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Effects of sodium fluoride on immune response in murine macrophages

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ABSTRACT

Excessive fluoride intake may be harmful for health, producing dental and skeletal fluorosis, and effects upon neurobehavioral development. Studies in animals have revealed effects upon the gastrointestinal, renal and reproductive systems. Some of the disorders may be a consequence of immune system alterations.
In this study, an *in vitro* evaluation is made of fluoride immunotoxicity using the RAW 264.7 murine macrophage line over a broad range of concentrations (2.5–75 mg/L). The results show that the highest fluoride concentrations used (50–75 mg/L) reduce the macrophage population in part as a consequence of the generation of reactive oxygen and/or nitrogen species and consequent redox imbalance, which in turn is accompanied by lipid peroxidation. A decrease in the expression of the antiinflammatory cytokine *Il10* is observed from the lowest concentrations (5 mg/L). High concentrations (50 mg/L) in turn produce a significant increase in the proinflammatory cytokines *Il6* and *Mip2* from 4 h of exposure. In addition, cell phagocytic capacity is seen to decrease at concentrations of ≥20 mg/L. These data indicate that fluoride, at high concentrations, may affect macrophages and thus immune system function – particularly with regard to the inflammation autoregulatory processes, in which macrophages play a key role.

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1. Introduction

Fluorine in the form of fluorides represents 0.06–0.09% of the minerals present in the earth's crust (Fawell et al., 2006). Drinking water fluoride concentrations of about 1 mg/L are considered beneficial for the prevention of dental caries and for bone development (Fawell et al., 2006); however, excessive fluoride intake has adverse consequences (Ozsvath, 2009). Drinking water is considered to be the main source of fluoride exposure in humans. The World Health Organization (WHO) recommends water fluoride concentrations of under 1.5 mg/L (WHO, 2011). Nevertheless, it has been estimated that 32% of the world population consumes water with fluoride concentrations in excess of the recommended levels. Food can also contribute to fluoride exposure – the highest concentrations (>1 mg/kg) being found in seafood products, tea and fluoridated salt (USDA, 2004; Fawell et al., 2006).

Some studies in animals exposed to fluoride have described adverse effects upon different organs (hepatomegalia, nephrosis, myocardial mineralization, gastrointestinal alterations, effects upon reproductive organ morphology and function) (Doull et al., 2006; Fawell et al., 2006). Epidemiological studies provide clear evidence that chronic fluoride exposure causes dental fluorosis and, to a lesser extent, skeletal fluorosis (Doull et al., 2006) and effects upon neurobehavioral development (Lu et al., 2000; Liu et al., 2008).

In vitro studies indicate that fluoride can affect cell processes involved in many signaling pathways and in the maintenance of cell homeostasis (Barbier et al., 2010). The perturbation of these processes can trigger a series of events that result in the diseases associated with the chronic exposures commented above. In this respect it has been shown that fluoride is an important modulator of the expression of genes implicated in apoptosis, amino acid phosphorylation, oxidative stress, cell cycle progression, chemotaxis, glycolysis, inflammation and signal transduction (Agalakova and Gusev, 2012). Furthermore, fluorides are known to inhibit the activity of a broad range of enzymes (Dousset et al., 1984; Da Motta et al., 1999; Reddy et al., 2009). They are also able to cause phase S cell cycle arrest in different types of cells (Wang et al., 2004; Zhang et al., 2008) and to stimulate or inhibit cell proliferation depending upon the dose (Thaweboon et al., 2003; Yan et al., 2007).

Regarding the immunotoxicity of fluoride, the underlying mechanisms of action may involve the initiation and maintenance of inflammatory processes – both having been evidenced *in vivo* and *in vitro* at millimolar concentrations (U.S. EPA, 2006; Hosokawa et al., 2009). Immune response suppression has also been reported (Sutton, 1991). These immunotoxic effects, which could constitute the basis of the diseases induced by fluoride, have been little studied to date. Recently, the United States Environmental Protection Agency has underscored the need to investigate the immunotoxicity of this element (U.S. EPA, 2006). The present study contributes information in this respect, based on the *in vitro* evaluation of the effects of fluoride exposure upon immune system cell response using the RAW 267.4 murine model. This cell line was established from ascites from a tumor induced

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in a male mouse by intraperitoneal injection of Abelson murine leukemia virus (Raschke et al., 1978). Since then it has been used as a murine macrophage-like cell model in numerous studies, in which a variety of macrophage functions such as phagocytosis, ROS/NOS generation, apoptosis, and cytokine production have been evaluated (Anand et al., 2007; McCall et al., 2010; Wilhelmi et al., 2013).

2. Materials and methods

2.1. Reagents

A fluoride standard (NaF, 1000 mg/L, Panreac, Spain) was used for performing the experiments. For the quantification of fluoride by ion-selective electrode (ISE), standards and samples were diluted with TISAB II (total ionic strength adjustment buffer). The TISAB solution was prepared using 58 mg/mL of NaCl (Panreac), 10 mg/mL of *trans*-1,2 diaminocyclohexane-*N*,*N*,*N*′,*N*′-acid tetraacetic monohydrate (Fluka, Spain) and 57 μL/mL of glacial acetic acid (Panreac). TISAB II pH was adjusted to values between 4.8 and 5.2 using 7% (*w*/*v*) NaOH (Prolabo, Spain). Analytical reagent grade chemicals were used, together with deionized water (18.2 MΩcm) obtained with a Milli-Q water system (Millipore Inc., Millipore Iberica, Spain).

2.2. Cell line maintenance

The RAW 264.7 cells were supplied by Dr. Sánchez, of the Instituto de Agroquímica y Tecnología de Alimentos (IATA-CSIC, Valencia, Spain). 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, supplemented with 10% (*v*/*v*) fetal bovine serum, 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). All the reagents used were obtained from Hyclone Laboratories (Scientific Thermo, Spain).

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 cell scraper in phosphate buffered saline (PBS, Hyclone) and reseeded at a density of 0.5–1 × 10^4 cells/cm^2. The assays were performed with cultures between passages 13 and 30.

2.3. Cellular fluoride accumulation studies

The RAW 264.7 cells were seeded in 6-well plates at a density of 5.2 × 10^4 cells/cm^2, and after reaching confluence were treated with sodium fluoride (5, 10, 20 and 65 mg/L, equivalent to 0.26, 0.53, 1.1 and 3.68 mM NaF, respectively) over 4, 24 and 48 h. Following the exposure time, the medium was recovered and the monolayers were washed and detached in PBS (1 mL). To obtain the cell lysate, 100 μL of Triton X-100 (1% in PBS, Merck) were added and three freeze–thaw cycles were performed, followed by sonication for 10 min at 4 °C. The cell suspension was then centrifuged at 11,000 rpm for 5 min.

The concentration of fluoride in medium and cell lysate was quantified using a potentiometric method with an ion-selective electrode (ISE) (DC219-F, Mettler Toledo, Spain) (Rocha et al., 2013). The pH and ionic strength of the samples were adjusted using a 20% (*v*/*v*) TISAB II dilution. Recovery assays were performed to verify that the culture medium did not interfere with fluoride quantification. The analytical characteristics of the methodology are as follows: limit of quantification 0.125 ng/mL; precision <8%.

The quantity of fluoride accumulated by the cell cultures was corrected per number of cells determined by the trypan blue exclusion method (Trypan Blue Solution, 0.4%, Sigma, Spain).

2.4. Measurement of mitochondrial activity

Mitochondrial activity was evaluated using resazurin sodium (10-oxide of 7-hydroxy-3-hydro-phenoxacin-3-one sodium salt, Sigma). The RAW 264.7 cells were seeded in 24-well plates at a density of 2.6 × 10^4 cells/cm^2, and after reaching confluence (at 3 days post-seeding) were exposed over 4, 24 and 48 h to different concentrations of fluoride (2.5, 5, 10, 20, 50, 65 and 75 mg/L, equivalent to 0.13, 0.26, 0.53, 1.1, 2.6, 3.7 and 4.1 mM NaF, respectively). The fluoride standards were prepared in minimum essential medium with Earle's salts (MEM, PAA) supplemented with 100 U/mL of penicillin, 0.1 mg/mL of streptomycin, 0.0025 mg/mL of amphotericin B, 1 mM of sodium pyruvate and 10 mM of HEPES.

Following exposure, the medium was removed and the cultures were washed twice with PBS (500 μL). Then 500 μL of resazurin solution (10 μg/mL in supplemented MEM) were added, followed by incubation for 1 h at 37 °C in an atmosphere with 95% relative humidity and a CO_2 flow of 5%. The decrease in resazurin was determined by spectrophotometry performing readings at 570 and 600 nm (PowerWave HT Microplate Scanning Spectrophotometer, Bio-Tek instruments, USA). The results were expressed as percentages with respect to the absorbance of cells not treated with fluoride.

2.5. Determination of reactive oxygen and/or nitrogen species

The cells were seeded in 24-well plates at a density of 2.6 × 10^4 cells/cm^2. After reaching confluence, cells were exposed over 4, 24 and 48 h to different concentrations of fluoride (2.5, 5, 10, 20, 50 and 65 mg/L, equivalent to 0.13, 0.26, 0.53, 1.1, 2.6 and 3.7 mM NaF, respectively) prepared in supplemented MEM. Cells treated with 2 mM H_2O_2 (Prolabo) were used as positive controls.

Following the treatments, the medium was removed and the cells were washed with PBS. Then 100 μL of 2′,7′- dichlorofluorescein diacetate 100 μM (DHCF-DA, Sigma) prepared in PBS were added, followed by incubation at 37 °C for 30 min. After this time the medium was removed and the cells were washed with PBS and lysed using 150 μL of a solution of Triton X-100 (1% *w*/*v* in PBS). After sonication for 10 min at 4 °C and centrifugation at 11,000 rpm for 3 min, 100 μL of cell lysate was transferred to a 96-well plate and the fluorescence was determined ($\lambda_{excitation}$ = 488 nm; $\lambda_{emission}$ = 530 nm) using a PolarSTAR OPTIMA microplate reader (BMG-Labtech, Germany). The fluorescence values obtained were standardized per mg of protein, quantified by the Bradford method (Bio-Rad Protein Assay, Bio-Rad, USA). The results (arbitraty units of fluorescence/mg protein) were expressed as percentages with respect to the cells not treated with fluoride or H_2O_2.

2.6. Evaluation of lipid peroxidation

This assay was carried out by determining thiobarbituric acid reactive substances (TBARS) using the colorimetric technique described by Aviello et al. (2011), with slight modifications. The RAW 246.7 cells were seeded in 6-well plates at a density of 5.2 × 10^4 cells/cm^2, and after reaching confluence were treated with fluoride (5, 20, 50 and 65 mg/L, equivalent to 0.26, 1.1, 2.6 and 3.7 mM NaF, respectively) over 4, 24 and 48 h. Cells treated with $FeSO_4$ 0.1 mM and H_2O_2 1 mM were used as positive controls.

After the established times, the cells were washed with PBS, mechanically detached in PBS, and centrifuged at 1400 rpm for 5 min. The pellet was resuspended and lysed with TCA 5% *w*/*v* (100 μL) and Triton X-100 1% *v*/*v* (15 μL) using sonication (10 min, 4 °C) and centrifugation (11,000 rpm, 3 min). Then 50 μL of thiobarbituric acid (Merck) 0.67% (*w*/*v*) were added to 100 μL of cell lysate and the mixture heated to 60 °C for 15 min. After cooling in ice, the malonyldialdehyde (MDA) equivalents were measured at a wavelength of 532 nm using a PolarSTAR OPTIMA microplate reader. Quantification was made against

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