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Arsenic and fluoride induce neural progenitor cell apoptosis

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ABSTRACT

The aim of the present study is to determine the effect of inorganic arsenic (As) and its metabolites on the viability of the neural progenitor cell (NPC) line C17.2, in order to evaluate cellular mechanisms involved in As developmental neurotoxicity. Moreover, we analyzed the effects of the coexposure to As and fluoride (F), a situation to which some populations are commonly exposed. Our results show that NPCs are not susceptible to pentavalent As species [arsenate, monomethylarsonic acid, and dimethylarsinic acid] and F alone. However, the trivalent metabolites of arsenate [arsenite, monomethylarsonous acid, and dimethylarsinous acid] are toxic at concentrations below 1 mg/l, and this susceptibility increases when there is coexposure with F (≥5 mg/l). Arsenite triggers apoptosis after 24 h of exposure, whereas monomethylarsonous acid produces necrosis at very short times (2 h). Arsenite leads to an increase in intracellular Ca levels and generation of reactive oxygen species, which may cause a decrease in mitochondrial transmembrane potential, release of cytochrome *c*, and consequent activation of caspases. A slight activation of calpain also takes place, which might favor activation of the mitochondrial pathway or might activate other pathways. The treatment with some antioxidants such as quercetin and α-tocopherol shows only a partial reduction of the cytotoxicity.

1. Introduction

Arsenic (As) is an element that is present in nature in a great number of chemical forms of varying toxicity. Inorganic arsenic [As(III) + As(V)] is considered a human carcinogen by the International Agency for Research on Cancer (IA group, IARC, 2004). Moreover, chronic exposure to inorganic As is associated with an increase in type 2 diabetes, cardiovascular and cerebrovascular problems, chronic obstructive respiratory diseases, and non-carcinogenic skin disorders (Haque et al., 2003; Wang et al., 2007; Parvez et al., 2010).

Intake of As through water and food is the main way of exposure for humans. A large part of the world's population consumes drinking water with an As concentration below 10 μg/l, the limit recommended by the World Health Organization (WHO, 2008), however, in some areas the concentration may exceed 100 μg/l. The predominant form of As in drinking water is arsenate [As(V)]. Its metabolism in the organism gives rise to other chemical species [arsenite, As(III), monomethylarsonic acid, MMA(V), dimethylarsinic acid, DMA(V), monomethylarsonous acid, MMA(III), and dimethylarsinous acid, DMA(III)], some of which, especially the trivalent forms, have greater toxicity than the initial compound (Petrick et al., 2000; Styblo et al., 2000).

Prenatal exposure to inorganic As could have a considerable effect on the health of the child population. Exposures to inorganic As since the moment of the child gestation have been associated with neurobehavioral disorders (Tsai et al., 2003; Wasserman et al., 2004). Some studies also describe the toxic effect of As during development of the nervous system. Arsenite affects neurite growth and complexity during development (Frankel et al., 2009). Dhar et al. (2007) showed that exposure to As(III) in the stage of rapid brain growth in rats (postnatal days 4–10) was associated with defects in migration, delayed maturation, and alteration in the nuclear area of the Purkinje cells of the cerebellum. Namgung and Xia (2001) and Chattopadhyay et al. (2002), using primary cultures of neonatal rat neural cells, showed a reduction of neuronal viability and morphological changes.

In some parts of the world, the presence of high concentrations of As in drinking water is combined with concentrations of fluoride (F) (Wang et al., 1997; Rocha Amador et al., 2007) that exceed the maximum recommended by the WHO (1.5 mg/l) (WHO, 2008). Fluoride is considered an essential trace element and its use is recommended to prevent dental caries and for bone development.

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However, studies conducted in China showed effects on neonatal neurobehavioral development after a high intake of F during pregnancy (Jing et al., 2004).

There are few studies on the effects of the coexposure to As and F. In animals, the exposure to both compounds does not necessarily lead to more pronounced toxicity in adult mouse brain and interestingly exhibits some antagonistic effects (Flora et al., 2009). In contrast, Rocha Amador et al. (2007) suggested that coexposure to these two elements may be associated with a reduction of cognitive capabilities in children aged 8–10. However, they did not show whether the neurobehavioral effect is due to individual exposure to one of these elements or is the result of coexposure.

The aim of the present study was to make an in vitro evaluation of the effects of inorganic As and its metabolites, and the coexposure to F on the viability of neural progenitor cells, in order to evaluate cellular mechanisms involved in As or As/F developmental neurotoxicity. For this purpose, it has been used C17.2 cell line, originally derived from the developing mouse cerebellum. This cell line has the ability to differentiate into neurons, astrocytes or oligodendrocytes (Snyder et al., 1992, 1997) and was employed as a model to evaluate the toxic effect on neural development of other elements, such as the methylmercury (Tamm et al., 2006) and manganese (Tamm et al., 2008).

2. Materials and methods

2.1. Arsenic solutions

The arsenic acid [As(V)] stock solution (1000 mg/l) was acquired from Merck (Merck, Germany). The stock solutions of As(III) (1000 mg/l) were prepared by dissolving 1.320 g of As_2O_3 (Riedel de Häen, Germany) in 25 ml 20% (m/v) KOH solution, neutralizing with 20% (v/v) H_2SO_4 and diluting to 1 l with 1% (v/v) H_2SO_4. The solutions of monomethylarsonic acid [MMA(V)] and dimethylarsinic acid [DMA(V)] were prepared by dissolving the appropriate amount of the following salts in water: $CH_3AsO(ONa)_2{\cdot}6H_2O$ for MMA(V) (Carlo Erba, Italy), and $(CH_3)_2AsNaO_2{\cdot}3H_2O$ for DMA(V) (Fluka Chemika Biochemika, Spain). The standards of MMA(III) (CH_3AsI_2) and DMAIII [$(CH_3)_2AsI$] were acquired from Argus Chemical (Italy). The fluoride standard (NaF, 1000 mg/l) was acquired from Panreac (Panreac, Spain). Throughout the entire text, concentrations of arsenic species are expressed as mg As/l.

2.2. Cell culture

The C17.2 cell line was donated by Dr. Evan Y. Snyder (The Burnham Institute for Medical Research; La Jolla, CA, USA) and was maintained in an undifferentiated state throughout the study. Cells were seeded at a density of 5×10^4 cells/cm^2 in 75-cm^2 flasks using 10 ml of Dulbecco's Modified Eagle Medium (DMEM) (Sigma, Spain), supplemented with 10% (v/v) fetal bovine serum (Gibco, BRL Life Technologies, Scotland), 5% (v/v) horse serum (Gibco), 1% L-glutamine 200 mM (Gibco), 1% (v/v) antibiotics (penicillin/streptomycin) (Gibco), and 1% (v/v) fungizone (Gibco) (DMEMc). The cells were incubated at 37 °C in an atmosphere with 95% relative humidity and a CO_2 flow of 5%. Medium was replaced every 2–3 days. When the cell monolayer reached 70%–80% confluence, the cells were detached with a solution of 0.05% trypsin with 0.02% EDTA (ethylenediaminetetraacetic acid, Gibco), and reseeded. All the treatments with As and/or F described here were applied with DMEMc medium free of fetal bovine serum and horse serum.

2.3. Cell viability assay

For the viability assays we used sodium resazurin (7-hydroxy-3H-phenoxazin-3-one-10-oxide sodium salt, Sigma). The assay is based on the ability of viable, metabolically active cells to reduce resazurin to resorufin and dihydroresorufin, measurable by colorimetric methods. This conversion is intracellular, facilitated by mitochondrial, microsomal and cytosolic oxidoreductases (O'Brien et al., 2000). Cells were seeded at a density of 45,000 cells/cm^2 in 24-well plates for 24 h and subsequently exposed to the various treatments.

The pentavalent species DMA(V) and MMA(V) were assayed at several concentrations (0.5, 2.5, and 5 mg/l) and times of exposure (24, 48, and 72 h). For As(V), concentrations of 0.5, 1, 5, and 10 mg/l were assayed at 24, 48, and 72 h. The trivalent As species were assayed at concentrations of 0.1, 0.5, 1, 2.5, and 5 mg/l and times of 4, 24, 48, and 72 h for As(III); 2, 4 and 8 h for MMA(III); 1, 2, 4, and 24 h for DMA(III). The trivalent methylated species were assayed at shorter times of exposure because of their chemical instability. The cytotoxicity assays for F were performed at the concentrations of 0.5, 2.5, 5, 10 and 25 mg/l for 24, 48 and 72 h.

The As–F coexposure studies were conducted at 24, 48, and 72 h, using three different concentrations of F (1, 2.5 and 5 mg/l) combined with three different concentrations of each arsenical species [As(V), MMA(V) and DMA(V): 0.1, 1, and 5 mg/l; As(III), MMA(III) and DMA(III): 0.1, 0.5, and 1 mg/l].

After exposure, the medium was withdrawn and the culture was washed with PBS (phosphate buffered saline). Then 500 μl of resazurin solution (10 μg/ml in DMEMc without serum) was added and it was incubated for 2 h at 37 °C in an atmosphere with 95% relative humidity and a CO_2 flow of 5%. A volume of 100 μl for each condition studied was transferred to a 96-well plate and resazurin reduction was measured colorimetrically (570 and 600 nm) using a PowerWave HT microplate scanning spectrophotometer (BioTek Instruments).

2.4. Apoptosis and necrosis assessment by Annexin V–FITC and propidium iodide (PI) staining

Apoptotic rates were analyzed by flow cytometry using Annexin V–fluorescein isothiocyarate (FITC)/propidium iodide (PI). Cells (6×10^4 cells/cm^2) were cultured in 24-well plates for 24 h to reach 60%–70% confluence, and were then incubated with the various treatments. The assays were performed with As(III) (0.1, 0.5, and 1 mg/l) at 24 h and MMA(III) (0.1, 0.5, and 1 mg/l) at 4 h. DMA(III) was not assayed because of its instability. Coexposure to As(III) (1 mg/l) and F (2.5 and 5 mg/l) at 24 and 48 h was also evaluated. A positive control treated with staurosporine (200 nM, Sigma) was also used.

Detached cells and trypsinized adherent cells were collected, pelleted, resuspended in 200 μl of binding Annexin buffer [10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, Sigma) pH 7.4, 140 mM NaCl (Panreac), 2.5 mM $CaCl_2$ (Panreac)]. Then 5 μl of FITC-conjugated Annexin V (Gibco) and 10 μl of propidium iodide (1 μg/ml, Sigma) were added to 100 μl of this suspension. Cells were incubated for 15 min in darkness at room temperature and, after making the volume up to 400 μl with Annexin binding buffer, analyzed by flow cytometry (Beckman Coulter Epics XL-MCL). The values were expressed as percentages with regard to the total of events evaluated, distinguishing viable cells [Annexin(−), PI(−)], necrotic cells [Annexin(−), PI(+)], and apoptotic cells [early: Annexin(+), PI(−) and late: Annexin(+), PI(+)].

2.5. Colorimetric detection of caspases activity

Colorimetric determination of the activity of caspases is based on the detection of colored *p*-nitroaniline (*p*NA) following a cleavage from chromogenic caspase substrates: Ac-DEVD-*p*NA (acetyl-Asp-Glu-Val-Asp-*p*-nitroaniline, Sigma), Ac-IETD-*p*NA (acetyl-Ile-Glu-Thr-Asp-*p*-nitroaniline, AnaSpec, AnaSpec Inc., San José, CA, USA), and Ac-LEHD-*p*NA (N-acetyl-Leu-Glu-His-Asp-pNA, AnaSpec), for caspase-3, -8, and -9, respectively.

To analyze caspase activity, cells were seeded into 6-well plates at a density of 8×10^4 cells/cm^2 for 24 h to reach 60%–70% confluence. The treatments employed were the following: As(III) 0.1, 0.5, and 1 mg/l at 24 and 48 h; As(III) 1 mg/l and F 2.5 mg/l at 24 and 48 h; As(III) 1 mg/l and F 5 mg/l at 24 and 48 h; MMA(III) 0.1, 0.5, and 1 mg/l at 2 and 24 h. A positive control was also used, treated with staurosporine (100–200 nM). After treatment, detached and trypsinized cells were combined, washed with PBS, pelleted, and incubated for 20 min in iced lysis buffer [50 mM HEPES (Sigma), 5 mM CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, Sigma), 5 mM DTT (Dithiothreitol, Sigma)]. After centrifugation, the supernatant was used for the caspase-3, -8, and -9 activity assay.

For caspase-3, 5 μl of the supernatant were incubated with 85 μl of the caspase assay buffer [HEPES 20 mM, 0.1% CHAPS, 2 mM EDTA (Sigma), 5 mM DTT, pH 7.4], and 10 μl of the chromogenic caspase substrate (2 mM). For caspase-8 and -9, supernatant (40 μl) were incubated with 50 μl of the assay buffer and 10 μl of substrate (2 mM). Plates were incubated for 180 min at 37 °C and absorbance was determined at 405 nm with a microplate scanning spectrophotometer (PolarSTAR OPTIMA, BMG-Labtech, Germany). The values were normalized by mg of protein, determined by Bio-Rad Protein Assay (Biorad, USA).

2.6. Mitochondrial transmembrane potential assay

The variations in membrane potential resulting from exposure to As(III) or As(III) and F were measured using rhodamine 123 (Rho 123, Sigma).

For this analysis, 4×10^4 cells/cm^2 were cultured in 24-well plates for 24 h to reach 60%–70% confluence, and were then incubated for 24 h with the following treatments: As(III) 0.5 and 1 mg/l; As(III) 1 mg/l and F 5 mg/l; As(III) 1 mg/l and F 10 mg/l. 30 min before eliminating the treatments, Rho123 was added at a final concentration of 5 μM. After treatment, detached and trypsinized cells were combined, pelleted, and resuspended in 200 μl of PBS. Then 10 μl of propidium iodide (1 mg/ml) was added to 100 μl of this suspension. Cells were incubated for 15 min in darkness at room temperature and, after making the volume up to 400 μl with PBS, analyzed by flow cytometry.

2.7. Fluorimetric detection of calpain activity

Cells were seeded into 24-well plates at a density of 3×10^4 cells/cm^2 for 24 h to reach 60%–70% confluence. The treatments employed at 6 and 24 h were the following: As(III) 0.5 and 1 mg/l; As(III) 1 mg/l and F 5 mg/l. After treatment, detached and