# Regenerative Characteristics of Apical Papilla–derived Cells from Immature Teeth with Pulpal and Periapical Pathosis

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## Abstract

**Introduction:** The purpose of this study was to ascertain the regenerative characteristics of apical papilla–derived cells (APDCs) from immature teeth with pulpal and periapical pathosis and thus to provide proof-of-principle evidence for further regenerative endodontic research. **Methods:** Pulpal and periapical pathosis was induced in immature permanent double-rooted premolars of beagles, which were randomly assigned to experimental treatment groups: group AO ($n$ = 14), pulp disruption and access left open; group PS ($n$ = 14), supragingival plaque suspension-soaked cotton pellet was introduced, and access was sealed; and control ($n$ = 7), untreated. The teeth were extracted at 2- and 4-week periods after experimental treatments. APDCs were cultured from the extracted teeth, and their cellular proliferation, differentiation characteristics, and stemness were assessed. The data were statistically analyzed. **Results:** After 4 weeks of intentional pulpal and periapical pathosis induction period, all teeth in group PS showed features of apical periodontitis with necrotic pulp, and their APDCs showed significantly increased proliferation rate and osteogenic/odontogenic differentiation capabilities ($P < .05$). The stemness was maintained in all APDCs, although the stem cell population was smaller in group PS at 2-week period when the inflammatory responses were most fulminant ($P < .05$). **Conclusions:** The APDCs from immature teeth retained the regenerative characteristics with the differences according to their pulpal and periapical pathosis. The results of this study partly provide the evidence for regenerative endodontic research. *(J Endod 2016;■:1–7)*

## Key Words

Apical papilla-derived cells, endodontic animal model, immature tooth, regenerative endodontic procedure

### Significance

Regenerative characteristics of apical papilla–derived cells (APDCs) from immature teeth with pulpal/periapical pathosis were investigated. The APDCs retained regenerative characteristics with differences according to their pulpal/periapical pathosis and maintained stemness regardless of inflammatory status of apical papillae.

Pulpal and periapical pathosis of various causes such as dental caries or trauma is one of the most prevalent oral diseases (1). Permanent mature teeth with necrotic pulp and apical periodontitis can be successfully treated by conventional endodontic treatment (2). However, the disease in immature teeth hampers normal root development, resulting in wide open apex and thin dentin wall, which jeopardize the life span of the teeth. Therefore, the treatment strategies pursue recovery from the disease and induction of continuous root growth such as apexogenesis and/or apexification rather than conventional endodontic treatment.

Recently, many dental studies have focused on the regenerative endodontic procedure (REP) of immature teeth, which uses the concept of tissue engineering allowing for continued development of the root and surrounding tissue. In brief, induction of intracanal bleeding after complete disinfection may attract osteogenic stem cells from surrounding tissues, thus resulting in mineralized tissue ingrowth in the canal lumen (3, 4). A more detailed understanding of REP and completion of the genuine pulpal and periapical regeneration inevitably require *in vivo* research in a suitable animal model, and consequently some *in vivo* and *in vitro* studies have described the underlying mechanism of the healing process after REP in immature teeth with necrotic pulp and apical periodontitis (5–10). To date, evidence of hard tissue formation on root canal walls, apical closure, and formation of vital tissue in the canal space after REP has been reported (5–10).

The REP mainly targets the teeth with necrotic pulp and apical periodontitis, and the treatment outcome largely depends on the regenerative capacity of undifferentiated mesenchymal stem cells (MSCs) from apical papilla (11, 12). It is noteworthy that the MSCs that are presumed to retain significant self-renewal capacities and pluripotency are heterogeneous population of cells derived from the apical papillae tissue under pulpal and periapical pathosis (11, 13–15). However, there is an important gap in knowledge regarding the exact features of the apical papilla–derived cells (APDCs) in the endodontic inflammatory microenvironment. Therefore, the purpose of this study was to ascertain the regenerative characteristics of the APDCs from immature

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teeth with pulpal and periapical pathosis and thus to provide the proof-of-principle evidence for further regenerative endodontic research.

## Materials and Methods

### Preparation of Apical Papillae from Immature Teeth with Pulpal and Periapical Pathosis

The animal experimental protocols were approved by the Institutional Animal Care and Use Committee of Seoul National University (authorization no. SNU-121210-5).

A total of 35 double-rooted premolars from 4 beagles (Orient Bio, Gyeonggi, Korea) aged approximately 5–6 months were used in this study. The beagles were anesthetized with an intravenous injection of ketamine at a dose of 1 mg/kg. Before any intervention, the teeth were radiographed with a bisecting-angle technique to confirm the incomplete root development. The teeth were randomly assigned to the experimental groups to induce pulpal and periapical pathosis ($n = 28$). In group AO ($n = 14$), the pulp was mechanically exposed with #2 round carbide bur and disrupted by using sterile #20 stainless steel K-file (Dentsply Maillefer, Ballaigues, Switzerland) without removing it. The access cavity was left open in oral cavity. In group PS ($n = 14$), after pulp exposure and disruption as described above, supragingival plaque scaled from the dog's teeth was mixed with sterile saline and used to soak cotton pellets that were inserted into the access chamber. The access cavities were sealed with intermediate restorative material (Dentsply Caulk, Milford, DE). The animals were given analgesics postoperatively (ketoprofen 1 mg/kg). All teeth were radiographed on a weekly basis. After intentional inducing of pulpal and periapical pathosis, the teeth in each group were subdivided into 2 different groups according to the duration and were extracted after 2 and 4 weeks, respectively. Seven teeth were left intact as controls ($n = 7$) (Supplemental Figure S1 is available online at www.jendodon.com). Radiographs made at the time of pulp exposure as the initial measurements were compared with those made at the time of extraction as the final measurements. Each individual root was blindly evaluated by 2 investigators for assessment of the size of periradicular radiolucent area (pixels).

### Validation of APDCs

The extracted teeth were washed 5 times with cold serum-free $\alpha$-minimum essential medium (WelGene, Daegu, Korea) supplemented with 4% penicillin-streptomycin (Hyclone, Logan, UT). Apical papilla, periodontal ligament (PDL), and pulp tissue were harvested from the extracted non-treated control teeth according to Akiyama et al (16). Apical papilla from root foramen area and PDL from root surface were gently separated by using a sterile blade. Apical 2 mm of the root tip was cut with a bone cutter, and coronal pulp tissue was extirpated with a sterile barbed broach. Each collected tissue that was further diced into pieces (smaller than 1 $mm^3$) was placed on 6-well plate and cultured in growth medium ($\alpha$-minimum essential medium containing 10% fetal bovine serum [Hyclone] and 1% penicillin-streptomycin) for 3 weeks. The cells extended out from tissue pieces were harvested and subcultured. The cells harvested from apical papilla, PDL, and pulp tissue were investigated on their capacities of proliferation and cellular differentiation to ensure the discrete characteristics of APDCs from the others.

### Cell Culture

The APDCs from immature teeth with pulpal and periapical pathosis were prepared and subcultured. We used the cells at passages 2–4, and each independent experiment was performed by using the cells at the same passages. For the proliferation tests, the cells were seeded at the density of $2.1 \times 10^4$ cells/$cm^2$, and the amounts of double-stranded DNA were measured by using PicoGreen reagent (Invitrogen, Eugene, OR) after 1, 3, 5, and 7 days of culture in growth medium. We seeded the cells at a density of $2.1 \times 10^4$ cells/$cm^2$ and cultured them in a differentiation medium (growth medium supplemented with 10 mmol/L $\beta$-glycerophosphate [Calbiochem, La Jolla, CA] and 50 $\mu$g/mL L-ascorbic acid [Sigma-Aldrich, St Louis, MO]) for 7 or 14 days for the osteogenic/odontogenic differentiation characteristics.

### Osteogenic/Odontogenic Differentiation

To investigate the osteogenic/odontogenic differentiation capabilities of APDCs, we performed the analyses of alkaline phosphatase (ALP) activity and biomineralization (alizarin red staining and calcium deposition quantification) as reported previously (17). For alizarin red staining, the cultured cells were fixed with 4% formaldehyde and thoroughly washed with double distilled water 2 times. The fixed cells were stained with 2% alizarin red stain (pH 4.1–4.3; Sigma-Aldrich) solution for 30 minutes at room temperature in dark condition. Thereafter, cell culture plates were washed, air-dried, and identified of alizarin red staining with light microscopy. For calcium deposition quantification, cultured cells were washed with cold phosphate-buffered saline 2 times and treated with 0.5 N HCl for 24 hours. The calcium content was measured by using a calcium assay kit (QuantiChrom; Bioassay Systems, Hayward, CA) according to the manufacturer's instructions. For ALP activity assay, the whole cell lysates were prepared, and ALP activity was measured by using *p*-nitrophenylphosphate (Sigma-Aldrich) as the substrate.

### Reverse-Transcription Quantitative Polymerase Chain Reaction Analyses

Total RNAs were extracted from the inflamed tissues and cultured cells by using RNAiso Plus reagents (Takara, Otsu, Japan), and cDNA was synthesized by using a PrimeScript RT reagent kit (Takara). Reverse-transcription quantitative polymerase chain reaction was performed by using a Real-time PCR system (Applied Biosystems, Foster City, CA). All reactions were run in triplicate and were normalized to the housekeeping gene, *Gapdh*, as a template. The primer sequences used in this study are listed in Supplemental Table S1 is available online at www.jendodon.com.

### Statistical Analysis

The results of radiographic analyses were assessed with Mann-Whitney *U* test. Data from the repeated *in vitro* experiments were presented as means and standard deviations (SD). Statistical analysis was performed with analysis of variance and Tukey post hoc test. The level of significance was set at 5%.

## Results

### Validation of APDCs

To validate current experimental protocol for the culture of APDCs, the APDCs, PDL-derived cells (PDLDCs), and dental pulp–derived cells (DPDCs) isolated from the non-treated control teeth were investigated on their capacities of proliferation and cellular differentiation. APDCs showed significantly higher proliferation than PDLDCs and DPDCs at day 7 ($P < .05$, Fig. 1*A*). Regarding the osteogenic/odontogenic differentiation, APDCs showed significantly higher ALP activity and osteocalcin (*Ocn*) expression than PDLDCs and DPDCs after 14 days of differentiation ($P < .05$, Fig. 1*B* and *D*). In contrast, dentin sialophosphoprotein (*Dspp*) expression of APDCs was about 8-fold to 20-fold lower than that of PDLDCs and DPDCs, respectively ($P < .05$, Fig. 1*C*). The proliferation capacity and the bone (dentin)-matrix