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EFFECT OF EXPOSURE TIME OF EDTA ON THE ENDOGENOUS RELEASE OF BONE MORPHOGENIC PROTEIN 2 FROM DENTIN SCAFFOLD

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ABSTRACT

Aim: The aim of this study was to demonstrate the amount of endogenous bone morphogenic protein 2 released under the effect of EDTA exposure for different time intervals.

Material and methods: The dentin scaffold was randomly assigned to the groups (n = 4). Group 1 received 17 % EDTA for 5 minutes, while Group 2 received 17 % EDTA for 10 minutes. For 15 minutes, Group 3 received 17 % EDTA. Distilled water was used as a control in Group 4. After each treatment, a PBS wash step was performed, Quantikine™ ELISA was used to quantify the amount of BMP2 liberated from the dentin scaffold after pretreatment with EDTA for 5, 10, and 15 minutes, the scaffolds were analyzed under SEM to investigate the cell attachment, morphology, the opening of dentinal tubules, and smear layer removal.

Results: Dentin scaffold 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 viability than the control group ($p < 0.001$), Moreover, dentin treated with 17% EDTA for 10 min manifested higher concentration of BMP2 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 the dentin treated with EDTA for 5 or15 min.

Conclusion: Maximum endogenous bone morphogenic protein 2 release was obtained when dentin scaffold treated with 17% EDTA for a period of 10 minutes.

KEY WORD: stem cell, dental scaffold, EDTA

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INTRODUCTION

Bacteria invasion and dental trauma can cause damage to the pulp tissue of developing teeth. Pulp tissue gets irritated in these instances, and pulp necrosis develops if the inflammation doesn't stop. This increases the death of odontoblasts, causing root growth to be disrupted [1], this makes teeth more fracture-brone.

Recently, regenerative endodontics field has provided new potentialities for necrotic pulp and immature-root permanent teeth. Three crucial factors are needed to be combined for successful endodontic tissue regeneration, stem cells and progenitor's cells, Bioactive signaling molecules and biocompatible scaffolds [2].

Dental pulp contains resident precursors of stem cells because the tooth can produce reparative dentin following damage [3]. Dental pulp stem cells (DPSCs) were discovered in permanent and deciduous teeth in 2000 and 2003, respectively, proving this theory[4]. These dental derived stem cells can differentiate and proliferate into odontoblasts like cells to form dentine matrix in dental pulp [5].

We noticed tremendous progress in DPSC studies over the last two decades, from functional characterization[6] and mechanistic investigation [7] to preclinical experiments [8] and clinical trials on dental pulp regeneration [9].

BMPs were first introduced as a growth factor for controlling molecular signals in cartilage and bone development, in addition to induction of developmental morphogenesis of a variety of organs and tissues during embryogenesis stages, including teeth[10]. BMPs are considered as a potent dentinogenetic factor and can induce dentin regeneration[11]. Dentin extracts, in particular, can trigger dentin regeneration through induction dental pulp stem cells into odontoblast like cells [12].

Transforming growth factor (TGF) superfamily includes BMP-2are involved in the growth, regeneration, and hemostasis of the skeleton [13]. BMP-2 can induce in vitro osteogenic/odontogenic differentiation[14] and in vivo bone formation significantly[15].

Scaffolds were first used in preclinical dental pulp regeneration trials because it was thought that scaffolds would bio mimic stem cells growth conditions. Biomaterials play a critical role in both tissue engineering and cell therapy. Scaffold's matrices mesh were constructed to link a porous structure together and support the cellular milieu to optimize tissue regeneration conditions. Synthetic and natural polymer scaffolds have been widely designed to support the proliferation rate and differentiation of dental pulp stem cells effectively into pulp-dentin complex regenerative tissue to retore the function of badly decayed teeth [16].

Tissue engineering, particularly rely on precise design of biocompatible scaffolds, to guide the role dental stem cells play in the regenerative dentistry, moreover, paved the way for their successful clinical use in endodontics cell therapy to counter the effects of endodontic infection and tooth decay. Scientists continue to develop tissue-engineering-based scaffold and cell therapies to obtain more convincing and predictable evidence of pulp-dentin complex regeneration.

MATERIAL AND METHODS

Preparation of dentin slices/tooth scaffolds and conditioning procedures

Dentin/scaffold material was extracted from healthy human third molars. The coronal dentin was cut into a square shape approximately 1 mm thick and 5 mm for two surfaces. Dentin slices were sterilized and disinfected with ultraviolet rays and 70% alcohol. Dentin slices were rinsed three times in PBS before being put in sterile 6-well plates. The dentin scaffold was assigned to 4 groups at random (n = 4 groups). **Group 1** get a 5-minute treatment

with 17% EDTA, while **Group 2** received a 10-minute treatment with 17% EDTA. Treatment with A 17 % EDTA for 15 minutes in **Group 3**. **In Group 4**, distilled water was used as control group. Following each treatment, a PBS wash step was performed. The EDTA was collected and stored at -20 ºc for ELISA study and the dentin slices were equilibrated in a culture media for 24 hours and placed in 6-well plates for cell seeding.

ELISA test to determine the amount of BMP2 secreted from dentin scaffolds

Quantikine™ ELISA (Catalog no. DBP200, R&D, USA) was used to determine the concentration of BMP2 released from the dentin scaffold following treatment with EDTA for 5, 10, and 15 minutes. Before using, bring all reagents and samples to reach room temperature (Fig. 4a-c). All standards, controls, and samples were prepared as recommended and measured twice. Each well received 100 µl of RD1-19 diluent. Following that, 50 µl of standard, control, of sample were added to each well. With the adhesive strip, the wells were covered. On a horizontal orbital microplate shaker set at 50 rpm to 500 rpm, the plate was incubated for 2 hours at room temperature. Each well was aspirated and cleaned four times with 400 µl of Wash Buffer. Following the last wash, any residual Wash Buffer was aspirated from the plate and inverted onto clean paper towels. Each well received 200µl of BMP-2 Conjugate and incubated for 2 hours at room temperature. After washing, each well received 200µl of Substrate Solution and set in the dark for 30 minutes at room temperature. Each well received 50µl of Stop Solution. The well's color changed from blue to yellow. Using a microplate reader set to 450nm determined each well's optical density in under 30 minutes. The mean absorbance for each standard was plotted on the y-axis against the concentration on the x-axis, and the best fit curve was drawn between the points on the graph to create a standard curve.

Cell attachments and infrastructure analysis Scan electron microscopy

The effect of chelating chemical compounds on removing the smear layer and disclosing the dentinal tubules were studied using scanning electron microscopy (SEM). The dentin scaffold was conditioned with the following chelating agent solutions prior to cell culture (3 dentine scaffolds per group): EDTA (17 % EDTA) for 5 minutes, EDTA (17 % EDTA) for 10 minutes, EDTA (17 % EDTA) for 15 minutes, and the distilled water-treated scaffold served as a control group. After one week of cell culture on the conditioned dentine scaffold, two other groups were available to test cell adhesion and proliferation. **Group I**: $1x10^6$ DPSCs were cultivated on a dentin scaffold treated with 17% EDTA. **Group II:** a dentin scaffold was prepared with 10% EDTA, $1x10^6$ DPSCs, and 25 ng/ml human recombinant BMP2. After removing dentin scaffolds and a 2.5% glutaraldehyde solution was used to fix at 4^{oc} for two hours. They were dried using hexamethyldisilane after being dehydrated in a succession of ethanol grades. After gold coating, scaffolds were examined by scanning electron microscope 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

1. ELISA assay for the BMP2 concentration released from dentin scaffold treated with EDTA at 3 different times

As shown in Table 1; Fig. 1, the concentration of BMP2 released from the untreated dentin scaffold and scaffold treated with EDTA for 5, 10 and 15 minutes was determined. Compared to the untreated dentin scaffold, scaffold treated with EDTA for 5min didn't show any statistical difference in BMP2 concentration ($p= 0.149$). Dentin treated with EDTA for 10 and 15 min showed higher concentration of BMP2 compared to the untreated dentin ($p < 0.001$).

TABLE (1) BMP2 concentration released from dentin scaffold after treatment with EDTA for 5, 10 and 15 minutes.

Groups	NT	EDTA 5 min	EDTA 10 min	EDTA 15 min
Conc (pg/ml)	69.01± 13.6	82.26± 20.4	123.02± 14.9*	120.53± 18.5*

Significant difference compared to the control group by one-way ANOVA followed by posthoc multiple comparisons (LSD test) at p* ≤0.05. NT: dentin scaffold without EDTA treatment.

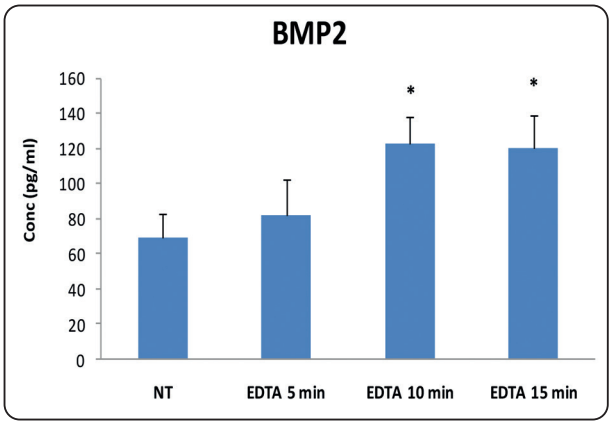

Fig. (1) BMP2 concentration released from dentin after 5, 10 and 15 minutes of EDTA treatment.

3. Cell attachment on dentin scaffold treated with EDTA for 5, 10 and 15 minutes.

Dentin scaffold was conditioned with 17% EDTA for 5, 10 and 15 min, then DPSCs were cultured on the conditioned dentin scaffold for 7 days and compared with the cultured cells on unconditioned scaffold and the negative control (Table 3; Fig. 9). Compared to the negative control group, unconditioned dentin scaffold with EDTA didn't show any statistical difference in the cell attachment ($p= 0.611$). In contrast, the conditioned scaffold with EDTA for 5 min showed a significant increase in the cell attachment compared to the control group ($p < 0.05$). Additionally, dentin conditioned 10 and 15 min with 17% EDTA showed more cell attachment compared to the control group ($p< 0.001$).

2. Cell proliferation and calcification with culturing with BMP2 and/or Dentin scaffold for 7, 14 and 21 days

Dental pulp cell lines were cultured alone as a negative control group, with dentin scaffold after treatment with EDTA 17% for 10 mins group, with BMP2 protein group, with dentin scaffold and BMP2 group for 21 days. Additionally, positive control group with osteogenesis inductive media was cultured. Under inverted light microscope, it was apparent that DPSCs cultured with dentin were growing normally at 7 days, which confirmed the good biocompatibility of the dentin scaffold (Fig. 12a-e). Moreover at 14 days, calcification was formed in all groups except the negative control group, and cells cultured on dentin showed cellular polarity in a regular direction (Fig. 13b-e). Furthermore, calcified nodules were more expanded and distributed in DPSCs cultured on dentin scaffold and treated with BMP2 more than the other groups (Figs. 1 b-e).

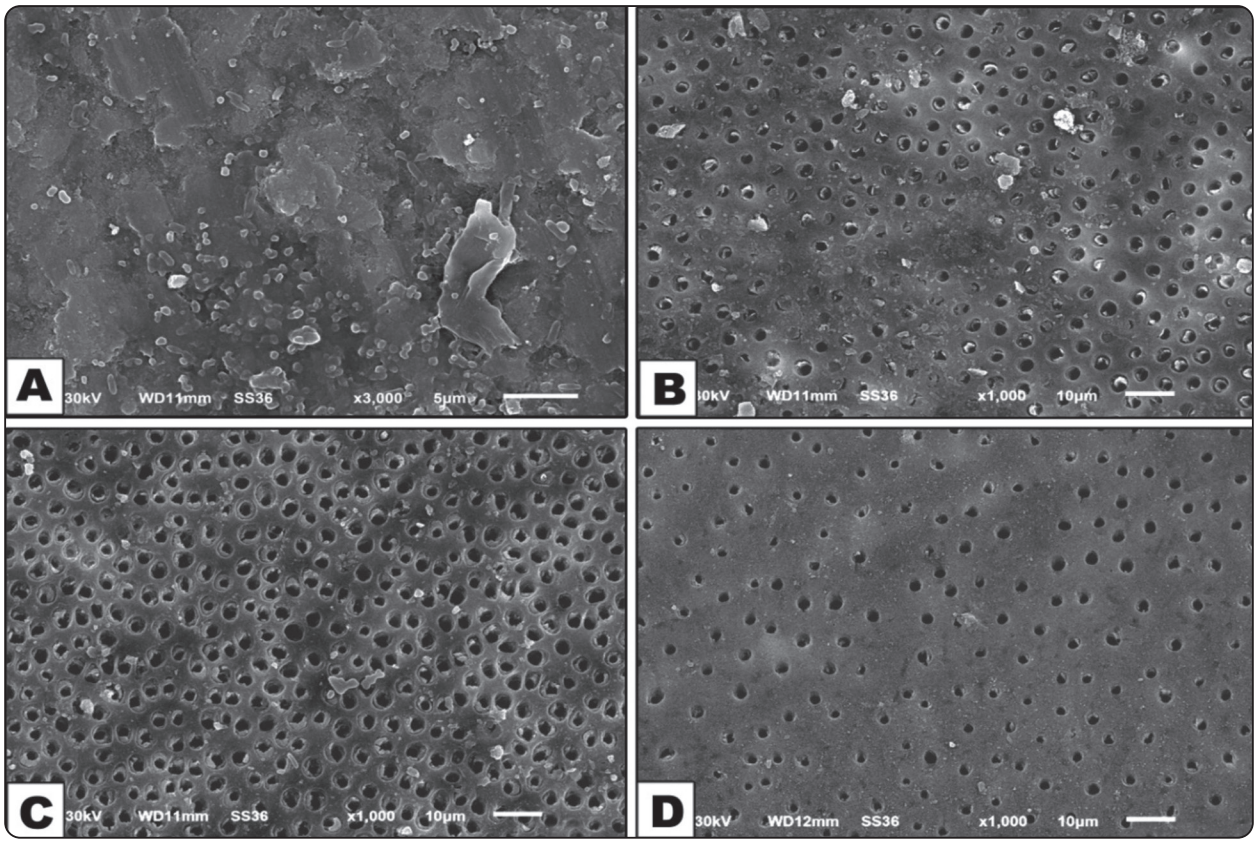

Fig. (2) Dentin scaffold examined by scanning electron microscopy showing; a) unconditioned dentin scaffold, b) dentin scaffold treated with EDTA for 5 min with opened dentinal tubules, c) scaffold treated with EDTA for 10 min, and d) scaffold treated with EDTA for 15 min.

DISCUSSION

The treatment of patients with joint replacements, dental and oral craniofacial surgeries, ranging from tooth restorations to significant renewal of facial soft and mineralized tissues, costs an enormous amount of money annually [17]. Many dental problems are expected to be resolved through the replacement or regeneration of oral tissues damaged by neoplastic and trauma.

Dental and endodontic breakthroughs are on the horizon since prosthetic teeth, bone, and oral tissues are now widely available, and endodontic regeneration can be stimulated [18].

Apexification is known as the process by which an environment conducive to the creation of a calcific barrier across the open apex is formed in the root canal and the periapical tissue. However, this approach has significant drawbacks, including the unpredictable establishment of the apical barrier and the lengthy treatment period, which frequently requires multiple visits [19]. These considerations have prompted an adjustment of the classic apexification process based on Ca(OH)2 in order to accomplish quick canal obstruction by introducing an artificial barrier of MTA [20]. Apexification of MTA shortens treatment time and promotes periradicular tissue repair [21].

Despite these benefits of MTA apexification, thin dentinal walls continue to be a clinical issue, and the expensive cost of MTA and the difficulty in manipulating the material to the apical 3–4 mm may limit its broad application [22].

Endodontic regenerative therapies are biologically based methods that are used to restore damaged tissues, as root and dentin structures, and pulp-dentin complex cells. The main goal of regenerative endodontics approaches is to regenerate pulp-like tissue, especially the pulp-dentin complex; repair damaged dentin, such as after a carious exposure; and regenerate off root or apical dentin [23].

Endodontic regeneration requires a high amount of disinfection [24]. However, it has been demonstrated that microorganisms penetrate deeper into the teeth of younger individual than older one [25], making the eradication of bacteria from immature diseased teeth a formidable issue [26]. Irrigation with NaOCl is often the most reported approach for root canal disinfection. Subsequently, studies advocated using 17% EDTA in regenerative endodontics due to its role in reversing the negative effects of NaOCl.

Dentin matrix may be preferable to standard materials for dentin regeneration due to its mechanical and non-immunogenicity property of acellular matrixes rich in dentinogenesis and proteins components[27]. Dentin from adult rats treated with EDTA at concentrations of 17% and 5% was shown to eliminate smear layers, funnel dentinal tubules, and alter dentin permeability, all of which allowed for appropriate protein and factor release from dentin [28]. Furthermore, Li et al reported that treatment of dentin with EDTA must be optimized, because insufficient demineralization may lead to a less odontogenic scaffold, while excessive demineralization can weaken the dentin's structure and obstruct the preservation and efficiency of odontogenic components [29].

In the current study, dentin/tooth scaffold treated with EDTA was used in the present or absent of BMP2 as a one of the dentin releasing growth factor to induce dental pulp stem cells odontoblastic differentiation, suggesting this model as a treatment for the pulp-dentin complex.

Dentin scaffold was treated with 17% EDTA for 5, 10 and 15 minutes and examined with scanning electron microscopy (SEM). Dentin treated with EDTA showed removal of smear layer with opened dentinal tubules compared to the untreated dentin. Notably, dentin tubules were larger and various in dentin scaffold treated with EDTA for 10 minutes.

One of the most challenging aspects of regenerative endodontics is obtaining progenitor pulp cells that multiply recurrently and produce cells or pulp tissues that can be introduced into the root canal [30]. Dental pulp stem cells (DPSCs) were chosen for oral regeneration due to their strong proliferative activity, considerable osteogenesis potential, and immunomodulatory properties [31]. In addition, using a pulp stem cell line eliminates the need for patients to provide their own cells through a biopsy. Moreover, the pulp tissue structures can be created in preparation for fast implantation [32].

The effect of dentin/ tooth scaffold on DPSCs adhesion and viability was detected in this study. Cells were cultured for 7 days with dentin after being conditioned with EDTA for 5, 10 and 15min. Compared to the untreated dentin, dentin treated with EDTA showed a significant viability increase, which was higher at 10 min. Besides, SEM examination revealed high attachment between DPSCs, and dentin scaffold treated with EDTA for 10min, detected by the dentinal tubules covered with large number of cells and their extracellular matrix. This finding is consistent with that of Li et al., who revealed that dentin increased the growth of dental stem cells [33].

After that, DPSCs were seeded for 7, 14 and 21 days with the dentin conditioned with EDTA for 10 min. At 7 days, cells showed normal growth and viability, while calcified nodules were observed at 14 days with a higher viability than the control cells. Dentin-cultured cells demonstrate a significant substantial survival, proliferation, and differentiation toward odontoblast cells after

21 days, indicating that dentin is a biocompatible scaffold, as Li and his team hypothesized [33].

On the other hand, Galler et al demonstrated that conditioning dentin with EDTA increased dental stem cell adherence and motility, as well as the morphogens expressed from the dentin that promote the differentiation of dental stem cells toward odontoblast-like cells [34]. Given that dentin contains bioactive molecules can activate the cellular responses needed for dentin regeneration [35], it is crucial to determine which dentin signals are necessary for odontoblastic differentiation. Such information would enhance our comprehension of the dentin regeneration mechanism and guide future research efforts in dental pulp engineering. Dentin-derived morphogenic signals, in particular BMP-2, were shown by Casagrande et al. to be required and sufficient for inducing odontoblast development in stem cells [36].

In the current study, we measured the concentration of bone morphogenic protein-2 (BMP2) that released from dentin using ELISA assay. ELISA tests have been utilized in a wide range of research to quantitatively detect cytokines and proteins[37]. Our data showed that dentin scaffold treated with EDTA for 5, 10 and 15 minutes, expressed BMP-2 with significantly high concentration at 10 minutes.

CONCLUSION

Although dentin can release BMP2 protein after being treated with EDTA 17 percent, this concentration alone with the scaffold does not induce significant odontogenic differentiation in DPSCs, whereas the combination of 25ng/ml BMP2 with the dentin scaffold significantly increases the differentiation capacity of DPSCs toward odontogenic linage and the formation of mineralization matrix. Similarly, recombinant protein alone had lower odontoblastic differentiation capability than the protein plus conditioned scaffold combination. Another function for EDTA acid is to remove bacterial film inside the root canal also, EDTA is considered non-toxic and safe for cells, so it can be considered an effective factor in engineering and activating stem cells. Finally, we prove the possibility of using stem cells grown on a scaffold of dentin in the presence of human bone morphogenic protein 2 in the regeneration of dental pulp tissue and root canal treatment.

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