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## Original

# Effect of Magnesium, Strontium or Fluoride Ions on *in vitro* Activities of Odontoblast-like Cells (MDPC-23)

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Abstract: Magnesium (Mg), strontium (Sr) or fluoride (F) ions have been shown to affect in vitro activities of bone cells and in vivo mineralization. The purpose of this study was to investigate the effect of Mg, Sr and F ions on the activities of odontoblast-like cells (MDPC-23) using an in vitro cell culture model. Mg, Sr or F ions were added to the medium in the following concentrations: Mg – 1, 5, 10 mM; Sr – 0.05, 0.1, 0.3, 1 mM; F- 0.005, 0.01, 0.05, 0.1 mM. Results were observed after 1, 7 and 14 days using Von Kossa staining, scanning and transmission electron microscopy. Preliminary results showed the following: Mg ions demonstrated a positive effect at low concentrations and a negative effect at high concentrations; Sr ions demonstrated a positive dose dependent effect up to a concentration of 0.1 mM and a negative effect at higher concentrations; and F ions demonstrated a positive dose-dependent effect up to a concentration of 0.005 mM and inhibitory effects at higher concentrations. This preliminary study showed that the positive or negative effects of Mg, Sr or F ions on odontoblastic activities (proliferation and mineralization) depend on their concentration levels in the environment.

Key words: Magnesium, Strontium, Fluoride, odontoblast

## Introduction

Magnesium (Mg), strontium (Sr) or fluoride (F) ions were shown to have an effect on *in vitro* activities of bone cells (osteoblasts and osteoclasts) and were reported to act on several levels to alter the complex cascade of events that regulate the mineralization process [1-4)]. These elements have also been involved in therapeutic agents in the management of osteoporosis [5-7)].

Mg has been associated with the mineralization of calcified tissues, mainly in bones and teeth[4)]. Mg indirectly influences mineral metabolism, for example through activation of alkaline phosphatase. Mg directly influences, or even controls, the crystallization processes of mineral substances as well as the pattern of mineral formation [8-11)].

Sr has long been known to cause rickets when fed to growing animals at a sufficiently high dose. Dietary Sr induces rickets in either calcium-replete [12, 13)] or calcium-deficient animals [(14)]. Sr acts on several levels to alter the complex cascade of events that regulate the mineralization process. In addition to displacement of $Ca^{2+}$ from an existing matrix-bound mineral phase, binding of $Sr^{2+}$ to a calcifiable collagenous matrix can also occur by its interaction with a site normally involved in the binding of $Ca^{2+}$ during the initial step of calcification[15)]. Sr also affects the organic matrix composition locally[16)] which implies an altered elaboration of matrix components by cells [12, 17)]. Sr was shown to affect bone formation *in vivo* and resorption *in vivo* and *in vitro*[7)]. *In vivo* studies showed that Sr salts stimulate parameters of bone formation in rodents and humans, and this result in increased trabecular bone volume. At low dosage levels, stable Sr was reported to improve the vertebral bone density in osteoporotic patients[18)].

F is an important therapeutic agent in the treatment of osteoporosis, but has toxic effects at higher doses or with chronic exposure [19)]. F influences bone growth both on a biological level, by acting as a mitogenic agent. The *in vivo* toxic effects of F are: hypomineralization of newly formed osteoid, hypermineralization of bone and, in endemic fluorosis, formation of woven bone[20)]. Lower doses of F, on the other hand, can be used to increase bone mass in a regular fashion without any noticeable formation of mineralization defects[19, 21)]. The effects of F on bone cells in

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monolayer culture are well known: fluoride has a dose-dependent effect on cell proliferation, extracellular matrix production, and alkaline phosphate activity [1, 3]. F-treated bones were reported to have an affect on cell activities of osteoblast-like cells[22] *in vitro* while F-substituted carbonatehydroxyapatite were shown to promote bone formation *in vivo*[23]. F-treated dentin and F-substituted apatite were shown to inhibit osteoclast resorption[24, 25].

While many studies have reported on the effect of Mg, Sr or F ions on bone cells (osteoblast-like and osteoclast-like), the effect of these ions on odontoblast-like cells have not been investigated to date. The purpose of this preliminary study was to investigate the effects of high and low levels of Mg, Sr, or F ion concentrations on *in vitro* activities (proliferation and differentiation) of odontoblast-like cells.

## Materials & Methods

*Cell cultures*

MDPC-23 cloned 3T6 cells of 18-day CD-1 fetal mouse molar dental papillae (odontoblast-like)[26] was inoculated at a density of 5000 cells/$cm^2$, and cultured in alpha modified medium (Cellgro Mediatech, Inc., Herndon, VA, USA), containing 10 % fetal bovine serum (FBS), ascorbic acid (50 mg/mL) and b-glycerophosphate (2 mM). $MgCl_2$, $SrCl_2$, and NaF reagents (Fisher Scientific, Springfield, NJ, USA) were used as the source of Mg, Sr or F ions, respectively. Mg, Sr, or F ions were added to the medium in the following concentrations: Mg - 1, 5, 10 mM; Sr - 0.05, 0.1, 0.3, 1 mM; F - 0.005, 0.01, 0.05, 0.1 mM. The media (control) included $MgSO_4$( 0.8 mM) and $CaCl_2$ (1.8 mM). The cells were passaged and incubated for a day prior to placement in medium, then changed treated medium, and sampled at 7 days (confluent of mono-layer) and 14 days (mineralization). The cells were maintained in a 5 % $CO_2$ atmosphere.

*Von Kossa Staining*

Each sample was washed three times with phosphate buffer saline (PBS), fixed with 10 % formaldehyde for 30 min at room temperature, and then washed three more times with PBS. Five percent aqueous silver nitrate was added to samples in the dark room for 30 min, and then washed three times with distilled water. The samples were exposed to UV light for 30 min and observed by light microscopy. The staining area was calculated using imaging analyzer (BIOQUANT NOVA, R&M Biometrics, Inc., Nashville, TN, USA).

*Cell count*

The cells were treated with trypsin, diluted with PBS, and counted using the Coulter counter (COULTER Z2 - Particle count & Size Analyzer).

*Scanning electron microscopy (SEM) analysis*

The specimens were fixed using solution consisting of 2 % glutaraldehyde in 0.1 M Na- cacodylate-HCl buffer with 0.1 M sucrose (pH 7.2) and dehydrated in 70 %, 80 %, 90 %, and 100 % ethanol for 30 min each. The plates were observed by SEM (JEOL/JSM-5400).

*Transmission electron microscopy (TEM) analysis*

The specimens were fixed in a mixture of 2 % glutaraldehyde in 0.1 M Na- cacodylate-HCl buffer with 0.1 M sucrose (pH 7.2), and post-fixed with 1% osmium tetroxide. After that, epon-embedded ultrathin sections were prepared, double-stained, and examined under H800 transmission electron microscope (Hitachi Co., Japan).

*Statistical analysis*

The Von Kossa staining data was compared to the control group and each group, and was subjected to non-parametric analysis. Statistical differences among group were determined using the Kruskal-Wallis test and the Mann-Whitney U-test. A P-value of 0.05 was considered to be statistically significant. All data were presented as means ± standard error.

## Results

*Cell culture and cell count*

Odontoblast-like cells (MDPC-23) in Mg- and Sr-containing media displayed no remarkable difference compared with those in control in any period of proliferation. In media containing 5 mM and 10 mM Mg or Sr, cells became detached after reaching confluence. Cell proliferation was initially inhibited but slowly increased in F-containing media in a dose-dependent manner. Most of the cells in F-containing media were dead after 2 weeks, but did not detach.

*Von Kossa staining*

After 7 days (Fig. 2A), with cells in the control group, Von Kossa staining revealed formation of mineralized nodules. The intensity of staining (indicating nodule formation) was similar for both control and cells in media containing with 1 mM Mg, but much less with cells in media containing 5 mM and 10 mM Mg. Less nodule formation was observed with cells in media containing 0.05 mM and 0.1 mM Sr compared to the control group (0.05 mM< 0.1 mM); no nodule or less formation was observed with cells in media containing 0.3 mM and 1 mM Sr (0.3 mM > 1 mM ). Compared to the control group, cells in media containing 0.005 mM F formed less number of nodules while cells in media containing 0.1 mM F formed more nodules. Nodule formation was not observed with cells in media containing 0.01 mM and 0.05 mM F.

After 14 days (Fig.1, Fig.2B), cells in the control group and 1 mmol/L Mg-containing media displayed increased mineralization and started to detach. Mineralized nodules were not observed and

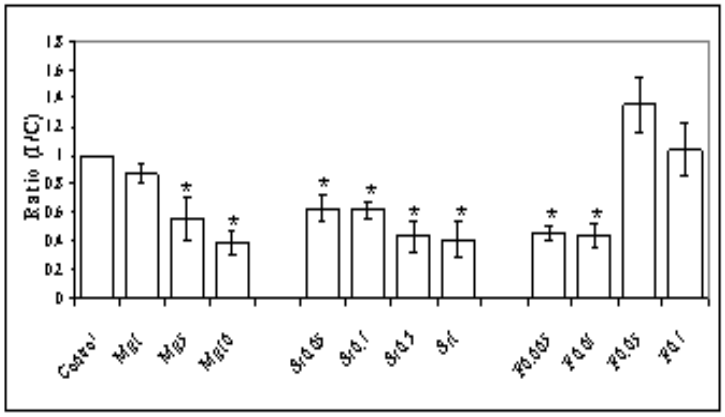


Fig.1 Ratio (I/C) of mineralized area in media with (I) and without (C) ions using Von Kossa staining after 14 days. Significant differences among groups using Kruskal-Wallis test: $P < 0.05$. Each bar represents means ± standard error. Comparison between the control group and each group were made separately using the Mann-Witney nonparametric U-tests. *$P < 0.05$

cells were detached in media containing 5 mM and 10 mM Mg. Cells in media containing 0.05 mM and 0.1 mM Sr also displayed increased number of nodules (0.05 mM < 0.1 mM), but less than the control group, and cells in media containing 0.3 mM and 1 mM Sr formed less number of nodules. Cells in media containing 0.05 mM and 0.1 mM F formed less nodules than those in the control group, while cells in media containing 0.05 mM and 0.1 mM F formed more nodules or deposited on the bottom of the wells.

*Morphological features of SEM*

The morphological features of the cells in control and in media containing Mg, Sr, or F ions are shown in Fig.3. MDPC-23 (odontoblast-like) cells after 1 day: cells in Mg- and F-treated

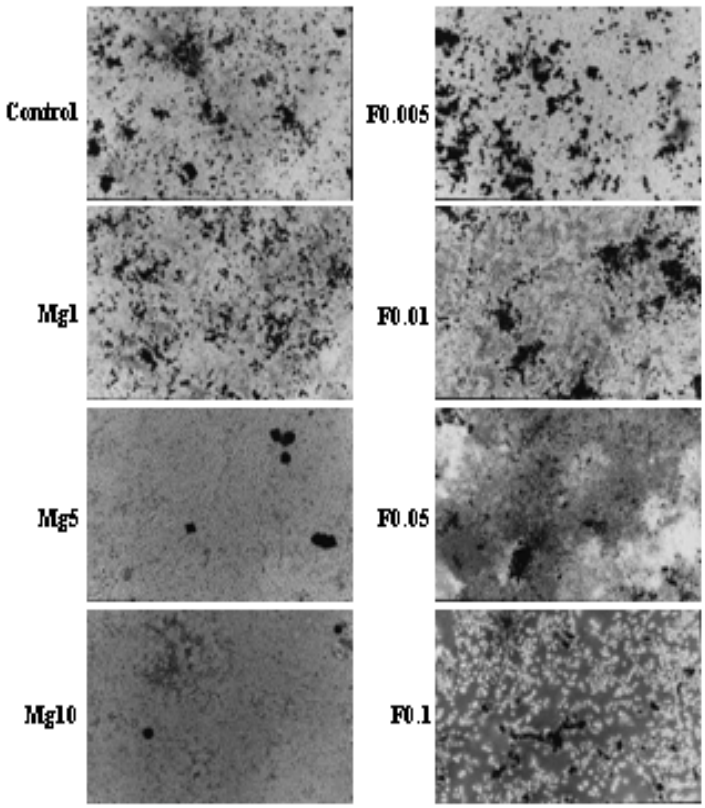


Fig.2 Results after Von Kossa Staining: After 14 days, black staining indicates mineralization.

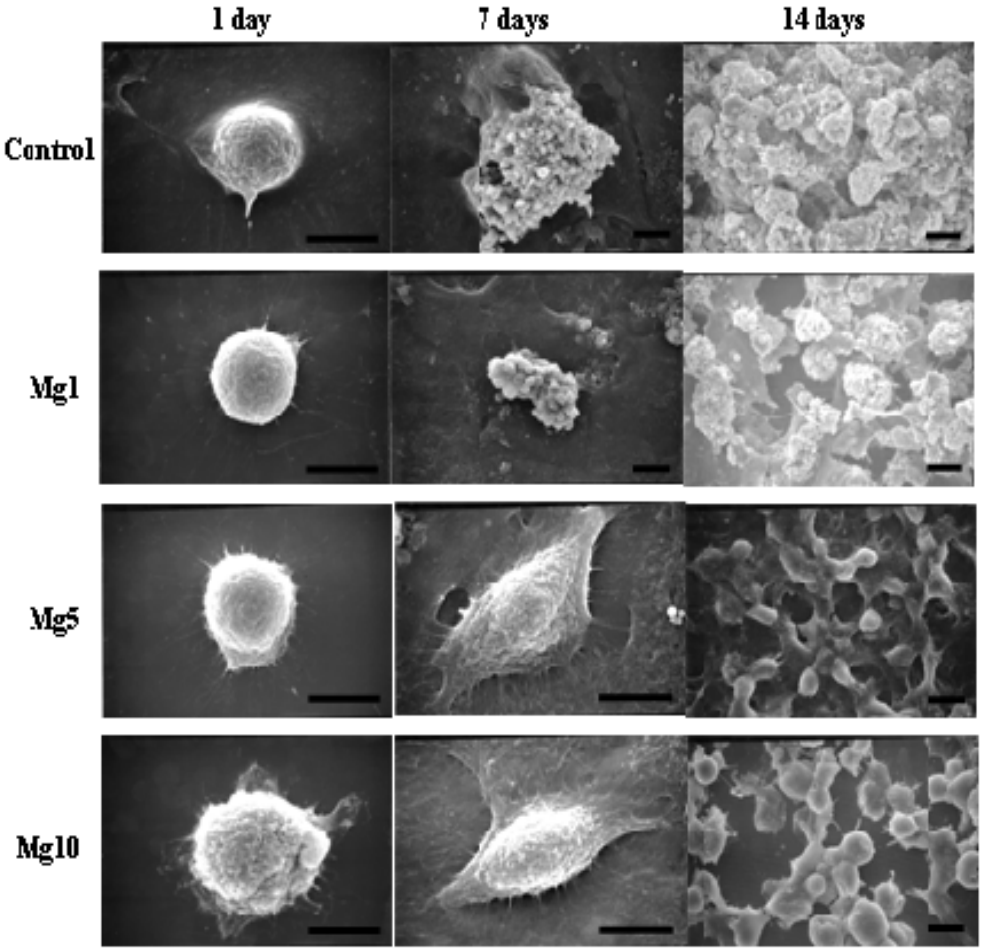


Fig.3(A). Scanning electron microscopy (SEM) micrographs showing the morphology of odontoblast-like cells (MDPC-23) in culture media containing: (A) Mg ions (1, 5, 10 mM) compared to control,

media displayed more filopodia than the control group. The abundance of filopodia was inversely related to the F concentration: the higher the concentration, the less number of filopodia observed.

*MDPC-23 cells in Mg-containing media after 7 days (Fig. 3A*) cells in media with 1 mM Mg formed smaller sized mineralized nodules as compared to the control group. Cells in media containing 5 mM and 10 mM Mg changed their shapes from round to oval and did not form mineralized granules on cell surface.

*MDPC-23 cells in Mg-containing media after 14 days (Fig. 3A)*

cells in media with 1 mM Mg formed numerous nodules, but the abundance of mineralized granules was less than those of the control group. Cells in media containing 5 mM and 10 mM Mg remained without any mineralized granules, and these cell surfaces became smoother, and the lamellipodia decreased as the Mg concentration increased.

*MDPC-23 cells in Sr-containing media after 7 days (Fig. 3B)*: cells also formed smaller sized mineralized nodules compared to those in the control. The surface of cells in media treated with 1 mM Sr appeared smoother compared to those in the control and to those in media treated with 0.1 mM and 0.3 mM Sr.

*MDPC-23 cells in Sr-containing media after 14 days (Fig. 3B)*

the number of mineralized granules on the cell surfaces decreased as the Sr ion concentration in media increased.

*MDPC-23 cells in F-containing media after 7 days (Fig. 3C*)

cells were arranged in clusters. Mineralized granules were not observed. In media with 0.1 mM F, mineralized granules were observed on lamellipodia and on the plate surfaces.

*MDPC-23 cells in F-containing media after 14 days (Fig. 3C)*

the number of mineralized granules on surfaces of cells in F-containing media was less than those in control and decreased

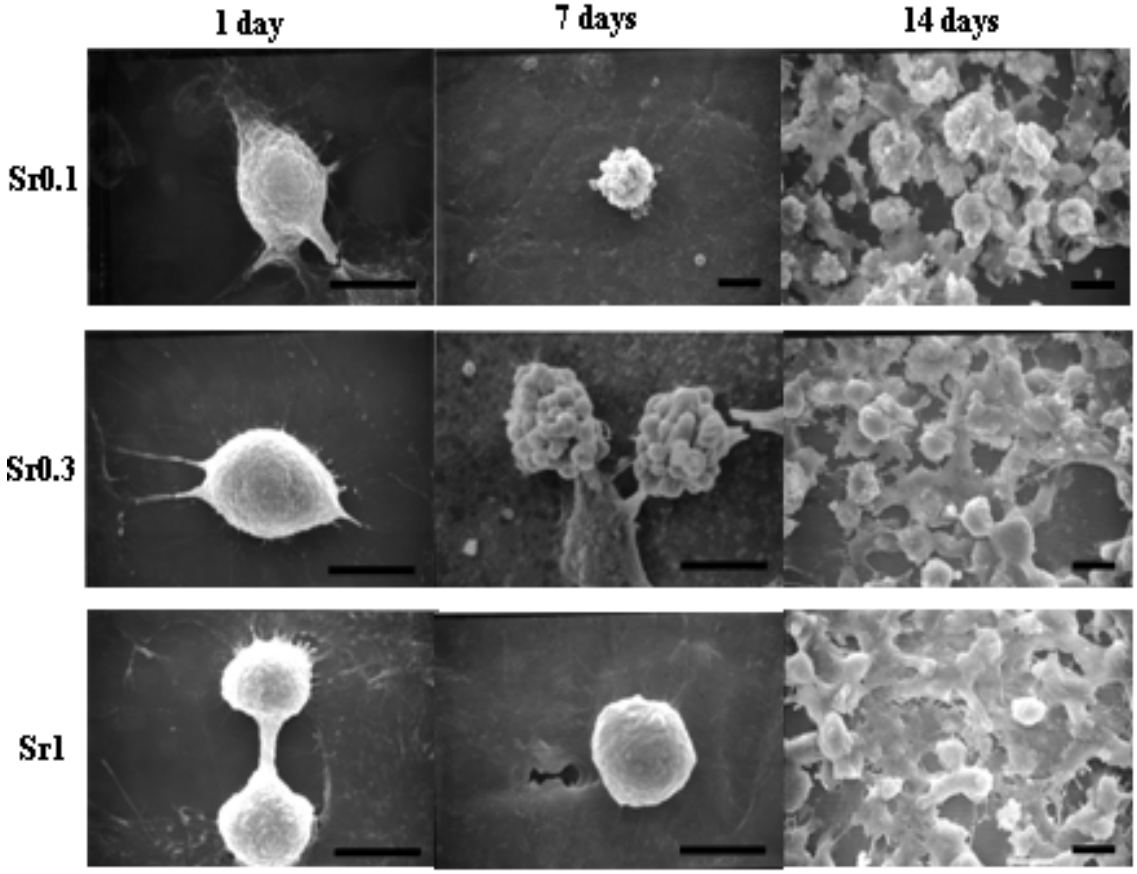


Fig.3 (B). Scanning electron microscopy (SEM) micrographs showing the morphology of odontoblast-like cells (MDPC-23) in culture media containing: (B) Sr ions (0.1, 0.3 and 1 mM) compared to control.

with increasing F ion concentration. Mineralized granules were also observed on the plate surfaces.

*Morphological features of TEM*

In the control group (Fig.4A,B) after 14 days, MDPC23 cells have the feature of extending a cellular process and polarity. There are lots of collagen fibers and nodules around the cell.

In 1mM Mg (Fig.4C), the cells showed a tendency to loose their cell polarities. The cell shape showed irregular process. Vacuolations of the rough endoplasmic reticulum were seen in the cytoplasm. On the other hand, collagen fibers and nodules were decreased.

In 10mM Mg (Fig.4D), many vacuoles were observed in the process as well as in the cytoplasm. The cellular membranes were disintegrated. No collagen fibers and nodules were produced.

In 1mM Sr (Fig.4E), lots of microvilli can be observed. The rough endoplasmic reticulum have little vacuole. No collagen fibers were produced.

In 0.1mM F (Fig.4F), the cell organelles disappeared. The cells have various size of intracytoplasmic vacuoles. The cells produced microvilli.

**Discussion**

This preliminary study showed that high Mg concentration in the cell culture in media had a toxic effect on the odontoblastic cells and thus prevented the formation of extracellular matrix. This result is consistent with a similar effect of Mg on bone cells [27]. Furthermore, although Mg did not affect the proliferation of MDPC-23 cells directly, a dose dependent effect on the mineralization was indicated by Von Kossa staining and SEM. In media containing 5 mM and 10 mM Mg, MDPC-23 cells tended to detach easily and lamellipodia were decreased. It seemed that Mg affected the cell structure and decreased their abilities to adhere. According to the observation on TEM, the nodules were less dose-dependent. This result was consistent with the results of Von Kossa staining and SEM. Interestingly, the observation on SEM was not so different between control and 1mM Mg, otherwise the observation of 1mM Mg showed deformity compared to control. This result was based on the assumption that cell deformity started from intracellular to extracellular.

Results of this study suggest that Sr ions do not affect the proliferation and the structure of MDPC-23 cells. However Sr

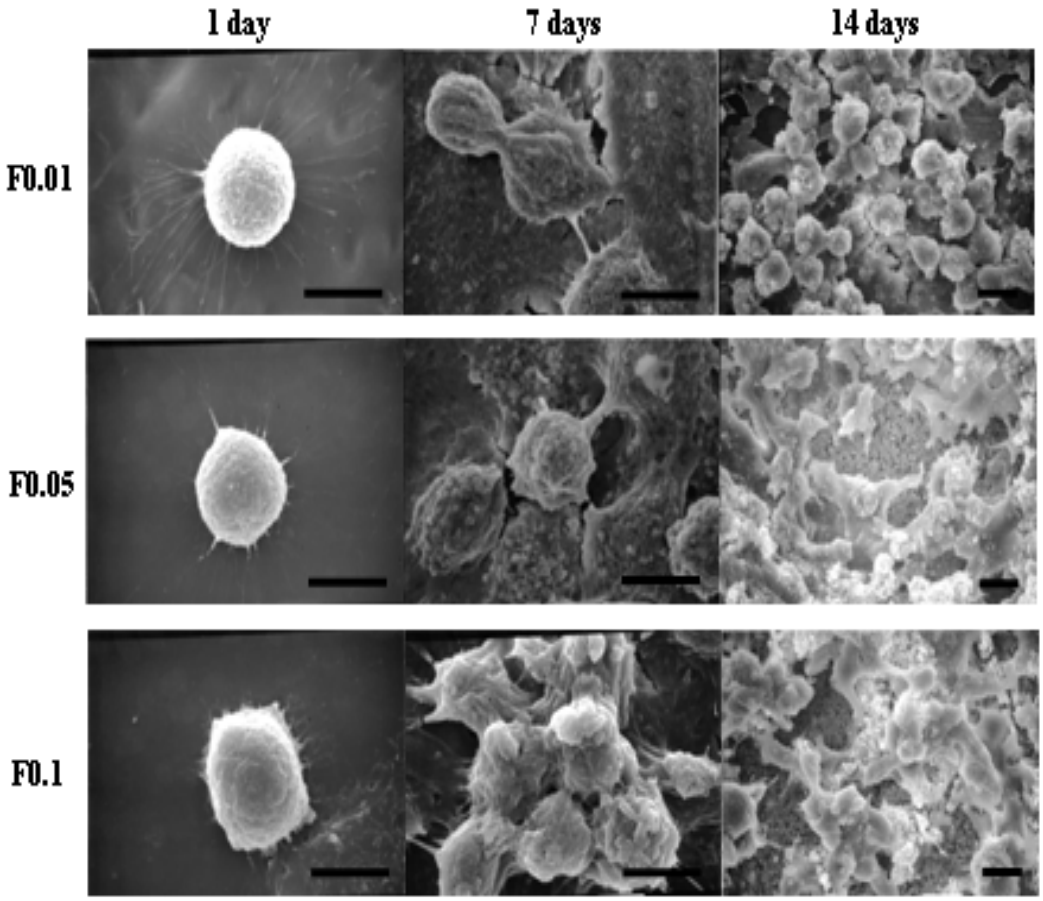


Fig.3 (C). Scanning electron microscopy (SEM) micrographs showing the morphology of odontoblast-like cells (MDPC-23) in culture media containing: (C) F ions (0.01, 0.05, 0.1mM) compared to control after 1 day, 7 days, and 14 days. Precipitation on the bottom of the well (arrows) was observed. Bar showed 10 mm.

had a dose dependent effect on mineralization. MDPC23 cells of higher concentration of Sr groups were more deformed compared to control group by TEM observation. This study showed that mineralization was promoted at low F concentration in a dose-dependent manner. However inhibitory effect of F on proliferation of MDPC-23 cells at higher concentration was observed. According to the results of Von Kossa staining and SEM analysis, cells in media containing high F concentration produced more nodules than the control group, but the mineralized granules were observed only on the plate and not on the cell surfaces. It is possible that the presence of mineralized granules on the plate may not have been produced by the cells. The cell shape by TEM observation was changed in high F concentration.

Odontoblast-like cells have not been extensively studied compared with osteoblast-like cells. Results from this preliminary study indicated that Mg, Sr, or F ions affect the activities (proliferation and mineralization) of odontoblast-like cells and these effects depend on their concentration levels in the environment.

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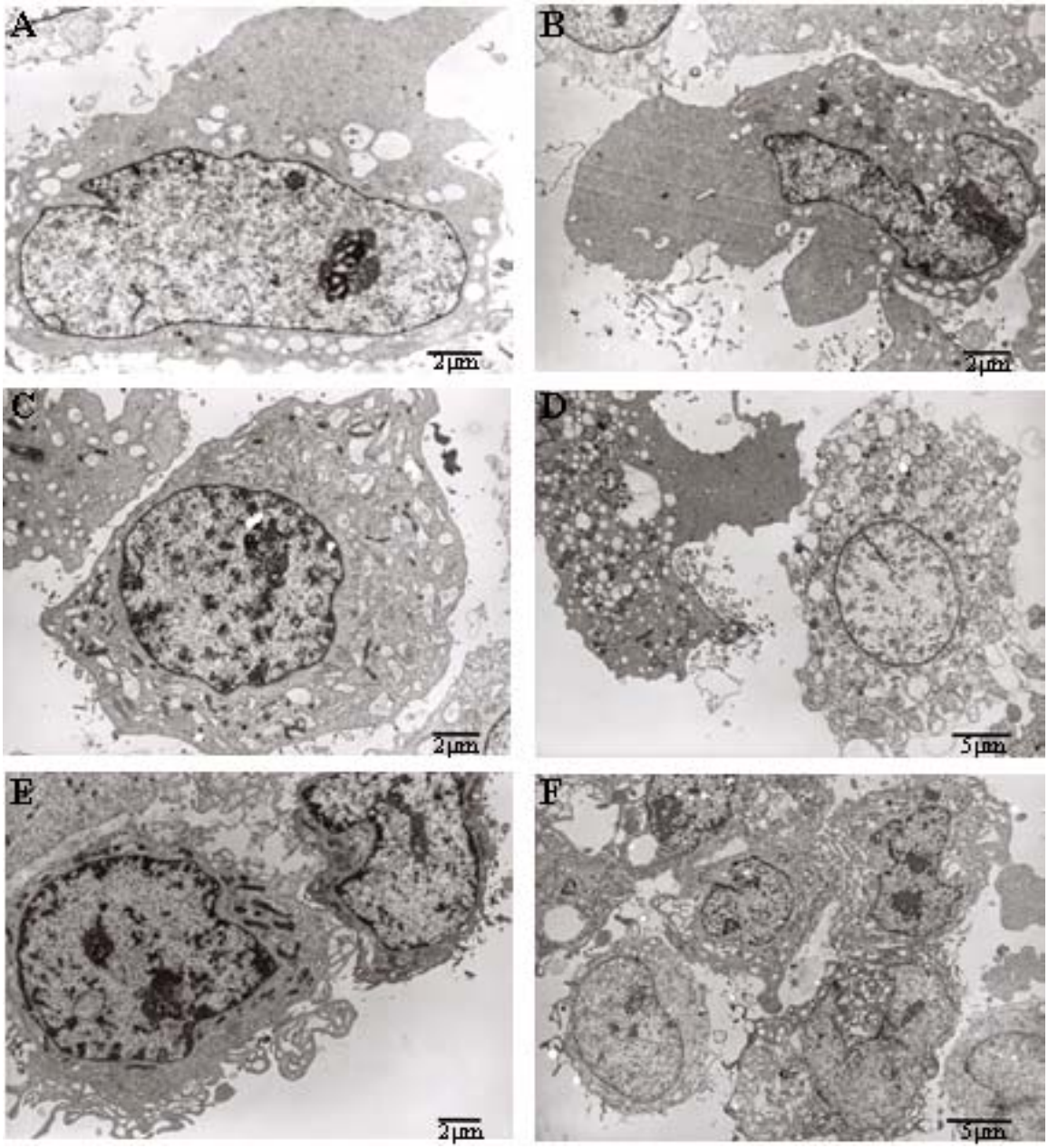


Fig.4 Transmission electron microscopy (TEM) micrographs showing the morphology of MDPC-23 cells in culture media containing: (A,B) control, (C) 1mM Mg ions, (D) 10mM Mg ions, (E) 1mM Sr ions, (F) 0.1mM F ions after 14 days.

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