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Research Article

Determination of Mesenchymal Stem Cell Origin during Bleeding-Induced Regenerative Endodontic Procedure Using 2-Step Real-Time Reverse-Transcription Polymerase Chain Reaction (qRT-PCR)

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Abstract

Introduction: This study evaluated possible stem cells origin during regenerative endodontic procedure, whether from surviving dental pulp stem cells within disinfected root canals walls or from stem cells of apical tissues or they act synergistically.

Methods: Immature necrotic permanent single rooted-teeth (n = 30) of patients (n = 24) 7-18 years old were divided into two groups according to presence or absence of apical radiolucency: group A with apical radiolucency and group B with healthy periodontium. After informed consent, two-sessions regenerative endodontic protocol was implemented. First session root canals were disinfected using 1.5% NaOCl irrigate and Bimix medicament for 3 weeks. Second session root canals were irrigated using 17% EDTA followed by a final saline rinse. Saline samples were collected from disinfected root canals using sterile paper points. Periodontium was apically stimulated using hand files until bleeding reached the cementoenamel junction for the assumed stem cells delivery. Blood samples were collected from root canals using sterile paper points. The expression of specific mesenchymal stem cell gene markers; CD105 and CD73 was assessed using 2-step qRT-PCR relative to infected root canals. Mann-Whitney U test was used for comparison. Statistical significance was set at "$p \leq 0.05$".

Results: In group A, the fold increase for CD105 and CD73 in saline samples were 19.6% and 7.6%, respectively, while the fold increase in blood samples were 26.6% and 17.8% with statistically significant difference, P-value < 0.001. Same trend was observed in group B, the fold increase for CD105 and CD73 in saline samples were 20.2% and 8%, respectively, while the fold increase in blood samples were 26.2% and 17.6% with statistically significant difference, P-value < 0.001.

Keywords: Mesenchymal; Regenerative Endodontics; qRT-PCR, Immature; Necrotic

Abbreviations

CD: Cluster of Differentiation; qRT-PCR: Quantitative Reverse Transcription-Polymerase Chain Reaction; EDTA: Ethylene Diamine Tetra-Acetic Acid

Introduction

The developing dentition is at risk for pulpal necrosis because of trauma and developmental dental anomalies such as dens evaginates. Management of tooth with open apex is very challenging due to various factors. These are thin dentinal walls liable to fracture, lack of minor apical constriction essential for optimum obturation and unfavorable crown-root ratio for restorative purpose [1]. Every effort is done to avoid loss of an immature permanent tooth in young patients with mixed dentition leading to loss of function, malocclusion, and inadequate maxillofacial development [2,3]. Traditionally, immature necrotic permanent teeth were managed by apexification using calcium hydroxide dressing for long time [4]. Unfortunately, the remaining dentin thickness of apexificated tooth is thin and fragile, suffering adverse effects of long-term calcium hydroxide application such as dentin embrittlement [5]. Others managed immature necrotic teeth through single visit mineral trioxide aggregate artificial barrier against which obturation material can be compacted [6]. These traditional treatment modalities neither reinforced root either vertically or laterally, nor regained neural or vascular supply.

It is generally agreed that pulpal regeneration is applicable for avulsed immature teeth with open apices after replantation [7-9]. Shifting apexification to apexogenesis even for non-vital pulps with apical periodontitis or abscess was found to be clinically applicable. According to the American Association of Endodontics [10], regenerative endodontics used the concept of tissue engineering to restore the root canals to a healthy state allowing for continued development of the root and surrounding tissues. Tissue engineering involves the identification of progenitor cells capable of tissue

regeneration when seeded in biodegradable scaffolds and exposed to morphogenic signals [11]. Pulp revascularization procedures have been advocated in immature teeth for decades, the foundation of regenerative endodontic procedures was established by Nygaard - Ostby in the 1960s, whereas the first case of regeneration of a permanent immature tooth with necrotic pulp and apical periodontitis was published in 2001 by Iwaya., *et al* [12].

Recently, several case reports showed superior outcome of tissue engineering strategy in managing immature teeth with necrotic pulps [13-15]. Tissue engineering tetrad includes stem cells, signaling molecules, appropriate scaffold, and finally conductive environment for proper regeneration of vital tissues. To date, four types of human dental stem cells have been isolated; Dental Pulp Stem Cells (DPSCs) [16], Stem cells from Human Exfoliated Deciduous (SHED) teeth [17], Stem Cells from Apical Papillae (SCAP) [18], and Periodontal Ligament Stem Cells (PDLSCs) [19]. Among them, all except SHED originate from permanent teeth. Signaling molecules regulate the division or specialization of stem cells to the desirable cell type, and mediate key cellular events in tissue regeneration including cell proliferation, differentiation, and matrix synthesis [20]. Dentin acts as a reservoir of signaling molecules [21]. Scaffold is a three-dimensional structure; which supports cells organization and vascularization. several published case reports induced bleeding inside the disinfected pulp canal space using different armamentarium such as, K File, endodontic explorer and sterile needle gauge to create blood clot scaffold and deliver stem cells from apical tissues [13-15,22-24].

Several studies assumed the presence of stem cells based on monitoring clinical and radiographic outcome of regenerative endodontic procedure [25-27]. All these studies did not decide whether stem cells arose from apical tissues (SCAP) or disinfected root canal walls (HDPSCs). Hence, there was a knowledge gap in verifying the actual source of mesenchymal stem cells in regenerative endodontic treatment during management of immature necrotic permanent dentition. This study investigated the origin of mesenchymal stem cells whether from SCAP or HDPSCs during management of immature necrotic permanent teeth with/without apical radiolucency. The expression of CD105 and CD73 gene markers were evaluated; being a dependent measure of the relative presence of stem cells using 2-step qRT-PCR.

Material and Methods

Patient Recruitment

This clinical study was conducted with approval of ethics committee of Faculty of Oral and Dental Medicine, Cairo University. An informed consent was formulated to include the aim of study, sequence of steps, benefits and risks, and probable prognosis of regenerative process. All data of the participants were stored securely in locked files in areas with limited access to ensure patients confidentiality. The exclusion criteria were; patients who received systemic antibiotic within 3 months prior to seeking treatment, systemic diseases that would interfere with healing, teeth with radiographic signs of external or internal resorption, and finally root fracture. The patient or his/ her parents was informed verbally about this modern treatment approach and its possible risks such as pain, swelling, sinus tract development, tooth discoloration, tooth fracture as well as treatment failure. They were also informed about possible shift in treatment plan from regenerative endodontics to traditional modalities such as apexification or surgical endodontics upon failure of the initiated regenerative treatment, and parental consent was obtained.

Twenty-four patients with thirty teeth were recruited from outpatient clinic of Department of Pedodontics and Endodontics, Faculty of Oral and dental medicine, Cairo University. Inclusion criteria were; patient age ranging between 7 and 18 years, medically free, the offending teeth were necrotic immature permanent maxillary or mandibular single rooted with open apices. The participants of this study were divided into two groups according to apical condition radiographically; group A with apical radiolucency (n = 12 patients with 15 teeth) and group B with healthy periodontium (n = 12 patients with 15 teeth).

Regenerative Procedure and Sample Collection

All patients were anesthetized, either through infiltration in case of maxillary teeth or mental nerve block in case of mandibular teeth. Rubber dam was applied; and access was prepared, where restorative diagnosis confirmed the initial clinical diagnosis of pulp necrosis. Root canals were irrigated with 5 ml saline rinse and single sterile paper point was placed till middle third of root canal estimated working length. The saline soaked paper points were immediately placed into RNA isolation lysis buffer (Bio Basic Inc. Markham, Ontario, Canada) to be sent to biochemistry laboratory in Faculty of Medicine, Cairo university for standardizing the calibrator (untreated samples). Root canals were copiously irrigated using 10 ml 1.5% sodium hypochlorite NaOCl delivered using 28 Gauge safety Steri Irrigation Tips (DiaDent Group International, Burnaby, BC, Canada) inserted 3 mm below cementoenamel junction. Working length was determined using electronic apex locator Root ZX II (J. Morita USA, Irvine, California) and confirmed radiographically using parallel technique with receptor holding device. Canals were irrigated again with 10 ml 1. 5% NaOCl, which was delivered 2 mm coronal to apical canal terminus. The irrigate hydro-dynamically agitated with EndoActivator (DENTSPLY MAILLEFER, Baillagues, Switzerland) device using blue tips 2 mm short of working length for 60 seconds. Root canals were lightly instrumented using rotary Ni- TiProtaper Universal files (# F3, F4) and finally dried with sterile paper points. Bimex; a mixture of ciprofloxacin 500 mg (European Egyptian Pharm. Ind. Alexandria, Egypt) and Metronidazole 500 mg (Sanofi-Aventis, Cairo, Egypt), was applied inside the root canals. Initially, tablets of both antibiotics were smashed using mortar and pestle and mixed with Isocaine 3% plain anesthesia to form a creamy mix. Bimex was applied inside root canal using Micro-Apical Placement system (MAP System, ProduitsDentaires SA, Vevey, Switzerland) and endodontic pluggers. The depth of penetration was controlled short of the working length using stoppers attached to pluggers to prevent medicament extrusion apically, Cotton pellet was placed

inside pulp chamber and access cavity was sealed using light cured Fuji IX (Fuji II LC; GC Corp, Sydney, Australia).

All patients returned asymptomatic after 23.7 days ± 2.5 days (21-29 days). The offending teeth were anesthetized with Isocaine 3% plain anesthesia (Mepivacaine HCl, 4 NOVOCOL PHARMACEUTICAL, Ontario, Canada) followed by rubber dam application. Access was reopened and Bimex was removed using 20 ml 1.5% NaOCl, mechanically agitated using Endo Activator. Root canal dentin was conditioned using 20 ml 17% EDTA applied 3 mm coronal to apical terminus via Steri Irrigation Tips for 2 minutes. This was followed by final saline rinse 5 ml. A single sterile paper point was placed into the canal till 2 mm short of working length, pressed against root canal dentin and allowed to absorb the saline for 2 minutes. The paper points were inspected under magnification using eye loupes 3.5X to ensure that they did not catch blood from apical tissues [28]. The saline soaked paper points were immediately placed into RNA isolation lysis buffer (Bio Basic Inc., Markham, Ontario, Canada) to be sent to biochemistry laboratory in Faculty of Medicine, Cairo university. Afterwards bleeding was evoked, as reported by Banch and Trope [13], by vertical strokes using K-File extending beyond the working length until bleeding was observed at cementoenamel junction. Blood was left for 3-5 minutes to allow clotting. Intracanal blood sample was taken using single sterile paper point inserted 2 mm of working length and were immediately placed in RNA isolation lysis buffer. The Eppendorfs for both saline and blood samples were coded with number per the key of participants, placed inside freezer until delivered within 5 hours to Biochemistry department to be stored in Ultra low freezer -80°C until investigation.

RNA Isolation and 2-step Real-time Reverse-Transcription Polymerase Chain Reaction

Samples were removed from the ultra-low freezer. Total RNA was isolated using Qiagen tissue extraction kit (Qiagen, USA) according to manufacturer's instructions. Samples assembled inside the rack were lysed in lysis Buffer RLT. The lysate was centrifuged using cooling centrifuge and transferred into a new micro centrifuge tube. Buffer RW1 added to the RNeasy spin column and Centrifuged. Then Buffer RPE was added to the RNeasy spin column and centrifuged to wash the spin column membrane. RNeasy spin column was placed in a new 1.5 ml collection tube. RNase-free water, 30 - 50 µl, was added directly to the spin column membrane and centrifuged.

After RNA extraction, paper points were removed from the Eppendorf's and the elution transferred to new Eppendorf's. The total RNA was used for cDNA conversion using high capacity cDNA reverse transcription kit (Fermentas, USA), it is an RNA dependent DNA polymerase that uses single stranded RNA as a template in the presence of a primer to synthesize a complementary DNA strand. First strand buffer provided preferred pH and ionic strength for reverse transcription. Deoxynucleotide triphosphate (dNTPs) dATP, dTTP, dGTP, dCTP were used for extension of primers. Three µl of random primers were added to the 10 µl of RNA which was denatured for 5 minutes at 65°C in the thermal cycler. The RNA primer mixture was cooled to 4°C. The cDNA master mix was prepared per the kit instructions and was added, (Table 1). Total volume of the master mix was 19 µl for each sample. This was added to the 31 µl RNA-primer mixture resulting in 50 µl of cDNA. The last mixture was incubated in the programmed thermal cycler one hour at 37°C followed by inactivation of enzymes at 95°C for 10 minutes, and finally cooled at 4°C.

Component	Volume (total 19 µl)
First strand buffer	5 µl
10 mMdNTPs	2 µl
RNase inhibitor (40 U/µl)	1 µl
MMLV - RT enzyme (50 U/µl)	1 µl
DEPC-treated water	10 µl

Table 1: Components of cDNA master mix.

The cDNA templates were then used in real-time PCR reaction in presence of specific primers for the mesenchymal stem cell markers: cluster of differentiation 105 (CD105), cluster of differentiation 73 (CD73).

For each cDNA sample the following reagents and volumes were added (Table 2), with following running condition using Step One Plus real-time PCR device (Table 3).

PCR reaction mix component	Volume
Forward Primer	1 µl
Reverse Primer	1 µl
Sybr green mix	12.5 µl
Cdna template	5 µl
RNAse free water	5.5 µl
Total volume	25 µl

Table 2: Master Mix preparation/sample.

Thermal cycling condition		
Stage	Temp.	Time
Hold	50°C	2:00
One cycle		
Denaturation	95°C	0:15
Annealing	60°C	1:00
Extension	72°C	1:00
40 cycles		

Table 3: Real-time PCR cycles for quantitative gene expression using applied Biosystems Step One Real-Time PCR System.

The experiment results report included the cycles thresholds (Ct) of calibrator, CD73, CD105 in addition to the endogenous control (GAPDH), which were used for calculating the relative quantification of both gene markers.

The expression of the CD105 and CD73 genes were evaluated; being a dependent measure of the relative presence of stem cells. The Relative Quantification (RQ) or fold change of both gene markers in saline and blood samples was calculated using the following equation:

Δ Ct = Ct gene test - Ct endogenous control

ΔΔCt = ΔCt sample1 - ΔCt calibrator

RQ = Relative quantification = $2^{-\Delta\Delta Ct}$

The RQ is the fold change compared to the calibrator, which was infected root canal.

Furthermore, the relative difference between RQ of blood samples and saline samples as well as percentage change were calculated.

Results and Discussion

The whole 30 teeth of the 24 patients included in this study were subjected to quantitative assessment of gene expression CD105 and CD73 using 2-step qRT-PCR.

CD105

Within each group, Comparison of CD105 fold change in disinfected root canal space versus those migrated by bleeding induction revealed that the fold increase in blood samples was statistically significant higher than in saline samples (P-value < 0.001).

Correlation between groups showed that in saline samples the fold increase of CD105 in group A (19 6 ± 1.7) was slightly lower than in group B (20.2 ± 2.0), indicating lower fold increase of stem cells in the disinfected root canals of necrotic teeth with apical radiolucency's (group A) as compared to absence of radiolucency (group B). On the other hand, in blood samples the fold increase of CD105 was slightly higher in group A (26.6 ± 3.2) than group B (26.2 ± 1.1), indicating more influx of stem cells from apical tissues for necrotic teeth with apical radiolucencies (group A), as compared to absence of radiolucencies (group B). Statistically, however, there was no significant difference concerning the fold increase of CD105 when comparing those detected inside disinfected root canal (P-value 0.405) or when correlating those migrated by bleeding induction from apical tissues (P-value0.763) among the two groups (Table 4).

		Group A		Group B		P-value
	Marker	Mean	SD	Mean	SD	
CD105	Blood	26. 6	3. 2	26. 2	1. 1	P_1 < 0. 001* P_2 = 0. 405
	Saline	19. 6	1. 7	20. 2	2. 0	P_3 = 0. 763
	% Change	36		30. 5		P_4 = 0. 205
CD73	Blood	17. 8	1. 5	17. 6	1. 1	P_1 < 0. 001* P_2 = 0. 433
	Saline	7. 6	1. 1	8. 0	1. 5	P_3 = 0. 762
	% Change	139		128		P_4 = 0. 481

Table 4: Descriptive statistics and test of significance comparing change and % change of CD markers in the two groups.

* Statistically significant difference

P_1 comparing between blood and saline in the same group

P_2 comparing between saline in group A and B

P_3 comparing between blood in group A and B

P_4 comparing between % changes in group A and B.

CD73

Within each group, Comparison of CD73 fold change in disinfected root canal space versus those migrated by bleeding induction revealed that the fold increase in blood was statistically significant higher than in saline samples (P-value < 0.001).

Correlation between groups showed that in saline samples the fold increase of CD73 in group A (7.6 ± 1.1) was slightly lower than in group B (8.0 ± 1.5), indicating lower fold increase of stem cells in the disinfected root canals of necrotic teeth with apical radiolucencies (group A) as compared to absence of radiolucency (group B). On the other hand, in blood samples the fold increase of CD73 was slightly higher in group A (17.8 ± 1.5) than group B (17.6 ± 1.1), indicating more influx of stem cells from apical tissues for necrotic teeth with apical radiolucencies (group A), as compared to absence of radiolucencies (group B). Statistically, however, there was no significant difference concerning the fold increase of CD73 when comparing those detected inside disinfected root canal (P-value 0.433) or when correlating those migrated by bleeding induction from apical tissues (P-value 0.762) among the two groups, table 4.

Change and percentage change between saline and blood samples

Though the absolute RQ values of saline and blood samples for CD73 were lower than CD105, the change and percentage change was more evident for CD73 than for CD105 via bleeding induction. In group A, the percentage change via bleeding induction was 36% for CD105 and 139% for CD73 relative to saline samples. Similar trend was observed for group B where, the percentage change by bleeding induction was 30.5% for CD105 versus 128% for CD73 relative to saline samples. Comparing both groups, the percentage change for either CD105 or CD73 was more in group A compared to group B, with no statistical significance, (P-values 0.205, 0.481 respectively).

Discussion

Several studies assumed the presence of stem cells based on monitoring the success of clinical and radiographic outcome of regenerative endodontic procedure [25-27]. However, they did not study the origin of these cells. Only one study quantitatively assessed the locally delivered mesenchymal stem cells from periarticular tissues into root canal space [30]. In the current study, saline samples were used as a representative measure for the residing stem cells inside disinfected pulp canal space and blood samples as a representative measure for stem cells form apical tissues.

Quantitative assessment of mesenchymal stem cells was conducted using molecular biology techniques, through investigating the gene expression of CD73 and CD105 using 2-step qRT-PCR; as a dependent measure of the relative presence of mesenchymal stem cells as stated by the Society of Cellular Therapy in a population of cells [28-31].The present study selected 2-step qRT- PCR due to its superior sensitivity, good reproducibility, and wide dynamic quantification range [32]. All patients participated in the current study were subjected to quantitative assessment of the

gene expression of CD105 and CD73 to monitor the impact of apical condition on concentration of mesenchymal stem cells through dividing our patients into two groups [33], to decide whether a series of inflammatory and immunological responses apically in group A adversely influenced the tissue engineering outcome.

Twenty-four patients with thirty teeth had been included in the study. The mean ± standard deviation values of age for all patients in the study were 11.1 ± 3.2 years with a minimum of 7.0 years and a maximum of 18.0 years of age [34]. Group A showed a statistically significant higher mean age value than Group B. This statistically significant difference mostly is attributed to apical bone resorption which is a characteristic feature of long standing apical diseases confirmed through dental history from patient. Several other studies showed a lower mean age value [26] may be due to ungrouping and pulp necrosis which was the only inclusion criteria used.

The findings of the current study showed the existence of both gene markers CD105 and CD73 in both groups with and without radiolucencies as well as in both saline and blood samples, however, with variable RQ values [28-31].

Presence of both gene markers CD105 and CD73 in saline samples in both groups supported the hypothesis of mesenchymal stem cells residence inside disinfected pulp canal space, irrespective to apical condition. One study found that hypoxia has no effect on stem cells proliferation, but it evoked the up-regulation of genes specific for osteogenic differentiation, neuronal differentiation and angiogenesis [35]. These cells are mostly dental pulp stem cells embedderd inside dentin matrix and retained their vitality despite hypoxia in group B or apical pathosis in group A.

The slightly non-significant lower concentration of both gene markers in saline samples in cases with apical radiolucencies (group A) may be attributed to longer standing pulp necrosis relative to cases with intact periodontium (group B), which might have adversely influenced the concentration of viable dental pulp stem cells inside root canals.

Blood samples in both groups revealed increased expression of both gene markers compared to the corresponding saline samples, where the fold change for CD105 increased by 36% in group A and 30.5% in group B with no statistically significant difference (P-value 0.205) while fold change for CD73 increased by 139% in group A and 128% in group B, also with no statistical significance (P-value 0.481). This confirmed the influx of cells from periarticular tissues containing mRNA transcripts encoding mesenchymal stem cell marker genes into disinfected pulp canal space via bleeding induction, irrespective to apical condition [28].

The slightly non-significant higher concentration of both gene markers in blood samples in cases with apical radiolucencies (group A) may be due to activation of healing of apical lesions after optimum root canal disinfection as one study showed activation of mesenchymal stem cells markers in healing apical lesions relative to the healthy control group [31].

It was noted that the absolute values for CD73 were lower than CD105 for both saline and blood samples. Similar trend was revealed by other study who didn't even find CD73 gene marker in their saline samples [28].

Conclusion

Under the limitations of this study, the following conclusions could be drawn:

1. 2-step qRT-PCR proved to be a valid method for determining mesenchymal stem cells origin during regenerative endodontic procedure.

2. The mesenchymal stem cells participating in pulp regeneration originate from both disinfected pulp canal space (HDPSCs) and the apical tissues (SCAP).

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Conflict of Interest

There is not any financial interest or any conflict of interest.

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