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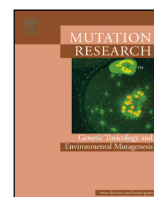

Arsenic and fluoride co-exposure affects the expression of apoptotic and inflammatory genes and proteins in mononuclear cells from children

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ABSTRACT

Humans may be exposed to arsenic (As) and fluoride (F) through water consumption. However, the interaction between these two elements and gene expression in apoptosis or inflammatory processes in children has not been thoroughly investigated. Herein, the expression of *cIAP-1*, *XIAP*, *TNF-α*, *ENA-78*, *survivin*, *CD25*, and *CD40* was evaluated by RT-PCR. Additionally, the surface expression of CD25, CD40, and CD40L on peripheral blood mononuclear cells was analyzed by flow cytometry, and TNF-α was measured by Western blotting. This study examined 72 children aged 6–12 years who were chronically exposed to As (154.2 μg/L) and F (5.3 mg/L) in drinking water and in food cooked with the same water. The urine concentrations of As (6.9–122.4 μg/L) were positively correlated with the urine concentrations of F (1.0–8.8 mg/L) ($r^2 = 0.413$, $p < 0.0001$). The *CD25* gene expression levels and urine concentrations of As and F were negatively correlated, though the *CD40* expression levels were negatively correlated only with the As concentration. Age and height influenced the expression of *cIAP-1*, whereas *XIAP* expression was correlated only with age. Additionally, there was a lower percentage of CD25- and CD40-positive cells in the group of 6- to 8-year-old children exposed to the highest concentrations of both As and F when compared to the 9- to 12-year-old group (CD25: 0.7 ± 0.8 vs. 1.1 ± 0.9, $p < 0.0014$; CD40: 16.0 ± 7.0 vs. 21.8 ± 5.8, $p < 0.0003$). PHA-stimulated lymphocytes did not show any changes in the induction of CD25, CD69, or CD95. In summary, high concentrations of As and F alter the expression patterns of CD25 and CD40 at both the genetic and protein levels. These changes could decrease immune responses in children exposed to As and F.

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1. Introduction

Arsenic (As) and fluoride (F) are elements widely distributed in nature and are frequently found together in diverse geological strata and aquifers. The main cause of human exposure to these two elements is the consumption of contaminated water and food [1].

Chronic exposure to As in drinking water is related to the development of arterial hypertension, peripheral vascular disease, diabetes mellitus [2,3], and an increased risk for several types of cancer [2,4–6]. The mechanisms through which As is thought to contribute to these pathologies include As-induced cellular damage, the induction of oxidative stress [4,7–9], and alterations in DNA methylation [10,11]. Studies of As-exposed populations have demonstrated unusual gene expression profiles for cell cycle control-related factors, transcription factors, and inflammatory molecules [9]. Additionally, changes have also been observed in the expression of genes related to immune system function in adults exposed to drinking water with 32 μg/L of As [12].

Fluoride is considered a pro-inflammatory factor because it induces reactive oxygen species and apoptosis in THP-1 monocytes [13] and lipoxygenase expression in peripheral blood monocytes

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[14]. An effect on regulatory T lymphocytes (Treg) was reported in populations exposed to F [15]. Moreover, hepatic and renal damage was observed in children exposed to drinking water with F concentrations higher than 2 mg/L [16].

In Mexico, there are regions with water containing As and F concentrations that exceed the current acceptable levels established by the Mexican Standard NOM-127-SSA1-1994 for human use and consumption (25 μg/L for As and 1.5 mg/L for F) [17,18].

Although As and F are found together in aquifers, the interaction between these two elements has not been investigated. A decrease of toxic effects at the biochemical level has been reported when As and F are administered together in drinking water to rats, suggesting a potential antagonistic effect of the two elements [19–21]. Using microarrays, our group determined that the *IL-6*, *IL-1β*, *TNF-α*, *TGF-β*, *CD40*, *IL-2RA*, *CD40L*, *CD25*, *ENA78*, *SURVIVIN*, *XIAP*, and *IAP-1* genes are differentially expressed in adults chronically exposed to high concentrations of As (22.5–148.9 μg/L) and F (2.3–5.4 mg/L) compared to the control group (As: 0.3–1.4 μg/L; F: 0.1–0.7 mg/L) [22]. However, the effects of exposure to drinking water containing As and F in children are unknown. Therefore, we analyzed the expression of genes and proteins associated with apoptotic and inflammatory processes in peripheral blood mononuclear cells from children exposed to drinking water contaminated with As and F.

2. Subjects and methods

2.1. Population

The study population consisted of 72 children from 6 to 12 years old in the community of Salitral de Carrera in the municipality of Villa de Ramos, which is located in the northeastern area of San Luis Potosí, S.L.P., México. The concentrations of As and F in the drinking water from that community were previously quantified at 22.5–148.9 mg/L and 2.3–5.4 mg/L, respectively [22]. The water consumption and nutritional and socioeconomic status of the children were evaluated using a questionnaire completed by the children's parents. Additionally, the health status of the population under study was evaluated through physical examination by a qualified physician. Hematic biometry and a general urine test were performed for each child. The children diagnosed with any disease or undergoing any drug treatment were excluded from the study. The bioethical committee of the Faculty of Medicine at the Autonomous University of San Luis Potosí approved this work. Informed consent was obtained from the parents of the children participating in the study.

2.2. Cells

A venous puncture was used to collect peripheral blood samples (10 mL) in vacutainer tubes coated with 15% EDTA to prevent coagulation. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll Hypaque density gradient centrifugation (Sigma–Aldrich, St. Louis, MO). The cells were subsequently cultured in RPMI 1640 medium (Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (FBS) (Sigma–Aldrich), 2 mM L-glutamine, 100 U penicillin, and 100 μg/mL streptomycin. For activation assays, the cells were cultured (1×10^6 cells/mL) in RPMI medium with or without 5 μg/mL phytohemagglutinin (PHA) (Sigma–Aldrich) for 24 h.

2.3. RT-PCR

RNA was isolated from PBMC with TRIzol (Invitrogen Life Technologies, Carlsbad, CA) and precipitated with isopropanol. The purity of the samples was determined by the 260/280 nm absorbance ratio using a spectrophotometer. To synthesize cDNA, 2 μg of total initial RNA was used with oligo(dT) primers and the SuperScript II Reverse Transcriptase enzyme (Invitrogen). The gene amplification was performed by mixing 20 ng/μL of cDNA with 20 μM sense and anti-sense primers, 1× PCR buffer, 0.05 mM dNTPs, and 0.02 μL of DNA Taq polymerase in a 25-μL total reaction volume. Amplification of the β-actin housekeeping gene was used as a control. The primer sequences are shown in Table 1 [22]. The amplification conditions were as follows: denaturation at 94 °C for 5 min, followed by 30–36 cycles of 94 °C for 30 s, 60–64.2 °C for 30 s, and 72 °C for 45 s, with a final extension step at 72 °C for 5 min. The products were separated on a 1.5% agarose gel and visualized after staining with ethidium bromide on a UV transilluminator. The resulting bands were further analyzed by densitometry using one-dimensional image analysis software (Kodak Digital Science, Rochester, NY).

Table 1
Primers sets used in PCR reaction.

Gene	Primer sequences
cIAP-1	Forward 5′-CAGACACATGCAGCTCGAATG-3′
	Reverse 5′-AAGCCACCATCACAACAAAAG-3′
XIAP	Forward 5′-GCCTTAGACAGGCCATCTGAGA-3′
	Reverse 5′-TTCCTCGGGTATATGGTGTCTGAT-3′
TNF-α	Forward 5′-GAAAGC ATGATCCGGGACGTG-3′
	Reverse 5′-GATGGCAGAGAGGAGGTTGAC-3′
CD40	Forward 5′-TGCCAGCCAGGACAGAAACT-3′
	Reverse 5′-GGGACCACAGACAACATCAG-3′
ENA-78	Forward 5′-GAACCCGCGACCGCTCGC-3′
	Reverse 5′-AGAAAAGGGGCTTCTGGATCAA-′3
IL2-RA	Forward 5′-AAGCTCTGCCACTCGGAACACAAC-3′
	Reverse 5′-TGATCAGCAGGAAAACACAGC-3′
Survivin	Forward 5′- GATTTGAATCGCGGGACCCGTTG-3′
	Reverse 5′-TCAAGACAAAACAGGAGCACAGT-3′
β-Actin	Forward 5-′CGGGACCTGACCGACTACCT-3′
	Reverse 5′-GGCCGTGATGTC CTTCTGC-3′

2.4. Flow cytometry

PBMC were incubated in phosphate-buffered saline (PBS) containing 2% FBS and anti-CD40-(FITC), anti-CD40L-(FITC), or anti-CD25-(FITC) (eBioscience, San Diego, CA) antibodies for 20 min at 4 °C. The cells were then washed and fixed with 1% paraformaldehyde in PBS. All the data were acquired with the FACSCalibur flow cytometer (Becton Dickinson, San José, CA) and analyzed with CellQuest Pro Software (Becton Dickinson). The results are expressed as the percentage of positive cells.

2.5. Western blotting

PBMC were lysed using a protease inhibitor cocktail (Sigma–Aldrich) in lysis buffer (25 mM Hepes, 150 mM NaCl, 1.5 mM $MgCl_2$, 0.200 mM EDTA, and 0.5% Triton X-100). The protein lysates were then incubated at 4 °C with shaking for 30 min and then subjected to centrifugation. The supernatant was subjected to the bicinchoninic acid method to determine the protein concentration present in the sample (Thermo Fisher Scientific, Rockford, IL). The protein concentration was measured using a microplate reader spectrophotometer (Multiskan Labsystems, MTX Lab Systems, Vienna, VA). The protein pellet (30 μg) was carefully dissolved in protein loading buffer and boiled at 95 °C for 5 min. The samples and the western C molecular weight protein standard (BIO-RAD Laboratories, Hercules, CA) were separated by 15% SDS-PAGE and transferred to a nitrocellulose membrane using the trans-blot cell system (BIO-RAD). The membranes were blocked in 1× TBS (0.5 M Tris-base and 9% NaCl, pH 8.4) containing 3% BSA and 0.1% Tween-20. The membranes were incubated with goat anti-human TNF-α (R&D Systems, Minneapolis, MN) diluted 1:500 and then with anti-goat IgG-HRP diluted 1:1000 (R&D Systems). The Strep Tactin-HRP conjugate (BIO-RAD) diluted 1:40,000 was also added to detect the unstained protein standard. After washing the membranes in TBS with 0.1% Tween-20, the membrane was incubated with an enhanced luminol reagent substrate (Pierce, Rockford, IL) according to the manufacturer's instructions. The protein bands were visualized using the v3 western workflow system (Bio-Rad). The TNF-α signal intensity was normalized to β-actin. The group means for the subjects exposed to As and F and for the control subjects were compared. All the assays were performed in duplicate.

2.6. Fluoride determination

Fluoride was quantified in solution as fluorine (F-) by a potentiometric method with a selective ion electrode using the Beckman ϕ350 potentiometer and Fluorine Ion Selective Electrode (Beckman, Brea, CA). Analyses were performed on three independent urine samples and were corrected for specific gravity [23]. Both the standards and samples were analyzed in triplicate.

2.7. Arsenic measurement

The quantification of total As was performed using atomic fluorescent spectrophotometry with hydride generation. The PS Analytical 10.055 Millennium Excalibur System (PS Analytical, Deerfield Beach, FL) equipped with an empty cathode lamp was used for the analysis. The samples were first treated with acidic digestion following the protocol described by Ruiz-Navarro et al. [24]. The measurements were performed on three independent urine samples (collected on different days) and were corrected for specific gravity [23]. The standards and samples were analyzed in triplicate.

2.8. Statistical analysis

A statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA), SPSS 15.0 for Windows and Minitab Release 14 (MiniTab Inc.,

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