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EFFECT OF EXPOSURE TIME OF EDTA ON THE ENDOGENOUS RELEASE OF TRANSFORMING GROWTH FACTOR BETA 1 FROM DENTIN SCAFFOLD

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ABSTRACT

Aim: The aim of this study was to demonstrate the amount of endogenous bone TGF-β1 release 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 TGF-β1 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 TGF-β1 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 Transforming growth factor beta 1 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

Dental caries/decay affects the permanent teeth of 2.43 billion individuals (36% of the world's population) in addition to the baby teeth of 620 million children (9% of the world's population) [1]. Dental caries is the most frequent chronic pediatric illness in the United States. A staggering 92% of adults in the United States showed a cavity development, and also more than a quarter of this population has untreated dental rot [2]. A person can suffer from a deep decay in tooth as a result of an accident or from recurring dental work trauma and his tooth then may become infected, inflamed or unhealthy, despite the fact that superficial decay can typically be repaired and stopped with a filling. An infection can spread to neighboring tissues if left untreated [3].

The novel biomaterials development in addition to stem cell (SC) technology has made tissue engineering based treatment which is a viable option for rooting the canal therapy by maintaining or replacement of dental pulp stem cells (DPCs) [4]. There are three main components of tissue engineering: SCs, growth factors (GFs) and scaffolds. They are managed in space and also time for tissue regeneration[5]. Controlled degradation, biocompatibility, physical and chemical stability, adhesion and mechanical strength are all requirements for an ideal scaffold[6]. Furthermore, the scaffold has to be incorporated with growth factors that encourage differentiation and proliferation of cells as an artificial carrier [7].

Gronthos et al identified human dental pulp stem cells (DPSCs) from third molar teeth which are adult stem cells with mesenchymal stem cell characteristics. [8]. Multiple connective tissue cell lineages which include the osteogenic and odontogenic phenotype, may be generated in DPSCs [9]. DPSCs are also extremely proliferative and may be safely cryopreserved [10]. When compared to differentiated cell types as well as embryonic stem cells, they showed a desirable source for the tissue engineering applications and regenerative medicine due to their features and quicker development route [11].

TGFs are considered as multifunctional regulators involved in different aspects of cell activity such as matrix secretion, proliferation, differentiation and apoptosis [12]. They are essential for modulating odontoblast activity since they are TGF growth factors super family members. TGF-s are also expressed by maturing odontoblasts [13], resulting in their sequestration in the dentin matrix and the formation of a matrix-associated reservoir of TGF-s that could be produced in response to changes in matrix that caused by caries or trauma [14]. Endogenous TGF-1 applied to exposed pulpal cells increases reparative dentin production, according to in vivo experiments [15]. TGF-1 and TGF-3, but not TGF-2, have induced both pulpal cell proliferation in the sub-odontoblast area and matrix secretion in a tooth slice organ culture model [16].

Achieving the features of biomaterials for tissue regeneration scaffolds is One of the challenges in development of new treatments, as they must be characterized by interconnected porosity, biocompatibility and conductivity for the attachment. Moreover, these biomaterials must have the ability to enhance the committed cells proliferation and differentiation and also to incorporate inductive factors, appropriate mechanical properties as well as biodegradability. In labs, several biomaterials have been created to meet these needs, the most significant of which is facilitating progenitor cell homing and differentiation by giving inductive signals for geographically and/or temporally directing tissue regeneration [17]. Extracellular matrix (ECM) components, other proteins, peptides, natural and synthetic polymers, polysaccharides and bio-ceramics, as well as numerous novel composites have all been explored for this purpose. Each material has its own structure, composition, chemistry and degradation profile and the ability to be modified for tissue regeneration [18].

Revascularization, innervation, growth factor incorporation, cell-matrix interactions, remineralization, controlled bio-degradation as well as contamination control should be adapted for regeneration of dentin pulp complex [19].

MATERIAL AND METHODS

Preparation of dentin slices/tooth scaffolds and conditioning procedures

We extracted dentin/scaffold material from healthy human third molars. On both sides, the coronal dentin was cut to a thickness of 1 mm and a width of 5 mm. (Fig 2. B). UV rays and 70% alcohol were used to sterilize and disinfect dentin slices. Three times in PBS, dentin slices were washed before being placed in sterile 6-well plates. After cell harvesting, they were seeded onto the dentin scaffold at a density of ($1.5x10^6$ cells/scaffold) and cultured in a DMEM medium. The dentin scaffold was separated to 4 groups at random (n = 4 groups). **Group 1** treated with 17 % EDTA for 5-minute treatment, while **Group 2** received a 17% EDTA treatment for 10-minute. **Group 3** received A 17 % EDTA for 15 minutes. In **Group 4**, distilled water was used as control group. PBS was used as washing buffer after each step. The EDTA was collected and stored at -20°C for ELISA analysis, and the dentin slices were equilibrated in culture medium for 24 hours before being seeded in 6-well plates.

ELISA test to determine the amount of TGF-1β secreted from dentin scaffolds

The concentration of TGF-1β released from the dentin scaffold after treatment with EDTA for 5, 10, and 15 minutes was determined using the Quantikine™ ELISA (Catalog no. DB100B, R&D, USA) (Fig. 3a-c). All samples and reagents were prepared at room temperature before use.

All samples, controls, and standards were prepared and measured twice. Each well was diluted with 100 l RD1-19 diluent, and we added 50 µl of example, standard, or control to each well. The sticky strip was used to cover the wells. Using a horizontal orbital microplate shaker set to 50–500 rpm, the plate was incubated at room temperature for 2 hours. Each well was aspirated and cleaned four times with 400 µl of Wash Buffer. Any remaining Wash Buffer was aspirated off the plate and inverted onto clean paper towels after the last wash. Each well was incubated for 2 hours at room temperature with 200 µl of TGF-1β Conjugate.

Following washing, 200 µl of Substrate Solution was added to each well and left in the dark for 30 minutes at room temperature. Each well was supplied with 50 µl of Stop Solution. The color of the wells shifted from blue to yellow.

In less than 30 minutes, the optical density of each well was assessed using a microplate reader set at 450nm. The standard curve was generated by using the absorbance mean of each standard that was graphed on the y-axis against the concentration on the x-axis.

RESULTS

Detection of TGFβ1concentration released from dentin/tooth scaffold conditioned with EDTA

The concentration of TGFβ1 protein released from dentin scaffold after treatment with 17% EDTA for 5, 10 and 15 min was measured using ELISA assay and compared with the untreated dentin scaffold (Table 2; Fig. 7). The results revealed that there was no statistical difference in the concentration of TGFβ1 between the untreated dentin and dentin treated for 5 min groups ($p= 0.068$). Whereas dentin scaffold treated with EDTA for 10 and 15 min showed significant increase in the TGFβ1concentration compared to the untreated dentin group ($p < 0.001$).

Cell attachments and infrastructure analysis Scan electron microscopy (SEM)

SEM microscopy was used to investigate the impact of chelating chemical compounds on eliminating the smear layer and exposing the dentinal tubule. The dentin scaffold was preconditioned using the following chelating agent solutions before cell culture (3 dentine scaffolds per group: 17 % EDTA for 5 minutes, 17 % EDTA for 10 minutes, 17 % EDTA for 15 minutes, and the distilled water-treated scaffold served as a

control group. Two other groups were provided to test cell adhesion and proliferation after one week of cell culture on the conditioned dentine scaffold. **Group I**:1x10^6 DPSCs were cultured on a dentin scaffold was received 17% EDTA. **Group II:** a dentin scaffold was treated with 10% EDTA, 1x10^6 DPSCs, and 5 ng/ml human recombinant TGF-1β. Following the removal of dentin scaffolds, a 2.5% glutaraldehyde solution was utilized to fix for two hours at 4°C. After dehydration in a series of ethanol, they were dried using hexamethyldisilane. SEM [JEOL JSEM-6510LV; JEOL, Tokyo, Japan] at faculty of Agriculture, Mansoura University was utilized to evaluate the scaffolds for cell adhesion, morphology, dentinal tubule opening, and the absence of the smear layer after gold coating.

TABLE (2): TGFβ1 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)	29.46 ±9.37	43.83 ±7.36	159.88 ±10.77*	122.39 ±20.2*

Data expressed as mean± SD, Significant difference detected by one-way ANOVA followed by post hoc (LSD test) at p ≤0.05, NT: Unconditioned dentin scaffold.*

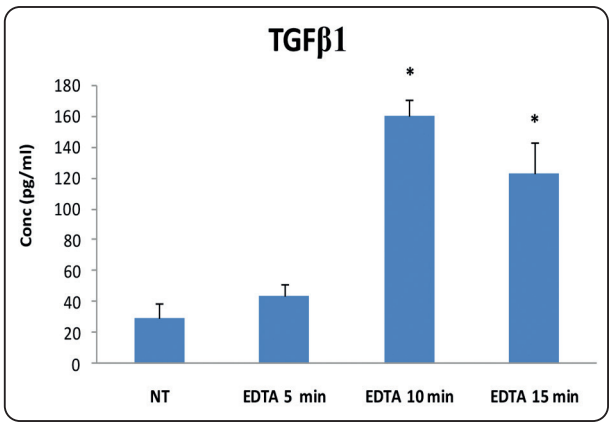

Fig. (1) TGFβ1 concentration released from dentin conditioned with EDTA for 5, 10 and 15 minutes.

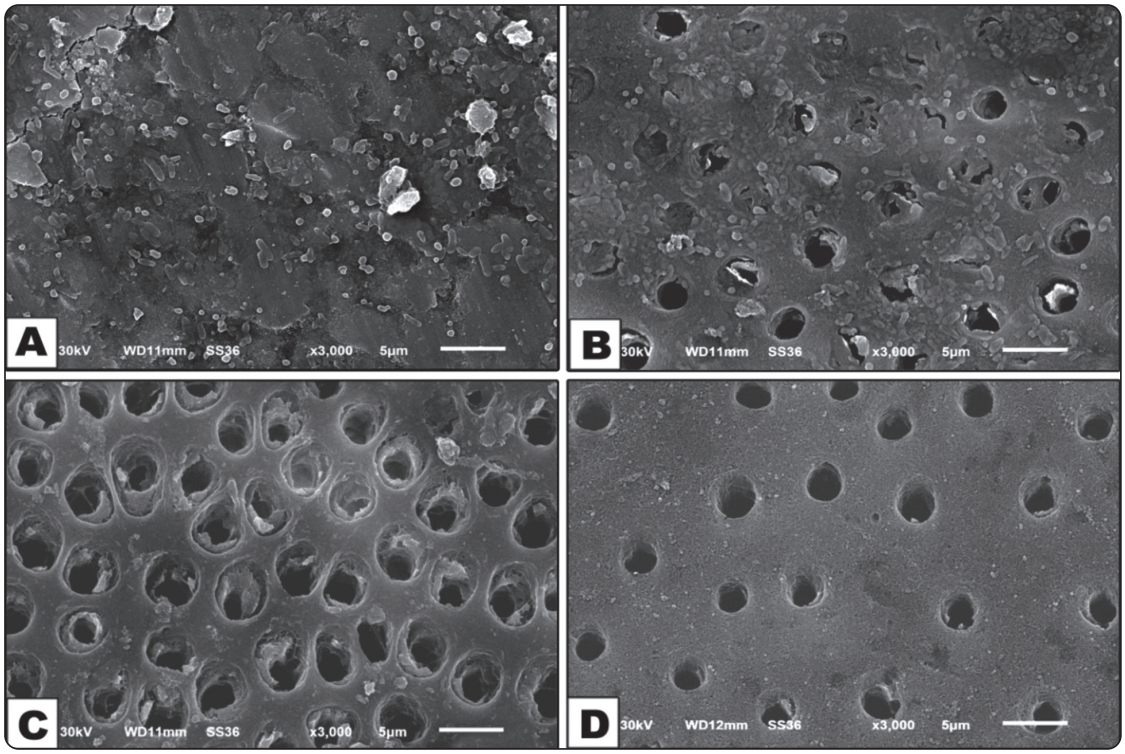

Fig. (2) Scanning electron microscopy showing a) unconditioned dentin scaffold, b) scaffold conditioned with EDTA for 5 min, c) 10 min, and d) 15 min.

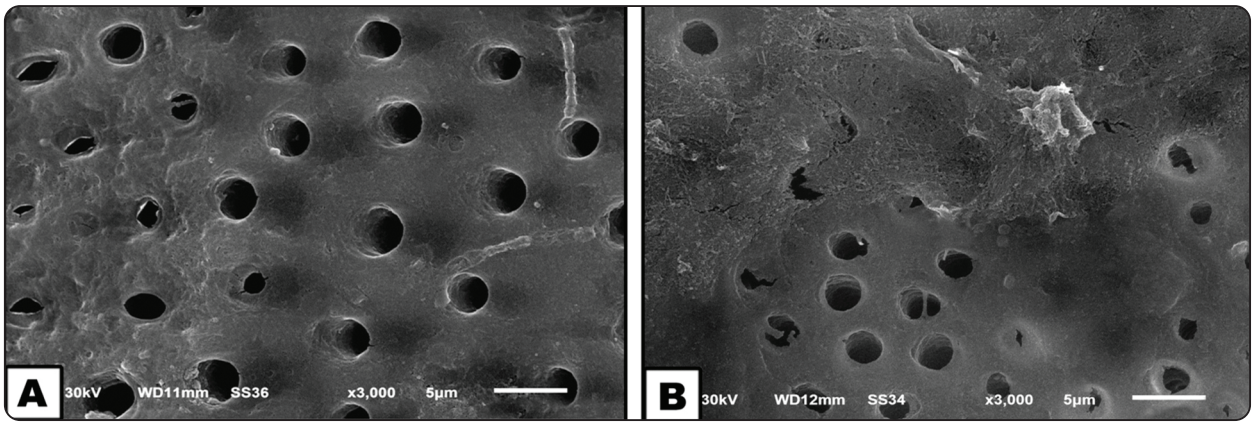

Fig. (3) Scanning electron microscopy for conditioned dentin scaffold with EDTA showing; a) dentin scaffold+ DPSCs group, and b) dentin scaffold+ DPSCs+ TGFβ1 group.

DISCUSSION

Conventional root canal therapy is removing inflammatory or necrotic pulp tissue and inserting a synthetic substance into the root canal system, therefore destroying the tooth pulp's natural shape and function. Dentin walls that are thin and funnel-shaped make obturation harder and root fractures are more frequent in teeth that have incomplete root. Calcium hydroxide or MTA can be used to create a calcified barrier as part of an apexification technique. But the length of therapy varies, the danger of fracture increases because of repetitive manipulation, and in most cases, the continuation of root growth cannot be maintained [20].

In these situations, complete dental pulp regeneration is very desirable. Dental revascularization procedures that involve non-symptomatic teeth, root creation, and healing even in patients with preoperative periapical lesions have recently been described in clinical trials [21]. Disinfection of the root canal system with provocation of bleeding into canals and coating with mineral trioxide aggregate are all part of the protocol. It is common practice in traditional root canal preparation to irrigate with NaOCl, which is commonly utilized at doses ranging from 0.5 percent to 6 percent [22]. NaOCl dissolves organic detritus and assists in the elimination of microorganisms as well as the clearance of necrotic tissue due to its high antibiotic and proteolytic action. Because NaOCl triggers an inflammatory response and causes significant damage when it comes into touch with important tissue, higher doses have the opposite effect of improving efficacy while also increasing toxicity [23].

After root canal preparation, NaOCl is not able to eliminate the smear layer that forms on the dentin. Toxic agents and the smear layer may generate adverse circumstances for apical stem cell migration, adhesion, and proliferation, which makes NaOCl an unreliable irrigation solution in this case since it might impair the outcome of any regeneration operation. Prior to obturation, the use of extra irrigates to ensure a clean and free of smear layer surface has frequently been recommended for standard root canal preparation [24]. Inorganic calcium phosphate crystal lattices are demineralized by chelating chemicals such as EDTA, which remove calcium from the crystal lattice. A clear dentin surface with exposed dentinal tubules is left behind after the loose smear layer is removed by EDTA [25].

Decalcification of dentin results in the emergence of collagen fibrils from the organic matrix, which contain adhesion motifs that allow cells to adhere to them via integrin receptors [26]. Additionally, EDTA treatment liberates growth factors that are trapped in the dentin matrix, such

as transforming growth factor-b (TFG-β1) [27]. Therefore, this study aimed to evaluate the impact of TFG-β1 released from dentin scaffold alone or combined with recombinant TFG-β1on DPSCs differentiation toward odontoblast- like cells.

In the present study, treatment of dentin scaffold with 17% EDTA for 5, 10 and 15 min showed removal of the smear layer with opened dentinal tubules using scanning electron microscopy (SEM). Dentinal tubules were various and wider in dentin treated with EDTA for 10 min.

Notably, dental pulp stem cells (DPSCs) play an important role in maintaining pulp homeostasis and repairing damage. According to mechanistic studies, the destiny of DPSCs is orchestrated by a regulatory network comprising of extrinsic and internal elements. As a result of these discoveries, we now have a better knowledge of how niche responsive progenitors' function in nature. Preclinical research has created a variety of DPSC transplantation-based techniques, among which preconditioned DPSCs and DPSC aggregates have shown significant promise as we investigate the potential of DPSCs in pulp regeneration. After pulpectomy, DPSC transplantation has effectively rebuilt the physiological pulp structure in situ, functionalized with neurovascularization, indicating an innovative method to treating pulp disorders [28].

In our study, DPSCs were grown on dentin scaffold after being conditioned with EDTA for 5, 10, and 15 min to assess the impact of dentin to organize the cell adhesion and differentiation into odontoblast-like cells. Dentin treated with EDTA for 10 min revealed the best cell viability. Moreover, DPSCs were cultured with dentin conditioned for 10 min for 7, 14, and 21. The significant viability was showed with cells cultured for 21 days.

In addition, Morphological observation by SEM showed large number of DPSCs with their extracellular matrix spread over dentinal tubules after 7 days as observed previously by Li et al. [29]. Besides, the normal shape of mesenchymal stem cells was seen at 7 days from culture. While at 14 and 21 days, calcified nodules was detected by inverted microscope. These findings corroborate Pang et al hypothesis that certain sticky and soluble substances that bind to cell surface receptors govern cell development and attachment to dentin. This adhesion is caused by EDTA conditioning, which extracts calcium from inorganic calcium phosphate and alters the surface characteristics of the dentin. As a result, the differentiation of cells adherent to dentin treated with EDTA different from cells linked to untreated dentin [30].

The findings of Galler et al. indicate that EDTA may promote dentin–pulp regeneration and enhance the attachment of newly formed tissue to the canal walls [31] , the dentin matrix is exposed and growth factors including transforming growth factor-b (TFG-β1) from the dentin matrix are released [32].

The transforming growth factor-(TGF-) superfamily consists of a collection of covalently linked growth factors that have been shown to influence cell differentiation, proliferation, and extracellular matrix (ECM) secretion[33]. Additionally, these growth factors may be involved in cellular signalling during odontogenesis and dental tissue healing. Differentiating odontoblasts express both transcripts and the protein TGF- 1 during tooth development [34]. The odontoblasts express transcripts encoding for TGF-βs, which may lead to confiscation of the growth factors from dentine matrix during matrix secretion [35]. The dentine matrix may contain a reservoir of TGF-β isoforms which may be released during carious demineralisation and could mediate dental tissue repair [36].

In this study, the concentration of TGF-β1 released from dentin matrix was evaluated using ELISA technique after dentin being treated with EDTA for 5, 10, and 15 min. With a high sensitivity, ELISA results showed a high concentration of TGF-β1 in dentin treated with EDTA for 10 min compared to the 5 and 15 min.

CONCLUSION

It has been shown thatTGF-Beta1 protein with DPSCs on dentin scaffold treated with EDTA 17 ٪, can efficiently produce odontoblast like cells with the functionality to produce dentin mineralization like matrix, on the contrary, this concentration of TGF Beta-1secreted by dentin scaffold after been conditioning by EDTA is not sufficient to induce the differentiation of DPSCs into odontoblast cells. the combination of 5ng/ml TGF beta-1 with the dentin scaffold significantly increases the differentiation capacity of DPSCs toward odontogenic linage and the formation of mineralization matrix. Another function for EDTA acid is to remove bacterial biofilm and smear layer inside the root canal also, EDTA is considered biocompatible with stem cells, and show no cytotoxicity 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 transforming growth factor beat-1 in the regeneration of dental pulp tissue and root canal treatment.

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