Fibrin Gel Improves Tissue Ingrowth and Cell Differentiation in Human Immature Premolars Implanted in Rats

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Abstract

Introduction: In pulpless immature human premolars implanted in rodents, this study investigated whether fibrin gel offered advantages over leaving the root canal empty regarding soft tissue ingrowth and cell differentiation. **Methods:** Root canals of extracted human immature premolars (n = 12) were accessed and then irrigated with 5% sodium hypochlorite followed by 17% ethylenediaminetetraacetic acid. Root canals were then either left empty or filled with a fibrin gel (n = 6 each) before being placed subcutaneously on top of the calvarial bone of rats (1 tooth per rat) for 12 weeks. After sacrifice, teeth were histologically assessed. Tissue ingrowth was quantified and compared between groups using the Mann-Whitney U test (P < .05). Cells adhering to the pulp canal wall were immunohistochemically screened for the presence of bone sialoprotein (BSP) and dentin sialoprotein (DSP). **Results:** More tissue grew into the pulp space when teeth were filled with fibrin gel (P < .05). The presence of fibrin gel affected not only the extent of tissue ingrowth but also tissue morphology and differentiation of cells contacting the dentinal wall. In the fibrin gel group, newly formed tissue was similar to normal pulp, constituted of inner pulp, cell-rich zone, cell-free zone, and an apparent odontoblast layer, which stained positive for BSP and DSP. Newly formed blood vessels were also more abundant compared with the initially empty root canals. **Conclusions:** Under the conditions of this study, fibrin gel improved cell infiltration and cell-dentin interaction. Both are necessary for pulp tissue regeneration. *(J Endod 2014;40:246–250)*

Key Words

Pulp, regeneration, revascularization

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Attempts to revascularize the pulp space in nontraumatized teeth date back to the second half of the last century (1). However, the concept was dropped for some decades because the procedure was unpredictable and only worked in teeth that contained a vital pulp before root canal treatment. Things changed when the first case reports appeared on revascularization of immature necrotic premolars in children (2). The multitude of case series that followed spurred the current clinical guidelines for such teeth (3). These include disinfection of the canal space followed by induced bleeding and covering of the blot clot with a biocompatible material. The concept of controlled bleeding is based on the fact that in immature teeth, despite the presence of a periapical lesion, the periapical tissues contain a significant amount of pluripotent cells with the potential to form new tissues inside the pulp space (4). However, animal experiments have raised some doubt as to whether these tissues reflect a functioning pulp. In immature teeth in dogs, periodontal tissues filled the root canal space after a revascularization procedure (5). Revascularization procedures based on overinstrumentation and the formation of a blood clot in the canal space are further limited by the fact that they are restricted to young individuals with an apical papilla at the apex of the affected tooth (6).

A potential alternative approach to current techniques for pulp revascularization is cell homing (ie, the controlled attraction of cells into a scaffold by chemotaxis (7) and the exposure to cues facilitating and guiding proper regeneration). In theory, cell homing with the correct sequence and spatial application of tissue factors could enable clinicians to regenerate a functioning pulp (8). This should be a significant improvement over current revascularization schemes. The concept of cell homing into human teeth has been tested by subcutaneously implanting single-rooted teeth in the dorsum of mice (9). However, after this first groundbreaking study, many questions remain to be addressed. The root canals were opened from the crown, a situation that is not encountered in the clinic. Moreover, the irrigating procedure applied before implantation was not specified. Studies on dentin cylinders implanted in mice have shown that irrigation greatly affects cell differentiation (10). It would appear that tissue factors present in dentin are liberated by decalcifying agents such as ethylenediaminetetraacetic acid (EDTA). However, it is not known whether a scaffold inside the canal space can improve tissue ingrowth compared with leaving the canal space empty. A simple scaffold would be fibrin gel, which can simulate a blood clot (11). A blood clot can be hard to obtain clinically (3). However, in contrast to a blood clot, fibrin gel can be modified to serve as a delivery device for the controlled release of tissue factors (12). The aim of this study was to investigate the effect of fibrin gel as a scaffold material on tissue ingrowth and cell differentiation in pulpless human immature premolars that were subcutaneously implanted in rats.

Materials and Methods

Experimental Teeth

Human premolars (n = 12) with immature roots from the department's collection of extracted teeth were used for the current experiments. Patients had given informed consent that their teeth could be used for scientific studies. All these teeth were extracted for orthodontic reasons and stored in 0.1% thymol at 5°C. Personnel handling the teeth applied all necessary precautions for infection control. The institutional review board approved the procedures.

Preparation of Teeth and Root Canals

Soft-tissue remnants at the root surface were removed using a curette. Subsequently, teeth were immersed in 5% sodium hypochlorite (NaOCl) for 5 minutes in an ultrasonic bath (TEC-25; Benzer Dental AG, Zürich, Switzerland) at 60 W and 33 Hz. Roots were standardized to a length of 9 mm by cutting the crown near the cementoenamel junction using a diamond-coated disc under water cooling. Access to the pulp cavity was obtained using a cylindrical diamond-coated burr in a counter-angle handpiece. The pulp tissue was removed using a barbed broach (Dentsply Maillefer, Ballaigues, Switzerland). No further mechanical treatments were performed. Root canals were irrigated with 5 mL of a technical-grade 5% NaOCl solution for 5 minutes. Subsequently, 5 mL of a 17% EDTA solution was applied for 5 minutes. Irrigants were delivered through a 26-G cannula (Sterican; B Braun Medical, Crissier, Switzerland). All root canals were finally irrigated with normal saline solution (5 mL). Access cavities were then dried with compressed air and filled with glass ionomer cement (Ketac Molar; 3M ESPE, Seefeld, Germany). Teeth were kept sterile in 70% ethanol in a safety cabinet at room temperature until implantation.

Fibrin Gel Preparation and Placement

The day before the implantation teeth were placed under the sterile bench to allow the evaporation of the 70% ethanol overnight; 1% fibrin gel was prepared and injected into the root canals before implantation in the rats. Frozen human fibrin and thrombin dilutes prepared from a Tisseel kit (Baxter, Zurich, Switzerland) were suspended in Tris-buffered saline solution at a pH of 7.4 to form a fibrin gel. The final gel was formed by mixing 8 mg/mL fibrinogen, 2.5 mmol/L Ca^{++}, and 2 National Institutes of Health U/mL thrombin. The gel was injected from a sterile syringe into the root canals of 6 teeth from the apical opening using a 26-G cannula (Sterican). The setting time was approximately 2–4 minutes. The other 6 teeth were left empty. Specimens were stratified (matched) between the test and control group so that the number of root canals and the size of the apical opening were similar between groups. Subsequently, the specimens were placed into a humid cell culture incubator at 37°C until implantation, which was performed within 2 hours.

Implantation

Twelve female Crl:CD Sprague-Dawley rats (Charles River, Raleigh, NC) weighing between 200 and 250 g were used for these experiments. All the following procedures were approved by the institutional ethics committee for animal research. The 9-mm tooth specimens were placed on top of the calvarial bone of the rats subcutaneously. Rats were given ketamine analgesic and were anesthetized by isoflurane inhalation. A longitudinal incision on the head was performed using a scalpel. The blunt end of surgical scissors was then used to dissect the underlying tissue to create space for the tooth specimen to be implanted. Each rat received 1 tooth specimen. The wounds were sutured using polyamide 6/0 (Supramid, Melsungen, Germany) to obtain primary closure.

Assessment of Tissue Ingrowth

Animals were sacrificed 12 weeks after implantation by CO_2 asphyxiation. Subsequently, teeth were explanted. Tooth specimens were fixed in 4% paraformaldehyde, embedded in Technovit 9100 New (Heraeus Kulzer, Wehrheim, Germany), sectioned longitudinally (Leica Microsystem, Heerbrugg, Switzerland), and stained with Goldner trichrome. Each section had a thickness of 0.5 μm. Three subsequent sections were prepared from the center of each tooth. Sections were placed on glass slides and then viewed and photographed in a digital light microscope (Leica Microsystem). Image analysis was performed using freely available software (Image J; National Institutes of Health, Bethesda, MD). The total area of the root canal was defined. Subsequently, the area of coherent soft tissue that was present in the pulp space was delineated. Soft tissue areas were normalized to the total pulp space area and averaged from the 3 central sections per specimen. These values were compared between groups using nonparametric statistics and the Mann-Whitney *U* test with the alpha-type error set at 5%.

Immunohistochemistry

Immunohistochemical localizations of bone sialoprotein (BSP) and dentin sialoprotein (DSP) were observed in additional serial sections from the center of the specimens. After Technovit 9100 New removal by a chloroform-xylene solution (1:1) and dehydration, sections were treated with 3% H_2O_2 in a solution of 0.01 mol/L phosphate-buffered saline (pH = 7.4) for 30 minutes at room temperature. For DSP-stained sections, antigen retrieval was performed with heat before 3% H_2O_2 treatment. Unspecific binding was blocked by goat serum (Dako, Glostrup, Denmark). Specimens were incubated with primary antibodies (Merck Millipore, Darmstadt, Germany) against BSP (1:200) at 4°C overnight and DSP (1:100) at room temperature for 5 hours. Specimens incubated in mere buffer solution were used as controls. The specimens were then incubated with biotinylated antibodies (Merck Millipore) against the primary antibodies. Peroxidase-conjugated streptavidin was applied before using 3,3′-diaminobenzidine tetrahydrochloride for detection. Slides were counterstained with hematoxylin and observed by light microscopy.

Results

The role of fibrin gel for cell homing in root canals irrigated with NaOCl and then EDTA was assessed. The fibrin gel improved tissue formation in the root canal after implanting the teeth in the rats for 12 weeks both quantitatively (Fig. 1) and qualitatively (Fig. 2*A–F*). Significantly ($P < .05$) larger areas in the root canal were filled with tissue in specimens filled with fibrin gel compared with the root canals that were left empty before implantation. The median area filled with newly formed tissue was 43% of the total root canal area in the teeth that had been filled

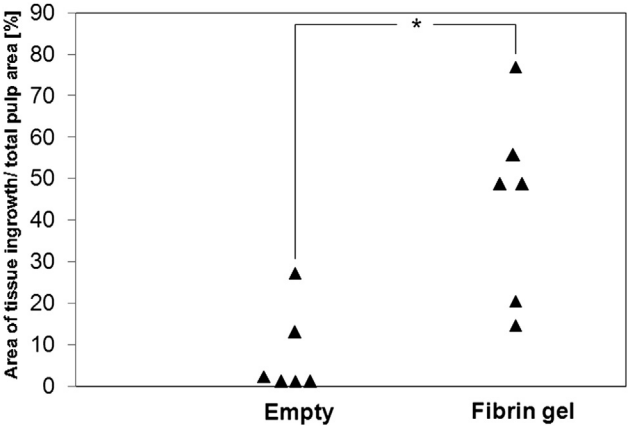

Figure 1. Tissue ingrowth into root canals of human premolars ($n = 6$ per group) implanted onto the calvarial bone of rats. Comparing the percentage of the tissue ingrowth area between groups revealed a significantly ($P < .05$) higher tissue formation inside root canals filled with fibrin gel compared with empty counterparts. Three sections per tooth were analyzed and the value averaged. For comparison between the 2 treatment groups, these mean values per tooth were used (nonparametric comparison between ranks of mean values per tooth).