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RELATION BETWEEN DENTIN SCAFFOLD/EDTA EXPOSURE TIME DURATION AND THE ENDOGENOUS BONE MORPHOGENIC PROTEIN 7 RELEASE

Ibrahim Abdalmonem Mohammed*, Rania Mohmoud Elbackly** *and* Amany El-Said Badr***

ABSTRACT

Aim: to demonstrate the amount of endogenous bone morphogenic protein 7 release under the effect of different times of EDTA exposure.

Material and methods: Dentin scaffolds were randomly assigned to the following groups (n = 4). Group 1 received 17 % EDTA for 5 minutes; Group 2 received 17 % EDTA for 10 minutes; Group 3 received 17 % EDTA for 15 minutes and distilled water was used as a control in Group 4. After each treatment, ELISA was used to quantify the amount of BMP7 liberated from the dentin scaffolds after pre-treatment with EDTA for 5, 10, and 15 minutes. The scaffolds were analyzed under SEM to investigate dental pulp stem cell (DPSCs) attachment, morphology, the opening of dentinal tubules, and smear layer removal in the different groups.

Results: Dentin scaffolds conditioned with 17% EDTA for 10 and 15 min showed the maximum degrees of enlargement in dentinal tubules and smear layer removal, and revealed higher DPSCs attachment than the control group ($p < 0.001$). Moreover, dentin treated with 17% EDTA for 10 min manifested higher concentrations of BMP7 release than the unconditioned dentin ($p < 0.05$). Using scanning electron microscopy, Dentin scaffold treated with EDTA for 10 min presented the highest degrees of enlargement in dentinal tubules and smear layer removal than dentin treated with EDTA for 5 or 15 min.

Conclusion: maximum endogenous bone morphogenic protein 7 release was obtained when dentin scaffolds were treated with 17% EDTA for a period of 10 minutes.

KEY WORD: dental pulp stem cells, dentin scaffold, EDTA, BMP-7.

* Msc Student, Endodontics Department, Faculty of Dentistry, Mansoura University, Mansoura, Egypt.
** Professor, Endodontic Department, Faculty of Dentistry, Alexandria University, Alexandria, Egypt.
*** Professor, Head of Department of Endodontic, Faculty of Dentistry, Mansoura University, Mansoura, Egypt

INTRODUCTION

In 2000, Gronthos et al.[1] discovered dental pulp stem cells and their capacity to differentiate into odontoblasts and generate dentin-pulp complex in animal models, which paved the path to dentin pulp regeneration and engineering in humans. Third molars are one of the most common sources of human dental pulps stem cells (DPSCs).[2] Dental pulp stem cells and morphogenic inductive signalling molecules are essential for dentin pulp complex regeneration. Various growth factors and inductive molecules have been identified to have a role in the regeneration and mineralization process, a critical step in dentin pulp complex regeneration and organization in in vitro and in-vivo models.[3]

The transforming growth factor-β (TGF-β) family, particularly Bone morphogenetic proteins (BMPs), are involved in many biological processes, mainly bone and tooth development.[4] For instance, during early tooth development in the initiation stage, Bone morphogenetic proteins 2, 4 &7 are expressed in dental epithelial and involved in the epithelia–mesenchymal crosstalk in tooth formation induction signalling pathways.[5]

Bone morphogenetic protein 7 (BMP-7) is among the growth factors implied in teeth development and regulation. Some studies depict that human recombinant BMP-7 stimulates reparative dentin formation upon applying it to freshly cut dentin. Moreover, BMP7 has been proven to induce osteodentin transformation of pulp tissue after direct implantation in in vivo animal models.[6] Therefore, the objective of the current study was to assess the release of endogenous BMP-7 from dentin scaffolds treated with EDTA with different exposure times. Additionally, the attachment of DPSCs to the treated dentin scaffolds was assessed as well as the morphological changes of the dentin surfaces following different treatments.

EDTA is one of the most commonly used chelating materials for conventional root canal treatment as it removes the smear layer[7], and enhances the release of different growth factors from the dentinal tubules as Bone morphogenetic proteins and vascular endothelial growth factors[8]. These factors play a vital role in enhancing revascularization and the calcification and formation of new dentine layers [9, 10] .

17% EDTA is also shown to enhance the proliferation and survival rate of Dental pulp stem cells compared to Sodium hypochlorite (NaoCl) as a chelating agent[11].

Moreover, dentin primed with 17% EDTA promotes cell adhesion, and this step is crucial for subsequent proliferation and differentiation of DPSCs to odontoblast in the canal [10]. So it is highly recommended when dealing with revascularization by over instrumentation bleeding to use EDTA before the procedures in order to provide the progenitor cells with the appropriate concentration of growth factors to provide the molecular signals needed for regeneration and survival [8, 9].

Although many studies have examined the role of EDTA concentration and addition of exogenous bone morphogenic proteins on stem cells differentiation, there are lack of research on the effect of EDTA exposure time on the indigenous release of BMP7, this will be illustrated in this study by several laboratory assays to demonstrate the amount of endogenous bone morphogenic protein 7 release under the effect of different times of EDTA exposure.

MATERIALS AND METHODS

This study was approved by the research ethics committee of the faculty of dentistry, Mansoura University (protocol ID# M08080120).

Dentin scaffold preparation:

Dentin scaffolds were generated from healthy third molars following informed written consent. The coronal dentin was sliced into 5x5 mm square discs with 1 mm thickness with a Cryostat

microtome and PBS irrigation. Ultraviolet rays (UV) were used to sterilize the dentin scaffolds, and then they were disinfected with 70% alcohol. Dentin slices were rinsed three times in PBS before being placed in sterile 6-well culture plates. Dental pulp stem cells (DPSCs) were obtained from LONZA (Basel, Switzerland). These PoieticsTM DPSCs were separated from adult third molars obtained following a donor's 'wisdom' teeth excision. These cells have extremely comparable phenotypic and functional properties to bone marrow-derived mesenchymal stem cells. DPSCs were thawed and subcultured according to Lonza's methods and growth media (DPSCsBulletKitTM Medium, PT-3005, Lonza). Upon 80% confluence, cells were trypsinized and DPSCs were subsequently seeded at a density of (1.5 *106 cells/scaffold) onto the dentin scaffolds for 7 days and grown in the previously mentioned supplemented media.

Groups:

The dentin scaffolds were randomly assigned to the groups (n = 4). Group 1 received 17 % EDTA for 5 minutes, while Group 2 received 17 % EDTA for 10 minutes, Group 3 received 17 % EDTA for 15 minutes. Distilled water was used as a control in Group 4. After each treatment, a PBS wash step was performed.

ELISA assay for BMP7 concentration released from dentin scaffolds:

QuantikineTM ELISA was used to quantify the amount of BMP7 liberated from the dentin scaffold after pre-treatment with EDTA for 5, 10, and 15 minutes. (R&D, USA, Cat. No. DBP700). Before starting the tests, all samples and buffers were allowed to reach room temperature and tests were performed according to manufacturer's instructions. Using an ELISA reader set at 450 nm, the optical density was detected within half an hour. The average duplicate results for standard, control, and sample were taken to generate a standard curve. The curve was generated between the points on the graph to produce a standard curve using the mean absorbance on the y-axis and the concentration on the x-axis.

Scanning electron microscopic (SEM) evaluation of cell attachment:

For SEM, dentin scaffolds were conditioned before the cells were cultured into one of the chelating agents' solutions (3 dentin scaffolds for each group): purified water (control group) (n=3), 17 % EDTA for 5 minutes (n=3), 17 % EDTA for 10 minutes (n=3), and 17 % EDTA for 15 minutes (n=3).This assay was used to investigate whether the chelating agent could remove the smear layer and reveal the dentinal tubules. The seeded pre-treated dentin scaffolds from each group were fixed by 2.5% glutaraldehyde at 4°C for two hours. Dentin scaffolds were dehydrated in an ethanol series, then dried with hexamethyl disilane to remove water from the scaffold. After coating with gold, the scaffolds were analysed under SEM to investigate the cell attachment, morphology, the opening of dentinal tubules, and smear layer removal.

RESULTS

BMP7 concentration released from dentin scaffolds treated with EDTA at different exposure times:

The concentration of BMP7 released from the dentin scaffolds after treatment with EDTA for 5, 10 and 15 minutes was detected (Fig. 1). Dentin treated with EDTA for 5 min did not show any statistical difference in BMP7 concentration compared to the untreated dentin ($p= 0.169$). In contrast, Dentin treated with EDTA for 10 min showed a significant increase in the BMP7 concentration compared to the untreated dentin ($p < 0.001$). Similarly, Dentin treated with EDTA for 15 min showed a significant increase in the BMP7 concentration compared to the untreated dentin ($p < 0.001$).

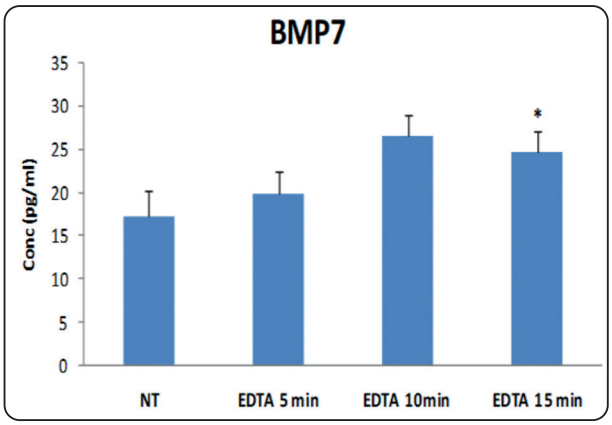

Fig. (1): BMP7 released from dentin after 5, 10 and 15 minutes of EDTA treatment.

Cell attachment and morphological examination of dentin scaffolds using SEM:

The infrastructure of dentin scaffolds was examined using scanning electron microscopy. Untreated dentin scaffold showed smear layer with no opening dentinal tubules (Fig. 2a). Dentin scaffolds treated with EDTA for 5, 10 and 15 min revealed removal of the smear layer with enlarged opened dentinal tubules (Fig. 2b, c, d). Interestingly, Dentin scaffolds treated with EDTA for 10 min presented the highest degrees of enlargement in dentinal tubules and smear layer removal than the dentin treated with EDTA for 5 or 15 min.

Additionally, DPSCs attachment after culturing for seven days on dentin scaffold treated with EDTA 17 % for 10 min was observed. Cells displayed a polygonal, rounded some stretch-type morphology, flattened and stretched (Fig. 3).

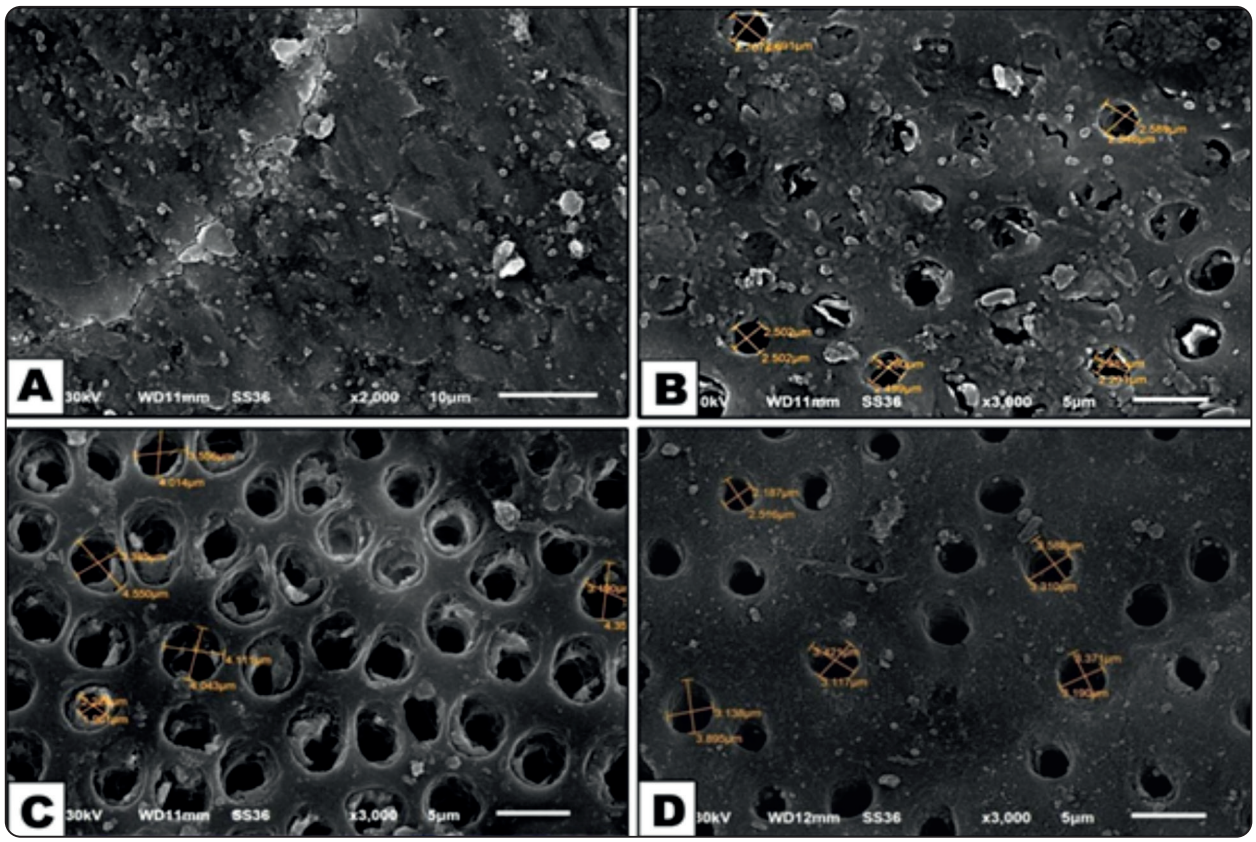

Fig. (2): Scanning electron microscopy illustrates morphological analysis of dentinal tubules opening and smear layer removal after dentin scaffold conditioning with EDTA, a) untreated dentin scaffold, b) scaffold treated with EDTA for 5 min, c) 10 min, and d) 15 min.

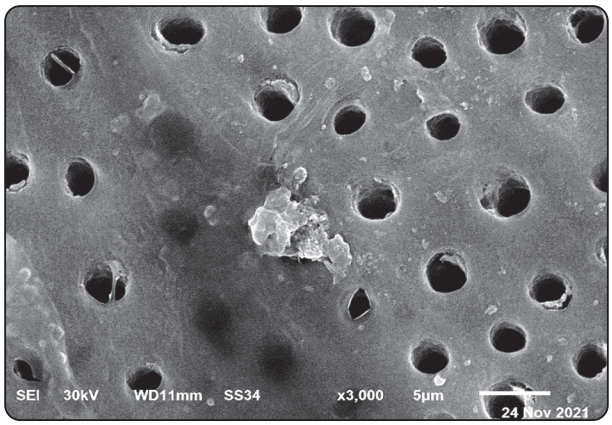

Fig. (3): Scanning electron microscopy illustrates cell attachment and different cellular morphology after dentin scaffold conditioning and DPSCs cultured for 7 days

DISCUSSION

It was discovered that EDTA treatment removes the smear layer from dentin matrix.[12] Previously, the effect of varying EDTA concentrations on smear layer removal and dentin tubule exposure was investigated.[13-15] Additionally, it is recognized that the duration of treatment has an effect on the eventual dentin structure.[16] Dentin treatment protocols must be well adjusted, as excessive demineralization can degrade dentin's structure and impair the retention and effectiveness of odontogenic components, whereas insufficient demineralization results in a less odontogenic scaffold.[17]

In our study, we tested conditioning dentin/tooth scaffolds with 17% EDTA for 5, 10 and 15 minutes using scanning electron microscopy (SEM). We found that EDTA could remove the dentin smear layer and opened the dentinal tubules. Moreover, these opened dentinal tubules were bigger in dentin treated with EDTA for 10 minutes.

Additionally, conditioning dentin with EDTA before regenerative endodontic treatment increased dental stem cell adherence and motility, as well as the growth factors released from the dentin and differentiation of stem cells toward odontoblast-like cells.[18] These factors are critical especially since currently employed strategies in regenerative endodontics rely on endogenous growth factor releases and a cell homing approach to recruit resident stem cells.

In the current study, we investigated whether seeding cells on dentin-tooth scaffolds could promote cell adhesion and differentiation in vitro or not. For cell-based bone and tooth regeneration, mesenchymal stem cells from the oral cavity are among the most promising. In addition to their simplicity of handling in vitro, these cells also have high proliferative activity, significant osteogenesis potential, and immunomodulatory features that make them good candidates for tissue regeneration applications.[19, 20, 21] Therefore, dental pulp stem cells (DPSCs) were used in our study.

Adhesion of DPSCs on dentin was evaluated after conditioning the dentin with 17% EDTA for 5, 10 and 15 minutes. The data revealed that dentin conditioned with EDTA for 10 minutes was the best for the cells adhesion.

In this study, protein concentration was analysed using ELISA kits. With great sensitivity, ELISA tests have been utilized in a wide range of research to quantitatively detect cytokines and proteins in blood or liquid samples.[22, 23] Dentin matrix has been shown to express morphogens that have a significant effect on dental stem cells differentiation into odontoblasts, as well as the formation of a network of dentin tissues and the regulation of mineralization during dentin regeneration and development. [24, 25] In the present study, we focused on one of the morphogens released from dentin matrix which is bone morphogenic protein-7 (BMP7). ELISA assay showed that dentin scaffold expressed BMP7 when preconditioned with 17% EDTA for 5, 10 and 15 minutes with significant high concentration of BMP7 at 10 minutes.

CONCLUSION

1- EDTA has great effectiveness in removing the insulating layer present in the dentin and thus the possibility of removing the largest amount of bacteria within the root canals and secreting proteins important for the differentiation of stem cells into dentin-secreting cells such as the morphogenic protein of human bone 7. However, the effect of EDTA appears to be time-dependent and should be optimized to enhance cell attachment.

2- EDTA acid is considered non-toxic and safe for cells, thus it is likely to be considered an effective factor in recruiting stem cells and activating them to regenerate dentin-pulp tissue.

Conflict of interest: The authors declare that they have no conflict of interest related to this study.

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Data availability: All raw data are available on request.

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