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Mini-review

Molecular mechanisms of fluoride toxicity

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ABSTRACT

Halfway through the twentieth century, fluoride piqued the interest of toxicologists due to its deleterious effects at high concentrations in human populations suffering from fluorosis and in *in vivo* experimental models. Until the 1990s, the toxicity of fluoride was largely ignored due to its "good reputation" for preventing caries via topical application and in dental toothpastes. However, in the last decade, interest in its undesirable effects has resurfaced due to the awareness that this element interacts with cellular systems even at low doses. In recent years, several investigations demonstrated that fluoride can induce oxidative stress and modulate intracellular redox homeostasis, lipid peroxidation and protein carbonyl content, as well as alter gene expression and cause apoptosis. Genes modulated by fluoride include those related to the stress response, metabolic enzymes, the cell cycle, cell–cell communications and signal transduction.

The primary purpose of this review is to examine recent findings from our group and others that focus on the molecular mechanisms of the action of inorganic fluoride in several cellular processes with respect to potential physiological and toxicological implications. This review presents an overview of the current research on the molecular aspects of fluoride exposure with emphasis on biological targets and their possible mechanisms of involvement in fluoride cytotoxicity. The goal of this review is to enhance understanding of the mechanisms by which fluoride affects cells, with an emphasis on tissue-specific events in humans.

Contents

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1. Introduction

The fluoride ion is derived from the element fluorine, a gas that never occurs in a free state in nature. Fluoride is abundant in the environment and exists only in combination with other elements as fluoride compounds, which are constituents of minerals in rocks and soil. Therefore, fluoride is commonly associated with volcanic activity.

Sources of fluoride include natural fluoride in foodstuffs and water, i.e., fluoridated water (usually at 1.0 mg/l), fluoride supplements (such as fluoride tablets), fluoride dentifrices (containing on average 1000 mg/kg), and professionally applied fluoride gel (containing on average 5000 mg/kg). The main source of fluoride for humans is the intake of groundwater contaminated by geological sources (maximum concentrations reaching 30–50 mg/l). The level of fluoride contamination is dependent on the nature of the rocks and the occurrence of fluoride-bearing minerals in groundwater. Fluoride concentrations in water are limited by fluorite solubility, so that in the absence of dissolved calcium, higher fluoride solubility should be expected in the groundwater of areas where fluoride-bearing minerals are common and vice versa [1].

Excessive fluoride intake over a long period of time may result in a serious public health problem called fluorosis, which is characterized by dental mottling and skeletal manifestations such as crippling deformities, osteoporosis, and osteosclerosis. Endemic fluorosis is now known to be global in scope, occurring on all continents and affecting many millions of people [2].

In some regions, artificial fluorides used to fluoridate community water supplies (mostly at around 1 mg/l) include silicofluoride compounds (sodium silicofluoride and hydrofluosilicic acid) and sodium fluoride (NaF). At neutral pH, silicofluoride is dissociated to silic acid, fluoride ion, and hydrogen fluoride (HF) [3]. The primary benefit associated with fluoride supplementation is linked to the potential to reduce the risk of dental caries due to the cariostatic effects of fluoride. Even in the past, fluoride was considered an essential element. In actuality, there is a lack of consensus as to the role of fluoride in human nutrition and optimal development and growth [4].

Additional risks of increased fluoride exposure are known; the most significant are effects on bone cells (both osteoblasts and osteoclasts) that can lead to the development of skeletal fluorosis. It is now recognized that fluoride also affects cells from soft tissues, i.e., renal, endothelial, gonadal, and neurological cells [5].

The minimal risk level for daily oral fluoride uptake was determined to be 0.05 mg/kg/day [6], based on a non-observable adverse effect level (NOAEL) of 0.15 mg fluoride/kg/day for an increased fracture rate. Estimations of human lethal fluoride doses showed a wide range of values, from 16 to 64 mg/kg in adults and 3 to 16 mg/kg in children [6].

Organofluoride compounds (carbon–fluoride bond) are increasingly used. These compounds have a wide range of functions and can serve as agrochemicals, pharmaceuticals, refrigerants, pesticides, surfactants, fire extinguishing agents, fibers, membranes, ozone depletors, and insulating materials [7]. An estimated 20% of pharmaceuticals and 30–40% of agrochemicals are organofluorines [8]. However, environmental and health issues are still a problem for many organofluorines. Because of the strength of the carbon–fluoride bond, many synthetic fluorocarbons and fluorocarbon-based compounds are persistent global contaminants and may be harming the health of wildlife [7]. Their effects on human health are unknown. However, the toxicity of fluorinated organic chemicals is usually related to their molecular characteristics rather than to the fluoride ions that are metabolically displaced.

The present review is focused on the molecular effects of inorganic fluoride with respect to potential physiological and toxicological implications. It addresses the current understanding of the signal transduction pathways and mechanisms underlying the sensitivity of various organs and tissues to fluoride. This review provides information on the cellular and molecular aspects of the interactions between fluoride and cells, with an emphasis on tissue-specific events in humans.

2. Uptake and accumulation

Fluoride is very electronegative, which means that it has a strong tendency to acquire a negative charge and forms fluoride ions in solution. In aqueous solutions of fluoride in acidic conditions such as those of the stomach, fluoride is converted into HF, and up to about 40% of ingested fluoride is absorbed from the stomach as HF [9].

Fluoride transport through biological membranes occurs primarily through the non-ionic diffusion of HF. Classic studies with artificial lipid bilayers and pH electrodes indicated that HF is a highly permeant solute with a permeability coefficient similar to that of water. The small neutral molecule of HF seems to penetrate cell membranes much faster than the dissociated fluoride ion, resulting in a more pronounced intracellular intake [9]. Membrane permeability to HF is five to seven orders of magnitude above that of fluoride [10]. Recent studies showed that approximately 45% of ingested fluoride is absorbed from the intestine, and that fluoride absorption from the intestine is less sensitive to pH and may occur via a carrier-mediated process (i.e., facilitated diffusion) [11]. It is not known whether such carrier proteins are also present in the membranes of other cells.

In addition, fluoride permeability via anion channels has been demonstrated in airway epithelial cells [12], but Gofa and Davidson [13] suggest that fluoride potentiates the activity of potassium-selective ion channels in osteoblastic cells. The activity of potassium and calcium channels may mediate many of the early events in fluoride-induced cell activation. Apparently, there are several pH gradient-dependent, carrier-mediated mechanisms for fluoride transport; one may involve fluoride uptake in the form of HF by diffusion; in other, fluoride appears to cross membrane by a F^-–H^+ cotransporter or F^-–OH^- exchangers in the presence of an inward-directed proton gradient cells [10]; however, further studies are needed to clarify this subject.

Relative to the amount of fluoride ingested, high concentrations of cations that form insoluble complexes with fluoride (e.g., calcium, magnesium and aluminum) can markedly decrease

gastrointestinal fluoride absorption causing hypocalcemia and inhibition of magnesium and manganese-dependent enzymes [14].

Fluoride combines with calcium to form calcium ionospheres that easily permeabilize the cell membrane [15]. The effect of fluoride depends on extracellular calcium and can be blocked by a combination of calcium-channel blocking agents, suggesting that the potentiation of channel activity is dependent on external calcium.

Once absorbed into the blood, fluoride readily distributes throughout the body, with the greatest amount retained in calcium-rich areas such as bone and teeth (dentine and enamel). In infants, about 80–90% of the absorbed fluoride is retained, but in adults this level falls to about 60% [6].

Fluoride is excreted primarily via the urine. Urinary fluoride clearance increases with urine pH due to a decrease in the concentration of HF. Various factors (e.g., diet and drugs) can affect urine pH and thus affect fluoride clearance and retention [6].

Inorganic complexes are formed between fluoride and metallic ions such as aluminum (Al) or beryllium (Be), these compounds are biologically effective having a potential role in physiological and toxicological processes. The fundamental action mechanisms for these inorganic molecules or the most explored at least, highlight their chemical structure that resembles the one of a γ-phosphate [16]. Therefore, several biochemicals and cellular effects of fluoride are explained by their interaction with enzymatic systems that have phosphoryl transfer activity like GTPases and ATPases. On the other hand, it has been reported the interaction between Al–F and Be–F to structural proteins such as actin [17] and troponin C [18]. Some studies showed the determinant role of amino acid residues in order to the establishment of hydrogen bridge between fluoride and some hemoproteins, such as Arg (peroxidases) and His (myoglobin) [19].

3. Cellular effects of fluoride

Fluoride exerts diverse cellular effects in a time-, concentration-, and cell-type-dependent manner. The main toxic effect of fluoride in cells consists of its interaction with enzymes. In most cases, fluoride acts as an enzyme inhibitor, but fluoride ions can occasionally stimulate enzyme activity. The mechanisms depend on the type of enzyme that is affected [20]. Fluoride at micromolar levels is considered an effective anabolic agent because it promotes cell proliferation, whereas millimolar concentrations inhibit several enzymes, including phosphatases, both *in vivo* and *in vitro* [21].

Metabolic, functional and structural damage caused by chronic fluorosis have been reported in many tissues. Research data strongly suggest that fluoride inhibits protein secretion and/or synthesis and that it influences distinct signaling pathways involved in proliferation and apoptosis including the mitogen-activated protein kinase (MAPK), p53, activator protein-1 (AP-1) and nuclear factor kappa B (NF-κB) pathways [22–24].

3.1. Redox status

Knowledge of the intracellular pathways involved in the cell–fluoride interaction is incomplete, probably due to the complexity and diversity of the molecular events underlying cell–fluoride interactions. However, significant conclusions are emerging from studies of the intracellular oxidative rate and of the gene expression and protein accumulation/traffic aspects of fluoride exposure.

Mitochondria are the key intracellular targets for different stressors including fluoride [25]. Fluoride alters the activity of many mitochondria-rich cells such as those of the human kidney [26] and the rat liver and pancreas [27]. However, information about the mechanism of fluoride-induced mitochondrial damage is scarce.

3.1.1. Cellular respiration

Fluoride ions can bind to functional amino acid groups surrounding the active centre of an enzyme to cause an inhibitory effect, as is the case for enzymes of the glycolytic pathway and the Krebs cycle, which are sensitive to inhibition by fluoride. Na^+/K^+-ATPases are also inhibited, leading to ATP depletion and a disturbance in cell membrane potential [20]. Therefore, fluoride ions inhibit cellular respiration and decrease the production of ATP.

In addition, NaF induces an increase in the release of cytochrome *c* (cyt C) from the mitochondria to the cytosol in human gingival fibroblasts [28]. Exposure causes decreased ATP production and, thus, increased cellular levels of ADP, AMP, GDP and Pi. However, strong long-term inhibition of cyt C is toxic to cells that cannot activate glycolytic ATP production sufficiently to supply energy for essential ATP-requiring reactions. Persistent inhibition results in endogenous induction and activation of nitric oxide (NO). On the other hand, NO inhibits mitochondrial respiration by decreasing the apparent affinity of cyt C for oxygen. NO induction has been documented in cases of fluoride exposure [15,29,30].

3.1.2. Inner membrane permeability and membrane potential

As mentioned above, fluoride can impair the function of mitochondria, diminishing cellular respiration and also promoting the release of cyt C and the induction of the caspase cascade. Fluoride-induced release of cyt C is a well-established mechanism during which fluoride acts as an uncoupling agent that induces the opening of the permeability transition pore [25].

Mitochondrial damage, as evaluated by the loss of mitochondrial membrane potential ($\Delta\Psi_m$), was observed in the mitochondria of pancreatic β-cells exposed to 1–2 mM fluoride. Moreover, linear regression analysis revealed a significant linear relationship between the loss of $\Delta\Psi_m$ and the increase in superoxide generation in β-cells exposed to 0.25–3 mM fluoride [31]. In a similar manner, subchronic exposure to fluoride in rats caused a decrease in the mitochondrial function and fertilization capability of spermatozoa [32].

It is known that mitochondria are the major source of reactive oxygen species (ROS) production and that the toxicity of fluoride is associated with ROS induction, the generation of NO and the reduction of cellular antioxidant defenses against oxidative damage (Table 1).

3.1.3. Generation of ROS

Fluoride exposure increase the generation of anion superoxide (O_2^-) [31,32]; increased O_2^- concentration and its downstream consequences such as hydrogen peroxide, peroxynitrite, hydroxyl radicals seem particularly important in mediating fluoride's effects. Moreover, fluoride increased NO generation [15,29,30] and it can react with superoxide to form peroxynitrite, and with thiols and metal centres in proteins to form nitrosyl adducts. It has also been shown interference with disulfide-bond formation and resulting in the accumulation of misfolded proteins in the endoplasmic reticulum (ER) causing ER stress and ROS production.

Oxidative stress is a recognized mode of action of fluoride exposure that has been observed *in vitro* in several types of cells and also *in vivo* in soft tissues such as the liver, kidney, brain, lung, and testes in animals and in people living in areas of endemic fluorosis (Table 1). Fluoride is thought to inhibit the activity of antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase, and catalase [31–33]. Moreover, fluoride can alter glutathione levels [34–37], often resulting in the excessive production of ROS at the mitochondrial level, leading to the damage of cellular components.

Table 1
Oxidative stress an oxidative damage associated to fluoride exposure. Arrows refer to increases (↑), or decreases (↓) regulation.

Model and dose of fluoride	Endpoint	Reference
***In vitro* (animals cells)**		
Mouse pancreatic beta-cells (βTC-6) at 1.35 and 2.5 mM for 12 h	↑Generation of O_2^-, ↓activity of SOD, ↓$\Delta\Psi_m$	[31]
Primary rat hippocampal neurons at 20, 40, and 80 mg/l, equivalent to 1.05, 2.1 and 4.2 mM for 24 h	↑Generation of ROS, ↓level of GSH, ↓activities of GSH-Px, and SOD, ↑lipid peroxidation	[34]
Murine hepatocytes at 100 mM for 1 h	↑Generation of ROS, ↓level of GSH, ↓GSH:GSSG ratio, ↓activities of SOD, and catalase, ↑lipid peroxidation, and oxidation of proteins	[35]
***In vitro* (human cells)**		
Hepatocellular carcinoma (HepG2) cells at 3 mM for 6 and 24 h	↓GSH/GSSG ratio, ↑gen expression of Mn-SOD	[43]
Neuroblastoma (SH-SY5Y) cells exposed at 0.05–5 mM for 24 h	↑Lipid peroxidation, and ↑protein oxidation	[160]
Human hair follicles exposed at 1.0, and 10 mM for 5 days	↑Lipid peroxidation	[161]
***In vivo* (animals)**		
Male albino guinea pigs exposed at 250 mg NaF/kg subcutaneously and sacrificed 8 h later	↑Generation of NO in blood	[15]
Male Wistar rats exposed at 5 mg/kg body mass/day, orally for 8 weeks	↑Generation O_2^-, ↓activity of SOD, ↓$\Delta\Psi_m$, ↑lipid peroxidation in spermatozoa	[32]
Male Swiss mice exposed at 50 mg/l in drinking water for 10 weeks	↑Generation of ROS, ↑lipid peroxidation, ↓activities of SOD, and catalase, ↑activities of GST, and GSH-Px, ↓ratio GSH:GSSG in brain	[49]
Albino rats exposed at 100 mg/l in drinking water for 4 months	↑Level of ascorbic acid ↓ level of uric acid in plasma ↑Lipid peroxidation, ↑level of GSH, ↑activity of GSH–Px, ↓activity of SOD in erythrocytes ↑Lipid peroxidation, ↑activities of GSH-Px, and GST, ↑GSH in brain and liver	[101]
Male albino Wistar rats exposed at 1, 10, 50, and 100 mg/l in drinking water for 12 weeks	↑Generation ROS, changes in levels of GSH in blood, ↑generation ROS in liver, kidney, and brain	[102]
Second generation of Male Albino adult Wistar rats exposed at 10, 50, and 100 mg/l in drinking water for 180 days	↑Lipid peroxidation, ↓activities of SOD, catalase, and GSH-Px in lung	[111]
Chicks exposed by diet to 100, 250, or 400 mg F/kg for 50 days	↑Generation of NO, ↑lipid peroxidation, ↓activities of SOD, catalase, and GSH-Px in serum	[29]
Male albino rats exposed at 10.3 mg NaF/kg body weight/day, orally for 5 weeks	↑Lipid peroxidation, ↑generation NO, ↓activities of SOD, and catalase, ↓Total antioxidant capacity, and ↓level of GSH in liver	[30]
Pig exposed to food supplemented with 250 mg F/kg for 50 days	↓Expression of gen Cu/Zn SOD in liver	[42]
Male rats exposed at 20 mg/kg/day for 29 days by oral gavage	↑Level of conjugated dienes in the testis, epididymis, and epididymal sperm pellet. ↓activities of GDH-Px, and catalase in the sperm	[162]
Male Wistar rats exposed at 50 and 100 mg/l in drinking water during 4 months	↓Activity of CuZn-SOD in pancreas	[50]
Male and female Wistar rats exposed at 50, 100, and 150 mg/l in drinking water during 3 months	↑Lipid peroxidation, ↓activities of SOD, and GSH-Px in liver	[163]
Barrows exposed at 250 and 400 mg/kg (from NaF) in their diets for 50 days	↑Generation of NO, ↑lipid peroxidation, ↓activities of GSH–Px, and SOD in serum ↑Lipid peroxidation, ↓activities of GSH-Px, and SOD in thyroid, liver, and kidney	[164]
Male Swiss mice exposed at 5 mg/kg body mass/day, orally for 8 weeks	↑ROS in erythrocytes, ↓level of GSH in blood, ↓activities of SOD, catalase, and GSH-Px, ↑lipid peroxidation, in kidney and liver	[36]
Female rats exposed at 100 mg/l in drinking water for 60 days	↑Lipid peroxidation, ↓activities of SOD, catalase, and GSH-Px in endometrium	[165]
Swiss albino male mice exposed at 50 mg/l in drinking water for 3 weeks	↑Generation of ROS, ↓GSH level, ↓activity of SOD in blood, ↑activity of catalase in liver	[152]
Male albino rats exposed at 10, 50 and 100 mg/l in drinking water for 10 weeks	↑Generation ROS in blood, liver, kidney, and brain ↓GSH/GSSG ratio in liver, kidney, and brain	[166]
Female Albino mice exposed 5 mg/kg body weight/day, orally for 30 days	↓Activities of SOD, catalase, and GSH–Px, ↓level of GSH, ↓total, dehydro and reduced ascorbic acid, ↑lipid peroxidation in ovary	[167]
Male Balb/c mice exposed at 200 mg/l, in drinking water for 7 days	↓Activities of SOD, GSH-Px, and catalase, ↑lipid peroxidation, in erythrocytes, and liver	[168]
Female Wistar rats exposed at 150 mg/l in drinking water for 28 days	↓Level of GSH, ↓activities of SOD, GPx, catalase and, glutathione reductase, ↑lipid peroxidation in brain	[169]
Wistar albino pups placentally and lactationally exposed from mother rats at 50, and 150 mg/l in drinking water	↑Lipid peroxidation, ↑protein oxidation in developing central nervous system	[170]
***In vivo* (human)**		
Residents from China-endemic area (mean urine concentration of 2 mg F/l)	↓Activities of SOD, catalase, and GSH-Px ↑Lipid peroxidation, in serum	[44]
Children with skeletal fluorosis from Indian-endemic area (mean water concentration of 5.53 mg F/l)	↑Level of ascorbic acid, ↓level of uric acid in plasma ↑Lipid peroxidation, ↓GSH, ↓activities of SOD and GSH–Px in erythrocytes	[37]

It is known that excessive ROS production leads to macromolecule oxidation, resulting in free radical attack of membrane phospholipids with resulting membrane damage via induction of lipid peroxidation (Table 1), mitochondrial membrane depolarization, and apoptosis.

Antioxidant treatment consistently protects cells from the lipid peroxidation caused by fluoride exposure [30,36], suggesting that oxidative/nitrosative damage is the major mode of action of fluoride.

Fluoride has been reported to activate the stress response signaling cascade involving MAP kinases [22,38]; however, the molecular events leading to such activation are poorly understood. Extracellular signal-regulated protein kinase (ERK) is activated by the Ras/MEKK/MEK-mediated pathway [39]. Ras, as a direct target for fluoride [40], provides a plausible mechanism for NaF-induced ERK activation. The PKC-dependent pathway and alterations in tyrosine phosphorylation are likely to be involved in NaF-induced MAPK activation [41].

Future investigations should be devoted to a deeper understanding of the molecular mechanisms underlying the effects of fluoride on mitochondrial gene expression and metabolism. Fluoride is an inducer of oxidative stress and modulates intracellular redox homeostasis, lipid peroxidation and protein carbonyl content. It also alters gene expression and causes apoptosis.

It is acknowledged that fluoride exposure induces the expression levels of genes encoding stress response factors, signal transduction components and apoptosis-related proteins (Table 2). Zhan et al. [42] found reduced hepatic SOD1 mRNA in young pigs fed with diets supplemented with high fluoride concentrations. There was some evidence of up-regulation of the stress response gene binding protein (BiP) upon exposure to fluoride [55]. BiP is an ER-resident molecular chaperone that is thought to prevent protein aggregation while maintaining a protein folding-competent state.

During cellular responses to environmental stress, the heat shock protein Hsp70 is rapidly activated and functions as part of the chaperone machinery. *In vitro* fluoride exposure in HepG2 cells [43] and chronic exposure to fluoride in humans resulted in increased expression of Hsp70 [44]. This finding suggests a possible role for Hsp70 as a mediator tolerance against chronic fluorosis.

In contrast, sperm cation channels (CatSper) are down-regulated in mice exposed to fluoride [45] and up-regulated in mice treated with the antioxidant selenium [46], suggesting that CatSper could represent an oxidative stress-related gene family. The CatSper family is exclusively expressed in the testis and plays an important role in sperm motility and male fertility.

Old and recent studies have shown that fluoride exerts different effects on the cell machinery leading to cell death, apoptosis and/or necrosis both *in vivo* and *in vitro*.

Table 2
Regulation of gene expression by fluoride exposure. Arrows refer to increases (↑) or decreases (↓) genes regulation.

Cells type and dose of fluoride	Gene expression	Ref.
***In vitro* (animals cells)**		
Primary rat hippocampal neurons at 40, and 80 mg/l, equivalent to 2.1, and 4.2 mM for 24 h	↑NF-κB	[24]
Porcine enamel organ cells and ameloblast-derived cell line (LS8 cells) at 2 mM NaF for 48 h	↑Growth arrest and DNA damage-inducible proteins GADD153, GADD45alpha, ↑binding protein, ↑the active X-box-binding protein-1, ↑non-secreted form of carbonic anhydrase VI	[51]
Mouse pancreatic beta-cells (βTC-6) at 1.35, and 2.5 mM for 12 h	↓Insulin	[31]
Primary rat hippocampal neurons at 40, and 80 mg/l, equivalent to 2.1, and 4.2 mM for 24 h	↓Neural cell adhesion molecules	[34]
Osteoblasts of Sprague–Dawley rats at 0.05 and 4 mM for 72, and 120 h	↑Osteoclast differentiation factor (ODF), ↑Osteoprotegerin	[106]
Mouse odontoblastos (MO6-G3 cells) at 1 mM for 5days	↓Extracellular matrix (asporin and fibromodulin), ↓Cell membrane associated proteins periostin, ↓the integrated transmembrane protein 2A, ↓signaling factor TNF-receptor 9, ↑Chemokine Scya-5	[107]
***In vitro* (human cells)**		
Ameloblast lineage cells at 10 μM for 24 h	↓Matrix metalloproteinase-20	[23]
Primary gingival epithelial cells at 5, and 50 μM for 24 h	↑Fibronectin, ↑Laminin (at 0.5 mM for 24 h)	[103]
Pulmonary epithelial (A549) cells at 5 mM for 8–24 h	↑Cyclooxygenase-2	[38]
Hepatocellular carcinoma (HepG2) cells at 3 mM at 6, and 24 h	↑p53, ↑heat shock protein (HSP)70, ↑Mn-SOD, ↓CYP1A1, ↓GAPDH	[43]
Embryonic hepatocytes (L-02 cells) at 40, 80, and 160 mg/l, equivalent to 2.1, 4.2, and 8.4 mM for 24 h	↑p53	[108]
Neuroblastoma (SH-SY5Y) cells at 40, and 80 mg/l, equivalent to 2.1, and 4.2 mM for 24 h	↑Apoptosis molecules Fas, Fas-L, and caspases (-3 and -8)	[57]
***In vivo* (animals)**		
Enamel epithelial cells of Wistar rats exposed to 100 mg F/l (5.25 mM) in drinking water for 8 weeks	↓Matrix metalloproteinase-20	[104]
Rib cartilage goat grazing in an industrial F pollution region of China, for 24 months	↑Type II collagen (COL2A1)	[105]
Liver of pig exposed to food supplemented with 250 mg F/kg for 50 days	↓Cu/Zn SOD	[42]
Sperm of Kunming mice exposed to 70, and 150 mg NaF/l (3.7, and 7.9 mM) in drinking water for 49 days	↓Plasma membrane Ca^{2+} channels, CatSper1	[45]
***In vivo* (humans)**		
Peripheral blood mononuclear cells from Mexican individuals drinking water with levels of 1.9–4.02 mg F/l	↓Inflammatory Chemokines (CCL1, CCL18, CCL19), ↓cytokines (IL-11; IL-2), ↓pro- and anti-inflammatory molecules (LTA, TNF-a, TGF-a, TGF-b1,and TGF-b3), ↓Apoptosis molecules (TNF-a, FasL, CD30L, 4-IBBL, TANK, TRAIL, DR3, Casp-2, Casp6, CIDE-A and CIDE-B), ↑survivine	[59]
Peripheral blood mononuclear cells from individuals living in endemic area in China (mean urine concentration of 2 mg F/l)	↑Heat shock protein, HSP-70	[44]

3.1.4. Necrosis

The cytotoxic effects of fluoride occur in all cell types. However, time- and concentration-dependent responses are different from one cell type to another. Necrosis has been observed as a primary mechanism of cell death in the presence of relatively high fluoride concentrations. Ghosh et al. [35] demonstrated that exposure of a primary culture of Swiss-strain mouse hepatocytes to 100 mM NaF for 1 h induced necrosis (propidium iodide positive/annexin V negative) via increased oxidative stress. These data coincide with the results of Matsui et al. who used a ten-fold lower concentration of NaF (10 mM) on a primary culture of rat thymocytes, although in that study an increase in intracellular calcium seemed to be involved [47].

3.1.5. Apoptosis

Programmed cell death is a complex phenomenon that includes delicate regulation of signaling proteins via gene expression and/or protein activity [48].

The role of oxidative stress in general, and of ROS in particular, in the induction of apoptosis seems to be concentration-dependent, and many works have concluded that fluoride induces apoptosis by elevating oxidative stress-induced lipid peroxidation, thus causing mitochondrial dysfunction and the activation of downstream pathways [22,25,46,49]. However, the central role of oxidative stress is now discussed because few studies have described the lack of ROS production during fluoride-induced apoptosis [28,50].

Many works have reported the role of intracellular calcium content in fluoride-induced apoptosis as a direct target of toxicity or an indirect consequence of altered cellular processes [51–56]. The possible mechanisms are described below.

Another component of apoptotic signaling is the expression/regulation of pro- and anti-apoptotic genes. For example, Bcl-2 has been demonstrated to be involved in fluoride-induced apoptosis. In the presence of 20 mM NaF, human gingival fibroblasts (HGF cells) showed a down-regulation of Bcl-2 followed by the activation of a mitochondrial cell death pathway through the enhancement of: (1) cyt C release from the mitochondria into the cytosol, (2) activation of the caspase cascade (with increased activities of caspase-3, -8 and -9), (3) the cleavage of Poly (ADP-ribose) polymerase (PARP) and (4) the expression of voltage-dependent anion channels [28].

The signaling pathway of the apoptotic response to fluoride exposure seems to also involve G proteins with varying effects. Thus, concentrations of 5–7.5 mM NaF are capable of inducing apoptosis with a concomitant increase in PKC, PKA, tyrosine kinase and calcium-dependent protein activities, whereas PI3 kinase acts in the opposite direction [41].

The second main finding of Lee et al. [28] involved the role of the death receptor-dependent pathway in fluoride-induced apoptosis. It was found that fluoride exposure resulted in the up-regulation of Fas ligand (Fas-L). The participation of the Fas-L pathway in fluoride-induced apoptotic cell death was also demonstrated with low-concentration exposure in osteoclast-like cells, where 0.1–0.3 mM NaF caused an increase of Fas and FasL and a decrease in NF-κB expression [28]. In addition, fluoride exposure in human neuroblast cells at 2–4 mM NaF increased caspase-3 activity and caused the mRNA up-regulation of Fas, Fas-L, and caspases (-3 and -8) [57].

The involvement of the death receptor led us to question the role of tumor necrosis factor-related apoptosis inducing ligand (TRAIL) in fluoride toxicity. The effect of NaF on TRAIL has been described; interestingly, the exposure of an adenocarcinoma cell line to 4-2(2-aminoethyl)benzylsulfonyl fluoride (AEBSF), an organic fluoride compound, did not promote apoptosis signaling, but did present morphological alterations associated with apoptosis. Park et al. [58] did not found the mechanism involved in this inhibition but that Cyt-C release from mitochondria was not decreased (when Cyt-C release is one of the main cellular event during TRAIL induced apoptosis) but apoptosis was arrest by this serine protease inhibitor in a late stage of apoptotic process; suggesting that this organic fluoride acts only on cell morphology (during post-mitochondrial events) and not on apoptosis signaling induced by TRAIL [58]. Considering that the main chemical form of fluoride used to induce apoptosis is NaF and that many studies reported fluoride-induced apoptosis, it is possible that AEBSF-induced mechanisms of cytoxicity are different from those of NaF.

Recent studies [59] performed in a human Mexican population show that exposure to fluoride can modify the expression of apoptotic genes in peripheral blood mononuclear cells (PBMC). In PBMC of subjects with urine concentrations in a range of 2.16–7.3 mg F/g creatinine, microarray experiments demonstrated a significant down-regulation of LT-b, CD40L, HVEM, caspase-6, TRAF-2 and TRAF-5 genes and an up-regulation of survivin when compared to the unexposed group.

A significant down-regulation of apoptotic genes of the TNF/TNFR superfamily (TNF-a, FasL, CD30L, 4-IBBL, TANK, TRAIL, and DR3) and the CIDE family (CIDE-A and CIDE-B) accompanied by up-regulation of survivin, which is a regulator of the cell-cycle and of apoptosis, was observed in PBMC of humans environmentally exposed to water contaminated with fluoride [59]. In addition, the expression of several pro- and anti-inflammatory genes was down-regulated in this group of residents from a fluoride-endemic area of Mexico [59]. In contrast, the *in vitro* exposure of A549 cells to NaF induced cyclooxygenase-2 (COX-2) expression, an important mediator of airway inflammation, at least partially by transcriptional up-regulation via the p38 and ERK pathways. Up-regulation of the COX-2 isoform is responsible for increased prostaglandin release and is also thought to be involved in inflammatory diseases. Furthermore, NaF-induced COX-2 expression was markedly suppressed by the Src family kinase (SFK) inhibitor but only partially suppressed by the epidermal growth factor receptor (EGFR) inhibitor, suggesting that SFK, and less significantly EGFR, may be the upstream tyrosine kinase responsible for NaF-induced COX-2 expression [38].

Another condition that can influence fluoride-induced cell death is pH. Thus, Hirano and Ando [54] showed that upon exposure of three different cell types, i.e., UMR106 (rat osteosarcoma cell line), rat alveolar macrophages and RAW264 (mouse macrophage cell line) cells to 5 mM fluoride for 8 h, the cytotoxicity was increased at acidic pH and decreased at alkaline pH. Interestingly, co-exposure to the calcium ionophore A23187 abolished the enhancing effect of acidic pH, confirming the important role of intracellular calcium in this phenomenon and suggesting that calcium-dependent endonucleases do not take part in fluoride-induced apoptosis. However, no precise mechanisms or participating proteins have been proposed [54].

All of these results confirm that fluoride induces apoptotic cell death through the modification of gene expression and protein activity by disturbing signaling messages through multiple mechanisms.

3.2. Secretion, trafficking and recycling vesicles

Vesicular traffic plays a key role in the transport of transmembrane proteins and secreted and soluble proteins when they are transported from one membrane compartment to another [60].

Protein transport occurs from ER to the plasmatic membrane via the Golgi apparatus (secretory proteins) or from the plasmatic membrane to endosomes and lysosomes (protein uptake). Consequently, vesicular traffic modulates exocytosis and endocytosis processes, both indispensable for preserves the homeostasis. The effect of fluoride exposure in vesicular traffic has been demon-

strated in some biological systems, through the interference with protein synthesis and secretion.

Dental fluorosis is a clear case in point of fluoride's influence on secretory pathways. It was quickly established that either acute or chronic exposure to NaF affects enamel formation, triggering dental fluorosis that manifests as mottled, discolored and porous enamel [23,61]. Aoba and Fejerskov [62] suggested that these effects are associated with precipitation of hydroxyapatite by fluoride ions, altering enamel mineralization. Alternatively, several authors concluded that these clinical signs are associated with the action of fluoride on the secretory functions of ameloblasts, epithelial cells responsible for enamel development [63].

The life cycle of these cells has three stages: secretory, transition and maturation. In the secretory phase there in an extensive ER that secretes large amounts of matrix proteins. In the transition phase, rough ER and Golgi complexes decrease. Finally, during the maturation phase the ameloblasts secrete serine proteases that induce protein degradation and removal. Several decades ago, morphological studies [64] showed that fluoride affects the secretory stage in ameloblasts. The biological mechanism was recently elucidated and involves the ER stress response [51], resulting in a reduction of protein synthesis, secretion and total protein concentration [65]. Similar effects of fluoride exposure have been shown in diverse cell types derived from tissues having a regulated secretion pathway, such as neurons and exocrine and neuroendocrine cells. A decrease in the total cellular protein concentration was observed in a cell line derived from anterior pituitary tumor cells exposed to NaF [21]. The effect of fluoride on insulin secretion has been studied for a long time, indicating either decrease [31,66] or increase of insulin secretion [67]. Neurotransmitter discharge is also affected by fluoride exposure. It has been reported that acetylcholine release is modified by this xenobiotic in both directions; release is increased in the brain [49] and decreased in the cervical ganglia [68]. The release of gamma-aminobutyric acid, another neurotransmitter in the cerebral cortex, is stimulated by fluoride exposure [69]. It is well-documented that fluoride's effects on exocytosis in neuronal tissue are mediated by G protein-coupled effector systems, inducing either an increase [70] or decrease [71] in neurotransmitter release. It has been observed that Al–F stimulates endocytosis via G protein activation as well [72]. Therefore, it is important to recognize that these biological effects of fluoride are closely associated with their ability to modify the activity of G-proteins.

On the other hand, the hormesis effect (phenomenon characterized by a low dose stimulation, high dose inhibition), has been proposed as a possible explanation for the paradoxical effects associated with fluoride exposure [5].

Nevertheless, until now the clinical evidence of fluoride exposure on vesicular traffic is mainly focus to dental fluorosis, it is important to contemplate that exocytosis or endocytosis function could be affected also in others target organ, although there is a lack of information the direct association with the development of other pathologies.

3.3. Effect on transport proteins

Among the wide variety of fluoride's effects, its effect on the activity of transport proteins is particularly important. As we previously mentioned, fluoride affects secretion and vesicular traffic via transport proteins that are synthesized in ER and subsequently transported to the Golgi and plasma membrane.

Many pathologies have been related to an alteration of activity, localization or expression of transport proteins such as inherited Fanconi Syndrome, characterized by uric acid, glucose, amino acids, phosphate and bicarbonate lost into the urine and caused by xenobiotics exposure [73]. Another example are Dent's Disease-like pathologies, that affects renal proximal tubule function, inducing hypercalciuria, proteinuria, calcium nephrolithiasis, nephrocalcinosis due to the alteration of endosomal channels [74].

However the direct impact of fluoride on the appearance of these pathologies have not been still investigated in spite of the existing experimental evidences that transport proteins could be affected by fluoride exposure.

3.3.1. Calcium and phosphate transporters

The relation between fluoride and calcium has been suggested since 1948 [75]. All over the years, more works have evidenced that fluoride could alters calcium homeostasis in human population [76] and that calcium also plays an important role in a wide range of cellular alterations induced by fluoride.

Calcium plays a key role in signaling mechanisms triggered by external or internal stimuli and regulates a variety of cellular processes [77]. Thus, calcium simultaneously represents an integrative signal and a central convergence point of many distinct signaling pathways in all cell types [78]. Cellular responses to changes in calcium concentrations are modulated by a tight regulation of the spatial and temporal occurrence of calcium and the intensity of the amplitude of such changes [79]. Plasmatic and organelle membranes separate compartments that have different free calcium concentrations, and the mechanisms that regulate the release and recovery of calcium are diverse and complex. These mechanisms include calcium channels, calcium-binding proteins, calcium-sequestering organelles (ER), sodium/calcium exchangers and calcium pumps. Effects of fluoride on calcium homeostasis and intracellular quantity have been described, although the mechanism and pathways have not been sufficiently established. It has been suggested that fluoride increases calcium retention by some tissues (redistribution process), as evidenced by hypocalcemia along with diminished urinary excretion and augmented intestinal absorption [80]. Chronic exposure to fluoride in drinking water (at concentrations of 0, 50, 100 and 150 mg/l) caused an increase in fluoride plasma levels (0.2, 2, 7 and 35 μmol/l, respectively) that was related to a reduction in calcium transport across the renal tubule ER and plasma membrane, as well as to a reduction of the amount of calcium pump proteins in isolated kidney membranes [52]. In contrast, increased cytosolic calcium concentrations have previously been established in several cells/tissues, including proximal tubules [53], fibroblasts [81] and osteoblasts [82]. Some reports have suggested the activation of G proteins as a possible mechanism [83]. Interestingly, both inhibitory and stimulatory effects of fluoride on the calcium pump have been shown in the cardiac sarcoplasmic reticulum. It was explained that the dissimilar responses were due to differential susceptibility of the conformational state of the calcium pump [84]. However, it is possible that the biphasic response could be due to a hormesic effect, as the fluoride concentrations at which inhibitory and stimulatory effects occurred were also different (10 and 2.5 mM, respectively). A lot of experiments still need to be performed to accounting for these apparently different effects of fluoride.

Because recent results have shown the relevance of the mitochondria, Golgi and peroxisomes as calcium stores [85], it is important to recognize that the action of fluoride on these organelles would also impair calcium homeostasis.

Calcium and phosphate homeostasis are stretch related in many physiological processes in general, and in kidney and bone metabolisms in particular. Besides calcium concentrations, phosphate is also regulated by hormonal factors (PTH or 1,25-dihydroxyvitamin) [86]. These mechanisms can also be targets of fluoride, nevertheless, few mechanisms have been proposed to explain a possible cellular effect on phosphate regulation.

Phosphorous is an essential element that enters the cell in the form of P_i P_i phosphate (P_i) by secondary active transport. Na^+/K^+-ATPase generates the electrochemical gradient trigger for the Na^+

ion flux necessary for P_i transport. The effect of fluoride on this co-transporter has been studied somewhat by the Peerce group, but the study was limited to organic compounds of fluoride. Inhibition of the transporter by mono- and di-fluorophosphates was observed [87].

3.3.2. Glucose transport

The effects of fluoride on glucose metabolism have been examined in both *in vivo* and *in vitro* studies. There is epidemiological evidence for a link between chronic exposure to fluoride and the development of glucose intolerance [88]. Our results [31] together with results from other studies [89] have shown that fluoride exposure may contribute to impaired glucose tolerance or increased blood glucose.

Studies evaluating toxic effects of fluoride on insulin secretion have been reported. Komatsu et al. [67] evaluated insulin secretion in RINm5F cells exposed to NaF and found that fluoride produced an increase of insulin release for up to 60 min as the fluoride concentration increased. In contrast, studies done in Langerhans islets isolated from rats found a relationship between fluoride exposure and decreased insulin secretion [66,90]. Menoyo et al. [90] found an inhibitory effect on insulin secretion at micromolar concentrations (5–20 μM), whereas Lin et al. [66] found the same effect at high millimolar concentrations (up to 17 mM). In contrast, Komatsu et al. [67] found increased insulin secretion at a millimolar range of fluoride exposure (3–20 mM) in RINm5F cells. We recently explored the effect of fluoride on glucose metabolism using *in vivo* and *in vitro* experimental models and confirmed that biologically relevant doses of fluoride result in impairment of an oral glucose tolerance test and decreased insulin synthesis without changes in GLUT-2 levels, respectively [31].

Recently, it was reported that Akt participates in the insulin signaling pathway because the substrate (AS160) is a Rab GTPase-activating protein. The suppression of AS160 phosphorylation promoted GLUT-4 retention in intracellular compartments. In addition, NaF activates GLUT-4 translocation [91]. It was reported that in renal tissue, the sodium/glucose co-transporter (SGLT) is not affected by fluoride exposure [92].

3.3.3. Na^+/K^+-ATPase

Na^+/K^+-ATPase is an ATP-powered ion pump that plays a central role in preserving the electrochemical membrane potential. Its activity establishes Na^+ and K^+ gradients across the plasma membrane that are essential to the life of the cell. Inhibition of this pump by NaF has been known for a long time [93]. The inhibitory effect has been demonstrated in different cell types, such as brush border cells and at the basolateral membranes of proximal tubule cells (35 mg/kg i.p.; [94]), in ascending limb cells [95], in erythrocyte cell membranes (500 mg/l; [96]) and in brain tissue [97]. Some pathological effects, e.g., natriuresis, polyuria and glucosuria in fluorosis poisoning, have been linked to the inhibition of Na^+/K^+-ATPase [92].

On the other hand, Anderson et al. reported an increase in Na^+/K^+-ATPase activity in osteoblast-like cells upon fluoride exposure (10 μM; [98]). At this time, a possible hormetic effect on this pump has not been explored, as no studies have shown both inhibitory and stimulatory effects on the activation of Na^+/K^+-ATPase within the same cell type.

3.3.4. Cystic fibrosis transmembrane conductance regulator (CFTR)

The cystic fibrosis transmembrane conductance regulator (CFTR) is an epithelial cAMP-dependent chloride channel and a modulator of ion channels and transporters involved in cellular mechanisms such as regulatory volume reduction or apoptotic volume decrease. Such processes involve effects on ATP release, intracellular calcium homeostasis, and potassium channel activation [99]. The CFTR protein is composed of five domains: two membrane-spanning domains, two nucleotide-binding domains (NBDs), and a regulatory domain [100]. NBDs have been shown to strongly interact with fluoride anions. Exposure to 20 mM of NaF resulted in increased activity of CFTR (enhanced chloride currents) due to increased burst duration of the channel. Such changes were prevented when NBD2, but not NBD1, was mutated, suggesting that the NBD2/fluoride interaction is responsible for this phenomenon [101]. In that study, it was also shown using GTPγS that G proteins did not participate in the fluoride-induced overstimulation of CFTR.

Since L'Hoste et al. [99] demonstrated that CFTR mediates apoptosis by controlling glutathione efflux and generation of ROS. Taking into account the results obtained by Berger et al. [100] and others[49,101,102], it is plausible to suppose that CFTR could participate in fluoride-induced apoptosis, acting on anti-oxidative system. However, experimental data still misses.

3.4. Transcriptional effects

Currently, no mechanisms of fluoride as an epigenetic agent have been recognized through its interaction with histone deacetylases, histone acetyltransferases, DNA methyltransferase, and microRNAs. Although, evidence indicates that fluoride is a transcriptional modulator for several cell types; both normal and pathological (Table 2). For instance, at low μM fluoride concentrations, increased expression of fibronectin and laminin have been found in epithelial cells, accompanied by a down-regulation of metalloproteinase expression as well as increased cell motility and migration, affecting the matrix assembly [23,103,104]. c-Jun is a key regulatory element for metalloproteinase expression, and human ameloblast lineage cells can respond to fluoride by down-regulating metalloproteinase transcription through the JNK/c-Jun signaling pathway [23]. *In vivo* [105] and *in vitro* fluoride exposure at mM concentrations also causes aberrant expression of genes implicated in bone modeling, tissue formation and growth [106,107], impairing the formation of the extracellular matrix and influencing cell communication, with the possible consequence of fluorotic patterns of standard and abnormal dentin.

Fluoride treatments have also been found to increase NF-κB mRNA levels in a concentration-dependent manner in neurons incubated with various concentrations of fluoride for 24 h [24]. NF-κB mediates, in part, the opposing signals of cell survival and cell death. Thus, up-regulation of NF-κB by fluoride could stimulate tumor cell growth, regulate the expression of early response genes mainly connected with the course of the inflammatory process, and regulate miRNAs.

Fluoride exposure was also shown to decrease the mRNA levels of neural cell adhesion molecules (NCAM) in a concentration-dependent manner in primary rat hippocampal neurons [34]. NCAM may serve a dual role by modulating intracellular signaling events in response to patterns of neuronal activity and by affecting changes in adhesion during the remodeling of synaptic structures.

The mRNA levels of p53 are up-regulated in embryonic hepatocytes incubated with fluoride [43,108]. p53 acts as a key regulatory molecule during cell stress, and affects cell emergency signaling by transcription or non-transcription. More recent studies have found that p53 can mediate the expression of various genes, such as p21, c-myc, Bcl-2, TGF-b, IL-2, Fas, and Bax. Depending on the cell type, cell environment and oncogenic alterations, p53 activation leads to the inhibition of cell cycle progression and differentiation, as well as induction of senescence or apoptosis [109].

It was recently reported that fluoride exposure regulates insulin gene expression in murine beta pancreatic cells, resulting in reduced insulin secretion [31]. Although several transcription factors have been implicated in glucose-induced transcription of the

insulin gene, the exact molecular mechanisms leading to regulation of insulin gene expression are unknown.

The mechanism leading to gene deregulation in response to fluoride exposure, as well as the functional significance of such alterations with respect to fluoride's adverse effects, are poorly elucidated at the current time. On the other hand, the sensitivity to fluoride-responsive genes varies from one cell type to another and is also highly dependent on fluoride concentration and the duration of exposure.

3.5. Inflammatory response

Inflammation is the first response of the immune system to infection or tissue damage, leading to the protection of the human body against these insults. However, chronic inflammation is harmful and has an important role in the development of several chronic diseases such as diabetes and atherosclerosis. Inflammation can be triggered by a chronic excess of neurotransmitters and/or metabolic factors, such as cytokines, lipids, and glucose. In many physiological or pathological settings, these stimuli can also elicit ER stress, which further disrupts metabolic functions, thereby causing more inflammation. Such vicious cycles could potentiate the signaling pathways that integrate stress and inflammation and could also cause metabolic deterioration in some cells.

Fluoride exposure has been implicated in inflammation. Human exposure to inhaled fluoride is implicated in acute respiratory failure-inducing inflammatory reactions in the respiratory tract [110]. In addition, rat lung tissues presented emphysema and lung parenchyma inflammation associated with loss of alveolar architecture in the second generation of adult rats exposed to 50 or 100 mg/l of NaF via the drinking water. The degree of lung damage was correlated with the dosage of fluoride [111].

Interleukin 8 (IL-8) has a pivotal role in several pathological conditions, such as chronic inflammation, fibrosis and cancer [112]. The inflammatory effect of fluoride exposure was evaluated in human lung epithelial cells, and an increase in the activity of IL-8 was found (with a 5–7-fold increase after 20 h exposure to 3.75 and 5 mM NaF, respectively) [113].

The expression of proinflammatory cytokines is controlled by proinflammatory regulatory transcription factors including the commonly present NF-κB, which exhibits fluoride-induced expression at the transcriptional level in hippocampal neurons [24].

The expression of COX-2, was increased markedly in A549 human pulmonary epithelial cells upon fluoride exposure [38]. In contrast, down-regulation of several inflammatory genes was observed in PBMC of residents from a fluoride endemic area [59]. However, the functional significance of such alterations due to fluoride exposure needs to be studied.

Accumulating evidence suggests extensive cross-talk between the inflammatory response and the ER stress response, a condition that produces an imbalance between the protein-folding load and ER capacity. Such processes disrupt the mechanisms by which proteins fold or increase the demand for protein folding, causing unfolded or misfolded proteins to accumulate in the ER lumen. Fluoride induces ER stress and initiates the unfolded protein response (UPR) in ameloblasts, which are responsible for dental enamel formation [51,65]. A growing body of evidence suggests that the signaling pathways in the UPR and inflammation are interconnected through various mechanisms, including the production of ROS, the release of calcium from the ER, the activation of NF-κB and the activation of the MAPK known as JNK (JUN N-terminal kinase).

As a general stress inducer, fluoride can activate kinases leading to the overactivation of transcription factors including JNK [22,23] and NF-κB [24]. These studies revealed that fluoride signals at multiple levels cause systemic metabolic changes.

In addition, UPR signaling is an important mediator of vascular inflammation and is possibly involved in the endothelial cell dysfunction observed in atherosclerosis.

Interestingly, NADPH oxidase (Nox), an important source of ROS in the vasculature, is activated by high levels of *in vitro* fluoride exposure [114]. Nitric oxide synthase (NOS) is involved in the formation of NO, a highly reactive, uncharged, membrane-permeable molecule that functions as a signal in many regulatory processes such as blood vessel dilation, immune responses and neurotransmission. NO is dependent on Nox; changes in Nox expression and activity have implications in endothelial dysfunction and vascular disorders. It is possible that endothelial dysfunction in coronary heart disease could be related to the chronic inflammation that coexists with atherosclerosis. Nox has been implicated as a rate-limiting step in signaling for inflammation and vascular remodeling following fluoride exposure.

Excessive production of NO can alter the oxidative state and calcium concentration in the ER and disrupt the electron transport chain, causing ER stress and ROS production. It is important to point out that ROS and NO levels are significantly increased after fluoride intoxication [29,30].

In addition, endothelial dysfunction and vascular disorders have been associated with fluoride exposure in cell lines [115,116] and in humans [117,118]. The data suggest an important role played by factors related to oxidative stress and vascular inflammation, providing future directions for research into the cardiovascular effects of fluoride exposure. However, most of these studies have been conducted using high levels of fluoride. Clearly, more studies are needed to elucidate the role of low to moderate fluoride exposure in vascular disease.

3.6. Cell migration

Directed motility and cellular migration are prominent features in the development and function of many organisms. These cellular events are characterized by a cycle of membrane protrusion during which new cell attachment processes form and the oldest processes detach. Several distinct extracellular and intracellular events must be coordinated so that migration can proceed; one of these events is actin cytoskeleton rearrangement.

The actin cytoskeleton is a dynamic structural component of cells that plays a key role in determining cell shape, motility and cytokinesis and is composed of actin filaments and specialized actin-binding proteins [119]. Filamentous actin is organized in structures such as actin stress fibers (bunches of actin filaments that span the cell and are linked to the extracellular matrix through focal adhesions), lamellipodia (cytoskeleton actin projections on the mobile edge of the cell) and filopodia (finger-like protrusions that are thin fibers containing parallel bundles of actin in the direction of the overhang). Cell migration involves the regulated polymerization and depolymerization of these actin fibers. Reorganization of the actin cytoskeleton is necessary for cell mobility and is mediated by Rho [120], Rac [121], and Cdc42 [33] proteins, all of which are small G proteins. Rho proteins regulate stress fiber formation, Rac proteins regulate lamellipodia formation and Cdc42 regulates filopodium formation [122]. The Sar1/Arf family belongs to the small G protein superfamily that has been associated with the regulation of intracellular vesicular traffic [123]. Further, Arf6 was reported to play a role in cell surface morphology because its activation by Al-F complexes induces the formation of actin-enriched protrusions [124].

Al-F complexes have also been used in the study of the structures and catalytic mechanisms of enzymes involved in phosphoryl transfer, because these complexes are considered phosphate analogs [125] and are therefore activators of heterotrimeric G-proteins [16]. Therefore, the biological effects of Al–F complexes

on cytoskeletal rearrangement have been explored in several models, and their implied mechanism of action is the activation of G proteins. In mast cells, resident cells of several types of tissues containing granules rich in histamine and heparin, Al–F complexes triggers the disassembly of the cortical actin filaments and/or their detachment from the plasma membrane [126]. In addition to Al–F, some studies have shown that other fluoride compounds, such as NaF, can cause actin cytoskeleton alterations. In endothelial cells, changes induced in the actin cytoskeleton by NaF were mediated by the time-dependent activation of Rho/RhoK, resulting in alterations in barrier function [116]. Similarly, Arakawa et al. [103] showed that in epithelial cells, NaF induces cell proliferation, cell migration and matrix protein production. Recent data suggest that fluoride exposure could have relevant implications in the behavior of tumor cells. In the GH4C1 cell line (derived from pituitary tumor cells), NaF induces cellular migration and proliferation; both effects could be associated with the malignant and invasive capacity of tumors [21]. Nevertheless, inhibitory effects of fluoride on cell migration have been described in embryonic neurons [127]. In addition, a significant decrease in sperm motility in adult male rats that received NaF was also observed [32].

The inductive effects of fluoride on cell migration are now beginning to be explored in medicine, specifically in dental treatment, using compounds that contain fluoride ions as bone graft materials with the aim of stimulating the migration of macrophages and proliferation of bone cells, leading to new bone formation [128].

Nevertheless, it has been recently shown that phenylmethylsulfonyl fluoride is a potent inhibitor of macrophage migration inhibitory factor [129]. The authors suggest that pursuing this line of research could lead to improved treatment opportunities for diseases like sepsis, cancer and certain immune diseases.

Inhibitory effects of fluoride on cell migration have been described also in embryonic neurons [127]. In addition, a significant decrease in sperm motility in adult male rats that received NaF was also observed [32]. These apparent contradictory effects of fluoride on cell migration, could be related with the cell type and the cell interactions with its environment.

Herein, we have showed several studies that relate the fluoride exposure and the induction of cellular migration. The tumor cells particularly have a capacity enhanced for migrate and invade either into adjacent or distant tissues. Therefore, the possibility would fit of thinking that the fluoride can change the migratory behavior of the tumor cells. At this respect, recently our group demonstrated in the GH4C1 cell line, that NaF induces cellular migration and proliferation; both effects could be associated with the malignant and invasive capacity of tumors [21]. Tatin et al. [130] lately showed the podosomes induction by NaF exposure in endothelial cells, this effect was mediated by the activation of G proteins like Cdc42 and Rac1. Podosomes are a kind of adhesion structures related with transformation cell. Therefore, although until now there is limited evidence of fluoride carcinogenicity; these data suggest that fluoride exposure could have relevant implications in the behavior of tumor cells.

3.7. Proliferation and cell cycle

In eukaryotic cells, the cell cycle is divided into two brief periods: interphase and mitosis. Interphase has three phases: G_1 (cells increase their size), S (DNA replication occurs) and G_2 (significant protein synthesis occurs, mainly for the production of microtubules). The mitosis (M) phase consists of karyokinesis (nuclear division) and finally cytokinesis (cytoplasm is divided into two cells). Quiescent and senescent cells remain in a phase of cellular arrest (G_0). It has been suggested that fluoride has differential effects depending on the cell type [131]. However, some studies provide evidence that the effects of fluoride exposure on cell cycle progression are closely related to the fluoride concentration. Fluoride's effect on cell proliferation has been investigated to some extent in both ameloblast and osteoblast cells. In ameloblasts, a clear biphasic effect on cellular proliferation was observed, with enhanced proliferation at micromolar fluoride concentrations and decreased proliferation at millimolar concentrations [61]. Similar results were reported by Thaweboon et al. [132] at concentrations of 5 and 80 mg/l (0.26 and 4.2 mM, respectively). The mitogenic effect of fluoride on osteoblast proliferation has been known for several decades [133], and this effect has been explored to find therapeutic alternatives for medical and dental applications [134]. In addition to the stimulation of osteoblast proliferation, fluoride also inhibits osteoclast activity [134]. Osteoblasts and osteoclasts are responsible for bone formation and resorption, respectively, and the effects of fluoride on these cells have been used to stimulate bone formation in several animal models [135]. Analysis of the effects of fluoride on cell cycle phases in cultured rat osteoblasts an increased number of cells at S phase and a decrease in cells at G_2/M phase, while the cells in G_0/G_1 remained unchanged [136]. In agreement with these results, it was found in GH_4C_1 pituitary tumor cells that at biologically relevant concentrations (10.7 μmol/l), fluoride induced DNA synthesis (S phase), whereas at high concentrations (1072 μmol/l), DNA synthesis was blocked [21]. On the other hand, a reduction in the number of cells in G_2/M phase was observed in the renal tissue of Wistar rats that received 50 mg NaF/l (2.62 mM) in their drinking water, which is similar to the Zhang study [136]. However, a significant reduction in DNA was also reported in this study [137]. The activation of mitogen activated protein kinases (MAPK), such as p38 and JNK, has been proposed as a possible mechanism downstream of G-protein activation by which fluoride exerts its effects on the cell cycle in lung epithelial cells [131]. It was recently found using proteomic analyses that in the livers of fish raised in water with high fluoride concentrations (35 mg/l), 24 proteins were highly expressed including Cyclin-D1, a protein involved in the modulation of cell cycle [138]. Fluoride can bind to Ran protein, a member of the GTP-binding G-proteins, which regulates nuclear-cytoplasmic transport during the G_1, S and G_2 phases and is implicated in the rearrangement of microtubules during M phase [122]. However, as of the writing of this paper, there is no information available on the activation of Ran by fluoride.

Cancer is a disease that is strongly linked with the cell cycle, and the well-known effects of fluoride on cellular proliferation, mainly described for bone cells, have motivated the development of different studies in this field, particularly in the case of osteosarcoma. Fluoride appears to be a mutagenic agent and induce chromosome aberrations, sister chromatid exchanges, and cytotoxic effects in cultured mammalian cells [139]. Moreover, low fluoride concentrations caused increased chromosomal anomalies along with primary DNA damage in human peripheral blood cultures (HPBC) [140]. However, results of toxicological and ecological studies remain controversial because some of these studies have not been able to establish a link between fluoride and osteosarcoma [141]. Other authors propose that high fluoride levels in the bone might be an important factor for osteosarcoma development [142], suggesting that exposure to high concentrations of fluoride could be a risk factor for the development of osteosarcoma [143].

In addition, DNA damage to hippocampal neurons, as determined by increased olive tail moment, was reported as a consequence of *in vitro* exposure to fluoride (2.1 and 4.2 mM for 24 h), indicating that primary DNA damage [24] causes increased chromosomal anomalies along with primary DNA damage in HPBC.

It is important to consider that previous reports showed that the exposure of tumor cells to fluoride could stimulate both their migration and proliferation [21], and consequently their invasive properties might also increase.

4. Consequences of co-exposure to fluoride and other substances

Drinking water is the primary source of fluoride exposure in humans. In this route of exposure, fluoride coexists with several other xenobiotics, frequently metals. Fluoride consumption within these mixtures could modify its kinetic and toxicity properties. Here, we present some mixtures that should be mentioned given their frequency and biological relevance.

4.1. Aluminum

In the earth's crust, it is unlikely to find free Al^{3+} because it is incorporated into minerals. However, with acid pluvial precipitation, a slight amount of free Al^{3+} is dissolved in water as a polluting agent [6]. In aqueous solutions where there is fluoride and traces of aluminum ions, Al–F complexes can form. The structure and proportion of the species that are formed is highly dependent on the pH and fluoride concentration. At pH >5.5, the predominant structure is $Al(H_2O)_6{}^{3+}$ (frequently abbreviated as Al^{3+}), and at pH <6.2 the predominant form is an aluminate, $Al(OH)_4{}^{1-}$. At pH >6.2 but <9, AlF_3 and $AlF_4{}^{1-}$ are the main species produced [144,145]. Complexes formed between fluoride ions and aluminum have central biologic relevance. $AlF_4{}^{1-}$ could be the most significant; due to its geometry, size and coordinance, it could function as an analog of the γ-phosphate activating G proteins [125,16]. As a consequence, it could affect several signaling pathways, resulting in changes in gene expression, cytoskeletal reorganization, intracellular vesicle trafficking, and nucleocytoplasmic transport [122]. Until now, practically all of the biological effects of fluoride ions have been associated with their capacity to form Al-F complexes, whose chemical and biochemical properties were extensively described in previous reviews [145,146]. Several other metals have been tested to evaluate their capacity to form fluoride complexes that could induce the activation of G proteins. Only Be has been reported to be as good of a cofactor as Al for signal transduction activation [16,125,146].

It has been described that Al-F complexes induce a wide range of health effects. Aluminum is considered to be a neurotoxin, and some studies have suggested that concentrations of Al in drinking water of 0.1–0.2 mg/l may increase the risk of Alzheimerĩs Disease (AD [147,148]). In an epidemiological study, Still [149] suggested that fluoride may be a protective agent against AD in populations exposed to both Al and fluoride ions. It was proposed that Al and fluoride compete for absorption in the gut and that this competition may serve as a mechanism for the possible protective effect [150]. However, neurotoxicity of Al-F complexes has also been shown [151].

4.2. Arsenic

Arsenic is a metalloid that occurs naturally in the earth's crust and is a component of more than 200 minerals. Its inorganic forms are mainly arsenite and arsenate compounds and are recognized worldwide as toxic to human health. The major source of exposure to arsenic or/and fluoride in the world is through food and drinking water contaminated with high levels of arsenic and/or fluoride [2]. Recently, the interaction of arsenic and fluoride received significant attention because concurrent exposure of these toxicants is frequent [36,49,59,152,153]. Although the toxic effects of arsenic and fluoride when administered alone have been widely studied and are relatively well-known, the biological effects and possible interactions when the exposure is simultaneous have only been scarcely studied, and the results of these studies are controversial. Antagonistic, synergistic, and independent effects have all been reported. Flora group [36] performed some studies in experimental models with the aim of evaluating the effect of arsenic and fluoride, individually or together, on oxidative stress and cell injury. It was concluded that the simultaneous administration of arsenic and fluoride is less toxic [36], or may even afford an antagonistic effect [49], compared to when these toxicants are administered individually. Although it has been suggested in humans that there are no interactions between the absorption and excretion of fluoride and arsenic when they are co-administered [154], some epidemiologic studies have shown that co-exposure to fluoride and arsenic increased the risk of reduced IQ scores in children [155]. Moreover, it was recently reported that simultaneous exposure to both xenobiotics increased the induction of genotoxic effects [140]. Therefore, it is clear that the toxic effects of simultaneous exposure to arsenic and fluoride remain controversial. This discrepancy could be explained by the differences in the biological systems used, or by the dosages or proportions of the combined doses of both pollutants. As a result, it is necessary to perform more studies to elucidate this issue.

4.3. Lead

Recently, a possible relationship between water fluoridation and elevated blood lead (PbB) concentrations in children was suggested. Ecologic associations have been reported between the use of silicofluoride compounds (sodium silicofluoride and hydrofluosilicic acid) and NaF as water fluoridation methods and elevated PbB concentrations among children [156]. Similar results were observed in children who live in old homes supplied by fluoridated water [157]. The increased lead in water is related to plumbing corrosion, especially when the water has been treated with both fluoridating and disinfectant (chloramines) agents [158]. It is therefore possible that the increased water lead levels observed when fluoride concentrations are between 1 and 2 mg/l, could be severely aggravated in places with high fluoride concentrations in the drinking water. However, a recent study found that fluoride increased PbB and lead levels in calcified tissues of animals exposed to low levels of lead, suggesting biological interactions as a possible cause of the epidemiological relationship between high PbB levels and the fluoridation of drinking water [159].

5. Conclusions

In this work, we focused on showing the effects of inorganic fluoride compounds on the cellular function of several biological systems. The studies described above demonstrated that fluoride can interact with a wide range of cellular processes such as gene expression, cell cycle, proliferation and migration, respiration, metabolism, ion transport, secretion, endocytosis, apoptosis/necrosis, and oxidative stress, and that these mechanism are involved in a wide variety of signaling pathways (Fig. 1).

Although many proteins involved in these alterations of the cell machinery have been identified, particularly phosphatases, many of the targets and the exact mechanisms/pathways taking part in these events are still unknown. However, the specific effect resembling that mimics the activation of G proteins by P_i, is frequently implicated in several of the biological effects described above. Currently, the increasing knowledge of the activation of G proteins by fluoride compounds suggests that such effects may result in corollary effects of fluoride on vesicle traffic, cell migration, cell proliferation, gene expression and other key functions in the cell. However, the complexity of fluoride's effects on these processes should be appreciated because the effects induced by fluoride are closely related to dose and concentration (hormesis effect). Furthermore, even though some studies report no clear evidence on the potential negative effects of fluoride exposure at permissible concentrations (e.g., studies that support water fluoridation), oth-

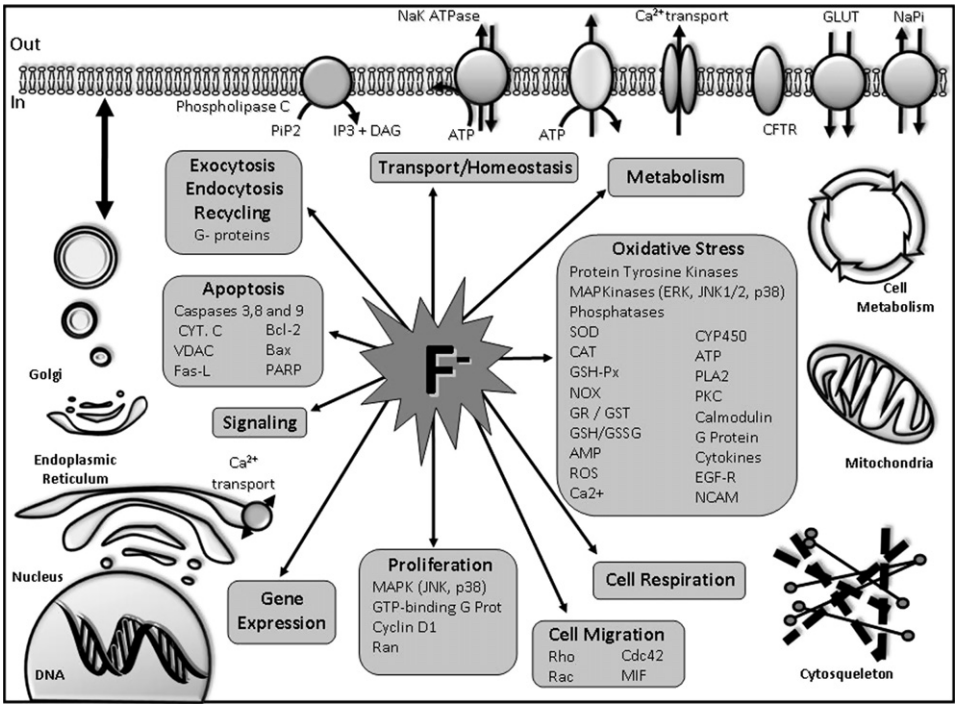

Fig. 1. General scheme of the biological consequence of fluoride (F^-) exposure on mammalian cells.

ers have shown evidence of fluoride's effects on cellular processes at biologically relevant concentrations. When discussing these controversial results, it is important to highlight that fluoride must be actively considered as a potent toxic compound in the field of toxicology, both in epidemiologic/ecological research and in fundamental or applied research. In conclusion, this evidence of the positive and negative effects of fluoride needs to be considered along with the ethical, environmental, ecological, financial, and legal issues that surround any decisions about water fluoridation. Any future research into the safety and efficacy of water fluoridation should be carried out with the appropriate methodology to improve the quality of the existing evidence base.

Finally, in drinking water, fluoride is frequently used with other elements (metals and/or metalloids), which does not necessarily lead to more pronounced toxicity; in some particular cases, antagonistic effects have been reported. Therefore, in the absence of clear proof to counter the known toxic effects of fluoride in combination with metalloids and metals, extensive studies are needed to conclusively determine the effects of such combinations on relevant cell types.

Conflicts of interest

None.

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