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Original Article

Responses of pulp vasculature after cavity preparation in rat molars

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ABSTRACT

Objectives: Our recent study showed that cavity preparation increases the number of proliferative cells in the dental pulp during postoperative days 2–5. This study aimed to clarify pulp vascular changes following cavity preparation.
Methods: Groove-shaped cavities were prepared on the medial surfaces of the upper first molars of 100-day-old Wistar rats. The animals were perfusion-fixed and tissues were collected during postoperative days 1–30, with subsequent India ink perfusion, immunohistochemistry for type IV collagen, CD31, and protein gene product (PGP) 9.5 and scanning electron microscopy with KOH digestion. The untreated upper first molars were used as controls.
Results: In the controls, blood vessels with large diameters were located in the center of the pulp tissue and ramified to make capillary networks and PGP9.5-positive nerves were extensively arborized to form the subodontoblastic nerve plexus beneath the odontoblast layer. Cavity preparation induced disturbance in injured odontoblasts and subodontoblastic capillaries and nerves. Blood vessel density and thickness subsequently increased in the center and periphery of the pulp tissues with the exception of the subodontoblastic capillary network during postoperative days 3–5. PGP9.5-positive nerves overlapped with CD31-positive blood vessels in the mesial coronal pulp. Until day 30, when the tertiary dentin formation was completed, the pulp blood vasculature showed the same distribution and morphological features as that of the controls.
Conclusions: These results suggest that increased vascular flow under neuronal regulation plays an important role in cell proliferation in the dental pulp following cavity preparation.

1. Introduction

The dental pulp has a relatively low interstitial compliance owing to its enclosure of rigid dentin. Even modest increases in pulpal fluid volumes increase tissue pressure, which might compress blood vessels and lead to ischemia and necrosis [1]. Tooth drilling (cavity preparation) induces destructive changes in odontoblasts at the affected site and acute inflammatory reactions [2]. Inflammation induces vasodilation and increased vessel permeability. Vasodilation increases the blood flow and, therefore, the blood volume. Additionally, vasodilation is typically localized to precapillary vessels, leading to an increase in capillary blood pressure, which causes an increase in interstitial fluid volume in the dental pulp [1]. Previous reports indicated that both the autonomic sympathetic nerves and the sensory nerves have a strong effect on the blood circulation in the dental pulp [3], because of the liberation of vasodilating neuropeptides such as CGRP and SP [4]. The majority of sensory nerve fibers containing CGRP and SP in the pulp seem to be located in the blood vessel walls, implicating these neuropeptides in blood flow regulation [5].

The dentin–pulp complex is capable of repair after tooth injuries such as caries, attrition, abrasion, and cavity preparation, resulting in tertiary dentin formation [6]. If the odontoblasts die, pulpal mesenchymal cells replace them to differentiate into odontoblast-like cells, resulting in the formation of reparative dentin. Our recent study showed that cavity preparation induces an increase in the number of proliferative cells in the dental pulp during postoperative days 2–5 [7]. Furthermore, the putative adult stem cells, which divided asymmetrically in the prenatal stages, reside in the center of the pulp tissue and associate with blood vessels [8], which suggests that drastic tissue reorganization subsequent to cavity preparation occurs because of the activation of putative adult stem cells in this stem cell niche. This study

Abbreviations: CGRP, calcitonin gene-related peptide; NFP, neurofilament protein; PAP, peroxidase-anti-peroxidase; PBS, phosphate buffered saline; PGP9.5, protein gene product 9.5; RT, room temperature; SEM, scanning electron microscopy; SP, substance P

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aimed to clarify pulp vascular changes following cavity preparation, using India ink perfusion, type IV collagen (a component of the basal lamina of the surrounding endothelial and Schwann cells), CD31 (a general endothelial cell marker), and PGP9.5 (a general neural marker) immunohistochemistry as well as scanning electron microscopy with KOH digestion.

2. Materials and methods

2.1. Cavity preparation

Forty-seven 100-day-old Wistar rats were used in this study. Animals were anesthetized via inhalation of sevoflurane gas followed by an intraperitoneal injection of chloral hydrate (at a maximum dose of 350 mg/kg). A groove-shaped cavity (the remaining dentin thickness was approximately 150–200 μm) was prepared on the mesial surface of the upper first molar via an air turbine with a tungsten carbide bur (diameter 0.6 mm) under water cooling (Supplementary Video S1). The cavity was left without any further treatment such as air-drying, etching, or filling. The untreated first molar of the animal was used as a control.

Supplementary material related to this article can be found online at http://dx.doi.org/10.1016/j.job.2015.05.003.

2.2. Histological procedure

Materials were collected from groups of two to five animals at intervals of 0, 1, 3, 5, 7, 14, and 30 days after cavity preparation ($n=47$). At each stage, the animals were perfused through the ascending aorta with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) or 100% methanol under deep anesthesia by intraperitoneal injection of chloral hydrate (350 mg/kg). To observe the blood vasculature, animals were further cardially perfused with India ink gelatin solution. The maxillae were removed and immersed in the same fixative overnight and subsequently decalcified in a 10% EDTA-2Na solution for 3 weeks at 4 °C and then rinsed in 0.01 M phosphate-buffered saline (PBS, pH 7.4) containing 30% sucrose for 6 h. For the India ink gelatin solution-perfused samples, 100 μm frozen sections were cut sagittally with a freezing microtome, mounted on glass slides, and counterstained with nuclear fast red.

2.3. Immunohistochemical analysis

Frozen 20–30 μm sections were cut sagittally. After endogenous peroxidase inhibition with methanol containing 0.3% H_2O_2 for 10 min, floating sections were processed with the PAP method. After rinsing in 0.01 M PBS containing 0.05% Triton X-100 (Triton), the sections were incubated in 2.5% normal porcine serum in Triton for 20 min at RT. The sections were then incubated overnight at 4 °C with rabbit anti-type IV collagen polyclonal antibody diluted to 1:6000. After rinsing with Triton, sections were incubated with porcine anti-rabbit IgG diluted to 1:60 (Dako, Glostrup, Denmark) at RT, followed by rabbit PAP complex diluted to 1:120 (Dako) at RT, and developed with 0.02% 3,3′-diaminobenzidine (Dohjin Laboratories, Kumamoto, Japan) in 0.05 M Tris–HCl buffer (pH 7.4) containing 0.005% hydrogen peroxide. Immunohistochemical controls were performed by replacing the primary antibodies with PBS. No specific immunoreaction was observed. For the double immunofluorescence staining for PGP9.5 and CD31, frozen sections were incubated with 0.25% trypsin solution at 37 °C for 20 min and treated with three consecutive incubations with an mouse anti-rat CD31 antibody (diluted 1:100; AbD Serotec, Oxford, UK) overnight at 4 °C, biotinylated anti-mouse IgG (diluted 1:100) for 60 min at RT, and Texas red-conjugated streptavidin (diluted 1:200; Vector Laboratories Inc., Burlingame, CA, USA) for 60 min at RT. After washing with PBS, sections were incubated with a rabbit anti-human PGP9.5 antibody (diluted 1:100; UltraClone Ltd., Isle of Wight, UK) overnight at 4 °C and FITC-conjugated anti-rabbit IgG (diluted 1:100; Vector Laboratories Inc.) for 60 min at RT. The sections were examined under a confocal laser-scanning microscope (FV300, Olympus, Tokyo, Japan).

2.4. SEM

The SEM analysis allows the observation of three dimensional tissue changes. Animals were transcardially perfused with saline followed by 2.5% glutaraldehyde in 0.06 M cacodylate buffer (pH 7.4) containing 0.5% sucrose. The maxillae were removed and immersed in the same fixative for an additional 24 h. Following decalcification in a 10% EDTA-2Na solution for 24 h at 4 °C, the first molars were cut sagittally and prepared according to the KOH digestion method to remove the extracellular matrices, including collagen components and intercellular substances [9]. The tissues were immersed in 30% KOH for 8 min at 62–65 °C and washed several times in 0.1 M phosphate buffer. The specimens were then stained by treatment with a 1% tannic acid solution and 1% OsO_4, dehydrated in a graded ethanol series, transferred to isoamyl acetate, and dried in a critical-point dryer (HCP-1, Hitachi, Tokyo, Japan) using liquid CO_2. These dried tissues were mounted on metal stubs and metal-coated with platinum-palladium using an ion-coater (IB-3, Eiko, Tokyo, Japan) and observed in an SEM (S-2380N, Hitachi) at an accelerating voltage of 10 kV.

2.5. Statistical analysis

The percentage of PGP9.5- and CD31-positive areas per unit area (287×84 μm^2 grid) was calculated in the control and prepared teeth. Furthermore, area ratio among different time points was compared by one-way analysis of variance (ANOVA) multiple comparisons adjusted by Bonferroni correction (SPSS 16.0J for Windows; SPSS Japan, Tokyo, Japan). Semi-quantitative analyses of the percentage of type IV collagen- and India ink-positive areas per unit area (514×154 μm^2 grid) were performed in the control and prepared teeth.

3. Results

3.1. Histological changes in the dental pulp following cavity preparation

Pulpal changes following cavity preparation have been previously described [2,7,10,11]. Cavity preparation caused the destruction of the odontoblast layer to form an edematous lesion and a shift of the injured odontoblasts toward the pulp core at the afflicted sites immediately after the operation. The degenerated odontoblasts were phagocytized by macrophages and, subsequently, the majority of the odontoblasts disappeared from the pulp–dentin border by day 1. The number of proliferative cells in the dental pulp significantly increased on day 2, while the newly differentiating odontoblast-like cells had already arranged along the pulp–dentin border and continued their proliferative activity across a wide range of the pulp tissue until day 5. Matrix secretion was initiated 3–5 days after cavity preparation. A large amount of reparative dentin was formed beneath the afflicted dentin until day 30.

3.2. Blood vasculature responses to cavity preparation demonstrated by the Indian ink perfusion method

The relationship between the vasculature and the surrounding tissue is evaluated by the Indian ink perfusion method. In the controls, blood vessels with large diameters were located in the