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Effect of different irrigation protocols on Transforming growth factor β1 release from dentin matrix and its relation to pulp regeneration (In –vivo Study)

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Abstract

Introduction: The aim of this study was to investigate the effect of different irrigation protocols on the release of Transforming growth factor β1 (TGF-β1) from the dentin matrix and on the regenerative potential of stem cells. Methods: A total of 48 human permanent anterior teeth were used in this study. Decoronation was done, two discs from each root were obtained for each evaluation part of the experiment and randomly subjected to 5 different irrigation protocols to be evaluated for TGF-β1 release using an ELISA test system. Stem cells from human deciduous exfoliated teeth (SHED) were isolated and root disc model construct for animal study was prepared and implanted into the subcutaneous space of rats. After 30 days, the root discs were retrieved and sent for histological staining for evaluation of regenerative potential of SHED. Results: Samples irrigated by (Chorohhexidine gluconate 2% & 17% EDTA) to have a significantly higher value of released (TGF-β1) than samples of other groups. The highest total blood count was shown in Chorohhexidine gluconate 2% & 17% EDTA group which had a significantly higher value than samples irrigated by 5.5% NaOCl & 17% EDTA. The highest area fration was showed in samples irrigated by deionized water which had a significantly higher value than samples irrigated by Chorohhexidine gluconate 2%. Samples of deionized water group appeared to show higher positive reaction than other groups. Conclusions: Chlorohexidine gluconate 2% could be used in regenerative endodontic procedures with EDTA 17% solution as a final rinse in purpose of increase TGF-β1 release and regenerated blood vessels number. SHED can be used as a postnatal source of stem cells for differentiation into odontoblast like cells.

Key Words

Growth factors, irrigation, regenerative endodontics, stem cells

Vital pulp is essential for maintaining normal tooth functions, such as sensory functions, formation of dentin, blood nourishment, and immune defense reactions(1). Regenerative endodontics by revascularization and tissue-engineering strategies have gained increased attention as a direct solution for this eternal challenge. Regeneration of a pulp-dentin complex needs all components of the tissue engineering triad: stem cells, growth factors, and scaffolds. SHED represent a population of multipotent stem cells that are perhaps more immature than other dental stem cells such as dental pulp stem cells (DPSC)(2). SHED present a remarkable advantage over DPSC as they can be retrieved from naturally exfoliated teeth, which are one of the only disposable post-natal human tissues(3). Several previous studies have focused on the regenerative potential of different cytokines and growth factors designed to improve cellular chemotaxis and cell homing into the emptied dental pulp space in vivo (4-7). The releasable growth factors from dentine matrix provide a possible source of cell signaling molecules for initiating dentine synthesis in trauma/necrosis situations. Differentiation of a new population of odontoblast-like cells is considered as the main objective of endodontic regenerative procedures. The release of TGF- β1 and other different growth factors can occur pathologically during dental caries and following restorative procedures(8). From a clinical perspective, the use of appropriate irrigation solutions could release the TGF- β1 stored in the dentin, thus contributing to successful regenerative endodontic treatment(7). Many irrigants have been used during mechanical root canal preparation, for example, but not as a limitation, sodium hypochlorite (NaOCl), ethylene diamine tetraacetic acid (EDTA) and chlorohexidine gluconate (CHX). Each of them has a distinct role in root canal surface treatment. NaOCl is the most commonly used antiseptic irrigating solution in root canal therapy. Sodium hypochlorite with concentrations of 1–6% have been used in RET(9). It is used during root canal instrumentation to promote debridement, lubrication, disinfection, tissue dissolution and collagen layer removal and dentin dehydration(10). EDTA conditioning of dentine promotes adhesion, migration and differentiation of the pulp stem cells from permanent teeth(8). In addition, various soluble proteins extracted from the dentine matrix are able to exert a direct biological effect on dental pulp stem cells in promoting mineralized tissue repair mechanisms(11). CHX was suggested to prevent microbial activity in vivo with residual effects in the root canal system up to 48 h(12). It has been used clinically in REPs at the concentrations of either 0.12% or 2% (9). It is important to know whether different endodontic irrigation protocols that could be used during endodontic regeneration procedures, have an effect on the amount of TGF- β1 released from dentine matrix into the root canal space and if these affect the regeneration potential of SHED in vivo. It is also beneficial to know if there is a direct relation between the amount of TGF- β1 released and the regenerative potential of SHED. The aim of this study was to investigate the effect of different irrigation protocols on the release of transforming growth factor β1 from the dentin matrix and the in vivo regenerative potential of stem cells.

Materials and Method

Part I: Effect of different irrigation protocols on TGF-β1) release from dentin matrix:

A. Sample selection and preparation:

Fourty eight freshly extracted human permenant anterior teeth were collected. The periodontal soft tissue were removed by scrapping the root surface with a scalpel blade and calculus was removed with a periodontal curette. For all teeth, the coronal portions were removed and root canal space of each

root segment was instrumented with hand files up to size 100 to achieve a standardized truncated cone-shaped canal with open apex of 1 mm in diameter (13) using step back technique by K-files. Root dentin discs were horizontally sectioned in mid root region using a diamond saw at low speed.

B. Immersion in different irrigation solutions groups:

Forty eight eppendorf tubes; each contained two discs, were randomly allocated to 5 groups with different irrigation protocols (Fig.1) as group 5 served as control group; 8 tubes in each group from 1 to 4 and 16 tubes in group 5, each disk was immersed in 20 mL test solution for 5 minutes(13):

1)5.5% NaOCl, 2)5.5% NaOCl followed by 17% EDTA, 3)Chorohhexidine gluconate 2% (14), 4)Chorohhexidine gluconate 2% followed by 17% EDTA, 5)Deionized water. (As control group)

C. Evaluation of TGF-β1 release:

48 dentin discs of part I samples were evaluated for TGF-β1 release using ELIZA. The discs were kept at 37º C for 1 day and at the end of the time point, medium from samples was collected and filtered (13). After completion of sample collection, all samples were thawed and subjected to growth factor quantification by using an ELISA test system for TGF-β1 following the protocol provided by the manufacturer.

Part II: Effect of different irrigation protocols on regenerative potential of stem cells from human exfoliated deciduous teeth (SHED):

1. Preparation of implant construct model:

This work was performed in the Unit of Biochemistry and Molecular Biology at The Medical Biochemistry Department, Faculty of Medicine, Cairo University.

A. Sample collection:

Normal human deciduous incisors were collected from 6 to 8 years old individuals at the dental clinic of pedodontics department, faculty of dentistry, Ain Shams University with strict adherence to the guidelines approved by the Ethics Committee of Ain Shams University, Cairo, Egypt. Consent of the guardians of the individuals was obtained prior to sample extraction.

B. Isolation and culturing of SHED:

SHEDs were isolated and cultured according to the guidelines from established protocols with certain adjustments(15). Under aseptic conditions, the dental pulp cavity of the crown was opened using drills. The pulp was extracted with a broach, immediately placed in Dulbecco's modified Eagle's medium (DMEM)/ F12 and transported to the laboratory.

C. SHED charachterization and Flow cytometry:

Cells were identified as being MSCs by their morphology and plastic adherence. Using flow cytometric analysis, expression of several mesenchymal stem cell markers was quantified. Cell analysis was performed using Flow Cytometer and analyzed using CXP Software.

D. Observation of cell morphology:

In order to observe cell morphology, cells were seeded at 1×10^5 cells/well and incubated in growth medium and observed using inverted light microscope with a digital camera for capturing images.

E. Scaffold and Root disc model preparation for animal study:

HydroMatrix™ Peptide Cell Culture Scaffold (16) was prepared according to manufacturer's instructions. 48 dentin discs of part II experiment (mentioned in part I) were used for preparation of implant construct model that were used in animal study. After socking the root discs in different irrigation protocols each root disc was ready to receive scaffold/ SHED mixture. Discs were allocated into 6 groups, the first five groups as in part I with scaffold/SHED mixture, and the sixth group with immersed in deionized water without scaffold/SHED mixture as negative control.

2. **Animal Study:**

The animal study outlined conformed to the medical research center, faculty of medicine, Ain shams university. Disposal of sacrificed bodies was done by the incinerator of Ain Shams University. The Research Ethics Committee of Ain Shams University accepted the proposal.

A. Surgical procedure:

After a week of acclimatization, eight rats were immunosupressed (17). The root disc constructs from the first five groups were then implanted into the subcutaneous space of the incision sites in rats. Eight rat received five tooth constructs, one from each group. The last 2 rats received 4 samples each from negative control group. The wounds were sutured to obtain primary closure.

B. Sample collection:

After 30 days, rats were euthanized and then root dentin discs were retrieved and freed from any skin attached carefully. The rest of rats' bodies were appropriately gotten rid of in the incinerator of Ain- Shams Hospital.

3. **Evaluation of regenerative potential of SHED: H&E and Masson Trichrome stain:**

Three sections obtained from each specimen were stained by H&E stain (18) and Masson's trichrome(19) for histological analysis of total number of vessels presented in disc lumen and area fraction of the newly formed collagen fibers. All stained sections were mounted and examined under light microscope. Numerical data were used for calculating the mean and median values and using Kolmogorov-Smirnov and Shapiro-Wilk tests.

Results

I. Release of transforming growth factor β1 (TGF-β1): Pairwise comparisons showed samples irrigated by (Chorohhexidine gluconate 2% & 17% EDTA) (124.98±8.36) to have a significantly higher value of released (TGF-β1) than samples of other groups ($p<0.001$).

II. Isolation and cultivation of Stem cells from human exfoliated deciduous teeth:

- SHED charachterization and Flow cytometry

The isolated cells were analyzed by flow cytometry to determine the expression of cell surface markers. The analysis indicated that the cells highly expressed CD90 (89.4±3.6%), CD29 (99.7±0.2%) and CD45 (1.0±0.6%) (Fig. 11).

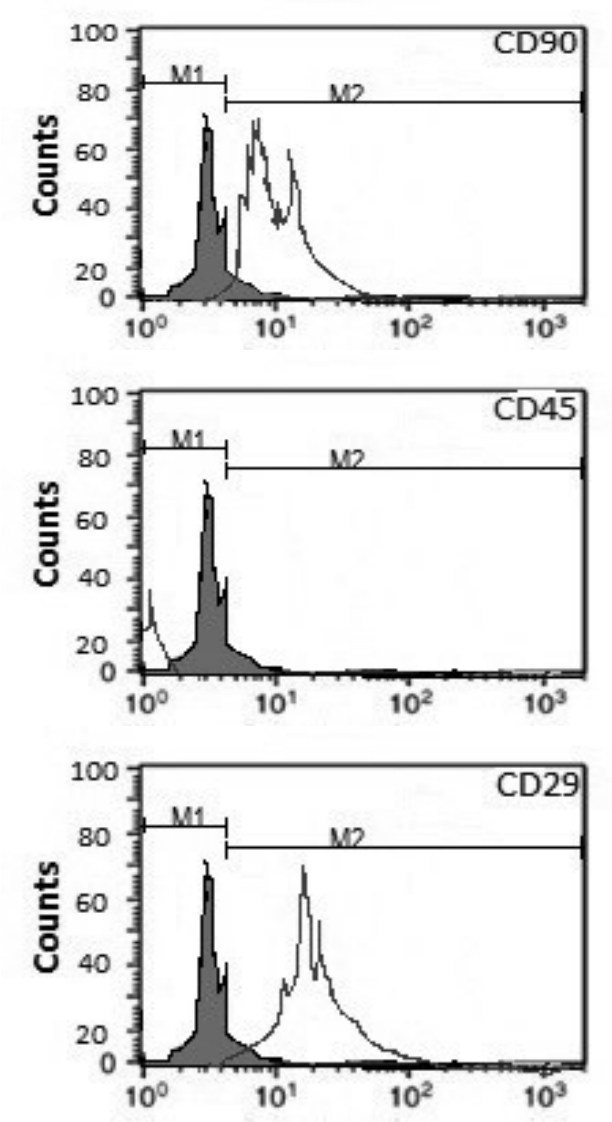
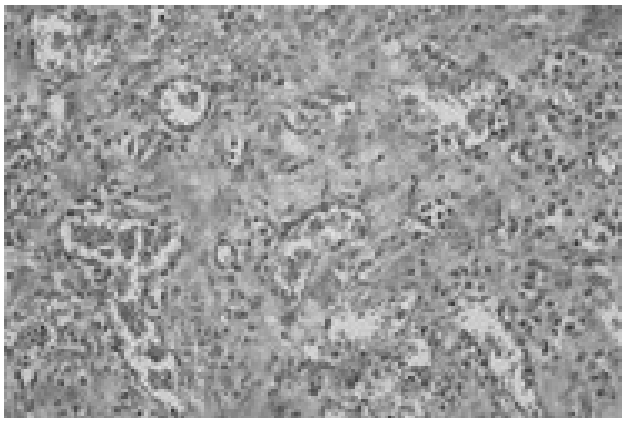


Fig. (11): *Flow cytometric analysis of the expression of mesenchymal stem cell markers CD90, CD29 and CD45 on isolated cells.*

III. The regenerative potential of stem cells

1. Histological assessment of cell/scaffold constructs in vivo with H&E:



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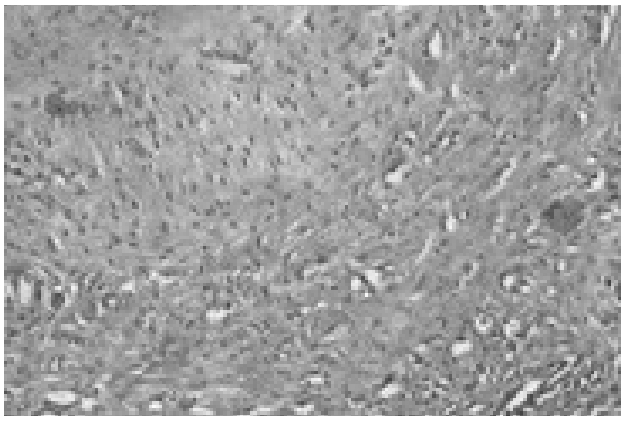

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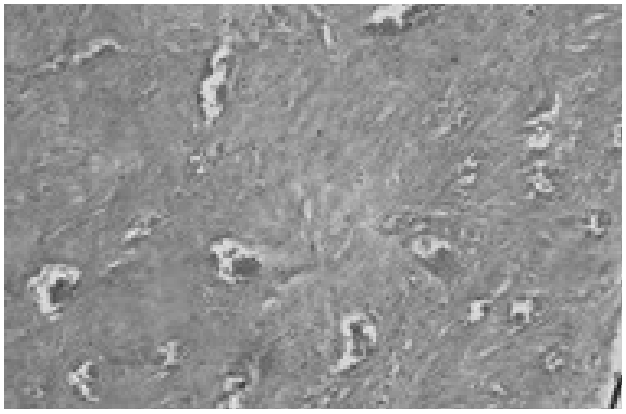

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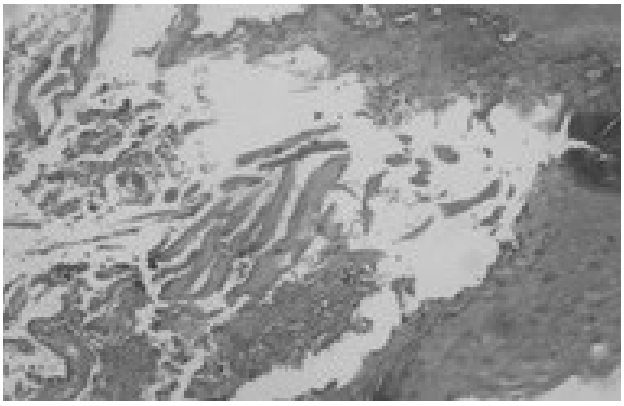

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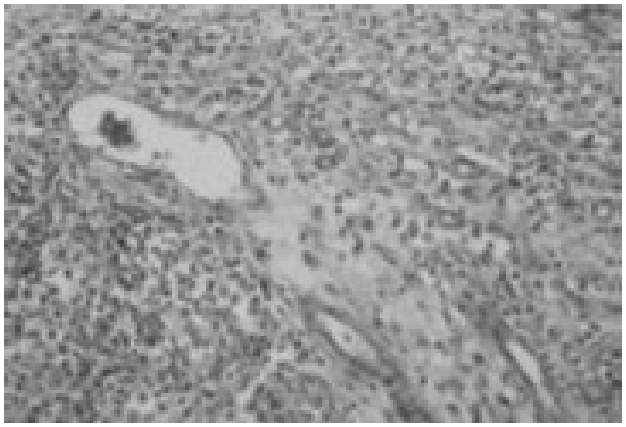

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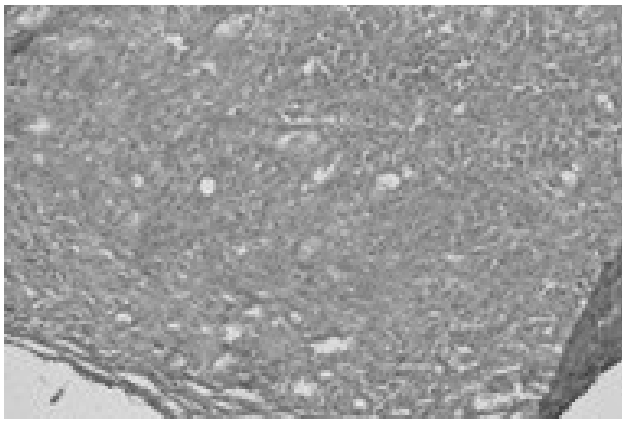
Fig.(12): Histopathological assessment of regenerated tissues. A detailed view of H&E stained sections of the canal lumen of groups 1-6 (A to F) (original magnification x200). Showing fibrous tissue fills the canal lumen with numerous congested and dilated blood vessels. (F) Negative control showing tissue appears to be irregular rat tissues inside pulp space (Epithelium, muscles and collagen fibres) (original magnification x200)



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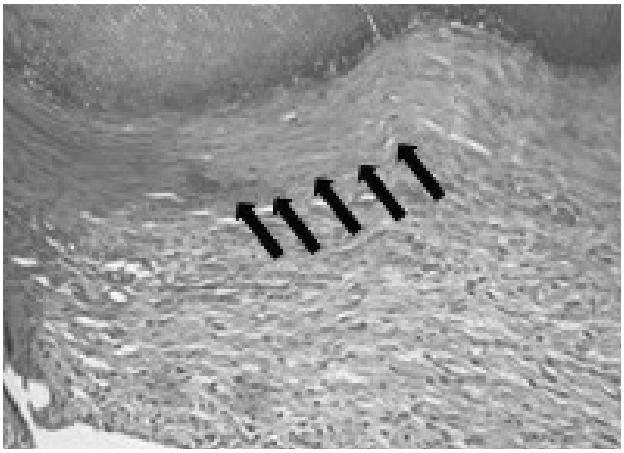

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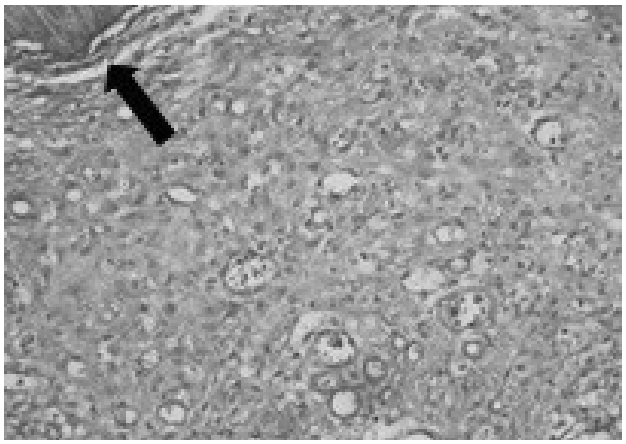

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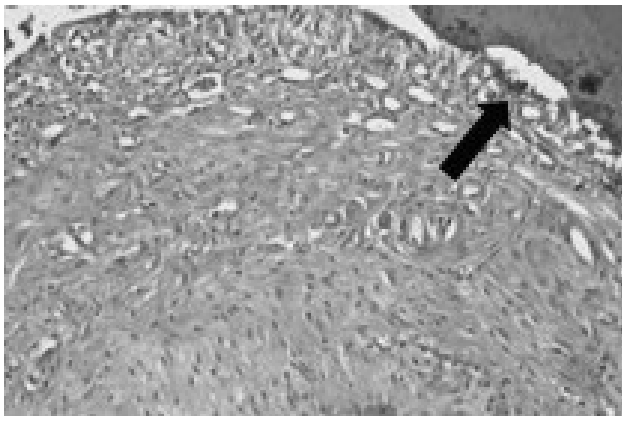

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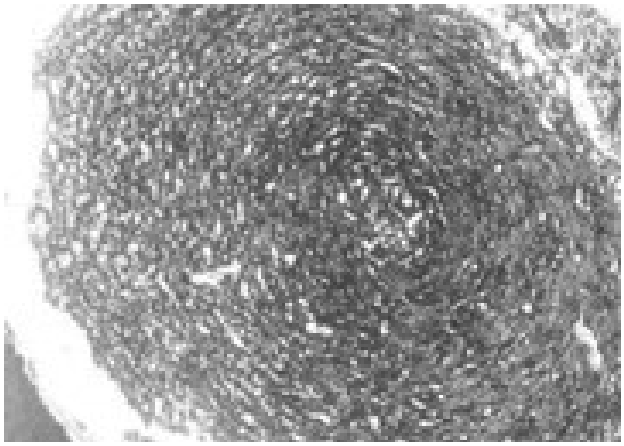
Figure (13): A detailed view of H&E stained sections showing (A) Black arrows indicate a layer of newly formed dentin like matrix.(B) Black arrow indicates area of palisading cell layer. (C) Black arrows indicate regeneration of continuous layer of uniform thickness of hyperchromatic newly formed dentin like matrix (original magnification x200).

2. Blood vessels count statistical analysis: There was a significant difference in the count of blood vessels found in samples of different groups ($p<0.001$). The highest count was found in samples irrigated by (Chorohhexidine gluconate 2% & 17% EDTA) (27.25±5.12), followed by samples irrigated by (5.5% NaOCl) (25.13±6.36), then samples irrigated by (Chorohhexidine gluconate 2%) (21.25±4.03), deionized water (20.75±4.50), (5.5% NaOCl & 17% EDTA) (19.50±4.84), while the lowest count was found in samples of the negative control group (1.75±1.49).

3. Histological assessment of cell/scaffold constructs in vivo with Masson Trichrome: Histological examination of the implants from different experimental groups, including the positive control group, stained with Masson Trichrome stain showed formation of massive amount of newly formed collagen



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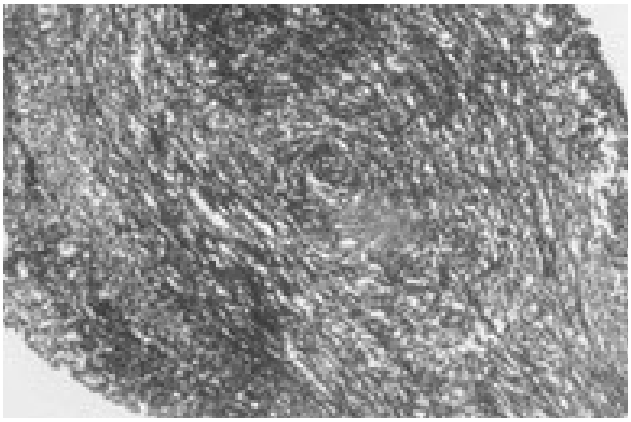

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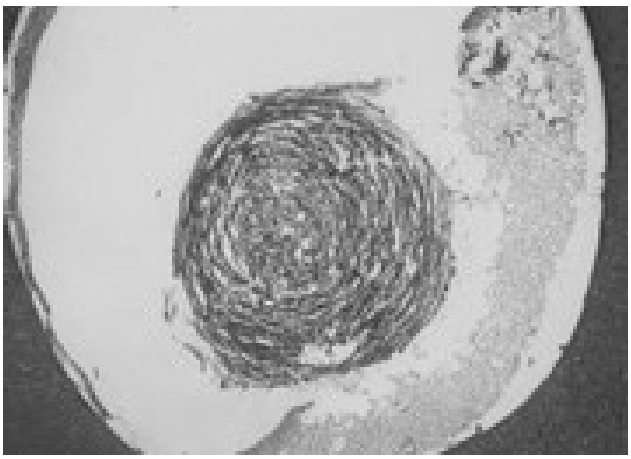

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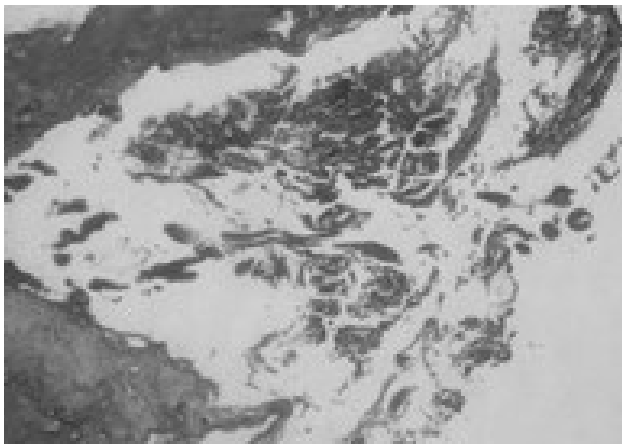

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Figure (15): Histological assessment of cell/ scaffold constructs in vivo with Masson's trichrome stain. (A, B)Showing canal lumen nearly filled with densely stained newly formed collagen fibres. (original magnification x200). (C) Showing the shrinkage artifact in some samples(original magnification x100).. (D) Showing the irregular tissue in control group with scarce amount of blue newly formed collagen fibres. (original magnification x200).

fibres inside the canal lumen. The newly formed fibres were deeply stained with the remarkable blue masson trichrome stain. All The empty spaces in some samples were shrinkage artifacts. The negative control group showed irregular tissues with scarce amount of newly formed collagen fibers. Fig.(15).

2. <u>Area fraction of newly formed collagen fibers:</u> There was a significant difference in the area fraction of newly formed collagen fibers formed in different groups ($p<0.001$). The highest area fraction was found in samples irrigated by deionized water (34.78 ± 3.86). Pairwise comparisons showed samples irrigated by deionized water (34.78 ± 3.86) to have a significantly higher value than samples irrigated by (Chorohhexidine gluconate 2%) (24.57 ± 7.17) ($p=0.030$). In addition, it showed samples of the negative control group (0.48 ± 0.11) to have a significantly lower value than other groups ($p<0.001$).

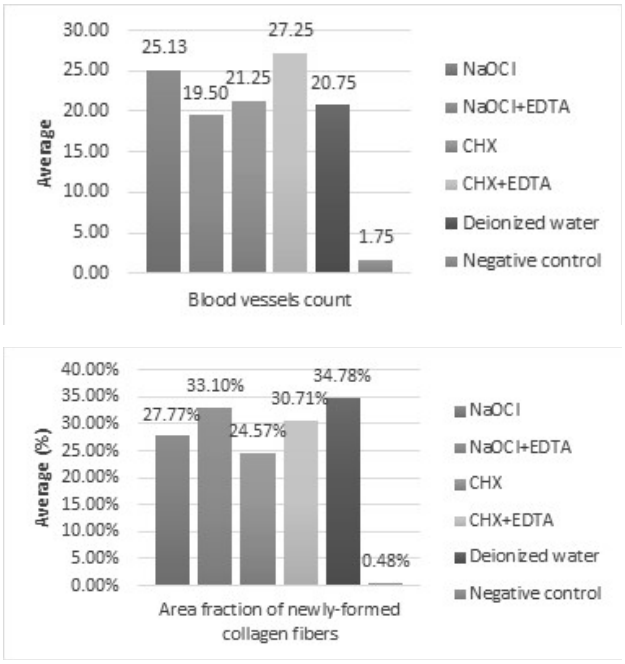

Figure (16): Bar chart showing average blood vessels count and average Area fraction (%) of newly-formed collagen fibers in different groups

Discussion:

Pulp tissue necrosis or injury from dental trauma and caries in teeth with incomplete apical development have provided unique opportunities to explore the regenerative potential in endodontics. The regenerative endodontic techniques (RET) is aiming to develop strategies for increasing tooth service and survival. Regeneration of damaged pulp tissues is based on the interplay of three essential components: stem cells, growth factors and scaffolds(20). In the present study, stem cells from human exfoliated deciduous teeth: SHEDs were selected because of their high potential to be differentiated into large number of tissues. SHED can differentiate into adipogenic, chondrogenic, neurons and endothelial cells (3, 21, 22). SHED can also differentiate into osteoblasts and are positive for TGF- β, FGF and VEGF receptors(22, 23). In the present study, SHED were isolated and cultured in complete aseptic environment to avoid contamination that is usually obtainable from tools, human skills, and materials. There are two methods to isolate SHEDs from pulp

tissue: Enzymatic dissociation of pulp tissue and outgrowth from tissue explants (24). Although enzyme digestion is considered to be the most common method used to acquire dental pulp stem cells (25, 26), it has been reported that the outgrowth method can also be used to acquire multipotent stem cells(27, 28). In the present study, the outgrowth method was used to isolate the cells from pulp tissue, as only a small amount of pulp tissue is available from deciduous teeth and the method was easy and convenient.

The results of the present study showed that isolated cells were identified as being MSCs by their morphology and plastic adherence. The first adherent cells appeared 4–6 d after initiation of the primary culture. Generally, the primary cells reached 80–90% of confluence at days 12–14 (data not shown).

The cells appeared to be rounded and spherical in morphology, but later most cells had a typical fibroblastlike appearance. At the seventh day of cultivation, a small percentage of cells were polygonal, spindle shaped or oval shaped. Each cell had 12 projections. SHEDs were cultured for 10 passages without any observed decreases of the cell growth rate.

As this previously observed morphology is not a special characteristic for mesenchymal stem cells, flow cytometric analysis was performed to quantify the expression of several mesenchymal stem cell markers. The isolated stem cells were shown to be CD90 positive (89.4±3.6%), CD29 positive (99.7±0.2%) and CD45 negative (1.0±0.6%). These results proved that the isolated cells (SHEDs) shared multiple characteristics with mesenchymal stem cells. SHEDs were demonstrated to have a marked capacity to proliferate.

Sodium hypochlorite with concentrations of 1–6% have been used in RET (9). EDTA is a chelating agent used to remove smear layer in conventional root canal therapy(29) and to cause release of growth factors from dentine matrix in RET (7). EDTA has also been proved to have weak antimicrobial activity(29).

Dentine matrix-derived growth factors released after EDTA treatment have been shown to be capable of signalling stem cells of apical papilla to differentiate into odontoblast-like cells(30). Thus, in our present study, EDTA solution with17% concentration was used.

In some clinical articles, NaOCl is combined with CHX at the first appointment of REPs, and the canal was irrigated with saline between the aforementioned irrigants (31-33). This combination is probably part of an effort to disinfect more adequately the root canal because of the antimicrobial activity and substantivity of CHX(34).

It was also proved that CHX used in clinically relevant concentrations affects SCAP survival directly and indirectly. But fortunately, these effects can be overcome by limitation of irrigation time (direct effects) and subsequent neutralization with L-a-lecithin (indirect effects) (35). CHX was postulated to be a promising and cell-friendly disinfection agent before REPs. Thus, in the current study, CHX was used with 2% concentration and in combination with EDTA 17%.

After root discs conditioning with different previously mentioned solutions, the discs were kept at 37º C for 1 day and at the end of the time point, medium from samples was collected, filtered and subjected to growth factor quantification by using an enzyme-linked immunosorbent assay (ELISA) test system for TGF-β1. TGF-β1was selected to be evaluated, as it has been proved previously that pulp cells can express their dentinogenic potential in response to an appropriate surface containing exogenous TGF- β1 and that the dentinogenic activity of dentin matrix may at least partly be ascribed to TGF- β1(36).

Statistical analysis of TGF-β1release after root discs conditioning reviled that there was a significant difference in the amount of the released (TGF-β1) from samples with different groups ($p<0.001$). The highest value of released (TGF-β1) was found in samples irrigated by (Chorohhexidine gluconate 2% & 17% EDTA) which have a significantly higher value of released TGF-β1 when compared with samples of other groups.

Although previous study has shown the

direct negative effect of chlorhexidine (CHX) on stem cell survival (37), this highest value could be attributed to the combination with EDTA which was the final conditioning media used. Therefore, a final rinse with EDTA before creation of a blood clot is advised. It was also shown with investigating the release of growth factors from dentine matrix after EDTA treatment when performed in non-infected root canals (7, 38). NaOCl 5% & EDTA 17% group has shown a significantly lower value than Chorohhexidine gluconate 2% & 17% EDTA group. This could be attributed to the previous study which showed that, the use of sodium hypochlorite before EDTA conditioning reduced transforming growth factor TGF- β1 release significantly (7). This effect is probably due to damage to the proteins including dentine growth factor. Unsurprisingly, the lowest value of released TGF-β1 was found in samples irrigated by deionized water which have a significantly lower value in comparison to other groups.

HydroMatrix™ is a peptide nanofiber 3-dimensional scaffold that promotes cell growth and migration. HydroMatrix utilizes specific peptides that self- assemble from fluid precursors into highly crosslinked peptide hydrogels in response to increases in temperature or ionic strength(16). After 30 days of implantation, the root construct was retrieved, histologically investigated and analyzed. The newly regenerated blood vessels in each root were counted and statistically analyzed in each group. The highest count was found in samples irrigated by Chorohhexidine gluconate 2% & 17% EDTA which was significantly higher value than samples irrigated by 5.5% NaOCl & 17% EDTA. This could be directly related to the previous results of TGF-β1 in our current study. Another interesting stain is Masson's trichrome. It is a three-colour staining protocol used in histology. It was used by Na et al to identify the newly formed collagen fibers. The lighter and darker blue stain represents new and old collagen, respectively(19). Area fraction of newly formed collagen fibers in disc lumen was analyzed. Area fraction was measured by calculating the percentage of pixels that have been highlighted in blue in images of masson trichrome stained sections using Image analysis software. There was a significant difference in the area fraction of newly formed collagen fibers formed in different groups ($p<0.001$).

Although EDTA conditioning of dentine promoted the adhesion, migration and differentiation of dental pulp stem cells towards or onto dentine(8), the highest area fraction was found in samples irrigated by deionized water. But there was no statistical difference between EDTA containing groups and deionized water group. Pairwise comparisons showed samples irrigated by deionized water to have a significantly higher value than samples irrigated by Chorohhexidine gluconate 2% alone. In addition, samples of the negative control group had a significantly lower value than other groups.

In our current study, it was concluded that: Chlorohexidine gluconate 2% could be used in regenerative endodontic procedures with EDTA 17% solution as a final rinse in purpose of increase TGF-β1 release and regenerated blood vessels number. SHED can be used as a postnatal source of stem cells for differentiation into odontoblast like cells.

Finally, we recommend using of EDTA as a final rinse in regenerative endodontic techniques. It is advisable to use Chlorohexidine gluconates 2% to enhance antibacterial efficiency during regenerative endodontic techniques. Moreover, larger animal models are needed and being planned in immunocompetent hosts to rigorously evaluate the pulp regenerative potential of SHED.

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