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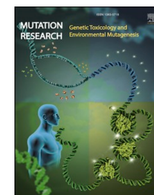

Fluorosilicic acid induces DNA damage and oxidative stress in bone marrow mesenchymal stem cells

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ABSTRACT

Excess fluoride in water can produce changes in tooth enamel mineralization and lead to diseases such as dental or skeletal fluorosis. The present study aimed to assess the genotoxic effects, oxidative stress, and osteoblastic mineralization induced by fluorosilicic acid (FA) in murine bone marrow-derived mesenchymal stem cells (BM-MSCs). BM-MSCs were isolated from the femurs and tibias of rats and cultured under standard conditions. Cells exposure occurred for 3, 7, 14, and 21 days to different concentrations of FA (0.6–9.6 mg/L). Cytotoxicity was observed in 14 and 21 days of exposure for all concentrations of FA (cell proliferation below 60%), and for 3 and 7 days, in which the proliferation was above 80%. Alkaline comet assay results demonstrated significant increased damage at concentrations of 0.3–2.4 mg/L, and the micronucleus test showed increased rates for micronucleus (1.2–2.4 mg/L) and nuclear buds (NBUDs) (0.3–2.4 mg/L) ($P < 0.05$/Dunnett's test). An alkaline comet assay modified by repair endonuclease (FPG) was used to detect oxidized nucleobases, which occurred at 0.6 mg/L. The oxidative stress was evaluated by lipid peroxidation (TBARS) and antioxidant activity (TAC). Only lipid peroxidation was increased at concentrations of 0.6 mg/L and 1.2 mg/L ($P < 0.001$/Tukey's test). The osteogenesis process determined the level of extracellular matrix mineralization. The mean concentration of Alizarin red increased significantly in 14 days at the 0.6 mg/L concentration group ($P < 0.05$/Tukey's test) compared to the control group, and a significant difference between the groups regarding the activity of alkaline phosphatase (ALP) was observed. Unlike other studies, our results indicated that FA in BM-MSCs at concentrations used in drinking water induced genotoxicity, oxidative stress, and acceleration of bone mineralization.

1. Introduction

Fluorine is one of the most important milestones in the history of dentistry due to its undeniable anti-cariogenic properties. Fluoridation occurs in several ways: in the fluoridation of public water supply, added to table salt, prescribed in drops; in topical applications; in community mouthwash programs; in dentifrices; among others [1]. However, its adverse effects are studied and known when its ingestion reaches levels of chronic or acute toxicity and when fluorosis develops.

The amount of fluoride used varies according to the region, taking into account the amount of fluoride present in water, food, and other beverages, plus some habits such as tea consumption and cooking and dehydrating foods with coal-rich in fluorides [2,3]. In the environment, fluoride is abundant in rocks, groundwater, and soils, originating in soils [4]. Increased concentrations of this element in soils are also a result of the use of groundwater for irrigation and/or increased capillarity of the ground [5]. The main Brazilian aquifers have shown high concentrations of fluoride in groundwater, detected through public wells and private use. Although considered as natural exposures, these waters require monitoring by management bodies related to the use of water resources

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and public health [6]. Terra et al. [7], when analyzing the groundwater quality in a region of the State of Rio Grande do Sul (RS; south of Brazil), observed quantities of fluoride that exceeded the limit established by the legislation with values up to 4.7 mg/L in samples collected from well water.

The World Health Organization (WHO) recommends water fluoridation as an appropriate method for caries prevention, and the amount of fluoride recommended in drinking water is between 0.5 and 1.5 mg/L [3]. Climatic conditions and habits of the local population alter the concentration values and must be considered once they can determine a daily water consumption above or below the limits estimated by WHO. Fluoride has been added to public water supplies since the 1950s in Brazil as a preventative measure against dental caries, becoming mandatory since 1975. In Brazil, the maximum allowed limit of fluoride in drinking water is 1.5 mg/L, set by Ordinance 2914/2009 of the Ministry of Health [8]. In a recent study, published by Krützmann et al. [9], the authors observed fluoride values upper to 1.4 mg/L in drinking water in samples collected at a hospital and a school in RS. Whenever the value of fluorides in water intended for human consumption exceeds 1.5 ppm, corrective measures must be taken to restore water quality, which rarely happens.

The decomposition of fluorine forms the fluorosilicic or fluosilicic acid (H_2SiF_6) (FA), which is a fluoride highly soluble in water [10] and used in drinking water treatment plants in RS. After the ingestion, the absorption of fluoride occurs *via* the digestive tract (75–90%) and the small intestine. The acidity of the stomach can increase this absorption, converting up to 40% of the ingested fluoride into hydrofluoric acid and subsequently distributed through the bloodstream [11,12]. The absorbed fluoride is rapidly distributed by plasma to all tissues and occurs in both ionic (reflects the recent fluoride intake) and non-ionic (fat-soluble fluoro compounds) forms [12,13]. Due to the high affinity for calcium, fluoride is associated with calcified tissue, like teeth and bones, through the crystalline network *via* isoionic and heteroionic exchanges, where approximately 99% of the total fluorine content of the human body is established, thus not accumulating in the soft tissues [4,14]. The plasma half-life is achieved within 20−60 min after oral ingestion and absorption, followed by a rapid decline as a result of both uptakes in calcified tissues and urine excretion. The percentage of fluoride excreted *via* urine and feces can be according to metabolism, age group, lifestyle, and genetic factors of each individual [10,12,15]. Human populations living in areas with fluoridated water (containing 1 ppm fluoride or −1 mg/L or −52.6 μM/L), plasma fluoride concentrations ranged between 0.5 and 1.5 μM/L [16]. Although plasma fluoride levels are modulated by several physiological factors regardless of fluoride intake, they constitute a significant biomarker of past and present exposures [17]. Moreover, fluoride could cross the placenta, and a consumption of 1.5 mg of fluoride/day can increase fetal blood concentrations by approximately twice, in addition to the presence of fluoride in breast milk at levels similar to blood [10–13].

Fluorosis is a developmental anomaly described as the first manifestation of toxicity due to the prolonged ingestion of fluoride. It can occur during the period of tooth formation and enamel maturation, characterized by increased enamel porosity, making it appears opaque [18], mainly affecting children during the period of dental development [11]. Bone fluorosis is the most severe case where bone deformations occur and weakening of bone tissue due to the excessive deposition of fluorine, determined by the ingestion of water over prolonged periods with a high fluoride content (in general above 4 mg/L) [10]. In regions where water has fluoride concentrations greater than 2.0 ppm (∼2 mg/L), or in workers in the aluminum or fertilizer industries, frequently exposed to fluoride, skeletal fluorosis is quite common, with a prevalence of more than 20% [19]. Bone fluorosis has shown higher severity at younger ages, children, and teenagers [1,10], which can be due to the faster fluoride absorption by bones in this age group compared to adults [20]. Fluoride presents effects not only on bones and teeth but also on other tissues, such as the kidneys and liver [18,21].

Studies with various *in vitro* and *in vivo* systems attempt to understand which cellular damage the fluorides can cause. Among these studies, some authors suggest that fluoride can increase the production of free radicals acting on the oxidative degradation of lipids and promoting changes in the cell membrane, leading to the formation of cytotoxic products and consequently cell death [4,22–25]. In addition, studies seek answers about the epigenetic or DNA repair system mechanisms, which are still not explained. Some of these studies relate the exposure time and amount of fluorides, animal and human cells, and animals and populations exposed to high fluoride levels with the expression of various genes [21,26–28]. Bone marrow-derived mesenchymal stem cells (BM-MSCs) are multipotent cells that can differentiate into a variety of mesenchymal tissues, including osteoblastic differentiation [29]. Due to many studies *in vitro* and *in vivo* using fluoride concentrations well above the established for human consumption in drinking water, besides controversial results regarding genotoxicity and mutagenicity damages, here we investigate the effect of FA on cytotoxicity, DNA damage, oxidative stress, and rate of osteoblastic mineralization in BM-MSCs in different concentrations of fluoride.

2. Material and methods

2.1. Chemicals and reagents

H_2SiF_6 (Fluorosilicic Acid = FA; concentration 22.10%, lot 1805181) was obtained from DMAE (Municipal Department of Water and Sewerage of Porto Alegre, RS, Brazil; kindly provided by the engineer André Petry). The plasticware used in this study were purchased from BD Falcon (São Paulo, Brazil). The reagents used in the culture of mesenchymal stem cells consisted of a complete culture medium (CCM), which was composed of Dulbecco's modified Eagle's medium (DMEM from Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 100 U penicillin ml^{-1}, and 10 mg streptomycin ml^{-1} solution. The cell washing occurred by Ca^{2+} and Mg^{2+} free Hank's balanced solution (Gibco Invitrogen). Tetrazolium bromide was purchased from Sigma-Aldrich (CAS 298-93-1, St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) (CAS Number: 67-68-5, >99.9%) was purchased from MERCK (Merck Biosciences, Schwalbach, Germany).

2.2. Culture and expansion of BM-BMSCs

Bone marrow cells from femurs and tibias of adult Wistar Kyoto rats (between four- and eight-week-old) were harvested, under sterile conditions. Bone marrow cells were harvested from the tibia and femur; bone marrow cavities of the rats were flushed with CCM. Cells were individualized by carefully passing through 18–25 gauges needles. BM-MSCs were cultured in DMEM in 5% CO_2 at 37 °C, grown to confluency, and were trypsinized and transferred onto a larger surface. This study was approved by the Ethics Committee of Cardiology Institute of Rio Grande do Sul (number UP 5472/17).

2.3. MTT assay

Cytotoxicity analysis was evaluated with 3-(4, 5-dimethyl thiazol-2 yl)-2, 5-diphenyl tetrazolium bromide (MTT assay). Cytotoxicity assay was performed according to the International Organization for Standardization ISO 10993–52009 [30] and aimed to determine the period and concentration for exposures. Cells were seeded at a density of 1×10^4 cells/cm^2 into 96-well plates and cultured for 24 h in 5% CO_2 at 37 °C. Considering that water fluoridation concentrations recommended by WHO [3] in drinking water are between 0.5 and 1.5 mg/L, the cells were exposed to different concentrations of FA (0.6–9.6 mg/L), positive control (20% DMSO), and negative control (DMEM) for 3, 7, 14, and 21 days. DMSO is used as a positive control for MTT assay because it inhibits the mitochondrial respiration and the release of cytochrome c

from the mitochondria to the cytoplasm and induces cell death [31]. After the treatment periods, the cells were washed with Hank's solution, and 50 μL of fresh culture medium (without FBS and no phenol red) containing MTT solution (1.0 mg/mL) was added per well, then the plate was incubated for 2 h. The resulting formazan crystals were dissolved in 100 μL of DMSO, and the absorbance at 540 nm was measured using a microplate reader (Multiskan, UNISCIENCE). The results of MTT determined the exposure time for the other tests. Values are shown in % of cytotoxicity.

2.4. Alkaline comet assay

Comet assay was performed under alkaline conditions, as described by Singh et al. [32], with modifications [33,34]. Cells were seeded at a density of 1×10^4 cells/cm^2 into 24-well plates and cultured for 24 h in 5% CO_2 at 37 °C. Cells were exposed to different concentrations of FA (0.15, 0.3, 0.6, 1.2, and 2.4 mg/L; selected according to the MTT assay and WHO recommendation), positive control (Methyl methanesulfonate, MMS; 200 μM) and negative control (DMEM) for 3, 7, and 14 days. After the treatments, the cells were washed with Hank's solution, trypsinized, and suspended in DMEM. Then, the cell suspension was gently mixed with 0.75% low melting point agarose and immediately spread onto microscopic slides pre-coated with a layer of 1.5% normal melting point agarose. Slides were incubated in ice-cold lysis solution (2.5 M NaCl, 10 mM Tris, 100 mM EDTA, 1% Triton X-100, and 10% DMSO, pH 10.0) at 4 °C for 1 h. Subsequently, they were placed in a horizontal electrophoresis box containing freshly prepared alkaline buffer (300 mM NaOH and 1 mM EDTA, pH −13.0) at 4 °C for 20 min to facilitate DNA unwinding. A 300 mA and 25 V (0.9 V/cm) electric current was applied for 20 min to perform the electrophoresis. Subsequently, the buffer solution was neutralized with 0.4 M Tris (pH 7.5), and the DNA was stained with silver nitrate. Images of 100 randomly selected cells (50 cells from each of the two replicate slides) were analyzed. Damage index (DI) was evaluated, and each cell was assigned to one of five classes (from no damage = 0 to maximal damage = 4); according to the tail size, the values obtained for each individual could range from 0 to 400 [35].

2.5. Enzyme-modified comet assay

The enzyme-modified alkaline comet assay enables the identification of oxidative DNA damage using specific enzymes to repair oxidative damage. For this study, formamidopyrimidine DNA glycosylase (FPG) sensitive sites were used, which recognizes the common oxidized purine, 8-oxoguanine (8-oxoG), and ring-opened purines [36,37]. This test was performed according to Collins et al. [38]. For this treatment, the cells were exposed to the three lowest concentrations of FA (0.15, 0.3, and 0.6 mg/L), and the modified comet assay was performed in the presence of the FPG enzyme. After the lysis step, the slides were removed from this solution and washed three times with buffer solution (Hepes 400 mM, KCl 1 M, EDTA 5 mM, BSA 2 mg/mL, pH 8.0), and then incubated at 37 °C in enzyme buffer supplemented with FPG (1 μg/mL solution) for 30 min in a humid chamber. After this step, the slides were placed in a horizontal electrophoresis chamber and covered with alkaline buffer solution (300 mM NaOH and 1 mM EDTA, pH > 13) freshly prepared and remained for 40 min. The DNA was then electrophoresed for 30 min at 25 V (0.90 V/cm) and 300 mA. The following steps were carried out, as previously mentioned in the comet assay.

2.6. Micronucleus test

The BM-MSCs were seeded at a density of 1×10^4 cells/cm^2 into 24-well plates and cultured for 24 h in 5% CO_2 at 37 °C. Cells were exposed to different concentrations of FA (0.6, 1.2, and 2.4 mg/L), positive control (MMS; 200 μM), and negative control (DMEM) for 7 days. After the treatment period, the cells were washed with Hank's solution and trypsinized. After centrifugation, the supernatant was discarded, and the cells were gently suspended in DMEM. Approximately 200 μL of cell suspension was transferred to cytocentrifuge cups and centrifuged for 5 min at 700 rpm to produce slides. The slides were removed, fixed, and stained with Instant Prov. After staining, the slides were air-dried and examined under 1000x magnification using a light microscope to evaluate the frequency of nuclear buds (NBUDs) and micronucleus (MN) per 1000 cells [39].

2.7. Lipid peroxidation assay (TBARS) and determination of total antioxidant capacity (TAC)

Cells were seeded at a density of 1×10^4 cells/cm^2 into cell culture flasks in cell growth areas of 75 cm^2 for 24 h in 5% CO_2 at 37 °C for lipid peroxidation assay and antioxidant activity. The cells were exposed to different concentrations of FA (0.6, 1.2, and 2.4 mg/L) and negative control (DMEM) for 7 days. After the treatment period, the cells were washed with Hank's solution and trypsinized. Lipid peroxidation was monitored by the formation of thiobarbituric acid reactive substances (TBARS) during an acid-heating reaction, according to Wills [40], with modifications. Specifically, 300 μL of supernatant from each sample was combined with 450 μL of 15% trichloroacetic acid and 0.67% thiobarbituric acid. The mixture was heated at 100 °C for 15 min. After cooling to room temperature, the samples were centrifuged at 5000 rpm for 10 min. The supernatants were isolated, and their absorbance was measured at 530 nm. Hydrolyzed 1,1,3,3-tetramethoxypropane (TMP) was used as standard, and the results were expressed as μmol TMP/mg of protein.

The screening of antioxidant activity was performed based on the sample ability to scavenge the radical $ABTS^+$ [2,2-azino-bis (3-etilbenzotiazolin)-6-sulfonic acid]. The method follows the procedures described by Re et al. [41]. The $ABTS^+$ solution is formed from the reaction of 7 mM ABTS with 2.45 mM potassium persulphate. This solution was kept in the dark at room temperature for 12–16 h before use. Then the solution ABTS $^{\bullet}+$ was diluted with 5 mM phosphate buffer saline (PBS pH 7.4) until an absorbance of 0.700 ± 0.35 at 734 nm. Then, 1.0 mL of ABTS $^{\bullet}+$ diluted solution was added to 10 μL of the sample. The absorbances were read precisely 6 min after the initial mixture. For quantification, a standard curve was used with Trolox solution, and the results were expressed as mM Trolox.

2.8. BM-MSCs differentiation

Bone marrow stem cells were seeded into the FA samples at 3×10^4 cells/cm^2 and maintained in the osteogenic induction medium (CCM-O). The CCM-O consisted of CCM supplemented with dexamethasone (10^{-8} M), ascorbic acid 2-phosphate (5 μg/mL), and betaglycerophosphate (10 μM). The medium was changed every 3 days, and calcium deposition was stained with Alizarin Red S. For the mineralization analysis and alkaline phosphatase assay, cells were exposed to a concentration of 0.6 mg/L of FA during all the treatment period. FA was added in each medium change during the test.

2.8.1. Mineralization analysis

After 14 days of osteogenic differentiation, cultures were fixed and stained with Alizarin Red S. Excess dye was removed, and 500 μL of isopropanol was added to dilute the staining. The content was transferred to a 96-well plate for spectrophotometric reading (Multiskan Ex original, Serial RS-232c) at 540 nm. A molar ratio of 1:2 (Alizarin Red S: calcium) was applied [42]. Results were expressed as Mm/mL.

2.8.2. Alkaline phosphatase (ALP) assay

After the osteogenic differentiation induction, the cultures were rinsed with phosphate-buffered saline (PBS) and incubated for 2 h at 37 °C with 200 μl of the BCIP/NBT substrate kit (Invitrogen). After the addition of 200 μl SDS/10% HCL, cells were incubated overnight at 37

°C in 5% CO_2 (v/v). The supernatant was transferred to a 96-well plate for spectrophotometric reading (Multiskan Ex original, Serial RS-232c) at 595 nm. Results were expressed as optical density (OD).

2.9. Statistical analysis

Statistical analysis of the obtained data was performed using One-Way Analysis of Variance (ANOVA) complemented by Dunnett's or Tukey test using GraphPad Prism 5.0 (GraphPad Inc., San Diego, CA, USA) program. Differences were considered statistically significant when the P-value was less than 0.05.

3. Results

The treatment of the mesenchymal stem cells with FA showed a reduction below 60% in cell proliferation in the MTT assay for 14 and 21 days of exposure for all the concentrations tested (0.6, 1.2, 2.4, 4.8, and 9.6 mg/L) and for 3 and 7 days the cell proliferation was above 80% (Fig. 1). Based on these results, we defined a maximum concentration of 2.4 mg/L for the other tests. The cytotoxicity percentual (mean and standard deviation) of the positive control was 14.24 ± 6.16 (3 days), 15.53 ± 12.69 (7 days), 7.98 ± 3.09 (14 days), 13.76 ± 13.67 (21 days), demonstrating that the test worked well. Therefore, for the analysis of alkaline comet assay, 3, 7, and 14 days were used as exposure time for the concentration of 1.2 mg/L, then assisting decide the cell exposure time.

Exposure of FA for 3, 7, and 14 days to BM-MSCs (Fig. 2A), using the alkaline comet assay, demonstrated a significant increase at the concentration of 1.2 mg/L exposed for 7 days ($P < 0.05$) in relation to the negative control. According to these results, cells were treated for 7 days using five concentrations: 0.15, 0.3, 0.6, 1.2, and 2.4 mg/L for the alkaline comet assay. Fig. 2B demonstrates the results for comet assay of cells treated according to this condition with FA, which is possible to observe a significant increase for all concentrations for DNA damage (dose-response) (except in 0.15 mg/L). Results for modified comet assay (Fig. 3) demonstrated increased DNA damage for the cells treated with FA without enzyme at concentrations 0.3 and 0.6 mg/L, and for the cells exposed to FA in the presence of the FPG enzyme at all concentrations when compared to the negative control. In addition, when comparing the comet assay with an enzyme in relation to the respective concentrations without enzyme, the concentration of 0.6 mg/L demonstrated a significant difference.

For the MN test, determination of lipid peroxidation and total antioxidant capacity, the cells were treated for 7 days with the three highest concentrations used in the comet assay: 0.6, 1.2, and 2.4 mg/L. Fig. 4A and B summarize the results of MNs and NBUDs frequencies. Significant induction of MNs was observed at 1.2 and 2.4 mg/L, and NBUD rates increased after the treatment at all concentrations tested.

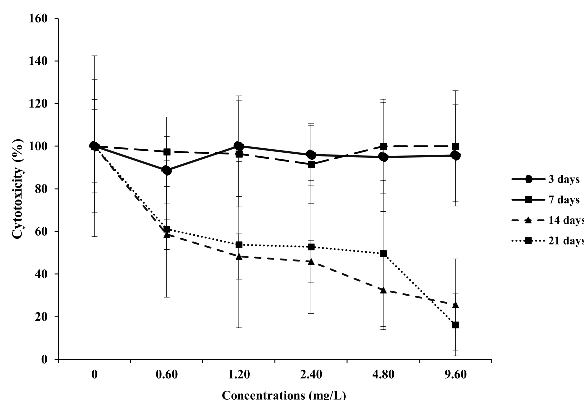

Fig. 1. Cell cytotoxicity. Evaluation of cytotoxicity of mesenchymal stem cells after treatments with different concentrations of fluorosilicic acid during 3, 7, 14 and 21 days (mean ± SD). Negative Control [0 mg/L]: culture medium. Experiments conducted in quadruplicate.

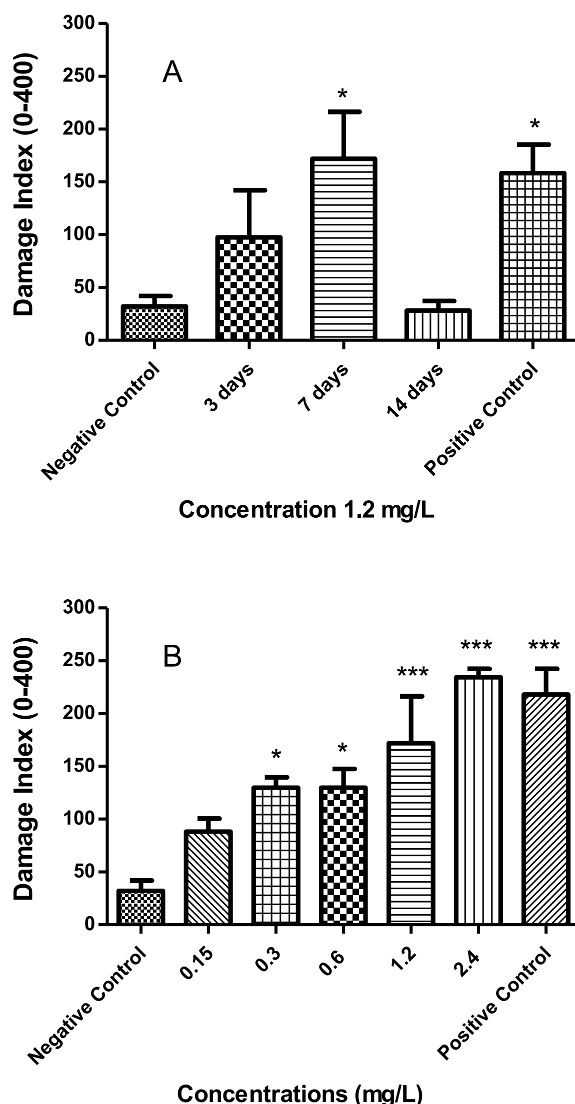

Fig. 2. DNA Damage. (A) Damage index (mean ± SD) using Comet assay on mesenchymal stem cells exposed during different periods (3, 7 and 14 days) to fluorosilicic acid at a concentration of 1.2 mg/L; (B) Damage index (mean ± SD) on mesenchymal stem cells exposed to five concentrations during 7 days to fluorosilicic acid. *Significant at $P < 0.05$, *** $P < 0.001$ in relation to the negative control group (Dunnett's test). Negative Control: culture medium. Positive control: methyl methanesulfonate: 200 μM. Experiments conducted in quadruplicate.

The results of lipid peroxidation and antioxidant capacity are shown in Fig. 5A and B. The lipid peroxidation levels were significantly higher at concentrations of 0.6 and 1.2 mg/L when compared to the negative control. Total antioxidant capacity levels were not significantly different between negative control and the concentrations tested.

Fig. 6A demonstrates the alkaline phosphatase activity (ALP) after 14 days of contact of the BM-MSCs with the FA. There was a significant difference between the CCM-O and the CCM-O with the addition of the FA. Fig. 6B shows the extracellular matrix mineralization of BM-MSCs exposed to FA. Higher extracellular matrix mineralization can be seen when BM-MSCs are cultured on the FA surface (CCM-O + FA). In the treated group, the mean concentration of Alizarin red significantly increased on day 14 compared to the CCM-O group.

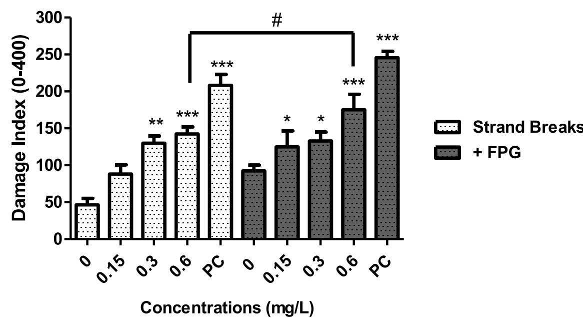

Fig. 3. DNA Damage with enzymes. Damage index in alkaline Comet assay (strand breaks) and modified comet assay with enzyme (+FPG - sensitive sites) (mean ± SD) on mesenchymal stem cells exposed to three concentrations during 7 days to fluorosilicic acid. *Significant at P < 0.05, ** P < 0.01, *** P < 0.001 in relation to the negative control group; # P < 0.05 in relation to strand break at the same concentration (Tukey's Multiple Comparison Test). Negative Control (0 mg/L): culture medium. PC (Positive control): methyl methanesulfonate in the concentration of 200 μM. The tests were carried out in quadruplicate and the experiment was performed twice.

4. Discussion

Studies revealed that fluoride consumption has the potential to interfere through molecular and genetic mechanisms in different organs and tissues of the body, interfering with signaling and apoptosis pathways [18,27,43]. In our study, FA induced genotoxicity (observed in both comet assay and MN test), probably by oxidative stress mechanisms, and the acceleration of bone mineralization in BM-MSCs in some concentrations also found in drinking water.

The MTT assay was performed for the evaluation of cell proliferation and to determine the FA concentrations and exposure time to be used for the other tests. To verify the potential cytotoxicity of the FA, concentrations of 0.60–9.6 mg/L were used. The lowest concentration value was chosen because it was close to the limit allowed by WHO, and the highest concentration was determined according to quantities found in drinking water in many countries worldwide [24,44–46]. The MTT assay indicates mitochondrial alteration and, consequently, respiratory chain damage by cytotoxicity [47]. At all concentrations tested for the exposure periods of 3 and 7 days, the FA was not considered cytotoxic to BM-MSCs at the concentrations tested (proliferation > 80%), but for 14 and 21 days, the proliferation reduced to less than 60%. Then, according to these data, the maximum concentration of 2.4 mg/L was determined. This definition is in concordance with *in vitro* guidelines [48,49], in which the highest concentration should aim to produce 55 ± 5% of cytotoxicity. Higher levels may induce chromosome instability as a secondary effect of cytotoxicity. Other studies also indicated that fluoride could induce cytotoxicity depending on the concentration and duration of treatment. Fu et al. [50], evaluating the effects of fluoride on human embryonic stem cells, observed a decrease in cell viability and proliferation at high concentrations (~80 mg/L) of sodium fluoride in 96 h (time-dependent).

Some studies observed that excessive chronic fluoride intake could directly affect the decrease in the expression of bcl-2 family members, even at low concentrations, activating stress pathways, and promoting apoptosis. Yang et al. [51] observed that osteoblast apoptosis was mediated by direct effects of fluoride on the expression of bcl-2 family members during fluoride treatment on MC3T3-E1 osteoblastic cells at 0.4−42 mg/L of fluoride for up to 48 h. The bcl-2 family of proteins acts in the regulation of apoptotic gene expression, preventing cytochrome C activation and caspases activity, and protecting the mitochondria [18]. In another study [27] performed with a population consuming water with 1.9–4.02 mg/L of fluoride, peripheral blood mononuclear cells showed alterations in the expression of the TNF/TNFR superfamily, responsible for the activation of genes of autoimmunity, inflammation,

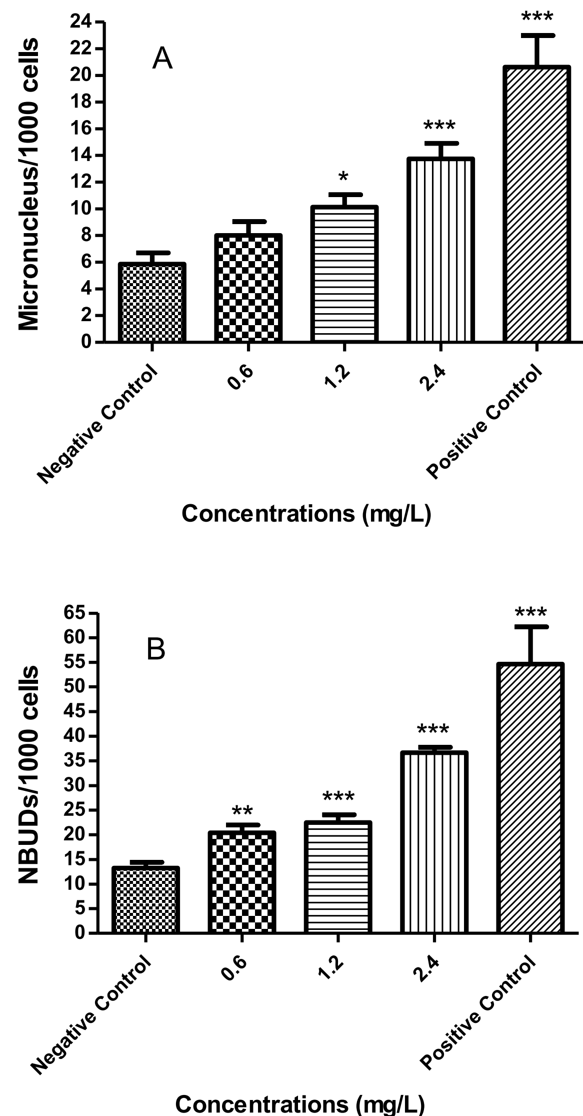

Fig. 4. Mutagenicity by the frequency of MN and NBUDs. Number of micronucleus and NBUDs results/1000 cells (mean ± SD) using micronucleus test on mesenchymal stem cells exposed to three concentrations of fluorosilicic acid for 7 days. *Significant at P < 0.05, ** P < 0.01, *** P < 0.001 in relation to the negative control group (Dunnett's test). Experiments conducted in quadruplicate. Negative Control: culture medium. Positive control: methyl methanesulfonate: 200 μM.

and apoptosis. Fu et al. [50] also observed that concentrations upper to 80 mg/L caused apoptosis besides suppressed proliferation, and half of this concentration could disturb the gene expression patterns. Therefore, apoptosis could be related to the cytotoxicity observed in this study. Our MTT results corroborate with this information, as well as the data found in the literature, which shows that the effects of long-term exposure to fluorides affect many mitochondrial proteins, induce the reduction of intracellular ATP, inhibit cellular respiration, generate free radicals, which results in oxidative stress, as well as apoptosis [52,53].

Based on the results of cell proliferation and before performing other tests, the alkaline comet assay was used to define the exposure time using only the concentration of 1.2 mg/L for 3, 7, and 14 days. The results of this test allowed us to define 7 days as the best treatment period in this study. This definition was based on one of our objectives, which evaluated the osteogenic differentiation. Then, 3 days would be a short time of exposure, and the non-significant DNA damage observed in 14 days would possibly be related to cytotoxicity. Comet assay can

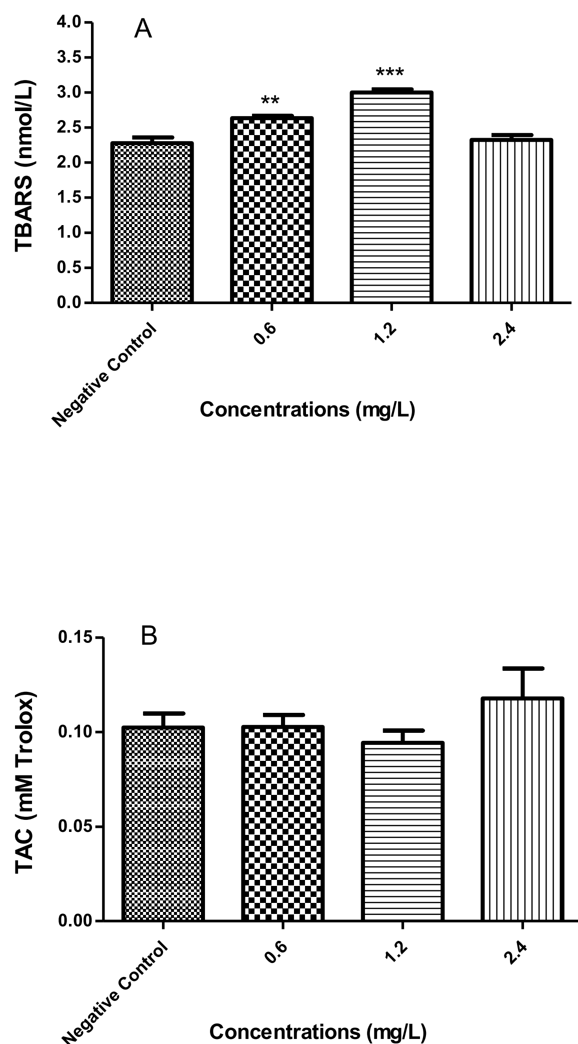

Fig. 5. Quantification of oxidative damage markers (mean ± SE). (A) Lipid peroxidation (TBARS - nmol/L). (B) Total antioxidant capacity (TAC - mM Trolox/L) in mesenchymal stem cells exposed to three concentrations of fluorosilicic acid for 7 days. **Significant at $P < 0.01$, *** $P < 0.001$ in relation to the negative control group (Tukey's test). Experiments conducted in quadruplicate. Negative Control: culture medium.

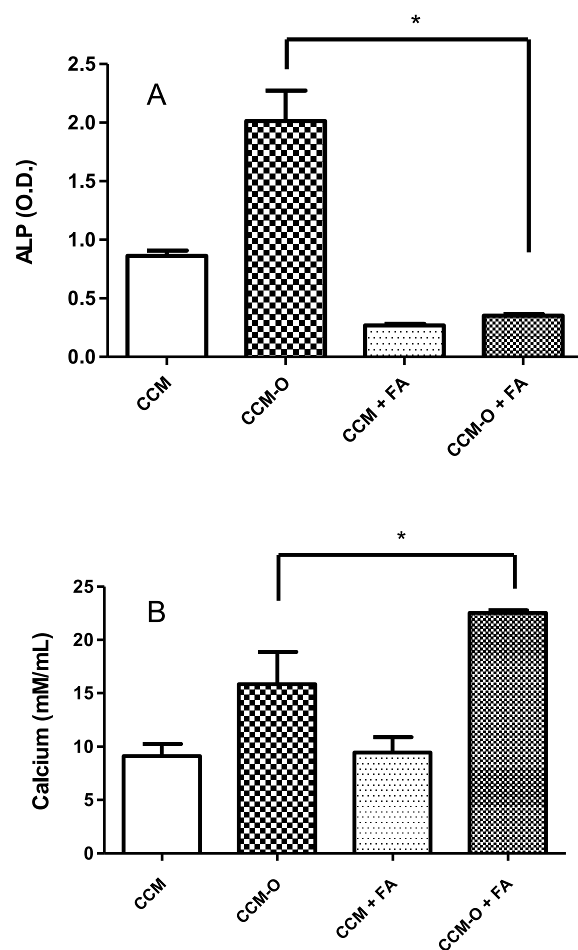

Fig. 6. Quantification of osteogenic induction markers (mean ± SD). (A) Alkaline phosphatase (ALP) activity after 14 days of exposure to fluorosilicic acid at a concentration of 0.6 mg/L; (B) Extracellular matrix mineralization after 14 days of contact of BM-MSCs with the fluorosilicic acid at concentration 0.6 mg/L. CCM: Culture medium, CCM-O: Osteogenic induction medium, CCM + FA: Culture medium + fluorosilicic acid, CCM-O + FA: Osteogenic induction medium + fluorosilicic acid. Experiments conducted in sextuplicate. *Significant at $P < 0.05$ (Tukey's test).

detect possible lesions to the DNA molecule, such as single- or double-strand breaks, DNA adducts, abasic sites, and incomplete repair sites, originated by an agent capable of being carcinogenic and/or mutagenic [35].

Comet assay results demonstrated genotoxic effects for concentrations upper to 3.0 mg/L of FA in BM-MSCs cells exposed for 7 days with a dose-response relationship. In concordance with the alkaline comet assay, MN test results obtained from BM-MSCs cells exposed for 7 days with FA showed mutagenicity by the increased frequency of MN (1.2–2.4 mg/L) and NBUDs (0.6–2.4 mg/L). Comet assay detects recent lesions that can be repaired, such as breaks and alkali-labile sites, while the MN test detects non-repairable damage. MNs are the result of the chromosomal disruption caused by DNA damage, loss of chromosomal segregation due to mitotic errors, oxidative stress, and changes in the cell cycle. Whereas the NBUDs originate from the imbalance of various proteins of the nuclear lamina, which represents the elimination of amplified DNA and/or DNA repair complexes [54]. Studies have shown that fluoride can modulate the activity of most stages of repair by base excision (BER) [23,55–57]. The use of these biomarkers is associated with genomic instability and the predictive risk of various chronic diseases caused by exposure to environmental contaminants [38,58].

Other studies also demonstrated DNA damage in cells exposed to fluoride using the comet assay on *in vivo* models with concentrations higher than those in our study and for longer periods [21,59]. Or also on *in vitro* assays with lower concentrations, 40 μg/mL of sodium fluoride for 24 h, in hepatocytes from human embryos [60]. Regarding the MN test, Manivannan et al. [61] observed an increase in MNs in mice that ingested fluoride in drinking water at high concentrations and for more lasting periods than those in our study. And Wang et al. [62] on an *in vitro* study using human embryonic stem cells (EBf-H9), also treated with fluoride at high concentrations (>4 mg/L). In addition to these studies, DNA damage was detected, possibly due to the ability of fluoride to form covalent bonds with DNA and to cause damage through its active chemical properties [21]. It is well established that changes in the antioxidant system can lead to damage to various macromolecules such as lipids, proteins, and DNA. Consumption of large amounts of fluorides over a prolonged period results in constant production of reactive oxygen species (ROS) [22,63,64].

The comet assay is a widely used method for assessing DNA damage and, with modifications, it possibly became the most sensitive and confirmatory assay regarding the mechanisms of oxidative damage [65]. Specific repair endonucleases induce additional breaks and increase the DNA damage of the comets through the recognition at the sites of oxidized bases [37,65]. To understand the mechanisms of genomic

instability induced by FA, we used the comet assay modified by repair endonuclease (with FPG). FPG can recognize oxidized purine bases, ring-opened purines, and especially 8-oxoG (mainly secondary oxidation products formed in DNA) [37]. In this study, our results demonstrated the formation of oxidation products in mesenchymal cells exposed to FA, at a concentration of 0.6 mg/L, compared to the same concentration of the alkaline comet assay, and this result might be related to the induction of ROS [66]. Collins et al. [67] demonstrated that the comet assay modified with enzymes like FPG could provide a value of the damage for the endogenous oxidative base, which is more than 10-fold lower than those most estimated from HPLC. Nevertheless, the FPG treatment showed a slightly higher level, although not significantly, at the other concentrations when compared to the level of DNA damage found in the alkaline comet assay. In concordance with our findings, studies with fluoride have shown a relation between DNA damage and oxidative stress [21,56,57]. In the alkaline comet assay without enzyme, we observed an increase at all concentrations tested in comparison to the respective negative control, but significantly at the concentration of 0.3 and 0.6 mg/L. This finding is because the type of FA that induced lesion in the mesenchymal stem cells may be caused by other pathways than those usually detected by the FPG enzyme, as oxidized pyrimidines.

Corroborating with these findings of the modified comet assay, we observed a significant increase in the levels of lipid peroxidation in BM-MSCs exposed to FA for 7 days, suggesting that free radicals could be involved in oxidative stress induction and DNA damage. However, we did not observe an increase at the highest concentration (2.4 mg/L) and a non-significant increase in TAC for all tested concentrations. Jain et al. [22] and Chouhan et al. [63], in *in vivo* studies with rats, obtained results similar to ours, demonstrating increased TBARS levels in the brain of the animals that drank water with a 3 mg/L fluoride solution for 60 days, and in the liver of the animals that drank water for 12 weeks with concentrations of 1, 10, and 50 mg/L. Bonola-Gallardo et al. [64] found a positive correlation between fluoride ion concentrations in drinking water and plasma malondialdehyde (MDA) concentrations (an increase in free radicals causes the overproduction of MDA). In another study, authors showed that children exposed to high concentrations of fluoride (0.5–12.6 mg/L) *via* water consumption in India demonstrated a significantly increased lipid peroxidation with elevated levels of MDA in their red blood cells, and a significant decrease in the activities of the antioxidant enzymes glutathione S-transferases (GST), catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx), as well as a reduction in glutathione (GSH) tripeptide concentrations, which may lead to imbalances of the cellular redox state [23].

In addition to the genotoxic effects found in the quantification of markers of osteogenic induction, due to FA treatment, we observed an increase in the alkaline phosphatase in the group consisting of osteogenic medium (CCM-O) compared to the CCM-O + FA group, as expected. However, there is an increase in bone mineralization in the CCM-O + FA group compared to the CCM-O group, which does not correspond to the expected results. The activity of alkaline phosphatase is associated with cell differentiation and acts as a marker of bone formation [68]. We suggest that the fluoride pathway that stimulates bone formation involves other unknown pathways and not only the induction of alkaline phosphatase. Studies suggested that fluoride acts directly on bone growth, speeding the osteogenic differentiation property of BM-MSCs [69], and leads to an increase in the mineralization ability of osteoblasts, which may result in abnormal remodeling patterns in bone mineralization caused by genotoxic changes, as observed in our study. There are reports available in the literature that suggest a correlation between fluoride exposure and osteosarcoma [70]. However, these findings relate to high fluoride levels above those used in this study. Moreover, a link between p53 mutations and fluoride bone content has been reported in tissue samples from osteosarcoma in Indian patients [71].

Our findings suggest that the exposure to FA (fluoride used in drinking water) in mesenchymal stem cells, in the conditions of this study, has the potential to cause a genotoxic effect demonstrated through increased DNA damage using alkaline comet assay and MN test. It also provided evidence of the relation of fluoride genotoxicity with oxidative damage in purines and oxidative stress in lipids. In addition, FA promoted an increase in calcium content at concentrations considered safe for consumption, which may be related to fluorosis and bone cancer reported in the literature. But it is important to emphasize that, for our highest concentration used, 2.4 mg/L, a plasma level of approximately 0.13 mg/L (our lowest concentration in this study) is expected. Despite this, populations are chronically exposed to FA, and our results help to understand the mode of action and mechanisms of FA causing DNA damage. In relation to the broad applications of fluoride in drinking water, we suggest that concentration and exposure time should be considered, and that particular protection is necessary to avoid its harmful effects on human health.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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