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Efficacy of Chitosan as Final Irrigating Solution on TGF-β1 Release from Root Canal Dentin and its Effect on Dental Pulp Stem Cells Response

Asmaa Abd El-Hady[1*], Wael H.Kamel[2], Mona H. Farid[3], Dina Sabry[4]

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azhardentj@azhar.edu.eg

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ABSTRACT

Purpose: To study the effect of Chitosan final irrigating solution compared with EDTA using different irrigation techniques, on TGF-β1 release from root canal dentin, using enzyme-linked immunosorbent assay (ELISA), and to evaluate the effect of released TGF-β1 on Dental Pulp Stem Cells. **Materials and Methods**: Human DPSCs were isolated from impacted third molars. Forty eight human root segments were prepared to a standardized truncated cone-shaped canal with an open apex of 1 mm diameter, the volume of prepared segments were determined using CBCT. Samples were randomized into two experimental groups (n=24) regarding the final irrigating solution; Group I: 1.5% NaOCl followed by 17% EDTA, Group II: 1.5% NaOCl followed by 1% Chitosan, then subdivided into subgroups A and B (n = 12) according to the final irrigation technique: A: Needle Irrigation, B: XP-Endo Finisher agitation. The released TGF-β1 were quantified using ELISA at 4 hours, 1 day, and 3 days following irrigation. DPSCs response to1.5% NaOCl, 17% EDTA, 1% Chitosan, and different concentrations of released TGF- β1 was assessed using MTT assay. **Results**: No statistically significant difference in the quantity of TGF-β1 released by 17% EDTA and 1% Chitosan following needle irrigation or XP Endo Finisher agitation. 1% Chitosan significantly increased DPSCs viability compared to17% EDTA ($P < 0.05$). TGF-β1 showed significant expansion of viable DPSCs in a positive correlation to its concentration ($P < 0.001$). **Conclusions:** 1% Chitosan effectively released TGF-β1 from root canal dentin as17% EDTA. The released TGF-β1 promoted DPSCs proliferation at picogram levels in a concentration dependent manner.

KEYWORDS

Chitosan, TGF-β1, Dental Pulp Stem Cells

- Paper extracted from Doctor Thesis titled "Efficacy of Chitosan as Final Irrigating Solution on TGF-β1 Release from Root Canal Dentin and its Effect on Dental Pulp Stem Cells Response"

1. Assistant Lecturer of Endodontics, Faculty of Dental Medicine for Girls, Al-Azhar University, Cairo, Egypt.
2. Professor and Head of Endodontic department, Faculty of Dental Medicine for Girls, Al-Azhar University, Cairo, Egypt.
3. Professor of oral and dental biology, Former Dean of the Faculty of Dental Medicine for Girls, Al-Azhar University, Cairo, Egypt.
4. Professor of medical biochemistry and molecular biology, Faculty of Medicine, Cairo University, Cairo, Egypt.

* Corresponding author email: dr.asmaa.amf08@gmail.com

INTRODUCTION

The paradigm of Regenerative Endodontics has gained researchers attention over the past few years, as it provides an alternative therapeutic option for the challenging immature permanent teeth with pulp necrosis. It follows principles of wound healing process including the recruitment and proliferation of dental stem cells, followed by their differentiation into mineralized tissue synthesizing cells along with regeneration of new pulp-like tissue inside the root canal space [1,2].

Pulp revascularization of immature permanent teeth is a possible cell homing strategy for pulp regeneration, proposed in clinical practice over the past decades. In this procedure, the root canal system is initially disinfected, then it is brimmed with a blood clot of provoked bleeding made from periapical tissues, in an attempt to revive tissues in the pulp space and continuation of root development [1, 3].

Successful pulp-dentin complex regeneration depends primarily on disinfection strategies of the root canal system and efficacy of smear layer removal, which promotes subsequent stem cell migration, adhesion and differentiation orchestrated by various signaling molecules and growth factors (GFs) at the dentin interface [4]. Although GFs have short half-life, the binding of bioactive GFs to dentin extracellular matrix proteins protects them from biodegradation and prolongs their life-time span, which could be released in active forms during carious process or following the use of certain dental materials having the ability to cause dentin demineralization; e.g.: Ethylene diamine tetraacetic acid (EDTA), etching adhesives, and calcium hydroxide[5-7].

A number of GFs have been identified and released from dentin matrix after dentin conditioning. Among them are; transforming growth factor- β (TGF-β) superfamily, fibroblast growth factor-2 (FGF-2), and several angiogenic growth factors as platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF). These GFs have been found to be effective at picograms level and play critical roles in wound healing and repair [4]. TGF-β1 is a multifunctional GF; it is chemotactic for stem cells of dental pulp and apical papilla and regulates their growth, odontoblast differentiation, mineral synthesis and apoptosis [4, 8-11].

The influence of various irrigating solutions on the release of GFs from dentin have been widely investigated over the past few years, with the findings of highest GFs being released following EDTA irrigation in comparison with sodium hypochlorite and chlorhexidine [4,7,8]. Also, the use of ultrasonic activation have been reported to enhance GFs release from dentin [12].

Studies aiming to find a more biocompatible solutions than EDTA were promising, with the natural substituents continually gaining more attention. Chitosan, besides being a natural polysaccharide obtained via partial deacetylation of chitin fond in crab and shrimp shells, and the second most abundant substance following cellulose, it possesses a high chelating property in acidic conditions in addition to being biocompatible, biodegradable, bio adhesive with lack of human toxicity and a broad spectrum antibacterial activity, collectively dependent on its chemical structure and the molecular size[13, 14].

The XP-Endo Finisher file has been innovated as an adjunctive tool to improve the efficiency of root canal irrigation. The ability of this MaxWire nickel-titanium rotary file to expand at body temperature and its tortuous motion have been demonstrated to have better smear layer removal[15,16] comparable to that of ultrasonic activation[17], in addition to better irrigation penetration into dentinal tubules with increased disinfection potency[18].

Hence, this study was directed to investigate the effect of Chitosan final irrigating solution compared with EDTA following needle irrigation or XP-Endo Finisher agitation, on TGF-β1 release from root canal dentine, using enzyme-linked immunosorbent assay (ELISA), and to evaluate the effect of released TGF-β1 on DPSCs. The first hypothesis was that

17% EDTA and 1% Chitosan would have similar effects on TGF-β1 release from root canal dentin and non-cytotoxic effects on DPSCs. The second hypothesis was that the released TGF-β1 would not promote DPSCs response.

MATERIAL AND METHODS

DPSCs isolation and identification

Four human impacted third molars from 3 donors (20 to 24 years of age, free of any systemic or oral diseases affecting dental tissues) were extracted and used for DPSCs isolation, following obtaining informed written consent considering usage of their donor of dental pulp; the study was approved by the Research Ethics committee, Faculty of Dental Medicine for Girls, Al-Azhar University (REC code EN-F-020-001). Immediately after extraction (at Oral and Maxillofacial Surgery Clinic, Faculty of Dental Medicine for Girls, Al-Azhar University.), teeth were cleaned with sterile gauze socked in 70% ethanol for 15 seconds, after which root surfaces were scrapped with a scalpel blade to remove any remnants of soft tissues. Each tooth was then externally sterilized using the following protocol: first, the tooth had several washes in sterile Phosphate Buffered Saline (PBS); next, immersed in 1% povidone iodine (PVP-I) for 2min, then immersed in 0.1% sodium thiosulfate (to neutralize the iodine) for 1 min, and finally washed with sterile PBS [19]. Pulp tissue was then obtained after splitting the tooth into two equal halves using Hand Held Pulp Isolator device (Shteiwi. A device, patent pending No. PCT/ EG2015 / 000026).

The obtained pulp tissue was then stored in sterile eppendorf containing 1ml a-MEM supplemented with 100 U/mL penicillin/streptomycin and transferred on ice to the Molecular Biology laboratory at the Faculty of Medicine, Cairo University, for isolation of DPSCs using enzymatic dissociation with 0.5mg Collagenase type I in 10ml PBS. DPSCs were characterized by their morphology using Inverted light microscope, and their expression of mesenchymal stem cell surface markers analyzed by flow cytometry. Cells at passage 3 were used in this study [19, 20].

Preparation of root segment model

Forty eight extracted human teeth that conformed to the following inclusion criteria: Single rooted permanent teeth without fractures, artificial alterations, or anatomic aberrations; were collected from the Oral and Maxillofacial Surgery Clinic, Faculty of Dental Medicine for Girls, Al-Azhar University, and this was performed with the approval of the Faculty Ethical Committee (REC code EN-F-020-001). Following sterilization according to the aforementioned protocol [19], teeth were decoronated, then root segments were standardized by cutting 12mm from apex with a double face diamond disk. All root segments were instrumented with hand files up to size 100 (Stainless steel K-file #100, MANI Inc. Japan.), using M4 Safety handpiece to guide hand files through the canals smoothly while applying very slight apical pressure, to acquire a standardized truncated cone-shaped canal having an open apex of 1 mm in diameter, and a total of 5 to 8 mL of 1.5% NaOCl (Clorox, Egyptian Company for household cleaners, 10th Of Ramadan city, Egypt.) per model was used as irrigating solution. The external root surfaces were painted with nail varnish, while leaving the inner root canal surface without varnish [21] (Fig. 1).

Root segments were embedded in a high-precision rubber base impression material that placed in plastic dental arches, and three-dimensional X-ray images using the i-CAT imaging system were obtained to determine the volume of each prepared root canal space, using field size of 16 cm diameter x 4 cm height, and the exposure time was 26.9 seconds, operating at 120 kV and 5 mA with slice thickness of 0.125 mm. The parameters including the length (L), coronal diameter (D), and apical diameter (d) were viewed, and measurements were performed by the in vivo 5 software (Anatomage, USA) (Fig. 2). The volume of canal space was determined as a truncated cone using the following formula: $V(\text{canal}) = \pi * L \{(D/2)^2 + (D/2)(d/2) + (d/2)^2\} / 3$ [21].

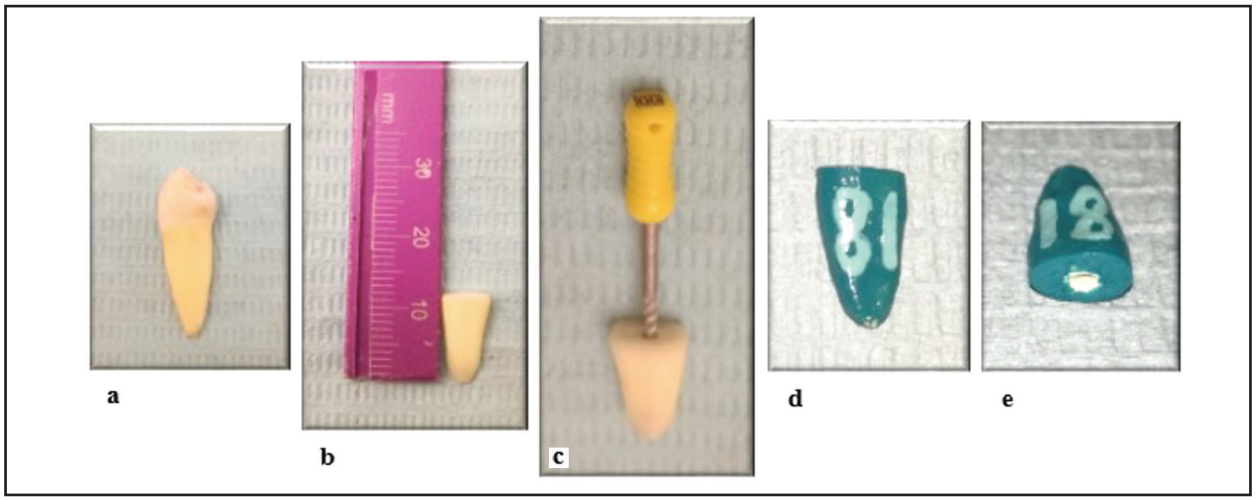

Figure (1)**:** A photograph showing root segment model preparation: a) sterilized single rooted permanent tooth, b) tooth was decoronated and the root segment standardized to 12mm, c) root canal was prepared up to size #100, d) and e) The external root surface was painted with nail varnish, leaving only the inner root canal surface without varnish.

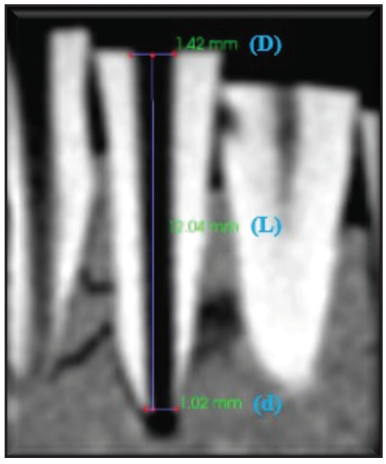

Figure (2)**:** CBCT post preparation image illustrating measurements used for calculating the volume of canal space. The length (L), coronal diameter (D), and apical diameter (d).

Samples grouping

Root segments were randomized into two experimental groups (n=24) according to the final irrigating solution; Group I: 1.5% NaOCl followed by 17% EDTA (Calix-E - EDTA Solution, Dharma Resources., USA.), and Group II: 1.5% NaOCl followed by 1% Chitosan (Chitosan from shrimp shells (low-viscosity Powder), Sigma-Aldrich., USA.), then subdivided into subgroups A and B (n = 12) according to the final irrigation technique: Subgroup A: Needle Irrigation, and Subgroup B: XP-Endo Finisher (0.0/25 Ni Ti Max Wire file, FKG Dentaire, La Chaux-de-Fonds, Switzerland) agitation. The sample size was calculated based upon the results of a study utilizing release of TGF-β1 as the primary outcome [21]. The minimum sample required was found to be 12 specimens per subgroup for a total of 48 specimens; which was based on 2 x 2 fixed effects analysis of variance; the first factor (Irrigating solution) includes 2 levels and the second factor (Irrigation technique) includes 2 levels, using alpha (α) level of 5% and power of 80%. Sample size calculation was performed using IBM® SPSS® Sample Power® Release 3.0.1.

Irrigation protocol and quantification of TGF-β1 release

The irrigation procedure were carried out under meticulous aseptic conditions inside the laminar flow hood (Thermo Scientific™ 1300 Series Class II, Type A2, Thermo Fisher Scientific Inc. Ohio, USA.), at the Molecular Biology laboratory, Faculty of Medicine, Cairo University. For Subgroups (SG IA and IIA): Root segments were first irrigated with (20 mL/5 min. each) of freshly prepared 1.5% sodium hypochlorite (NaOCl) solution (PH 12)

dispensed through a 30-gauge Navi-Tip flexible irrigation needle with closed end and side-vents, where the needle was inserted to 1 mm from root segment end. Then each root segment of SG IA received a final irrigation with 17% EDTA, (20 mL/5min) solution (PH 9.5). While SG IIA was finally irrigated with freshly prepared 1% Chitosan solution (PH 7), prepared by diluting 1gm of Chitosan powder in 100 mL of 1% acetic acid under magnetic stirring for 2 h [22].

For Subgroups (SG IB and IIB): final irrigating solutions were agitated for 1 minute using the XP-Endo Finisher file (size 25, taper 0.00) mounted in a 17:1 reduction handpiece that was powered by a torque-limited electric motor (E CONNECT; Eighteeth Medical Technology Co., Ltd.); at a rotational speed of 800 rpm and a torque-control of 1 N/cm as recommended by manufacturer's instructions. The XP-Endo Finisher file was inserted till 1 mm from root segment end with a gentle up and down motion.

Following irrigation, paper points were used to dry each root segment before being placed into 1 mL alpha-minimum essential medium (a-MEM) supplemented with 100 U/mL penicillin and 100 U/mL streptomycin; and incubated at 37°C for 4 hours, 1 day, or 3 days. At the end of every period, medium from 4 samples of each subgroup was obtained and the released quantity of TGF-β1 was assessed using enzyme-linked immunosorbent assay (ELISA), according to the manufacturer protocol (Human TGF-β1 ELISA Kit. Chongqing Biospes Co., Ltd. China). The optical density (O.D.) absorbance was immediately determined using a Microplate ELISA reader (STATFAX 2100, Wiener Lab Group, Argentina) reader at 450 nm, then the concentration of TGF-β1 was calculated. The obtained values were in accordance with observations from ELISA Kit standards provided by the manufacturer.

TGF-β1 concentration in each root canal space was then calculated using the formula:

$$C\ (canal) = C\ (ELISA) \times V\ (collecting\ Medium) / V\ (canal)$$ [21].

Data were collected and checked for normality using (Kolmogorov-Smirnov and Shapiro-Wilk tests). For each experimental group data were displayed as mean and standard deviation (SD) values, and analyzed using Mann-Whitney U test. Kruskal-Wallis test was used to compare between different time periods. Dunn's test was used for pair-wise comparisons when Kruskal-Wallis test is significant. The significance level was set at $P \leq 0.05$.

Viability and Proliferation of DPSCs (MTT Assay [23])

Cell proliferation and survival in response to 1.5% NaOCl, 17% EDTA, 1% Chitosan, and different concentrations of released TGF-β1 (517, 193, 165, 137.7, 109.6, 82 pg./ml) were studied using the MTT cell proliferation kit (Chongqing Biospes Co., Ltd. China); which is a rapid colorimetric assay that could be applied to evaluate cell proliferation and cytotoxicity. 3x 10^3 human DPSCs (3000 cells/well) were cultured in 96-well plate in a-MEM with 10%FBS. Following cell attachment to the plate, 100µl/well test solutions were added to the plate and incubated at 37 °C and 5% CO2 atmosphere, medium without GFs served as the control, then the MTT assay were performed following the protocol provided by the manufacturer and photometric detection of the absorbance was accomplished using micro plate ELISA reader (STATFAX 2100, Wiener Lab Group, Argentina.) at 570 nm, where higher optical absorption density (O.D) indicates that the cell proliferation is more sooner; while, the cell cytotoxicity is more bigger when the O.D. value is much lower. Data of light absorption of different test solutions were presented as mean and standard deviation (SD) values, and analyzed using Repeated measure ANOVA. The significance level was set at $P < 0.05$.

RESULTS

Morphology and flow cytometry of isolated DPSCs: (Fig. 3)

Regarding cellular morphology, Inverted light microscope showed DPSCs having rounded appearance early in culture. After 1 week, colonies of stellate-shaped cells having polygonal cell bodies with multiple long-stretching processes were formed. At day 14, DPSCs showed typical elongated cellular appearance. Concerning the expression of stem cell markers, flow cytometric analysis showed high levels of positive expression for CD90 and CD105 (97% & 94% respectively) while being negative for CD34 and CD45 hematopoietic markers (0.7% & 1.1% respectively).

Released TGF-β1concentration: (Table 1) and (Fig. 4)

The volume of each canal space which was calculated as a truncated cone shape, has been used to convert the concentration of growth factors from ELISA to that in canal space; and the average volume of the prepared root canal space was 14.08 ± 2.76 mm^3.

The comparison between mean TGF-β1 concentrations at different time periods revealed that at 4 h after irrigation, 1.5% NaOCl + 17% EDTA using XP-Endo Finisher agitation had released the greatest amount of TGF-β1 (159 pg/ml) followed by 1.5%NaOCl + 1%Chitosan Needle irrigation (148.9 pg/ml), however the result was not statistically significant. At 1 day, 1.5%NaOCl + 1%Chitosan using XP-Endo Finisher agitation released the greatest amount of TGF-β1 (150.3pg/ml), which was also not statistically significant. At 3 days, both 1.5% NaOCl + 17% EDTA and 1.5%NaOCl + 1%Chitosan using XP-Endo Finisher agitation had released the greatest amounts of TGF-β1 (151 pg/ml and 148.9 pg/ml) respectively, but also the result was not statistically significant.

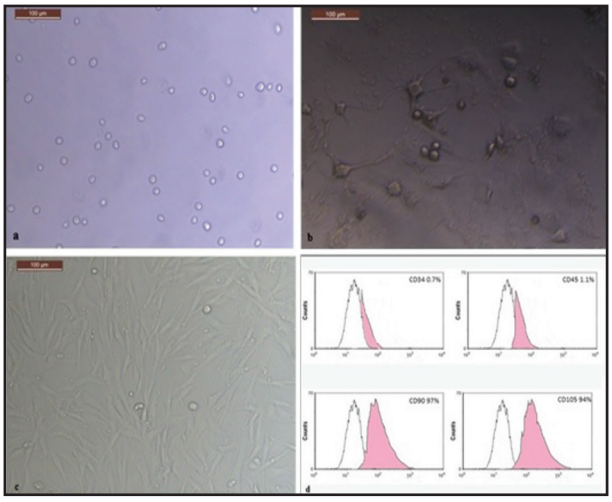

Figure (3): Morphology of DPSCs isolated by means of enzymatic digestion (DPSC-EZ) using inverted light microscopy. (a) Rounded cells early in culture (1 day). (b) Typical colonies of stellate-shaped pulp cells having polygonal cell bodies with multiple long-stretching processes. (c) Typical elongated cellular appearance, late in culture (14 days). Bars 100μm. (d) Cytometric graph represents negativity of CD34 & CD45, and positivity of CD90 & CD105 markers.

Table (1). *Comparison of the mean and standard deviation (SD) for TGF-β1 concentrations at different time periods.*

Irrigation solution	Irrigation technique	4 hours		1 day		3 days		*P*-value	Effect size *(Eta squared)*
		Mean	SD	Mean	SD	Mean	SD		
NaOCl + EDTA	Needle irrigation	139.5	20.5	136.8	47.5	136.9	16.9	0.944	0.209
	XP-Endo Finisher	159	25.5	136.4	25.8	151	9.8	0.492	0.073
NaOCl + Chitosan	Needle irrigation	148.9	20.6	112.4	11.3	120.8	30.1	0.105	0.278
	XP-Endo Finisher	139.9	28.4	150.3	30.6	148.3	23.5	0.668	0.132

**: Significant at P ≤ 0.05*

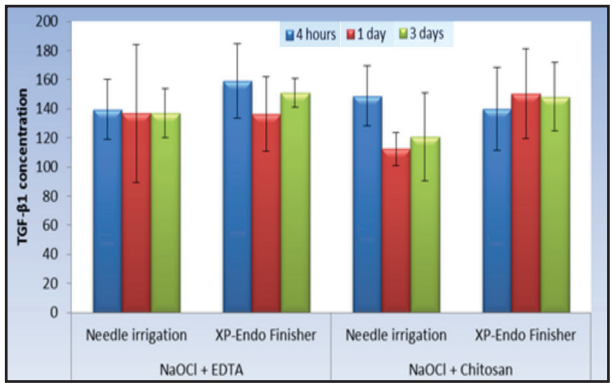

Figure (4): Bar chart representing mean and standard deviation values for released TGF-β1 concentrations at different time periods.

Viability and Proliferation of DPSCs (MTT Assay): (Fig. 5 and 6)

Comparisons of the mean Optical density (OD) obtained after 24h of treatment with1.5% NaOCl, 17% EDTA, and 1% Chitosan revealed statistically significant differences among all tested solutions ($P < 0.05$). 1% Chitosan showed significant increase in the mean viable DPSCs (0.73) compared to other test and control groups. While treatment with 17% EDTA resulted in slight increase in the mean viable DPSCs (0.164), which was not significant than the control group (P-value = 0.314). Contrary1.5% NaOCl showed the lowest mean DPSCs survival (0.042) that was significantly lower than the control group (0.122) with P-value <0.001.

Considering DPSCs response to different concentrations of the released TGF-β1, the results revealed a gradual increase in the mean absorbance with increasing TGF-β1 concentration. The difference between mean absorbance for the control group was significantly lower than that of TGF-β1 Conc. 193 pg/ml (P-value = 0.004), and TGF-β1 Conc. 517 pg/ml (P-value = 0.001). Also, the mean absorbance values for TGF-β1 Conc. 517 pg/ml were significantly higher than 193 pg/ml (P-value = 0.032), as well other tested TGF-β1 Conc. (P-value = 0.001). While the mean absorbance values for TGF-β1 Conc. 193 pg/ml were only significantly higher than TGF-β1 Conc. 109.6 pg/ml (P-value = 0.008). The mean absorbance values for TGF-β1 concentrations of (165, 137.7, 109.6, 82 pg. /ml) were not significantly different than the control group.

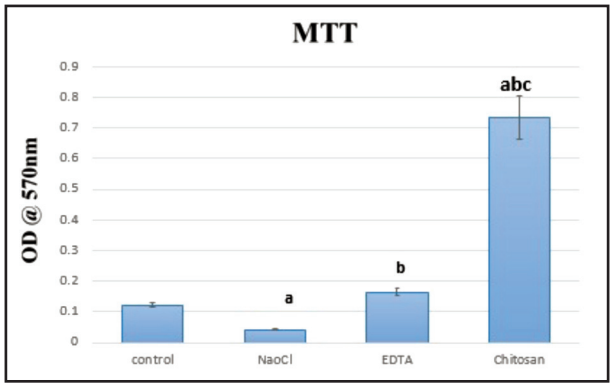

Figure (5): Means of Optical density at 570 nm measured on micro-plate reader at 24h for control and test groups (1.5% NaOCl, 17% EDTA, and 1% Chitosan); small letters denote significant difference at $P < 0.05$.

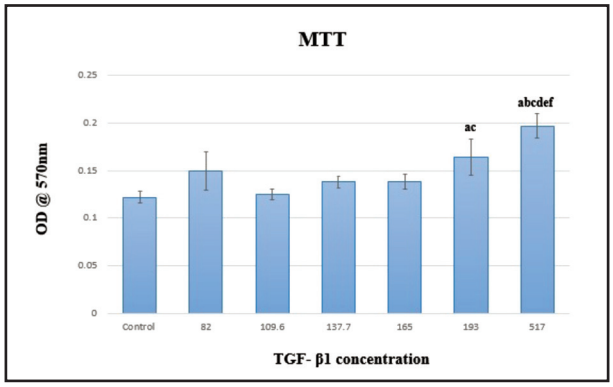

Figure (6): Means of Optical density at 570 nm measured on micro-plate reader for different concentrations of released TGF-β1; small letters denote significant difference at $P < 0.05$.

DISCUSSION

In order to optimize conditions for cellular recruitment, adhesion, proliferation differentiation and new tissue regeneration, a final irrigation step using biocompatible solutions having the ability of smear layer removal, exposure of dentinal tubules

and collagen fibrils, besides releasing growth factors fossilized in the dentin matrix might be crucial for the success of current regenerative endodontic protocols.

The chelating properties of EDTA and Chitosan solutions, and their effects on root canal dentin have been widely investigated [14, 24, 25]; however no study has been reported in the literature comparing their ability to release growth factors from root canal dentin. In the present study, 1.5% NaOCl followed by 17% EDTA was used based on the latest clinical considerations for Regenerative Endodontic Procedures proposed by the American Association of Endodontists (AAE) [26]. While 1% Chitosan solution was chosen owing to its ability to remove smear layer as EDTA, besides being antibacterial and biocompatible natural material[13,14]. In addition, the XP-Endo Finisher file has been used for activation of the final irrigants used because of its ability to enhance smear layer removal and disinfection properties of irrigating solutions [15-18].

TGF-β1 was evaluated as a model for released growth factors firstly because of its regularity effects in immune response and repair processes, including cell proliferation, migration differentiation, and apoptosis, besides influencing extracellular matrix synthesis and degradation [5, 8]. Secondly it was investigated owing to its surpassing release from demineralized dentin matrix compared to other GFs, as described in previous studies [7, 10, 12]. The results demonstrated that 17% EDTA and 1% Chitosan either with needle irrigation, or XP-Endo Finisher agitation released comparable amounts of TGF-β1, accordingly the first null hypothesis was affirmed. This could be attributed to the similarity in demineralizing efficiency of both solutions that have been reported in previous studies testing their abilities for smear layer removal following root canal preparation [14, 25].

The amount of released TGF-β1 assessed in the present study may conceivably minimal compared to previous studies that evaluated growth factors release following dentin conditioning with EDTA, which could be attributed to differences in methodology, donor age, and volume of the prepared root canal space [10,21]. The majority of previous studies have used either dentin powder, or dentin disks that released GFs from all surfaces into immersing conditioning solutions in which GFs have been quantified [7,10]. While the current study assessed TGF-β1 released into root canal space using root segment models that have been painted with nail varnish except for its inner root canal surfaces, which may closely resemble the clinical scenario. Also, the use of NaOCl before 17% EDTA and 1% Chitosan final irrigation, could be another contributing factor due to its detrimental effect on proteins, thus lowers subsequently released growth factors, hence it was recommended to restrict its use to the first visit [7,26].

The use of XP-Endo Finisher gitation showed an increase in the released TGF-β1 concentration, which might be related to an improved irrigation penetration into the dentinal tubules. However such increase was not statistically significant, which could be attributed to the large diameter of the prepared root canal space that facilitated contact of chelating agents along the entire canal surface area.

Returning to regenerative endodontic literature, DPSCs have been recognized for its high proliferative and multilineage differentiation capacities that made dental pulp tissue engineering possibly achieved. In the present study DPSCs were isolated from impacted third molars in the range of 20 to 24 years of age where they remain undifferentiated and dormant, since third molars start to develop at the age of six and the last permanent teeth to fully develop; concurrently with previous studies suggesting 25 years old as a maximum donor age in order to isolate impeccable stem cells from permanent teeth [27, 28]. As regards morphological characteristics of cultured DPSCs, polygonal cells with multiple long-stretching processes, and elongated fibroblast-like cells seen in culture were consistent with observations reported in previous

studies. With respect to the expression of stem cell markers, DPSCs were strongly positive for CD90 and CD105, while showed extremely low levels of expression for CD34 and CD45 hematopoietic markers. These observations were in agreement with previous studies and general characteristics of mesenchymal stem cells [27, 29].

In terms of cytotoxic effect of irrigating solutions used in this study, DPSCs directly exposed to 1.5% NaOCl showed the least viable cells, whereas 17% EDTA showed similar DPSCs viability as the control. These findings were in accordance with a previous study evaluating the cytotoxic effects of different irrigating solutions on stem cells from the apical papilla (SCAP) [30]. On the other hand, 17% EDTA and NaOCl in concentrations of 1% or higher have been reported to be severely cytotoxic which could be related to differences in experimental conditions and used cell lines [31]. As regards 1% Chitosan, the results partially rejected the null hypothesis since it showed significant increase in DPSCs viability compared to 17% EDTA and control, which indicated an increase in DPSCs metabolic activity. This was in agreement with a previous study that cultured DPSCs in mineralizing medium supplemented with 5 or 10 μg/ml chitosan and reported that chitosan could stimulate in vitro DPSCs proliferation and early osteogenic differentiation [32]. Another recent study revealed conflicting results, where 0.2% Chitosan and 17% EDTA showed similar increase in the number of viable cells. This might be explained by the use of lower concentration of chitosan, besides using cytotoxic test model other than DPSCs that might have different sensitivity towards the tested solutions [33].

Within this study, the released TGF-β1 in the concentration range of 82–517 pg. /ml were shown to enhance DPSCs metabolic activity suggesting DPSCs proliferation and increased cell numbers. This could be explained by biological interaction of TGF-β1 molecule with DPSCs cell surface receptors with activation of signaling pathways that balances DPSCs self-renewal and proliferation. The effect was concentration dependent with 517 pg. /ml demonstrating the greatest metabolic activity, which demonstrates that the released TGF-β1 was functioning and able to stimulate DPSCs proliferation at very low concentrations, hence rejecting the null hypothesis. The observed expansion in cell numbers was in line with previous studies which reported the presence of a variety of growth factors in conditioning solution following dentin matrix demineralization, and these GFs were effective and elicited cell recruitment, proliferation and differentiation at the picogram levels [4, 5, 8-10].

CONCLUSIONS

Within the limitations of this study it could be conclude that:

1. Final irrigation step with 1% Chitosan was able to release TGF-β1 from root canal dentin comparable to 17% EDTA, but additionally having more biological activity.
2. The use of XP-Endo Finisher agitation would clinically improve growth factor release in regenerative procedures.

Although the released TGF-β1 was functioning, further investigations of its functionality should be considered.

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CONFLICT OF INTEREST

The authors declare no potential conflicts of interest regarding specific financial interests, the authorship and/or publication of this article.

REFERENCES

1. Eramo S, Natali A, Pinna R, Milia E. Dental pulp regeneration via cell homing. Int Endod J. 2018; 51:405-19.
2. Saoud TMA, Ricucci D, Lin LM, Gaengler P. Regeneration and Repair in Endodontics-A Special Issue of the Regenerative Endodontics-A New Era in Clinical Endodontics. Dent J (Basel). 2016; 4:3.
3. Galler KM, Krastl G, Simon S, Van Gorp G, Meschi N, Vahedi B, et al. European Society of Endodontology position statement: Revitalization procedures. Int Endod J. 2016; 49:717-23.
4. Galler KM, Widbiller M, Buchalla W, Eidt A, Hiller KA, Hoffer PC, et al. EDTA conditioning of dentine promotes adhesion, migration and differentiation of dental pulp stem cells. Int Endod J. 2016; 49:581-90.
5. Smith AJ, Duncan HF, Diogenes A, Simon S, Cooper PR. Exploiting the Bioactive Properties of the Dentin-Pulp Complex in Regenerative Endodontics. J Endod. 2016; 42:47-56.
6. Gougousis K, Giannakoulas DG, Taraslia V, Agrafioti A, Anastasiadou E, Kontakiotis EG. Number of Dental Stem Cells on Root Canal Dentin after Application of Triple Antibiotic Paste or Calcium Hydroxide: An In Vitro Study. Eur J Dent. 2019; 13:161-5.
7. Galler KM, Buchalla W, Hiller KA, Federlin M, Eidt A, Schiefersteiner M, et al. Influence of root canal disinfectants on growth factor release from dentin. J Endod. 2015; 41:363-8.
8. Galler KM, D'Souza RN, Federlin M, Cavender AC, Hartgerink JD, Hecker S, et al. Dentin conditioning codetermines cell fate in regenerative endodontics. J Endod. 2011; 37:1536-41.
9. Ivica A, Zehnder M, Mateos JM, Ghayor C, Weber FE. Biomimetic Conditioning of Human Dentin Using Citric Acid. J Endod. 2019; 45:45-50.
10. Sadaghiani L, Gleeson HB, Youde S, Waddington RJ, Lynch CD4, Sloan AJ. Growth Factor Liberation and DPSC Response Following Dentine Conditioning. J Dent Res. 2016; 95:1298-307.
11. Wattanapakkavong K, Srisuwan T. Release of Transforming Growth Factor Beta 1 from Human Tooth Dentin after Application of Either ProRoot MTA or Biodentine as a Coronal Barrier. J Endod. 2019; 45:701-5.
12. Widbiller M, Eidt A, Hiller KA, Buchalla W, Schmalz G, Galler KM. Ultrasonic activation of irrigants increases growth factor release from human dentine. Clin Oral Investig. 2017; 21:879-88.
13. Yadav P, Chaudhary S, Saxena RK, Talwar S, Yadav S. Evaluation of Antimicrobial and Antifungal efficacy of Chitosan as endodontic irrigant against Enterococcus Faecalis and Candida Albicans Biofilm formed on tooth substrate. J Clin Exp Dent. 2017; 9:e361-e7.
14. Kmiec M, Pighinelli L, Tedesco MF, Silva MM, Reis V. Chitosan-properties and applications in dentistry. Adv Tissue Eng Regen Med Open Access. 2017; 2:205-11.
15. Zand V, Mokhtari H, Reyhani MF, Nahavandizadeh N, Azimi S. Smear layer removal evaluation of different protocol of Bio Race file and XP-endo Finisher file in corporation with EDTA 17% and NaOCl. J Clin Exp Dent. 2017; 9: e1310-e4.
16. Elnaghy AM, Mandorah A, Elsaka SE. Effectiveness of XP-endo Finisher, EndoActivator, and File agitation on debris and smear layer removal in curved root canals: a comparative study. Odontology. 2017; 105:178-83.
17. Zhao Y, Fan W, Xu T, Tay FR, Gutmann JL, Fan B. Evaluation of several instrumentation techniques and irrigation methods on the percentage of untouched canal wall and accumulated dentine debris in C-shaped canals. Int Endod J. 2019; 52:1354-65.
18. Azim AA, Aksel H, Zhuang T, Mashtare T, Babu JP, Huang GT. Efficacy of 4 Irrigation Protocols in Killing Bacteria Colonized in Dentinal Tubules Examined by a Novel Confocal Laser Scanning Microscope Analysis. J Endod. 2016; 42:928-34.
19. Perry BC, Zhou D, Wu X, Yang FC, Byers MA, Chu TM, et al. Collection, cryopreservation, and characterization of human dental pulp–derived mesenchymal stem cells for banking and clinical use. Tissue Eng Part C Methods. 2008; 14:149-56.
20. Elgendy AA, Fayyad DM. Cell viability and apoptotic changes of dental pulp stem cells treated with propolis, chitosan, and their nano counterparts. Tanta Dent J. 2017; 14:198-207.
21. Zeng Q, Nguyen S, Zhang H, Chebrolu HP, Alzebdeh D, Badi MA, et al. Release of Growth Factors into Root Canal by Irrigations in Regenerative Endodontics. J Endod. 2016; 42:1760-6.
22. Silva PV, Guedes DF, Pécora JD, Cruz-Filho AM. Time-dependent effects of chitosan on dentin structures. Braz Dent J. 2012; 23:357-61.

23. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods. 1983; 65:55-63.

24. Mittal A, Dadu S, Yendrembam B, Abraham A, Singh NS, Garg P. Comparison of new irrigating solutions on smear layer removal and calcium ions chelation from the root canal: an in vitro study. Endodontology. 2018; 30:55-61.

25. Mathew SP, Pai VS, Usha G, Nadig RR. Comparative evaluation of smear layer removal by chitosan and ethylenediaminetetraacetic acid when used as irrigant and its effect on root dentine: An in vitro atomic force microscopic and energy-dispersive X-ray analysis. JCD. 2017; 20:245-50.

26. American Association of Endodontists (AAE). Clinical Considerations for a Regenerative Procedure:Revised2018. https://www.aae.org/specialty/wpcontent/uploads/sites/2/2018/06/ConsiderationsForRegEndo_AsOfApril2018.pdf (accessed on 30 March 2020)

27. Rodas-Junco BA, Villicaña C. Dental Pulp Stem Cells: Current Advances in Isolation, Expansion and Preservation. Tissue Eng Regen Med. 2017; 14:333-47.

28. Aydin S, Şahin F. Stem Cells Derived from Dental Tissues. Adv Exp Med Biol. 2019; 1144:123-32.

29. Weiss JB, da Silva Gonçalves F, da Costa Maranduba CM, de Resende LM, do Carmo AM. Isolation and characterization of stem cells derived by human dental pulp from harvest based in rotary and manual techniques used in endodontic therapy. Braz Dent Sci. 2020; 23:10.

30. Trevino EG, Patwardhan AN, Henry MA, Perry G, Dybdal-Hargreaves N, Hargreaves KM, et al. Effect of irrigants on the survival of human stem cells of the apical papilla in a platelet-rich plasma scaffold in human root tips. J Endod.2011; 37:1109–15.

31. Koulaouzidou EA., Margelos J., Beltes P. and Kortsaris AH. Cytotoxic effects of different concentrations of neutral and alkaline EDTA solutions used as root canal irrigants. J Endod. 1999; 25:21-3.

32. Amir LR, Suniarti DF, Utami S, Abbas B. Chitosan as a potential osteogenic factor compared with dexamethasone in cultured macaque dental pulp stromal cells. Cell Tissue Res. 2014; 358:407-15.

33. Pivatto K, Pedro FL, Guedes OA, Silva AF, Piva E, Pereira TM, et al. Cytotoxicity of chelating agents used in endodontics and their influence on MMPs of cell membranes. Braz Dent J. 2020; 31:32-6.