

THE INDUCTION OF DENTIN BRIDGE-LIKE STRUCTURES BY CONSTRUCTS OF SUBCULTURED DENTAL PULP-DERIVED CELLS AND POROUS HA/TCP IN PORCINE TEETH

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ABSTRACT

The purpose of this study was to investigate dentin-bridge formation in teeth following the transplantation of dental pulp-derived cells seeded on hydroxyapatite/tricalcium phosphate (HA/TCP) scaffolds. The dental pulp tissues were removed from the extracted first molar teeth of miniature pigs and single cell populations were subcultured. Second-passage cells that had alkaline phosphatase activity were combined with scaffolds. Cell-scaffold constructs were placed in contact with the exposed pulp tissue. The dimensions of the exposed pulp site were approximately 1–2.5 mm in diameter and 2–3 mm in depth from the tooth surface. After placing the constructs, the tooth was restored with composite resin. Six weeks after transplantation, hard tissue formation was observed on the pulp tissue in histology. Dentinal tubule-like structures were observed in most of the hard tissue generated, and columnar cells, which showed positive immunoreactions with dentin sialoprotein (DSP) and heat shock protein (HSP)-25, were aligned beneath the hard tissues. When only scaffolds were placed on the pulp tissues, particles of hard tissue were formed, however dentinal tubule-like structures and odontoblasts were not observed despite the formation of hard tissue. In conclusion, the implantation of dental pulp constructs into pulp exposed stimulates the formation of calcified dentin-like structures.

Key Words: Dentin bridge, Dental pulp cells, Hydroxyapatite/ β -TCP, Tissue engineering, Pulp capping

INTRODUCTION

During odontogenesis, primary dentin is secreted by post-mitotic odontoblasts which are differentiated from the dental papilla derived from the cranial neural crest.^{1,2)} Once the odontoblasts suffer injuries, the differentiation of mesenchymal cells is induced from the precursor cell popula-

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tion in the dental pulp,^{3,4)} and these cells are recruited to the injured site to differentiate into odontoblasts. Subsequently, the newly-differentiated odontoblasts produce a dentin matrix which is referred to as a “dentin bridge” following direct pulp capping or partial pulpectomy.⁵⁾ However, even a small exposure of dental pulp after removal of softened dentin can occasionally cause a severe inflammatory reaction. This can then require a total pulpectomy because the odontoblasts are often irreversibly damaged. Since successful direct pulp capping therapy plays a key role in maintaining the function of teeth,^{3,4,6)} substitute materials enabling effective generation of dentine bridges have been extensively investigated for the improvement of pulp-capping therapy.

Recently, applications of bone morphogenetic proteins (BMPs)⁷⁻⁹⁾ or gene transfer of Bmp11/Gdf11¹⁰⁾ have been reported to induce dentin-bridge formation on exposed pulp tissues in animal models. Even more recently, the first report on the cell-based therapy demonstrated that a pellet culture of dental pulp-derived cells placed onto exposed pulp tissue induced dentin-bridge formation.⁷⁾ It is still unclear whether this approach is effective in the case of a larger exposure of dental pulp because pellet culture therapy needs a great number of cells for complete restoration. Since these previous studies clearly demonstrate the potential for dentin-bridge formation by use of pulpal cells after dental pulp exposure,^{3,7,10)} we assume that pulp capping therapy based on tissue-engineering technology using both cells and a scaffold have an advantage of effectively generating a dentin bridge when the area of pulp exposure is relatively large.

Recently, the field of tissue engineering has introduced a novel therapy for the fabrication of neo-tissues using optimized cells in a three-dimensional scaffold for replacement of tissues and organs.¹¹⁻¹³⁾ In the field of dental tissue engineering, numerous studies have shown successful periodontal tissue engineering^{14,15)} and managed to generate immature tooth structures by conservative tissue engineering techniques.¹⁶⁻¹⁸⁾ The regenerative endodontics field is dedicated to biological regeneration of dental pulp tissues, but little progress has been made using scaffolds and cells to generate newly dental pulp tissues.¹⁹⁾ Then, tissue constructs were creating by seeding subculture dental pulp-derived cells onto HA/TCP scaffolds and transplanting them into the pulp-exposed teeth of miniature pigs to investigate their ability to regenerate dental tissues.

MATERIALS AND METHODS

All animal experiments were approved by the “Animal Experiment Advisory Committee of Nagoya University School of Medicine” and performed in accordance with the “Guidelines for Animal Experimentation of Nagoya University, JAPAN.”

Preparation of scaffolds for dental pulp-derived cells

Inorganic porous block scaffolds for the seeding of dental pulp-derived cells were composed of hydroxyapatite and β -tricalcium phosphate (HA/TCP) ceramic. The scaffolds were circular in shape with a diameter of 6 mm and a thickness of 3 mm (Fig. 1a) (HA:TCP=2:8, 60% porosity, kindly provided by NGK Spark Plug Co., Ltd., Nagoya Japan). The average pore diameter of HA/TCP blocks was approximately 300 μ m, measured by scanning electron microscope (SEM) (VE-7800, KEYENCE, Osaka, Japan) following sputter coating of scaffolds with gold (Fig. 1b and c).

Isolation, culture and differentiation of dental pulp-derived cells

The dental pulp-derived cells were harvested and cultured as described in our previous studies.²⁰⁻²¹⁾ Briefly, the dental pulp tissues were removed from the extracted first molars of six- to seven-month-old miniature pigs (NIBS, Chubu Kagaku Shizai Co., Ltd. Nagoya, Japan) under

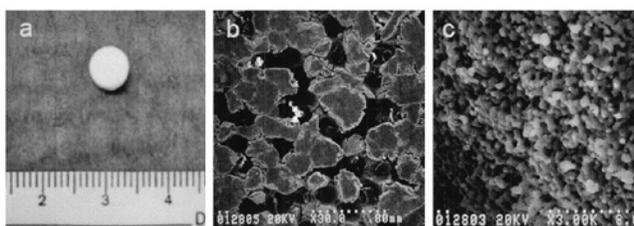


Fig. 1 (a) Stereomicroscopic view of a porous HA/TCP scaffold (diameter: 6 mm). (b) Scanning electron microscopic (SEM) view of porous HA/TCP for dental pulp-derived cells ($\times 30$). The average size of pores is $300\ \mu\text{m}$. (c) More highly magnified view ($\times 3000$) of the HA/TCP scaffold.

general anesthesia using endotracheal intubation with fluothane. A local anesthetic (2% xylocaine with 1:100,000 epinephrine; Astra, Sodertalje, Sweden) was also administered by infiltration at the buccal and lingual gingivae in the alveolar ridge of the mandible before the operation. The obtained dental pulp tissues were minced into less than $1\ \text{mm}^3$ pieces and digested in a solution of 2 mg/ml collagenase type I (Wako Pure Chemical Industries, Osaka, Japan) in 0.1 M PBS for 50 min. Single cell populations were seeded into T-75 flasks (Greiner Bio-one, Frickenhausen, Germany) in a complete culture medium (CCM) consisting of a mixture (1:1) of Dulbecco's modified Eagle's minimum essential medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA) and Nutrient Mixture F-12 Ham (Sigma-Aldrich) supplemented with 15% Fetal Bovine Serum (Thermo Trace Ltd. Melbourne, Australia), 2% Antibiotic-Antimycotic (Sigma-Aldrich), and $100\ \mu\text{M}$ L-ascorbic acid 2-phosphate (Sigma-Aldrich). After reaching 80% confluence, the cells were subcultured for the purpose of the following analysis. The cell features in the CCM were routinely evaluated using phase-contrast inverted microscopy (Olympus Optical Co., Ltd., Tokyo, Japan) (Fig. 3a).

Effect of osteogenesis induction medium on dental pulp-derived cells

To investigate the osteogenic differentiation capacity of subcultured dental pulp-derived cells, an alkaline phosphatase (ALPase) activity assay and alkaline phosphatase staining were performed. To induce osteogenic differentiation, the subcultured cells were plated at the optimal density (1×10^4 cells/ml into 12-well polystyrene dishes [BD Biosciences]) in CCM for 3–4 weeks until 80% confluency, and CCM was replaced with an osteogenesis induction medium (OIM) in which CCM is supplemented with 10% FBS, 10 mM β -glycerophosphate (Sigma-Aldrich), 0.2 mM ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA) and 10^{-8}M dexamethasone (Sigma-Aldrich) as described in the previous report.¹¹⁾ The medium was replaced every 2–3 days.

The quantitative analysis of ALPase activity was performed according to the previous protocol.²²⁻²³⁾ Briefly, ALPase activity in *p*-nitrophenol was measured at 37°C for 6 min in Milli-Q water using a Fast *p*-Nitrophenyl phosphate tablet set (Sigma-Aldrich) as a substrate. The relative amount of *p*-nitrophenol was estimated from the light absorbance at a wavelength of 405 nm at days 1 and 10 (Model 650 Microplate reader; Bio-rad Laboratories, Hercules, CA, USA) after CCM was replaced with OIM. To measure cell numbers, the subcultured dental pulp-derived cells in each well were counted using a WST-8 kit (Cell-counting Kit-8; Dojindo Laboratories, Kumamoto, Japan). For counting the cell number, a tetrazolium salt that produces a highly water-soluble formazan dye was employed. One hour after incubation with the reagent according to the manufacturer's instructions, the average cell number was determined by measuring light absorbance at a wavelength of 450 nm at days 7 and 10 (Bio-rad Laboratories).

After 2 weeks in CCM or OIM, subcultured dental pulp-derived cells were washed in a 0.1 M PBS and incubated with the alkaline-dye mixture including Fast Violet B Salt (Sigma-Aldrich) plus Naphthol AS-MX Phosphate Alkaline Solution (Sigma-Aldrich) at 18–26°C for 15 minutes to determine alkaline phosphatase activity.

Cavity preparation and pulp capping procedure

The cell-HA/TCP constructs were prepared before beginning the pulp capping experiments. The subcultured dental pulp-derived cells (approximately 5.0×10^6 cells at second passage) were resuspended into 100 μ l of CCM and then inoculated into the HA/TCP blocks. The cell density was selected because the ALP activity was most effective to subcultured dental pulp-derived cells in the preliminary experiments. The cell-HA/TCP constructs were placed in a 96-well plate in CCM for 2 weeks, and the constructs were cultured for an additional 2 weeks in OIM.

The operation was performed as an auto graft under the same conditions of general anesthesia as described in the above section. The standard procedures for cavity preparation and pulp capping therapy described in previous studies using monkeys^{24–26}) were modified for the experimental model using miniature pigs. Ten molars from six males of 6- to 7-month-old miniature pigs (NIBS, Chubu Kagaku Shizai Co., Ltd.) were used. Class V-type cavities were created by means of a size 330 carbide burr with a high-speed dental handpiece under sterile water-cooling. Briefly, a cavity of 2–3 mm in depth was created in the distal half of the occlusal surface. After washing the cavities with sterile saline, HA/TCP scaffolds with (N = 6) or without (N = 4) the subcultured dental pulp-derived cells were crushed and forced into the root canal space. Dry cotton was pressed onto the pulp for 1 min each time and continued until bleeding onto the underlying the dental pulp tissues stopped. They were then covered with adhesive cement (Super-Bond, Sun Medical Co., Ltd., Moriyama, Japan) and light cured composite resin (UniFil Core, GC Co., Ltd., Tokyo, Japan) to seal up the cavities according to the manufacturer's instructions.

Micro-computed tomography

At 6 weeks post-transplantation, teeth were extracted and evaluated using micro-computed tomography to determine whether dentin-bridge formation was accomplished as described in a previous study.²⁷) Briefly, extracted teeth were fixed in 10% neutral-buffered formalin and scanned using a conebeam tomography system (CBSTAR MCT-100CB; Hitachi Medico Technology, Chiba, Japan) with a micro-X-ray source (60 μ m, 85kV, 100 μ A) directed towards the sample of entire teeth.

Histological observations

Tooth specimens were collected 6 weeks after transplantation and were fixed with 4% paraformaldehyde solution in 0.1 M PBS and decalcified in ethylenediamine tetraacetic acid disodium salt (pH 7.4) (EDTA) for 10 days. Following decalcification, the samples were processed for embedding in paraffin. The specimens were cut sagittally at about 5 μ m. The sections were stained with hematoxylin and eosin to evaluate the hard tissue formation at the surface of exposed pulp and the general cellular architecture. In addition, to determine the effect of the subcultured dental pulp-derived cells, the average thickness of newly-formed dentin bridge from both cell-HA/TCP constructs and HA/TCP alone on exposed pulp tissues was measured using Scion Image picture-imaging software (Scion Corp, Frederick, MD, USA).

Immunohistochemistry

An immuno-peroxidase procedure using the avidin-biotin peroxidase complex (ABC) method was used for immunohistochemistry. The antibodies used were chicken anti-pig dentin sialoprotein

(DSP) polyclonal antibody (diluted 1:5000; gift from Dr. J. P. Simmer and Dr. Y. Yamakoshi, University of Michigan, USA) and rabbit anti-human HSP25 polyclonal antibody (diluted 1:1000; StressGen Biotechnologies Corp., Victoria, BC, Canada). Decalcified teeth were equilibrated in a 30% sucrose solution for cryoprotection. The specimens were cut sagittally at a thickness of about 50 μm on a freezing microtome (FX-801; Yamato Kohki, Tokyo, Japan), collected into cold phosphate-buffered saline (PBS), and treated as free-floating sections. The sections were treated by two consecutive incubations with biotinylated anti-chicken IgG for DSP or anti-rabbit IgG for HSP25 antibodies and ABC complex (Vector Laboratories Inc., Burlingame, CA) for 12 hrs each at room temperature. The sites of antigen-antibody reaction were made visible by placing sections in 0.05 M Tris HCL buffer (pH 7.6) containing 0.04% 3-3'-diaminobenzidine tetrachloride and 0.002% H_2O_2 . The immunostained sections were sequentially post-fixed in 1% OsO_4 reduced with 1.5% potassium ferrocyanide, dehydrated in an ascending grade of ethanol, and embedded in Epon 812 (Taab, Berkshire, UK). Semi-thin sections (1 μm in thickness) were prepared and counter-stained with 0.03% methylene blue.

Statistical analysis

All data were presented as the means and standard deviation (SD). The number of cells between the different mediums was compared using Student's t-test. A *P*-value of <0.05 was considered to be statistically significant.

RESULTS

Osteogenic differentiation potential of dental pulp-derived cells in vitro

Since ALPase activity is a typical (non-specific) marker of hard tissue formative cells such as osteoblasts and odontoblasts,^{28,29)} we measured the ALPase activity in the dental pulp-derived cells under culture conditions with CCM or OIM. We measured the cell numbers on days 7 and 10 in culture. The growth rates of dental pulp-derived cells cultured in CCM was similar to that in OIM (Fig. 2a). In contrast, dental pulp-derived cells treated with OIM showed a significantly higher rate of ALPase activity compared with those cultured in CCM on day 10 (Fig. 2b).

Furthermore, histochemical staining of ALPase was clearly positive in dental pulp-derived cells treated with OIM (Fig. 3b), but negative in dental pulp-derived cells with CCM (data not shown). The number of positive cells was less than 10% in the total number of subcultured dental pulp-derived cells.

Histological and immunohistochemical analyses in pulp-capping therapy

To determine the effect of adding subcultured dental pulp-derived cells, autogenous transplantation was performed on exposed pulp tissue from miniature pig teeth. Six weeks after pulp capping with HA/TCP seeded with dental pulp-derived cells, hard tissue formation was observed in all samples (6/6) (Fig. 4). Both tubular and atubular calcified matrices were observed in the hard tissues generated on the exposed pulp (Fig. 5a and b), and columnar cells and cell polarity were arranged beneath the deposited dentin bridge-like matrix (Fig. 5b and d). Moreover, we performed immunohistochemistry for DSP and HSP-25 to determine whether these cells had the phenotype of odontoblasts or not, since both DSP³⁰⁾ and HSP-25^{31,32)} are considered to be phenotypic markers of odontoblasts. The columnar cells were positive for both DSP (Fig. 6a and b) and HSP-25 immunoreactions (Fig. 6c and d).

The cells of the subjacent reorganizing tissue were comprised of a mixed range of cells with differing morphologies, including fibroblasts and inflammatory cells. These cells were

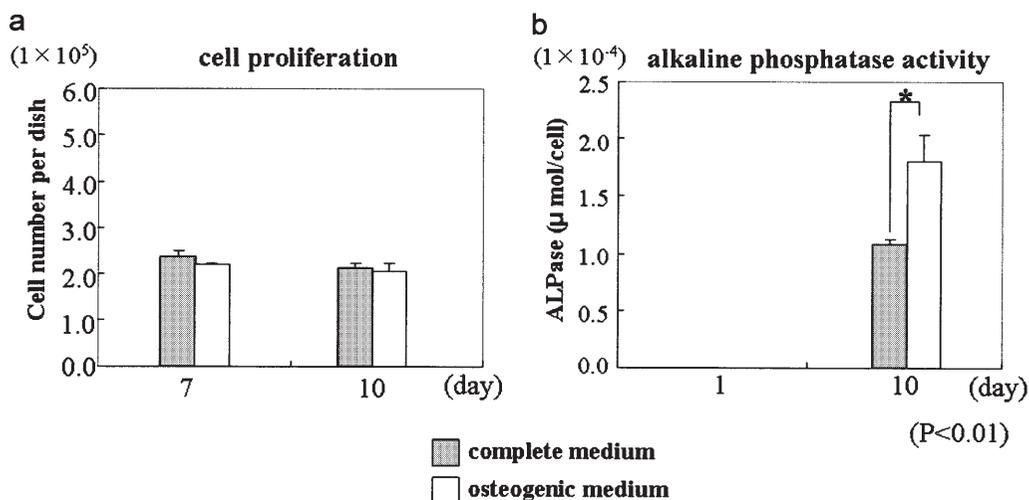


Fig. 2 Effect of osteogenic induce medium (OIM) on cell proliferation and differentiation in porcine subcultured dental pulp-derived cells (a and b). (a) Cell growth of subcultured dental pulp-derived cells in complete culture medium (CCM, n=3) and osteogenic induce medium (n=3) on days 7 and 10. Cell growth in CCM is similar to that in OIM. (b) Alkaline phosphatase (ALPase) activity in the subcultured dental pulp-derived cells with CCM or OIM. ALPase activity with OIM (n=3) becomes twice as large as that with the CCM (n=3) on day 10. Asterisk indicates a significant difference ($*p<0.01$).

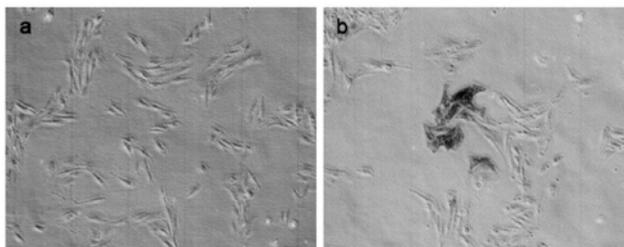


Fig. 3 (a) Phase contrast microscopic view of subcultured dental pulp-derived cells after two passages. These cells show either spindle or polygonal shapes. (b) ALPase in the dental pulp-derived cells subcultured for 2 weeks in OIM. Positive reactions are observed in portions of the subcultured dental pulp-derived cells after two passages.

distinguished from odontoblast-like cells (Fig. 5b and d). Although the success rate of hard tissue formation was 100% (6/6), dentinal tubules were observed in four out of six samples, resulting in a success rate of 66.7%. The remaining samples (2/6) only showed atubular dentin matrix in the generated hard tissues.

In the control group, capping by HAP/TCP alone gave rise to hard tissue formation, but neither dentinal tubules nor odontoblast-like cells were observed in the generated hard tissues. In addition, continuity of hard tissue was not maintained in the control teeth, resulting in leakage and subsequent severe inflammation of the remaining dental pulp (Fig. 5e and f).

The average thickness of the generated hard tissues was evaluated in both experimental (n=6)

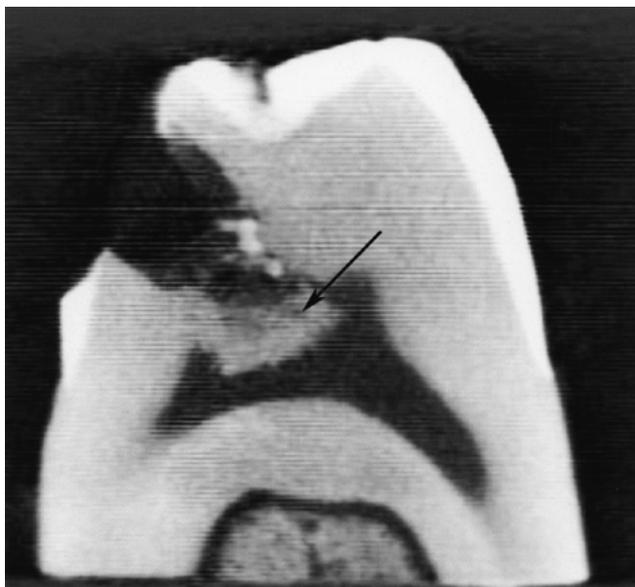


Fig. 4 Sliced μ CT image of an extracted tooth at 6 weeks after transplantation in the experimental group. Newly formed hard tissue is observed at the interface between the prepared cavity and the remaining dental pulp chamber (arrow).

and control (n=4) exposed pulp tissue groups. The average thickness in the experimental group (capping by the cell-HA/TCP constructs) was approximately 1.1 mm by histological sections, whereas that in the control group (capping by the HA/TCP without cells) was approximately 0.7 mm, showing a significant difference between the two groups ($P < 0.05$).

DISCUSSION

The present study is one of the first to establish that implanting dental pulp constructs into a pulp-exposed tooth can promote mineralization. The era of regenerative endodontics is expected to involve the introduction of biological therapies to revitalize teeth.¹⁹⁾ The present study has demonstrated the proof-of concept that tissue formation following the implantation of dental-pulp constructs into teeth may be a possible future regenerative endodontic therapy.

The density of odontoblast-like cells was suggested to be the most important factor influencing dentin-bridge formation²⁵⁾ whose potential depends on several pre- and post-operative factors such as pulp status, the prevention of bacterial infection, and the exposed pulpal size.^{4,33)} Despite the widely-accepted usage of calcium hydroxide products for direct pulp-capping therapy,^{24,34-36)} these materials frequently cause necrosis, inflammation, and dystrophic calcification in exposed pulp tissue.^{37,38)} Prevention of bacterial microleakage is one of the most important factors in achieving good prognosis over the long term.²⁵⁾ In the present study, we proposed a novel pulp-capping therapy based on tissue-engineering technology, which has the advantage of completely sealing the cavity even though the exposed site is relatively large.

Three main factors, cells, scaffolds, and growth factors are required for creating an acceptable tissue-engineered construct. Recent studies have demonstrated that human dental pulp from adult teeth and exfoliated deciduous teeth contain dental pulp stem cells that are capable of forming

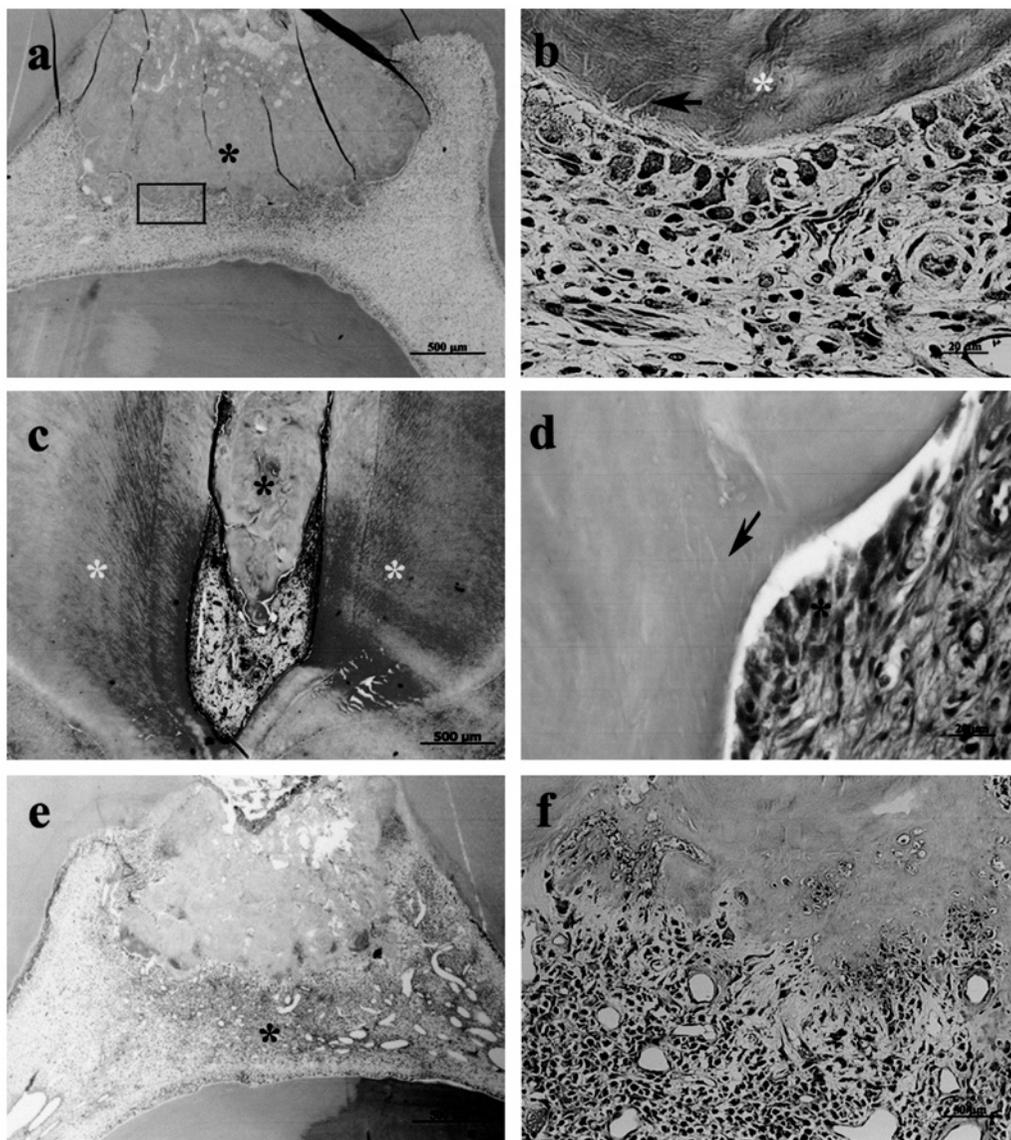


Fig. 5 H&E stained section of the teeth in the experimental (direct pulp-capping therapy with cell-HA/TCP) (a-d) and control groups (with HA/TCP) (e, f) at 6 weeks post-transplantation. (a) A large amount of generated hard tissue is observed (asterisk). The generated matrix occupies most of the pulp chamber on the exposed dental pulp tissue. (b) More highly magnified view of box in a. Odontoblast-like columnar cells (black asterisk) are aligned along the edge of the generated dentin-like tissues including dentinal tubules (arrow). Atubular dentin-like tissue is also observed in the generated tissues (white asterisk). (c) Dentin bridge-like tissue (black asterisk) occupies the coronal pulp chamber to reach the roots (white asterisks). (d) More highly magnified view of c. Dentinal tubules (arrow) are associated with the odontoblast-like cells which are columnar in shape (asterisk). (e) Although hard tissue formation is recognized in the pulp chamber, inflammatory reactions remain beneath the generated hard tissue (asterisk). (f) More highly magnified view of e. Neither typical odontoblast-like cells nor dentinal tubules are recognized in the pulp tissue beneath the generated hard tissue.

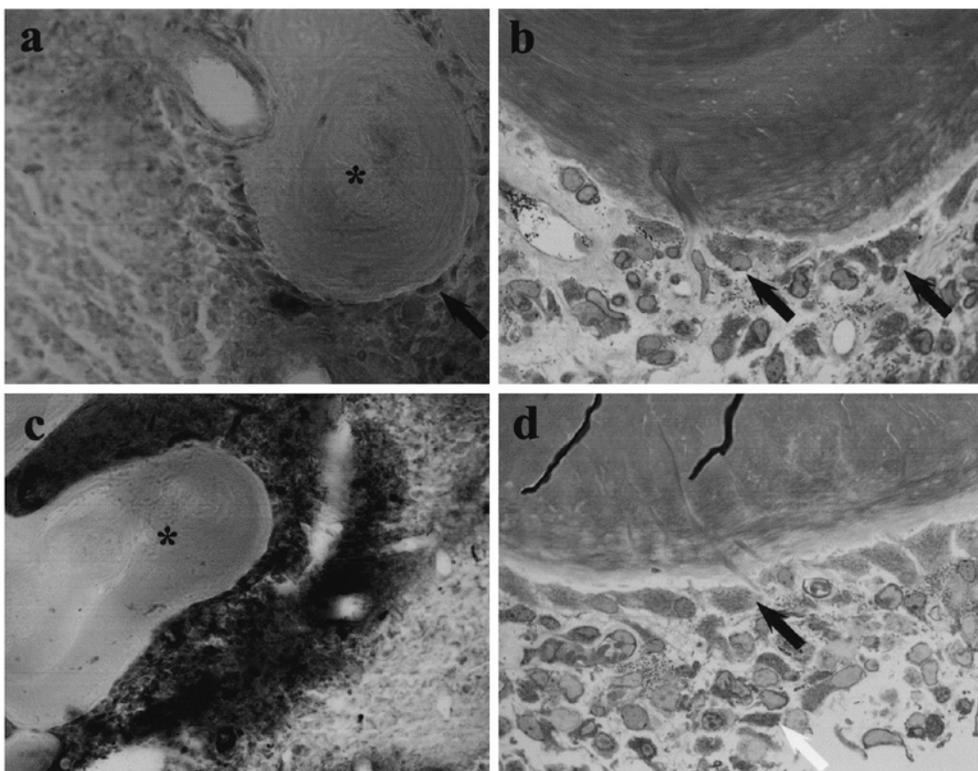


Fig. 6 DSP- (a, b) and HSP-25- (c, d) immunoreactivity in the generated tissue in resin blocks (a, c) and semi-thin sections (b, d) at 6 weeks post-transplantation. (a) Columnar cells which align beneath the generated hard tissue (asterisk) are positive for DSP-immunoreactivity (arrows). (b) More highly magnified view of the same position indicated by arrows in a. odontoblast-like columnar cells are positive for DSP-immunoreactivity in their cytoplasm (arrows). (c) Intense HSP-25-immunoreactivity is observed around the generated hard tissue (asterisk). (d) More highly magnified view of the same position indicated by asterisk in c. HSP-25-immunopositive reactions are observed in the odontoblast-like columnar cells (black arrow) and the subodontoblastic cells (white arrow) beneath the generated hard tissue.

dentin when these cells are subcultured in osteogenic medium and transplanted with HA/TCP.³⁹⁻⁴¹⁾ The previous and current evidences suggest that the subcultured dental pulp-derived cells promote the differentiation of osteogenic lineages in the osteogenesis induction medium, and dental pulp-derived cells from miniature pig molars appear to have the potential to regenerate hard tissues in the exposed pulp tissues.

A 2:8 constitutive ratio of HA/TCP scaffold is found to have the greatest effect on mesenchymal stem-cell-induced bone formation, whereas other HA/TCP ratios had less effect on bone formation.⁴²⁾ Furthermore, β -tricalcium phosphates are able to induce hard tissue formation if used for pulp-capping agents,^{43,44)} although HA alone has no dentinogenetic effect on pulp tissue when used as a pulp-capping agent.⁴⁵⁾ Stanley (1989) identified the possible risk that particles of the capping agent may be the cause of the inflammation and/or necrosis.⁶⁾ From this evidence we have chosen the 2:8 constitutive ratio of HA/TCP block as the scaffold for dental pulp-derived cells in this study.

Our pulp-capping therapy using cell-HA/TCP constructs induced the effective formation of dentin bridge-like structures after 6 weeks transplantation. The reason why the period of transplantation was decided was that the dentin-bridge formation was shown in the pulp capping therapy in dog for 4 weeks in another group experiment.⁷⁾ Therefore, six weeks is considered a sufficient period in which to induce the dentin-bridge formation in dental pulp of pig. In these structures, there were two types of hard-tissue matrix with or without dentinal tubules associated with the columnar cells showing intense immunoreactivity for DSP and HSP-25. DSP and dentin phosphoprotein (DPP), the cleavage products of DSPP, which is regarded as an odontoblast marker, constitute the major part of the noncollagenous dentin matrix and a highly phosphorylated protein secreted by odontoblasts.⁴⁶⁾ Recent studies have suggested an intimate relationship between the termination of cell proliferation and expression of HSP-25.^{47,48)} In addition, newly-differentiated odontoblasts also express intense HSP-25-IR under pathological conditions such as cavity preparation and tooth replantation using experimental animal models,^{32,49)} suggesting that this protein is a useful marker for the differentiation of odontoblasts during the pulpal healing process after exogenous harmful injuries. With respect to the hard tissue formation on the exposed pulp tissue, the average amount of generated hard tissue in the control group was significantly smaller than in the experimental group using cell-HA-TCP constructs. These results clearly suggest that subcultured dental pulp-derived cells are important for effective induction of dentin bridge-like structures on the exposed pulp tissue. Combination of subcultured dental pulp-derived cells with preexisting pulp cells in addition to the HA/TCP scaffold may induce a large quantity of calcified matrix in the early stage, resulting in prevention of bacterial microleakage. However, the exact mechanism by which transplanted dental pulp-derived cells are committed into the odontoblast-like cells remains to be clarified. It is noteworthy that necrotic tissues appeared on part of the exposed pulp tissue in 2 out of 6 samples from the experimental group. This phenomenon may be attributed to a defect in the continuity of dentine bridge-like structures, allowing the penetration of microorganisms into the pulp tissue via microleakage from the cavity margin.

In conclusion, complete dentin-bridge formation is a prerequisite for the long-term success of direct pulp-capping therapy to maintain the viable dental pulp tissue.^{6,50)} The transplanted dental pulp-derived cells may survive and regenerate the dentin bridge-like structures on exposed dental pulp tissue. The generative potential of the subcultured dental pulp-derived cells may indeed be important for tissue engineering pulp-capping therapy in the future.

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