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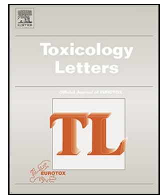

Full Length Article

Immunotoxicological effects of arsenic bioaccumulation on spatial metallomics and cellular enzyme response in the spleen of male Wistar rats after oral intake

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HIGHLIGHTS

- Arsenic is immunotoxic and accumulated in the spleen tissues.
- Arsenic causes spatial disturbances of metals in the spleen tissues.
- Arsenic modifies the activity of redox enzymes in splenocytes.
- Arsenic, metals, redox enzymes and spleen tissues are related.

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ABSTRACT

Arsenic (As) is a worldwide environmental contaminant, which compromises immunity and causes various associated disorders. To further investigate its immunotoxicity, male Wistar rats were exposed to 100 ppm of sodium arsenite (inorganic AsIII) in drinking water for 2 months. Given that metals are signi ficant immune regulators, their content and distribution were analysed in spleen tissues, to then evaluate subsequent changes of redox enzyme responses in spleen parenchyma cells (splenocytes). X-ray fluorescence spectrometry demonstrated As accumulation in both white and red pulps ($p < 0.005$), and As-related pulp-dependent modifications of the content of Cu, Ca, Zn and Fe ($p < 0.01$). Correlational path analysis revealed direct effects of As on their spatial distribution (Cu: −0.76, Ca: −0.61, Zn: 0.38; $p < 0.02$). As-exposed splenocytes showed γ-glutamyltranspeptidase inhibition, peroxidase induction, and variable responses of nitric oxide synthase ($p < 0.05$). Concanavalin A-treated splenocytes (T cell mitogen) were more susceptible *in vitro* to these As-related enzymatic changes than those treated with lipopolysaccharide (B cell mitogen) ($p < 0.05$). The study thus established the impact of As bioaccumulation on metallic spatial homeostasis in the spleen, and then identified enzymatic dysfunctions in splenocytes. This suggested that arsenic disrupts biometal-dependent immune pathways and redox homeostasis, with mitogen exposure modifying the toxicological response.
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1. Introduction

Arsenic (As) is a toxic metalloid that is widely present in the environment (20th element in the Earth's crust) and in the human body (12th element) (Jomova et al., 2011). Non-occupational human exposure is mainly by ingestion of contaminated food and

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drinking water. Groundwater contamination is known in 35 countries, with more than 130 million people in Asia and 14 million in Latin America being exposed (Bundschuh et al., 2012; Chakraborti and Nriagu, 2011).

Chronic exposure to arsenic causes, among others, skin lesions, vascular disorders, metabolic pathologies, neuropathies, nephropathies and many types of cancer (Grasso et al., 2011; Paul et al., 2013). Although these diseases are well-recognised, their molecular mechanisms need further study. Several hypotheses have been suggested about As-induced pathology (Hong et al., 2014), including overproduction of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Ray et al., 2014; Sharma et al., 2014). Arsenic also induces immune dysfunctions, such as chronic inflammation, autoimmunity, and immunodeficiency with opportunistic illnesses (Schulz et al., 2002; Tarantino et al., 2013). However, its specific immune effects remain only partly understood.

After intake, As is well absorbed in rats, reaching the bloodstream and binding to erythrocytes (Biswas et al., 2008). Rats exhibit a unique haemoglobin alpha chain with a cysteine residue, which strongly binds and retains trivalent As (Lu et al., 2007). It then deposits in the liver, kidneys, lungs, skin, and spleen (Cui and Okayasu, 2008; McClintock et al., 2012; Rubatto Birri et al., 2010), targeting lymphocytes (Ullah et al., 2015). The spleen has two areas (white lymphocyte-enriched pulp, and red erythrocyte-enriched pulp with macrophages) that may accumulate arsenic (Cui and Okayasu, 2008). This accumulation in the spleen alters the level and distribution of elements such as Fe, Cu and Zn in different rat organs, such as the kidney and brain (Rubatto Birri et al., 2010; Rubio et al., 2008). Other species also show effects of As on the tissue content of metals (Abdel-Gawad et al., 2016; García-Sevillano et al., 2013).

Therefore, our aim was to analyse arsenic-induced effects on the redox and metal homeostasis of the spleen and its cells, as possible mechanisms of immune impairment. To accomplish this, we had to assess the spatial distribution of metals in the spleen after sixty days of exposure to sodium arsenite in drinking water (100 ppm), using X-ray fluorescence analysis (XRF), which is non-destructive and performs multi-element analysis without prior preparation (Rubatto Birri et al., 2010; Rubio et al., 2008).

2. Materials and methods

2.1. Chemicals and supplies

Sodium arsenite ($NaAsO_2$) was purchased from Anedra (Argentina), rodent food from GEPSA Grupo Pilar SA (Argentina), and other reagents, standard reference materials and general chemicals from Sigma-Aldrich Co. (USA). Governmentally-certified water (As $<10\,\mu g/L$) was supplied by Aguas Cordobesas SA (Argentina). The possibility of food contamination with arsenic was assessed using conventional wavelength dispersive XRF spectrometry (with levels below the detection limit −1 μg/g-).

The standard reference material 1577c (National Institute of Standards & Technology, USA) and TORT-2 (lobster hepatopancreas reference material for trace metals with 21 mg/kg of As, Institute for National Measurement Standards, Canada) were used for SR-μXRF (micro X-ray fluorescence spectrometry using Synchrotron radiation). TraceCERT® standards in the cellulose matrix (iron −Fe-, copper −Cu-, zinc −Zn-, arsenic −As-; with 1 g/L As in nitric acid, Sigma-Aldrich Co., USA) were used for EDXRF (energy dispersive X-ray fluorescence spectrometry).

2.2. Animal model and samples

Optimal experimental conditions (dose, time, species, sex, age) for developing an animal model of chronic arsenic poisoning to emulate human exposure were previously published (Rubatto Birri et al., 2010). These conditions had no effect on feeding, body weight, organ weights (brain, spleen, kidneys, heart, liver, pancreas, and testes), behaviour, and survival. This model is according to the high chronic exposure to As of 4.5 million people throughout their lives in Latin America (McClintock et al., 2012). Animals (2-month-old Wistar male rats) were divided into two equal groups of eight. One group received drinking water *ad libitum* containing 100 ppm of sodium arsenite (equivalent to 57.7 ppm of As) for 60 days (≈5.5% of lifespan). The control group received drinking water without As (0 ppm) under the same conditions. Water was changed daily. The animals were sacrificed the next day by isoflurane inhalation, with all procedures in accordance with ethical concerns and good laboratory practices (86/609/CEE). After autopsy, spleens were obtained, cut into 0.5 mm slides and lyophilised for X-ray-based techniques. Spleen fractions were used to isolate splenocytes (from C and As-treated animals) for other assays according to a reported procedure (Soria et al., 2014), with cellular viability (>95%) being confirmed by trypan blue exclusion.

2.3. Splenocyte culture and *in vitro* assay

In order to confirm the effects of arsenic on spleen parenchyma cells, splenocytes from unexposed rats (200 μL with 1000 cells/μL in 96-well plates) were treated *in vitro* with 0 or 7.5 μg/mL of As (corresponding to As concentrations found by SR-μXRF in the white pulp of exposed spleens, with other authors supporting similar values in blood) (Nandi et al., 2008), for 72 h in accordance with a previous study of dose-response effects on cells (Soria et al., 2014), by using isolated cells maintained at 37 °C in a 5% CO_2 atmosphere (1000 cells per 1 μL of RPMI-1640 medium with 10% foetal bovine serum and 100 μM ciprofloxacin). Additionally, subsets were co-treated with 5 μg/mL of concanavalin A or 5 μg/mL of lipopolysaccharide, to evaluate the effects of arsenic on the mitogenic response of splenocytes (Soria et al., 2014).

2.4. Markers of redox response

The specific activity of three redox-related enzymes was measured in mechanically homogenised splenocyte cultures from all experimental groups by colourimetric methods, and protein content was determined by the Bradford method:

γ-Glutamyltranspeptidase (GGT, EC 2.3.2.2): 100 mM pH 8.5 Tris buffer with substrates (2.9 mM L-γ-glutamyl-3-carboxy-4-nitroanilide 100 mM glycylglycine) was added to the sample at a 1:10 ratio. Results were calculated as follows: ΔA/min (average difference of absorbance at 405 nm per minute) × 1158 (constant)/cellular proteins, to be expressed as pNA nmol/min/mg, under conditions of initial velocity and linearity (Quiroga et al., 2010).

Nitric oxide synthase (NOS, EC 1.14.13.39): L-citrulline formation was assayed by mixing samples (5% trichloroacetic acid-deproteinised supernatant) with chromogenic solution (1 part 0.5% diacetylmonoxime plus 0.01% thiosemicarbazide, 2 parts 0.025% $FeCl_3$ in a solution of 25% sulphuric acid and 20% phosphoric acid) at a 0.1:3 ratio. After heating for 5 min (96 °C) and cooling, 530-nm absorbance was recorded to calculate L-citrulline μmol/mg using a standard curve (Boyde and Rahmatullah, 1980).

Peroxidase (PO, EC 1.11.2.2): Chromogenic solution (ethanol 1% *O*-phenyldiamine (OPD) stable for 4 h), 0.05% H_2O_2 and sample were mixed (0.1/4.9/5, respectively) at 25 °C in darkness. The reaction was measured at 570 nm after 5-min incubation (linear kinetics up to 15 min). The ΔA/min was converted to OPD μmol/min/mg prot by multiplying by 0.0111 (derived from the extinction molar coefficient 9.9 $cm^{-1}\,mM^{-1}$ in 110 μL) (Bovaird et al., 1982).

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