Tissue Engineering of Necrotic Dental Pulp of Immature Teeth with Apical Periodontitis in Dogs: Radiographic and Histological Evaluation

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Aim: *To evaluate tissue engineering technology to regenerate pulp-dentin like tissues in pulp canals of immature necrotic permanent teeth with apical periodontitis in dogs. Study design: The study was performed on 36 teeth in 12 dogs. The experiment was carried out using split mouth design. In each dog 3 teeth were selected for implementing the study procedure. Apical periodontitis was induced in Group A and B teeth. Group (A): immature upper left 2nd permanent incisors that were transplanted with a construct of autologous dental pulp stem cells with growth factors seeded in a chitosn hydrogel scaffold. Group (B): immature upper right 2nd permanent incisor that received only growth factors with scaffold. A third tooth in each dog was selected randomly for isolation of dental pulp stem cells (DPSCs). Both groups were closed with a double coronal seal of white MTA (Mineral trioxide aggregate) and glass ionomer cement. Both groups were monitored radiographically for 4 months and histologically after sacrificing the animals. Results: There was no statistically significant difference in radiographic findings between group (A) and group (B) for healing of radiolucencies, while there was statistically significant difference between group (A) and group (B) regarding radicular thickening, root lengthening and apical closure. Histologically, group (A) teeth showed regeneration of pulp- dentin like tissue while group (B) teeth did not show any tissue regeneration. Conclusion: Dental pulp stem cells and growth factors incorporated in chitosan hydrogel are able to regenerate pulp- dentine like tissue and help in complete root maturation of non-vital immature permanent teeth with apical periodontitis in dogs.*

Keywords: *Tissue engineering, dental pulp stem cells, necrotic immature permanent teeth.*

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INTRODUCTION

The discovery of dental stem cells and recent cellular and molecular biology studies have spurred the development of novel therapeutic strategies in engineering and regeneration of diseased or injured oral tissue. Tissue engineering relies on identifying the appropriate cells, developing conductive scaffolds, and understanding the morphogenic signals required to induce cells for tissue or organ regeneration. In recent years, the dental medicine field has begun to explore the potential applications of stem cells and tissue engineering in repairing and regenerating dental structures [1,2].

Yamaucbi *et al* [3] reported promising results in bioroot and dentin-pulpal complex engineering and regeneration strategies; however, the application of this technique to necrotic root canal spaces is limited. Dental pulp stem cells are the most accessible source of postnatal stem cells. They have the potential to differentiate into several cell types, including odontoblasts, neural progenitors, osteoblasts, chondrocytes, and adipocytes. Their multipotency, high proliferation rate, and accessibility make dental pulp stem cells an attractive source of cells for tissue regeneration.

The increasing demand to maintain pulp vitality has increased the need for investigations into new methods of tissue regeneration. Potentially, damaged odontoblasts can be replaced by newly regenerated populations of odontoblast-like cells derived from stem cells within the dental pulp, and previous studies have identified and isolated highly proliferative stem cells from the dental pulp[4,5]. These cells have the ability to differentiate into odontoblasts and regenerate dentin and pulp-like tissue resembling normal physiologic tissue [6,7].

Irreversible injury to the dental pulp of an immature permanent tooth from infection or dental trauma before complete root development terminates dentine formation and tooth maturation. It is difficult to endodontically manage a necrotic immature permanent tooth with apical periodontitis. Not only is it difficult to debride and obturate the root canal in these cases, but the thin dentinal walls additionally increasing the risk of subsequent fracture. Furthermore, conventional root canal treatment cannot regenerate new dentin and pulp tissue [8,9]. Apexification enables a calcified barrier to form at the root apex by placing a biocompatible material against the periapical tissues via the root canal. Calcium hydroxide and mineral trioxide aggregate (MTA) are the materials of choice for apexification procedures. MTA has better sealing properties, biocompatibility, and ability to induce cementoblast attachment to the barrier [10,11]. Internal bonding techniques using composite resin materials within the root canal have demonstrated improved fracture resistance in immature teeth and decreased treatment time when combined with MTA apexification. However, no apexification can produce apical maturation with increased root thickness [12,13].

Developments in tissue engineering and stem cell biology potentially allow regeneration of pulp and dentine-like tissue. The isolation and characterization of dental pulp stem cells (DPSCs), stem cells from exfoliated deciduous teeth (SHED), and stem cells from apical papilla (SCAP) has furthered the possibility for pulp and dentine regeneration [11].In 2008, Cordeiro *et al* [14] seeded SHED and endothelial cells onto biodegradable scaffolds in sectioned human teeth and then implanted the sections into immuno-compromised mice. They found that the stem cells differentiated into odontoblast- and endothelial-like cells in vivo, producing tissue closely resembling the dental pulp with a viable blood supply.

Huang *et al* [15] subsequently performed an experimental study regenerating vascularized human dental pulp in an emptied root canal space. They were able to produce new dentin on existing dentinal walls using stem and progenitor cells from the apical papilla, and dental pulp using a human root fragment in an immune compromised mouse model. They showed that pulp-like tissue can be regenerated in emptied root canal space using stem cells from apical papilla and DPSCs. They also concluded that these stem cells were able to generate odontoblast-like cells producing dentin-like tissue on existing dentinal walls.

Regeneration of pulp tissue relies on the provision of appropriate biodegradable scaffolds, which contain or can be seeded with growth factors and bioactive signaling molecules, to support cell organization and angiogenesis. Natural scaffolds such as collagen offer good biocompatibility and bioactivity; however, synthetic scaffolds such as polylactic acid, polyglycolic acid, foams, and hydrogels have more predictable mechanical properties and offer greater control of degradation time [16].

Through knowledge of the developmental biological processes occurring during dental tissue repair will help in creating future clinical strategies to regenerate lost or diseased dental tissue. The dream of many patients and clinicians may be realized in the near future.

The purpose of this study was to employ and evaluate novel tissue engineering strategies directed to create a vital pulp-dentin complex structure within a root canal space. This will enable the complete root maturation of immature non vital permanent teeth with apical periodontitis, in otherwise healthy dogs by a combination of dental pulp stem cells, growth factors and scaffolds.

MATERIALS AND METHOD

This is an experimental study performed on 36 teeth in 12 dogs. The inclusion criteria : 6 month old male dogs, with immature upper permanent incisors teeth (with open apices) and with orally and systemically healthy condition. These dogs were treated in accordance with the guidelines approved by the Institutional Animal Care and Use Committee of Faculty of Medicine, Cairo University, Egypt. All procedures were carried out under aseptic conditions and general anesthesia. All animals were given analgesic for pain relief after each procedure.

The experiment was carried out using split mouth design. In each dog 2 teeth were selected for implementing the study procedure, 1 in each side. They were classified as follows:

Group (A): Immature maxillary left second permanent incisors for transplantation with construct of autologous dental pulp stem cells with growth factors seeded in a scaffold.

Group (B): Immature maxillary right second permanent incisor received only growth factors with scaffold.

One tooth in each dog was selected randomly for isolation of the dental pulp stem cells (DPSCs).

All the dogs were treated under general anesthesia induced by Pentothal(Abbott Laboratories, North Chicago, IL) 13.5 mg/kg intravenously and intubation and maintenance with isoflurane (Halocarbon Laboratories, River Edge, NJ) supplemented with local anesthesia, bupivacaine plain 0.5%(Abbott Laboratories, North Chicago, Illinois, USA). All operative and postoperative procedures

were monitored by staff of the department of Laboratory and Animal Medicine during the postoperative period. The clinical work was implemented in seven phases:

Due to their much similarity to human in disease presentation as well as in the body physiology compare to other animal model, dogs were selected as the subject for this study.

Phase I: Isolation of dental pulp stem cells

The pulp tissue of the randomly selected teeth (one tooth per dog) was mechanically exposed with a #2 round carbide bur in a high-speed hand-piece under aseptic conditions. The pulp tissue was removed from the crown and root of each tooth using sterile barbed broaches, files, reamers, or an excavator. The extracted pulp tissue was digested in a solution comprising 3mg/mL collagenase type I and 4mg/ml dispase in Dulbecco's modified Eagle's medium (DMEM) for 1 hour in a CO_2 incubator. The single-cell suspensions were then separated by passing the cells through a 70-mm cell strainer (Falcon, BD Labware, Franklin Lakes,NJ, USA). The cell suspensions were cultured in a 25-cm^2 plastic flask containing DPSC medium (MEM, Gibco BRL, Carlsbad, CA,USA),10% fetal bovine serum (FBS, Gibco BRL, Carlsbad, CA,USA), 100U/mL penicillin, 100 mg/mL streptomycin (Gibco BRL, Carlsbad, CA,USA), and amphotericin B (BiochromAGBiotechnologie, Leonorenstra ße2, Berlin, Germany), and then incubated at 37°C and 5%CO_2.The medium was refreshed every 2days until achieving 80% confluence for optimal cell harvesting [4].

Phase II: Characterization of Isolated DPSCs:

Characterization of DPSCs as being mesenchymal stem cells (MSCs) was identified by their fusiform–shaped morphology.

Differentiation potential of DPSCs

To induce osteogenic differentiation, the culture medium was replaced with a differentiation medium (alpha-MEM supplemented with 50 mg/ml ascorbic acid2-phosphate [Sigma Chemical Co.], 10nM dexamethasone [Sigma Chemical Co.], 10mM b-glycerophosphate [Sigma Chemical Co.], and 10% fetal bovine serum) three times per week. After 3 weeks, osteogenic differentiation was examined by Alizarin red staining(Sigma Chemical Co.).

Reverse–transcriptase Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed to confirm that the DPSCs expressed stem cell markers. Briefly, the total RNA was extracted from the cells using RNeasy purification reagent (Qiagen, Valencia, CA, USA), and 1µg of the extracted RNA was reverse transcribed using AMV reverse transcriptase (RT) for 30 min at 42°C in the presence of oligo-dT primer. Polymerase chain reaction (PCR) was performed using specific primers for stem cell markers (Oct4 and Nanog; Table 1). Glyceraldehyde 3 phosphate dehydrogenase (GAPDH) and water were used as internal and negative controls for all samples, respectively. PCR was performed for 35 cycles as follows: denaturation at 95°C for 30 seconds, annealing at 5°C to 63°C for 30 seconds, and elongation at 72°C for 1 min, with final 10-min incubation at 72°C after completing the last cycle. The PCR products were separated by electrophoresis through a 1% agarose gel, stained, and photographed under ultraviolet light.

Table 1: Primer sequences used in RT-PCR

Gene	Primer sequence
NANOG	Forward: CCTTCTCCAATGCCTGAGGTT Reverse: CATTGGCAAGGATGCAGGAT
Oct4	Forward : TGCAGCTCAGTTTCAAGAATAT Reverse : AATAGTCACTGCTTGATCGTTT
GAPDH	Forward: TGTCCCCACCCCCAATGTATC Reverse: CTCCGATGCCTGCTTCACTACCTT

Flow cytometry analysis

Adherent cells (at the end of the 3rd passage) were washed and resuspended in phosphate-buffered saline. Monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) against CD14, CD73, and CD146 were added directly to the suspension and incubated for 1 h at 4°C. The cells were then incubated with anti-mouse immunoglobulin G fluorescein conjugated secondary antibody (Millipore Corp, Temecula, CA, USA) for 45 minutes on ice. The same species isotypes will be served as a negative control. The cell suspensions were washed twice and analyzed using a BDFACS Calibur flow cytometry system (Becton Dickinson, San Jose, CA, USA).

Phase III: Induction of apical periodontitis

The pulps of groups (A) and (B) teeth were mechanically exposed with a #2 round carbide bur in a high speed hand-piece under non aseptic conditions. A sterile #20 stainless steel endodontic hand file was used to disrupt the pulp tissue in the canal spaces without removing it from the canals. Supra-gingival plaque scaled from the surrounding teeth was mixed with sterile saline (0.9% sodium chloride; Hospira Inc., Lake Forest, IL, USA),and the suspension was soaked with sterile sponges and sealed temporarily in the pulp chambers using intermediate restorative material (IRM). The animals were administered analgesia (butorphanol tartrate 0.2 mg/kg; Torbugesic; Fort Dodge Animal Health, Fort Dodge, IA, USA) postoperatively.

Teeth in groups (A) and (B) were radiographed using radiograph paralleling devices. These aids were used for all subsequent radiographs at the identical angle and magnification. The teeth were monitored radiographically every week until there was radiographic evidence of apical periodontitis (approximately 3 weeks).

Phase IV: Disinfection

The teeth in groups (A) and (B) teeth were re-entered under aseptic conditions, isolated with rubber dams and retractors, and the surfaces disinfected with 0.12% chlorhexidine and iodine (Humco, Texarkana, TX,USA) with the animals under general and local anesthesia. After removing the IRM and sponge, the canals were irrigated with 10 mL of 2.5% NaOCl. No mechanical instrumentation was performed in the canals. The canals were dried with sterile paper points and disinfected with a solution comprising equal parts metronidazole, ciprofloxacin, and minocycline in sterile saline (0.9% sodium chloride, Hospira Inc) [17] .The paste was applied to the canal spaces with a sterile lentulo spiral in a slow speed hand-piece. The teeth were closed temporarily with a sterile sponge, followed by IRM and glass ionomer for 2 weeks to

allow disinfection of the canal spaces. After 2 weeks, the temporary restorations and sponges were removed from the experimental teeth under aseptic conditions, and general and local anesthesia. The antibiotic mixture was irrigated from the canals with 10mL of 2.5%NaOCl and 10mL of sterile saline per tooth. The teeth were now ready for stem cell transplantation.

Phase V: Transplantation of stem cell-scaffold construct into root canals

The root canals of group (A) teeth were transplanted with their own previously expanded cells (autologous) at a dose of 5×10^5cells(Ishizaka et al. 2012) [18] supplemented with several growth factors:10ng/mL vascular endothelial growth factor (VEGF-2) (R&D, Minneapolis, MN, USA);100 ng/mL basic fibroblast growth factor (bFGF) (R&D); 10 ng/mL platelet-derived growth factor (PDGF) (R&D); 50 mg/mL nerve growth factor (NGF); and 100 ng/mL bone morphogeneticprotein-7 (BMP7).The growth factors (2mg/mL) were adsorbed onto a chitosan hydrogel scaffold that was seeded with undifferentiated DPSCs. Thechitosan hydrogel scaffold was fabricated as follows.Chitosan (1 g; 77% deacylation, high molecular weight; Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 2% acetic acid under agitation. Sodium alginate powder (Sigma-Aldrich; St. Louis, MO, USA) was dissolved and thoroughly mixed in 25mL of distilled water, and the chitosan solution was added and mixed homogenously. The construct comprising DPSC, growth factors, and the chitosan hydrogel scaffold was injected into the pulp chambers and root canals of group(A) teeth, while the root canals of group (B) teeth received only the chitosan hydrogel scaffold with growth factors.

Finally, teeth in both groups were closed with a double coronal seal of white MTA (Dentsply Tulsa Dental; Johnson City, TN, USA) and composite (P60; 3M Dental Products; St. Paul, MN,USA).

Phase VI: Radiographic analysis

Teeth in both groups (A) and (B) were monitored radiographically once a month for 4 months. Initial radiographs made at the time of disinfection were compared with the follow-up radiographs after transplantation. Each tooth root was taken as the unit of measure. Two examiners evaluated the radiographs independently of one another after a training session explaining the gold standard of the three evaluation parameters. Each examiner graded each root for the following parameters: diminished size or absence of periapical radiolucency, presence or absence of continued thickening of radicular walls, presence or absence of continued root lengthening and presence or absence of apical closure. The kappa statistic values comparing the two evaluators were as follows: 0.66 for evaluation of periapical radiolucencies, 0.62for evaluation of both thickness and lengthening of radicular walls, and 0.60 for evaluation of apical closure, all indicating good agreement between the two evaluators. When there was not agreement between both evaluators, a discussion was undertaken until a consensus was reached. The data were analyzed with chi-square tests, with the level of significance set at $p < 0.05$, to determine if there were any significant differences between the experimental groups.

Phase VII: Histopathological study

The animals were sacrificed under general anesthesia. The jaws with the treated teeth were resected and placed in formaldehyde (Fisher Scientific).After removing the soft tissue and excess hard tissue, the specimens were decalcified in Formical (Decal Chemical Corporation, Congers, NY, USA) for 6 days and Immunocal (Decal Chemical Corporation, Tallman, NY, USA) for 2 months. The teeth were removed from the jaw, embedded in paraffin, sectioned longitudinally, stained with hematoxylin and eosin, and evaluated under light microscopy for the presence or absence of healthy and vital tissue, and new mineralized tissue (pulp and dentin-like tissue).

Statistical analysis

Statistical analysis was performed using SPSS 18.0 (Statistical Package for Social Studies Inc., Chicago, IL, USA) for Windows. The groups were compared using the Chi square test. Statistical significance was set at $P < 0.05$.

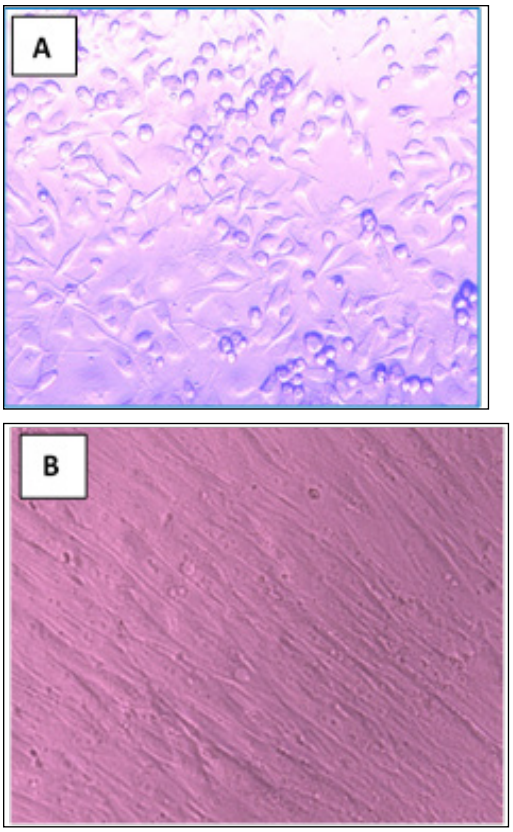
RESULTS

Characterization of Isolated DPSCs

The isolated DPSCs exhibited a spindle-shaped morphology (Figure 1)

Figure. 1: Examination of isolated dental pulp stem cells (DPSCs).

(A)The isolated dental stem cells exhibited a fibroblastic spindle shape. (B) Cells were harvested after reaching 80% confluence.



Differentiation potential of DPSCs

Representative images of the differentiated dental stem cells are shown in (Figure 2A). The dental stem cells that were grown in osteogenic inductive medium for 21 days were positive on Alizarin red staining, (Figure 2B). These results confirmed that the isolated DPSCs could differentiate into other phenotypes.

Expression of stem cells markers on RT-PCR

DPSCs were assessed by RT-PCR for the presence of undifferentiated stem cell markers including Oct-4 and Nanog, with GAPDH and water as the positive and negative controls, respectively. All DPSCs showed expression of these markers (Figure 3).

Expression of DPSC markers on flow cytometry

The results of the flow cytometric analysis are shown in figure 4 (DPSCs were weakly positive for CD14 (1.73%) and CD73 (2.16%), and strongly positive for CD146 (92.1%).

Radiographic findings

Chi-square test analysis of the radiographic results showed no statistically significant differences between groups (A) and (B) in the healing of radiolucency($P = 1.000$). However, the radicular thickening differed significantly ($P = 0.001$) between groups (A) and (B). In addition, root lengthening was significantly greater in group (A) than in group (B) ($P = 0.004$), and the rate of apical closure was significantly higher in group (A) than in group (B) ($P = 0.004$) (Table 2 and Figure 5).

Histopathological findings

When longitudinal sections of the group (A) teeth, which were transplanted with chitosan hydrogel scaffold seeded with DPSCs and growth factors, were assessed, we found that the emptied canal was filled with regenerated pulp-like tissue. Fibrous tissue with detached pulp stone and multiple large and small blood vessels were present; some vessels were empty or showed fatty degeneration,

Figure. 2: Differentiation of dental pulp stem cells (DPSCs).

(A) Undifferentiated stem cells. (B) Three weeks after induction in osteogenic condition media, the cells have differentiated into osteoblasts, as indicated by the Alizarin red staining.

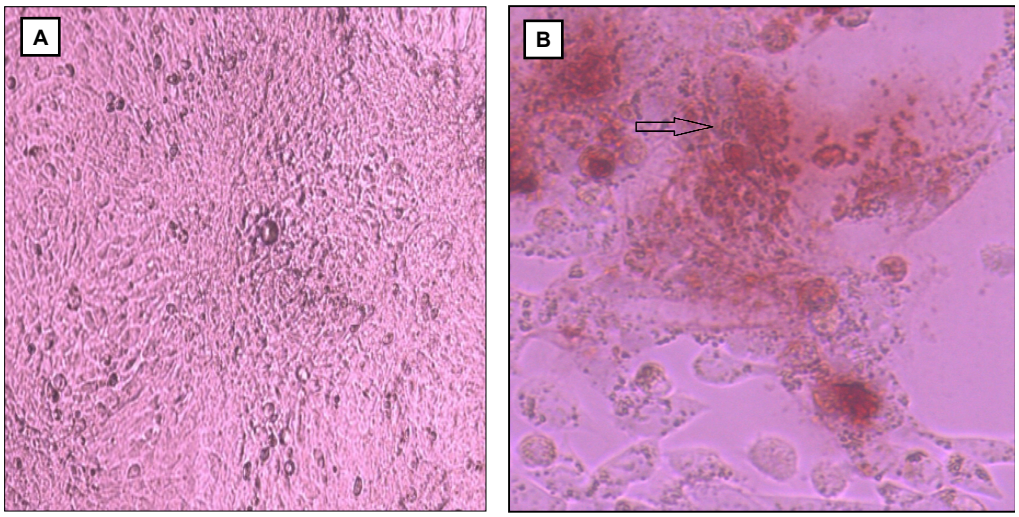


Figure 3: Gene expression of stem cell markers in dental pulp stem cells (DPSCs).

The agarose gel electrophoresis shows the RT-PCR products of stem cell markers in dental pulp stem cells (DPSCs). Lane M:DNA ladder with 100-bp DNA ladder (Fermentas Canada Inc., Canada),

Nanog (425bp), Oct-4 (245bp), and the positive control GAPDH (156 bp) in lanes1, 2, 3 respectively. The negative control (water) is shown in lane 4.

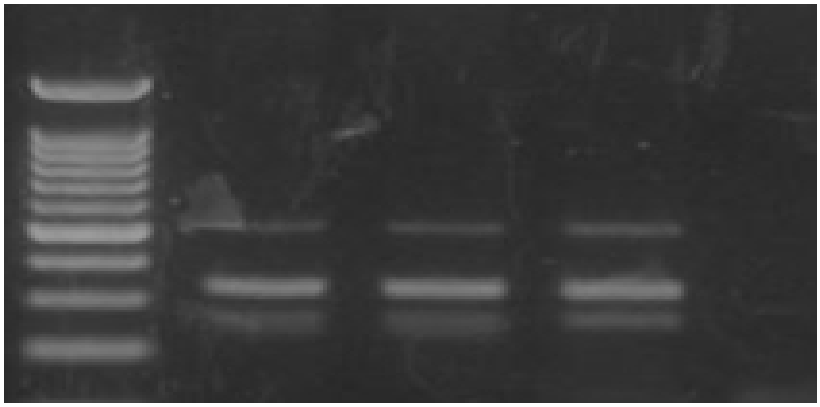


Table 2: Comparison of radiographic findings post-transplantation

Parameter	Group A (n = 12)		Group B (n = 12)		*P* value
	Yes	No	Yes	No	
Healed periapical radiolucency	10(83.33%)	2(16.67%)	10(83.33%)	2(16.67%)	1.000
Radicular thickening	11(91.67%)	1(8.33%)	3(25%)	9(75%)	0.001*
Radicular lengthening	9(75%)	3(25%)	2(16.67%)	10(83.33%)	0.004*
Apical closure	9(75%)	3(25%)	2(16.67%)	10(83.33%)	0.004*

*Data expressed as the number (percentage). Groups were compared using the Chi-square test, with P < 0.05 considered significant.

Figure. 4: Flow cytometry of the isolated dental pulp stem cells (DPSCs).DPSCs were weakly positive for CD14 (1.73%) and CD73 (2.16%), and strongly positive for CD146 (92.1%).

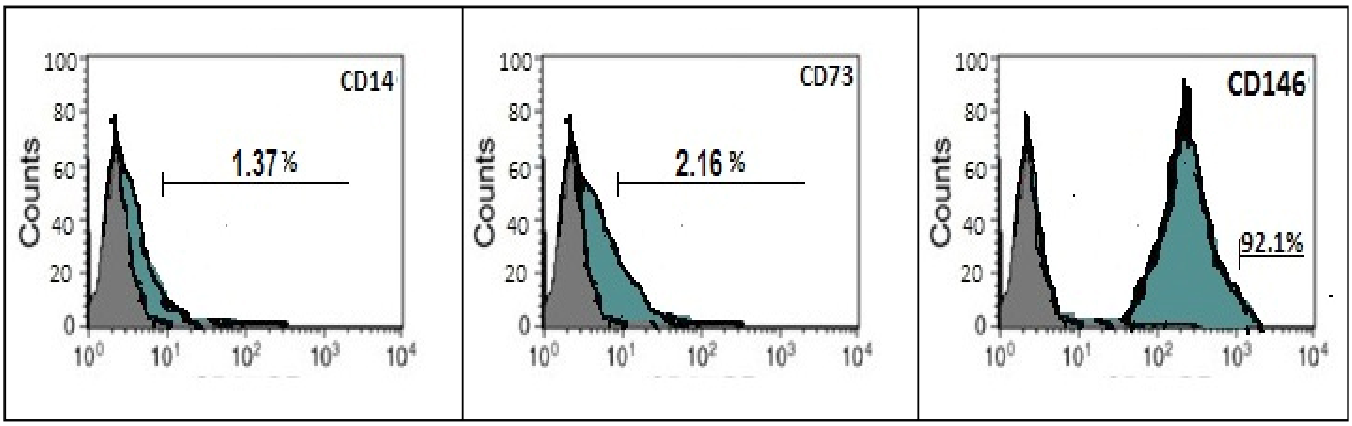
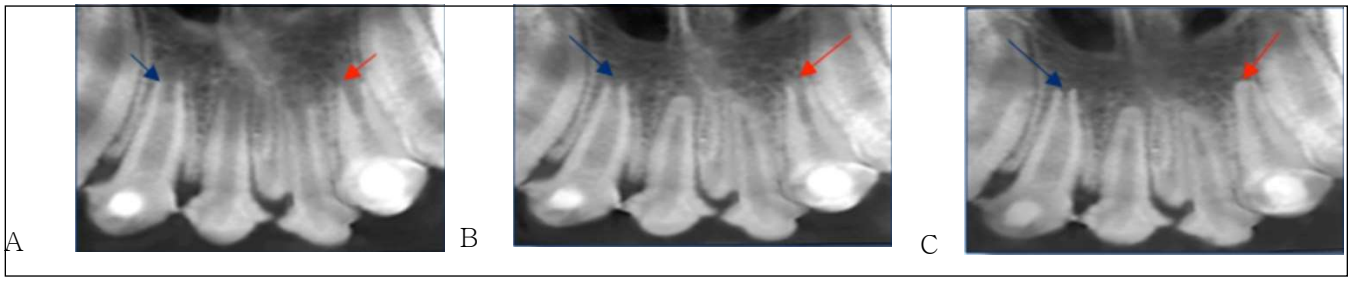


Figure. 5: Radiographic follow up. (A) Immediate postoperative radiograph showing immature upper permanent incisors with periapical radiolucent areas in the maxillary right and left 2nd incisors. (B) Follow-up radiograph (2 months) post-transplantation showing reduction of the periapical lesion and narrowing of the apex. (C) Follow-up radiograph (4 months) showing thickening, lengthening, and root apex closure of teeth in group (A) red arrow, with no thickening, lengthening, or root apex closure in group (B) blue arrow.



while others contained fluid. The vascularized pulp-like tissue resembled the natural pulp (Figure 6). In group (B) teeth, no soft tissues were observed (Figure 7).

A layer of regenerated dentin-like tissue was deposited onto the canal walls in both groups. The newly deposited dentin-like tissue was tightly adhered to the original dentin, except for gaps caused by histologic processing artifacts. The regenerated dentin-like tissue did not form well-organized dentinal tubules, except in a few regions where the odontoblast-like cells were better aligned. The regenerated dentine was entrapped by fibrous tissue and degenerated blood vessels. Apical closure was observed in 16.67% of teeth in group (B).

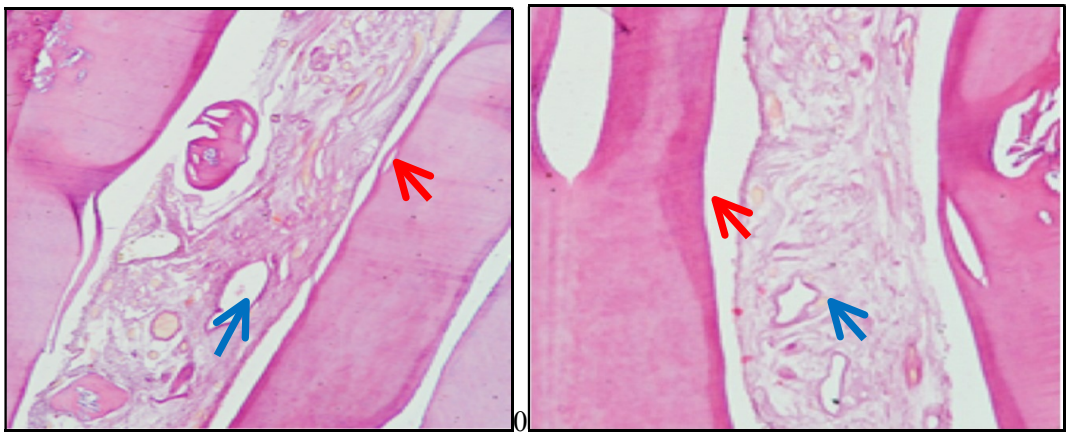
DISCUSSION

The present study simulates the management of patients with necrotic immature permanent teeth and apical periodontitis due to trauma or caries. This study is one of the few demonstrating evidence of the potential role of DPSCs in the regeneration and synthesis of vascularized pulp and dentin-like tissues in necrotic immature root canals with apical periodontitis.

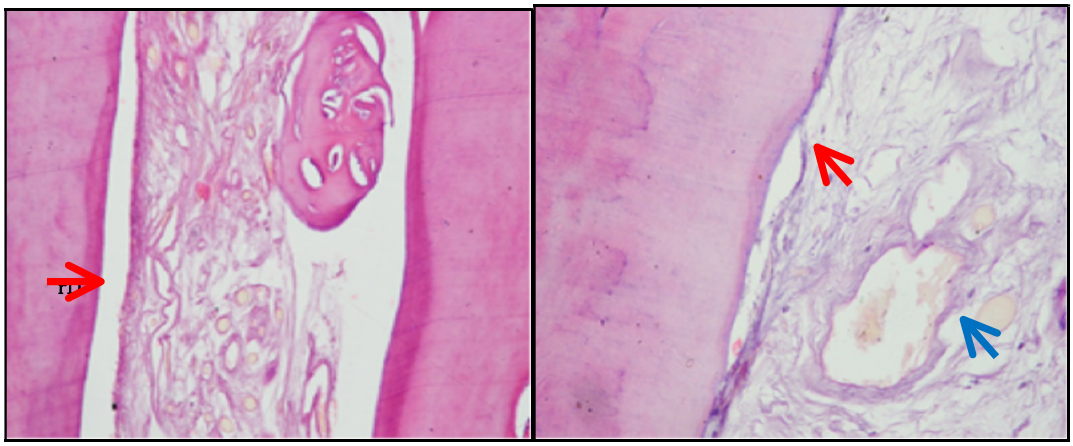
The three main components required to create a tissue-engineered construct are cells, scaffolds, and growth factors. DPSCs are clonogenic and proliferate rapidly. They can differentiate into odontoblast-like cells, making them the most promising candidate for regeneration of the dentin-pulp complex. They are also similar to other types of adult stem cells, self-renewable, and have multi-lineage differentiation potential, including the ability to differentiate

Figure 6: Showing delicate fibrous tissue resembling the pulp tissue inside the root canal containing multiple large and small blood vessels (some blood vessels are free of RBCs, some are empty or show fatty degeneration while the others are filled with fluid) (blue arrows).Newly formed dentin like tissue was shown (red arrows) H& E.

Group A: Teeth transplanted with construct of the dental pulp stem cells, growth factors in chitosan scaffold



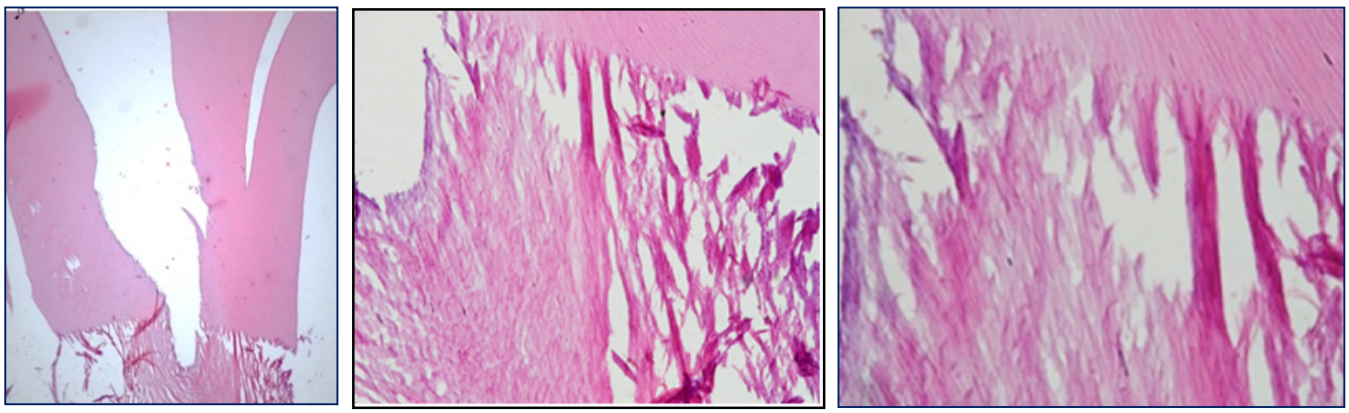
X100X100



X200X400

Group B: Teeth Transplanted with Only Chitosan Scaffold and Growth Factors

Figure. 7: Showing pulp canal free of any tissues with the formation of reparative dentin, (rD) also shows continued root development with incomplete bridging of the apical (X100) foramen formed of fibrous calcified tissues,(X200 and higher magnification of X400), H& E.



X100 X200 X400

into neurons of the peripheral nervous system. Dental pulp is derived from migrating neural crest cells, suggesting that DPSCs may also be an appropriate candidate for nerve regeneration. Thus, DPSCs are capable of generating a tissue with morphological and functional characteristics closely resembling those of dental pulp [19-21].It has been reported that DPSCs differentiate into the endothelial cells that form blood vessels [4, 22].

In the current study, the isolated DPSCs were characterized by gene expression and flow cytometry. The cells expressed the pluripotency markers Oct-4 and Nanog, which are transcription factors. This is in agreement with findings by Vishwanath *et al* [22],who found that Oct-4 and Nanog were expressed as stem cell markers and indicated their pluripotent behavior.

The flow cytometric analysis showed that cells expressing CD14 were few (1.37%), indicating that the population primarily comprised mesenchymal stem cells and not hemopoietic cells. This agrees with findings by Vishwanath *et al* [22], who reported that dental pulp stem cells do not express hemopoietic stem cell markers. Also in the current study the cDPSCs expressing CD73 comprised only 2.16% of the population however, it shows high CD146 expression, these results consistent with those of Dissanayaka *et al*,[23] who similarly observed high CD146 expression in cDPSCs in contrast to human bone marrow mesenchymal stem cells while they were negative for CD73,

The second essential component of tissue engineering is the scaffold. In this study, a chitosan hydrogel type scaffold was used. This material has several characteristics that ensure successful tissue regeneration as follows: the ability to accept seeding of stem/progenitor cells and supplementation with growth factors; does not induce an acute or chronic inflammatory response; biodegradable, allowing the cured tissue to replace the biomaterial; adequate biomechanical properties; bioactivity facilitating attachment to soft and hard tissues; biocompatibility with the host tissue; osteo-inductivity, and angiogenic potential. Chitosan hydrogel type scaffolding can replace missing or damaged tissues and organs, promote cell attachment and proliferation, and accelerate tissue regeneration. [24,25] Ultimately, the goal of employing this injectable scaffold is to allow for stem cell transplantation in the entire root canal and pulp chamber system.

The third component of the construct is growth factors. Proliferation, differentiation, and survival of DPSCs are modulated by multiple growth factors in the pulp tissue. These factors have potentially important roles in signaling during dentin and pulp tissue repair, and thus, in pulp regeneration[16,26,27].

In the present study, multiple growth factors were integrated into the scaffold, which was already seeded with stem cells. This protocol was based on previous reports stating that the combination of several growth factors such as beta-Fibroblast Growth Factor (β-FGF), Vascular Endothelial Growth Factor (VEGF), or Platelet-Derived Growth Factor (PDGF), with Nerve Growth Factor (NGF) and Bone Morphogenetic Protein-7 (BMP7) plays an important role in the induction and differentiation of DPSCs into odontoblast-like cells and building of new pulp and dentin-like tissues[28,29].

One major concern in dental pulp tissue engineering is the generation of a functional vascular network that can rapidly establish an efficient blood vessel network. A rich vascular network can help maintain the high metabolic demands of DPSCs, as the blood supply for the space only comes from the apex. VEGF was used in the current study because it is considered the most important regulator of vasculo genesis and angiogenesis. When seeded into three-dimensional collagen gels, VEGF induces endothelial cells to form capillary structures and triggers the differentiation of DPSCs into endothelial cells [30,31]. Basic fibroblast growth factor and PDGF were added for their chemotactic and angiogenic roles. NGF was included because of its role in promoting the survival and growth of nerve fibers, while BMP7 was added to promote mineralized tissue formation [32-35].

Some of the growth factors embedded in the dentin matrix, such as transforming growth factor β,bone morphogenic proteins (BMPs), PDGF, fibroblast growth factor, and VEGF, can release, attract, and induce odontoblast differentiation of seeded stem cells during pulp regeneration [14] ; however chemical disinfection of the root canal space may damage these embedded growth factors [7].Notably, Cordeiro *et al* suggested that existing dentin is sufficient to guide stem cells within the canal space to differentiate into odontoblast-like cells, and chemical treatment of dentin did not appear to affect this capacity [14].

In this study, the radiographic and histologic investigation revealed that the construct comprising dental pulp stem cells, growth factors, and chitosan scaffold transplanted in the group (A) teeth survived well and were able to regenerate new pulp and dentine-like tissues. Radiography demonstrated that 91.67% of teeth in group (A)had radicular thickening, and 75% had root lengthening and apical closure. This prevalence are significantly higher than those observed in the teeth of group (B), which were transplanted with growth factors and scaffold only, with 25% of teeth showing radicular thickness and only 16.67% showing increased root length and apical closure.

Although the group (B) teeth were transplanted with the growth factors and chitosan alone,16.67 %of teeth still showed continued root development, which may be caused by the presence of stem cells residing in the apical papilla of incompletely developed teeth (SCAP). These cells have collateral circulation enabling them to survive during pulp necrosis and under the influence of the surviving epithelial cells in the Hertwig's root sheath, they can differentiate into odontoblast-like cells and continue root formation[13,36-39].

The histological investigation showed that in the group (A) teeth, a pulp-like tissue was regenerated in the root canals, while the group (B) teeth did not show any soft tissue formation within the root canals. The group (A) teeth also contained a layer of new dentin-like tissue with dentinal tubule-like structures along the dentinal walls of the root canal. The disorganization of the newly synthesized dentin-like tissue and the alignment of the odontoblast-like cells may be explained by the occupation of the dentinal tubule by a newly differentiated odontoblast-like cell with lessened potentiality.

These findings consistent with those of Huang *et al* [15], who examined the potential regeneration of vascularized human dental pulp and new dentin production within the emptied root canal space using a stem/progenitor cell-mediated approach using a human root fragment and immune compromised mouse model. He stated that the tertiary dentin produced by the natural pulp less organized dentinal tubules and possible cell entrapment within the dentin, and that the newly regenerated dentin-like tissue may be similar to tertiary dentin. He showed that pulp-like tissue can be regenerated. The present findings are also in agreement with those of other studies that directly delivered autologous or allogeneic stem cells into the root canals in animals and show promising results for pulp and dentin regeneration[40,41].Similarly, Zhu et al [42] using fifty-six immature roots from mandibular premolars of four beagles dogs investigated the new tissues growing into the pulp space of dog teeth that were infected, disinfected and filled with blood clot (BC), dental pulp cells (DPCs), platelet-rich plasma (PRP) or a combination of DPCs and PRP. He concluded that a combination of DPCs + PRP increased vital tissue regeneration within the root canals of immature teeth associated with apical periodontitis.

CONCLUSION

Transplantation of processed autologous dental pulp with growth factors embedded in chitosan hydrogel scaffold may prove useful in regenerating pulp and dentin-like tissues in necrotic immature permanent teeth with apical periodontitis in dogs. These findings can be added to other evidence that processed autologous dental pulp has the potential to survive well, differentiate into odontoblast-like cells, and regenerate pulp and dentin-like tissue in necrotic immature teeth with apical periodontitis. Further studies are needed to support these findings.

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CONFLICT OF INTEREST

None. The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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