



Research Article

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In vivo and immunohistochemical study of dentin and pulp tissue regeneration in the root canal

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ABSTRACT

The ultimate goal of endodontics therapy is the regeneration of tooth supporting tissues. This case study evaluated whether the transplantation of autologous dental pulp stem cells with chitosan scaffold into pulpless root canals could mediate pulp regeneration in human mature teeth. The dental pulp stem cells isolated from the patient's pulp tissue. The cells were then seeded onto a chitosan scaffold and then transported into the root canal of the patient. After eight weeks the tooth was extracted and prepared for histological and immunohistochemical examination in purpose to identify the nature of tissues formed in the canal space. After endodontic regeneration procedures, pulp-like tissue characterized by the presence of odontoblast-like cells aligning dentin-like hard tissue was observed. This is the first clinical and histological evidence in the dental literature that shows that pulp and dentin like tissue can be generated in a human tooth using dental pulp stem cells.

Key words: Dental pulp stem cells, Chitosan, Pulp regeneration, Human root canal.

INTRODUCTION

The health of teeth depends on the integrity of the hard tissue and the activity of the pulp and periodontal tissues, which are responsible for supplying nutrition to teeth. The Lack of nutrition provided by the pulp tissue can increase the risk of tooth fracture.[1] Regeneration of the dentin and pulp tissues in the pulp space of teeth serves the ultimate goal of preserving teeth via endodontic approaches. Attempts to induce tissue regeneration in the pulp canal space have been a longstanding investigation.[1, 2] Pulp tissue regeneration has been explored using the biodegradable synthetic material polyglycolic acid seeded with dental pulp stem cells (DPSCs), and results showed pulp-like tissue formation in both in vitro and in vivo models.[1, 3] These previous approaches were the proof-of-principle studies that only tested the formation of a pulp-like soft tissue without dentin formation. From a clinical perspective, the following issues must be considered when attempting to regenerate functional pulp and dentin tissues in a root canal space: (1) regenerated pulp tissue must be vascularized although the blood supply is only available from the apical end; (2) newly differentiated odontoblasts should form on the existing dentinal wall in the root canal space, and (3) new dentin should be produced by the new odontoblasts onto the existing dentin.[4, 5]

To date, there is a lack of evidence demonstrating that the human pulp tissue can be regenerated in an emptied root canal space with only one opening to the blood supply. Using human DPSCs from permanent teeth seeded onto a dentin surface, small amounts of discontinuous dentin-like mineralized tissue on the existing dentin surface have been observed in vivo.[6] Stem cells can have an embryonic or adult origin.[7, 8] Adult stem cells are present in a wide range of tissues such as the pancreas, bone marrow, adipose tissue and umbilical cord. Because they are

obtained from the patients themselves, these cells have the advantages of not triggering immune rejection, responding to growth factors inherent in the host, and not incurring ethical or moral objections.[8] Recently, researchers have tried to use tissue engineering techniques in pulp regeneration, with some promising results. Huang *et al*; reported that pulp-like and dentin-like tissues were formed on subcutaneous root canal implants containing synthetic scaffolds seeded with stem-progenitor cells from apical papilla and dental pulp.[9] Kodonas *et al*; seeded swine DPSCs on organic or synthetic scaffolds before implanting them as hybrid root implants in the jaw bones of mini pigs. The results showed the formation of dentin-like structures.[10] In another research a tooth slice model was used, and showed that the stem cells from human exfoliated deciduous teeth seeded onto the synthetic scaffolds which were fabricated in the pulp chamber space formed well-vascularized pulp-like tissue in an *in vivo* study model. In addition, odontoblast-like cells derived from the pulp-like tissue were localized against the existing dentin surface.[11]

As essential elements in tissue engineering, scaffold materials have shown their importance in supporting newly formed tissue in pulp regeneration studies. A number of biodegradable materials including synthetic and natural polymers have been successfully used as supportive scaffolds for dental pulp tissue engineering.[12-15] Among them, naturally derived polymers are of special interest due to, as natural components of living structures, their biological and chemical similarities to natural tissues.[16] In recent years, many studies have been investigated the application of scaffold materials for regenerative medicine and tissue engineering.[17] Chitosan is one of the widely recommended scaffold materials for its unique features, such as no toxicity, biodegradability, and ability to be produced in different forms for tissue engineering to repair and regenerate injured tissues and organs.[18-20] Separate studies have also demonstrated that three-dimensional (3D) scaffolds can create a microenvironment for the cells to attach and grow *in vitro* and *in vivo*. The scaffold can transport the cells by holding them in their particular 3D structure, as well as regulate biological functions of cells.[21] Chitosan can be processed into interconnected porous 3D structure by freeze-drying, freeze-gelation, and other techniques.[22, 23]

However, no studies have investigated the nature of the tissue present in mature teeth after regeneration procedure in humans. The aims of this clinical case study is that dental pulp stem cells combined with chitosan as the scaffold material might mediate dental pulp regeneration in mature permanent teeth.

EXPERIMENTAL SECTION

Ethics

An eighteen year old female patient has been considered to extract her lower first right premolar for orthodontics therapy. The patient had an unremarkable medical history, no other oral or systemic diseases. She was not on medications and denied any allergies. Informed consent was obtained from the patient and this study was reviewed and approved by Ethics Committee of the dental collage of Damascus University (no.8; 30/01/2011).

Tissue collection

Radiographic and intraoral examination of the premolar tooth did not show any carious lesions. Pulp sensibility tests were performed with Endo-Ice (Coltene, Switzerland) and heated gutta-percha, and the premolar tooth was responded to the tests within normal limits and was not sensitive to palpation or percussion.

Human dental pulp stem cells were obtained as previously described by other studies.[24-26] The procedure requires strict control of the aseptic chain due to widespread presence of microorganisms in the oral environment. Scaling with ultrasonic devices and hand instruments, extraoral asepsis and intraoral prophylaxis were performed and rinsing with chlorhexidine 2%. local infiltrating anesthesia (2% lidocaine with 1:100,000 epinephrine) was applied, followed by rubber dam isolation and access to the pulp chamber was made using sterilized diamond burs (komet, Germany) at high speed handpiece under constant distilled water irrigation, and the pulp tissue removed with endodontic files k-file#20 (Mani, Japan), and were immediately placed in individual containers filled with a-Minimum Essential Medium (a-MEM; Gibco-Invitrogen, Uk), with 100 units/mL penicillin-G, 100 µg/mL streptomycin, and 0.25 mg/mL fungizone (Gibco-Life Technologies, Usa), stored under controlled temperature 4°C, and sent to a laboratory for cell isolation (Fig.1).



Figure 1. The pulp tissue after isolation from the canal space of the tooth

Root canal preparation

Working length was estimated with an apex locator Root Zx II (Jmorita, Japan) and confirmed with periapical radiograph. Then, the root canals were prepared using Protaper universal rotary files (Dentsply Maillefer, Switzerland) up to file F5 with 17% EDTA (Pulpdent, Usa) irrigation. The prepared canal received a final rinse with 5 mL physiological saline and was dried with sterile paper points and the access cavity was sealed with 4 mm of Vitrebond (3M ESPE, Usa) as a temporary restoration.

Culture of dental pulp stem cells

The entire procedure of cell culture was performed in a laminar flow unit (Microflow, Advanced Bio Safety Cabinet-Class II). the pulp tissue was placed in culture dish containing a-MEM and minced (Fig. 2,A), then digested in a solution of 3mg/mL collagenase type I and 4mg/mL dispase (Sigma-Aldrich, Usa) for 30–60 minutes at 37° C (Fig. 2,B). The digested mixtures were passed through a 70 μ m cell strainer (Falcon; BD, Usa) to obtain single-cell suspensions. Cells were seeded in 25-cm² culture flasks and cultured with a-minimum essential medium (Gibco-Invitrogen, Uk) supplemented with 15 % fetal bovine serum (FBS, Gibco-Life Technologies, Usa), 2mM L-glutamine, 100 mM L-ascorbic acid-2-phosphate, 100U/mL penicillin-G, 100 μ g/mL streptomycin, and 0.25 mg/mL fungizone (Gibco-Life Technologies, Usa) and cultured under 5% CO₂ at 37°C. The culture was monitored by means of an inverted optical microscope (Nikon, Eclipse-TS100, Japan) combined with digital camera (Sony, DSC-H1, Canada).

The medium was replaced every three days during a period of approximately 21 days when cells culture reached about 80-90 % confluence, and was passage at 1:3 ratio. Cells were expanded for experimentation at passage 3.

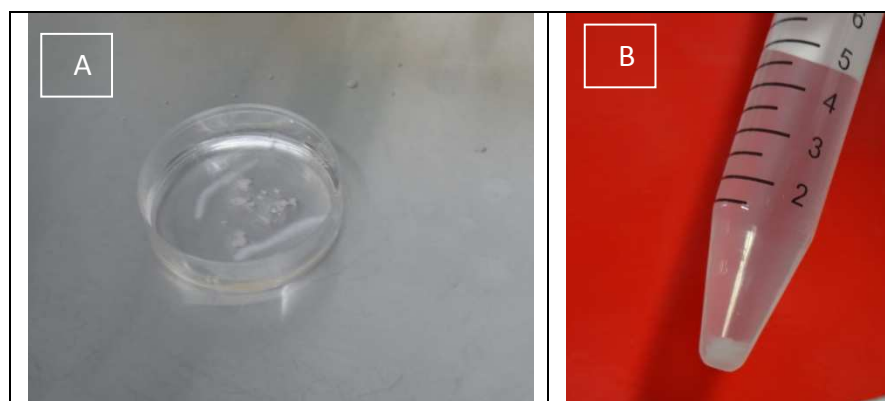


Figure 2. (A): the isolated pulp tissue was cut into small segments. (B): after digestion of the pulp tissue

Morphological study of the DPSCs culture

Colony formation units of fibroblastic cells were normally observed within 1–2 weeks after cell seeding (Fig. 3,A). After adherence to the plastic surface, stem cells initially exhibited an ovoid shape that evolved early during the first 24 hours to a typical fibroblast-like form, which remained until confluence (Fig. 3,B).

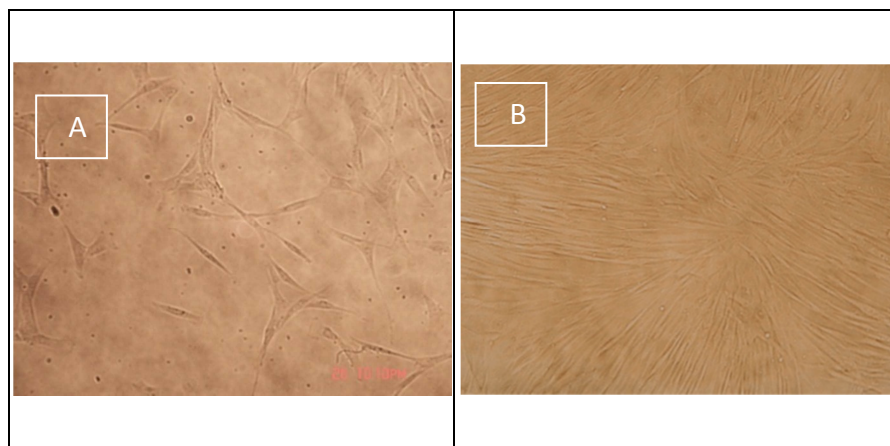


Figure 3. (A): Primary culture of Dpscs during the first week. (B): Culture of Dpscs with total confluence

Fabrication of chitosan porous scaffolds

Chitosan scaffolds were prepared by a combination of freeze gelation and leaching out technique of Sodium chloride crystals used as porogen.[27-30]

Chitosan (76 % deacetylated; Sigma Aldrich, Usa) (2 g) was dissolved in 1% (v/v) acetic acid solution (100 ml) and agitated with magnetic stirring for 48 h at room temperature (Fig. 4,A). The resulting solution was filtered and stored at 4°C until use. Sodium chloride salt (125–250 μm) was mixed in the chitosan acidic solution to 90 wt%. The mixture frozen at -80°C for 24 h, and then lyophilized for 24 h at -56°C by a freeze-dryer (Jlabtec, Korea) (Fig. 4,B). The chitosan–salt mixture was washed in ethanol (100, 90, 80, 70 and 50%, v/v) sequentially for 2 h each. Finally, after salt-leaching for 48 h in distilled water, the scaffold was frozen at -80°C for 24 h and then lyophilized for 24 h.



Figure 4. (A): dissolving chitosan powder in acetic acid solution with magnetic stirring. (B): lyophilizing the chitosan with freeze-dryer

Dental pulp stem cells and scaffold construct

The size of scaffold used for cell culture was 7 mm of length and 2 mm of thickness so as it resemble the prepared root canal space. Before culture, the scaffold was sterilized by placing under an ultraviolet lamp (Herolab, Germany) for 30 minutes, and then was soaked in 70% ethanol to pre-wet for 30 minutes and then was exchanged with phosphate-buffered saline (PBS, Gibco–Life Technologies, Usa) for three times (30 minutes each). The scaffold was then washed twice with culture medium (1 hour each). 10^6 DPSCs were seeded on the scaffold and the cell-scaffold constructs were cultured in 5 mL culture medium for 24 hour; and then transported into the patient's tooth.

Transplantation of DPSCs and scaffold construct to the patient

Four weeks later the second visit was scheduled, the tooth was asymptomatic subjectively. Objectively, the tooth was functional with normal periodontal condition and insensitive to percussion and palpation, and there was no presence of swelling. However, the tooth was not responsive to cold testing.

Local anesthesia was obtained by using 2% lidocaine with 1:100,000 epinephrine. After rubber dam isolation, the temporary restoration was removed, and the root canal was irrigated with saline and 17% EDTA (5 mL, 5 minutes) (Fig. 5,A), then a final rinse with 5 mL saline and was dried the canal with sterile paper points (Fig. 5,B).

Immediately, the Dpscs/chitosan construct were inserted into the root canal space of the tooth, and the accessed cavity was sealed with ProRoot white Mineral Trioxide Aggregate (MTA) (Dentsply Tulsa Dental, TN, Usa) (Fig. 5,C), and Vitrebond (3M ESPE, Usa) (Fig. 5,D) followed by final restoration using adhesive nanocomposite resin (Filtek Z350; 3M ESPE, Usa) (Fig. 5,E).

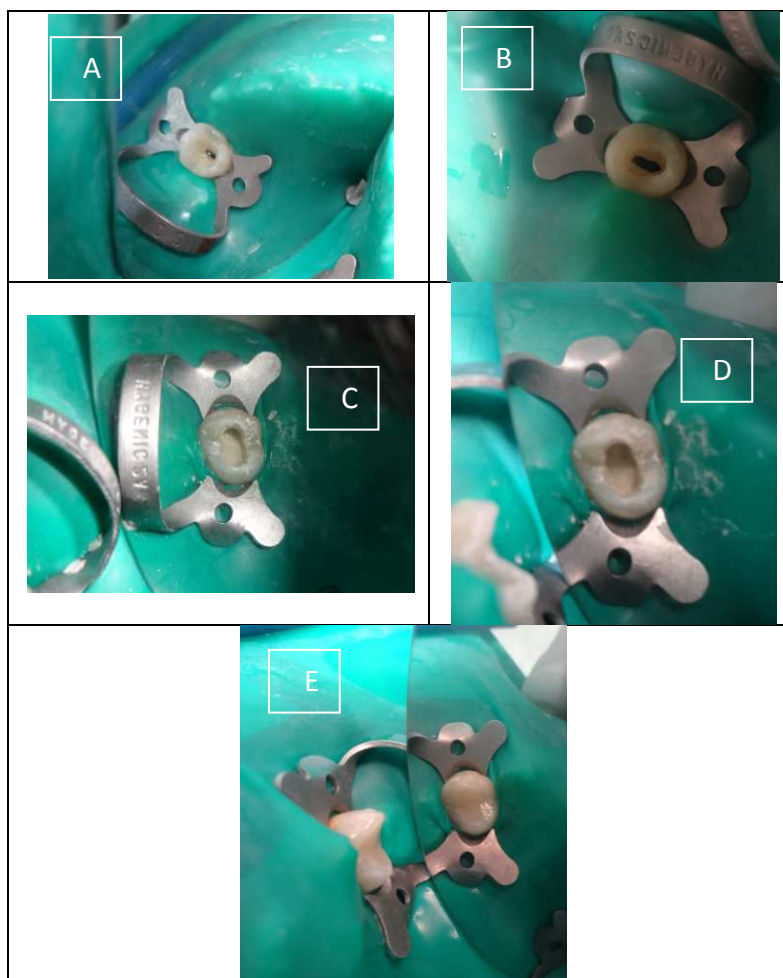


Figure 5. (A): irrigating the canal with EDTA 17 %. (B): after drying the canal with paper point. (C): MTA placement at the orifice of the canal. (D): after Vitrebond placement above the MTA. (E): the final restoration with adhesive composite

Extraction the tooth and histological analysis

At the third visit 8 weeks later, the tooth continued to be asymptomatic and negative to pulp sensitivity, showed no obvious change in shade or color, and postoperative radiographs were taken. The presence or absence of apical radiolucency was evaluated by two blinded examiners.

Next, infiltrating anesthesia was applied, followed by extraction of the tooth with forceps and processed for histological and immunohistochemical evaluation. Soft tissue remnants and crown restorations were removed to ensure adequate penetration of fixative into the canal. And the tooth was immediately placed in individual containers filled with formol 13 %.

The tooth decalcified in 10% Nitric acid for 4 weeks. The specimen was then soaked with ethanol (100, 95, and 90%) sequentially for 3 hours each, followed in xylol for 1 hour, and embedded in paraffin. Serial sections of approximately 5 μ m thickness were cut along the long axis of the tooth, and stained with routine hematoxylin and eosin (Merk, Germany) for histological analysis and examined under a light microscope.

Immunohistochemical staining

To evaluate the nature of the tissue immunohistochemical staining was performed. The sections were washed in phosphate-buffered saline (PBS) and immersed in methanol containing 3% hydrogen peroxide to block endogenous peroxidase activity and incubated with primary antibodies overnight at 48°C. Primary antibodies used were as

follows: dentin sialoprotein (DSP, LF-21), and bone sialoprotein (BSP II; LF-25), from (Santa Cruz Biotechnology, Santa Cruz, Usa).

For enzymatic immunohistochemical staining, Zymed SuperPicTure polymer detection kit DAB (Invitrogen, Usa) was used according to the manufacturer's protocol. Sections were counterstained with Mayer's Hematoxylin and eosin.

RESULTS

Clinical and radiographic findings of the tooth showed no signs of mobility, no swelling or sinus tracts, negative response to pulp sensitivity and the restoration was intact.

Histological findings of vital tissues in the canal space

The sample showed a regeneration of a vascularized connective tissue, capillaries were not congested and the fibers scattered randomly as in normal healthy connective tissue (Fig. 6.A). Surprisingly, in the superficial area of the connective tissue and just next to the dentin a layer of cells were clearly distinguished, these cells were cuboidal, centered ciphured nuclei and had a visible process penetrating a newly formed dentin-like structure and bypassing to the old dentin of the tooth (Fig. 6,B-C).

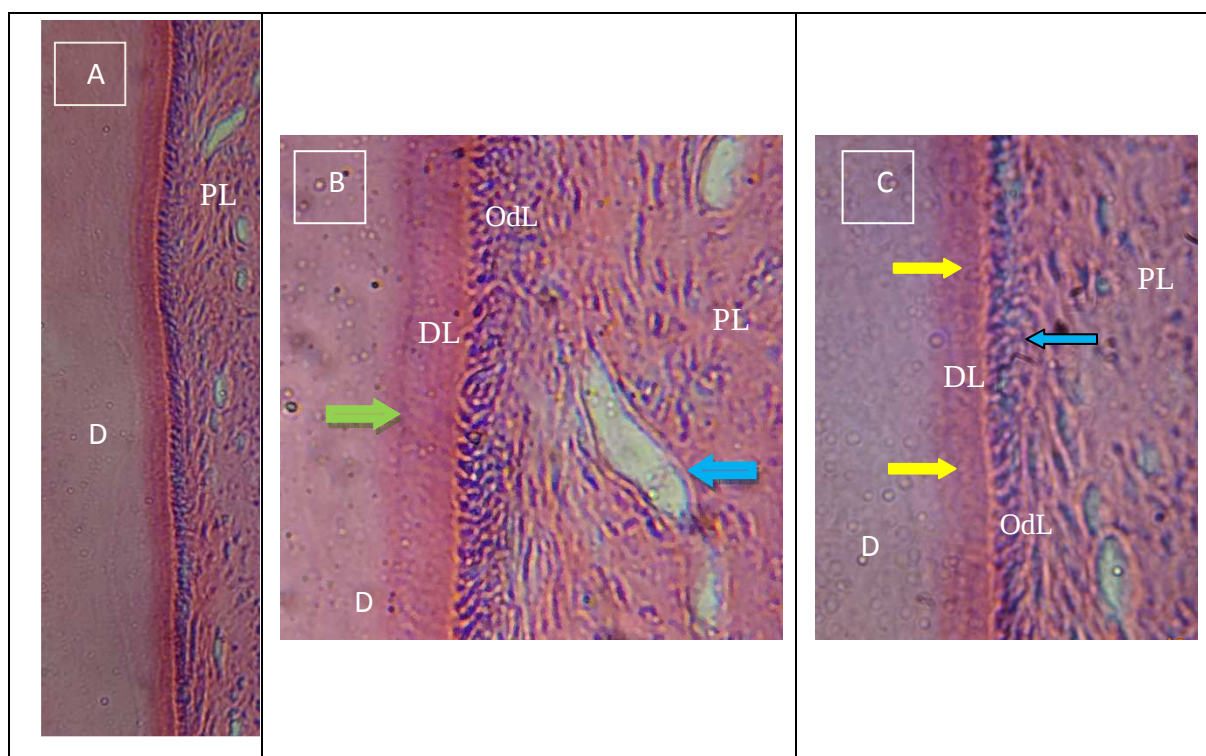


Figure 6. Histological analysis of pulp/dentin regeneration. (A): regeneration of pulp-like and dentin-like tissue. (B): the junction of original dentin and dentin-like tissue (green arrows), in addition the blood vessels in the pulp-like tissue (blue arrows). (C): continuous layer of uniform thickness of dentin-like tissue (yellow arrows), well aligned odontoblast-like cells (blue arrows). D, original dentin. DL, dentin-like tissue. PL, pulp-like tissue. OdL, odontoblast-like cells

We applied immunohistochemical staining to clarify the nature of this layer of cells. They were 100% positive for Dentin sialoprotein, while they were negative for Bone sialoprotein (Fig. 7,A-B).

Meaning that we had a new pulp formation and moreover we had a newly-formed odontoblast cells layer, which does resemble morphologically odontoblast, but also function as odontoblasts.

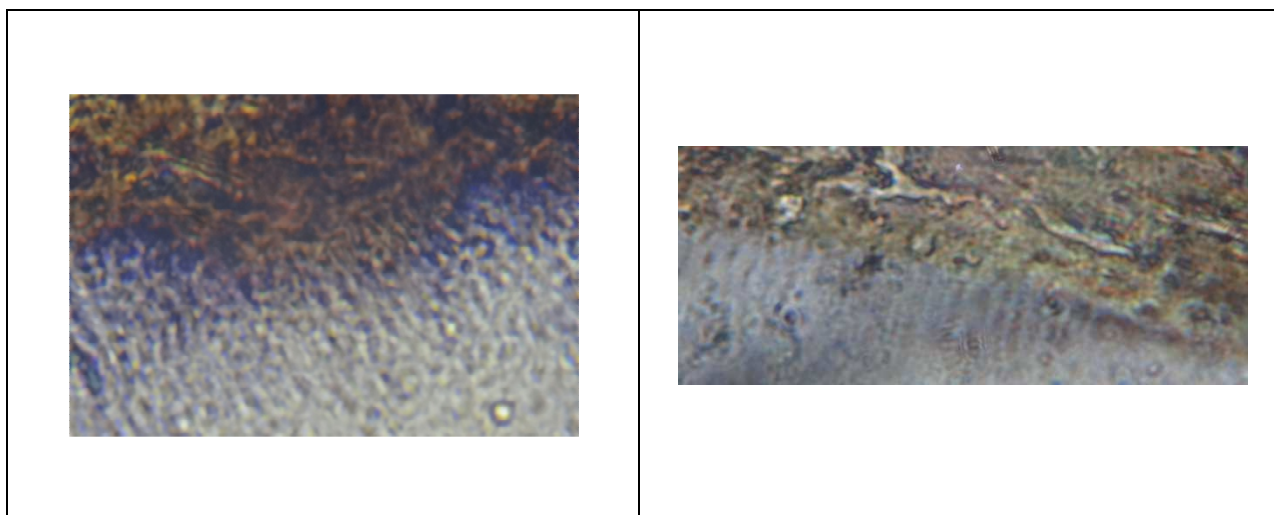


Figure 7. Immunohistochemical analysis of pulp/dentin regeneration. (A): dentin sialoprotein. (B): bone sialoprotein

DISCUSSION

The normal dental pulp is a loose connective tissue surrounded by dentin. The dental pulp and dentin function as a unit, and the odontoblasts represent a crucial element in this system. The odontoblasts are located in the periphery of the pulp tissue, with extensions into the inner part of dentin. Dentin would not exist unless produced by odontoblasts, and the dental pulp is dependent on the protection provided by the dentin.[3] Regeneration of dental pulp tissue is not only necessary to the construction of a fully functional bio-root but also represents an alternative treatment for dental pulp diseases.[9] To date, researchers have tried to replicate vital reproductive pulp tissue by seeding stem cells from dental tissue into various biodegradable scaffolds.[31-33] The purpose of this case study is to describe the histological observation of a human mature permanent tooth after regeneration procedures. In our knowledge, this is the first histological observation of a human regenerated mature permanent tooth. Our study demonstrated the synthesis of vascularized human pulp/dentin-like tissues in human root canal space after application of endodontic regeneration procedures. The promise and potential of regenerative endodontic therapies in teeth was first explored by Nygaard-Østby in 1961 with limited success.[34] Histological analysis demonstrated that a functional pulp-dentine complex was not routinely induced. Current and future research into regenerative endodontics is focused on tissue engineering principles including root canal revascularization, postnatal stem cell therapy, pulp implantation, scaffold implantation, injectable scaffold delivery, three-dimensional cell printing and gene delivery.[3] The challenge for the clinician is to recognize the potential of these new therapies and where appropriate incorporate them into everyday practice. In patients, the outcomes of teeth with previous necrotic pulps and open apices that have been treated with regenerative endodontics have always been evaluated clinically and radiographically.[35-37] These clinical cases show that continued hard-tissue deposition inside the canals of these teeth can occur under certain conditions. There are no histological reports regarding the nature of tissues present in the canals of these teeth treated with regenerative endodontics.

In this study, clinical examination, histological and Immunohistochemical analyses were used to evaluate DPSCs-mediated pulp regeneration. On the basis of tissue engineering theory, the first step in tissue regeneration is to obtain appropriate stem cells.[3] In this study, the autologous DPSCs were isolated from human dental pulp tissue.

Previous experiments have shown that an important feature of the DPSCs is their odontoblastic differentiation potential.[26] In early research with DPSCs, human DPSCs showed the *ex vivo* potential for self-renewal and differentiation into osteoblasts, adipocytes, chondrocytes, and neurons; transplantation of human DPSCs mixed with hydroxyapatite/tricalcium phosphate formed an ectopic pulp-dentin-like tissue complex in nude mice.[26] The formation of a pulp-dentin-like complex in immunocompromised mice suggested that these cells have odontoblastic differentiation potential. A recent *in vivo* study targeting root dentin perforation repair with the use of DPSCs and dentin matrix protein 1 (DMP1) carried by collagen matrix showed that soft connective pulp-like tissue was formed in the perforated site, but no hard tissue was generated.[33]

The results of this study showed eight weeks after the regeneration procedure a loose connective tissue filled the canal space up to the coronal third. The tissue in the canal space was rich in cells and well vascularized. This tissue is similar to the connective tissue in the normal canal space. A layer of flattened cells similar to odontoblasts were polarized along the dentin-like in the root canal. It is evident that these odontoblast-like cells were newly

differentiated odontoblasts from the dental pulp stem cells after regeneration procedure of the mature permanent tooth.

To generate new dentin, stem cells must first differentiate into odontoblasts.[9] In vivo studies have demonstrated that stem cells from human exfoliated deciduous teeth were able to differentiate into odontoblast-like cells lining against the existing dentin surface.[31] These findings suggest that existing dentin is sufficient to guide stem cells in the canal space to differentiate into odontoblast-like cells. Even the chemical treatment of dentin did not appear to affect this capacity. From our results, it appears that dental pulp stem cells seeded in the chitosan scaffold are attracted to the dentinal wall, differentiate into odontoblast-like cells, and extend their cellular processes into the dentinal tubules. The mechanism underlying this phenomenon has been speculated to be the release of growth factors such as transforming growth factor- β (TGF- β) that is known to be embedded in dentin, which attracts and induces odontoblasts differentiation of the seeded stem cells.[25, 38]

The most interesting and important finding of this study is the formation of a continuous layer of dentin-like tissue with uniform thickness on the existing canal dentin walls. This fulfills a major requirement of functional tissue engineering/regeneration because dentin production is one of the major functions of pulp. Regeneration is defined as the replacement of damaged tissue by the same cells.[3] However, regeneration is a histological observation and cannot be determined radiographically. Based on the present case study, the tissue in the canal space appeared to be an extension from dental pulp stem cells. Because we did not create bleeding before placing DPSCs in the root canal of the present case, we believe whatever tissue has been produced in the canal is a result of the presence of DPSCs. Expression analysis of DSP, BSP key genes indicates that the regenerated pulp tissues closely resemble natural pulp tissue.

We agree with previous in vitro studies which demonstrated that DPSCs seeded onto a processed transformed into odontoblast-like cells each with a cellular process extending into the dentinal tubule which is very similar to the natural pulp tissue.[25, 39]

Taken together, our findings show that pulp/dentin regeneration can be accomplished with stem cells-mediated tissue engineering approach. Our in vivo study showed that autologous DPSCs combined with chitosan scaffold transplanted into pulpless root canals laid to pulp regeneration. Furthermore, a histological analysis showed that these cells demonstrated an excellent capacity to form dentin and blood vessels, results supporting the concept of using DPSCs together with scaffolds as a potential way to treat mature teeth. Another key component in tissue engineering is the scaffold.[3] Scaffolds have been used in tissue regeneration to facilitate the formation and maturation of new tissues or organs, where a balance between temporary mechanical support and mass transport (eg, degradation and cell growth) is ideally achieved.[3] Since the discovery of human DPSCs, the regeneration of dentin-pulp complex has been mediated by using different scaffolds such as hydroxyapatite/tricalciumphosphate ceramic powder, hydrogels, synthetic scaffolds, ceramic scaffolds, organic scaffolds and platelet-rich plasma.[40-43] However, none of these scaffold materials is ideal for pulp regeneration, because they are either not suitable for soft tissue regeneration or are too complicated for synthesis and manipulation. Chitosan is one of the most commonly used dressings for the promotion of wound healing.[27] Previous reports have suggested that chitosan could be used as a scaffold material in soft tissue regeneration and tested in clinical regenerative medicine.[44, 45] The chitosan scaffold is a porous scaffold that can be molded into any shape.[46] several studies have demonstrated that chitosan is a nontoxic, biodegradable, and biocompatible material that can potentially be used in various biomedical applications, including drug delivery, wound dressing, tissue engineering, skin substitutes, nerve regeneration, hemostatic action, implants, and antibacterial coating.[27, 46] In addition, it is commercially available and easy to manipulate, therefore we used chitosan as the scaffold material in our study.

Our results corroborate these findings and further show that DPSCs can adhere to and proliferate on the surface of the chitosan scaffold, demonstrating the chitosan biocompatibility and lack of cytotoxicity. The ability of chitosan to support cell attachment and proliferation is attributed to its chemical properties and hydrophilic character.[47, 48] our findings proved that chitosan is considered a suitable scaffold for dental pulp regeneration. Further studies are required to understand the mechanisms of DPSC mediated pulp regeneration and to explore strategies for enhancing pulp tissue regeneration, including the application of growth factors.

CONCLUSION

Based on histological observation of the present in vivo case study, regeneration of the pulp-like tissue is possible after an endodontic regeneration procedure. Using human DPSCs and chitosan scaffolds showed for the first time the regeneration of vascularized pulp-like tissue and the formation of dentin-like structures in the root canal space of the human teeth.

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