants were divided into aliquots and kept frozen until the enzymatic activity determinations were performed.

TRAP. The reaction mixture contained the free radical generating compound AAPH 10 mM and 200 μM luminol in 50 mM sodium phosphate buffer, pH 7.4. Aliquots of 10 to 20 μL of the crude homogenates were added to 3 mL of the reaction mixture to assess their antioxidant potential. The mixture was incubated at room temperature to generate luminescence once the antioxidant potential was overcome. The induction time for

chemiluminescence was measured in a liquid scintillation counter Wallac LC1010. Trolox was used as a standard reference for the induction time [19].

GSH content. Aliquots of 200 µL of the crude homogenates were added to 200 µL of trichloroacetic 10% and the mixture was centrifuged at 10000 *x g* during 15 min. GSH content was immediately measured in duplicate as acid-soluble thiols, using 100 µL of the supernatant and 1 mL of 1.5 mM DTNB in 0.25 M sodium phosphate buffer, pH 8.0. The mixture was incubated for 10 min and the absorbance at 412 nm was measured. Acid soluble thiols (AST) were quantified using a calibration curve with pure GSH as standard [20,21].

Enzymatic determinations

Glutathione S-transferase (GST). GST activity was assayed in a final volume of 1 mL of 100 mM phosphate buffer pH 6.5 containing 0.5 mM CDNB dissolved in 1% v/v acetonitrile and 2.5 mM GSH as substrate. Baseline (non enzymatic reaction) was continuously recorded at 340 nm, and 10 µL of the 10000 *x g*-supernatant was added. The changes in the absorbance were recorded and corrected for spontaneous reaction, and the mIU of enzymatic activity were calculated using a molar extinction coefficient of 9.6 mM^{-1} cm^{-1} [22].

Catalase (CAT). CAT activity was determined according to Beers and Sizer [23] with slight modifications [24]. The reaction was performed in 1 mL sodium phosphate buffer 50 mM pH 7.0 containing 25 mM H_2O_2. Baseline absorbance was controlled to be stable and equal to one unit of absorbance and 20 µL of supernatant were added to initiate the reaction. Specific activity (at 25mM H_2O_2) was expressed as IU/ mg protein using a molar extinction coefficient of 40 M^{-1} cm^{-1}.

Protein determination.

Protein content was determined according to Lowry et al. [25] using BSA as standard.

Data analysis

For the statistical analysis, data from the three experiments were used (n = 6 for each treatment). A block design was applied to include clutch effects on the results, and statistical differences between clutches and between treatments were assessed by ANOVA and Tukey *Post hoc* test. The LC50 for As in toad embryos was calculated by non-linear regression fitting of a logistic model to data [26]. The LC50 values for each stage were compared by

Student´s T-test. The No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC) values were statistically assessed from experimental data by ANOVA- Dunnet's test [14].

Probabilistic risk analysis

A probabilistic analysis for *R. arenarum* embryos was performed for acute risk taking into account maximum As levels detected in Chronic Endemic Regional Hydroarsenicism regions from Argentina [4,27]. Briefly, the reported values were ordered and probit regression was applied on their percentile distribution [28]. The probability to find environmental concentrations exceeding the LC50 , LOEC and NOEC values for As acute toxicity in *R. arenarum* was estimated from this environmental distribution curve using the fitted model.

RESULTS

Arsenic toxicity in toad embryos.

Arsenic caused a dose-dependent mortality in toad embryos exposed from fertilization until complete embryonic development. Mortality reached 100% at HB stage when embryos were exposed to the highest As concentrations (30, 40 and 50 mg/L As) (Figure 1). The LC50 value for As, assessed by non-linear regression fitting, was 24.1±1.2 mg/L at HB stage. No further increase in the percentage of mortality was observed as the embryonic development progressed to later stages, in spite of the increase on the exposure time. Consequently, the LC50 values at OR and CO stages remained similar to the one observed at HB stage (Table 1; $p>0.05$). The homogeneity of the response to As was also similar in the three stages, as observed from the fitted slopes (Figure 1, Table 1). The NOEC (mortality) value for the continuous exposure to As was 10 mg/L, determined at the end of embryonic development ($p=0.05$, vs. controls). For the concentration ranges used in this work, the LOEC was estimated in 20 mg/L As. On the other hand, As toxicity was significantly lower when embryos were exposed from HB stage until one day after the end of embryonic development. Embryos exposed to 50 mg/L As showed a mortality of 17.5% when they reached CO + 1 ($p=0.012$ vs. control), a value that is clearly lower than the one registered when embryos were exposed to As from fertilization until complete embryonic development (100% mortality) (Figure 1). No particular malformations were observed in As-treated toad embryos. Neither embryos exposed to 10, 20 and 25 mg/L As from the beginning of the development nor those exposed to 20 and 50 mg/L As

from HB stage on displayed significant differences in the percentage of malformations when compared to the control group (data not shown).

A probabilistic distribution of sites in Argentina containing As in superficial water was performed (number of data *N*= 66), showing a very good fitting for probit model (R^2=0.993; Figure 2). The probability of exceedance estimated from the model equation was 1.0 % of sites for the LC50 determined in continuously exposed *R. arenarum* embryos. The LOEC (mortality) was exceeded in 1.3 % of sites, while the NOEC was exceeded in 3.5 % of sites in probabilistic terms.

Biochemical responses to arsenic

The effects of exposure to As on biochemical markers were evaluated at the CO stage. The statistical analyses by ANOVA in block design for all the biomarkers indicated that there were no significant effects between the different clutches (experiment replicates; $p>0.05$). The exposure to the sublethal concentration of 10 mg/L As caused a significant decrease of 20% (p=0.038) in TRAP (Figure 3A). Embryos exposed to As concentrations around the LC50 (20 and 25 mg/L) also showed a significant decrease of 24% (p=0.025) and 33% (p=0.008) in TRAP, respectively. Endogenous GSH measured as AST content showed a significant increase of 1.5 fold and 2.5 fold in embryos exposed to 10 and 20 mg/L As, respectively, when compared to control values (Figure 3B; p = 0.00034 and 0.00016 respectively). However, AST content in embryos exposed to 25 mg/L As was similar to control values. The contribution of GSH (AST) to the total antioxidant capacity showed a significant increase in embryos exposed to 10 and 20 mg/L As (0.6 and 2.25-fold vs. control; p=0.033 and 0.00018 respectively), but was similar to control values in embryos exposed to 25 mg/L As (Figure 3C). Control CAT activity was high and averaged 106.6 IU/ mg protein. CAT activity tended to decrease in embryos exposed to all As concentrations, reaching a maximum reduction of 20% in embryos exposed to 20 mg/L (Figure 4A). However, none of these differences was statistically significant. The activity of GST showed a significant increase in embryos exposed to all the concentrations of As tested, reaching a maximum increase of 30 % (p=0.00056) in embryos exposed to 25 mg/L, when compared to the control group (Figure 4B).

DISCUSSION

In the present study, we report that As causes mortality at relatively high concentrations in *R. arenarum* toad embryos continuously exposed from fertilization until complete embryonic development. Noticeably, LC50 remained the same from HB stage to CO stage, averaging 24.3 mg/L As. This result suggests that early developmental stages previous to HB stage are the most sensitive to the toxic effects of As. Afterwards, no additional signs of acute toxicity or mortality were observed. Thus, the embryos surviving the acute exposure to As are able to overcome its toxicity and/or to develop mechanisms of resistance. This is also supported by the fact that the exposure from day 4 of development on (HB stage) showed a significantly higher resistance to As compared with exposure from fertilization. To illustrate this point, the LC50 for embryos exposed from fertilization was on average 24.3 mg/L, and 50 mg/L caused 100% mortality, while the same concentration hardly caused mortality in embryos exposed to As from day 4. The decrease in toxicity observed when exposure to As started in advanced embryonic stages may be suggesting a substantial change in As toxicokinetics, which might involve new transport and/or excretion mechanisms being expressed with the progression of development, and which are absent in the first embryonic stages. LC50 could not be determined in embryos exposed to As from HB stage, because the mortality values registered were below 50%, even when exposed to the highest concentration tested (50 mg/L As). Similar results were reported for *R. arenarum* tadpoles exposed to sodium arsenite from CO stage on (after the embryonic phase is concluded), where LC50 values varied between 46 and 57 mg/L As and 4 to 17 d of exposure were evaluated [15]. Other aquatic organisms exposed to sodium arsenite show LC50 values in the order of mg/L As: the 96-h LC50 of sodium arsenite for juvenile rainbow trout (*Oncorhynchus mykiss*) is 21.0 mg/L [29], and the 7-day LC50 of sodium arsenite for Japanese medaka (*Oryzias latipes*) is 15 mg/L [30].

We estimated a NOEC value of 10 mg/L As for the continuous exposure to sodium arsenite and a LOEC value for survival of 20 mg/L As. These values are similar to those reported by Brix et al. [31] in nauplii of the crustacean *Artemia franciscana* exposed to different concentrations of sodium arsenite (NOEC: 8 mg/L As, LOEC: 15 mg/L As). Taking into account that hydroarsenicism is widely distributed in Argentina, we could estimate from a probabilistic distribution approach a relevant percentage of sites where the embryonic development of *R. arenarum* would be affected. According to our study, there is a probability of 1.0 % to find sites contaminated with

As exceeding the LC50 value for toad embryos and a probability of 3.2% to find sites that exceed the NOEC-lethality value. If sublethal adverse effects resulting from lower As levels are considered, a higher risk probability would be expected. We are currently investigating the effects of chronic exposure to low levels of As, ranging from 0.01 to 1 mg/L As, in toad embryos and larvae; according to the estimated exceedance probability (from 98.8% to 32.6%, respectively), it is likely that toad embryos which inhabit contaminated sites suffer from chronic-sublethal effects due to As exposure. No macroscopic alterations in *R. arenarum* embryos could be observed along their embryonic development, even at the highest concentrations of As evaluated. On the contrary, other toxicants such as pesticides are able to cause malformations along with oxidative stress in *R. arenarum* embryos [32].

Our results suggest that the exposure to As alters the oxidative metabolism of toad embryos while embryonic progression to larvae seems normal. Oxidative stress in *R. arenarum* embryos exposed to As is manifested by the decrease of the total antioxidant capacity along with a tendency of CAT activity to decrease. Depending on the intensity of ROS production, CAT activity may be either increased due to enzyme induction or inactivated through a direct inhibitory effect on the heme group of the active center [33]. The significant decrease in TRAP in *R. arenarum* embryos suggests a decrease in the ability of the antioxidant system to overcome the oxidative stress elicited by the toxicant. Similarly, Guidi et al. [34] have reported that bivalves (*Unio pictorum*) transplanted to different sites of a metal-polluted river basin showed a significant decrease in the ability to eliminate oxyradicals. On the other hand, an antioxidant response is evidenced by the induction of GST activity and the increase in GSH content. This antioxidant response would prevent a deeper decline of the total antioxidant potential in embryos exposed to As. Reduced glutathione plays an important role in the preservation of cellular redox status and defenses against ROS and xenobiotics [35]. One of the mechanisms involved in the antioxidant response is the transcription factor-mediated activation of genes containing an "Antioxidant Response Element" (ARE), the Nrf2-ARE pathway; the antioxidant enzyme GST-2 is up-regulated by oxidative stress through this mechanism [36]. Ferrari et al. [32] described the induction of GST activity due to pesticide exposure at the end of *R. arenarum* embryonic development (CO stage). They reported that the role played by the GSH-dependent system as an antioxidant defense is quite different from that of CAT and SOD along the embryonic development in *R. arenarum* [24,32], as suggested in other anuran species [37]. CAT constitutes one of the most important

antioxidant defenses during the early stages of development in anuran, while the GSH-dependent system gains importance at the end of the embryonic development. Thus, GST induction due to As exposure would protect *R. arenarum* embryos at the end of their embryonic development by preventing oxidative stress-induced damage at relatively low and sublethal levels. The change in As toxicity suggested by our results and the hypothesis of an activation of excretion mechanisms may be linked to the increase in GSH levels. Reduced glutathione participates as a reducing agent in As biotransformation [38], and in its excretion as arsenic-glutathione conjugates by multidrug resistance-associated protein-2 (MRP2) [39]. Active embryonic organogenesis is coincident with embryo hatching [37] that occurs one stage before HB stage. At this point of development, gills become fully active and GSH-dependent metabolizing enzymes gain relevance together with the increase in GSH levels [24]. These metabolic and physiological changes may thus contribute to the differential sensitivity to As exposure observed in the first embryonic stages and after HB stage. It has also been reported that low levels of As affect the antioxidant responses by increasing glutamate cysteine ligase activity and GSH levels in zebrafish, even at a concentration considered safe for the environment [40]. Whether low levels of As, closer to the environmental concentration limits considered safe, are able to cause oxidative stress and disturbance of cell metabolism in *R. arenarum* embryos, is currently a matter of study in our laboratory.

We conclude that *R. arenarum* embryos are more sensitive to As during early developmental stages and that relatively high concentrations of the toxic element are required to elicit mortality. At sublethal As concentrations, oxidative stress is observed and the increase in GSH levels and GST activity at late embryonic stages would probably act as a protective response to prevent further oxidative damage and help As excretion by conjugation.

Acknowledgment—This work was supported by grants from the Universidad Nacional del Comahue 04 I 004 and from the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT) PICT-2007-00214.

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Figure 1. Mortality in embryos of *R. arenarum* continuously exposed to As from fertilization (closed symbols), or from heart beat stage on (HB, 4 days of development) (open symbols). Data represent the mean ± standard error of 3 independent experiments, with treatments in duplicate (n = 6). No significant differences were found between the stage responses in the mortality profiles, either for the "fertilization on" exposed embryos (see Table 1) or for the "HB on" exposed embryos (p=0.05). OR: operculum at right, CO: complete operculum, CO+1: one day after CO stage.

Figure 2. Environmental exceedance probabilities determined from sites containing As in superficial water in Argentina. Data of maximal As concentrations at diverse sites in Argentina published by Bundschuh et al. and Farnfield et al. [4,27] were ordered to calculate the percentile distribution, and a probit regression was applied using the corresponding log-transformed concentration data.

Figure 3. Effects of As on the antioxidant capacity (TRAP) (A) and on the GSH content (B) measured in CO embryos of *R. arenarum* continuously exposed from fertilization until CO stage to 0, 10, 20 and 25 mg/L As. C. Contribution of GSH (AST) to the total antioxidant capacity. Data represent mean ± standard error of 3 independent experiments, with treatments in duplicate (n = 6). Different letters indicate significant differences: p <0.05 determined by ANOVA- Tukey *Post-hoc* test.

Figure 4. Effects of As on CAT (A) and GST (B) activity measured in CO embryos of *R. arenarum* continuously exposed from fertilization until CO stage to 0, 10, 20 and 25 mg/L As. Data represent mean ± standard error of 3 independent experiments, with treatments in duplicate (n = 6). Different letters indicate significant differences: p <0.05 determined by ANOVA- Tukey *Post-hoc* test.

Table 1: Fitted parameter values for As mortality in the logistic curves shown in Fig. 1 at HB, OR and CO stages.

Stage	LC50 (mg/L)	Slope	Control survival (%)
HB	24.1±1.2	7.9±2.3	84.1±4.4
OR	24.7±1.2	9.2±2.8	79.0±3.6
CO	24.0±1.2	7.0±2.2	77.0±4.2

Data represent mean ± standard error of 3 independent experiments, with treatments in duplicate (n=6). No significant differences were observed (p=0.05; Student's T test)

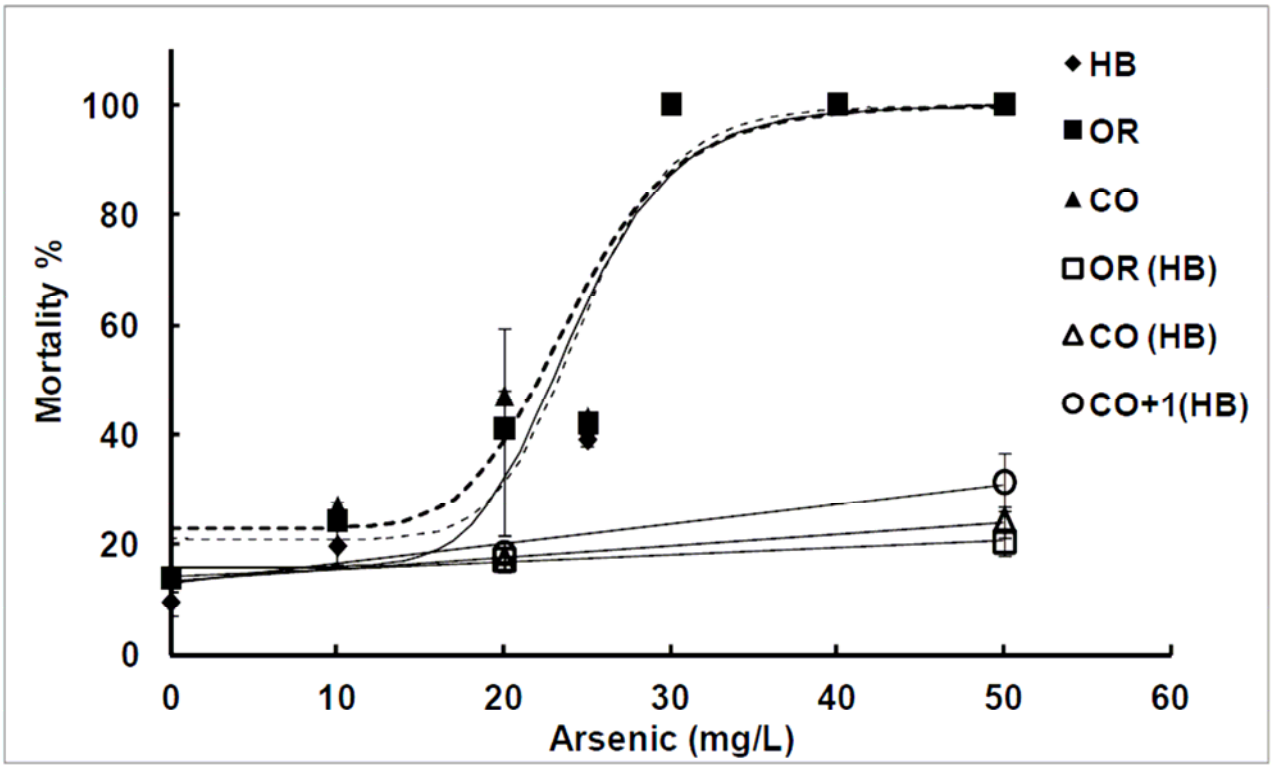


Figure 1

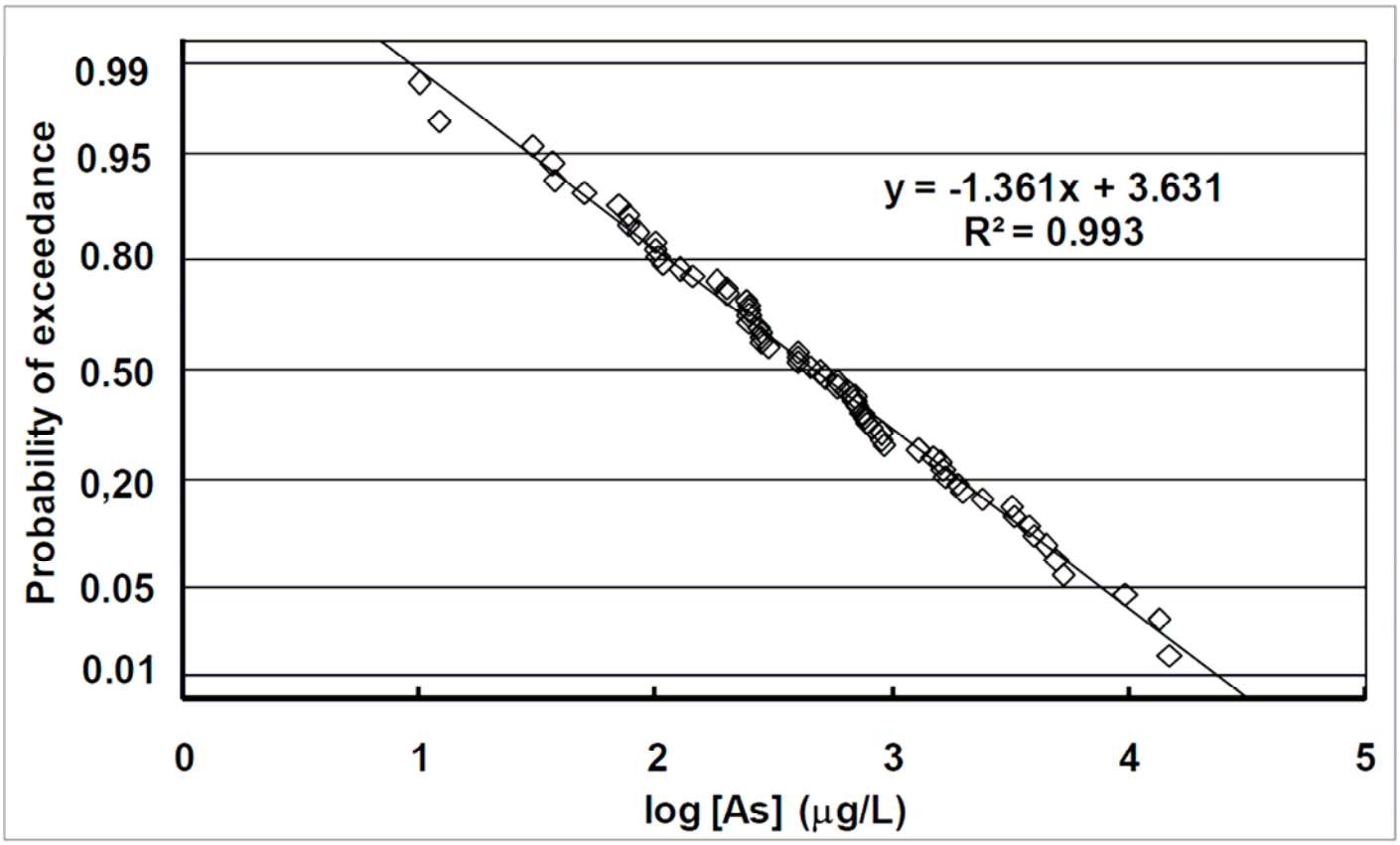


Figure 2

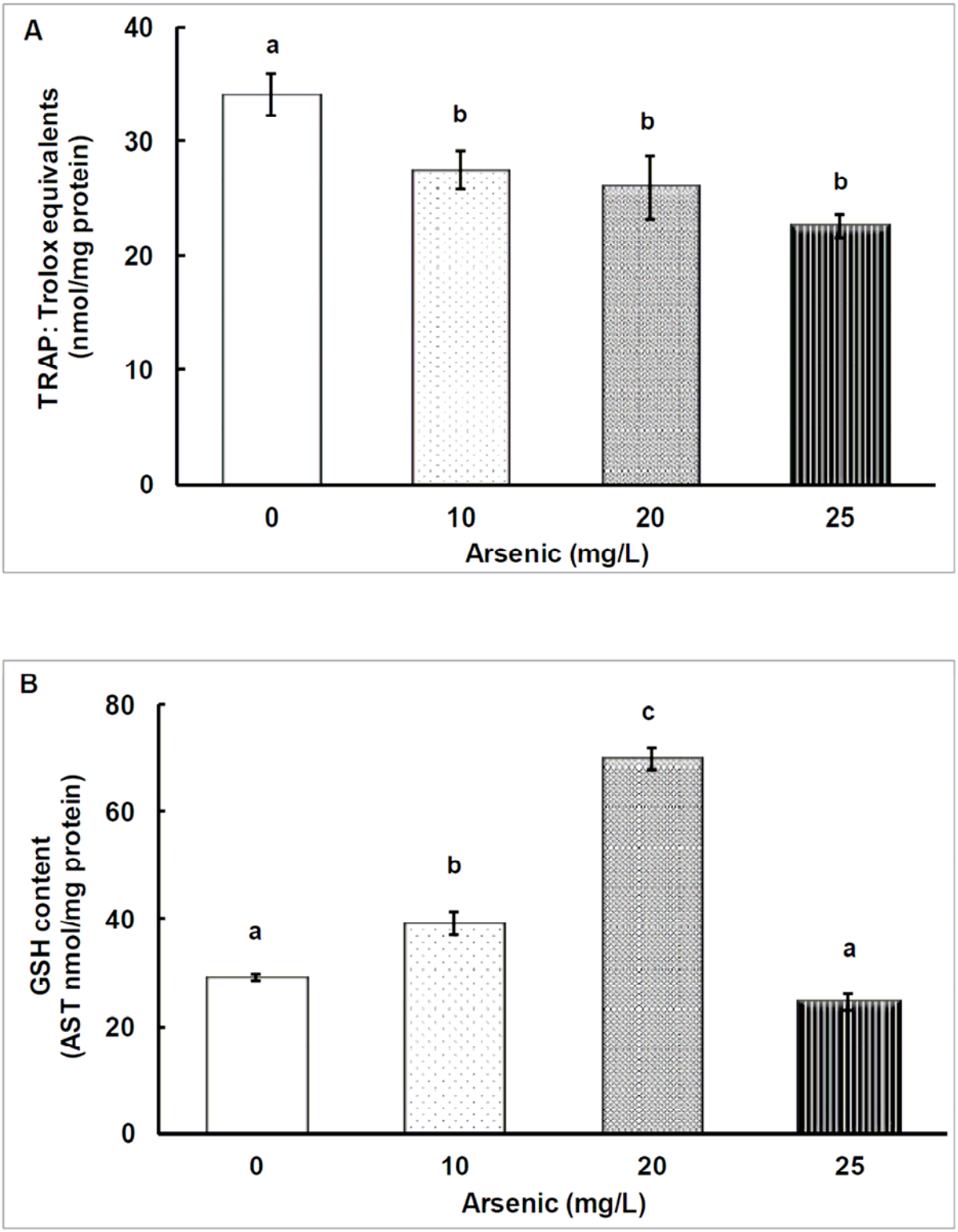


Figure 3a

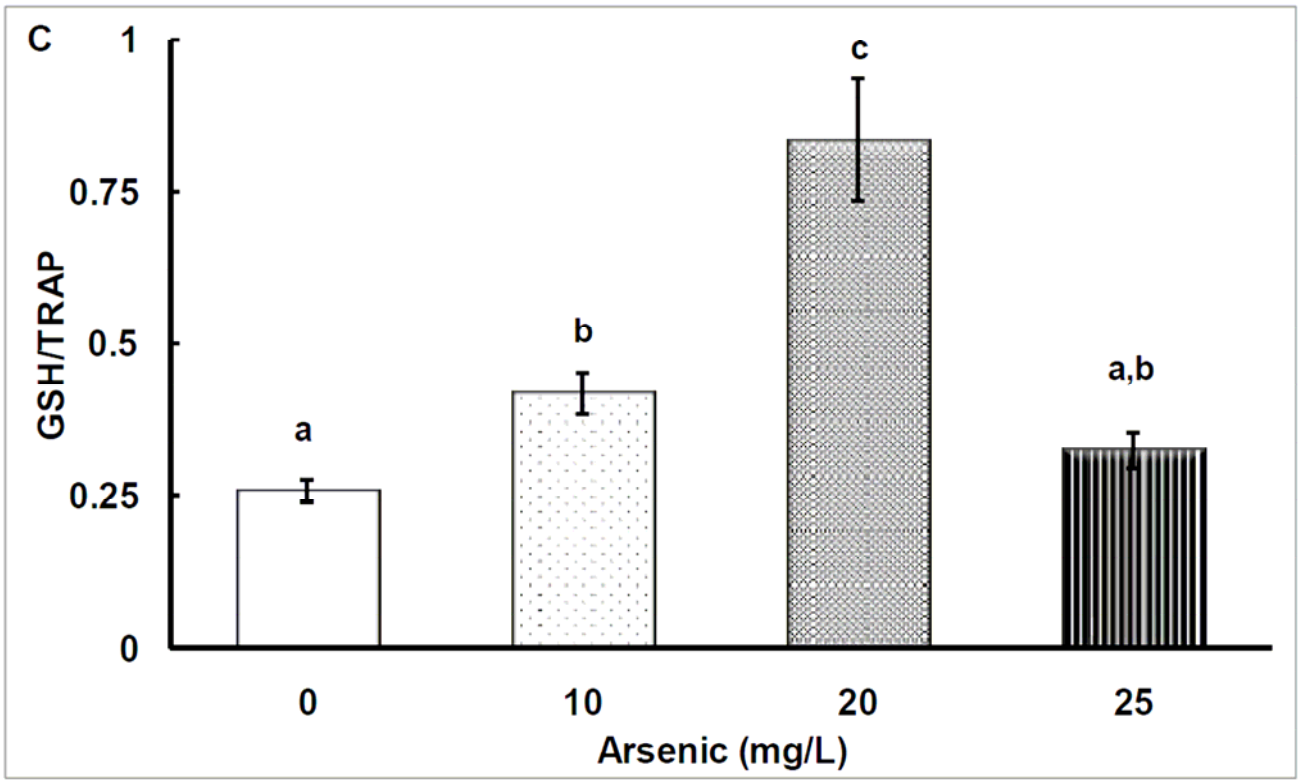
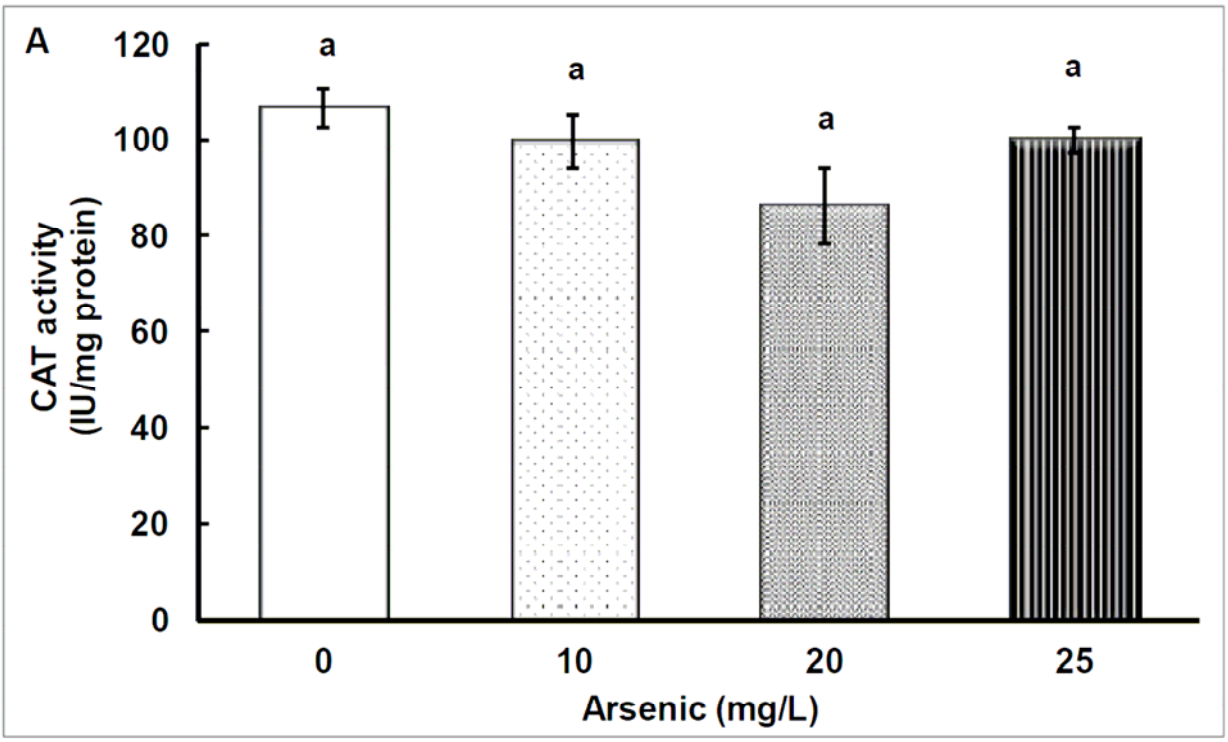


Figure 3b



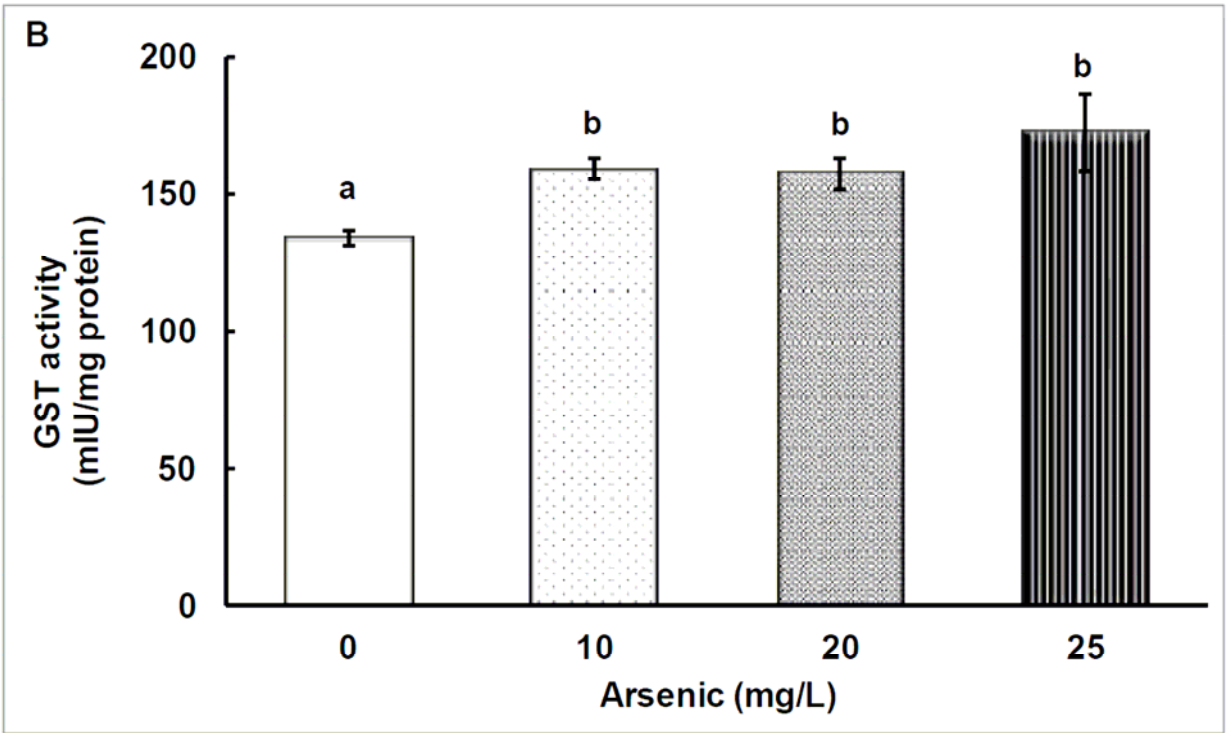


Figure 4