Silver diamine fluoride: A stem cell cytotoxicity and dentin growth factor release in-vitro study

M.M. Khalil[1*], A. Bashir[1,2*], Y. Monjazebi[1], M. Shah[1], A. R. Tawfik[1], I. Hussein[1], M. Jamal[1]

[1]*Hamdan Bin Mohamed College of Dental Medicine, Mohammed Bin Rashid University of Medicine, and Health Sciences, Dubai Health Care City, Dubai, United Arab Emirates*
[2]*Fatima College of Health sciences, Sheikh Maktoum bin Rashid Raod, Ajman, United Arab Emirates*

** The authors equally contributed to this work*

e-mail: mohamed.jamal@mbru.ac.ae

DOI 10.23804/ejpd.2023.1928

Abstract

Aim To investigate the effect of 38% SDF and its serial dilutions on the Stem cells from Human Exfoliated Deciduous teeth (SHED) and its ability to release growth factors from deciduous dentin.

Methodology The viability of SHED post-exposure to 38%, 3.8%, 0.38%, 0.038%, and 0.0038% SDF were assessed at 2, 5, and 7 days using the CyQuant assay, and results were validated using the MTT assay. The osteogenic differentiation of the cells was also investigated post-exposure to 0.0038% SDF. The release of the growth factors; TGF-β1, FGF-b, BMP-2, and VEGF from deciduous dentin discs exposed to 38% SDF, 0.0038% SDF, Ca(OH)2, MTA, and 17% EDTA were examined using ELISA. Statistical analysis was performed using means and standard deviations ($p < 0.05$). Two-way ANOVA compared the means of more than two groups with Tukey's multiple comparison test. The unpaired t-test was also used to compare the differences between the two data sets.

Results In the presence of SDF concentrations of 38%, 3.8%, and 0.38%, SHED were not viable, 0.038% SDF reduced the cell viability, and 0.0038% SDF enhanced the cellular proliferation at day 5 of exposure. The growth factors released from deciduous dentin in response to 38% SDF and 0.0038% SDF were not statistically significantly different for TGF-β1, BMP-2, and FGF. While VEGF showed a significantly higher release with 0.0038% SDF.

Conclusions 38% SDF released dentinogenic growth factors from dentin discs, potentially explaining its role in reactionary dentinogenesis. Moreover, 0.0038% SDF resulted in a non-cytotoxic concentration that enhanced cellular proliferation and released bioactive molecules from dentin comparable to the 38% concentration. After further investigations, the 0.0038% dilution of SDF could present itself as a clinical concentration.

KEYWORDS silver diamine fluoride, dentin growth factor, in-vitro study

Introduction

Silver diamine fluoride (SDF) is an inexpensive topical solution used in Japan for over 50 years for caries treatment among patients of different ages [Horst, 2016]. The clear, odorless solution consists of antimicrobial silver, remineralizing fluoride, and stabilizing ammonia at an alkaline pH of 10 [Horst, 2016; Kim et al., 2021]. Recently, SDF underwent a renaissance worldwide, especially after the Food and Drug Administration (FDA) approved its use as a desensitizing agent in 2014. In 2020, the American Dental Association (ADA) supported its use for caries management [Zaeneldin et al., 2022].

SDF has been clinically successful in arresting and preventing caries and treating dentin hypersensitivity [Horst, 2016; Kim et al., 2021; Mei et al., 2013]. Applying the topical medicament to dentin forms a squamous layer, increasing its resistance to acid dissolution and enzymatic digestion and partially blocking the tubules resulting in reduced hypersensitivity [Horst, 2016; Shah S et al., 2014]. The effectiveness of SDF in controlling caries has resulted in its use on lesions of different depths. Applying the solution in deep lesions raises concerns due to minimal data on its cytotoxicity and effect on the dentin-pulp complex [Kim et al., 2021; Srisomboon et al., 2022; Zaeneldin et al., 2022].It is known that SDF can penetrate dentin and cause the death of pulp cells [Hu S et al., 2022]. Despite that, recent animal and human studies have claimed that SDF has the potential to heal reversibly inflamed pulp tissue and induce new dentin formation [Bimstein and Damm, 2018; Korwar et al., 2015; Vanegas et al., 2014].

Studies on pulp capping materials have proven that they can stimulate pulp and dentin regeneration through material-directed repair (reactionary dentinogenesis); a possible cause is the release of growth factors when the materials interact with dentin (Figure 1)[Graham et al., 2006a; Sadaghiani et al., 2016; Smith, 2003; Tomson et al., 2017]. In addition, recent studies have suggested that diluted SDF can be successful clinically as an indirect pulp capping material in primary teeth [Shafi et al., 2022].

However, supporting the extension of the use of SDF as a deep cavity liner and indirect pulp capping agent would require further investigation into its exact mechanism of action on an application to dentin and its effect on vital pulp and the mesenchymal cellular component. Therefore, our study aimed to investigate the effect of 38% SDF and its serial dilutions on the stem cells from human exfoliated deciduous teeth (SHED) and its ability to release growth factors from human dentin.

Material and methods

Sample collection

The cell and tooth samples were freshly collected from consenting patients (5–12 years old) after obtaining institutional review board (IRB) approval. All human subjects' legal, ethical, and welfare rights were respected. Immediately after extraction, the teeth were kept in collection media (α-modification of Eagle's Medium (α-MEM) (GIBCO) with 1% Primocin® (InvivoGen®) at 4 oC.

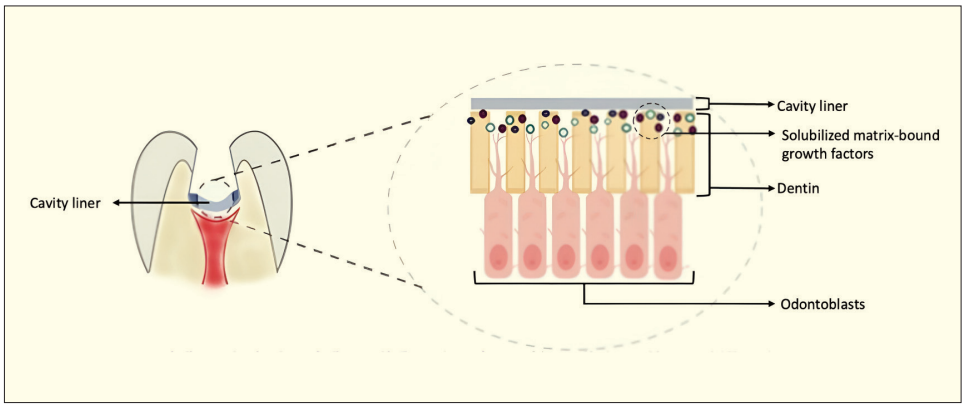

FIG. 1 A schematic diagram showing cavity liners and indirect pulp capping materials placed in deep cavities may solubilize and expose matrix-bound growth factors. Upon release, the growth factors may move toward the odontoblasts and the cavity surface.

Isolation and culture of SHED

The pulp was extracted from intact and caries-free deciduous teeth, minced, and digested in a buffer (3 mg/mL collagenase type I and 4 mg/mL dispase (Roche®)) for 30 min. Isolated cells were then plated in culture dishes containing mesenchymal cell (MSC) media (α-MEM, 10% Fetal bovine serum (FBS) (Gibco®), 1 mM l-alanyl-l-glutamine and 1% Primocin).

The cells were incubated and at 70% confluence, the cells were trypsinized and passaged. The experiments used cells between passages 3 and 5, and three cell lines created a biological triplicate [Gronthos et al., 2000; Jamal et al., 2015].

Cell viability and cytotoxicity assays

The CyQuant cell proliferation assay kit (Invitrogen®) tested the cytotoxicity of 38% SDF and its dilutions 3.8%, 0.38%, 0.038%, and 0.0038% on SHED. In 96-well plates, ~1,000 cells were grown for 24 hrs then the media was replaced with media supplemented with 38% SDF and its serial dilutions. The cell proliferation was assessed at 2, 5, and 7 days, where they were incubated with CyQuant dye according to the manufacturer's instructions. The fluorescence was measured and quantified using a microplate reader (~480 nm excitation, ~520 nm emission) [Horton et al., 1999; Quent et al., 2010].

The study further validated the results with MTT (methyl thiazolyl diphenyl-tetrazolium bromide) assay on the SDF dilutions that showed the lowest cytotoxicity. Cells were seeded (~10,000 cells) in 96-well plates for 24 hr, then media was replaced with media supplemented with SDF dilutions. The viability was assessed on days 2 and 5, where they were incubated with MTT solution for 4 hours, then it was replaced with dimethyl sulfoxide (DMSO). The absorbance was measured (~560 nm) using a microplate reader [Liu et al., 1997; Rai et al., 2018] The viability assays were performed in biological and technical triplicates.

Cell differentiation assay

The osteogenic differentiation assay investigated non-cytotoxic SDF dilution. Cells (~10,000) were seeded in 96-well plates and 24 hrs later the media was replaced with media supplemented with 0.0038% SDF. Differentiation was initiated at 90% confluency by incubating the cells in mineralisation differentiation media(MDM) (STEMPRO® Osteogenesis Differentiation Kit-Gibco). After ten days cells were fixed with 4% paraformaldehyde and stained with 2% Alizarin Red (AR) (Lifeline cell technology®) solution.

A light microscope visualized positively stained calcifications and quantified them using image processing software (ImageJ®, NIH, USA). The experiment was performed on technical and biological triplicates [Kobayashi et al., 2020].

Dentin supernatant extraction

The dentin supernatant extraction procedure is based on published protocols [Aksel et al., 2020; Atesci et al., 2020]. Briefly, a total of 15 intact and caries-free human deciduous teeth were made into ≈ 300 +/- 20 mg dentin discs. The discs were divided into 5 groups according to the treatment; 38% SDF, 0.0038% SDF, calcium hydroxide (Ca(OH)2), mineral trioxide aggregate (MTA), and 17% Ethylenediaminetetraacetic acid (EDTA). The 38%, 0.0038% SDF, and 17% EDTA were placed on one dentin surface using a micro brush and re-applied 5 min later. The MTA and Ca(OH)2 were prepared according to the manufacturer's instructions and applied on one dentin surface. After 15 minutes, all groups were placed with the material side facing up in a 24-well plate containing PBS with 5mM/ml phenylmethanesulfonylfluoride (PMSF). The 24-well plate was placed in a shaking incubator at 37 °C for 24 hrs, and the supernatant was collected separately and frozen. The process was repeated with fresh PBS with PMSF using the same dentin discs. The supernatants from the first and second 24-hrs were pooled together.

Sandwich ELISA (enzyme-linked immunosorbent assay)

The supernatant was tested for the four growth factors: Human Transforming Growth Factor Beta 1 (TGF-β1), human Fibroblast Growth Factor basic (FGF-b), Human Bone Morphogenic Protein 2 (BMP-2), and Human Vascular Endothelial Growth Factor (VEGF).

The solid-phase sandwich ELISA kits (Thermofisher scientific®) were used according to the manufacturer's instructions to determine the growth factor concentrations. The absorbance was measured (450 nm) using a microplate reader (Hidex Sense®), and concentration was calculated as per kit protocol.

Statistical analysis of the data

Statistical analysis for the different experiments was performed using SPSS for Windows software; $p < 0.05$ was considered significant. Means and standard deviations were calculated from numerical data and presented in figures. Two-way ANOVA compared the means of more than two groups with Tukey's multiple comparison test. The unpaired t-test was also used to compare the differences between the two data sets.

Results

1.Assessing cell proliferation and differentiation

a. CyQuant assay (Figure 2a)

The cells cultured with 38%, 3.8%, and 0.38% SDF showed no proliferation. The 0.038% SDF showed significantly lower proliferation than the control on days 2 and 7. On the other hand, 0.0038% SDF showed significantly higher proliferation

than the control on day 5.

b. MTT assay (Figure 2b)

The SHED remained viable after 2 and 5 days of culturing with 0.038% SDF and 0.0038% SDF and viability was not significantly different from the control.

c. Osteogenic differentiation (Figure 2c)

After differentiating the cells, calcification nodules were detected by AR stains in both tested groups. There was no significant difference in the number of nodules in both 0.0038% SDF and the control.

2.Assessing growth factor release from dentin supernatant (Fig.3)

The deciduous dentin supernatant from the different groups (38% SDF, 0.0038% SDF, Ca(OH)2, MTA, and 17% EDTA) was quantified for the four growth factors; TGF-β1, FGF-b, BMP-2, and VEGF over 48 hrs. The results were presented as the mean concentration of total quantified growth factors.

The only dentin discs that released TGF-β1 were those treated with 38% SDF and 17% EDTA. The dentin discs with 38% SDF released significantly lower concentrations than those with 17% EDTA ($p < 0.0001$).

All the treated dentin discs released BMP-2 at different concentrations. The discs with 17% EDTA showed the highest growth factor release, and there is no significant difference between the concentration of BMP-2 released from discs with 38% and 0.0038% SDF.

The highest amount of FGF was released from the dentin discs treated with MTA. The MTA's liberation of FGF was comparable to that of discs treated with Ca(OH)2. There was no significant difference between the concentration of FGF released from discs with 38% and 0.0038% SDF.

All the treated discs released VEGF; the 0.0038% SDF significantly ($p < 0.05$) showed the highest release among the tested materials, except when compared with 17% EDTA, there was no significant difference. The release of VEGF from the dentin discs treated with 0.0038% SDF was significantly higher than those treated with 38% SDF ($p < 0.05$).

Discussion

The use of SDF in children is increasing as it is recommended for caries control by influential organisations such as the American Academy of Pediatric Dentistry (AAPD) and the ADA [American Academy of Pediatric Dentistry, 2021]. There have even been suggestions that "diluted" SDF (1:10) could be used as an indirect pulp capping agent in deep carious primary teeth lesion in place of Ca(OH)2 [Shafi et al., 2022]. This in-vitro study focuses on the cellular response and biological effect of 38% SDF and its different dilutions. The evidence supporting its use has mainly focused on its impact on dental hard tissues.

Very limited data on its biological effect is available despite its frequent application in moderate to deep carious lesions [Srisomboon et al., 2022]. This study found that the clinical concentration of SDF (38%) was cytotoxic when directly applied to cells. On the other hand, the dilution of 0.0038% SDF was non-cytotoxic, enhanced cellular proliferation, and did not inhibit osteogenic dif-

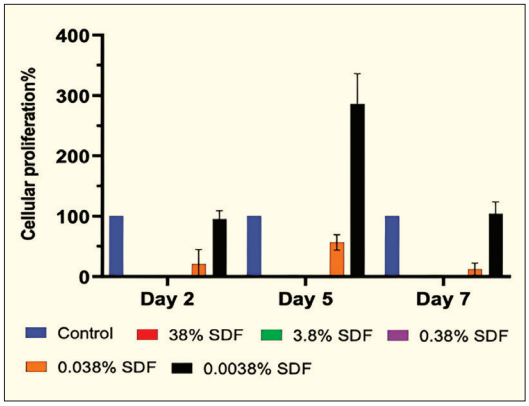

FIG. 2A The CyQuant assay indicates the viability of SHED grown in MSC media (control) and MSC media with 38%, 3.8%, 0.38%, 0.038%, and 0.0038% SDF after 2, 5, and 7 days.

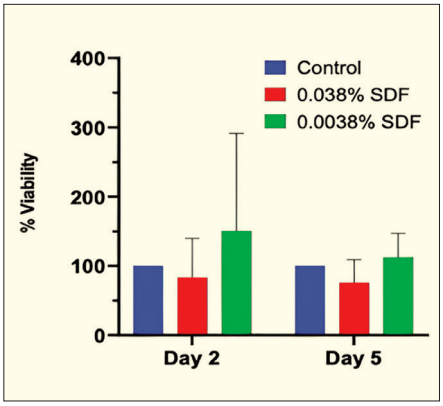

FIG. 2B The MTT assay expresses the percentage of viable SHED after being grown in MSC media (control) and MSC media with 0.038% SDF and 0.0038% SDF for 2 and 5 days.

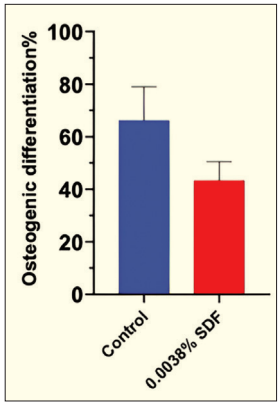

FIG. 2C The cell differentiation assay investigated the percentage of osteogenic differentiation of SHED grown in MSC media and 0.0038% SDF.

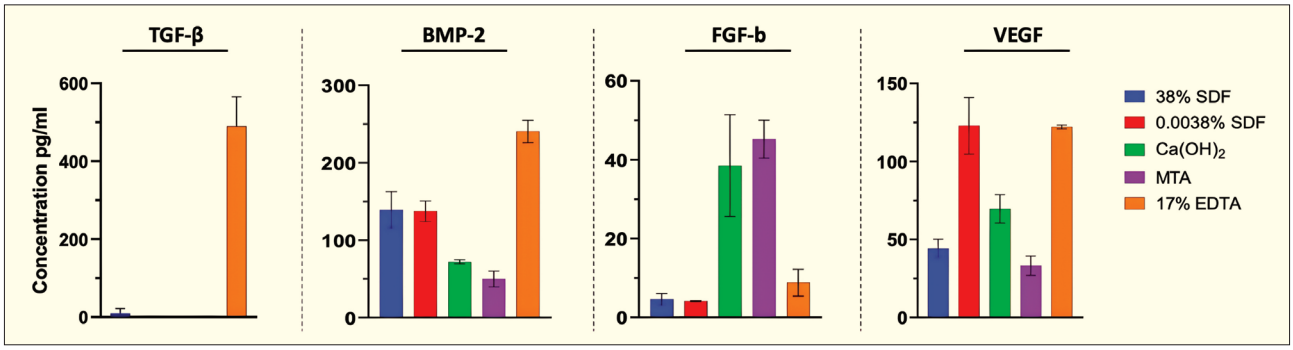

FIG. 3 ELISA assay showing the release of different growth factors (TGF-β1, BMP-2, FGF-b, and VEGF) from deciduous dentin discs supernatant over 48 hrs of exposure to different agents.

ferentiation. This study also investigated different growth factors related to the repair and regeneration of the dentin pulp complex. The clinical (38%) and non-cytotoxic (0.0038%) concentrations of SDF released comparable bioactive molecules from deciduous dentin. These findings provide additional valuable information regarding the safety of extending the use of 38% SDF to deeper lesions closer to the pulp.

On evaluating the cytotoxicity of 38% SDF and its dilutions, it was found that 38%, 3.8% and 0.38% SDF adversely affected cellular viability. This could be due to the high concentrations of silver and fluoride ions found in 38%, 3.8% and 0.38% SDF solutions that may have been toxic to the cells on direct contact. These ions, in combination, have a synergistic effect and may enhance oxidative stresses, triggering inflammatory reactions and leading to cell death [Inkielewicz-Stepniak et al., 2014; Kim et al., 2021].

On the contrary, the more diluted solutions, 0.038%, and 0.0038% proved less cytotoxic. The cells grown in media supplemented with 0.0038% SDF at all different time points and in both viability assays', showed higher proliferation and viability than those grown in non-supplemented MSC media. The results of this study conclude that low silver and fluoride ions concentrations could enhance cellular proliferation. Similarly, a study on different concentrations of silver nanoparticles on sheep primary mesothelial cells found that they induced the proliferation of the cells at low concentrations [Arsenopoulou ZV et al., 2018]. Additionally, the effect of low fluoride concentrations on proliferation, differentiation, and extracellular-matrix synthesis in normal human dental pulp cells in-vivo was found to stimulate the proliferation and differentiation of dental pulp cells and increase dentin regeneration [Nakade et al., 1999].

Conflicting with our results, 38% SDF was diluted to 0.038% and 0.0038% in another study and applied directly on rat pulpal cells and evaluated at 6 and 24 hrs; the reported results were unfavourable. The groups treated with SDF showed lower viability and metabolic activity than the control group, suggesting that SDF has an inhibitory effect on pulp cells even when diluted. The morphology of the cells also changed; the SDF-treated groups exhibited a contracted, spherical shape with increased intercellular spaces [Kim et al., 2021]. Possible causes for the differences in the viability between our study and this study could be the type of cells used and the time points of the evaluation.

To further evaluate the non-cytotoxic concentration, this study investigated whether 0.0038% SDF would inhibit SHED's ability to differentiate towards osteogenic lineage when stimulated. It was found that the cells could still differentiate and produce calcification nodules comparable to those grown in MSC media. These results indicated that 0.0038% SDF did not interfere with the postnatal stem cells' ability to differentiate, maintaining a unique property of stem cells that allows them to regenerate and replace damaged cells that are fully functional [Hoang et al., 2022; Jamal et al., 2011; Wang et al., 2018].

The growth factors solubilised from dentin by the clinical and non-cytotoxic concentrations of SDF were investigated and compared to those released from pulp-capping materials. As far as the authors know, no other studies have reported the growth factors released from deciduous dentin.

Previous studies have demonstrated that EDTA, Ca(OH)2, and MTA solubilise and mobilise a range of bioactive proteins from the permanent dentin matrix [Kucukkaya Eren et al., 2021; Smith, 2003; Tomson et al., 2017; Wattanapakkavong and Srisuwan, 2019]. These molecules are sequestrated in the dentin during dentinogenesis and remain protected until the dentin contacts acids or certain materials. On release; the bioactive molecules play a major role in signaling different aspects of tissue regeneration [Tomson et al., 2017; Wattanapakkavong and Srisuwan, 2019].

This study investigated TGF-β1, FGF-b, BMP-2, and VEGF released from deciduous dentin. This study aimed to replicate the clinical scenario and find out the bioactivity of the tested materials. These specific growth factors are significant in their ability to signal the repair and regeneration of the pulp-dentin complex [Atesci et al., 2020]. TGF-β1 and BMP-2 stimulate odontoblast-like cell differentiation contributing to regeneration [Graham et al., 2006b; Liu et al., 2022; Smith, 2003]. While VEGF signals lymphangiogenesis, vasculogenesis, and angiogenesis, this is important to promote blood vessel formation in the injured dental pulp. On injury, VEGF and FGF-2 signal the recruitment, proliferation, migration, and differentiation of progenitor cells in the pulp, initiating the repair [Smith, 2003].

Our results demonstrated that each material applied to dentin liberated different amounts of tested growth factors. Differences in the released bioactive molecules from the materials are probably because, in their application to dentin, they release different ions and produce ionic dissolution products that create an environment that promotes the release of other sequestrated bioactive molecules [Tomson et al., 2017].

Despite differences between the bioactive molecular profiles of the tested materials, the 17% EDTA and MTA released the highest amounts of proteins. Remarkably, the 38% SDF and the 0.0038% SDF were similar in their growth factor release from deciduous dentin, with no significant difference for the TGF-β1, BMP-2, and FGF. While for VEGF, the deciduous dentin discs released a higher growth factor concentration with 0.0038% than the 38% concentration. Therefore, the bioactive molecular profile of 38% and 0.0038% SDF is comparable with a significantly higher release with the diluted solution in VEGF.

The analysis of dentin components released by Ca(OH)2, grey and white MTA using ELISA analysis, shows the release of a rich cocktail of growth factors. MTA liberated a broader profile of growth factors compared to Ca(OH)2 due to the material's capacity to extract dentin matrix components resulting in differences in their biocompatibility and dentin bridge formation [Tomson et al., 2017, 2007].

Our findings suggest that application of 0.0038% SDF to deciduous dentin can also trigger the same repair and regeneration response from the dentin and pulp found with the 38% solution in previous studies [Bimstein and Damm, 2018; Korwar et al., 2015; Vanegas et al., 2014].

Using this concentration in deep cavities or directly on the pulp can be considered a safe alternative to the 38% solution. In the future, it could provide an alternative to pulp-capping materials in the market that are expensive and difficult to manipulate and apply [Gurcan and Seymen, 2019]. While a diluted SDF was found to be a successful indirect pulp capping agent in primary teeth, this warrants further research as the concentration used clinically (1:10 dilution) was much higher than that used in this research project (1:10000), where favorable biological outcomes were observed [Shafi et al., 2022].

Although 0.0038% SDF holds promise, this study has several limitations that would require further investigation. The solution's silver and fluoride ions will be reduced by diluting SDF 10,000 times. This ion reduction will most likely affect the favorable properties that rely on the presence of these ions in high concentrations.

Therefore, 0.0038% SDF should be further assessed to understand how diluting the solution has affected its remineralisation, anti-cariogenic and antimicrobial properties [Shafi et al., 2022].

Conclusions

This study demonstrated the cytotoxicity of the clinical concentration of SDF (38%) and its dilutions and identified a non-cytotoxic concentration obtained by diluting 38% SDF 10,000 times. The non-cytotoxic solution (0.0038%) enhanced the cellular proliferation of SHED significantly in culture after five days.

The growth factor liberation of 0.0038% SDF from dentin discs occurred and was comparable to the bioactive molecular profile of the 38% solution. This study indicated that 38% SDF is cytotoxic on cellular contact, and careful application in deep cavities is recommended. This study raises the possibility of using SDF in a diluted form (0.0038%) in deep cavities and direct contact with the pulp cells for pulp-dentin repair and regeneration.

Acknowledgment

This study was supported by an internal grant from MBRU.

Conflict of interest

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

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