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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.

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

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Key words: revascularization, pulp, regeneration

Running title: Impact fibrin gel on tissue ingrowth

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Abstract

Introduction: In pulpless immature human premolars implanted in rodents, this study investigated whether a 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% EDTA. 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 (one tooth per rat) for 12 weeks. After sacrifice, teeth were histologically assessed. Tissue ingrowth was quantified and compared between groups by Mann-Whitney U test, $p<0.05$. Cells adhering to the pulp canal wall were immunohistochemically screened for the presence of bone and dentin sialoprotein (BSP and DSP).

Results: More tissue grew into the pulp space when teeth were filled with a fibrin gel ($p<0.05$). The presence of a 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, constituting 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 as compared to the initially empty root canals.

Conclusions: Under the conditions of this study, a fibrin gel improved cell infiltration and cell-dentin interaction. Both are necessary for pulp tissue regeneration.

Attempts to revascularize the pulp space in non-traumatized 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 prior 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). Animal experiments, however, 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 over-instrumentation 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, i.e. 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). After this first groundbreaking study, however, 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 prior to 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 EDTA. However, it is not known whether a scaffold inside the canal space can improve tissue ingrowth compared to leaving the canal space empty. A simple scaffold would be a fibrin gel, which can simulate a blood clot (11). A blood clot can be hard to obtain clinically (3). In contrast to a blood clot, however, a 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 a 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% NaOCl for 5 min 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 cemento-enamel junction using a diamond-coated disc under water cooling. Access to the pulp cavity was obtained using a cylindrical diamond-coated bur 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 min. Subsequently, 5 mL of a 17% EDTA solution was applied during 5 min. Irrigants were delivered through a 26-gauge 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 (TBS) at pH 7.4 to form a fibrin gel. The final gel was formed by the mixing 8 mg/mL fibrinogen, 2.5 mM Ca^{++}, and 2 NIH Units/mL thrombin. The gel was injected from a sterile syringe into the root canals of 6 teeth from the apical opening using a 26-gauge cannula (Sterican, B.Braun Medical). Setting time was approximately 2-4 min. The other 6 teeth were left empty. Specimens were stratified (matched) between test and control group, so that number of root canals and 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 (SD) Sprague Dawley rats (Charles River, Raleich, 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 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 one 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's 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 Institute of Health). 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 non-parametric statistics, 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. Following 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 M PBS, pH 7.4 for 30 min 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 h. Specimens incubated in mere buffer solution were used as controls. The specimens were then incubated with biotinylated antibodies (Merck Millipore, Darmstadt, Germany) against the primary antibodies. Peroxidase-conjugated streptavidin was applied before using 3,3' – diaminobenzidine tetrahydrochloride (DAB) for detection. Slides were counterstained with haematoxylin and observed by light microscopy.

Results

The role of a fibrin gel for cell homing in root canals irrigated with NaOCl and then EDTA was assessed. The fibrin gel improved tissues formation in the root canal after implanting the teeth in the rats for 12 weeks both quantitatively (Fig. 1) and qualitatively (Fig. 2). Significantly ($p<0.05$) larger areas in the root canal were filled with tissue in specimens filled with fibrin gel compared to 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 with the fibrin gel, compared to a median of 11 % in counterparts that did not contain this scaffold. Highly developed small blood vessels extended throughout the inner tissues. Moreover, the structure of the newly formed tissues showed a pattern similar to normal pulp, constituting of inner pulp, cell-rich zone, cell-free zone, and an apparent

odontoblast layer (Fig. 3). Empty pulp space also revealed some tissue growth in the root canal. However, tissues were apparently unable to grow towards coronal part of the pulp space (Fig.2). In the roots filled with fibrin gel, odontoblast-like cells were abundantly found close to the dentinal wall. These cells extended their processes into the dentinal tubules. Strongly BSP-positive areas were identified in the cells integrated to the dentinal wall (Fig. 4). DSP-immunoreactive areas were localized at the dentinal wall (predentin) in roots filled with fibrin gel, while they were absent in the formerly empty root canals.

Discussion

This study on human immature premolars implanted in rats showed that, compared to leaving the canal space empty, a fibrin gel has a beneficial effect on both, ingrowth of soft tissue into the root canal space and cell differentiation in that tissue towards a functioning pulp.

The current study is limited by the fact that the natural immature teeth were difficult to control in terms of the stage of the development and the shape of the root canal. Width of the opening at the root apex was diverse among the sample as well as the shape and number of the root canals. However, the tooth specimens were stratified in all experiment. Structural diversities were controlled for by matching similar characteristics between the experimental groups. As this was an animal study, albeit with human teeth, there can be no direct clinical conclusions. Nevertheless, it could be stated that the model for pulp regeneration studies presented in this communication is clinically relevant. It stands out from published material by the fact that immature human teeth were used, and thus the spatial and molecular environment was more realistic compared to published material on this topic. 5% NaOCl and 17% EDTA irrigants, which are easily available and commonly used by dentists, were used to reflect normal clinical practice. NaOCl dissolves biofilm, necrotic soft tissue remnants, and the organic aspect of superficial canal wall dentin, while EDTA removes smear layer and inorganic aspects in the pulp canal wall (13). The model presented here has yet another new

feature, which was placing the tooth on top of calvarial bone of the rat. This might be closer to the condition of the tooth in alveolar bone compared to implantation in the dorsum region of test animals (9).

The concept of cell homing depends on the controlled delivery of tissue factors, which attract and differentiate circulating cells (7). In the current study, however, no such factors were applied. Consequently, the ingrowing tissue had to be triggered either by the fibrin gel itself or by tissue factors released from the dentinal wall. Both cell-based and cell-homing approaches showed various aspects necessary for pulp regeneration (14-16). Some of these studies already focused on the use of irrigating solutions (10, 17) and the type of scaffold (18-20). In the current study, the fibrin gel showed distinctive tissue in-growth without cell transplantation. In the presence of a fibrin gel, structured tissues similar to normal pulp were observed after 12 weeks of implantation. Previous studies demonstrated either adipose tissues or ectopic mineral deposition in the canal space of whole teeth or tooth fragments implanted in rodents (9, 21). A recent study in immature, formerly infected teeth in dogs, suggested that an injectable scaffold is unnecessary for pulp regeneration. These authors observed less regenerated tissues in root canals filled with a fibroblast growth factor (bFGF) in a gelatin hydrogel compared to normal blood clot induction (22). They also noted that the newly formed tissue inside the root canal resembled periodontal tissues. In contrast, our histologic slides showed that a mere fibrin gel can promote the formation of pulp-like tissues in the immature teeth. Although no cells were applied in this study, the in-growth tissue properly formed the structure as regular pulp tissues. No ectopic hard tissue formation was observed in the current experiments. Differences between the current and some published results may be seen in species-related features of pulpal repair. Furthermore, the fibrin gel that was used here may be superior to scaffolds used in former studies. Unlike a synthetic hydrogel, fibrin is a biodegradable fibrinolytic system (11) and naturally a provisional regeneration matrix. Compared to other natural scaffolds, fibrin is the first scaffold to form as blood clot after

injury to which cells can adhere and can invade during tissue repair. Additionally, many growth factors in the blood and various cell type integrin can interact and bind to several sites of the fibrin (11). These properties make fibrin an attractive matrix for cell migration and differentiation.

The current model monitored soft tissue formation at one point in time. However, immunoreactivity to BSP and DSP showed that mineralized tissues might be formed at the dentinal wall. Specimens were embedded in Technovit, which might impede the detection of some proteins. Although no immunohistochemistry on Technovit previously showed DSP protein detection (23, 24), we also investigated rat molars as positive controls. Immunoreactivity against DSP rarely showed in the odontoblast cell layer in these rodent teeth, but rather in the subjacent dentin and pre-dentin (data not shown). These expression patterns were similar to the counterparts observed in the implanted, formerly pulpless, human teeth. This DSP expression strongly suggests that the newly formed pulp-like tissue did not only resemble a pulp morphologically, but apparently also performed some of the steady-state functions of a normal pulp. Further studies are necessary to evaluate whether (and if yes, when) new dentin formation is possible in the model described in this study.

In conclusion, the current communication presents a cell homing model for pulp regeneration/ cell homing studies that mimics clinical conditions. In this first investigation, it was shown that a mere fibrin gel as scaffold has beneficial effects on soft tissue ingrowth and cell differentiation into a treated pulpal space. In fact, the new pulpal tissue that formed was astonishingly similar to a normal pulp, without any addition of tissue factors into the implanted fibrin scaffold. Further studies should now focus on the question whether this rather interesting, and to a large extent surprising, result could yet be improved upon by implementing specific growth factors for cell recruitment and differentiation into the treatment protocol.

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Figure captions

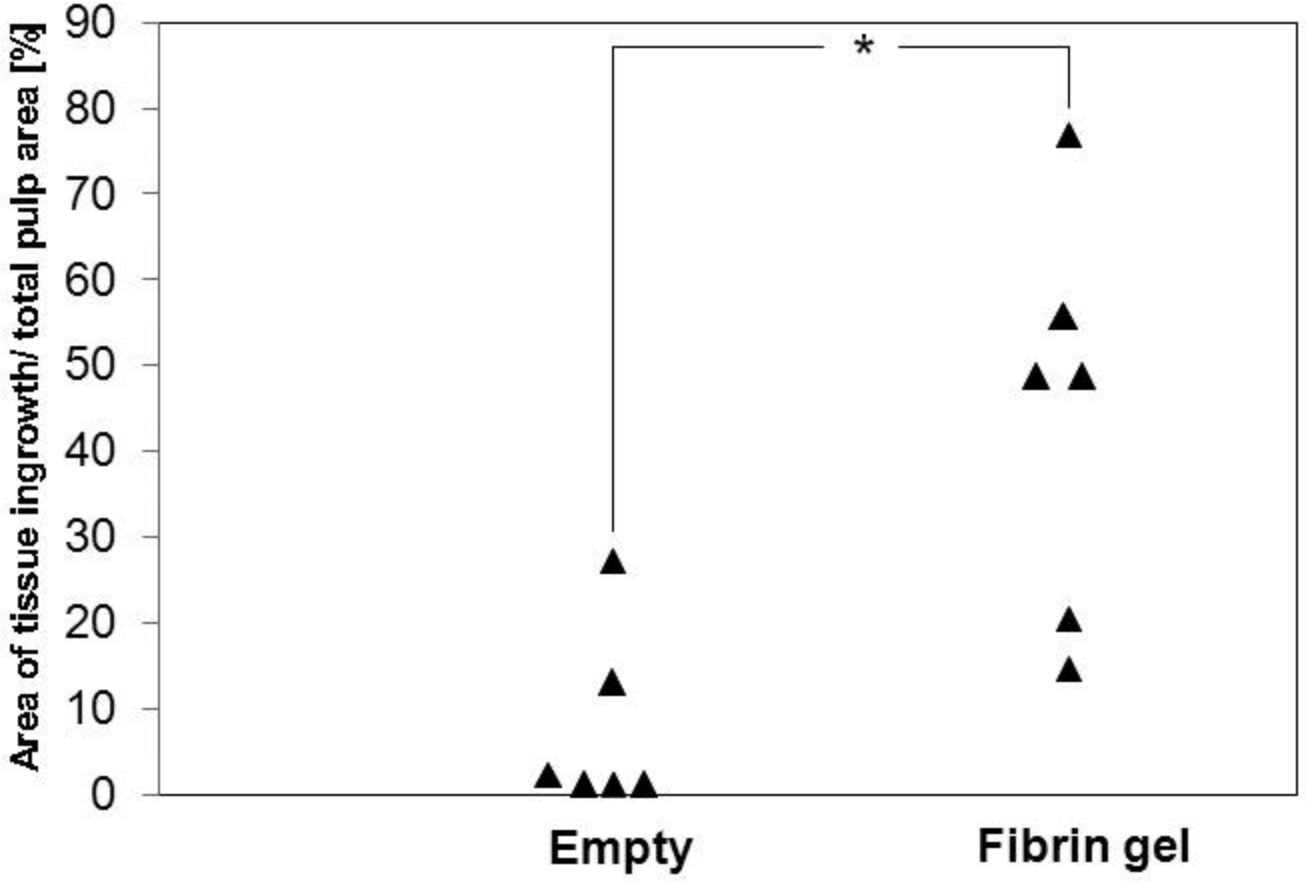
Figure 1. Tissue ingrowth into root canals of human premolars (N = 6 per group) implanted onto the calvarial bone of rats. Comparing the percentage of in-growing tissue area between groups revealed a significantly ($p<0.05$) higher tissue formation inside root canals filled with fibrin gel compared to empty counterparts. Three sections per tooth were analyzed and the value averaged. For comparison between the two treatment groups, these mean values per tooth were used (non-parametric comparison between ranks of mean values per tooth).

Figure 2. Histologic analysis of representative root canal filled with fibrin gel and empty counterpart after implantation on calvarial bone of the rat for 12 weeks. EDTA was used to irrigate the root canals in both groups. In-growing tissues (A, D), cells integrated with the dentinal wall (B, E) and vascularization (C, F) were assessed under the light microscope.

Figure 3. Fibrin gel as scaffold showed pulp like tissue formed inner the root canal of immature permanent tooth after implantation on calvarial bone of the rat for 12 weeks (panel A: overview, panel B: higher magnification of root canal wall). Center of the pulp (CN) located at the innermost of the root canal, constituted of large blood vessels and dense connective tissue. Cell rich zone (CR) the next layer toward to dentinal wall, presented abundance of cells before reaching cell free zone (CF) which contained capillaries network, loose connective tissue and fewer cells localized in this area. Odontoblast like cells, which integrated with the dentinal wall, showed in odontoblast cell layer (OD) the outermost layer of the pulp tissue.

Figure 4. Localization of dentin sialoprotein (DSP) and bone sialoprotein (BSP) in immature teeth after 12 weeks of implantation in the rat. Irregular soft tissue was observed in the empty group (A, B). Cells located at dentin were faintly positive to BSP (A1, A2), while no immunoreaction to DSP was localized in the same area (B1, B2). Odontoblast-like cells were

found at the dentinal wall of root canals filled with fibrin gel (C, D). In this group, the immunohistochemical process revealed intense immunoreactivity of BSP in odontoblast-like cells and their subjacent area (C1, C2). Inner pulp tissues were weakly positive for BSP. DSP was localized underneath odontoblast-like cells in the predentin (D1, D2).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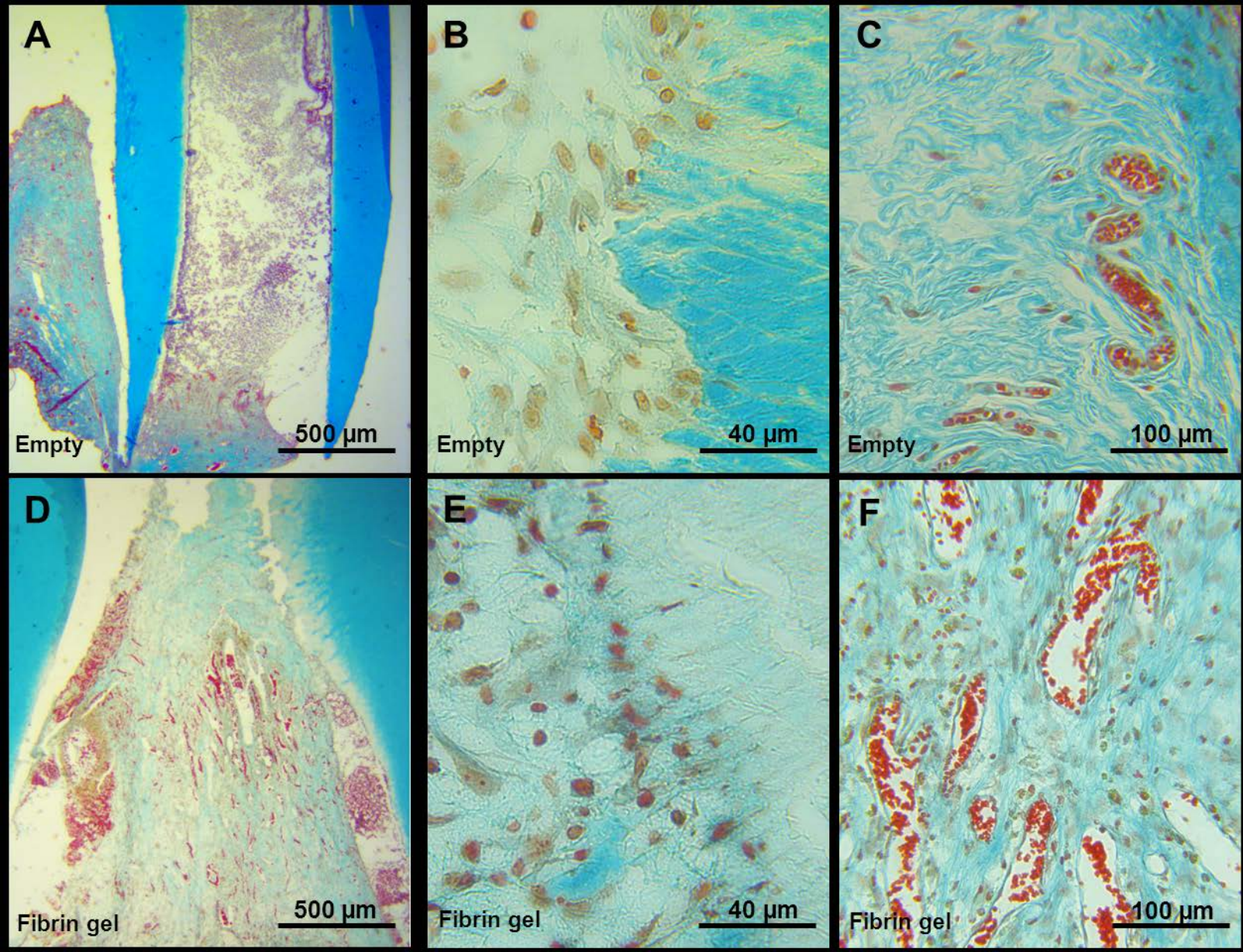
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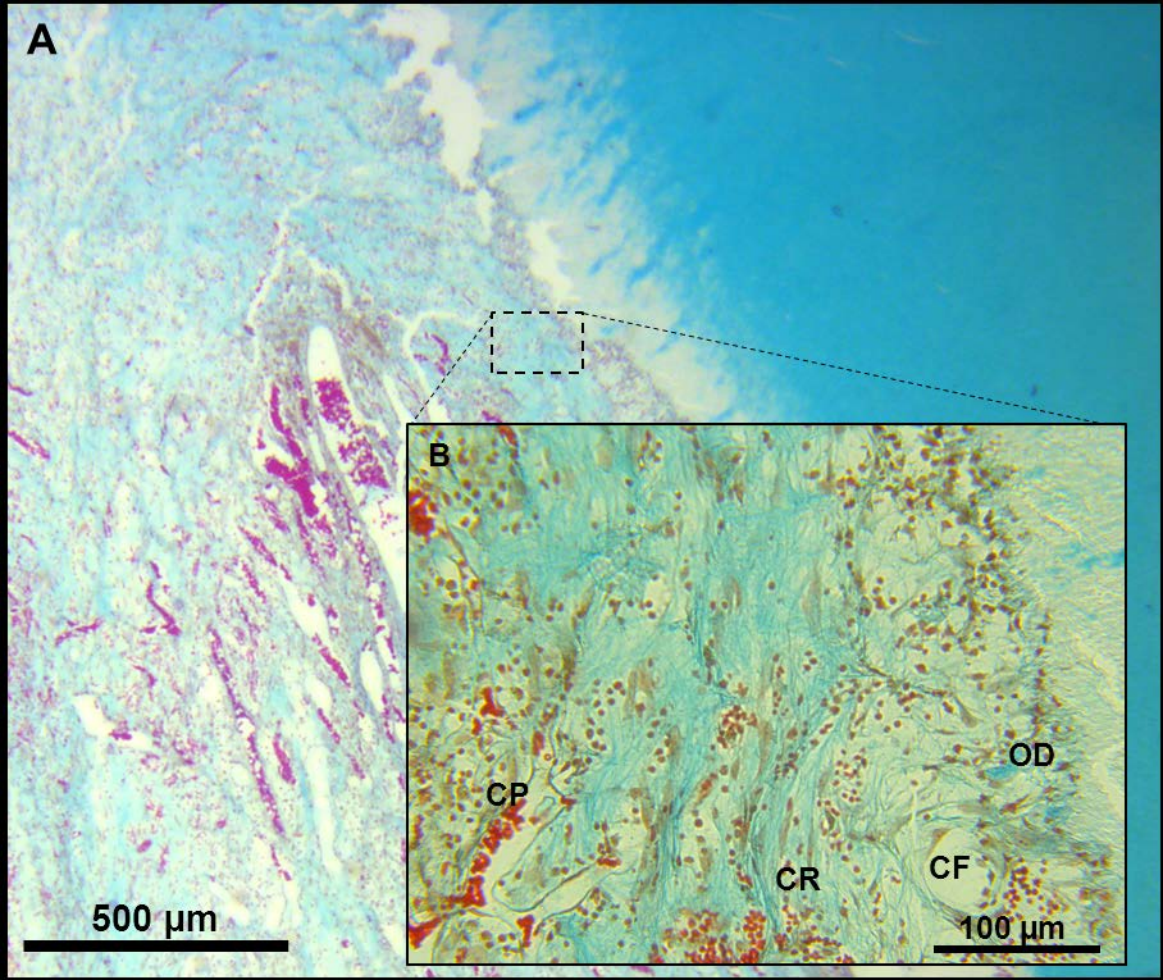
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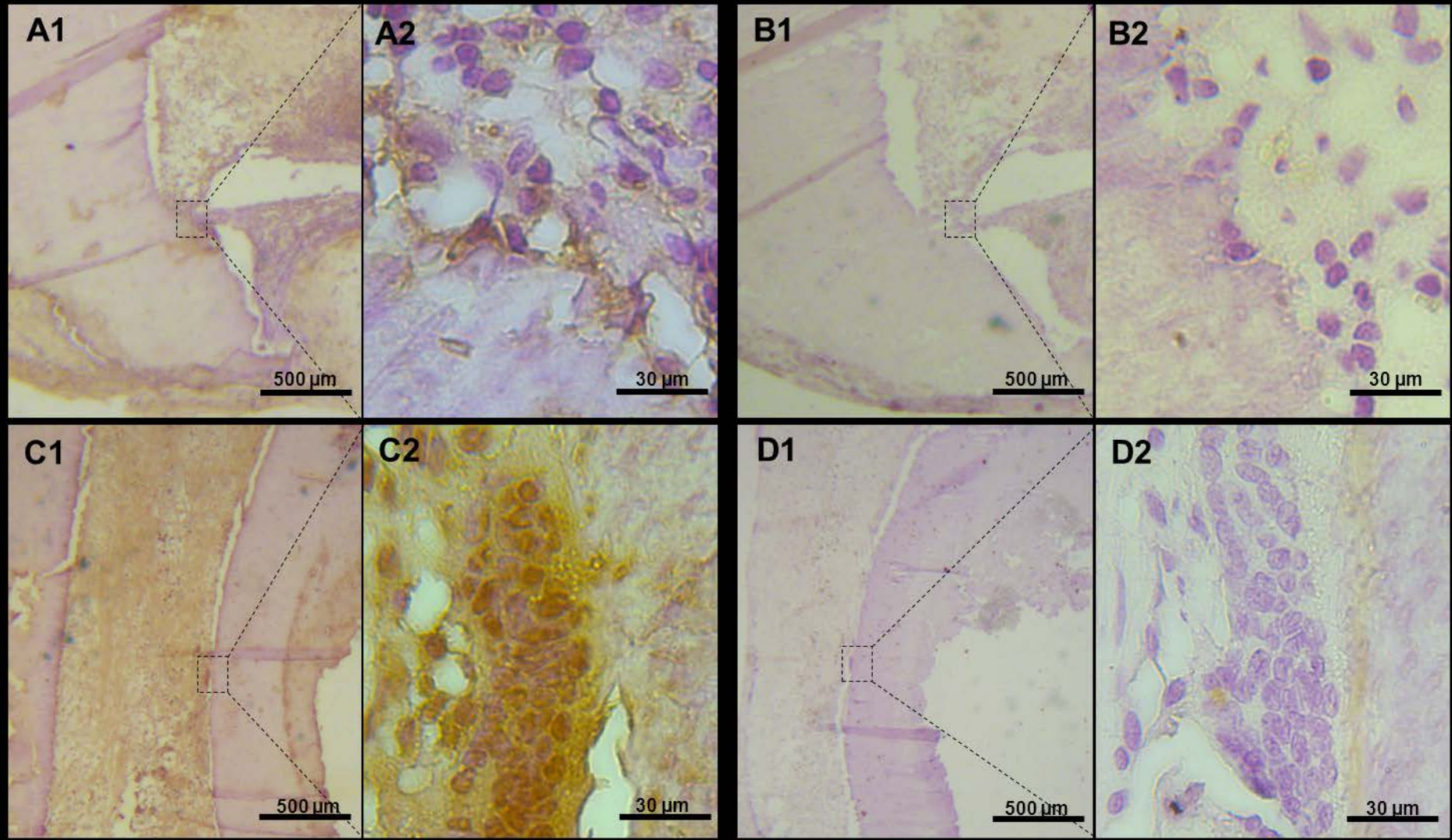
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