[Now published in full after the initial publication as an Epub ahead of print August 1, 2023 at www.fluorideresearch.online/epub/files/211.pdf]

EFFECT OF FLUORIDE ON OSTEOINDUCTIVE PROCESSES IN HUMAN OSTEOBLAST hFOB1.19

Laura Vázquez-Ayala,[a] Amaury Pozos-Guillén,[a] Hector Flores,[a] Gabriela Palestino-Escobedo,[b] Diana Maria Escobar-García[a,*]

San Luis Potosí, Mexico

ABSTRACT: Fluorine (*F*$^-$) is considered a toxic chemical element, the exposure to this chemical compound in humans goes almost unnoticed; at present, an important number of people are exposed to water sources contaminated with *F*$^-$ causing health damage, and the effects on bone tissue are considered the most significant. *Objective:* **To evaluate the effect of *NaF* in different osteoinduction processes.** *Materials and methods*: **An ATCC hFOB1.19 cell line was used which were cultured with the different *NaF* concentrations (1, 1.5, 3, 5, 10, 15 mg/l). Quantitative viability through the Alamar-Blue test and qualitative cytotoxicity according to ISO10993-5:2009, intra and extracellular *Ca*$^{+2}$ concentrations through the Arsenazo III test, qualitative and quantitative osteogenesis processes in osteoblasts with the Millipore osteogenesis kit, immunocytochemistry to determine the expression of *RUNX-2*, and Mito-Tracker assay to evaluate the quantity of mitochondria in the treated cells were performed.** *Results and conclusions:* **Fluoride significantly altered cellular morphological patterns in sub-chronic exposure, it was also capable of reducing the expression of the *RUNX2* protein and the *Ca*$^{+2}$ concentration of intracellular and extracellular as well as being able to reduce the number of mitochondria in a dose-dependent way.**

Key words: *Ca*$^{+2}$*;* Mitochondria; Morphological changes; Osteoinduction; *RUNX2.*

INTRODUCTION

Chronic consumption of Fluorine in higher concentrations than those recommended by the WHO produces various alterations at the cellular, biochemical, and genetics expression levels; in addition, alter the proper functioning of systems and organs.[1] The synthesis and calcification of the bone matrix are directed by osteoblasts; they are responsible for the development, growth, and regeneration of the bones; also, to having the bone matrix synthesis function and control of their mineralization.[2] The osteoinductive process consists of the ability of a substance to recruit and differentiate the cells needed for bone growth and regeneration. In general, it refers to the different steps by which pluripotent or multipotent mesenchymal stem cells are recruited to a certain site for repair, and thus be stimulated into early preosteoblasts.[3,4] This process commonly occurs in any type of bone regeneration development, such as fractures or implants.[5] They create an intercellular organic substance called osteoid,[6] this organic substance is formed by an amorphous matrix with components such as collagen fibers type I; thus, regulating the general bone maintenance process.[7] Another molecule associated with osteoinduction is the transcription factor *RUNX2* which is essential for osteoblastic differentiation and skeletal morphogenesis and acts as a scaffold for nucleic acids and regulatory factors

[a]Basic Sciences Laboratory, Faculty of Dentistry, San Luis Potosi University, 78290 San Luis Potosí, SLP, México.
[b]Biopolymers and Nanostructures Laboratory, Faculty of Chemistry, San Luis Potosi University, 78290 San Luis Potosí, SLP, México.
*Corresponding author:
Diana María Escobar-García; Faculty of Dentistry, San Luis Potosi University. 2 Manuel Nava, Zona Universitaria, 78290, San Luis Potosí, SLP, México. Phone: 52 444 8262300 X 5134 E-mail: diana.escobar@uaslp.mx

involved in skeletal gene expression, and is essential for the maturation of osteoblasts and both intramembranous and endochondral ossification.[8]

The objective of this study was to evaluate the behavior of osteogenic markers, the degree of cytotoxicity and the effect on the mitochondria of osteoblasts treated with different *NaF* concentrations in human osteoblast hFOB1.19

MATERIALS AND METHODS

Cell culture

An ATCC cell line of human osteoblasts hFOB1.19 was used in DMEM culture medium enriched with 5% fetal bovine serum and 1% antibiotic (with 10,000 units of penicillin, 10 mg of streptomycin, and 25 µg of anphotericin B per mL SIGMA) 37°C, 5% CO_2 and, 95% humidity. The cells were cultured with different *NaF* concentrations (1, 1.5, 3, 5, 10, 15 mg/L) and two controls, osteoblasts in culture medium as negative control and osteoblasts treated with osteoinductive medium (*OIM*) as osteoinductive control; incubation times were adapted to each of the experiments. Finally, the culture medium was replaced every three days.

Qualitative cytotoxicity gradient evaluation

This test was based on ISO 10993-5:2009, related to *"In vitro cytotoxicity tests"*. The cells were kept in incubation for 21 days with the different *NaF* concentrations; after this period, the cells were examined through an inverted optical microscope where the morphological changes were recorded and compared with a control group of cultured osteoblasts without *NaF.* Tests were performed in triplicate

Cell viability assay

To evaluate the cytotoxic effect of the different *NaF* concentrations, the Alamar Blue test (Alamar Blue kit Cell Viability Reagent of Thermo Fisher) was performed; subsequently, the culture medium containing the different *NaF* concentrations was replaced; under these conditions, the cells were incubated for a period of 48hr additional. After this incubation period, 10 µL of the Alamar Blue reagent was added to each 96 plate well per 100 µL sample, incubated for an additional 3hr under the same conditions, a reading of the absorbances at 450 nm was made in a microplate reader (Multiskan Thermo Scientific). For this test, there was a life control corresponding to cells cultured with culture medium enriched with FBS and antibiotic and a death control which 30 min prior to adding the alamar blue reagent, 50% of the culture medium was replaced by methanol to cause cell death. Tests were performed in quintuplicate

Ca^{+2} concentration

The Ca^{+2} concentration in the cells treated with the different *NaF* concentrations was determined by means of the Ca^{+2} Arsenazo III kit by Byosistems, according to the manufacturer's specifications. The determination of extracellular Ca^{+2} was made in the culture medium containing those of the cells treated with the different *NaF* concentrations for 21 days. The intracellular calcium concentration was determined in the cells treated with the different *NaF* concentrations. The treated cells were subjected to ultrasound (30 sec, 3 times) with an ultrasonic tip. The calcium concentration was reported in mg/dL/mg of protein. The Bradford method was used,

which is based on the formation of a complex between the bright blue Coomassie dye and the proteins in solution; the absorbance is read at 590 nm. A calibration curve was made with bovine albumin at different concentrations. Tests were performed in triplicate

Osteogenesis quantification assay

The cells were incubated in 13mm discs (24 well boxes) with a culture medium containing the different *NaF* concentrations for 21 days (in triplicate). Millipore osteogenesis kit was used in accordance with the manufacturer's instructions. The concentration of Alizarin Red is indicated in mg/mL/ mg of protein.

Immunocytochemistry RUNX2

The detection of *RUNX2* was performed in osteoblasts treated with different *NaF* concentrations for a period of 21 days; at the end of this incubation period, the samples were fixed with 10% neutral formalin for 30 min at 25°C, they were washed with PBS twice, were blocked with 1% bovine albumin Tween for 1 hr at 25°C, washed with PBS-Tween 20 to 0.025%; then, the cells were put in contact with the first mouse anti *RUNX2* antibody (Invitogen) at a concentration of 1:100, incubated overnight at 4°C. Subsequently, the cells were washed with PBS-Tween three times and the second monoclonal antibody IgG2a Alexa Fluor 488 (Santa Cruz) was added for 2 hr at 25°C at a concentration of 1:500 in conditions of darkness. Finally, the last wash was performed with PBS-Tween 20. Additionally, a NucRed solution of Invitrogen was prepared (according to the manufacturer's specifications). Finally, dyed cells were observed on the Leica confocal laser fluorescence microscope (CLFM). The fluorescence units were determined for each sample with the help of the Leica Microsystem LAS-AF TCS-SPE SOFWERE. Tests were performed in triplicate

MitoTracker Deep Red FM assay

For the evaluation of mitochondria of cells exposed to different *NaF* concentrations, osteoblasts exposed to different treatments (1, 1.5, 3, 5, 10, and 15 mg/L) were seeded in 24-well plates provided with discs of 13 mm diameter, for a period of 21 days. The MitoTracker Deep Red FM kit was used (according to the manufacturer's specifications). Each sample was observed in the confocal laser fluorescence microscope (CLFM). The fluorescence units were determined for each sample with the help of the Leica Microsystem LAS-AF TCS-SPE SOFWERE. Tests were performed in triplicate

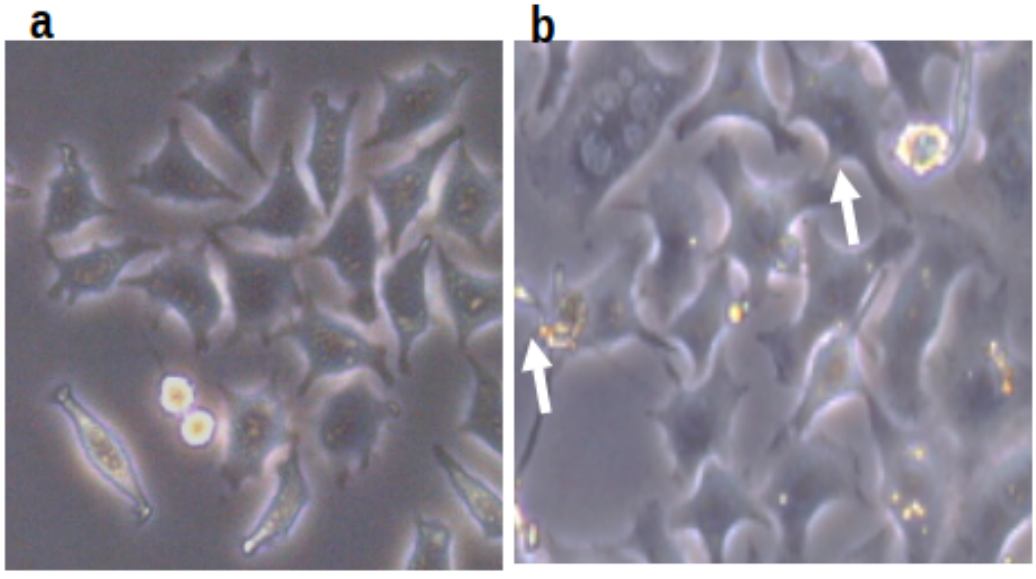
Statistical analysis

Results were shown as means ± standard deviations (SDs). The statistical analyses were performed using SigmaPlot v 11.0 statistical package. One-way ANOVA was used to analyze the difference among the multiple groups. The student's t-test was used to evaluate the difference between two groups. $p < 0.05$ was regarded as statistical significance.

RESULTS

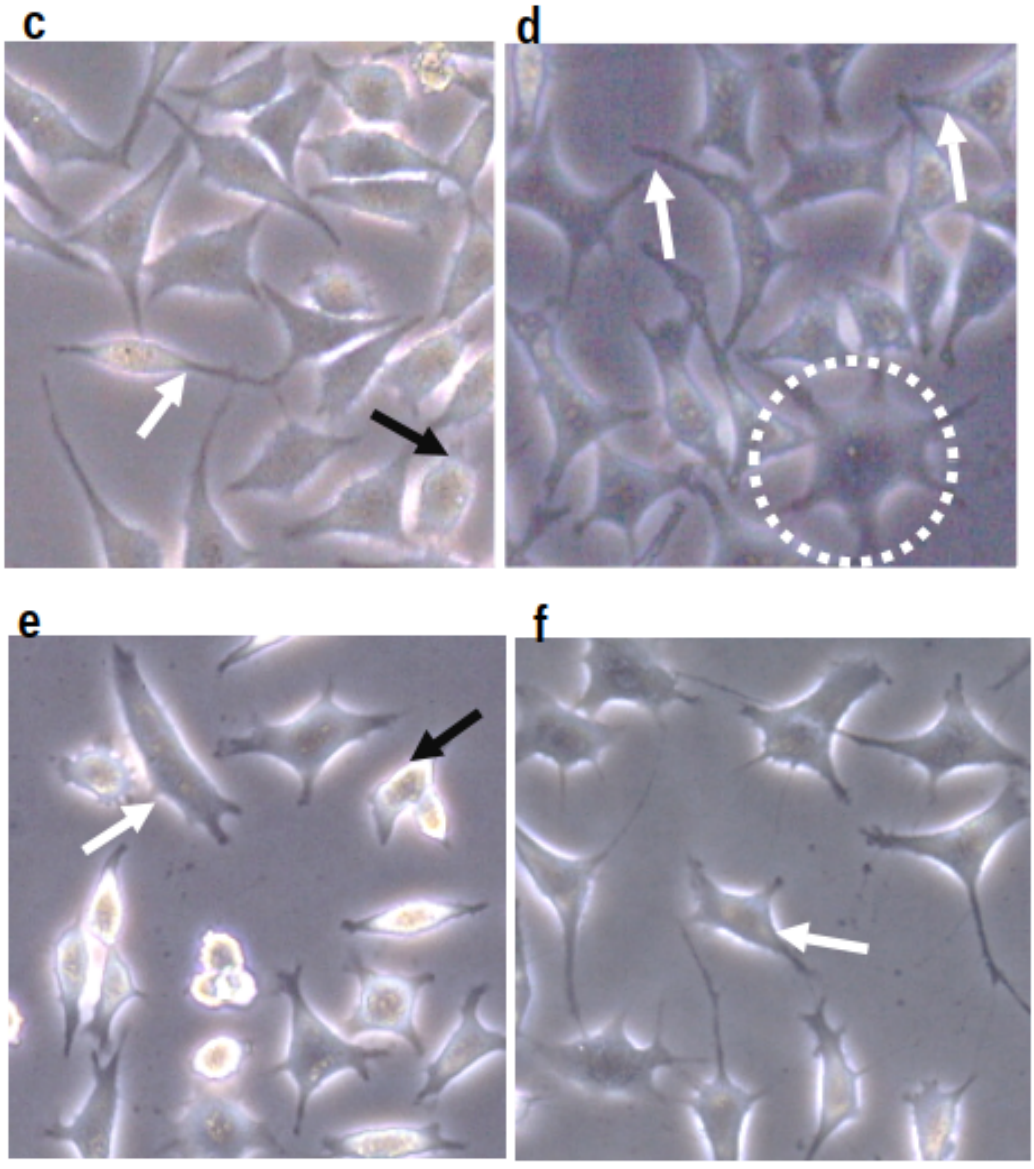
Qualitative/Quantitative Cytotoxicity

Qualitative Assay: Figure 1. shows the morphology of the osteoblasts treated with the different *NaF* concentrations. When the osteoblasts were exposed to *NaF* concentrations of 1, 1.5, and 3 mg/L, a mean level of damage in cell morphology was observed, with less than 50% of the round cells, lacking intracytoplasmic granules, there was no extensive cell lysis; there was less than 50% growth inhibition (Figure 1 C, D, and E), unlike cells treated with 5mg/L *NaF* concentrations, 10 and 15 mg/L showed moderate level of damage where more than 30% of cell layers contain rounded cells or were lysed; cell layers are not completely destroyed, but no significant inhibition of growth was observed (Figure 1 F, G, and H). Cell morphology was modified by the presence of *NaF*, which was corroborated by the increase in cell volume (Figure 1D, white circle) and by the decreased number of focal contacts (Figure 1 C, E, and G, white arrows). Also, when *NaF* concentration increased, it generated cytoplasmic vacuolization, nuclear abnormalities, and pyknosis (Figure 1 c, e, and h, black arrow).



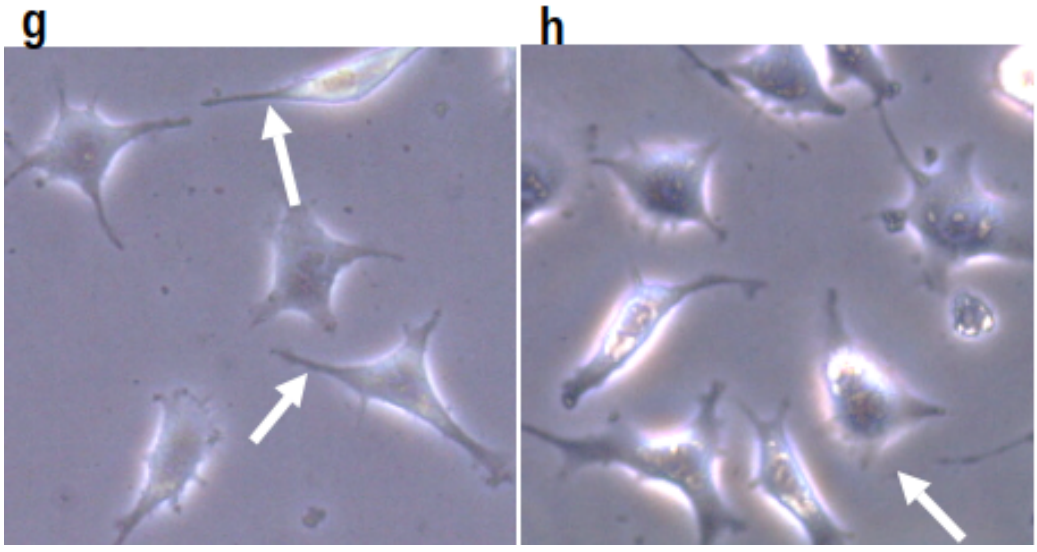
Figures 1a and 1b: Cytotoxicity assay. Osteoblast treated at different NaF concentration for 21days and 24 hr respectively Qualitative cytotoxicity based on the international standard ISO 10993-5 tests for in vitro cytotoxicity. a. Control; b. Osteoinductive medium (OIM); c. 1 mg/L; d. 1.5 mg/L; e. 3 mg/L; f. 5 mg/L; g. 10 mg/L; h. 15 mg/L. Cell morphology is modified by the presence of NaF, this assumption was corroborated by the increase in cell volume (Figure 1D white circle) and the decreased number of focal contacts (Figure 1 c, e and, g, white arrows,). On the other hand, when NaF concentration increases, they generate cytoplasmic vacuolization, nuclear abnormalities and picnosis (Figure 1: c, e and, h, black arrow).

[Now published in full after the initial publication as an Epub ahead of print August 1, 2023 at
www.fluorideresearch.online/epub/files/211.pdf]



Figures 1c–1f: Cytotoxicity assay. Osteoblast treated at different NaF concentration for 21days and 24 hr respectively Qualitative cytotoxicity based on the international standard ISO 10993-5 tests for in vitro cytotoxicity. a. Control; b. Osteoinductive medium (OIM); c. 1 mg/L; d. 1.5 mg/L; e. 3 mg/L; f. 5 mg/L; g. 10 mg/L; h. 15 mg/L. Cell morphology is modified by the presence of NaF, this assumption was corroborated by the increase in cell volume (Figure 1D white circle) and the decreased number of focal contacts (Figure 1 c, e and, g, white arrows,). On the other hand, when NaF concentration increases, they generate cytoplasmic vacuolization, nuclear abnormalities and picnosis (Figure 1: c, e and, h, black arrow).

[Now published in full after the initial publication as an Epub ahead of print August 1, 2023 at www.fluorideresearch.online/epub/files/211.pdf]



Figures 1g and 1h: Cytotoxicity assay. Osteoblast treated at different NaF concentration for 21days and 24 hr respectively Qualitative cytotoxicity based on the international standard ISO 10993-5 tests for in vitro cytotoxicity. a. Control; b. Osteoinductive medium (OIM); c. 1 mg/L; d. 1.5 mg/L; e. 3 mg/L; f. 5 mg/L; g. 10 mg/L; h. 15 mg/L. Cell morphology is modified by the presence of NaF, this assumption was corroborated by the increase in cell volume (Figure 1D white circle) and the decreased number of focal contacts (Figure 1 c, e and, g, white arrows,). On the other hand, when NaF concentration increases, they generate cytoplasmic vacuolization, nuclear abnormalities and picnosis (Figure 1: c, e and, h, black arrow).

Quantitative assay (cell viability): *NaF* increased cell proliferation in a dose-dependent way when incubated for 48 h since none of the treatments reduced viability by more than 30% (Figure 2).

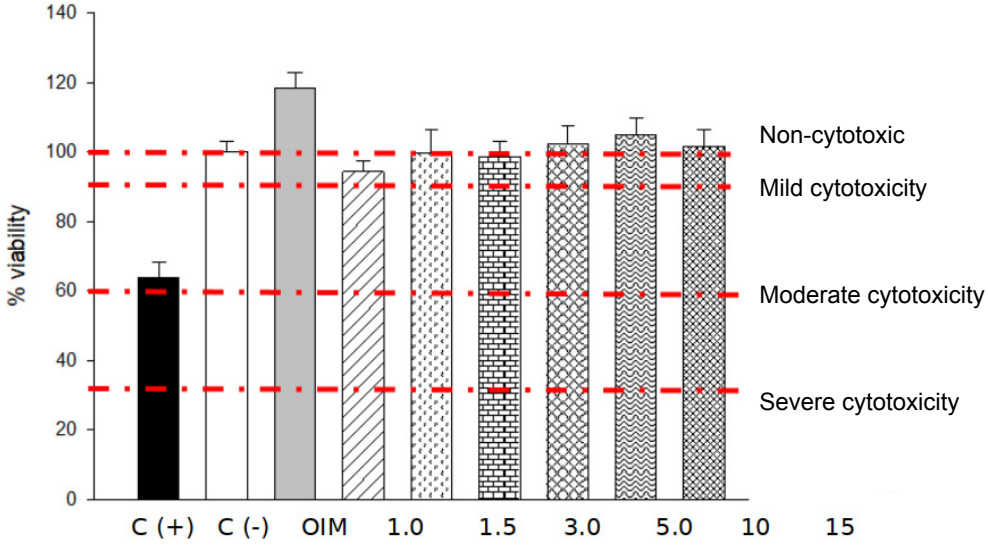
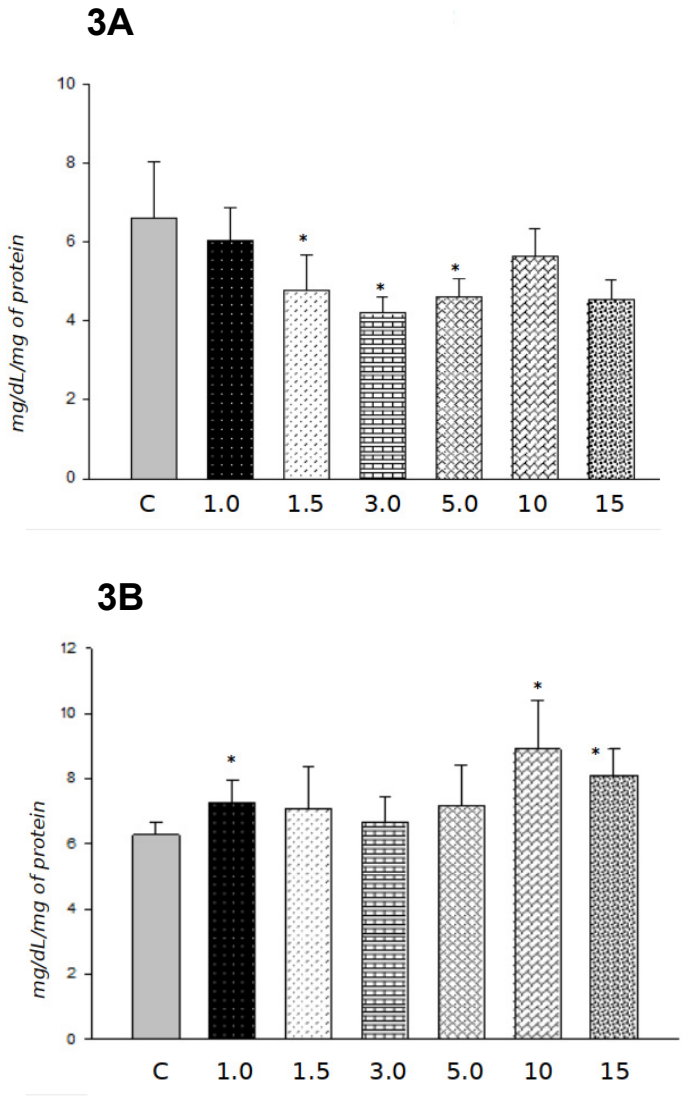


Figure 2. Quantitative cytotoxicity Alamar blue assay: Dead control (C+); Live control (C-), OIM, 1.0 mg/L, 1.5 mg/L, 3.0 mg/L, 5.0 mg/L, 10 mg/L and 15 mg/L. * $p<0.05$. The red dotted lines indicate the degree of toxicity, ranging from non-cytotoxic to severe cytotoxicity. The cells treated with the different concentrations of NaF are in the range of non-cytotoxicity.

[Now published in full after the initial publication as an Epub ahead of print August 1, 2023 at www.fluorideresearch.online/epub/files/211.pdf]

Intracellular/extracellular Ca $^{+2}$ concentration

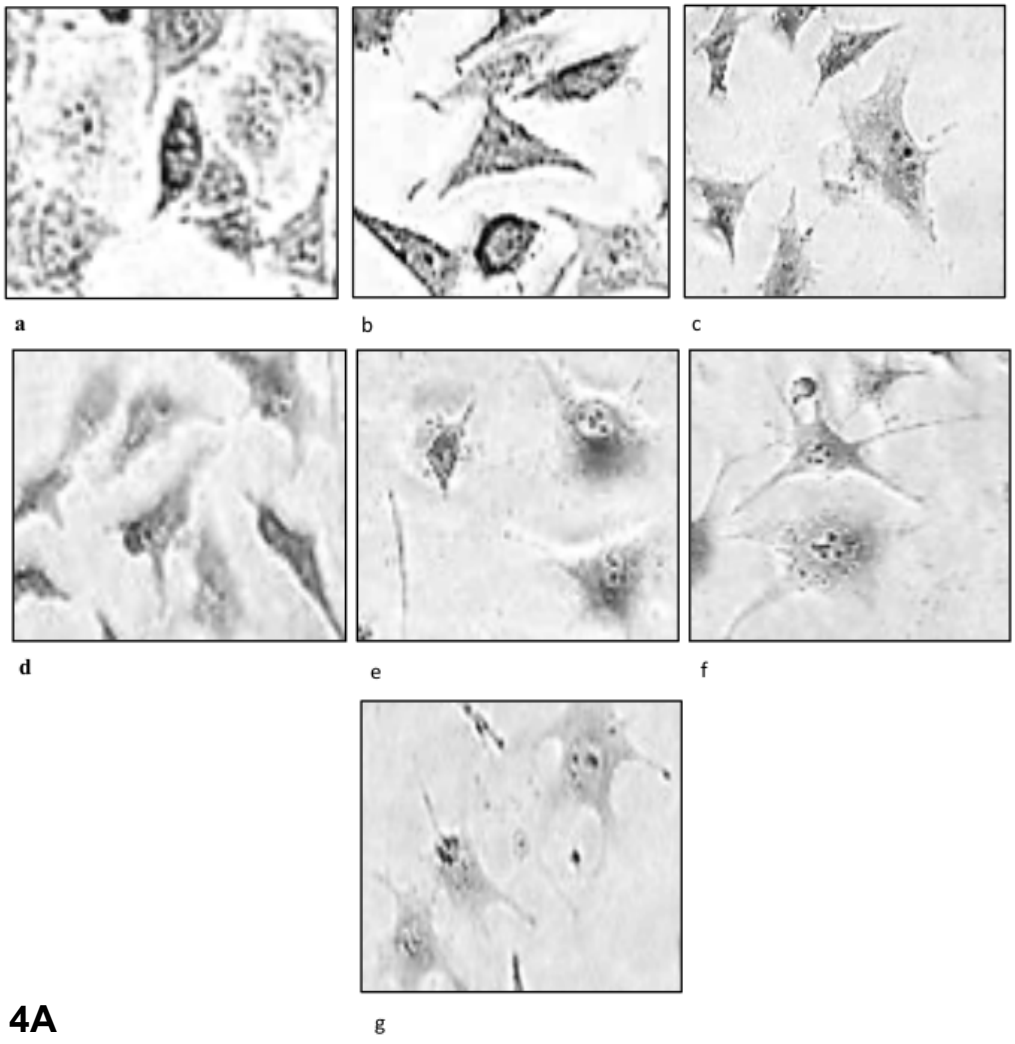
Intracellular calcium concentration decreased significantly in *NaF* treatments to 1.5, 3, and 5 mg/L ($p<0.05$). The greatest reduction in the Ca^{+2} concentration occurred in osteoblasts treated with 3 mg/L (~35%) (Figure 3A). The concentration of the extracellular $Ca^{+}2$ quantified in the culture medium of osteoblasts treated with the different NaF concentrations increased in a dose-dependent way and this increase was discreet in the lowest concentrations (1–5 mg/L); however, these differences were statistically significant. In addition, when *NaF* concentrations increased to 10 and 15 mg/L, extracellular calcium also increases in both treatments by a little more than 30% relative to control (Figure 3B), these differences were also statistically significant ($p<0.05$).



Figures 3A and 3B. Ca^{2+} concentration in osteoblast treated with different NaF concentration (mg/L). 3A: Intracellular Ca^{2+}, 3B: Extracellular Ca^{2+}. *p<0.05.

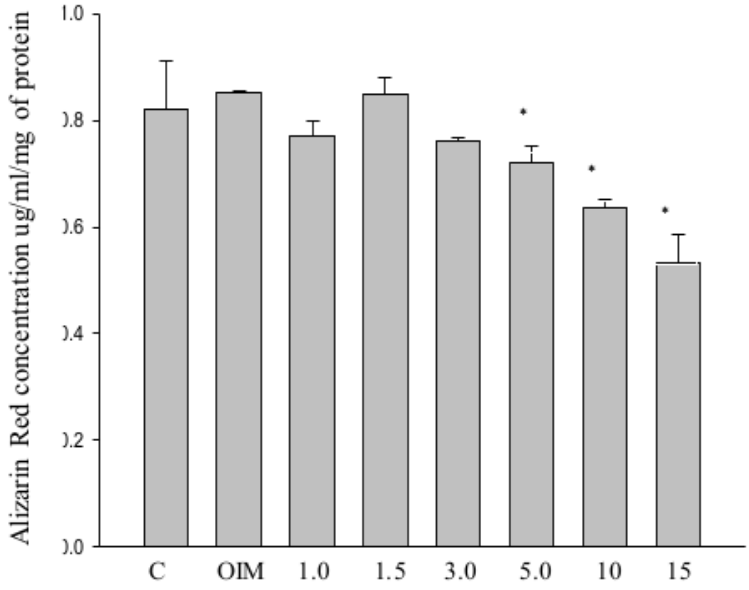
Osteogenesis Quantification assay

Qualitative osteogenic analysis showed that color intensity increases when cells were treated with *NaF* at 1.0 and 1.5 mg/L (Figure 4A); however, the detection of mineralized deposits measured as color intensity decreases in a concentration-dependent way in osteoblasts treated with *NaF* concentrations between 3 and 15 mg/L. In relation to the results obtained in the osteogenic analysis quantitative (Figure 4B), it was observed that a higher concentration of red Alizarin occurs in osteoblasts treated with *OIM* and *NaF* at 1.5 mg/L, than in the control osteoblasts not treated with *NaF*.



4A

Figure 4A. Alazarin Qualitative Red Assay. NaF effect into osteoblast mineralization. a. Control; b. 1 mg/L; c. 1.5 mg/L; d. 3 mg/L; e. 5 mg/L; f. 10 mg/L; g. 15 mg/L. At higher concentrations of calcium, the cells have a brown coloration, as can be seen in the control group.



4B

Figure 4B. Alazarin Red Quantitative Assay. NaF effect into osteoblast mineralization. C = Control; OIM = Osteoinductive medium; NaF concentrations (mg/L) = 1.0, 1.5, 3.0, 5.0, 10 and 15. * $p<0.05$.

RUNX2 immunocytochemistry

In the immunocytochemical assay, the expression of *RUNX2* was evidenced by the presence of fluorescent green color in the cells. In osteoblasts treated with low *NaF* concentrations (1 and 1.5 mg/L), *RUNX2* expression is observed with a pattern like the control (Figure 5). *RUNX2* expression decreased in a dose-dependent manner. This effect was specific for osteoblasts stimulated with high *NaF* concentrations (5-15 mg/L), where the green fluorescence characteristic of the presence of *RUNX2* is becoming getting dimmer; this effect was specific for the osteoblast stimulated with high *NaF* concentrations (5–15 mg/L).

MitoTracker Deep Red FM assay

The results of the cells treated with *NaF* showed variations in the intensity of the dye accumulated in the mitochondria, these changes were dependent on the *NaF* concentration with respect to the control. In cells treated with 1, 1.5, and 3 mg/L the fluorescence intensity decreased slightly, presenting a good number of mitochondria (Figure 6). In the cells treatment with 5.0 mg/L of NaF the intensity of fluorescence decreased, exhibiting a reduction in the number of mitochondria.

[Now published in full after the initial publication as an Epub ahead of print August 1, 2023 at
www.fluorideresearch.online/epub/files/211.pdf]

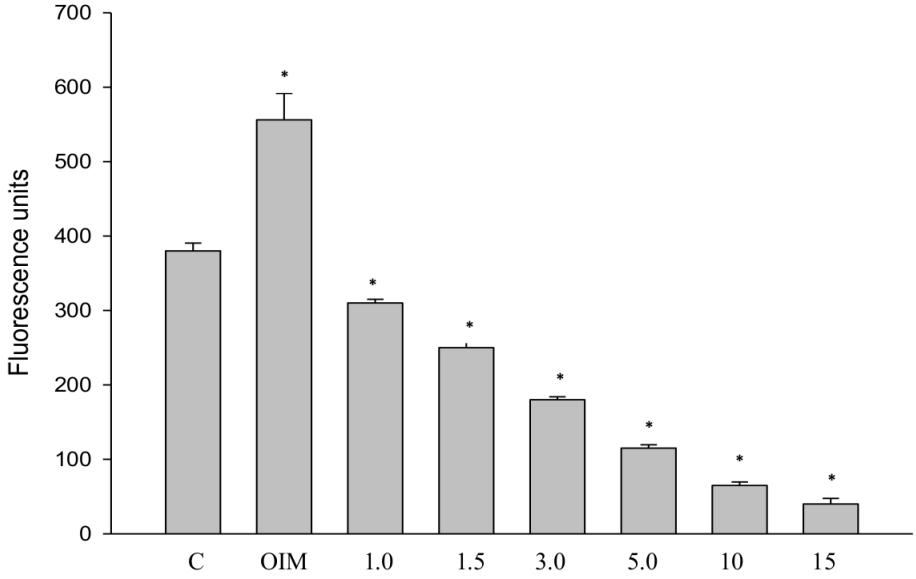


Figure 5. RUNX2 immunocytochemistry. (Fluorescence units). Differential expression of RUNX2 after treatment with different NaF concentration Control; Osteoinductive medium (OIM); NaF concentrations: 1 mg/L; 1.5 mg/L; 3 mg/L; 5 mg/L; 10 mg/L; 15 mg/L. in this figure can be observed clearly decrease in the expression of RUNX2 in a concentration-dependent manner. *p<0.05

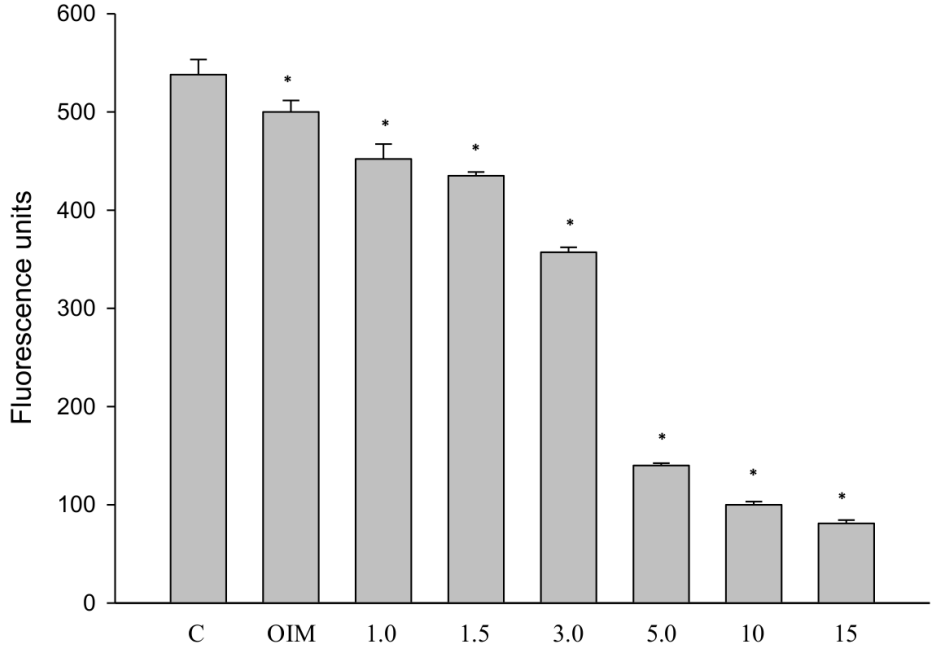


Figure 6. Effect of different NaF concentration into the mitochondria in Osteoblasts: Control; Osteoinductive medium (OIM); NaF concentrations: 1 mg/L; 1.5 mg/L; 3 mg/L; 5 mg/L; 10 mg/L; 15 mg/L. In this figure, a decrease in the number of mitochondria can be clearly observed in a concentration-dependent manner. *p<0.05.

DISCUSSION

The results obtained in this study show that high *NaF* concentrations can significantly alter the *RUNX2* expression and Ca^{+2} deposits, essential molecules for the correct functioning of the main support system of the body, such as the bone system. These results, encourage futures studies related to the effects of consuming high *NaF* concentrations.

NaF does not significantly affect cell disruption when the incubation period is for 48 h of treatment; the *NaF* connections used in the present study are in a non-cytotoxic range (quantitative assay) (48 h exposure) compared to the control of cells cultured in DMEM medium, since none of the treatments reduced viability by more than 30%. These results demonstrate the importance of qualitative cytotoxicity determination, as this test showed large changes in cell morphology but no decrease in cell clearance. Opposing, when the treatments were carried out subchronically for 21 days, a dose-dependent decrease in cell elimination was qualitatively observed; Thus, F^- can favor the formation of bone organs rich in this mineral, which generates hard and inflexible bone compared to the healthy bone without F^-, which has the characteristic of being resistant and flexible.[9]

In cell treatments with *NaF,* Ca^{+2} ions seem to be replaced by fluorine, causing a possible replacement of hydroxyapatite (calcium phosphate mineral) by fluorapatite; making the bones more compact, small, and less flexible, which can result in a reduction in the elasticity of the calcified matrix.[5] When there are changes in intracellular concentration, Ca^{+2} alters the activity of many cellular proteins that respond to Ca^{+2} such as insulin-1 and osteocalcin; in the same way, the low concentration of Ca^{+2} induces intracellular signaling pathways involved in cell proliferation to perform their functions inadequately.[10] The skeletal structure becomes a storage system for 98% of body calcium. Osteoblasts participate indirectly in calcium homeostasis, integrating it into the bone matrix, which causes an increase in mineral density and bone strength. Therefore, Ca^{+2} is universally known as the basis for the formation of a healthy skeletal structure.[11]. Extracellular Ca^{+2} concentration slightly increased in cells treated with *NaF* concentrations from 1 to 5 mg/L. On the other hand, when *NaF* concentrations increase to 10 and 15 mg/L, extracellular Ca^{+2} also increases in both treatments, these results may be due to the substitution of Ca^{+2} by fluorine that causes calcium to be in the extracellular space. An imbalance in Ca^{+2} concentrations can generate oxidative stress and induce mechanisms of apoptosis, affecting intracellular organelles causing future damage to the good functioning of tissues.[10] Adequate concentrations of Ca^{+2} is required for the activation of different metabolic enzymes such as pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, and isocitrate dehydrogenase. Calcium influx into the mitochondrial matrix leads to increased metabolism through interaction with these metabolic enzymes, increasing ATP production for the cell.[12] Alterations in the concentration of this mineral put at risk multiple cellular functions in which Ca^{+2} is critical, such as the correct functioning of the mitochondria, which is an organelle responsible for regulating oxidative stress, ATP generation and the correct mineralization of bone tissue.[11]

There was a statistically significant osteogenic decrease in cells treated with *NaF* at concentrations greater than 1.5 mg/L, leading to demineralization of bone tissue,

which can result in a reduction in bone density; causing a fragile and porous bone, prone to fractures and leading to diseases related to loss of bone density such as osteopenia and osteoporosis.[13] These results coincide with those obtained with qualitative osteogenic analysis.

RUNX2 is an important protein for the proper development and formation of bone and cartilage. In the immunocytochemical assay the expression of *RUNX2*, was evidenced by the presence of fluorescent green color in the cells, this indicates a correct regulation of osteogenic differentiation by regulating the cell cycle; in the same way, this molecule functions as a scaffolding protein, which through its different domains allows interaction with other bone matrix proteins. The fact that *RUNX2* decreases indicate that the normal function of the bone cell is altered;[26] it can trigger interruptions in osteoblast maturation,[14] as well as cause changes in the morphology and function of osteoblasts.[15] Excessive consumption of fluoride can destroy the processes of bone formation and resorption, altering the process of bone turnover and causing skeletal fluorosis, a condition in which several lesions occur, such as degenerative changes in the joints, osteosclerosis, and osteoporosis.[16]

Mitochondria are the major source of energy for cellular activity, by ATP generation via oxidative phosphorylation. Emerging evidence of the last decade indicates that mitochondria form a highly dynamic intracellular network that executes the "quality control" of the organelle's population in a process that implies their fusion, fission and autophagic degradation.[17] Cells treated with 1, 1,5 and 3 mg/L have a good number of mitochondria, therefore, it can be said that these cells have a correct production of ATP, that assures the generation of the energy needed to carry out its functions, such as the cell cycle, differentiation, proliferation, and proper storage of Ca^{+2}; which will allow the maintenance of bone health.[18] On the other hand, from the 5.0 mg/L treatment the intensity of fluorescence decreases, exhibiting a reduction in the number of mitochondria, which can trigger a decrease in mitochondrial function, this effect may be due to the increase in the process of mitophagy;[19] or inhibition of mitochondrial biogenesis;[20] these processes are directly associated with the appearance of neurodegenerative and cognitive disorders observed in the population exposed to high concentrations of F^- in drinking water.[21] Similarly, when mitochondria are injured, they become highly reactive and harmful to themselves and to the surrounding mitochondria, causing cell death. Although mitochondria are indispensable for correct cell functioning and therefore for life, there are also important factors in the release of numerous signals that activate signaling pathways that can lead to apoptosis or cell necrosis;[22] therefore, it is necessary to develop new research that could provide evidence of the mechanisms by which the mitochondrial state is affected when cells are treated with high concentrations of *NaF*.

Although experiments in cell line cultures such as osteoblasts are suitable experimental model, that clarifies the effects caused by the consumption of high concentrations of F^- for human beings, it would be very interesting to measure some of the parameters analyzed in this study, such as *RUNX2* expression and other osteoinductive indicator molecules for molecular techniques, in an animal model where the results obtained are subjected to an integral metabolism of F^-, which the cell culture model lacks.

CONCLUSIONS

The results of this study showed that an increase in the concentration of F^- in the cell can produce harmful effects, constituting a significant health risk since it was shown that the F^- altered cellular morphological patterns in sub-chronic exposure, it is also capable of reducing the expression of the *RUNX2* protein and the concentration of intracellular and extracellular Ca^{+2} as well as being able to reduce the number of mitochondria in a dose-dependent way.

REFERENCES

[1] Valdez-Jiménez L, Soria Fregozo C, Miranda Beltrán ML, Gutiérrez Coronado O, Pérez Vega MI. Effects of the fluoride on the central nervous system.Neurologia 2011;26(5):297–300. doi:10.1016/j.nrl.2010.10.008

[2] Rutkovskiy A, Stensløkken KO, Vaage IJ. Osteoblast Differentiation at a Glance.Med Sci Monit Basic Res 2016;22:95-106. doi:10.12659/msmbr.901142

[3] Miron RJ, Sculean A, Shuang Y, et al. Osteoinductive potential of a novel biphasic calcium phosphate bone graft in comparison with autographs, xenografts, and DFDBA. Clin Oral Implants Res 2016;27(6):668.75. doi:10.1111/clr.12647

[4] Genitiempo M, Perna A, Santagada D, et al. Single-level Bryan cervical disc arthroplasty: evaluation of radiological and clinical outcomes after 18years of follow-up.European spine journal: official publication of the European Spine Society, the European Spinal Deformity Society, and the European Section of the Cervical Spine Research Society 2020;29(11), 2823-2830. https://doi.org/10.1007/s00586-020-06486-5

[5] Fillingham Y, Jacobs J. Bone grafts and their substitutes. Bone Joint J 2016;98-B (1 Suppl A):6–9.

[6] Thiel A, Reumann MK, Boskey A, Wischmann J, von Eisenhart-Rothe R, Mayer-Kuckuk P. Osteoblast migration in vertebrate bone.Biol Rev Camb Philos Soc 2018;93(1):350–363. doi:10.1111/brv.12345

[7] Alonso V, de Gortázar AR, Ardura JA, Andrade-Zapata I, Alvarez-Arroyo MV, Esbrit P. Parathyroid hormone-related protein (107-139) increases human osteoblastic cell survival by activation of vascular endothelial growth factor receptor-2. J Cell Physiol 2008;217(3):717–727. doi:10.1002/jcp.21547

[8] Thaweesapphithak S, Saengsin J, Kamolvisit W, Theerapanon T, Porntaveetus T, Shotelersuk V. Cleidocranial dysplasia and novel RUNX2 variants: dental, craniofacial, and osseous manifestations. Journal of applied oral science: Revista FOB 2022 30, e20220028. doi:10.1590/1678-7757-2022-0028

[9] Colombo M, Poggio C, Dagna A, et al. Biological and physico-chemical properties of new root canal sealers. J Clin Exp Dent 2018;10(2):e120–e126. doi:10.4317/jced.54548

[10] Khammissa RAG, Fourie J, Motswaledi MH, Ballyram R, Lemmer J, Feller L. The Biological activities of vitamin D and its receptor in relation to calcium and bone homeostasis, cancer, immune and cardiovascular systems, skin biology, and oral health. Biomed Res Int 2018;2018:9276380. doi:10.1155/2018/9276380

[11] Zayzafoon M. Calcium/calmodulin signaling controls osteoblast growth and differentiation. J Cell Biochem 2006;97(1):56–70. doi:10.1002/jcb.20675

[12] Gollihue JL, Rabchevsky AG. Prospects for therapeutic mitochondrial transplantation. Mitochondrion 2017;35:70–79. doi:10.1016/j.mito.2017.05.007

[13] Murshed, Monzur. Mechanism of bone mineralization. Cold Spring Harbor perspectives in medicine2018;8(12): a031229.

[14] Wysokinski D, Pawlowska E, Blasiak J. RUNX2: A master bone growth regulator that may be involved in the DNA damage response.DNA Cell Biol 2015;34(5):305–315. doi:10.1089/dna.2014.2688

[15] Zhang J, Jiang N, Yu H, et al. Requirement of TGF signaling for effect of fluoride on osteoblastic differentiation.Biol Trace Elem Res 2019;187(2):492–498. doi:10.1007/s12011-018-1387-x

[16] Rojas A, Aguilar R, Henriquez B, et al. Epigenetic control of the bone-master Runx2 gene during osteoblast-lineage commitment by the histone demethylase JARID1B/KDM5B. J Biol Chem 2015;290(47):28329–28342. doi:10.1074/jbc.M115.657825

[17] Yang Chen, Wang Yan, Xu Hui. Treatment and Prevention of Skeletal Fluorosis. Biomed Environ Sci 2017, 30(2): 147–149.doi:10.3967/bes2017.020

[18] Golpich M, Amini E, Mohamed Z, Azman Ali R, Mohamed Ibrahim N, Ahmadiani A. Mitochondrial dysfunction and biogenesis in neurodegenerative diseases: Pathogenesis and treatment. CNS Neurosci Ther 2017;23(1):5–22. doi: 10.1111/cns.12655

[19] Tan P, Zhou B, Zhao W, Jia L, Liu J, Wang H. Mitochondria-mediated pathway regulates C2C12 cell apoptosis induced by fluoride. Biol Trace Elem Res 2018;185: (2):440–447. doi:10.1007/s12011-018-1265-6

[20] Kerr JS, Adriaanse BA, Greig NH, et al. Mitophagy and Alzheimer's disease: Cellular and molecular mechanisms. trends Neurosci 2017;40(3):151–166. doi: 10.1016/j.tins.2017.01.002.

[21] Waugh DT. Fluoride exposure induces inhibition of Sodium/Iodide Symporter (NIS) contributing to impaired iodine absorption and iodine deficiency: Molecular mechanisms of inhibition and implications for public health.Int J Environ Res Public Health 2019;16(6):1086. doi:10.3390/ijerph16061086

[22] Popov LD. Mitochondrial biogenesis: An update. J Cell Mol Med 2020;24(9):4892–4899. doi: 10.1111/jcmm.15194