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 regards to the inflammation autoregulatory processes, in which macrophages play a key role.
Keywords: Fluoride; immunotoxicity; macrophages; oxidative stress; cytokines; phagocytosis.

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 (hepatomegaly, 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 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. MATERIAL 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 $5.2 \times 10^4$ 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-phenoxyacin-3-one sodium salt, Sigma). The RAW 264.7 cells were seeded in 24-well plates at a density of $2.6 \times 10^4$ 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 37ºC in an atmosphere with 95% relative humidity and a CO₂ 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 × 10⁴ cells/cm². 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 H₂O₂ (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 37ºC 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 4ºC and centrifugation at 11000 rpm for 3 min, 100 µL of cell lysate was transferred to a 96-well plate and the fluorescence was determined (λ_{excitation} = 488 nm; λ_{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 (arbitrarily units of fluorescence/mg protein) were expressed as percentages with respect to the cells not treated with fluoride or H₂O₂.

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/cm², 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 FeSO₄ 0.1 mM and H₂O₂ 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 (11000 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 malondialdehyde (MDA) equivalents were measured at a wavelength of 532 nm using a PolarSTAR OPTIMA microplate reader. Quantification was made against an MDA standard curve (0.5, 1, 2.5, 5 and 10 µM). The results (MDA equivalents = pmoles of MDA equivalents/mg protein) were expressed as percentages with respect to the cells not treated with fluoride or H₂O₂/FeSO₄.

2.7. Evaluation of differential cytokine expression

We evaluated the gene expression of proinflammatory [tumor necrosis factor-alpha (Tnfα), Il6 and macrophage inflammatory protein (Mip2)] and antiinflammatory (Il10) cytokines in
RAW 264.7 cells treated with fluoride (5, 10, 20 and 50 mg/L, equivalent to 0.26, 0.53, 1.1 and 2.6 mM NaF, respectively). The assays were carried out in cells seeded in 6-well plates at a density of $5.2 \times 10^4$ cells/cm$^2$ and exposed to fluoride over 4, 24 and 48 h. Gene expression was evaluated by reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

After exposure, the cells were washed twice with PBS and recovered with cell scraper in PBS. RNA was extracted using a NucleoSpin RNA II kit (Macherey-Nagel, Germany). The extracted RNA was quantified spectrophotometrically using a NanoDrop ND-1000 system (NanoDrop Technologies, USA). First-strand complementary DNA (cDNA) was obtained from 200 ng of total RNA using a reverse transcriptase core kit (Eurogentec Headquarters, Belgium).

qPCR was performed using the LightCycler® 480 Real-Time PCR system (Roche Diagnostics, USA). Reactions were carried out in a final volume of 10 µL containing 5 µL LightCycler® 480 SYBR Green I Master Mix (2X, Roche), 2.5 µL cDNA (50 ng/µL), 1 µL of each forward and reverse primer (10 µM; Biolegio, The Netherlands) (Table 1), and nuclease-free water. No-template controls were run to verify the absence of genomic DNA. Hypoxanthine-guanine phosphoribosyltransferase ($Hprt$) was employed as reference gene. PCR efficiency curves for each gene were calculated using triplicates of a two-fold dilutions curve.

The qPCR conditions were 95°C for 5 min, followed by 40 cycles: 10 s denaturation at 95°C, 10 s annealing at 55°C, and 20 s elongation at 72°C. The melting curve of each sample was analyzed after each PCR run to confirm PCR product specificity. The data were analyzed with the Relative Expression Software Tool (REST 2006, QIAGEN), in standard mode.

2.8. Evaluation of phagocytic capacity

Cells were plated in 96-well plates at a density of $1 \times 10^5$ cells/well and incubated overnight. The cells were then treated with different concentrations of fluoride (5, 10, 20, 50
and 65 mg/L, equivalent to 0.26, 0.53, 1.1, 2.6 and 3.7 mM NaF, respectively) over 4, 24 and 48 h. Cells treated with lipopolysaccharide (LPS) from Salmonella enterica serotype typhimurium (Sigma) (100 ng/mL) were used as positive controls. After treatment, the medium was removed and the cells were washed with PBS. To quantify phagocytosis, cells were incubated with fluorescein-labeled bioparticles (Vybrant Phagocytosis Assay, Life Technologies Invitrogen, Spain) for 3 h. The bioparticles were removed and trypan blue was added to the wells for 2 min to quench extracellular bioparticles. The trypan blue was then removed and the amount of bioparticles engulfed by the cells was quantitatively measured using a PolarSTAR OPTIMA microplate reader (λ_{excitation} = 480 nm; λ_{emission} = 520 nm). The results were corrected for total protein content and were reported as percentages with respect to the controls.

2.9. Statistical analysis

All the treatments were performed at least in quadruplicate in independent cultures. The statistical analysis was performed using one-way analysis of variance (ANOVA) with multiple comparisons using the Tukey HSD test (Sigma Plot 2012). Differences were considered significant for p<0.05.

3. RESULTS

3.1. Cellular accumulation of fluoride

The intracellular fluoride contents of the cells treated with 5 mg/L were not detectable at any of the evaluated exposure times. For the rest of the concentrations (10, 20 and 65 mg/L) the contents were generally low (0.4-1% of the total added amount). Fluoride within the cells increased with rising concentration, though not in a linear manner (Figure 1), and differences were observed according to the duration of exposure. In the case of the 10 and 20 mg/L
concentrations, the intracellular contents decreased over time, though the cells exposed to 65 mg/L showed maximum accumulation after 48 h. It should be noted that in all the assays, the sum of the fluoride contents in the medium and cell lysate was close to the added fluoride concentration, with a mass balance of 95-106%.

3.2. Mitochondrial activity of cells treated with fluoride

Figure 2 shows the results relating to mitochondrial activity of the cells treated with fluoride versus the untreated controls, at the different timepoints. Fluoride reduced mitochondrial activity only at the highest concentrations tested (≥50 mg/L). This decrease was found to be significant from 24 h for 50 mg/L (15%), and from 4 h of exposure for 65 and 75 mg/L (25% and 39%, respectively). It should be noted that complete suppression of mitochondrial activity was observed in the cells treated for 48 h with 75 mg/L.

3.3. Generation of reactive oxygen and/or nitrogen species

Figure 3 shows the formation of 2',7'-dichlorofluorescein (DCF) from DCFH-DA by the reactive oxygen (ROS) or nitrogen species (RNS) generated during the treatments with fluoride. Only the highest fluoride concentration (65 mg/L) caused an increase in ROS/RNS generation with respect to the untreated cells. This increase was observed from 4 h of exposure. The increase in ROS/RNS generation produced by fluoride (138-164%) was significantly smaller than those observed in cells treated with H2O2, a potent oxidant (185-337%), except for cells exposed to 65 mg/L of fluoride over 4 h.

3.4. Evaluation of lipid peroxidation

Malonyldialdehyde (MDA) is one of the low molecular weight end products generated through the primary or secondary decomposition of lipid peroxidation products (Janero,
1990). Figure 4 shows the MDA equivalents obtained in the different treatments. The highest fluoride concentrations (50 and 65 mg/L) were seen to significantly increase the intracellular MDA levels after 24 h of treatment. Although important, the increments (50 mg/L: 365 ± 145%, 65 mg/L: 869 ± 18%) were significantly lower than those observed in the cells treated with FeSO₄/H₂O₂ (1955 ± 105%).

3.5. Differential expression of cytokines using RT-qPCR

Table 2 shows the changes in the expression of cytokines Il10, Il6, Tnfa and Mip2 by the cells treated with fluoride versus the untreated controls, after 4, 24 and 48 h of exposure. In general, at concentrations of ≤ 20 mg/L exposure to fluoride resulted in a decrease in the expression of the evaluated cytokines after most of the considered time periods. However, these reductions were only consistently significant for the proinflammatory cytokine Il10, with 2.11- to 5.02-fold reductions versus the control cells after times of over 24 h.

Changes in cytokine expression of the cells treated with 50 mg/L were observed from 4 h of exposure. The proinflammatory cytokines Il6 and Mip2 showed significant up-regulation, especially after 48 h of treatment. At this concentration, the effect upon the antiinflammatory cytokine Il10 varied over time: after 24 h its expression was reduced with respect to the controls, while Il10 up-regulation was recorded after 48 h.

3.6. Macrophage phagocytic capacity

The data obtained (Figure 5) indicate a decrease in phagocytic capacity of the cells treated with fluoride versus the untreated control cells. The greatest reductions corresponded to the cells treated with concentrations of ≥ 50 mg/L. At 4 hours of exposure a significant reduction in the phagocytic capacity is observed for all fluoride treatment with respect to the non-treated cells (15-46%). This significant reduction is only evident at the highest concentrations at 24 h.
(50 mg/L: 32%; 65 mg/L: 81%) and 48 h (20 mg/L: 32%; 50 mg/L: 86%; 65 mg/L: 80%). The decrease in phagocytic capacity due to LPS was not significant until 24 h of exposure (24 h: 27%; 48 h: 33%).

4. DISCUSSION

Food and drinking water are the main sources of fluoride for human populations. Fluoride in drinking water generally does not exceed 1 mg/L, except for endemic areas, where concentrations ≤ 15 mg/L have been found (Fawell et al., 2006; Liu et al., 2014). Regarding food products, fluoride contents are only relevant for tea and seafood products, with concentrations up to 6 mg/kg (USDA, 2004; Fawell et al., 2006). Intake of fluoride usually ranges from 0.1 to 4.4 mg/day; however, in fluoride endemic areas, intakes of up to 27 mg/day have been reported (Fawell et al., 2006). These high intakes are associated with high levels of fluoride in urine (up to 11 mg/L) and in many cases with clinical manifestations (Wang et al., 2007).

Fluoride ingested through the diet can affect the calcified and non-calcified systems of the body. In non-calcified tissues there have been reports of diseases that could be a consequence of alterations of immune system function. Some studies indicate that the element may act as an immunosuppressor by reducing the peripheral blood lymphocyte, monocyte and neutrophil populations (Das et al., 2006; Giri et al., 2013). It has also been reported that fluoride can increase the inflammatory response of certain proinflammatory substances (Loftenius et al., 1999), and that in combination with aluminum it constitutes a potent activator of protein G (Strunecka et al., 2007). Most of these studies have been carried out using millimolar sodium fluoride doses (U.S. EPA, 2006). However, few data evidence an immunotoxic effect at concentrations commonly found in drinking water and food. Such studies should be encouraged, as underscored by the USEPA in its latest report, which recommends evaluating
the immunotoxicity of exposures to 4 mg/L of fluoride through drinking water (U.S. EPA, 2006).

Excessive exposure to fluoride can damage cell redox balance (Shivarajashankara et al., 2001; Bouaziz et al., 2007), reduce antioxidant capacity (Bharti and Srivastava, 2009) and increase the toxic effects mediated by the generation of reactive species (ROS/RNS) (Rzeuski et al., 1998). A close relationship has been shown between chronic exposure to fluoride and increased oxidative stress in experimental animal models and in highly exposed populations (Saralakumari and Rao, 1991; Shivarajashankara et al., 2001; Barbier et al., 2010). Although reactive species may play a relevant role in normal cell functions (Le Belle et al., 2011), they are more closely associated to pathological effects leading to cell damage and ultimately cell death. In our study, macrophage exposure to fluoride concentrations of ≥ 50 mg/L (2.6 mM NaF) resulted in the generation of reactive species, which in turn appeared to cause a significant increase in lipid peroxidation.

Such exposures, even over short time periods, cause macrophage redox imbalance, resulting in a decrease in mitochondrial activity and phagocytic cell viability. However, no such effects were observed at concentrations in the micromolar range (2.5-10 mg/L, equivalent to 0.13-0.53 mM NaF). These data are consistent with those found in the literature. In this sense, Sato et al. (1985) observed no effects upon the viability, morphology or enzyme activity of lymphocytes exposed to sodium fluoride concentrations of under 1.05 mM. It has even been reported that low fluoride concentrations (2.5 µM) favor important immune processes such as monocyte differentiation into macrophages (Stachowska et al., 2005).

In this study, a downregulation of the expression of the inflammatory cytokine IL10 was observed. The main activity of this interleukin includes the inhibition of cytokine production by macrophages and inhibition of the accessory functions of macrophages during T cell activation. Such functions indicate that IL-10 mainly plays an antinflammatory role in the
immune system (Moore et al., 2001). This may be the reason why co-exposure of fluoride with other proinflammatory agents results in an increase in their inflammatory response. Loftenius et al. (1999) found that 0.62 mM of fluoride increases the production of cytokines in the presence of hemagglutinin – a known mitogen with a potent proinflammatory capacity.

Regarding the expression of the proinflammatory cytokines, a biphasic behavior has been evidenced. Macrophages exposed to sodium fluoride concentrations between 5 and 20 mg/L (0.26-1.1 mM) presented downregulations, while cells treated with 50 mg/L (2.6 mM) showed in general upregulations, especially on $\text{Il6}$ and $\text{Mip2}$. Previous studies have reported different responses on the part of the cells of the immune system in relation to cytokine expression and release in the presence of fluoride. In this respect,Refsnes et al. (1999) found that treatment with fluoride at high concentrations (5 mM during 24 h) can increase the expression of cytokines $\text{IL6}$ and $\text{IL8}$. However, exposure to low fluoride concentrations (2.25 mg/L) results in a decrease in the expression and release of certain cytokines in ameloblasts, which are responsible for the production of dental enamel (Riksen et al., 2011). Our study also evidences a concentration-dependent response and confirms the role of fluoride in modulation of the immune response.

The inflammatory process is necessary for correct cell and tissue function, and is usually kept under strict regulatory control. An imbalance between the triggering, maintenance and arrest signals causes uncontrolled spread of the inflammatory process. Resolution of inflammation depends on the removal of apoptotic cells and on active suppression of inflammatory mediator production. Aberrations in either mechanism are associated with chronic inflammatory conditions and autoimmune disorders (Zhang et al., 2010). Macrophages play three key roles in the autoregulation of inflammation: antigen presentation, phagocytosis, and immune modulation through the production of cytokines and growth factors (Fujiwara and Kobayashi, 2005). Uptake of apoptotic cells by macrophages is thought
to suppress autoimmune responses through the release of antiinflammatory cytokines such as IL-10 and the inhibition of proinflammatory cytokines (Chung et al., 2006), facilitating the return to normal physiological conditions. As has been shown in our study, exposure to fluoride could affect this process, impeding normal function of the host defense system by reducing the macrophage population, increasing the expression of proinflammatory cytokines at high concentrations, and affecting the production of antiinflammatory cytokines at lower concentrations. A decrease in macrophage phagocytic capacity was also observed in the present study. Although the RAW 264.7 cell line was used to assess the immunotoxic effect of contaminants and in many cases a good correlation with data from primary human cells has been obtained (Shoham et al., 2001; Lyu and Park, 2005), the effects observed in this study must be confirmed by in vivo and in vitro studies using primary cell cultures.

On the other hand these effects have been evidenced after acute exposure, though it is not clear what may happen with repeated exposures over time. Approximately 200 million people are continuously exposed to fluoride concentrations equal to or greater than 0.1 mM through drinking water and food cooked with such water (Ayoob and Gupta, 2006). According to our own results and those of previous studies, these concentrations do not appear to have an important immunotoxic effect in the context of point fluoride exposures. However, there are no studies on the chronic effects of fluoride upon the immune system and its functions. Further research is therefore needed to clarify the immunotoxic effects of fluoride in the context of chronic exposures to concentrations representative of those found in the diet.

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**Conflict of interest**

The authors did not report any conflict of interest.

**References**


### Table 1. Sequences and efficacies of the oligonucleotides used in qPCR.

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<th>Gene</th>
<th>GenBank #</th>
<th>Oligonucleotide sequence</th>
<th>Amplicon size (bp)</th>
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<td>Il6</td>
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<td>Mip2</td>
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Table 2. Relative expression of *Tnfa*, *Il6*, *Mip2* and *Il10* (fold changes) of RAW 276.4 cells treated with F (5, 10, 20 and 50 mg/L) for 4, 24 and 48 hours (mean ± SD, n=3). Significant differences with respect to control (in bold letters) are marked with an asterisk (*) (p < 0.05).

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<th>Fluoride concentration (mg/L)</th>
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<tr>
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<td>-1.23 ± 0.12</td>
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<td>-1.40 ± 0.16</td>
<td>1.11 ± 0.18</td>
<td>-5.02 ± 0.23*</td>
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<td>48</td>
<td>-1.34 ± 0.15</td>
<td>-1.25 ± 0.20</td>
<td>-1.05 ± 0.16</td>
<td>-4.69 ± 0.14*</td>
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<td>4</td>
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<td>2.69 ± 0.58*</td>
<td>21.99 ± 3.37*</td>
<td>-1.29 ± 0.17</td>
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<td>11.32 ± 2.54*</td>
<td>-3.83 ± 0.07*</td>
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<td>230.5 ± 31.5*</td>
<td>50.63 ± 7.06*</td>
<td>15.24 ± 2.34*</td>
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Figure 1. Intracellular accumulation of fluoride in RAW 246.7 cells treated with fluoride (10, 20 and 65 mg/L) over 4, 24 and 48 hours. Values expressed as ng F/10^6 cells (mean ± SD, n=4).
Figure 2. Measurement of mitochondrial activity. Reduction of resazurin in RAW 246.7 cells treated with fluoride (2.5, 5, 10, 20, 50, 65 and 75 mg/L) over 4, 24, and 48 hours. Values expressed as percentage versus control (mean ± SD, n=8). The asterisks (*) indicate significant differences versus control for each of the assayed time periods (p< 0.05).
Figure 3. Generation of reactive oxygen and/or nitrogen species. Measurement of the formation of dichlorofluorescein (DCF, UAF/mg protein) in the RAW 246.7 cells treated with fluoride (2.5, 5, 10, 20, 50 and 65 mg/L) and with 2 mM H$_2$O$_2$ over 4, 24 and 48 hours. Values expressed as percentage versus control (mean ± SD, n=4). The asterisks (*) indicate significant differences versus control for each of the assayed time periods (p< 0.05).
Figure 4. Evaluation of lipid peroxidation. Malonyldialdehyde (MDA) equivalents of the RAW 246.7 cells treated with fluoride (5, 10, 20, 50 and 65 mg/L) and with 0.1 mM FeSO₄/1 mM H₂O₂ over 4, 24 and 48 hours. Values expressed as percentage versus control (mean ± SD, n=4). The asterisks (*) indicate significant differences versus the untreated controls for each of the assayed time periods (p< 0.05).
Figure 5. Phagocytic capacity of RAW 246.7 cells treated with fluoride (5, 10, 20, 50 and 65 mg/L) or with LPS (100 ng/mL) over 4, 24 and 48 hours. Values expressed as percentages versus control of the cells treated with fluoride (mean ± SD, n ≥ 4). The asterisks (*) indicate significant differences versus control for each of the assayed time periods (p< 0.05).