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Human dental pulp stem cells cultured onto dentin derived scaffold can regenerate dentin-like tissue in vivo

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Abstract Regeneration of dentin tissues in the pulp space of teeth serves the ultimate goal of preserving teeth via endodontic approaches. In recent times, many studies suggested that human dentin scaffolds combined with dental stem cells was a potential strategy for the complete dentin tissue regeneration. In this study, human dental pulp stem cells (DPSCs) were isolated and cultured. Dentin specimens were prepared from human third molars and treated with ethylene diamine tetra-acetic acid and citric acid to remove the smear layer. Then, DPSCs were cultured onto human treated dentin (hTD) and implanted in mouse model for 4, 6 and 8 weeks. The resulting grafts were assessed by hematoxylin and eosin stain and immunohistochemical stains. As a result, DPSCs were supported and induced to regenerate of dentin-like tissues which expressed specific dentin markers such as dentin sialophosphoprotein and dentin matrix protein 1 by combination with hTD in vivo. Furthermore, cells existed in the newly-formed dentin-like tissues also expressed typical human mitochondria antibodies, demonstrated that new tissues originated from human. In conclusion, the obtain results extend hopefully newly-established therapy to apply in endodontics and traumatic dental hard tissues.

Keywords Dentin · Dental pulp · Tissue engineering · Mesenchymal stem cells · Extracellular matrix protein

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Introduction

Stem cell research has become a promising field for tissue regeneration and implementation of regenerative medicine. Since the discovery and characterization of multipotent mesenchymal stem cells (MSCs) from bone marrow, similar MSCs populations derived from other tissues have now been characterized. Postnatal stem cells have been isolated from a variety of tissues including bone marrow, brain, skin, skeletal muscle and the gastrointestinal tract (Baroffio et al. 1996; Slack 2000; Campagnoli et al. 2001; Gronthos et al. 2003; Zannettino et al. 2008). Recent studies have revealed the presence of adult stem cells in dental tissues (Handa et al. 2002; Huang et al. 2008; Rodríguez-Lozano et al. 2011). They are described as multipotent stem cells, capable of self-renewal and differentiation into various cell types, such as osteocytes, adipocytes, chondrocytes, cementoblasts, odontoblasts and neural cells (Gronthos et al. 2000, 2002; Batouli et al. 2003; Kamata et al. 2004).

Adult dental pulp contains a heterogeneous population of cells, including fibroblasts, nerve cells,

undifferentiated mesenchymal cells or dental pulp stem cells (DPSCs), vascular cells. DPSCs are often found in highly vascularized sites and collected from the pulp tissue of clinically extracted human teeth by various isolation methods (Huang et al. 2006). These cells are expanded and expressed a heterogeneous assortment of markers associated with MSCs. Besides, DPSCs also express nestin and GFAP, which are molecules related to the neural crest-cell origin of the dental pulp (Lovschall et al. 2007). DPSCs are capable of differentiating into neuron-like cells, odontoblast-like cells, osteoblasts, chondrocytes, adipocytes, smooth and skeletal muscle cells (d'Aquino et al. 2007; Graziano et al. 2008). In particular, the importance of DPSCs is able to differentiate and secrete a matrix that produces osteogenesis and dentinogenesis in vitro and in vivo. Furthermore, DPSCs are potentially superior to other types of adult stem cell are extraction routinely throughout life. Even of more importance is the ability to secure DPSCs at a young age and store it for the future usage. A personalized stem cell can then be created from DPSC without using procedures that may cause ethical concerns (Batouli et al. 2003; Shi and Gronthos 2003; Miura et al. 2003).

Many kinds of synthetic and natural polymers have been utilized for dentin tissue engineering. However, few were able to regenerate the complete dentin tissue. Dentin, which is a mineral tissue, is included approximately 70 % hydroxyapatite and collagenous and noncollagenous organic matrix components. Dentin is formed by odontoblasts which establish a continuous single layer along the peripheral dental pulp tissue and deposit new layers of dentin throughout human life (Thesleff and Vaahtokari 1992; Stern et al. 2009). Dentin is an essential component for dental tissue engineering because makes up most of the tooth structure. Moreover, the soluble proteins of human dentin are bioactive proteins considered to be necessary for dentinogenesis (Chun et al. 2011; Li et al. 2011). Scaffolds from natural dentin, which were collected freely and eliminated as medical waste, have been suggested to be useful in dentin regeneration (Lluch et al. 2009).

Previous researches demonstrated that hDPSCs cultured on mechanically and chemically treated dentin appeared to establish an odontoblast-like morphology, with cytoplasmic processes extending into dentinal tubules. Their results suggest that isolated hDPSCs may differentiate into odontoblasts on dentin in vitro (Huang et al. 2006). However, it is not clear whether dentin scaffolds induce hDPSCs to regenerate the complete dentin tissue. Therefore, in this study we combined human treated dentin (hTD) and cultured DPSCs to assess regeneration of human dentin-like tissue in vivo.

Materials and methods

Isolation and culture of human dental pulp stem cells (DPSCs)

DPSCs were isolated and cultured followed protocol described previously (Tran et al. 2011). In brief, the human third molars were obtained from healthy donors at the Maxillofacial Faculty, under the approval of the Ethical Committee of Ho Chi Minh City Medicine and Pharmacy University, and stored in Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, MO) supplemented with antibiotics (300 U/ml penicillin and 300 mg/ml streptomycin, Sigma). Small fragments of dental pulp tissues were seeded onto 35-mm dishes (NUNC, Roskilde, Denmark) with Dulbecco's modified Eagle's medium/nutrient mixture F-12 Ham medium (DMEM/F12, Sigma) supplemented with 10 % fetal bovine serum (FBS, Sigma) and maintained in 5 % CO_2 at 37 °C. The fourth passage cells were used for following studies.

Characterization of cell proliferation

The DPSCs at the fourth passage were seeded with a density of 10^3 cells/well of 96-well culture dish (NUNC) with culture medium. Cell density was identified everyday, continuously in 10 days. Growth curve was determined from counted cell quantity. To assess the time of duplication or doubling time (DT), the following equation was applied: $DT = CT/\log N/N0 \times 3.31$, where CT = culture time, N = final number of cells, N0 = initial number of cells (Piera-Velazquez et al. 2002).

Cell-surface-marker characterization

The DPSCs at the fourth passage were detached and identified specific surface antigen expression by flow

cytometry. After the cells were harvested and transferred, they were fixed for 15 min in 4 % paraformaldehyde. The cells were incubated with 3 % bovine serum albumin and then with primary antibodies (BD Biosciences) raised against CD44, CD90, CD73, CD34, CD45, and HLA-DR for 1 h. The cells were washed with wash buffer, and the secondary antibody was added for 45 min at room temperature. Finally, the cells were washed three times and analyzed with a flow cytometer (FACSCalibur, BD Biosciences).

Multilineage differentiation

The fourth passage cells were seeded into 35 mm dishes and cultured until they reached subconfluence. After that, cells were cultured in the adipogenesis medium [DMEM/F12 medium containing 10 % FBS, 0.2 mM indomethacin, 0.5 mM isobutyl-methylxanthine, 100 nM dexamethasone, 10 mM insulin, 100 U/ml penicillin, and 100 mg/ml streptomycin (Sigma)] and osteogenesis medium [DMEM/F12 medium containing 10 % FBS, 100 nM dexamethasone, 100 mM β-glycerol phosphate and 50 μg/ml α-ascorbic acid 2-phosphate, 100 U/ml penicillin, and 100 mg/ml streptomycin (Sigma)]. After 2–3 weeks of induction, the cells were stained with oil red O or alizarin red to evaluate adipogenesis and osteogenesis, respectively.

Fabrication of human treated dentin

Dentin tissues were prepared from collected human third molars after the pulp tissue had been removed to obtain DPSCs. Periodontal tissues were completely eliminated by using mechanical method. Dentin tissues were cut into a cube shape (approximately 3 mm long × 2 mm wide × 1 mm thick). Dentin specimens were treated with 19 % citric acid (Sigma) for 1 min and 17 % diamine tetra-acetic acid (EDTA) (Sigma) for 10 min to remove the smear layer (Tran et al. 2011). hTDs were then sterilized by gamma radiation. Mineral trioxide aggregate (MTA, Tulsa Dental Products, Tulsa, OK) scaffolds were used as a control group.

Regeneration of dentin-like tissue in vivo

In order to prove the role of hTD scaffolds in induction for regeneration of dentin tissue of DPSCs in vivo, we subcutaneously implanted samples into the dorsum of Nude mice. All in vivo experiments were carried out in accordance with the ethical guidelines for Animal Care and Use Committee of University of Medicine and Pharmacy at Ho Chi Minh City. Nude mice were divided into three groups including one experiment group (hTD combined with DPSCs) and two control groups (MTA combined with DPSCs and single hTD). hTD and MTA scaffolds were soaked in culture medium at 37 °C overnight. Then, DPSCs were seeded onto scaffolds at density of 3×10^4 cells/scaffold and incubated in 5 % CO_2 at 37 °C for 3 days. Surgical operation was performed under deep anesthesia. After 4, 6 and 8 weeks, Nude mice were deeply anesthetized to collect all structures (one experiment group and two control groups). Structures were fixed with 10 % formaldehyde for 24 h at 4 °C and stained with hematoxylin and eosin (H&E) stain and immunohistochemical stains. Immunohistochemical antibodies included dentin matrix protein 1 (DMP-1), dentin sialophosphoprotein (DSPP) and human mitochondria (Sigma).

Results

Isolation and culture of human dental pulp stem cells (DPSCs)

Characterization of cell proliferation

After four times of subculturing, DPSCs strongly proliferated from the second day to the seventh day, peaked in the seventh day which was confluence, and gradually decreased in some days after that (Fig. 1).

Cell-surface-marker characterization

Surface markers expressed on the DPCs at the fourth passage were analyzed by flow cytometry. As a result, the DPSCs strongly expressed markers CD44 (99.84 %), CD73 (99.77 %) and CD90 (97.34 %) and lacked expression markers CD34 (0.42 %), CD45 (0.08 %), and HLA DR (0.18 %) (Fig. 2).

Differentiation into adipocytes and osteoblasts

The DPSCs at the fourth passage were differentiated into adipocytes and osteoblasts. The differentiation was achieved after 14–21 days. After 21 days, the

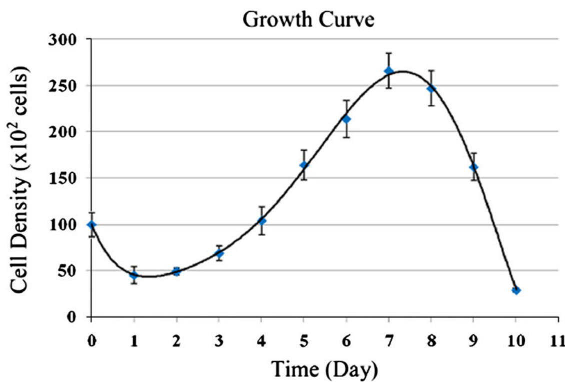


Fig. 1 Growth curve of DPSCs

result of oil red O and alizarin red staining showed that there were mineralized nodule (Fig. 3b) and lipid droplets (Fig. 4b) formation inside the differentiated cells.

Fabrication of human treated dentin

Based on results of SEM, dentin surface was readily visible in all specimens as evidenced by lack of smear layer and abundance of patent dentinal tubules (Fig. 5a). Furthermore, after three cultured days in vitro, DPSCs began spreading onto hTD surface (Fig. 5b).

Regeneration of dentin-like tissue in vivo

After surgical operation, all experimental mice rehabilitated quickly. Their normally activities were not affected by implanted materials. Based on results of H&E stain, in the experiment group, there was the formation of new tissues at 4, 6 and 8 weeks. Newly-formed tissue thickness and cell density increased from 4 to 6 weeks. However, cell density did not increase at 8 weeks. At 6 and 8 weeks, area adjacent to hTD took form concentrated matrix and expand over time. Furthermore, most cells were buried in concentrated matrix at 8 weeks (Fig. 6a–c). In the MTA control group, only a thin dentin-like matrix layer was formed on MTA scaffold surfaces at 8 weeks (no cells were observed) (Fig. 6d). On the other hand, no new tissue formed by using a hTD without DPSCs (single hTD control group) at 4, 6 and 8 weeks (Fig. 6e).

Expression of DSPP and DMP-1 was detected in the new tissues formed in hTDs at 4 (data not shown), 6 and 8 weeks (Fig. 6f–i). These results demonstrated

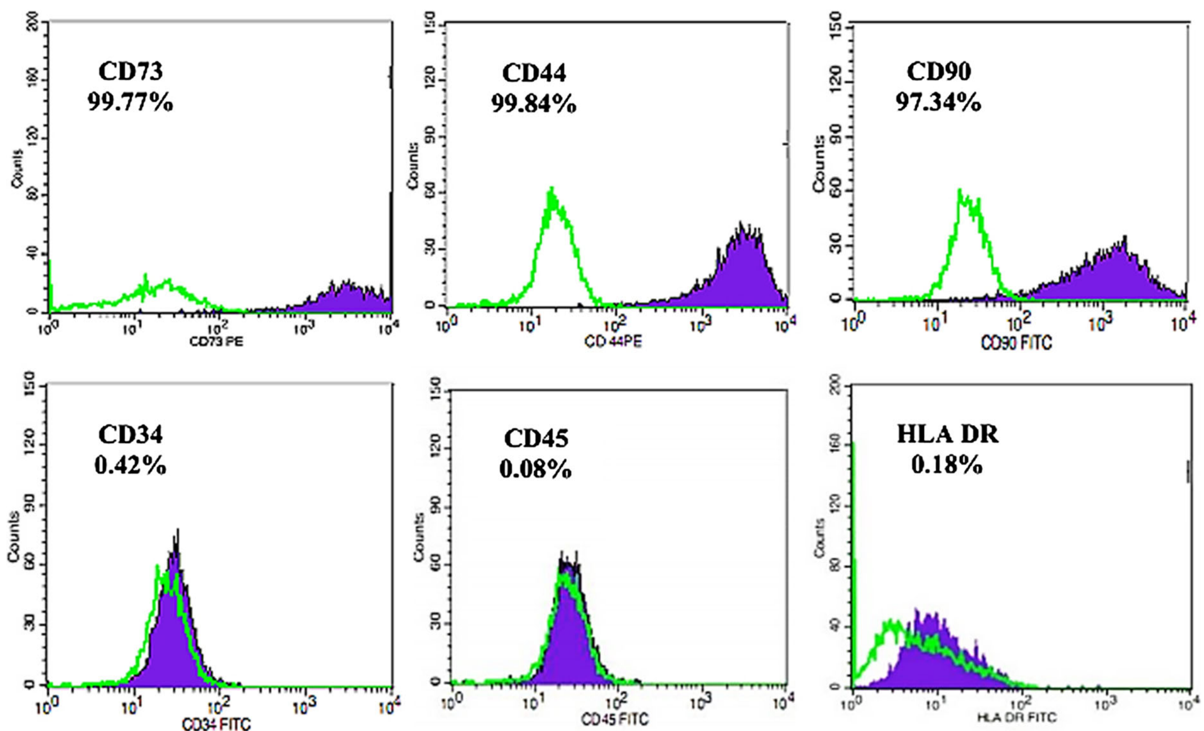


Fig. 2 Flow cytometry analysis of the DPSCs

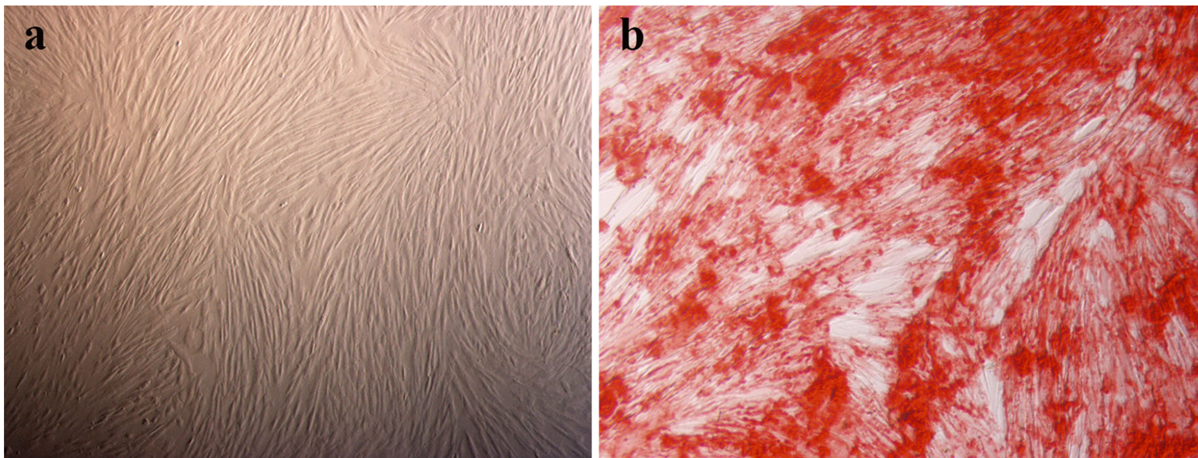


Fig. 3 Result of differentiation into osteoblasts of the DPSCs. **a** Negative control, **b** the differentiated cells after being stained with alizarin red. Images magnified: ×200

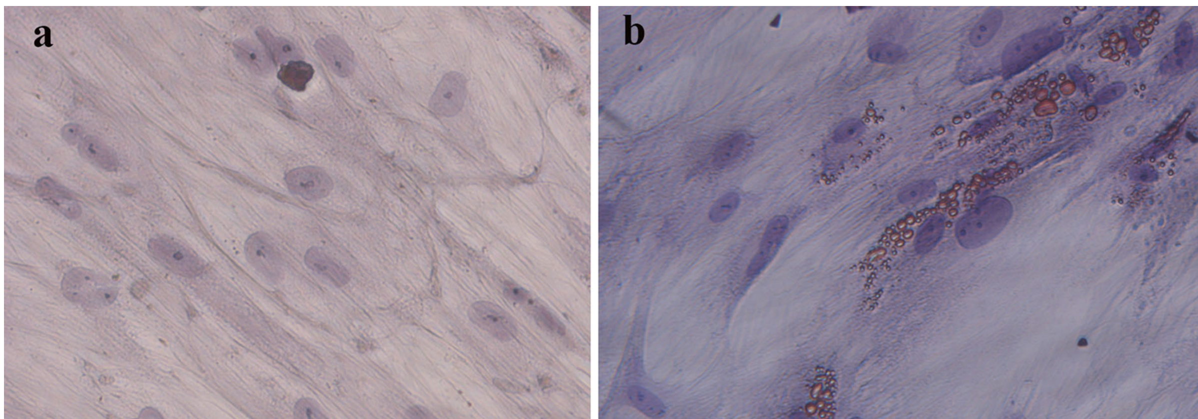


Fig. 4 Result of differentiation into adipocytes of the DPSCs. **a** Negative control, **b** the differentiated cells after being stained with oil red O. Images magnified: ×400

that the newly-formed tissues were dentin-like tissues. In order to demonstrate whether participation of the implanted DPSCs induced to regenerate dentin-like tissues, human mitochondria antibody, which only responded to a human source, was used. All cells in regenerated tissues were positive with human mitochondria antibody, proven that implanted DPSCs participated in human dentin-like tissues regeneration in vivo (Fig. 6j, k).

Discussion

Five types of human dental stem cells have been isolated and characterized: DPSCs (Gronthos et al. 2000), stem cells from human exfoliated deciduous teeth (SHED) (Miura et al. 2003), periodontal ligament stem cells (PDLSCs) (Seo et al. 2004), dental follicle progenitor cells (DFPCs) (Morsczeck et al. 2005) and stem cells from apical papilla (SCAP) (Sonoyama et al. 2008). Dental pulp, entrapped within the 'sealed niche' of the pulp chamber, is an extremely rich site for stem cells isolation. Dental pulp tissue has been reported to contain MSC-like population referred to as DPSCs (Gronthos et al. 2000). Several studies have reported that DPSCs express MSC markers such as CD10, CD13, CD29, CD44, CD59, CD73, CD90 and CD105, and do not express CD14, CD34, CD45, HLA-DR. Furthermore, DPSCs are capable of differentiation into odontoblast-like cells, osteoblasts,

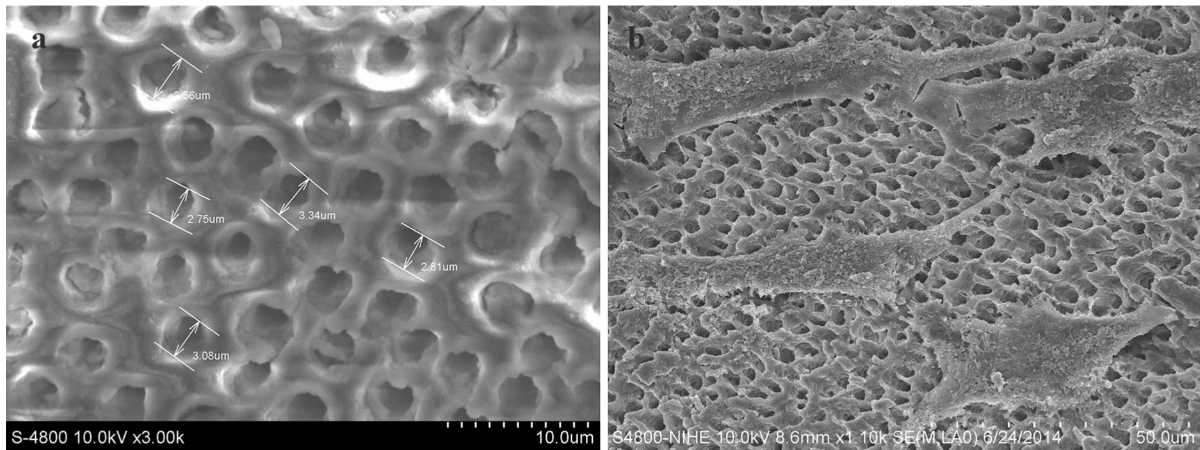


Fig. 5 SEM of dentin surface. **a** Surfaces of dentin slices were treated, **b** DPSCs spreaded on hTD surface. Images magnified: ×3000 (**a**), ×1100 (**b**)

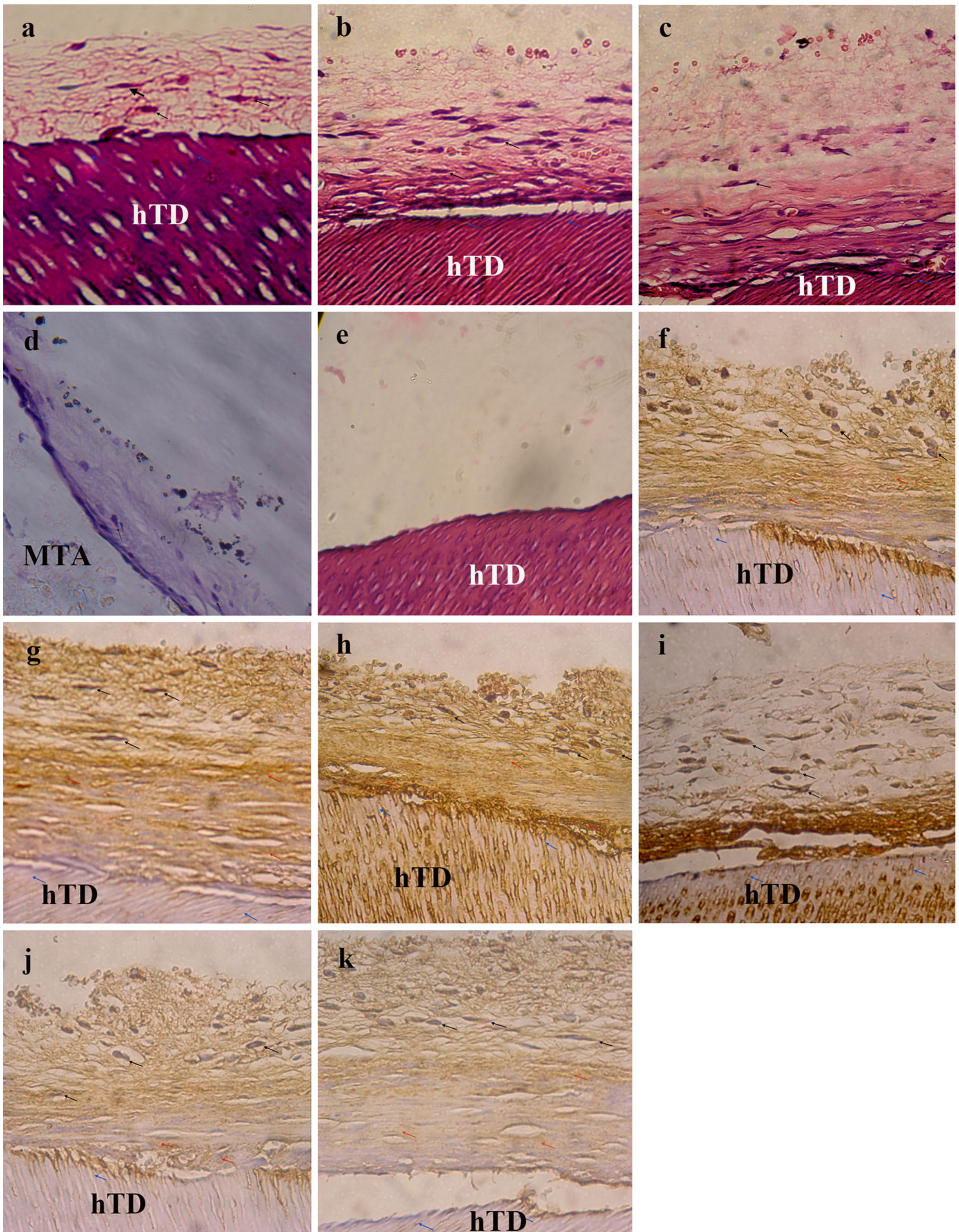
adipocytes, smooth and skeletal muscle cells (d'Aquino et al. 2007; Karaöz et al. 2009; Nakamura et al. 2009).

In this study, we demonstrated that isolation of human DPSCs by outgrowth method, allowed the recovery of a population of dental MSCs, which showed a notable proliferation potential, multipotency. Furthermore, DPSCs exhibited a DT of 54.38 h which revealed that DPSCs have higher proliferation capacities than bone marrow stem cells (BMSCs) (61.2 h) and slower proliferation capacities than umbilical cord stem cells (UCSCs) (24 h) and adipocyte stem cells (ADSCs) (45.2 h) (Hass et al. 2011). In addition, our data also showed that, DPSCs were expressed over 95 % positive for the CD44, CD73 and CD90 antigens, less than 2 % of the leucocyte marker CD45, the primitive haematopoietic progenitor and endothelial cell marker CD34, the B cell markers HLA-DR by flow cytometry analysis.

Until now, many materials have been used to create scaffolds for dentin regeneration. However, few have succeeded in obtaining complete dentin tissue (Li et al. 2011; Honda et al. 2007; Scheller et al. 2009). Previous studies have demonstrated that hTD could be a conformable scaffold for dental tissue engineering because of its non-immunogenicity, suitable mechanical properties, and releasing dentinogenetic factors. Previous studies also have shown that soluble proteins extracted from dentin, such as DSPP, DMP-1, proteoglycans (decorin and biglycan), as well as growth factors such as TGF-β, bone morphogenic protein (BMP), can regulate dentinogenesis and mineral formation (Chun et al. 2011; Li et al. 2011; Guo et al. 2009). In our experiment, we sterilized hTD by gamma radiation. Gamma radiation is mainly used for the sterilization of pharmaceuticals and biomaterials in graft. Results showed that hTD scaffolds sterilized by gamma radiation remained high level of bioactive protein such as TGF-β and DMP-1 (data not shown).

Fig. 6 Histological results of regeneration of dentin-like tissue ▶ in vivo. **a–c** Formation of new dentin-like tissues at 4, 6 and 8 weeks, **d** only dentin-like matrix were formed on MTA scaffold surfaces at 8 weeks, **e** no new tissue was formed on single hTDs at 8 weeks. Regenerated tissues on hTD were positive with DMP-1 and DSPP at 6 weeks (**f**, **h**) and 8 weeks (**g**, **i**). Regenerated tissues and its cells were positive with human mitochondria antibodies at 6 weeks (**j**) and 8 weeks (**k**)

EDTA, which is widely used in endodontic therapy, is a chelating agent capable of removing inorganic material to enlarge root canals, removing the smear layer, and preparing the dentinal walls for better adhesion of filling materials. The combined use of EDTA and acid citric has been shown to be particularly effective for smear layer and debris removal (Tran et al. 2011; Nakashima and Terata 2005; Huang et al. 2006). The result of treatment removed completely smear layer and exposed dentin tubules on hTD surfaces. Besides, we demonstrated that DPSCs could adhere and proliferate on hTDs in vitro (Tran et al. 2011). This indicated that hTD had good biocompatibility and supported cell growth.


a
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Mineral trioxide aggregate (MTA) is biomaterial that has been investigated for endodontic applications since the early 1990s. MTA was first described in the dental scientific literature in 1993 and was given approval for endodontic use by the U.S. Food and Drug Administration in 1998. MTA has been shown to induce hard-tissue repair of exposed pulps in experimental animals and to generate a greater frequency of dentin bridge formation than earlier materials (Lee et al. 1993; Schmitt and Bogen 2001; Andelin et al. 2003).

hTD scaffolds seeded with DPSCs were implanted into nude mice at the dorsum where non-affected normal activities of experimental animals, non-potential mineralization and abundant vasculargenesis. Implantation immediately after seeding DPSCs to the hTD scaffolds did not perform because DPSCs needed more time to adhere and spread on scaffold surfaces. According to growth curve of DPSCs (Fig. 2), the lag phase tended to be about 2 days for culture meaning that DPSCs could start to proliferate 2 days after being seeded onto hTD scaffolds. Furthermore, less than 3 days in vitro culture of hTD/DPSCs also did not obtain satisfactory regenerated results; concurrently, 3 days in vitro culture were proposed because risk of contamination for in vitro culture likely increased with time (Li et al. 2011).

During the last years, several studies investigated that hDPSCs formed multilayer and secreted extracellular matrix onto the treated dentin surface. Furthermore, hTD potentially induced DPSCs to establish an odontoblast-like morphology with a cytoplasmic process extending into a dentinal tubule after 14 days (Huang et al. 2006), 10 days (Shao et al. 2011) or 28 days (Neunzehn et al. 2014) in vitro. Through an in vivo study, pieces of root canals of the extracted teeth, containing collagen or PLA scaffolds seeded with the autologous cryopreserved DPSCs, were implanted into the fresh post-extraction socket of the mini pig jaw. The results showed an odontoblast-like cell construct with an organic matrix formation on the root canal wall surface of porcine teeth after 6 and 10 weeks. More specifically, the newly formed odontoblastic-like cell layer lining along the existing canal walls stained positive for DMP-1 antibody (Kodonas et al. 2012).

In this study, implantable structures were evaluated at 4, 6 and 8 weeks that identified growth and change of newly-formed tissues. Mineralization of new tissues occurred at the junction with hTD and developed on the opposite side; similar to the natural formation of human dentin. Specially, all hTD scaffolds and DPSCs derived from human; therefore this study was step ahead in dental tissue engineering. Two control groups lacked one of important elements (hTD or DPSCs), therefore no regeneration of new tissues. This suggested that hTD and DPSCs played essential role in the regeneration of dentin-like tissues in vivo.

Histological examination of implanted DPSCs/hTD structures showed that newly-formed tissues were positive with two specific markers for dentin matrix (DMP-1 and DSPP). During dentin formation, odontoblasts synthesize several noncollagenous proteins and deposit into the dentin extracellular matrix. One of these proteins is DSPP, which is believed to play a regulatory role in the mineralization of reparative dentin; it also serves as a specific marker for the odontoblastic phenotype (Papagerakis et al. 2002). Besides, DMP-1 is a multifunctional protein, prominent member of one category of non-collagenous proteins and plays an essential role in biomineralization. More recently, DMP-1 was found to induce cytodifferentiation of DPSCs into odontoblast-like cells during dentinogenesis, indicating that it could act as a morphogen with the potential to regenerate dentin-like tissue and to form reparative dentin (Toyosawa et al. 2004).

Results of our study indicated that hTD scaffolds induced and supported DPSCs to secrete extracellular matrix and regenerate dentin-like tissue. In addition, cells of newly-formed tissues were positive with human mitochondria; concurrently, no new tissue formed by using single hTD, suggested that the seeded DPSCs were essential role to participate in the regenerated dentin-like tissues.

Conclusion

This research described the successful combination of hTD and human DPSCs. This is one of potential strategies for dentin-like tissue regeneration based on tissue engineering principle.

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Conflict of interest The authors declare that there is no personal or financial conflict of interests in the current research.

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