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ENDOGENOUS RELEASE OF BONE MORPHOGENIC PROTEIN 4 FROM DENTIN SCAFFOLDS IN RESPONSE TO DIFFERENT EDTA SURFACE TREATMENT DURATIONS

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ABSTRACT

Aim: to quantify the amount of endogenous bone morphogenic protein 4 release under the effect of EDTA exposure for different time intervals.

Materials 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 BMP4 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 cells (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 BMP4 release than the unconditioned dentin ($p < 0.05$).

Using scanning electron microscopy, dentin scaffolds 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: endogenous bone morphogenic protein 4 release was the highest when dentin scaffolds were treated with 17% EDTA for a period of 10 minutes.

KEY WORD: dental pulp stem cells, dentin scaffold, EDTA, BMP-4

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INTRODUCTION

The inconsistent clinical outcome of traditional apexification procedures in teeth with incompletely formed roots has recently shifted the treatment of such necrotic immature permanent teeth toward regenerative endodontics to allow for periapical healing while at the same time allowing for continued root development with the possibility to regenerate the pulp-dentin complex. Regenerative endodontic therapy (RET) delivers dental stem cells with the blood clot as a scaffold as well as bioactive growth factors to the root canal to reform the pulp-dentin complex tissue matrix and compromised dental tissues and allow root development.[1]

Bioactive, biocompatible scaffolds provide stem cells grafts with an optimal 3D microenvironment and extracellular matrix support that promotes stem cell proliferation and differentiation.[2] A well-designed scaffold for supporting the survival and development of dental pulp stem cells should provide physical and biochemical support to the surroundings of the root canal and enhance the formation of a new tissue matrix for dentin pulp complex regeneration. Bioactive growth factors and other signalling compounds are among the conditions that need to be added to the target scaffold in a spatiotemporal relevant manner to ensure the total capacity of stem cell differentiation.

The transforming growth factor-beta (TGF-beta) superfamily includes a variety of peptide growth factors such as bone morphogenic proteins BMPs. BMPs got their name from their capacity to create ectopic bone matrix and cartilage tissue upon being transplanted into muscle tissue.[3] Bmp2, 4 and 7 are from these proteins that are commonly used in bone regeneration .[4, 5] In addition to that, BMP6 shows a great capacity to induce the osteoblast differentiation and survival and hypertrophic cartilage tissue formation that expressed in the condensed mesenchyme of bone periosteum and the perichondrium.[6] Particularly, BMP 2,4 and 7 have shown extensive potential as both regulators of tooth development and due to their contribution to the induction of reparative dentin in in vivo animal experiments. Therefore, the objective of the current study was to assess the release of endogenous BMP-4 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.

MATERIALS AND METHODS

This study was approved by the research ethics committee of the faculty of dentistry, Mansoura University (protocol ID# M06080120).

Preparation of dentin slices:

Ultraviolet rays (UV) were used to sterilize dentin scaffolds, which 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. Dentin slices were disinfected with 70% alcohol and 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 Poietics^{TM} DPSCs were obtained from adult third molars obtained following a donor's 'wisdom' teeth extraction. DPSCs were thawed and subcultured according to Lonza's methods and growth media (DPSCsBulletKit^{TM} 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.

Detection of Endogenous BMP4 release via ELISA assay

The concentration of BMP4 released from the dentin scaffolds after treatment with EDTA for 5, 10, and 15 minutes was detected by Quantikine™ ELISA. (Cat. no.DBP400, R&D, USA) All assays were carried out in duplicate. According to the manufacturer, all buffers, samples working, and standards were prepared.

One hundred µL of the diluent assay (RD1-61) was dispensed to every well. Then, 50 µL of control, standard, or specimens were added per well. The strip was covered with an adhesive ribbon provided and then incubated at room temperature for 2 hours. The reagent was removed from each well and then washed four times with 400 µL Wash Buffer, and Wash Buffer was discarded. Two hundred µL of Human BMP-4 Conjugate was loaded to every well, and in the dark, the strip was set for 2 hours at room temperature. A 200 µL of Substrate Solution was loaded to each well after removing the BMP-4 Conjugate reagent. Then, the strip was set for 30 minutes at dark and room temperature.

Finally, to each well, a 50 µL of Stop Solution was loaded, and the well's color was changed from blue to yellow. After 30 minutes, the optical density of each well was detected using an ELISA reader at 450 nm. The average duplicate results for standard, control, and sample were obtained to create a standard curve. The perfect curve across the points on the graph was used to generate a standard curve by graphing the mean absorbance on the y-axis against the concentration on the x-axis.

Cell attachment and morphological surface analysis using scanning electron microscopy (SEM):

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.

Two other groups were ready to assess cell attachment after one week of cell culture on the conditioned dentin scaffolds. Group 1: 1x106 DPSCs cultured onto 17%EDTA treated dentin scaffolds, and group 2: 1x106 DPSCs cultured onto 17%EDTA treated dentin scaffolds with 25ng/ml human recombinant BMP4. The seeded pre-treated dentin scaffolds from each group were then fixed with 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 [JEOL JSEM-6510LV; JEOL, Tokyo, Japan] at faculty of Agriculture, Mansoura University for cell adhesion, morphology, dentinal tubules opening and smear layer removal.

RESULTS

Detection of BMP4 concentration released from dentin/tooth scaffolds conditioned with EDTA

Dentin-tooth scaffolds were conditioned with 17% EDTA for 5, 10 and 15 min, and the supernatants were taken to measure the BMP4 protein concentration using ELISA assay (Fig.1). BMP4 released from dentin after conditioned with EDTA for 5 min did not show any statistical difference compared to its concentration in the unconditioned dentin group ($p > 0.05$). On the other hand, BMP4 concentration was significantly higher in dentin conditioned with EDTA for 10 min compared to the unconditioned dentin group ($p < 0.001$). Additionally, dentin scaffold conditioned with EDTA for 15 min showed higher BMP4 concentration compared to the unconditioned dentin ($p < 0.001$).

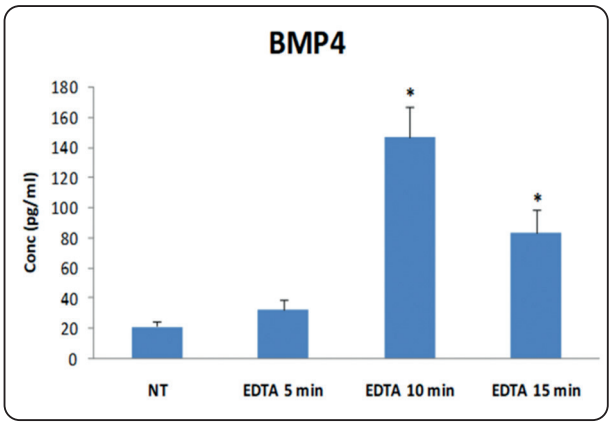

Fig. (1): BMP4 concentration released from dentin conditioned with EDTA for 5, 10 and 15 minutes.

Ultrastructural observation of treated dentin/ tooth scaffolds and cell attachment:

Dentin/tooth scaffolds were examined using scanning electron microscopy. Unconditioned dentin scaffolds with EDTA showed closed dentinal tubules (Fig. 2a). Dentin scaffolds conditioned for 5, 10 and 15 min with EDTA showed numerous opened dentinal tubules (Fig. 2b, c, d). Dentin scaffolds conditioned for 10 min with EDTA showed the most enlarged opened dentinal tubules among the other conditioned dentin samples.

Moreover, DPSCs were cultured for 7 days on dentin/tooth scaffolds after being conditioned with EDTA for 10 min. Cells appeared round and flattened (Fig. 3a). Furthermore, DPSCs were treated with BMP4 during culturing for 7 days on conditioned dentin/tooth slices. Cells appeared more flattened with elongated cytoplasmic processes than the DPSCs cultured on dentin scaffolds without BMP4 (Fig.3b).

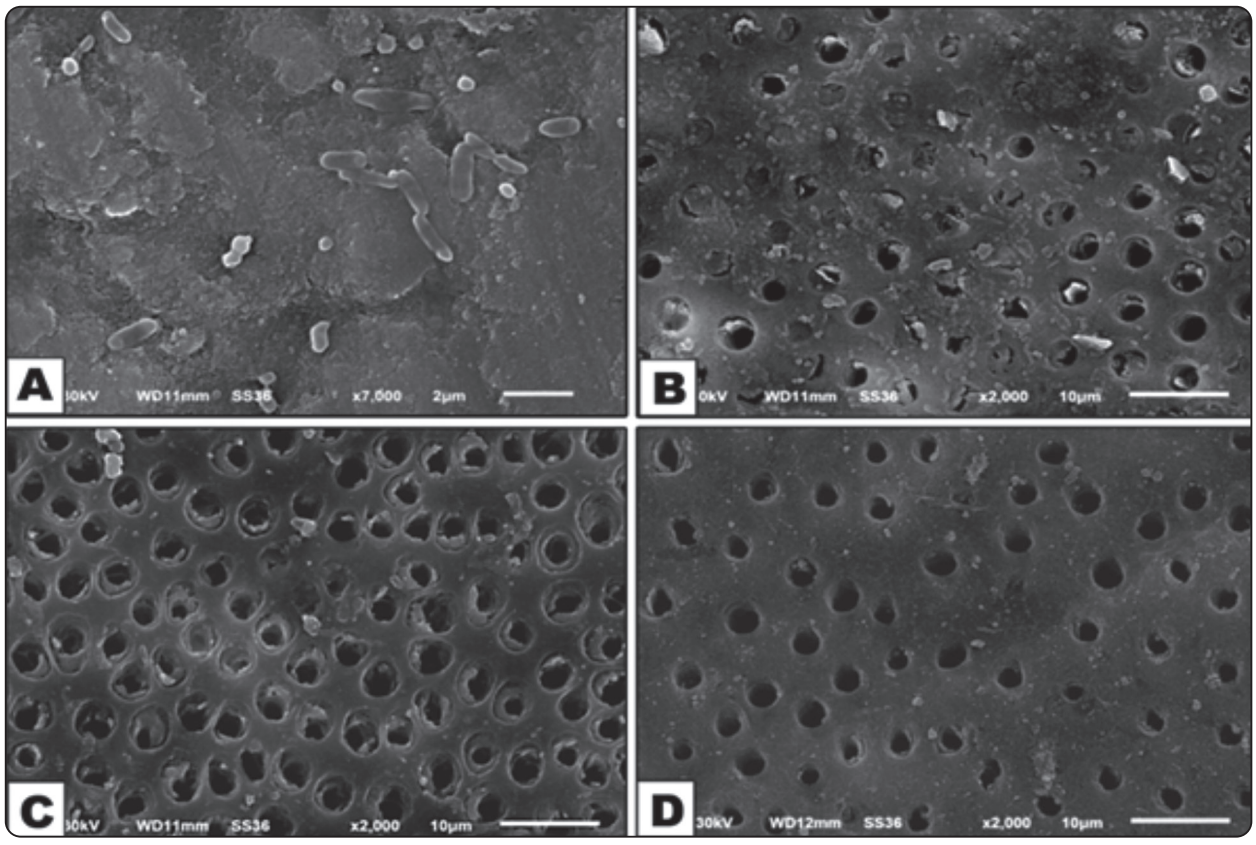

Fig. (2): Scanning electron microscopy showing a) unconditioned dentin scaffold, b) scaffold conditioned with EDTA for 5 min, c) 10 min, and d) 15 min.

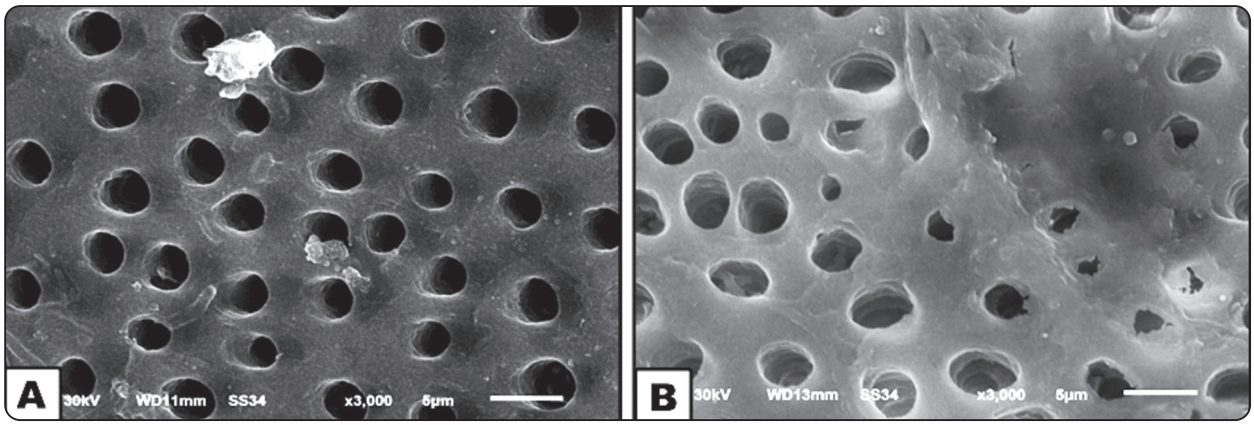

Fig. (3): Scanning electron microscopy of conditioned dentin scaffolds treated with EDTA showing; a) dentin scaffold+ DPSCs group, and b) dentin scaffold+ DPSCs+BMP4 group.

DISCUSSION

The first step and the most important prerequisite for regenerative therapy is intracanal disinfection. This is done by using different irrigants and medications.[7] For example, sodium hypochlorite (NaOCl) is used commonly for regenerative therapy. However, it has been shown that when the surfaces of root canal treated with NaOCl, this leads to reduction of stem cells survival rate and their attachment because of its toxicity.[8] In contrast, when EDTA is used after NaOCL, it promotes the stem cells survival and their adhesion on the dentin.[9, 10] Moreover, EDTA-treated dentin releases some growth factors that may lead to better stem cell survival rate at the same time contributing to stem cell homing, proliferation and differentiation.

The chelating agent EDTA has the ability of loosening the structure of dentin and it can remove the smear layer of in-organic as well as organic debris developed in the treatment of root canal.[11, 12] Previous studies pointed out that choosing the optimum concentration and time for dentin treatment with EDTA is critical as, too much EDTA could destroy the dentin structure, while low concentration generates a less odontogenic scaffold.[13, 14]

In the current study, dentin/tooth scaffolds were treated with 17% EDTA for 5, 10 and 15 minutes. The effect of different times of EDTA treatment was investigated using scanning electron microscopy (SEM). Dentin treated with EDTA illustrated removal of the smear layer with open dentinal tubules. The number of open dentinal tubules was greater in dentin treated with EDTA for 10 min also showing dentinal tubules with the largest diameter.

Besides, DPSCs were seeded on dentin/tooth scaffold after conditioning with EDTA for 5, 10, and 15 min to evaluate the capability of dentin to promote the migration and adhesion of DPSCs. Our data showed that cell attachment was enhanced on surfaces treated with EDTA for 10 min.

Furthermore, the morphology of DPSCs on dentin was observed by scanning electron microscopy. SEM analysis showed large numbers of DPSCs with their extracellular matrix spread over dentinal tubules after 7 days especially with the adjunctive addition of BMP4 highlighting the crucial role of this growth factor in improving cell survival and attachment. This observation is in accordance with Li et al.[15], who pointed out that dentin enhanced the proliferation of DFCS when examined by SEM. These findings are in parallelism with Pang et al.[16] who suggested that some adhesive and soluble factors which bind to cell-surface receptors regulate differentiation and attachment of cells on the surface of dentin. This adhesion is due to EDTA conditioning that extracts calcium from the in-organic calcium phosphate and changes

the dentin surface characteristics. As a result, the differentiation of cells, which attached to the dentin treated with EDTA, may differ from that attached to the EDTA-untreated dentin.[16]

In order to evaluate the effect of BMP4 protein released from dentin scaffold on DPSCs differentiation, the concentration of BMP4 released from dentin scaffold after being conditioned with EDTA for 5, 10 and 15 minutes was detected using ELISA assay. With a high degree of sensitivity, ELISA tests have been utilised quantitatively and qualitatively to identify components or proteins included in blood or liquid extracts.[17, 18] ELISA results illustrated that dentin scaffold expressed BMP-4 when preconditioned with 17% EDTA for 5, 10 and 15 minutes with a higher concentration at 10 minutes.

CONCLUSIONS:

The use of 17% EDTA has a time dependent effect on the release of endogenous BMP-4 from dentin scaffolds with the maximum effect being obtained after 10 minutes of exposure. Furthermore, DPSC attachment is enhanced following EDTA surface treatment and is favoured with further exogenous addition of BMP-4.

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 will be available upon request.

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