

Fluoride Modulates Preosteoblasts Viability and Matrix Metalloproteinases-2 and -9 Activities

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This study evaluated the influence of fluoride on cell viability and activity of matrix metalloproteinases (MMP) -2 and -9 secreted by preosteoblasts. Preosteoblasts (MC3T3-E1 murine cell line) were cultured in MEM medium supplement with 10% Fetal Bovine Serum (FBS) and nucleosides/ribonucleosides without ascorbic acid. Adherent cells were treated with different concentrations of F (as sodium fluoride-NaF) in medium (5×10^{-6} M, 10^{-5} M, 10^{-4} M and 10^{-3} M) for 24, 48, 72 and 96 h at 37°C, 5% CO₂. Control cells were cultivated in MEM only. After each period, preosteoblast viability was assessed by MTT assay. MMP-2 and -9 activities were performed by gel zymography. Also, alkaline phosphatase (ALP) activity was quantified by colorimetry in all experimental groups. It was shown that cultured cells with the highest dose of F (10^{-3} M) for 96 h decreased preosteoblast viability while lower doses of F did not alter it, when compared to untreated cells. No differences were observed in ALP activity among groups. Moreover, compared to control, the treatment of cells with F at low dose slightly increased MMP-2 and -9 activities after 24 h. It was concluded that F modulates preosteoblast viability in a dose-dependent manner and also may regulate extracellular matrix remodeling.

Key Words: metalloproteinase, phosphatase, fluoride.

INTRODUCTION

Bone tissue is a specialized form of connective tissue with reparative and remodeling properties (1). Osteoblasts are mononuclear cells that are responsible for bone formation, producing most of the proteins present in the bone extracellular matrix (ECM) and control its mineralization. Osteoblasts arise from osteoprogenitor cells located in the periosteum and bone marrow (2). Fluoride promotes bone formation and it has been used for treating osteoporosis in some countries (2,3). However, its beneficial effect remains unclear because F is mitogenic, enhancing osteoblast number in the bone tissue while promoting a cytotoxic effect, reducing mineralization and mineral apposition rate (2). In this context, it was shown that the treatment with F enhanced

both cortical bone thickness and bone mass (4).

F may alter osteoblast activity, acting either in a systemic way or in fluorapatite crystals (5), modulating the amount of bone mass in rats in a dose-dependent manner (6).

ECM turnover is clearly involved during bone remodeling and matrix metalloproteinases (MMPs) govern these events (7). MMPs are zinc-dependent endopeptidases. Collectively they are capable of degrading all kinds of extracellular matrix proteins. Also, they play an important role in tissue remodeling associated with various physiological and pathological processes such as morphogenesis, angiogenesis, tissue repair, cirrhosis, arthritis and metastasis (8,9). Normal human osteoblasts express MMP-2 and *in vitro*, they secrete abundant MMP-2. MMP-9 is produced by both

osteoblasts and osteoclasts in developing and remodeling bone (10). Recently, osteoblast-derived MMPs were shown to play a role in the bone metabolism by the degradation of bone matrix (10).

Despite the numerous studies undertaken on osteoblast/fluoride interactions, their regulation on ECM remodeling remains to be understood. The present study was undertaken to evaluate the effect of F on osteoblast viability by MTT assay, and MMP-2 and -9 activities by zymography gel analysis.

MATERIAL AND METHODS

Cell Culture

MC3T3-E1 preosteoblasts (ATCC murine cell line) were cultivated in MEM (Minimum Essential Medium) (Invitrogen, Carlsbad, CA, USA) medium supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 1% penicillin/streptomycin (Sigma, St. Louis, MO, USA) and nucleosides/ribonucleosides (Sigma), with no ascorbic acid (11). Cells plated at the density of 5×10^2 in 96-well plates (TPP-Techno Plastic Products, Trasadingen, Switzerland) were incubated at 37°C, 5% CO₂. After 8 h (time to allow cellular adherence), the adhered cells were treated with different concentrations of F (as NaF) (5×10^{-6} M, 10^{-5} M, 10^{-4} M and 10^{-3} M) prepared in MEM 10% FBS. The medium containing F was replaced every other day. Control cells received only MEM medium 10% FBS.

MTT Assay

Cells were plated and treated as described above for 24, 48, 72 and 96 h. After each time point, the supernatants were removed and new medium (no serum) was added followed by the addition of 10 µL of MTT (Sigma) (0.5 mg/mL in PBS) and incubation for 4 h at 37°C. Formazan precipitate was solubilized with 100 µL/well of 10% SDS-HCl 0.01 M. The plates were read in spectrophotometer at 540 nm. Each condition was done in triplicate (3 wells).

Extraction and Quantification of Secreted Proteins

Cells were treated with or without different concentrations of F in MEM 10% FBS (10^{-3} , 10^{-4} and 10^{-5} M) for 24 and 48 h (12). Supernatants were collected and the secreted proteins were quantified by Hartree

modified-Lowry (13).

Zymography Gel Analysis

Conditioned supernatants were obtained and 15 µg of protein were used for zymography gel loading. As controls, molecular weight standards and recombinant MMP-2 and MMP-9 proteins (Calbiochem; Merck, Darmstadt, Germany) were used. The gels were stained with Coomassie Blue G-250 (0.5%), scanned using the Imagemaster scanner (GE Healthcare®, Uppsala, Sweden) followed by Kodak Molecular Imaging Software (Kodak Co., Rochester, NY, USA) for analysis. The results show the mean of 3 independent gels, as reported by Hannas et al. (14).

ALP Activity Measurement

The commercial kit “Doles” (Dr. Jarbas Doles Laboratories Ltda., Goiânia, GO, Brazil) was used. Samples, standards and reagents were used according to manufacturer’s instructions. Samples were obtained by collecting total cellular extracts. The 96-well plates were read at 410 nm (FLUOstar OPTIMA; BMG Labtech, Offenburg, Germany). The data are shown as mean of UI/L where 1 UI is equivalent to 0.01667 µkat/L.

Statistical Analysis

Parametric analysis was performed by analysis of variance followed by Tukey’s test. Statistical significance was considered if $p < 0.05$.

RESULTS

Fluoride Alters Cell Viability in a Dose- and Time-Dependent Manner

In order to evaluate the effect of fluoride on cell viability, MTT assay (Fig. 1) was performed. As expected, preosteoblast presented a linear increased viability when cultured in medium during the experimental period (up to 96 h) (Fig. 1). The treatment of the cells with 10^{-4} and 10^{-5} M doses of NaF for 24 h decreased their proliferation compared to untreated cells ($p < 0.05$). However, lower doses of NaF (1×10^{-5} and 5×10^{-6} M) had no effect on preosteoblast viability at 72 and 96 h. Moreover, NaF at a higher dose (10^{-3} M) presented a tendency to decrease cell proliferation, which was significantly after 96 h

($p < 0.01$) (Fig. 1).

Fluoride Slightly Modulates MMP-2 and -9 Activities

Untreated preosteoblasts induced MMP-2 and -9 activities as judged by zymography gels, where the former was more pronounced than the last after both 24 and 48 h time points. Although not statistically significant ($p > 0.05$), treating the cells with high concentrations of NaF (10^{-3} M and 10^{-4} M) lead to greater MMP-2 activity after 24 h compared to untreated cells, while the lowest dose (10^{-5} M) barely altered its activity (Fig. 2). In this period, for MMP-9 (Fig. 3) only the group treated with 10^{-4} M seemed to increase its activity ($p > 0.05$). Moreover, the treatment of cells with F for 48h did not modify both MMP-2 and -9 activities when compared to control groups (Figs. 2 and 3).

Fluoride does not Modulate ALP Activity in Preosteoblasts

As shown in Table 1, the treatment of cells for 24 and 48 h with either low or high concentrations of NaF was not able to modify ALP activity.

DISCUSSION

The present study demonstrates the important role that fluoride plays in preosteoblast metabolism. A high dose of fluoride (10^{-3} M) decreased cell viability while low doses did not change it, judging by the MTT assay. Moreover, fluoride at both 10^{-3} M and 10^{-4} M presented a tendency to enhance MMP-2 and MMP-9 activity after 24 h and no differences were observed up to this period. Similarly, it has been shown that fluoride at low doses (as NaF) increases osteoblast proliferation (15) and bone mass (4). Also, proliferation of caprine osteoblasts was up-modulated after treatment with fluoride at 10^{-8} to 10^{-5} M and down-regulated at 10^{-4} to 10^{-3} M of NaF (16). The effect of fluoride on proliferation has also been shown in other cell types. In this context, Yan et al. (17) showed that NaF at doses of 4, 16 and 64 μ M led to increased ameloblast proliferation, while the dose of 1,024 μ M promoted apoptosis impairing osteoblast survival. The obtained results corroborate others, showing that fluoride affects cell proliferation in a dose-dependent

fashion. No significant enhancement of preosteoblast viability was found after treatment of cells with low doses. In this context, it is known that MTT assay has limitations that may impair its sensibility (18). Fluoride has been used in clinical trials for osteoporosis (2,3). Several treatment profiles, including different doses and long-term protocols, have been tested resulting in both beneficial (primarily at low doses) and impaired effects (3). The latter has been reported after long-term excessive fluoride intake (i.e. endemic areas) leading to what it is known as skeletal fluorosis (19). Recently, Inoue et al. (20) showed that treating osteoblasts and odontoblasts with low concentration of fluoride promoted greater and faster bone formation than with high concentration, in a dose-dependent fashion. The dual fluoride impacts on skeletal health, promoting bone formation or skeletal fluorosis can be highlighted by its dose-dependent effect in preosteoblasts. This biphasic effect of fluoride

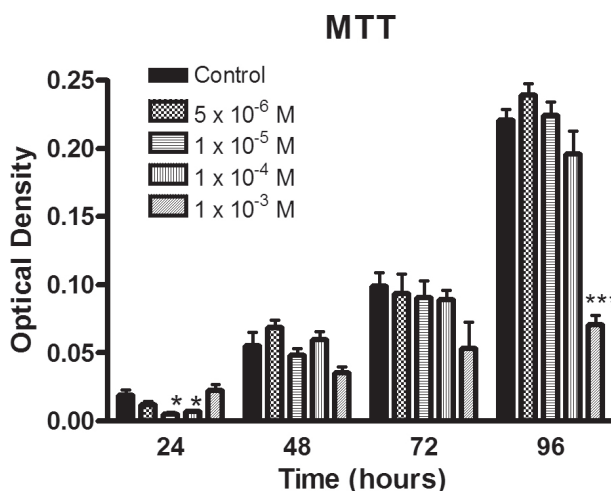


Figure 1. Effect of NaF on preosteoblast viability. MC3T3-E1 preosteoblasts were cultured in medium in an absence (control group) or presence of different concentrations of NaF (10^{-3} , 10^{-4} , 10^{-5} and 5×10^{-6} M). After 24, 48, 72 and 96 h MTT assay was performed. * $p < 0.05$; *** $p < 0.01$.

Table 1. Alkaline phosphatase activity (UI/L).

Period	Group			
	Control	10^{-5} M F	10^{-4} M F	10^{-3} M F
24 h	6.182 ^a ± 1.952 ^b	6.191 ± 1.949	6.164 ± 1.979	6.155 ± 1.957
48 h	6.165 ± 1.944	6.195 ± 1.974	6.179 ± 1.974	6.167 ± 1.974

^aMean. ^bStandard deviation.

suggested that fluoride can affect cells by multiple pathways, some of which are more sensitive to lower concentrations of fluoride and others that require higher fluoride concentrations. Several studies have shown that millimolar levels of fluoride might induce apoptosis in many cell types, including hepatic cells, epithelial lung cells, human leukemia HL-60 cells and ameloblast-lineage cells (17,21). In addition, other authors showed that fluoride does not induce DNA damage (22). In this study it is proposed that F at 10^{-3} M might trigger apoptosis by activating caspases signaling pathways.

The impact of fluoride on ALP activity is still controversial and it seems to be dependent on the

experimental design. Both up and down modulation have been found after fluoride treatment using osteoblasts (15-17). In the present study, there were no differences among the control and treated preosteoblasts. It is well known that ALP activity is up-regulated when culturing preosteoblasts under differentiation (23). Thus, this may be explained by the fact that the undifferentiated preosteoblasts have such low ALP activity that impairs either up or down modulation by fluoride.

As fluoride is also known to alter mineralization within the bone, it was examined whether fluoride has impact on remodeling matrix activity. In control preosteoblasts, MMP-2 activity was more pronounced

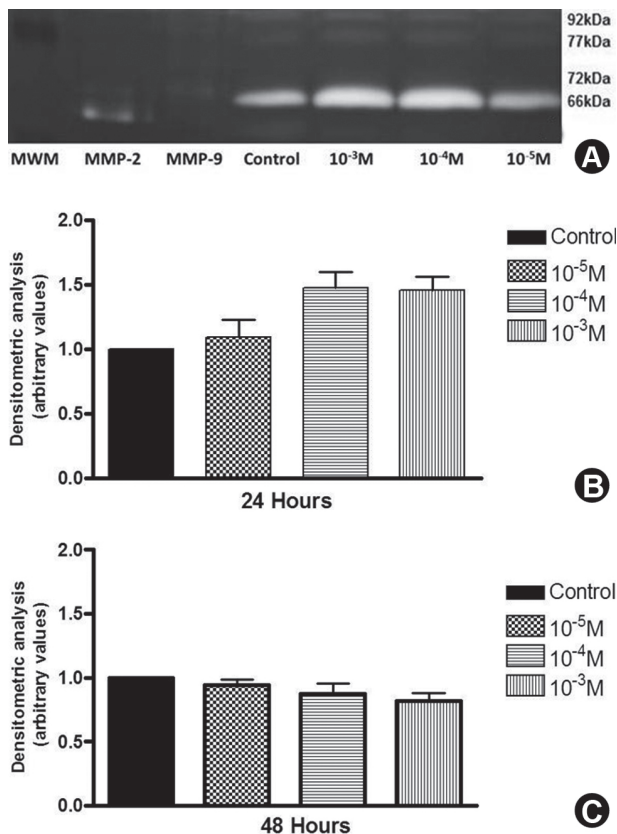


Figure 2. Effect of NaF on MMP-2 activity. Representative gel (24 h) is shown in A. MC3T3-E1 preosteoblast were cultured in medium only (control) or in presence of different doses of NaF (10^{-3} , 10^{-4} and 10^{-5} M). The supernatants were obtained at (B) 24 and (C) 48 h and used for zymography gel loading (15 μ g protein). The gels were scanned and the images were used for densitometric analysis using the Kodak Molecular Imaging Software. The active form (~66 kDa) was used for the densitometric analysis. The results are shown as arbitrary numbers (Mean \pm SD). The gels were conducted in triplicate. Control bands were standardized as number 1.

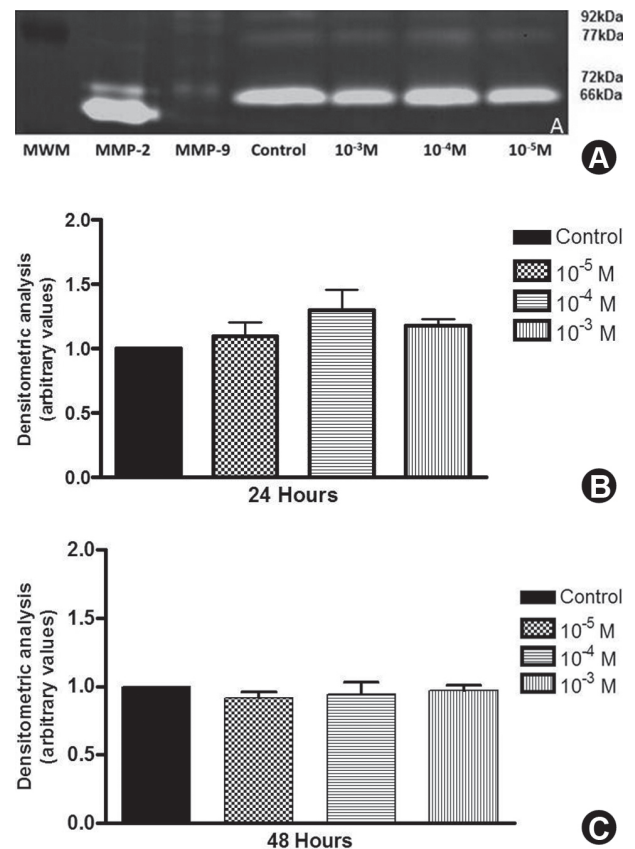


Figure 3. Effect of NaF on MMP-9 activity. Representative gel (48 h) is shown in A. MC3T3-E1 preosteoblast were cultured in medium only (control) or in a presence of different doses of NaF (10^{-3} , 10^{-4} and 10^{-5} M). The supernatants were obtained at (B) 24 and (C) 48 h and used for zymography gel loading (15 μ g protein). The gels were scanned and the images were used for densitometric analysis using the Kodak Molecular Imaging Software. The active form (~77 kDa) was used for the densitometric analysis. The results are shown as arbitrary numbers (Mean \pm SD). The gels were conducted in triplicate. Control bands were standardized as number 1.

than MMP-9. Also, treating the cells with fluoride slightly increased both MMP-2 and -9 after 24 h incubation with no effect up to this time point, compared to untreated control cells. It is worth emphasizing that, since this difference was not statistically significant ($n=3$), fluoride might have some biological effect on ECM remodeling. Little is known about the effect of fluoride on bone-producing MMPs. Waddington and Langley (24) showed that the presence of fluoride altered expression of MMP-1 and MMP-9 when mineralizing bone cells *in vitro*. The impact of fluoride on individual MMP-2 and -9 activities in bone formation has been reported in previous studies conducted by our research group. It was shown that the treatment of rats with 5 ppm F increased MMP-2 activity in alveolar bone on day 7 and of MMP-9 on day 30 (unpublished data). The possibility that fluoride could have greater affect in the osteogenic environment cannot be ruled out, since an increase in MMP activities has been reported during MC3T3-E1 preosteoblast differentiation (11). Also, there is a possibility that other MMP, besides MMP-2 and -9, have been modulated by fluoride in these cells. Moreover, the MMPs are inhibited by specific endogenous tissue inhibitors of metalloproteinases (TIMPs), which comprise a family of four protease inhibitors: TIMP1, TIMP2, TIMP3 and TIMP4. Enhancement of MMP activity may be a consequence of functional MMP stimulation or of TIMP deactivation (25). In this study, TIMP expression was evaluated because no significant modulation of MMP-2 and -9 after treatment was found. Therefore, the little influence of fluoride on MMP-2 and MMP-9 activity is likely to influence the composition of the remodeling matrix and subsequent mineralization. These results suggest that in response to fluoride, preosteoblasts may contribute to the process of active matrix degradation followed by bone formation by secretion and activation of MMPs.

RESUMO

Neste estudo, buscou-se avaliar a influência do fluoreto (F) na viabilidade celular e atividade das metaloproteinases de matriz (MMP)-2 e -9 secretado pelos pré-osteoblastos. Pré-osteoblastos (linhagem celular MC3T3-E1 murina) foram cultivados em meio MEM suplementado com 10% de soro fetal bovino (FBS) e nucleosídeos/ribonucleosídeos sem ácido ascórbico. Células aderidas foram tratadas com diferentes concentrações de F (na forma de fluoreto de sódio-NaF) em meio (5×10^{-6} M, 10^{-5} M, 10^{-4} M e 10^{-3} M) por 24, 48, 72 e 96 h a 37°C, 5 % de CO₂. Células do grupo controle foram cultivadas apenas em MEM. Após cada período, a viabilidade dos pré-osteoblastos foi avaliada por MTT. A

atividade das MMP-2 e -9 foram analisadas pela zimografia. Além disso, a atividade da fosfatase alcalina (FA) foi quantificada por colorimetria em todos os grupos experimentais. Foi demonstrado que as células cultivadas com a maior dose de F (10^{-3} M) no período de 96 h tiveram sua viabilidade comprometida, enquanto doses mais baixas de F não alteraram significativamente, quando comparado com células não tratadas. Não foi observada diferença na atividade da FA entre os grupos. Além disso, o tratamento de células com F em baixas doses, comparado ao grupo controle, promoveu um pequeno aumento da atividade das MMP-2 e -9 após 24 h. Pode-se concluir que o F modula a viabilidade de pré-osteoblastos de uma maneira dose-dependente e também pode regular a remodelação da matriz extracelular.

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REFERENCES

1. Davies JE. Understanding peri-implant endosseous healing. *J Dent Educ* 2003;67:932-949.
2. Dequeker J, Declerck K. Fluor in the treatment of osteoporosis. An overview of thirty years clinical research. *Schweiz Med Wochenschr* 1993;123:2228-2234.
3. Haguenaer D, Welch V, Shea B, Tugwell P, Adachi JD, Wells G. Fluoride for the treatment of postmenopausal osteoporotic fractures: a meta-analysis. *Osteoporos Int* 2000;11:727-738.
4. Cadir B, Kürkcü M, Öz IA, Benliday ME. Effects of vitamin K1 on fluoride-induced bone changes in growing rats: A histomorphometric and radiodensitometric study. *Arch Oral Biol* 2009;54:512-517.
5. Cheng K, Weng W, Wang H, Zhang S. *In vitro* behavior of osteoblast-like cells on fluoridated hydroxyapatite coatings. *Biomaterials* 2005;26:6288-6295.
6. Ohmi K, Nakagaki H, Tsuboi S, Okumura A, Sugiyama T, Thy TT, et al. The effect of fluoridation and its discontinuation on fluoride profiles in the alveolar bone of rat. *Calcif Tissue Int* 2005;77:226-232.
7. Garlet GP, Martins WJr, Fonseca BA. Matrix metalloproteinases, their physiological inhibitors and osteoclast factors are differentially regulated by the cytokine profile in human periodontal disease. *J Clin Periodontology* 2004;31:671-679.
8. Peres RC, Line SR. Analysis of MMP-9 and TIMP-2 gene promoter polymorphisms in individuals with hypodontia. *Braz Dent J* 2005;16:231-236.
9. Ruiz PA, Toledo OA, Nonaka CF, Pinto LP, Souza LB. Immunohistochemical expression of vascular endothelial growth factor and matrix metalloproteinase-9 in radicular and residual radicular cysts. *J Appl Oral Sci* 2010;18:613-620.
10. Nyman JS, Lynch CC, Perrien DS, Thiollou S, O'Quinn EC, Patil CA, et al. Differential effects between the loss of MMP-2 and MMP-9 on structural and tissue-level properties of bone. *J Bone Miner Res* 2011;26:1252-1260.
11. Zambuzzi WF, Yano X, Cavagis AD, Peppelenbosch MP, Granjeiro JM, Ferreira CV. Ascorbate-induced osteoblast differentiation recruits distinct MMP-inhibitors: RECK and TIMP-2. *Mol Cell Biochem* 2009;322:143-150.
12. Roomi MW, Monterrey JC, Kalinovsky T, Rath M, Niedzwiecki A. Patterns of MMP-2 and MMP-9 expression in human cancer cell lines. *Oncol Rep* 2009;21:1323-1333.

13. Hartree EF. Determination of protein: a modification of the Lowry method that gives a linear photometric response. *Anal Biochem* 1972;48:422-427.
14. Hannas AR, Pereira JC, Granjeiro JM, Tjäderhane L. The role of matrix metalloproteinases in the oral environment. *Acta Odontol Scand* 2007;65:1-13.
15. Yan X, Feng C, Chen Q, Li W, Wang H, Lv L, et al.. Effects of sodium fluoride treatment *in vitro* on cell proliferation, apoptosis and caspase-3 and caspase-9 mRNA expression by neonatal rat osteoblasts. *Arch Toxicol* 2008;83:451-458.
16. Qu WJ, Zhong DB, Wu PF, Han B. Sodium fluoride modulates caprine osteoblast proliferation and differentiation. *J Bone Minerar Metab* 2008;26:328-334.
17. Yan Q, Zhang Y, Li W, Denbesten PK. Micromolar fluoride alters ameloblast lineage cells *in vitro*. *J Dental Res* 2007;86:336-340.
18. Johnson JE. Methods for studying cell death and viability in primary neuronal cultures. *Meth Cell Biol* 1995;46:243-276.
19. Shashi A, Kumar M, Bhardwaj M. Incidence of skeletal deformities in endemic fluorosis. *Trop Doct* 2008;38:231-233.
20. Inoue D. Musculoskeletal rehabilitation and bone: Mechanical stress on the skeletal system. *Clin Calcium* 2010;20:503-511.
21. Anuradha CD, Kanno S, Hirano S. Fluoride induces apoptosis by caspase-3 activation in human leukemia HL-60 cells. *Arch Toxicol* 2000;74:226-230.
22. Ribeiro DA, Scolastici C, Marques ME, Salvadori DM. Fluoride does not induce DNA breakage in Chinese hamster ovary cells *in vitro*. *Braz Oral Res* 2004;18:192-196.
23. Ohta T, Wergedal JE, Matsuyama T, Baylink DJ, Lau KH. Phenytoin and fluoride act in concert to stimulate bone formation and to increase bone volume in adult male rats. *Calcif Tissue Int* 1995;56:390-397.
24. Waddington RJ, Langley MS. Altered expression of matrix metalloproteinases within mineralizing bone cells *in vitro* in the presence of fluoride. *Connect Tissue Res* 2003;44:88-95.
25. Brew K, Nagase H. The tissue inhibitors of metalloproteinases (TIMPs): an ancient family with structural and functional diversity. *Biochim Biophys Acta* 2010;1803:55-71.

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