ANTICANCER RESEARCH *37*: 4767-4777 (2017)
doi:10.21873/anticanres.11883

Review

Fluoride Induces Apoptosis in Mammalian Cells: *In Vitro* and *In Vivo* Studies

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Abstract. *Apoptosis is genetically programmed cell death, an irreversible process of cell senescence with characteristic features different from other cellular mechanisms of death such as necrosis. In the last years, apoptosis has been extensively studied in the scientific literature, because it has been established that apoptosis plays a crucial role following the time course of chronic degenerative diseases, such as cancer. Thus, several researchers have strugged to detect what chemical agents are able to inter fere with the apoptotic process. Thus, the purpose of this literature review is to assess if fluoride induces apoptosis in mammalian cells using in vivo and in vitro test systems. Certain mammalian cell types such as oral cells, blood and brain were exetensively investigated; the results showed that fluoride is able to induce apoptosis in both intrinsinc and extrinsic pathways. Moreover, other cells types have been poorly investigated such as bone, kidney and reproductive cells with conflicting results so far. Therefore, this area needs further investigation for the safety of human populations exposed to fluoride in a chronic way, as for example in developing countries.*

Fluoride is naturally present in the environment, being found in rocks, coal, and clay. Fluoride is an essential trace element encountered in small quantities in water, air, plants, and animals (1). It has long been recognized that fuoride in low concentrations is essential for teeth and bone development being an effective agent for optimizing mineralized tissues (2).

For many years ago, the daily exposure of human organisms to fluoride depended on the intake of this ion with drinking water being closely associated with topographical regions. Since fluoride has been discovered as a chemical agent capable of inhibiting dental caries, it has been widely used in dentistry as a prophylactic agent (3). Herein, sodium fluoride (NaF) has been incorporated to diverse applications, such as toothpastes, gels, drinks, salts, and others (4). As a consequence, the levels of fluoride exposure in human populations are increasing drastically, particularly in developing countries, where the control of exposure to the ion is not properly regulated by local government agencies. In fact, many side health effects have been attributed to excessive fluoride intake (5). This pathological condition is defined as fluorosis, which is characterized as a chronic long-term exposure to high levels of fluoride, clinically manifested by skeletal fragility and subsequent damage to internal organs, including kidneys, liver, and brain (6).

Apoptosis is genetically programmed cell death, an irreversible process of cell senescence with characteristic features (cell shrinkage, chromatin condensation, DNA fragmentation, apoptotic bodies) different from other cellular mechanisms of death such as necrosis (7). Two signaling pathways characterize the event: intrinsic or mitochondrial pathway mediated mainly by proteins belonging to the cl-2 family and the extrinsic pathway based on Fas type death receptors (7). Independent of the pathway involved in the process, apoptosis triggers the activation of caspases.

Nowadays, apoptosis has been extensively studied in the scientific literature, because it has been established that apoptosis plays a crucial role following the time course of chronic degenerative diseases, such as cancer (8). Thus, several researchers have struggled to clarify what chemical agents are able to inter fere with the apoptotic process. Some studies have demonstrated that excess fluoride can cause

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Key Words: Fluoride, apoptosis, *in vitro* studies, *in vivo* studies, review.

DNA damage, and change cell-cycle regulatory proteins that in turn induce apoptosis (9). However, the underlying mechanisms of apoptosis induced by fluoride are still unclear; especially to identify the signaling pathways that are closely involved to this process.

Thus, the purpose of this literature review is to assess whether fluoride induces apoptosis in mammalian cells using *in vivo* and *in vitro* test systems. Certainly, our critical analysis will provide new knowledge regarding the issue for future perspectives, specifically because there is limited information concerning the mechanism of fluoride-induced cell death so far.

In Vitro Studies

As expected, most *in vitro* studies have investigated the effects of fluoride on mineralized cells such as odontoblasts, ameloblasts and bone cells. Certainly, this is because fluoride interacts directly with mineralized tissues. Herein, it has been purposed several signaling pathways regarding apoptosis process. Such information was very important for better understanding the cytotoxicity induced by fluoride. However, such mechanisms needed to be confirmed by *in vivo* studies since *in vitro* assays do not consider the complex homeostatic condition that occurs *in vivo*, especially when investigating systemic exposure to chemical agents. The results are shown below.

Oral cells. First molar germs from 1-day-old Balb/c mice exposed to increasing concentrations of fluoride (0 mM, 1 mM and 5 mM) revelated disorganization in ameloblast and odontoblast-papilla zones (10). Caspase 8, caspase 3, Bax, Bid increased expression and subsequent more TUNEL positive cells in fluoride groups were noticed (10). Eluates derived from glass ionomer cements releasing fluoride continously also showed the presence of apoptotic process as depicted by high annexin V expression in human dental pulp cells (11).

When odontoblast cells were exposed to NaF at 3 mM, cells detached from their support and did not proliferate (12). Odontoblast like cells incubated with NaF at 5 mM exhibited caspase-3 activation, cleavage of poly(ADP-ribose) polymerase, DNA fragmentation, and the presence of cytoplasmic nucleosomes as well as ultrastructural alterations (13, 14). This resulted in the translocation of Bax to the mitochondria and the release of cytochrome *c* from the mitochondrial inter-membrane space into the cytosol, indicating that fluoride-mediated apoptosis ismitochondria-dependent (14). NaF exposure induced a biphasic phosphorylation of extracellular signal-regulated protein kinase (ERK) (13). Thus, exposure to NaF induces apoptosis in odontoblast-like cells, mainly through extrinsic apoptotic pathways depending on JNK and, less significantly, ERK pathways (13, 14). However, necrosis was detected to this concentration of fluoride showing that fluoride leads to an insult of high magnitude to the cellular apparatus (12).

Ameloblasts also showed high sensitivity to fluoride with respect to cytotoxicity, especifically apoptosis. Flow cytometry showed that both 10 μM and 20 μM NaF significantly increased the apoptotic index of ameloblast-lineage cells (15). Morphologically, high fluoride incubation damaged the ameloblast ultrastructure manifesting a series of intracellular stress responding cell organelle destruction, and a marked increase in the expression of apoptotic genes (16-18). Ameloblasts were growth-inhibited by as little as 1.9-3.8 ppm fluoride, whereas higher doses of this one induced endoplasmic reticulum stress and caspase-mediated DNA fragmentation increased intracellular Ca^{2+} *in vitro* in a dose related fashion (19-21). The endoplasmic reticulum stress has been closely related to X-box binding protein 1 (XBP-1) (21). On the other hand, some authors have revealed that excessive fluoride inhibited cell proliferation of ameloblasts (22). Others have yet reported that fluoride had a dual effect on cell proliferation, with enhanced proliferation at 16 μM, and reduced proliferation at greater than 1 mM F (23).

At protein and molecular levels, fluoride induced ameloblast apoptosis *via* activation of the FasL/Fas signaling pathway (Wang *et al*. 2016). Fluoride promoted cytochrome-*c* release, up-regulation of UCP2, attenuation of ATP synthesis, and H2AX phosphorylation (γH2AX) (18). High amounts of fluoride induced more apoptosis/dead cells and reduced the expression of Bcl-2 decreased expression along with up-regulated expression of CD68 (15, 22). Furthermore, a decreased expression and increased phosphorylation of Foxo1 in NaF-treated ameloblast cells has been shown (24). Recently, it has been demonstrated that NaF prevented apoptosis of ameloblast cells, by decreasing protein levels of cytoplasmic cytochrome *c*, cleaved caspase-9, and cleaved caspase-3 and caspase 8 while increasing the Bcl-2/Bax ratio and JNK expression level (21, 24). Fluoride treatment of ameloblast-derived cells significantly increased SIRT1 expression and induced SIRT1 phosphorylation resulting in the augmentation of SIRT1 deacetylase activity (17). Such findings demonstrated that fluoride is able to inhibit apoptosis *via* p53 acetylation pathway. Recently, western blot revealed that the protein expression of p-ERK and p-JNK were decreased, while the expression of p38 was increased (21). Taken as a whole, it seems that fluoride has a dual role on apoptosis in ameloblasts.

Expression of beclin1, which is required for autophagosome formation, decreases the expression of mTOR increased at 1.2 mmol/l NaF (25). Fluoride promoted cytochrome-c release, up-regulation of UCP2, attenuation of ATP synthesis, and H2AX phosphorylation (γH2AX) as far as DNA damage and cell death (18). Additionally, immunohistochemical analysis was performed on paraffin-embedded rat incisor sections to identify the expression of Beclin1 and mTOR proteins *in vitro*. Fluoride induced highly significant differences between non-exposed groups (25).

These findings suggest that NaF-induced apoptosis of ameloblast cells *via* inhibiting the mitochondrial pathway and activating caspases as a putative mechanism of dental fluorosis. Therefore, these results suggest that fluoride causes mitochondrial damage, that may lead to impairment of ameloblast function.

NaF reduced the cell viability of human gingival fibroblasts in a dose- and time-dependent manner (26). NaF increased TUNEL-positive cells and induced apoptosis by means of concomitant chromatin condensation and subsequent DNA fragmentation (26). In addition, NaF increased the release of cytochrome *c* from the mitochondria into the cytosol, as well as enhanced the caspase-9, -8 and -3 activities, the cleavage of poly (ADP-ribose) polymerase (PARP), and up-regulated the voltage-dependent anion channel (VDAC) 1 (26). Furthermore, NaF up-regulated the Fas-ligand (Fas-L), and Bcl-2 was down-regulated. Expression of Bax was unaffected in the NaF-treated human gingival fibroblasts (26). These results suggest that NaF induces apoptosis in human gingival fibroblasts through both the mitochondria-mediated pathways regulated by the Bcl-2 family and death receptor-mediated pathway (26).

When rat oral epithelial cells were exposed to NaF, a relatively high concentration of NaF (2 mM) induced cell death concomitant with decreases in mitochondrial membrane potential, chromatin condensation and caspase-3 activation (27). Such findings suggest that NaF did not activate caspase 3 in oral mucosa cells.

The gene networks Up-I and Up-II included many up-regulated genes that were mainly associated with the biological function of induction or prevention of cell death, such as *Atf3, Ddit3* and *Fos* (for Up-I) and *Atf4* and *Hspa5* (for Up-II). Interestingly, knockdown of Ddit3 and Hspa5 significantly increased and decreased the number of viable cells, respectively (27). Moreover, several endoplasmic reticulum stress-related genes including, *Ddit3, Atf4* and *Hapa5*, were observed in these gene networks (27). These findings will provide further insight into the molecular mechanisms of NaF-induced cell death accompanying endoplasmic reticulum stress in oral epithelial cells (27). When human squamous cell carcinoma cells were exposed to NaF, activated caspase 3 and subsequent DNA fragmentation was observed (28, 29). Also, NaF interferes with Bad-Bcl-2 complex by means of carbonic anhydrase II detachment from Bad protein, contributing, therefore, to the apoptosis process (30).

Bone cells. To date, some mechanisms have been purposed with regard to apoptosis induced by fluoride in bone cells. For example, osteoblasts were exposed to different concentrations of NaF (10(-6)-5 × 10(-4) M); the results revealed that NaF inhibited proliferation and arrested cell cycle at S phase as far as induced apoptosis (31-38). This was detected by activation of caspase 3 and 9 in osteoblast cells (32). Nevertheless, others have failed to detect caspase 3 activation after fluoride exposure to bone cells (39).

MTT assay showed that NaF at concentrations of 10^{-8} to 10^{-5} M promoted cell proliferation, whereas at 10^{-4} to 10^{-3} M it suppressed cell proliferation and induced apoptosis in caprine osteoblasts (33, 36). Such findings lead to the putative dual role of fluoride on cell cycle regulatory proteins, which is dose-dependent. Apoptosis induced by fluoride was due to alterations in the expression of both pro-apoptotic Bax and anti-apoptotic Bcl-2 (33). Particularly, there was a decrease in the Bcl-2/Bax ratio, which was found at both the mRNA and protein levels (33). Others have revealed that fluoride induced apoptosis through the MAPK/ERK signaling pathway (37).

NaF increased oxidative stress and decreased protein expression of IGF-I contributing to proliferation and apoptosis (31). Such findings suggest that the administration of NaF affects the rat osteoblast survival (33). Alkaline phosphatase (ALP) activity and mineralization ability increased in cells treated at 10^{-8} to 10^{-5} M with sodium, but decreased at 5.0×10^{-4} to 10^{-3} M dosage in caprine osteoblasts (36). Other authors have demonstrated no increase of alkaline phosphatase activity in bone cells exposed to fluoride (39). Conversely, calvaria cells were cultured for 28 days in the presence of several doses of NaF (0, 5, 10, 25, 50, and 75 μM), the results showed no effects on alkaline phosphatase activity but decreased mineralized nodule formation (40). These negative effects include decreased *COL1A1* mRNA, down-regulating the synthesis of COL1 protein. The results suggest that COL1 protein degradation following fluoride toxicity is due to a depletion of COL1A1 mRNA and not COL1A2 (33).

Osteoblasts also showed a reduction in the mineralization pattern only after 50 μM of NaF with a slight increase of type-I collagen and down-regulation of MMP-2 activity during the mineralization period (40). In conclusion, fluoride affects the production and degradation of the extracellular matrix during early onset and probably during the mineralization period.

The PCR chip detection found 13 up-regulating genes and 15 down-regulating genes, among which the expression of Bim, caspase 9, caspase 14, B-cell lymphoma-2 (Bcl2) and Bax increased with the doses of sodium fluoride, while the expression of caspase 3 was down-regulated in 5 mg/l sodium fluoride but up-regulated at the concentration of more than 10 mg/L sodium fluoride (41). Caspase 7 expression showed no obvious difference between the different concentration groups (41). However, caspase 10 decreased with the increasing doses of sodium fluoride (41). The underlying mechanisms of apoptosis induced by fluoride may be through the mitochondrial pathway (including endoplasmic reticulum stress pathway) and death receptor pathway (41). After scrutinizing the genome, it plausible to believe that fluoride induces apoptosis in osteoblast cells (38).

Blood cells. Human myeloma peripheral blood cells were cultured with increasing doses of fluoride (10, 20, 40, 80, 160, 320 μM). After 48 h exposure, fluoride increased cells viability at relatively low levels (10-160 μM); however, when the concentration reached 320 μM, the cell proliferation was significantly inhibited (42) and subsequent cell hemolysis occurence within 24 h in the presence of 0.5-16 mM of NaF (43). Morphologically, this change was characterized by shrunken echinocytes after 1 h and swollen spherocytes within 24 h (43). The development of NaF-induced erythrocyte death was accompanied by progressive positivity of annexin V-staining in response to 16 mM NaF within 24 h with a small cell population exhibiting necrotic features (43). The results suggest that overexposure to fluoride (160-320 μM) can induce cytotoxicity and regulate relevant genes expression (42, 44). Taken together, all findings confirm earlier reports on mechanisms involved in NaF-induced apoptosis in blood cells (45).

At the molecular level, mRNA expression of genes, including *ANKRD1, CRSP6, KLF2, SBNO2, ZNF649, FANCM, PDGFA, RNF152, CDK10,* and *CETN2* changed in a concentration-dependent manner and increased with fluoride exposure concentration in blood cells (42). Treatment of rat erythrocytes with 5 mM NaF for 1-24 h caused progressive accumulation of cytosolic C^{a2+} in the outer membrane surface (43). Incubation of erythrocytes with 0.1-10 mM NaF for 1 h produced a dose-dependent PKCα translocation from cytosol to membranes with appearance of active PKM fragment. NaF exposure for 14h led to complete loss of cytosolic PKCα and proteolysis of membrane PKCα (43). Besides, NaF weakly stimulated membrane PKCζ, although its subcellular distribution was not altered. Thus, transient PKCα activation/translocation positively contributes to NaF-induced apoptosis *in vitro* (43).

Splenic lymphocytes from mice were exposed to NaF (0, 100, 200, and 400 μmol/l) *in vitro* for 24 and 48 h (46, 47). NaF induced lymphocyte apoptosis, which was promoted by decreasing mitochondria transmembrane potential, up-regulation of Bax, Bak, Fas, FasL, caspase 9, caspase 8, caspase 7, caspase 6 and caspase 3 protein expression and down-regulation of Bcl-2 and Bcl-xL protein expression (46, 47). The above-mentioned data suggested that NaF-induced apoptosis in splenic lymphocytes could be mediated by mitochondrial intrinsic pathway and death receptor pathways.

Fluoride also induced apoptosis in human leucemia cells in a dose- and time-dependent manner (48). Many authors have proven that fluoride led to the activation of caspase-3 and caspase-9 (49-52) at higher concentrations (100-250 ppm), reduced cell viability, and decreased DNA and protein biosynthesis capability in cultured HL-60 cells. Morphologically, NaF promoted internucleosomal DNA fragmentation, and increased the proportion of hypodiploid cells (48). After exposure to NaF, there was an increase in MDA and 4-HNE and a loss of mitochondrial membrane potential (deltaPsi(m)) was also observed in NaF-treated cells (50). There was a significant increase in cytosolic cytochrome *c*, which is released from the mitochondria (50). This leads to down-regulation of Bcl-2 protein in NaF-treated cells in human leukemia cells (48, 50). However, others have assumed that NaF enhanced the expression of Bad protein, but not that of Bcl-2 and Bax proteins, and reduced HIF-1alpha mRNA expression (51).

Brain cells. The levels of apoptosis and c-Jun N-terminal kinases (JNK) in brain cells exposed to different concentrations of sodium fluoride (NaF) were detected (53). At a concentration of 50 ppm of fluoride, the increased apoptotic death rate was evidenced (53).

Fluoride induced apoptosis in hippocampal cell line (54). Primary rat hippocampal neurons were incubated with 20, 40, and 80 mg/l sodium fluoride for 24 h *in vitro* (55). The results showed that the cell survival rate in the 80 mg/l fluoride-treated group was significantly lower (55). As expected, the significant differences of intracellular Ca^{2+} concentration and apoptotic peaks were found in 5.0-40.0 mg NaF/L groups in rat hippocampal neurons (56). With respect to NCAM mRNA expression levels, a significant dose-dependent decrease was observed with 40 and 80 mg/l fluoride in hippocampal cells (55). In addition, protein expression levels of NCAM-180 in 40 and 80 mg/l fluoride-treated groups, NCAM-140 in all fluoride-treated groups, and NCAM-120 in the 80 mg/l fluoride-treated group were significantly decreased (55). Therefore, it is a concensus that fluoride could cause apoptosis, and decreased mRNA and protein expression levels of NCAM in rat hippocampal neurons. Such findings clarify part of the biological mechanisms of neurotoxicity induced by fluoride.

When brain tumor cells were exposed to fluoride, similar findings were found. For example, human neuroblastoma cells were incubated with 0, 20, 40, and 80 mg/L sodium fluoride (NaF) for 24 h *in vitro*. The data show that cell viability in the 40 and 80 mg/l fluoride groups were significantly lower than that of the control group (57). The percentages of apoptosis in the 40 and 80 mg/l fluoride groups were markedly higher in a dose-dependent manner. The activity of caspase-3 and mRNA expression levels for Fas, Fas-L, and caspases (-3 and -8) in the 40 and 80 mg/l fluoride groups were significantly higher as well (57). The results indicate that fluoride exposure could induce apoptosis in brain tumor cells, and the Fas/Fas-L signaling pathway may play an important role in the process (57).

Reproductive cells. When Sertoli cells were exposed do fluoride *in vitro*, endoplasmic reticulum stress, decreased cell viability and apoptosis was detected at varying doses (58).

The endoplasmic stress as evidenced by up-regulated glucose-regulated protein 78 kDa (GRP78), PKR-like ER kinase (PERK), phosphorylation of eukaryotic translation initiation factor 2α (p-eIF2α) and CCAAT/enhancer-binding protein-homologous protein (CHOP), without affecting total eukaryotic translation initiation factor 2α (eIF2α) (59). In addition, NaF facilitated the accumulation of ROS and increased nuclear translocation of nuclear factor erythroid 2-related factor 2 (Nrf2) in Sertoli cells (58, 59). The levels of FasL protein and mRNA decreased with the increase of fluoride concentration in mouse sertoli cells (60). The caspase 8 and caspase 3 mRNA levels were also decreased in fluoride groups in a dose-dependent manner (60). These findings indicated that fluoride triggered apoptosis by the Fas/FasL system, but it was not able to activate caspase 3.

The chemical effects of NaF on Leydig cells were also demonstrated since high dosage of NaF inhibited cell proliferation by stress-induced apoptosis (61). The intrinsic signalling apoptotic pathway was activated by fluoride exposure including caspase-3/caspase-9, B-cell lymphoma 2 (Bcl-2), and Bax (61).

Liver cells. Few studies have demonstrated apoptosis induced by fluoride in liver cels. Fluoride caused apoptosis and increased cell numbers in S phase of cell cycle in human embryo hepatocytes exposed to sodium fluoride at different doses (40 microg/mL, 80 microg/mL, and 160 microg/mL) for 24 h (62). There is a significant positive correlation between fluoride concentration and these pathological changes (62). Other findings showed that the percentage of apoptosis and c-Fos mRNA and protein expression levels in 40 and 80 mg/l NaF-treated groups were higher in liver cells (63). c-Fos methylation levels were decreased in 20, 40, and 80 mg/l NaF-treated groups against the control group (63). Further, Dnmt1 mRNA expression level was significantly decreased in the 80 mg/l NaF-treated groups; Dnmt3a and Dnmt3b mRNA expression levels were significantly decreased in 40 and 80 mg/l NaF-treated groups (63). These results suggest that NaF could induce apoptosis and up-regulate mRNA and protein expression level of c-Fos as well as decrease mRNA expression levels of Dnmt1, Dnmt3a, and Dnmt3b in L-02 cells. The decrease in c-Fos methylation levels might be involved in the early phase of apoptosis induced by NaF liver cells (63).

Incubation of hepatocytes with NaF increased apoptosis of HepG2 cells (64). Proliferation slightly increased and then decreased with a high fluoride treatment (64).

Lung. Lung epithelial cells were exposed to sodium fluoride (NaF-5 mM) alone for 24 h induced apoptosis as well as inhibited cell proliferation (65, 66). Furthermore, fluoride activated changes in mitochondrial membrane potential, permeability transition pore opening, cytochrome-*c* release, Bax/Bcl-2 ratio, caspase-3 and PARP-1 expressions (67).

In Vivo Studies

Oral cells. *In vivo* studies failed to investigate the effects induced by fluoride regarding the apoptotic process. Nevertheless, some pathobiological mechanisms have been purposed so far. Rats exposed to fluoride at 150 mg/L contributed to cell cycle disruption of oral cells as depicted by a lower cell number at G_0/G_1 as well as mitotic cells (68). In humans, exfoliate cytology in oral mucosa cells of individuals with diagnosed fluorosis pointed out apoptosis process as demonstrated by the increased nuclear size and decreased cell size (69). Toppical application of 1.23% acidulated phosphate fluoride to oral mucosa of rabbits after 1. 5 and 8 days of continuous exposure revealed genetic damage and morphological alterations indicative of apoptosis by transmission electron microscopy as depicted by loss of cell-to-cell contact, nuclear chromatin condensation and apoptotic bodies (70). At the molecular level, rats treated with fluoride at 10 mg/kg for 5 weeks showed that caspase-9 and caspase-3, and the gene expressions of Bax were significantly increased (71).

In enamel organs from rats or mice treated with 50, 100, or 125 ppm fluoride for 6 weeks, cytochrome-c release was increased (18).

It has been widely documented *in vitro* that ameloblasts are very sensitive to the toxic effects of high dose fluoride in drinking water (19). However, some results are conflicting so far. Activated Ire1 initiates an endoplasmic reticulum stress response pathway, and mouse ameloblasts were shown to express activated Ire1 (19). Ire1 levels were induced by fluoride treatment, indicating that endoplasmic reticulum stress may play a role in dental fluorosis (19). It is assumed that low dose fluoride, such as that present in fluoridated drinking water, did not induce endoplasmic reticulum stress (19).

Bone. Surprisingly, only one study has been dedicated to fluoride cytotoxicity induced by apoptosis in bone cells so far. Wistar rats exposed to increasing concentration of fluoride (50, 100, and 150 mg/l) for 8 weeks showed that expression of GRP78, XBP1, caspase-12, and CHOP were increased in a dose-dependent manner (72). Fluoride-induced apoptosis in osteoblasts was also dose-dependent. High concentrations of fluoride induced endoplasmic reticulum stress and osteoblast apoptosis *in vivo* (72). Caspase-12 and CHOP activation was associated with osteoblast apoptosis (72). To understand the real biological effects that are involved with the cytotoxicity induced by fluoride in bone cells remains to be elucidated.

Blood. Since peripheral blood reflects a systemic exposure to several chemical compounds, there are many studies investigating the effects of fluoride in these cells Children chronically exposed to fluoride at 5.3 mg/l in drinking water

and in food cooked with the same water showed a lower percentage of CD25- and CD40-positive cells (73). The same findings were observed in others (74).

Broilers exposed to high-fluoride diets containing 0, 400, 800, and 1,200 mg/kg fluorine showed that apoptotic lymphocytes were significantly increased with high-fluoride (75). Meanwhile, immunohistochemical tests showed that the Bcl-2 protein expression decreased, Bax and caspase-3 protein expression increased in lymphocytes of the high-fluoride groups (75).

In rats exposed to increasing concentrations of fluoride (0-500 ppm), p53, Bcl-2, and caspase-3 up-regulation was noticed in leukocytes (76). These results indicate that NaF intoxication can be an apoptosis inducer in rat leukocytes treated with the compound for eight weeks (76). However, others have mentioned that chronic exposure to fluoride at high concentrations (up to 100 ppm) did not produce any sign of apoptosis (77). Further studies are necessary to better understand what pathways closely to apoptosis are activated after fluoride exposure, because it is not possible to predict if fluoride is closely involved with the apoptosis process.

Liver. Rat hepatocytes exposed to fluoride revealed the presence of apoptosis when exposed to 150 mg/L for 4 weeks (68). Fluoride-induced morphological changes and significantly increased apoptosis and DNA damage in rats exposed to fluoride, by administering varying concentrations of fluoride (0, 50, 100, 200 mg/l) for 120 days (78). This was confirmed by high expression of caspase-3, caspase-9 protein expression with increasing NaF concentration in rat liver (78). The same results were confirmed in pigs exposed to high doses of fluoride (79).

Furthermore, cell cycle disruption as result of a lower number of liver cells undergoing cell divsion was noticed (68). An earlier study conducted by Campos-Pereira *et al.* (80) have affirmed that fluoride is not able to induce apoptosis in rat hepatic cells since no remarkable differences were noticed with a TUNEL assay. Nevertheless, morphological changes indicative of cytotoxicity such as empty nuclear spaces, cytoplasm degeneration, nuclear pyknosis, karyorrhexis and karyorrhexis followed by karyolysis were detected (80).

Fluoride induced up-regulation of some key genes closely related to oxidative damage, apoptosis, and mitochondrial stress (81). The expression of PI3K and Akt1 mRNA and proteins was significantly increased in fluorosed rat hepatocytes (81). The apoptosis and intracellular calcium concentration were also increased (81). The protein levels of Fas as well as FasL in NaF were significantly increased compared with those in the control group (82). Therefore, such published data have purposed several signaling pathways invoved to the pathogenesis of liver injury caused by fluorosis.

Spleen. Spleen of mice exposed to fluoride increased lymphocytes apoptosis, which was consistent with NaF-caused endoplasmic reticulum stress (46). Broilers exposed to high doses of fluoride presented endoplasmic reticulum stress in spleen cells that in turn leads to apoptosis as well (83). This was due to up-regulation of glucose-regulated protein 78 (BiP) and glucose-regulated protein 94 (GRP94), and by activating unfolded protein response (UPR) (46). Moreover, high expression of caspase 12 followed by, growth arrest, DNA damage and phosphorylation of JUN N-terminal kinase (p-JNK) were detected (46). Increased apoptosis ratio following caspase-9 and caspase-3 up-regulation in the spleen of rats exposed to an excessive amount of fluoride was noticed (84).

Rats exposed to excessive fluoride had lower levels of IL-1β, IL-2, IL-6, TNF-α, and IFN-γ in the spleen (84). Light and transmisison electron microscopy revealed the irregularly arranged lymphocytes, few lymph nodules and the apoptotic characteristics of lymphocytes (84).

Reproductive system. NaF exposure induced an enhanced testicular apoptosis, as manifested by caspase-3 activation, chromatin condensation and subsequent DNA fragmentation in rats (85). Further studies revealed that fluoride exposure elicited significant elevations in the levels of cell surface death receptor Fas with a parallel increase in cytoplasmic cytochrome *c*, indicating the involvement of both extrinsic and intrinsic apoptotic pathways (85). Overall, it seems that extrinsic and intrinsic signaling apoptotic pathways are closely actived by fluoride exposure.

Testis of male offspring were studied at 8 weeks of age exposed to fluoride during gestation. The results demonstrated that fluoride treatment enhanced germ cell apoptosis (86). In addition, fluoride elevated mRNA and protein levels of glucose-regulated protein 78 (GRP78), inositol requiring ER-to-nucleus signal kinase 1 (IRE1), and C/EBP homologous protein (CHOP), indicating activation of ER stress signaling (86). Furthermore, fluoride also induced testicular inflammation, as manifested by gene up-regulation of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), in a nuclear factor-κB (NF-κB)-dependent manner (86). These were associated with marked histopathological lesions including injury of spermatogonia, decrease of spermatocytes and absence of elongated spermatids, as well as severe ultrastructural abnormalities in testes, such as endoplasmic reticulum stress (86).

Testis of mice exposed to fluoride revealed 763 differentially expressed genes, including 330 up-regulated and 433 down-regulated genes, which were involved in spermatogenesis, apoptosis, DNA damage, DNA replication, cell differentiation, protease inhibitor activity, ubiquitin mediated proteolysis, and the signaling pathways of calcium,

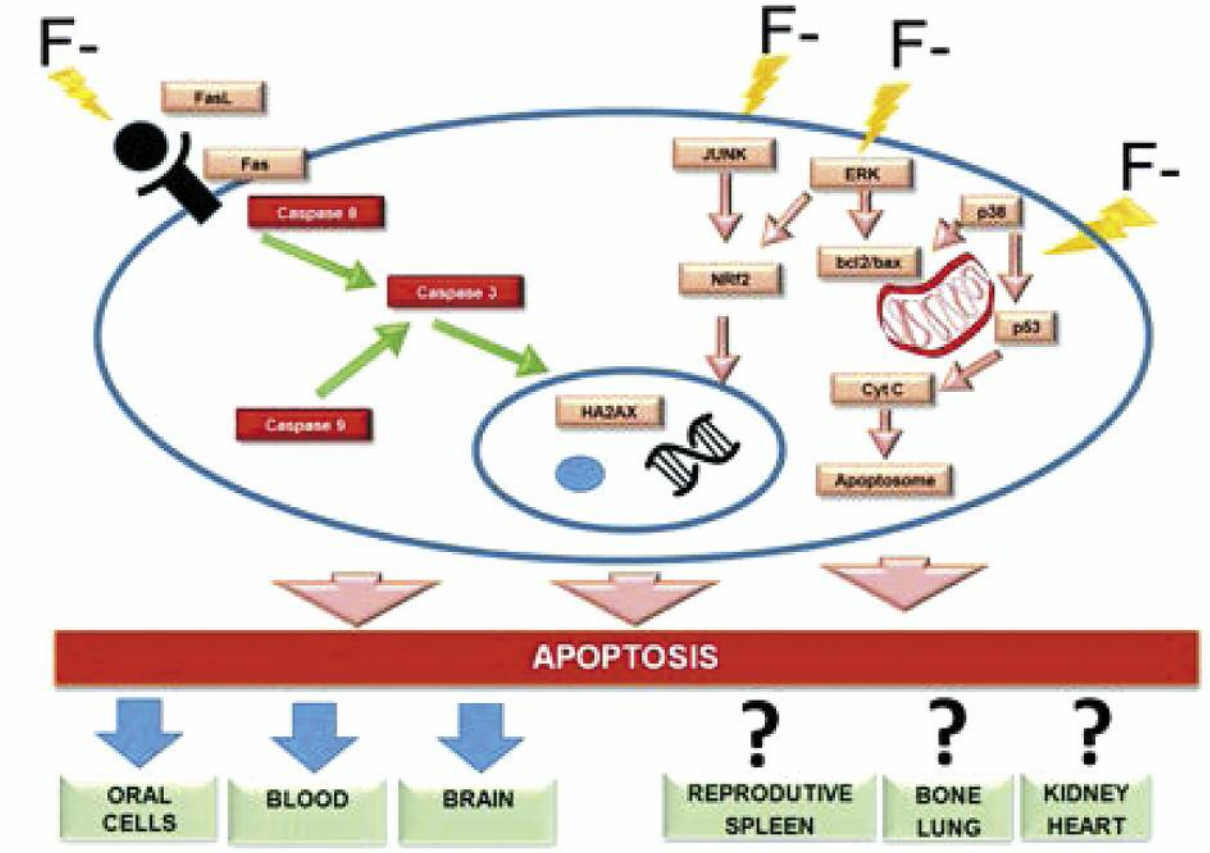

Figure 1. *Pathobiological mechanisms of the apoptosis process induced by fluoride in mammalian cells.*

JAK-STAT, MAPK, p53, Wnt, which were proved to be directly related to sperm quality (87, 88). Furthermore, more apoptotic spermatogenic cells were observed in the fluoride group, and the spermatogonium was markedly increased in S phase and decreased in G_2/M phase by fluoride (87). Taken as a whole, global genome microarray provides an insight into the reproductive toxicity induced by fluoride. However, additional molecular pathway analysis is needed (88).

Diffuse apoptosis in glandular epithelium and stromal cells were found in endometrial tissues of fluoride treated rats by the TUNEL method (89). Rats exposed to 100 or 200 mg/L NaF in their drinking water for 6 months showed ovarian apoptosis (90). Further investigations in ovarian granular cells showed that exposure to NaF activated extracellular regulated protein kinase (ERK) and c-Jun NH2 kinase (JNK), disrupting the ERK and JNK signaling pathways, while p38 and PI3K remained unchanged (90). Therefore, we assume that the majority of published papers are dedicated to the inflammatory host response induced by fluoride rather than apoptosis *in vivo*. Further studies are necessary to elucidate the issue.

Central nervous system. It has been established that fluoride is an important target when investigating the central nervous system because it induces oxidative stress, glial activation and inflammation which leads to neurodegeneration (91). All of those changes lead to abnormal cell differentiation and the activation of apoptosis through changes in the expression of neural cell adhesion molecules (NCAM), glial fibrillary acidic protein (GFAP), brain-derived neurotrophic factor (BDNF) and MAP kinases (91). Excessive exposure to this element can cause harmful effects such as permanent damage of all brain structures, impaired learning ability, memory dysfunction and behavioural problems (91).

Bcl-2 expression levels significantly decreased while caspase 12 levels were increased, in brain cells of rats offspring exposed to fluoride during gestation (87). Moreover, the number of apoptotic cells, the expression levels of cytochrome *c* and the expression levels of caspase-9 and caspase-3 significantly increased (87). By contrast, caspase-9 and caspase-3 protein levels significantly decreased in the rat brain exposed to fluoride (92). TUNEL assay revealed apoptosis of rat neurons with increasing

fluoride concentrations (93). Bax protein expression increased and Bcl-2 protein expression decreased in fluoride-treated rat brain compared with that of the control rat brain (93). The levels of IL-1β and IL-6 protein expression in microglial cells were significantly increased in the cortex and hippocampus of rats exposed to fluoride, and TNF-α immunoreactivity in microglial cells of the hippocampus was significantly higher in the 120 ppm fluoride-treated group than that in the control group (93).

Kidney. Male Sprague-Dawley rats were treated with 0, 50, 100, and 200 mg/l of NaF, *via* drinking water for 120 days (94). The results showed that NaF treatment increased apoptosis and subsequent DNA damage (94). In addition, NaF treatment increased the protein expression levels of cytosolic Cyt *c* and cleaved caspases 9, 8, and 3 (94). These results indicated that NaF induces apoptosis in rat kidneys through caspase-mediated intrinsic apoptosis pathway activation.

Avian broilers showed that the percentage of renal cell apoptosis was increased with increasing of dietary fluoride, when compared with that of the control group (95). Renal cells in G_0/G_1 phase were much higher, and renal cells in S phase, G_2+M phase, and proliferation index value were much lower in the high fluoride groups (95). Kidneys of rats exposed to fluoride showed the same results (96).

Conclusion

In this review, we present the published results regarding apoptosis induced by fluoride exposure in mammalian cells *in vitro* and *in vivo* (Figure 1). Some cell types such as oral cells, blood and brain were exetensively investigated; the results showed that fluoride is able to induce apoptosis in both intrinsinc and extrinsic pathways. Nevertheless, other cells types have been poorly investigated with conflicting results such as bone, kidney and reprodutive cells. Further studies using different end-points, as well as to evaluate the role of cell cycle regulatory proteins, are important to understand the cytotoxicity as a result of apoptosis induced by fluoride. Such information is very important to elucidate what cell types are more sensitive to fluoride exposure. Therefore, this area needs further investigation for the safety of human populations exposed to fluoride in a chronic way, as for example in developing countries.

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Received June 19, 2017
Revised July 4, 2017
Accepted July 6, 2017