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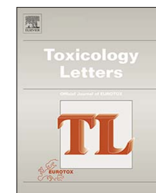

Effect of subchronic exposure to inorganic arsenic on the structure and function of the intestinal epithelium

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ABSTRACT

Inorganic arsenic (As), the most toxic form of As found in water and food, is considered a human carcinogen. Numerous studies show its systemic toxicity, describing pathologies associated with chronic exposure. The main pathway of exposure to inorganic As is oral, but many of the events that occur during its passage through the gastrointestinal tract are unknown.

This study evaluates the effect of subchronic exposure to inorganic As [As(III): 0.025–0.1 mg/L; As(V): 0.25–1 mg/L, up to 21 days] on the intestinal epithelium, using Caco-2 cells as *in vitro* model. Inorganic As produces a pro-inflammatory response throughout the exposure time, with an increase in IL-8 release (up to 488%). It also causes changes in the program of cell proliferation and differentiation, which leads to impairment of the cell repair process. In addition, subchronic exposure affects the epithelial structure, causing loss of microvilli, fundamental structures in the processes of intestinal absorption and digestion. Moreover, the exposure affects the epithelial barrier function, evidenced by an increase of Lucifer Yellow transport (103–199%). Therefore, it can be concluded that subchronic exposure to inorganic As can alter intestinal homeostasis, affecting the mucosal layer, which performs the most important functions of the intestinal wall.

1. Introduction

Arsenic (As) is a metalloid that is widely distributed in the environment. About 200 million people are affected by consumption of water contaminated with levels of inorganic As that exceed the maximum limits recommended by the World Health Organization (10 μg/L; WHO, 2012). Food is also an important source of exposure to inorganic As. Seaweed is the food with the greatest concentrations of this form of As, which can reach 140 mg/kg in *Hizikia fusiforme* brown seaweed (Almela et al., 2002). However, cereals, especially rice and rice products, are the foods that make the greatest contribution to intake of inorganic As because consumption of them is so high (EFSA, 2009). Moreover, in areas with chronic arsenicism, cooking with contaminated water can also cause substantial increases in levels of this metalloid in food (Diaz et al., 2015).

Arsenic has been classified by the Agency for Toxic Substances and Disease Registry as one of the 20 most dangerous substances present in the environment (ATSDR, 2000). Furthermore, the inorganic form of As is considered a carcinogenic agent for humans (IARC, 2004). Various target organs of inorganic As have been described: liver, kidney, bladder, skin, and central nervous system. Numerous *in vitro* and *in vivo* studies have been conducted on these systems, which have made it possible for knowledge about the toxicity of this metalloid to advance. On the other hand, there are few studies on its toxicity on a gastrointestinal level. However, the digestive system is the gateway through which As enters the blood circulation, where it exerts its systemic toxicity; moreover, the digestive system is in continuous contact with the contaminant, especially in cases of chronic exposure.

In populations chronically exposed to inorganic As through drinking water, symptoms of dyspepsia, gastroenteritis and chronic diarrhea have been reported (Borgoño et al., 1977; Guha Mazumder and Dasgupta, 2011). In rodents chronically exposed to high concentrations of monomethylarsonic acid [104 weeks; MMA(V) 10–1000 mg/L], a metabolite of inorganic As, it has been shown that the large intestine is the target organ (Arnold et al., 2003), and there have been observations of enlargements of the intestinal wall, edemas, hemorrhages and necrosis, ulcerations or perforations of the mucosa, as well as a significant increase in the incidence of squamous metaplasia of absorptive epithelial cells of the colon and rectum. *In vitro* studies have shown that acute exposure to trivalent forms of As generates a pro-inflammatory response (increase of cytokines IL-6, IL-8, and TNFα) and produces oxidative stress (Calatayud et al., 2013, 2014, 2015) in human colonic epithelial cells.

All the data reported in the studies cited indicate that inorganic As

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can have a toxic effect on a gastrointestinal level. The effects on the digestive system can affect important functions such as absorption or digestion of nutrients, and may also cause loss of the intestine's barrier function, which is necessary to avoid the passage of undesirable substances and microorganisms into the systemic circulation. The toxic effects on intestinal cells that have been demonstrated in acute exposures may be exacerbated in chronic exposures, even at lower concentrations. That is why it is necessary to evaluate the intestinal toxicity of As in these situations, which are frequent in many parts of the world, and also to determine what mechanisms are responsible for it.

The main aim of this study is to determine the alterations produced in the intestinal epithelium by continuous exposure to inorganic As, using Caco-2 cells as a cell model.

2. Materials and methods

2.1. Standard solutions of arsenic

The As(V) standard solution (1000 mg/L) was acquired from Merck (VWR). The standard of As(III) (1000 mg/L) was prepared by dissolving 1.320 g of As_2O_3 (Riedel de Haën) in 25 mL of 20% (m/v) KOH (Panreac), neutralizing with 20% (v/v) H_2SO_4 (Merck), and diluting to 1 L with 1% (v/v) H_2SO_4.

2.2. Conditions of cell culture and treatments with arsenic

The human colon carcinoma Caco-2 cell line was obtained from the European Collection of Cell Cultures (ECACC, number 86010202). The cells were maintained in 75 cm^2 flasks to which we added 10 mL of Dulbecco's Modified Eagle Medium (DMEM) containing 4.5 g/L glucose and 0.87 g/L glutamine. The medium was supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) non-essential amino acids (NEAA), 1 mM sodium pyruvate, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), 100 U/mL of penicillin, 0.1 mg/mL of streptomycin, and 0.0025 mg/L of amphotericin B (DMEMc). During the maintenance, the cells were incubated at 37 °C in an atmosphere with 95% relative humidity and a CO_2 flow of 5%. The medium was changed every 2–3 days.

When the cell monolayer reached 80% confluence, the cells were detached with a solution of trypsin (0.5 g/L) and EDTA (ethylene diamine tetraacetic acid, 0.22 g/L) and seeded in different supports for As exposure. For these treatments, we used DMEM supplemented as described above except for FBS. The quantity of FBS and the concentrations of As(III) and As(V) to be used were determined by evaluating two parameters: cell viability, by analyzing the reduction of resazurin sodium salt (Sigma), following the protocol described by Rocha et al. (2011), and the monolayer integrity, by microscopic visualization. After establishing the conditions for the study, we evaluated the toxicological parameters that are detailed below (Sections 2.3–2.8; Fig. S1 of Supplementary data). In all the cases, the cells were treated with As(III) or As(V) from the moment of seeding and the medium was replaced every 2 days by fresh As-spiked medium.

The assays were performed with cultures between passages 20 and 35. All the reagents used were obtained from HyClone Laboratories.

2.3. Pro-inflammatory response

The cells were seeded in 6-well plates at a density of 2.6×10^4 cells/cm^2, and they were exposed to As(III) (0.025, 0.075, and 0.1 mg/L) and As(V) (0.25, 0.75, and 1.0 mg/L) in DMEMc with 7.5% FBS. At the times stipulated (7, 14, and 21 days), the medium was recovered for analysis of the pro-inflammatory cytokine IL-8, using the ELISA Human IL-8/CXCL8 kit (Sigma), following the manufacturer's instructions.

2.4. Cell proliferation

Cell proliferation was evaluated by determining the number of cells at each stage of the cell cycle by analyzing the quantity of DNA. For this purpose, 6-well plates were seeded at a density of 2.6×10^4 cells/cm^2, and they were exposed to As(III) (0.025, 0.075, and 0.1 mg/L) and As (V) (0.25, 0.75, and 1.0 mg/L) in DMEMc with 7.5% FBS from the moment of seeding. The cells were analyzed at 2, 5, and 7 days post-seeding. After the treatment the cells were washed with PBS and recovered with trypsin/EDTA. The cell cycle was evaluated using the BD Cycletest™ Plus DNA kit (BD Bioscience), following the manufacturer's instructions. The samples were analyzed by flow cytometry (Beckman Coulter Epics XL-MCL).

2.5. Cell differentiation

The progress of intestinal cell differentiation was evaluated by analyzing gene expression of intestinal epithelium brush border proteins [isomaltase (*SI*), dipeptidyl peptidase 4 (*DPP4*), and villin 1 (*VIL1*)], transcriptionally up-regulated during enterocyte differentiation (Devriese et al., 2017) and of *MYC*, whose down-regulation is considered one of the essential mechanisms that allows the exit from the cell cycle and the onset of differentiation of Caco-2 cells (Leoni et al., 2012).

The cells were seeded in 6-well plates at a density of 2.6×10^4 cells/cm^2, and they were treated with As(III) (0.025, 0.075, and 0.1 mg/L) and As(V) (0.25, 0.75, and 1.0 mg/L) in DMEMc with 7.5% FBS for 5, 7, 14 and 21 days. After the exposure time the cell monolayer was washed with PBS and the cells were recovered with trypsin/EDTA for extraction of RNA using a NucleoSpin RNA II kit (Macherey-Nagel). The extracted RNA was quantified spectroscopically using a NanoDrop ND-1000 system (NanoDrop Technologies). First-strand complementary DNA (cDNA) was obtained from 200 ng of total RNA using a reverse transcriptase core kit (Eurogentec Headquarters).

qPCR was performed using the LightCycler® 480 Real-Time PCR system (Roche Diagnostics). Reactions were carried out in a final volume of 10 μL containing 5 μL LightCycler® 480 SYBR Green I Master

Table 1
Sequence and efficiency of the oligonucleotides used in the study of cell differentiation.

Gene	GenBank ID	Sequence 5′–3′	Amplicon (bp)	Efficiency
SI	*NM_001041*	F: AATCAGACACCCAATCGTTTCC R: GGGCAACCTTCACATCATACAA	134	2.12 ± 0.17
DPP4	NM_001935.3	F: GTGGCGTGTTCAAGTGTGG R: CAAGGTTGTCTTCTGGAGTTGG	111	2.01 ± 0.32
MYC	NM_002467.4	F: GTCAAGAGGCGAACACACAAC R: TTGGACGGACAGGATGTATGC	173	2.02 ± 0.07
VIL1	NM_007127.2	F: CTGAGCGCCCAAGTCAAAG R: AGCAGTCACCATCGAAGAAGC	127	2.04 ± 0.01
RN18S	NR_003286.2	F: CCATCCAATCGGTAGTAGCG R: GTAACCCGTTGAACCCCATT	151	2.00 ± 0.01

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