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Direct Pulp Capping Effect with Experimentally Developed Adhesive Resin Systems Containing Reparative Dentin Promoting Agents on Rat Pulp
-Mixed Amounts of Additives and Their Effect on Wound Healing -

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Abstract:

This study examined the wound healing process of exposed rat pulp when treated with experimental adhesive resin systems. The experimental direct pulp capping adhesive resin systems were composed of primer-I, primer-II and an experimental bonding agent. Primer-I was Clearfil SE Bond (CSE) primer containing 1.0 or 5.0wt% $CaCl_2$, and primer-II was CSE primer containing 0.1, 1.0 or 5.0wt% compound of equal mole of pA and pB with synthetic peptides derived from dentin-matrix-protein 1 (DMP1). Primer-I containing 1.0wt% and 5.0wt% $CaCl_2$ were assigned to the experimental groups 1 to 3, and 4 to 6, respectively. Primer-II containing 0.1, 1.0 or 5.0wt% compound of pA and pB were assigned to the experimental groups 1 and 4, 2 and 5, and 3 and 6, respectively. In all experimental groups, CSE bond containing 10wt% hydroxyapatite powder was used as the experimental bonding agent. The positive control teeth were capped with calcium hydroxide preparation (Dycal), and the negative control teeth were capped with CSE. The specimens were alternately stained with Mayer's H&E and the enhanced polymer one-step staining method. Experimental groups 1, 4, 5 and 6 showed a higher level of reparative dentin formation compared to the negative control 14 days postoperatively. At 28 days postoperatively, all experimental groups showed the formation of extensive reparative dentin, and experimental groups 4, 5 and 6 demonstrated similar dentin bridge formation as that of the positive control. How quickly reparative dentin formation occurs might depend on the concentration of $CaCl_2$ and pA and pB in the experimental primer.

Introduction:

Accidental pulp exposure may occur during cavity preparation as well as removal of deep carious dentin in clinical practice. The direct pulp capping procedure may be the most important factor for successfully preserving dental pulp, when pulp exposure occurs accidentally. Calcium hydroxide (CH) is the most eligible candidate for direct pulp capping because of its superior ability to form a dentin bridge.[1-3] However, there is a possibility that direct pulp capping with CH might decrease pulp tissue capacity because CH is strongly alkaline and forms a wide necrosis layer on the surface of pulp tissue.[4] In addition, CH has other problems such as a lack of adhesion to tooth structure, inadequate mechanical strength, and solubility causing a dead space. These may result in an increase in possible microleakage and infection of the dental pulp.[5]

An ideal direct pulp capping material should tightly adhere to dentin to prevent bacteria invasion and microleakage,[6,7] be clinically simple to handle, and promote dentin bridge formation. When the adhesive resin system is applied to direct pulp capping, good results are expected because of its high bond strength to dentin and mechanical properties. This concept is supported by the results of studies showing good pulp healing in resin capped teeth.[8-10] On the other hand, some studies showed poor results of direct pulp capping with adhesive resin even if no bacterial invasion by microleakage occurs.[11-14]

Our laboratory has been studying adhesive resin systems for direct pulp capping.[6, 15-23] Medina et al.[6] compared the pulp response to seven adhesive resin systems and their companion resin composites with that of a commercial CH material when applied to exposed primate pulp, and they found that the pulp response to four of seven adhesive resin systems was not significantly different from the CH application. However, CH could induce earlier and more consistent formation of reparative dentin compared with the other adhesive resin systems. Additional studies[24,25] have also reported that several adhesive resin systems applied to exposed pulp demonstrated no irritation to the pulp, but the dentin bridge formation with the adhesive resin systems was significantly slower than that using CH. This delayed dentin bridge formation of these adhesive resin systems may provide the critical situation for pulp to be exposed to bacteria through the resin dentin interface.

Therefore, we have developed a new adhesive resin system containing dentin promoting agents such as calcium phosphate for direct pulp capping.[26] Suzuki et al.[27] examined the wound healing process of rat pulp directly capped with experimental adhesives containing calcium phosphate, and showed that the addition of calcium phosphate to adhesive resin systems was effective in promoting dentin bridge formation, and the amount of reparative dentin depended on the concentration of the additives. Kato et al.[28] reported that experimental adhesives containing each type of calcium phosphate (hydroxyapatite,

brushite, whitlokite and octacalcium phosphate) were useful materials as reparative dentin promoting agents.

He et al.[29] reported that dentin matrix protein 1 (DMP1) can nucleate the formation of hydroxyapatite *in vitro* in a multistep process that begins by DMP1 binding calcium ions and initiating mineral deposition. They analyzed the peptide arrangement of DMP1, and predicted that the ESQES peptide (pA) and QESQSEQDS peptide (pB) were concerned with the formation of hydroxyapatite. Matsuzaki et al.[30] found that a compound of pA and pB coagulated each other, and formed hydroxyapatite crystals when combined with calcium ions *in vitro*. Furthermore, they proved that the compound combined with calcium ions possessed high calcification promoting ability by culture tests using MC3T3-E1 cells. Consequently, quicker dentin bridge formation could be expected when this compound with calcium ions is applied to exposed pulp. On the basis of these reports, our laboratory developed an experimental adhesive resin system containing 10wt% calcium chloride ($CaCl_2$), a 10wt% compound of pA and pB, and 10wt% hydroxyapatite for direct pulp capping in cooperation with Kuraray Medical Inc. The results of an animal study in which the new experimental adhesive resin system was used for direct pulp capping showed that the addition of $CaCl_2$ and a compound of pA and pB to the self-etching primer, and hydroxyapatite to the bonding agent were fairly effective in promoting dentin bridge formation.[31]

This experimental adhesive resin system has one problem: the price of pA and pB is too costly to produce for clinical use. However, the effects of this experimental adhesive system on direct pulp capping could be reduced if the compound of pA and pB would be adjusted to a lower concentration in the primer. Therefore, it is necessary to confirm the effect of various low concentrations of the compounds of pA and pB in the self-etching primer to reduce the production cost on wound healing and dentin bridge formation when the experimental adhesive resin system is used for direct pulp capping.

The purpose of this study was to examine the wound healing process of exposed rat pulp when using experimental adhesive resin systems containing $CaCl_2$ and a compound of pA and pB adjusted for various low concentrations in the respective primer, and to compare the results with positive (Dycal) and negative (Clearfil SE Bond : CSE) control. The null hypothesis of this study was that the low concentration of $CaCl_2$ and a compound of pA and pB to the respective primer would show good wound healing and dentin bridge formation of the exposed rat pulp compared to the positive control.

Materials and methods:

Experimental animals

A total of 62 rats (Sprague-Dawley male rats, 6 weeks old and about 180g in weight) were stocked. The rats were fed with solid food (MF, Oriental Yeast Co, Tokyo, Japan) and water for 2 weeks in the cages of the breeding house affiliated with our university. A total of 80 non-carious teeth, upper first molars, were treated by direct pulp capping when the rodents were 8-9 weeks old and weighed 300-400g. Five teeth were assigned to each experimental group.

This study was approved by the Ethics Committee of The Nippon Dental University School of Life Dentistry at Niigata. (Receipt & permission number: 87, June 23th, 2008)

Experimental groups and observation terms

The experimental materials used in this study are summarized in Table 1. Synthetic peptides derived from dentin matrix protein 1 (pA and pB) were produced by Kuraray Medical Inc.. Clearfil SE Bond (CSE, Kuraray Medical Inc., Tokyo, Japan) primer and CSE bond were used as the base for the experimental self-etching primer and bonding agent, respectively. The experimental direct pulp capping adhesive resin system was composed of primer-I, primer-II and the experimental bonding agent. Primer-I was CSE primer containing 1.0 or 5.0wt% $CaCl_2$, and primer-II was CSE primer containing 0.1, 1.0 or 5.0wt% compound of equal mole of pA and pB. The experimental bonding agent consisted of CSE Bond containing 10wt% hydroxyapatite. A summary of the experimental groups is shown in Table 2. Primer-I containing 1.0wt% and 5.0wt% $CaCl_2$ were assigned to the experimental groups 1 to 3, and 4 to 6, respectively. Primer-II containing 0.1, 1.0 or 5.0wt% compound of pA and pB were assigned to the experimental groups 1 and 4, 2 and 5, and 3 and 6, respectively. In all experimental groups, the experimental bonding agent was used after application of the primer. The teeth capped with calcium hydroxide preparation (Dycal, Dentsply Caulk, Milford, USA) and the teeth capped with CSE were used as a positive control and a negative control, respectively.

Two postoperative observation terms were set: 14 and 28 days, and rats were sacrificed on these days after direct pulp capping to make specimens for histopathological and immunohistochemical examination.

Specimen preparation

The rats were sedated with ether (Diethyl Ether; Wako Pure Chemical Industries Ltd., Osaka, Japan) and were then deeply anesthetized by an intraperitoneal injection of 5% pentobarbital sodium (Pentobarbital; SIGMA Aldrich Co., St. Louis, USA) at a dose of

40mg/kg. After each rat was fixed on an operating board, the mouth was kept in an open position with a jaw prop. The teeth were cleaned with 3% hydrogen peroxide (H_2O_2, Oxydol; Yoshida Pharmaceutical Co., Tokyo, Japan) and rinsed with a physiological saline solution (Physisalz PL-D; Fuso Pharmaceutical Industries Ltd., Osaka, Japan), and then disinfected with diluted iodine tincture (Yoshida Pharmaceutical Co., Tokyo, Japan).

Bowl-shaped cavities with a diameter of approximately 0.5 mm were prepared on the mesial marginal ridge of the right and left maxillary first molars with a FG #440SS regular cut diamond point (Shofu Inc., Kyoto, Japan) in a high-speed handpiece (Air-turbine handpiece, Super Load 9000; Yoshida Co., Tokyo, Japan) under copious water spray. The pulps were then exposed with a CA #1/2 steel round bur (Hager & Meisinger GmbH, Neuss, Germany) in a low-speed handpiece (Micromotor handpiece, Micro-Mega; Yoshida Co., Tokyo, Japan) under copious spray of distilled water. 10% sodium hypochlorite gel (NaOCl gel, AD Gel; Kuraray Medical Inc.) was applied to the cavity for five minutes to stop bleeding from the exposed pulp and to produce sterility. Reapplication of AD Gel was made to pulps that continued to bleed. This was followed by alternate irrigation with 3% H_2O_2 and 6% NaOCl solution (Purelox; Oyalox Co., Tokyo, Japan) three times to remove dentin chips and AD Gel. The cavity was then rinsed with a physiological saline solution, excess water was removed with sterilized small cotton pellets, and then the cavity was blown dry with a gentle air stream.

For each group, the experimental direct pulp capping adhesive resin system was applied to the cavities according to the bonding procedures shown as follows. Primer-I was applied to the cavity, and the surface was left undisturbed for twenty seconds, followed by gently air-blowing. Primer-II was applied in the same manner and then photopolymerized with a light-curing unit (Candelux; J Morita Co., Tokyo, Japan) for 10 seconds. Each experimental bonding agent was applied, and the surface was gently air blown and photopolymerized with a light-curing unit for 10 seconds.

After direct capping and bonding procedures, all the cavities were restored with a hybrid restorative resin composite (Clearfil AP-X A3; Kuraray Medical Inc.) and photopolymerized with a light-curing unit for 40 seconds.

Perfusion fixation

The rats were sacrificed by an intraperitoneal injection of an overdose of 5% pentobarbital sodium after each observation period. Pulp was fixed by transcardial perfusion with 4% paraformaldehyde phosphate buffer solution (4% PFA: pH 7.4). The maxillae containing the experimental teeth were carefully removed and immersed in 4% PFA at 4ºC overnight for further fixation.

Tissue preparation and serial sectioning

The specimens were trimmed of excess tissue and decalcified with 10% EDTA-2Na solution (pH 7.4) at room temperature for four weeks. After decalcification, AP-X was carefully removed from the cavity and rinsed with running water for 24 hours. The specimens were dehydrated in ascending grades of ethanol, dealcoholized by xylene, and then embedded in paraffin. Serial sections of 6μm thickness were cut with a sliding microtome (Jung Histoslide 2000R; Leica Microsystems Vertrieb GmbH, Wetzlar Germany) and alternately stained with Mayer's Hematoxylin-Eosin staining (HE), modified NF Watanabe silver impregnation staining (NF), and Hucker-Conn bacterial staining. Regarding immunohistochemical staining, the enhanced polymer one-step staining methods of TGF-beta1 staining and DMP1 staining were used.

Observation items and evaluation criteria

The stained sections were observed under a light microscope (Eclipse E1000; Nikon Co., Tokyo, Japan) and the following items were evaluated: pulp tissue disorganization (PTD), inflammatory cell infiltration (ICI), reparative dentin formation (RDF), and bacterial penetration (BP). The findings were evaluated according to the following criteria established by Medina III-Katoh.[6]

Pulp Tissue Disorganization

1. Normal or almost normal tissue morphology (none).
2. Odontoblast layer disorganization, but the deep part of the pulp was normal (mild).
3. Loss of general tissue morphology (moderate).
4. Necrosis in the coronal one-third or more of the pulp (severe).

Inflammatory Cell Infiltration

1. Absence or presence of a few scattered inflammatory cells in the pulp (none).
2. Mild acute/chronic cell lesions (mild).
3. Moderate inflammatory cell lesions seen as abscesses or densely stained infiltrates of polymophonuclear leucocytes, histiocytes and lymphocytes in one-third or more of the coronal pulp and/or the mid-pulp (moderate).
4. Pulp necrosis due to a severe degree of infection or lack of tissue in one half or more of the pulp (severe).

Reparative Dentin Formation

1. No dentin bridge formation (none).

2. Initial dentin bridge formation extending to not more than one-half of the exposure site (initial).
3. Partial/incomplete dentin bridge formation extending to more than one-half of the exposure site but not completely closing the exposure site (partial).
4. Complete dentin bridge formation (complete).

Bacterial Penetration

1. Absence of stained bacterial profiles in any of the sections (none).
2. Presence of stained bacterial profiles along the coronal or apical walls of the cavity (mild).
3. Presence of stained bacterial profiles within the cut dentinal tubules or axial wall of the cavity (moderate).
4. Presence of stained bacterial profiles within the dental pulp (severe).

In addition, the following histological features were recorded: hemorrhage, dentin chips, (location, size and number) and reactionary (irritation) dentin formation.

Immunohistochemical staining

In this study, the expression of TGF-beta1 and DMP1 were investigated as an index or marker for comparing the difference in the recovery process induced by the various direct pulp capping methods. The sections were deparaffinized with xylene, hydrated in ascending grades of ethanol, and then rinsed briefly with tap water and phosphate buffered saline (PBS: pH 7.4). The sections were incubated with primary rabbit antibodies, such as polyclonal anti-TGF-beta1 (TGF-beta1 (V): sc-146; Lot #F0809, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) working dilution, 1: 1000 for 12 hours at 4ºC, or polyclonal anti-DMP1 (Lot #005FD, Takara Bio Inc., Shiga, Japan) working dilution, 1: 4000 for 12 hours at 4ºC, and then rinsed with PBS three times for 5 minutes. They were immunochemically stained with Enhanced polymer one-step staining (EPOS) methods, Simple Stain Rat MAX-PO(R) (Lot #H0901, Nichirei Biosciences Inc, Tokyo, Japan). The antibody localized antigen was then detected by peroxidase activation of 3,3'-diaminobenzidine, Simple Stain DAB (Lot #H0906, Nichirei Biosciences Inc) for 10 minutes. Finally, the sections were lightly counterstained with Mayer's hematoxylin.

Measurement of the diameters of exposed pulp area

The diameters of the exposed areas were measured with a stereomicroscope (Measuring Microscope MM-40, Lot #2104048; Nikon Co., Tokyo, Japan) and the widest dimension

was recorded as the pulp exposure size of the specimen.

Statistical analysis

The diameters of the exposed pulp areas were statistically analyzed by one-way ANOVA and the Bonferroni *post-hoc* test for differences among the groups during each observation period at a significance level of 0.05.

The results of histopathological evaluation were statistically analyzed by the Mann-Whitney U-test for differences between each experimental group and the positive control as well as the negative control during each observation period at a significance level of 0.05.

Moreover, the correlation between ICI and BP was investigated by Kendall correlation analysis. Statistical procedures were performed at a significance level of 0.05 using the statistical software (SPSS 14.0J Base System SC; SPSS Japan Inc., Tokyo, Japan).

Results:

The diameters of pulp exposure area

The mean values for the pulp exposure sizes of each group and during each observation period ranged from 0.200±0.030 mm to 0.322±0.078mm. There was no significant difference among the pulp exposure sizes of the groups (P>0.05).

Histopathological and immunohistochemical findings

The summary of the results of the histopathological evaluation is shown in Figures 1 and 2. Representative histological and immunohistopathological images of some groups are shown in Figures 3-11.

1) Histopathological and immunohistochemical findings after 14 days

The Mann-Whitney U-test for data of PTD showed no significant difference between each experimental group and the positive control as well as the negative control ($P > 0.05$) except between Group 3 and the negative control ($P = 0.032$) (Table 3). In the specimens in which reparative dentin was formed at the surface of the exposed pulp, the odontoblastic layer was observed just beneath the reparative dentin.

The Mann-Whitney U-test for data of ICI revealed no significant difference between each experimental group and the positive control as well as the negative control ($P > 0.05$). ICI could be observed when the diameter of dental pulp exposure was wide. Inflammatory cells (mostly mononuclear leukocytes and macrophages) were scattered among the fibroblasts (Fig. 3).

The Mann-Whitney U-test for data of RDF revealed a significant difference between negative control and groups 1, 4, 5 and 6, respectively ($P = 0.008$) (Table 4). The layer of reparative dentin induced at the pulpal dentin wall of the periphery of the exposed site tended to be thicker when the concentration of dentin promoting agents was higher. Reparative dentin was observed at a comparatively deeper position from the pulp exposure site, and included denticle-like tubular dentin including pulp cells (Fig. 4a). Immunohistochemical staining with polyclonal anti-DMP1 showed a positive reaction in the denticle-like tubular dentin (Fig. 4b). In this case, RDF seemed to be initiated by the presence of dentin chips. Dentin chips were at the core of induced reparative dentin (osteodentin). In some cases, there were dentin bridge formations at the exposed surface, but the dentin bridges were incomplete (Fig. 5).

The negative control showed no evidence of RDF at the exposed surface (Fig. 6). The positive control showed dentin bridge formation at the exposed surface by tubular dentin at a comparatively deeper position from the pulpal exposed site, and a dead space was observed between the Dycal applied surface and the dentin bridge (Fig. 7).

No specimens were stained positive for bacteria in any of the groups at each observation period.

2) Histopathological and immunohistochemical findings after 28 days

No specimens from any of the groups showed PTD. Normal pulp morphology was observed in all specimens. No ICI was observed in any of the groups. Histopathological evaluation of all groups at each observation period showed that no specimen exhibited severe inflammation of the pulp.

RDF was observed in all of the groups. Groups 4, 5 and 6 had complete dentin bridges in all cases. The Mann-Whitney U-test for data of RDF revealed no significant difference between each experimental group and the positive control as well as the negative control ($P>0.05$). Dentin bridges had a two or three layered construction with a specific configuration (Fig. 8, 9). Immunohistochemical staining with polyclonal anti-DMP1 showed a positive reaction in the dentin bridge and denticle-like reparative dentin (Fig. 8b), however that with polyclonal TGF-beta1 showed no reaction in the reparative dentin (Fig. 8c). In the specimens which showed three layered construction, the superficial layer of the dentin bridge was composed of osteodentin, the middle and deep layers were composed of tubular dentin, and pulp tissue was sandwiched between them (Fig. 9). The reparative dentin observed in the groups containing 5.0wt% $CaCl_2$ (Groups 4, 5 and 6) tended to be thicker than that containing 1.0wt% $CaCl_2$ (Groups 1, 2 and 3).

The negative control showed complete dentin bridge formation at the exposed surface by reparative dentin composed of tubular dentin (Fig. 10). Complete dentin bridge formation was observed at the exposed surface in the positive control. However, the layer of applied Dycal was observed above the dentin bridge (Fig. 11).

No specimens were stained positive for bacteria in any of the groups and at each observation period.

Discussion:

Many studies have focused on hard tissue regeneration and tooth pulp regeneration, which are different from conventional treatment of pulp capping utilizing the ability of spontaneous pulpal healing.[32,33] In these methods, hard tissue and pulp tissue are repaired or regenerated by offering growth factors and a scaffold for cell differentiation.

Dentin matrix protein 1 (DMP1) is a non-collagen acidic phosphoprotein belonging to the SIBLING protein, and possesses strong affinity for OHAp.[34] DMP1 exists in hard tissue forming cells such as osteoblasts, osteocytes, ameloblasts, odontoblasts and cementoblasts, and is considered to play an important role in hard tissue regeneration and mineralization. Therefore, DMP1 is expected as a growth factor which is able to regenerate hard tissue.[35, 36] The concept of dentin bridge formation in this study was that primer-I containing $CaCl_2$ was first applied to the exposed pulp to supply a growth factor for OHAp formation, and primer-II containing DMP1 was secondly applied to promote odontoblast-like cell differentiation as a growth factor, and finally, experimental bonding resin containing OHAp was applied as both a growth factor and a scaffold for RDF.

In the present study, RDF was observed in all the experimental groups during all periods. Some specimens exhibited a complete dentin bridge 14 days postoperatively, and the quantity of the dentin bridge increased 28 days postoperatively. This result might be due to the significant promotion effect of RDF, in which the experimental adhesive resin system acted on all experimental groups. It was speculated that the dentin promoting agents containing the experimental primer penetrated into the exposed pulp tissue, and the dentin promoting effect continued acting on the pulpal exposure site. Experimental groups 1, 4, 5 and 6 showed a higher level of RDF compared to the negative control 14 days postoperatively. At 28 days postoperatively, all experimental groups showed the formation of extensive reparative dentin, and experimental groups 4, 5 and 6 demonstrated similar dentin formation as the positive control. These results indicated that the experimental adhesive resin system used in this study possessed the ability to promote RDF compared to Dycal during the early stage of direct pulp capping.

The rate of the RDF might depend on the concentration of the dentin promoting agents contained in the experimental primer. Specifically, the amount of RDF tended to increase when the concentration of the $CaCl_2$ was high. Ishikawa et al. reported that Ca ions are an important factor in controlling OHAp formation.[37] He et al.[29] and Matsuzaki et al.[30] reported that a compound of pA and pB exhibited the ability of OHAp formation when combined with calcium ions *in vitro*. Accordingly, this study showed abundant RDF due to the compound of pA and pB which generated OHAp using extensive calcium ions released from $CaCl_2$. The dentin bridges observed in groups 1, 2 and 3 containing 1wt%

$CaCl_2$ were incomplete and included tunnel defects, and were thinner than those of the positive control at 28 days postoperatively. Thus the ability to promote RDF may be inadequate in case of the primer containing 1wt % $CaCl_2$.

On the other hand, the difference among the concentrations of a compound of pA and pB did not affect the quantity of RDF. Katoh et al.[31] reported that either application of the primer containing $CaCl_2$ or that a compound of pA and pB demonstrated slight RDF, however, application of both them generated reparative dentin in good condition. Therefore, a low concentration of the compound of pA and pB in the primer could show the promoting effect of RDF when combined with calcium ions at a high concentration.

From the results of the histopathology observations, it was identified that the induced dentin bridges showed various structures. Reparative dentin was generated at a central part of the exposure area or the pulpal dentin wall of the periphery of the exposed site, and was finally connected with reactionary dentin. When their connection was incomplete, tunnel defects appeared between them. The cause of tunnel defect formation in teeth capped with calcium hydroxide has been clarified as not only due to the capping materials but also the persistence of vascular canals. Cox et al.[38] reported that tunnel defects existed in 89% of dentin bridges in pulps capped with calcium hydroxide, and these structural defects gave a bridge incapable of providing a long-term barrier to bacterial infection. However, in this study, tunnel defects were detected in 16.7% of the dentin bridges. This result is considered as one advantage of this adhesive system compared with calcium hydroxide.

The dentin bridge observed 28 days postoperatively exhibited a specific configuration which was constructed of three layers in the experimental groups. The superficial layer of the dentin bridge was composed of osteodentin, and the middle and deep layer was composed of tubular dentin. Pulp tissue was sandwiched among the three layers of dentin bridges. Suzuki et al.[27] reported that a pulpal exposure was closed with a dentin bridge composed of osteodentin when the experimental bonding agent containing 10wt% OHAp was applied to the exposed rat pulp. In addition, it has been reported that the wound healing processes after OHAp application are more desirable than those after CH application, and when an OHAp layer is used as the scaffolding for the newly formed mineralized tissue.[39] Therefore, it was speculated that the reparative dentin observed in the superficial layer might be induced by the bonding agent containing 10wt% OHAp. The reparative dentin observed in the middle and deep layer of the dentin bridge might be induced by the experimental primer-I containing $CaCl_2$ and the primer-II containing a compound of pA and pB, respectively. Both dentin promoting agents might diffuse into

deeper parts of pulpal exposure site with penetration of the primers. In some cases, denticle-like reparative dentin was observed at a comparatively deep position in the pulp.

In the area of pulp tissue sandwiched between layers of the dentin bridge, the collagen fibrils and reticular fibrils were stained by modified NF Watanabe silver impregnation staining, and a positive reaction by DMP1 staining was recognized. This positive reaction by DMP 1 staining might result from DMP 1 generated by newly differentiated odontoblast-like cells which were induced by pA and pB contained in the primer-II. These pulp tissue areas tended to decrease over time. Hence, in the future, these pulp tissue areas would be mineralized and a thick dentin bridge would be completed. On the other hand, TGF-beta1 staining showed no reaction in the area of pulp tissue sandwiched between the layers of the dentin bridge. Several studies have reported an application of TGF-beta1 for pulp injury induced wound healing processes such as pulp tissue regeneration, dentin extracellular matrix production, cytodifferentiation of odontblast-like cells and RDF.[40, 41] It was conjectured that inflammatory reactions in the rat pulp due to injury might almost disappear at an early stage of the wound healing process when considering the high recovery speed and capacity of rat pulp tissue.[27]

In some cases, the pulpal exposure site was closed by reparative dentin which was of osteodentin formed around the dentin chips remaining at the surface of the exposure site. Several studies reported that the dentin chips possess an RDF inducing ability.[42, 43] However, dentin chips have been known to possibility include bacteria, and they should be removed from the pulpal exposure area in order to prevent bacterial infection.[44, 45] In this study, the application of 10% NaOCl gel followed by alternate irrigation with 3% H_2O_2 and 6% NaOCl solution were practiced to stop the flow of blood, produce disinfection, and remove dentin chips. In our previous studies[46], 10% NaOCl (AD gel) was applied to the surface of the exposed pulp with the aim of improving dentin bond strength, achievement of hemostasis, disinfection, debridement of the exposure site, and irrigation of dentinal tubules inside. Adverse effects of AD gel were never observed in the pulp tissue. Senia[47] and Rosenfeld[48] have reported that the influence of NaOCl on the pulp tissue was limited to the surface of the exposed pulp due to the low osmotic ability of NaOCl to pulp tissue.

Many researchers indicate that the control of bleeding and exudation of tissue fluid from the exposed pulp were the key points of succeeding in direct pulp capping, and imperfect control might result in disturbing hardening of the capping materials and the persistence of protruding pulpal tissue.[49,50] Severe PTD and ICI observed in some specimens might result from the insufficient control of exudation of tissue fluid due to a large exposure site, the inadequate polymerization of the bonding agents and severe irritation from cavity

preparation. It has been reported that the unpolymerized components of adhesive resin systems showed more cytotoxicity to pulp cells than polymerized ones.[51]

Bacterial invasion was not observed in any of specimens at either of the observation periods. Inflammatory changes were observed in some specimens after 14 days, but not in any after 28 days. It has been shown that pulp inflammation is a consequence of bacterial invasion by microleakage rather than irritation from the material itself.[52] CSE was used as the base for the experimental agent in this study. Cytotoxicity of CSE is relatively lower than that of other adhesive resin systems.[53] Some studies have reported that CSE was able to elicit favorable pulp responses in direct pulp capping.[9,10] On the basis of these results, pulp irritation of the experimental adhesive resin system might be only slight during experimental periods. Furthermore, we confirmed that bond strength of the experimental adhesive resin system used in this study was sufficient for human dentin.[54]

Composite restorations may not last, and microleakage of restoration may occur over time due to degradation of adhesion.[55] Therefore, the authors consider that direct pulp capping should generate a complete dentin bridge at the pulpal exposure area in order to prevent invading bacteria and keep the pulp stable. In this study, experimental groups 4, 5 and 6 generated complete dentin bridges in all specimens after 28 days. These results supported our consideration for successful direct pulp capping.

The results lead us to the conclusion that the null hypothesis, that a low concentration of $CaCl_2$ and a compound of pA and pB to the respective primer would show good wound healing and dentin bridge formation of the exposed rat pulp compared to the positive control, was accepted. In conclusion, experimental groups 1, 4, 5 and 6 showed a higher level of RDF compared to the negative control 14 days postoperatively, and RDF showed no significant difference between each experimental group and the positive control as well as the negative control 28 days postoperatively. Furthermore the experimental groups containing 5wt% $CaCl_2$ (Groups 4, 5 and 6) regardless of the concentration of the compound of pA and pB demonstrated similar dentin bridge formation effects to the positive control after 28 days.

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Tables:

Figures:

Fig. 1. Results of the histopathological evaluation (14 days)

Fig. 2. Results of the histopathological evaluation (28 days)

Fig. 3a,b. Representative histologic image of Group 3 (14 days)
a Although reactionary dentin was generated at the pulpal dentin wall of the periphery of the pulp exposed site, there was no evidence of RDF at the exposed surface. Moderate pulp disorganization and infiltrations of inflammatory cells can be seen at the pulp exposed surface, but mid-pulp tissue was almost normal. (H&E, 100x)

b Inflammatory cell (mostly mononuclear leukocytes and macrophages) were scattered among the fibroblasts. There were reticular fibers beneath the exposed surface. (NF, 100x)

Fig. 4a,b. Representative histologic image of Group 5 (14 days)
a Two denticle-like reparative dentins are observed at the comparatively deeper position from the pulpal exposed site, and these were constituted of a tubular dentin type including pulp cells. Pulpal morphology was normal. (H&E, 100x)

b A strong positive reaction by DMP1 staining can be recognized in the denticle-like reparative dentin. (DMP1, 100x)

Fig. 5. Representative histologic image of Group 4 (14 days)
There were dentin bridge formations at the exposed surface, but the dentin bridges were incomplete. Pulpal morphology was normal. (H&E, 100x)

Fig. 6. Representative histologic image of the negative control (14 days)
Reactionary dentin was induced at the pulpal dentin wall of the periphery of the exposed site. There was no evidence of RDF at the exposed surface. (H&E, 100x)

Fig. 7. Representative histologic image of the positive control (14 days)
There was a thick complete dentin bridge which was composed of tubular dentin at a comparatively deeper position from the pulpal exposed site, and a dead space was observed between the Dycal applied surface and the dentin bridge. (H&E, 100x)

Fig. 8a-c. Representative histologic image of Group 4 (28 days)
a There was a thick complete dentin bridge which was composed of tubular dentin. There were restructured odontoblast-like cells just beneath the dentin bridge. Pulpal morphology was normal. (H&E, 100x)

b A strong positive reaction by DMP1 staining can be recognized in the dentin bridge and denticle-like reparative dentin. (DMP1, 100x)

c TGF-beta1 staining showed no reaction in the dentin bridge. (TGF-beta1, 100x)

Fig. 9. Representative histologic image of Group 6 (28 days)
The complete dentin bridge exhibited a construction of three layers with a respective specific configuration. The superficial layer was incomplete. The middle and deep layer were composed of tubular dentin. Pulp tissue was sandwiched between the two layers of the dentin bridge. (H&E, 100x)

Fig. 10. Representative histologic image of the negative control (28 days)
There was a complete dentin bridge which was composed of tubular dentin. (H&E, 100x)

Fig. 11. Representative histologic image of the positive control (28 days)
Complete dentin bridge formation was observed at the exposed surface. (H&E, 100x)

Table 1. Materials used in the study

Materials	Abbr	Lot#	Composition	Manufacturer
Clearfil SE Bond	CSE			Kuraray Medical Inc.
Primer		00812A	2-Hydroxyethyl Methacrylate	
		0299A	Hydrophilic Dimethacrylate	
			10-Methacryloyloxydecyl Dihydrogen Phosphate	
			N, N-Diethanol-p-Toluidine	
			d,I-Camphorquinone	
			Water	
Bond		01185A	Silanated Colloidal Silica	
		0373A	2-Hydroxyethyl Methacrylate	
			Hydrophobic Aliphatic Dimethacrylate	
			10-Methacryloyloxydecyl Dihydrogen Phosphate	
			N, N-Diethanol-p-Toluidine	
			d,I-Camphorquinone	
Hydroxyapatite Powder	OHAp	30605		Ube Material Industries , Ltd
Calcium chloride	$CaCl_2$	PKG3983		Wako co.
Synthetic peptide derived of dentin matrix protein 1 (DMP1)			pA (residues 386-390) pB (residues 414-422)	Kuraray Medical Inc.
Dycal		030729	Base paste: ester glycol salicylate, calcium phosphate, Ca tungstate, ZnO Catalyst paste: ethylene toluene sulfon amide, $Ca(OH)_2$ ZnO, Ti_2O, Zn stearate	Dentsply Caulk

Table 2. Experimental groups

Groups	wt% of $CaCl_2$ contained in primer-I	wt% of pA&pB contained in primer-II
1	1.0	0.1
2	1.0	1.0
3	1.0	5.0
4	5.0	0.1
5	5.0	1.0
6	5.0	5.0

Table 3. Results of statistical analysis for
pulp tissue disorganization (14 days)

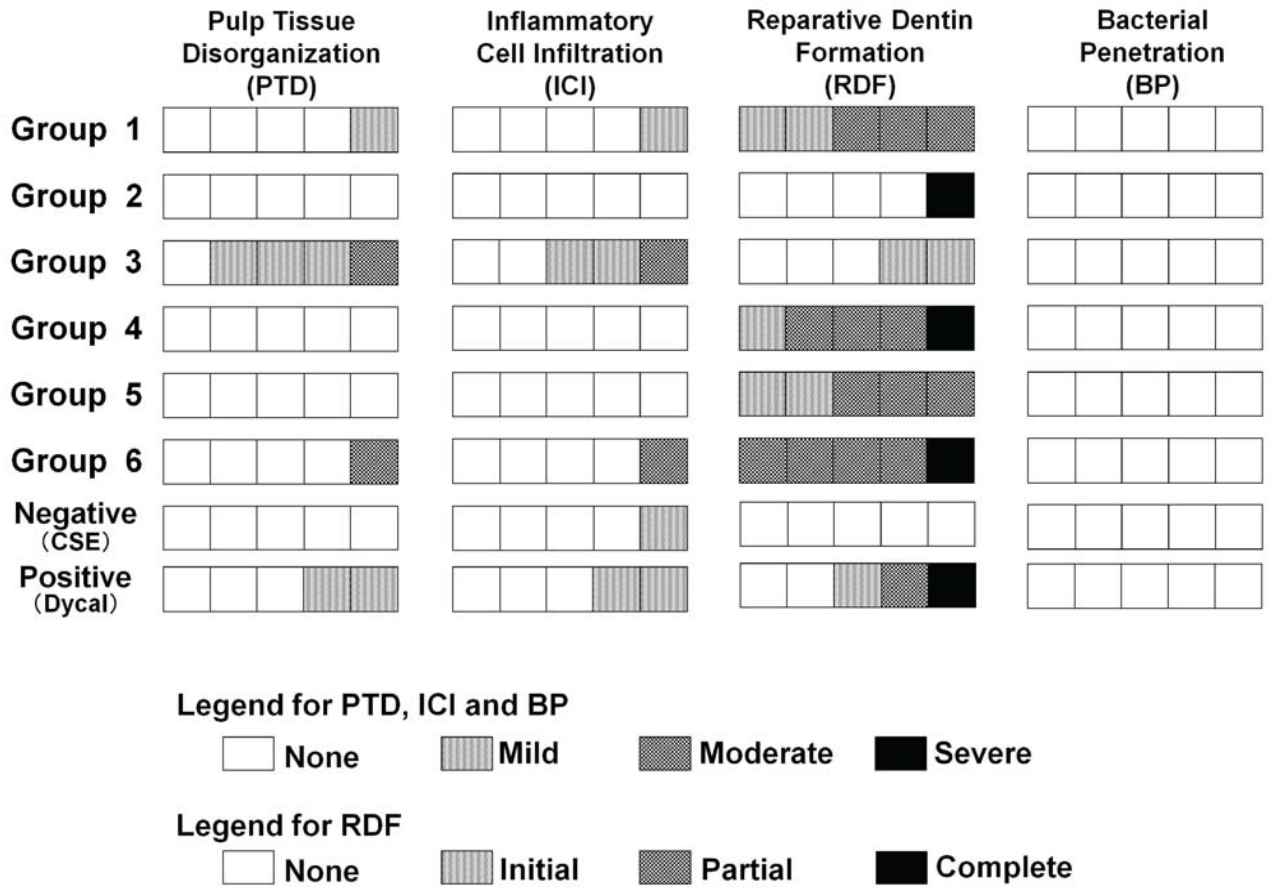
	Experimental groups					
	1	2	3	4	5	6
Negative control	NS	NS	* (0.032)	NS	NS	NS
Positive control	NS	NS	NS	NS	NS	NS

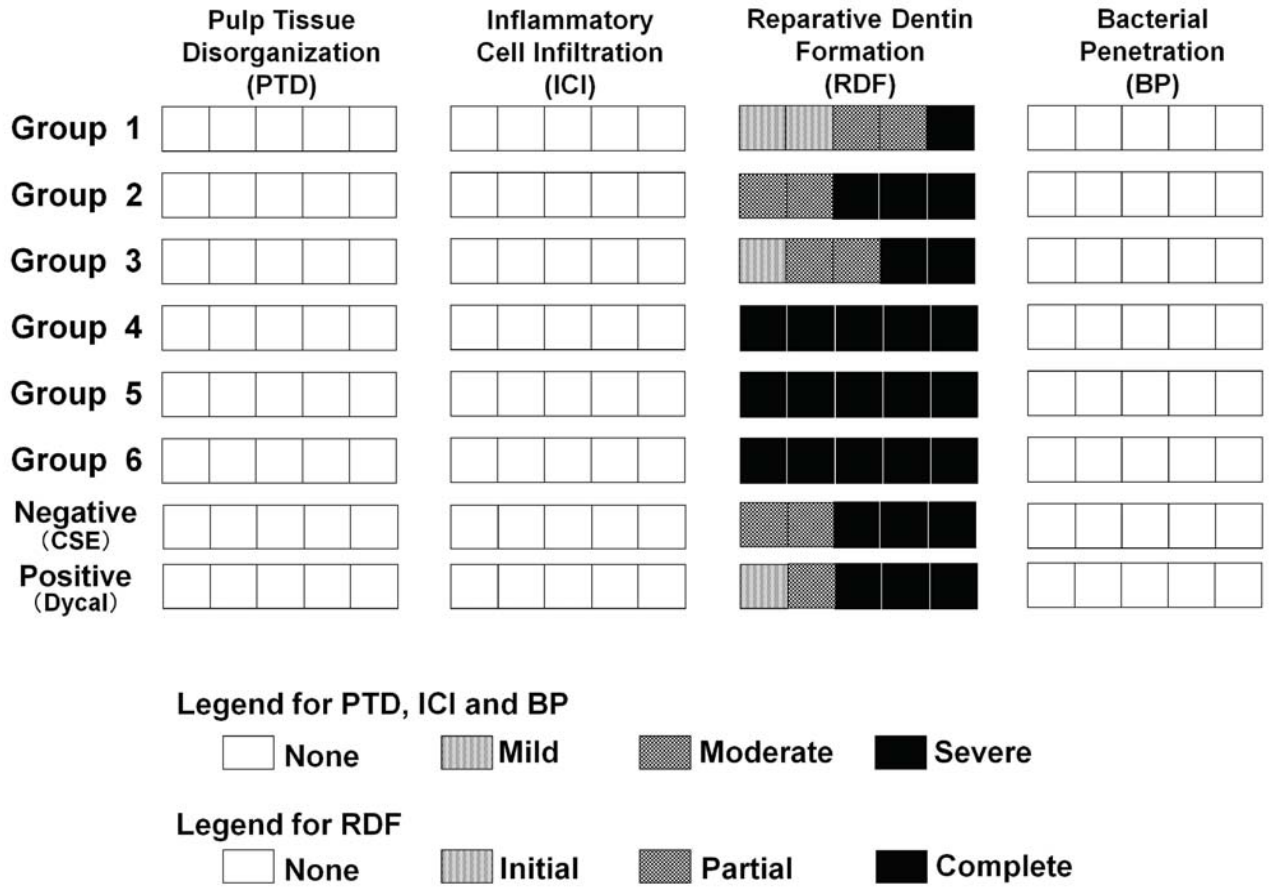
NS:Not significant
*:Significant difference (P＜0.05)

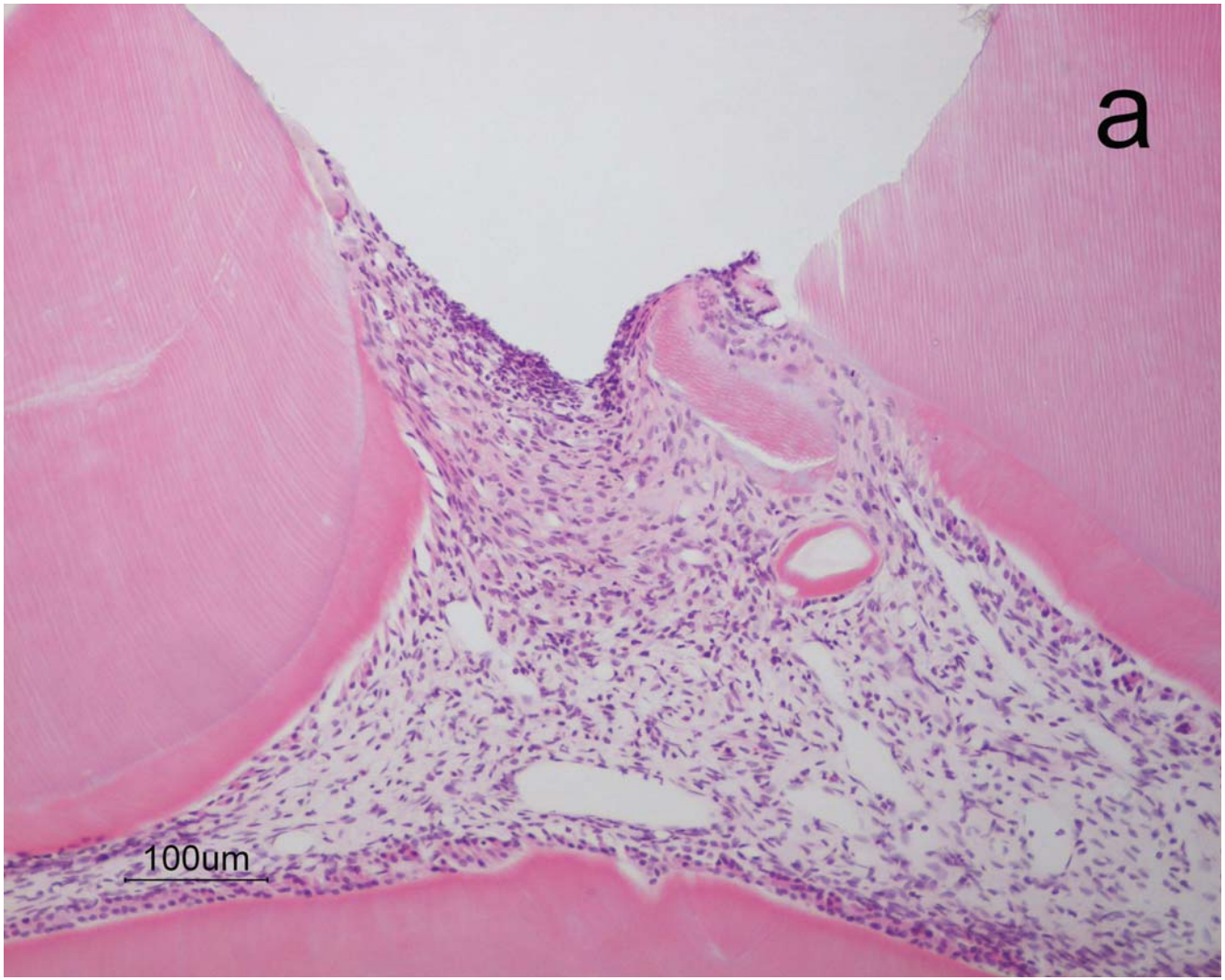
Table 4. Results of statistical analysis for
reparative dentin formation (14 days)

	Experimental groups					
	1	2	3	4	5	6
Negative control	* (0.008)	NS	NS	* (0.008)	* (0.008)	* (0.008)
Positive control	NS	NS	NS	NS	NS	NS

NS:Not significant
*:Significant difference (P＜0.05)


Pulp Tissue Disorganization (PTD)
Inflammatory Cell Infiltration (ICI)
Reparative Dentin Formation (RDF)
Bacterial Penetration (BP)
Group 1
Group 2
Group 3
Group 4
Group 5
Group 6
Negative (CSE)
Positive (Dycal)
Legend for PTD, ICI and BP
None
Mild
Moderate
Severe
Legend for RDF
None
Initial
Partial
Complete


Pulp Tissue Disorganization (PTD)
Inflammatory Cell Infiltration (ICI)
Reparative Dentin Formation (RDF)
Bacterial Penetration (BP)
Group 1
Group 2
Group 3
Group 4
Group 5
Group 6
Negative (CSE)
Positive (Dycal)
Legend for PTD, ICI and BP
None
Mild
Moderate
Severe
Legend for RDF
None
Initial
Partial
Complete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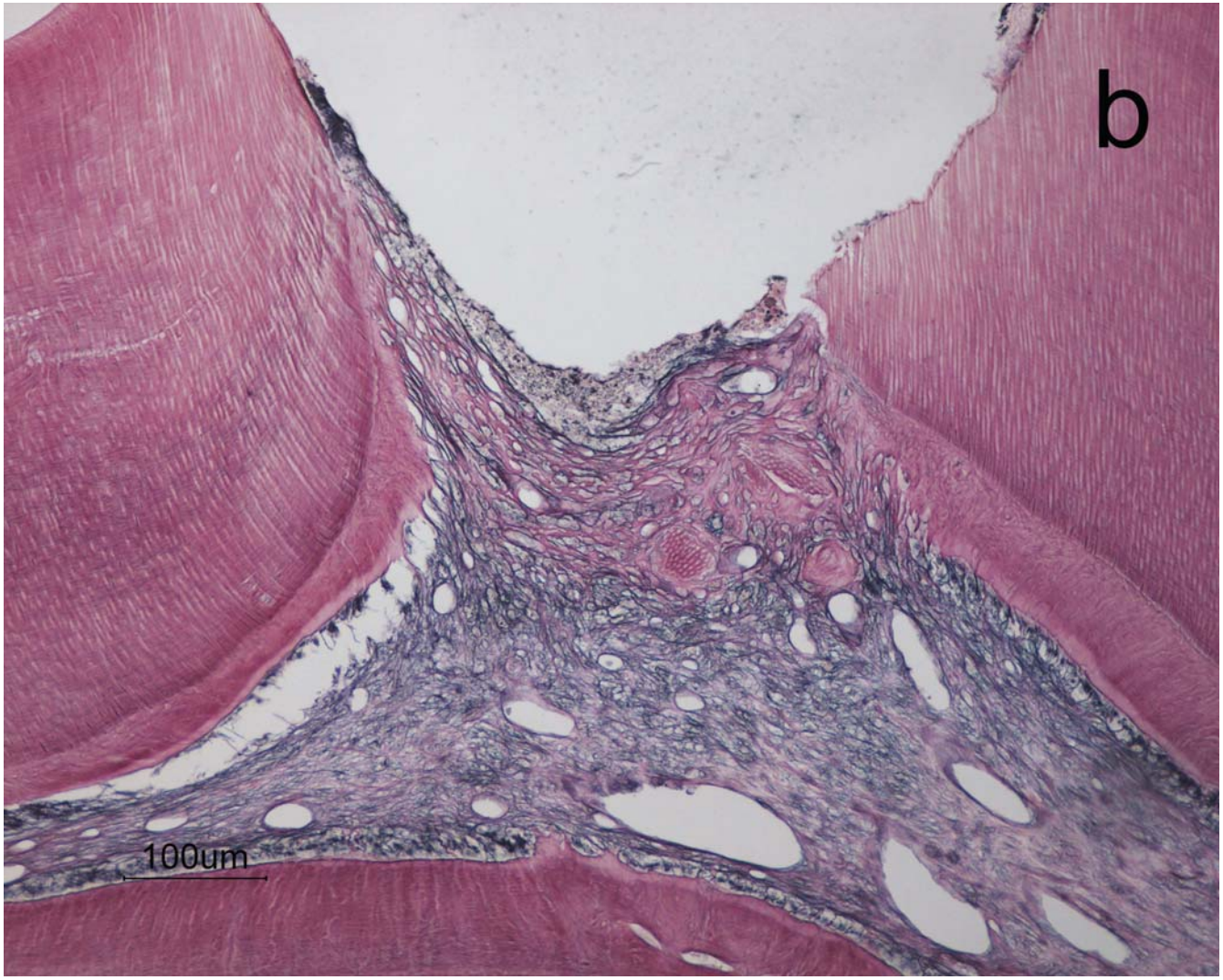
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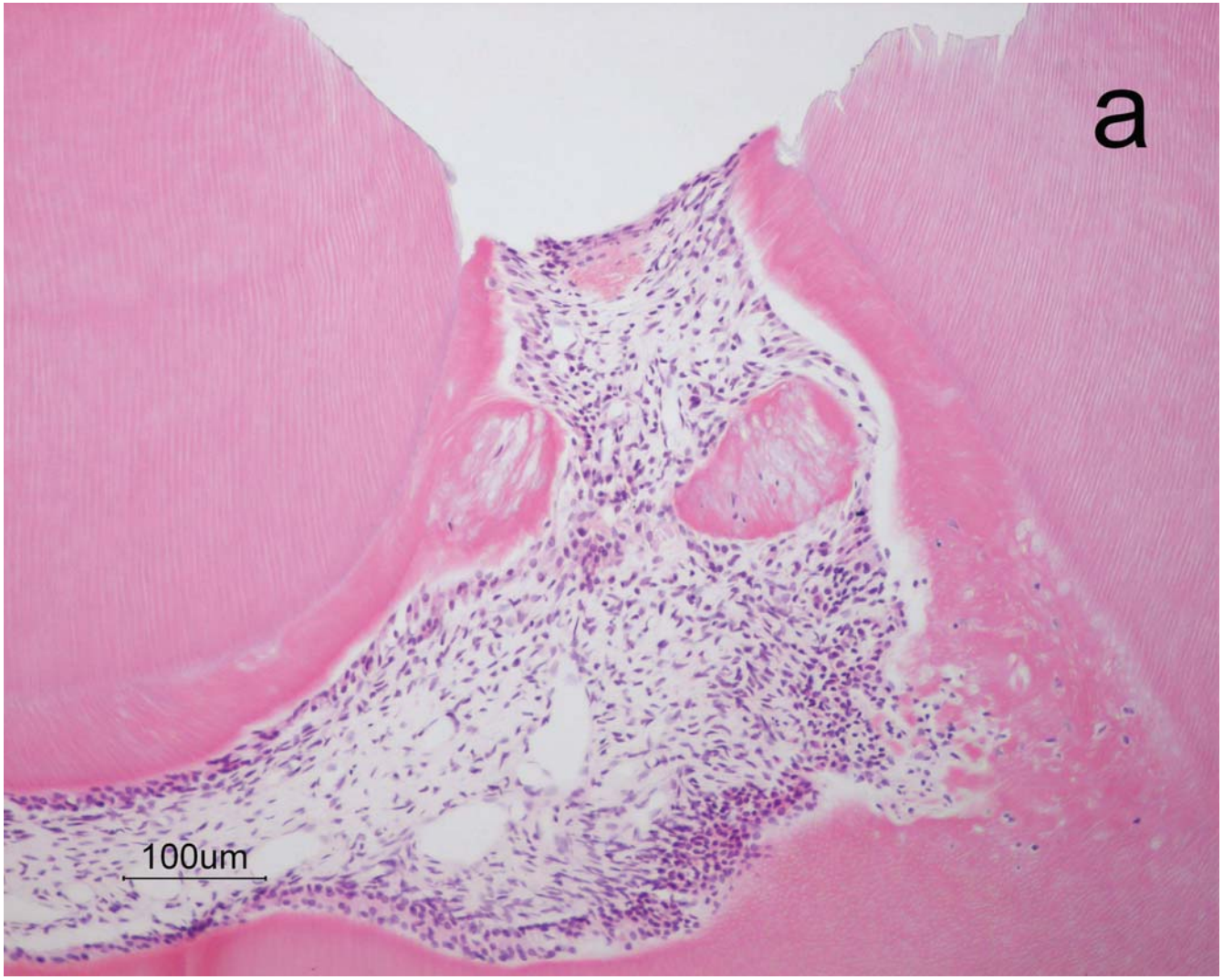
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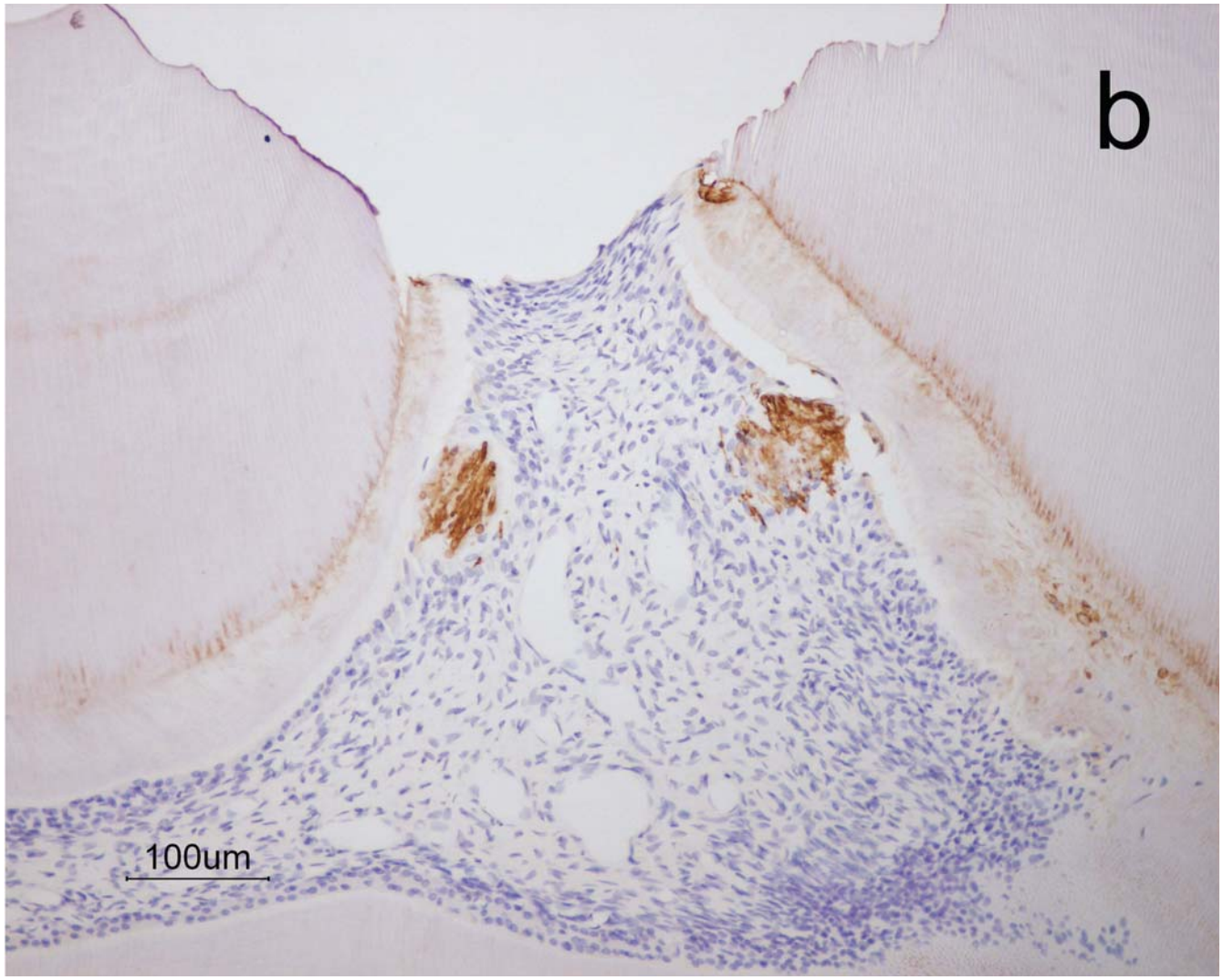
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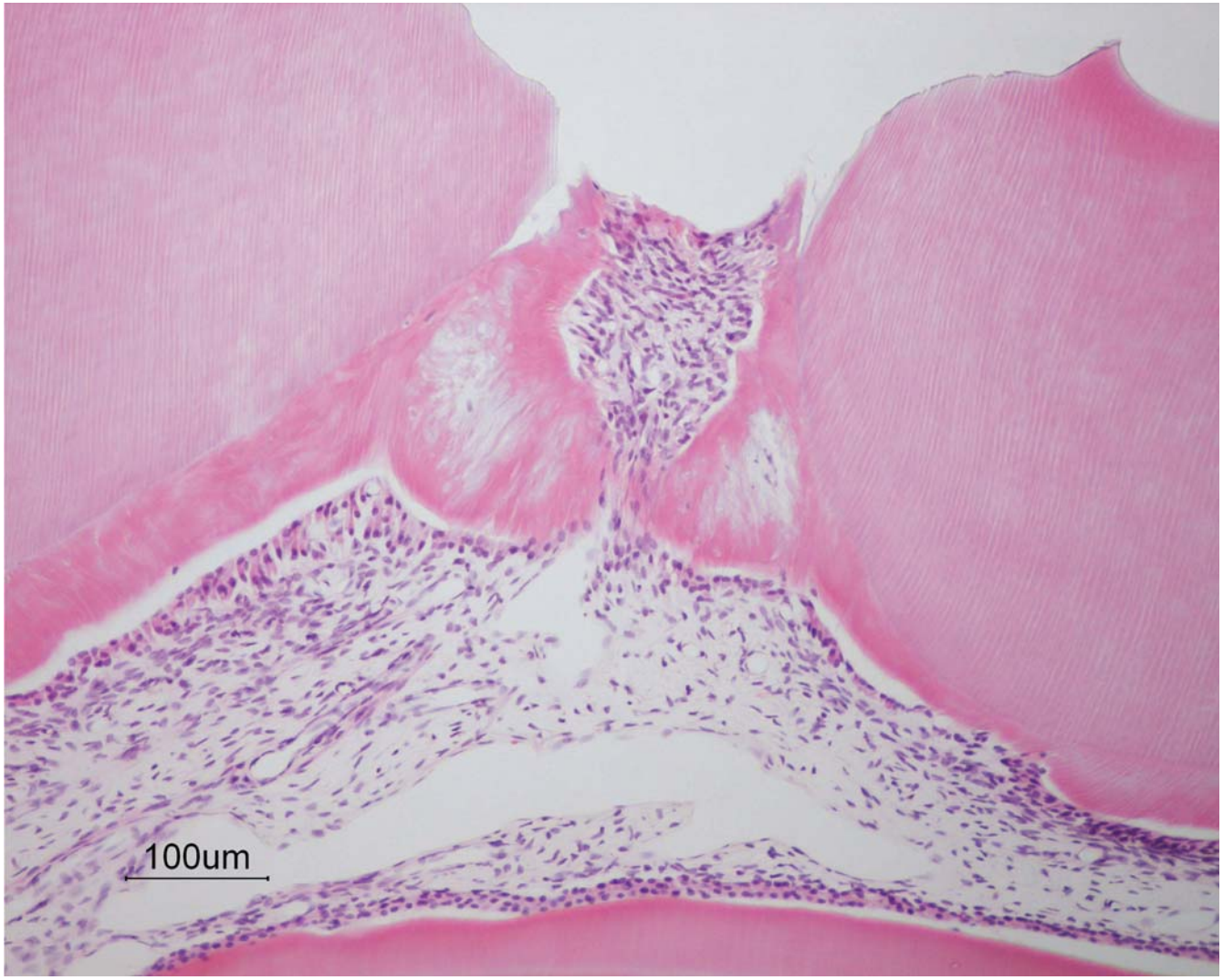
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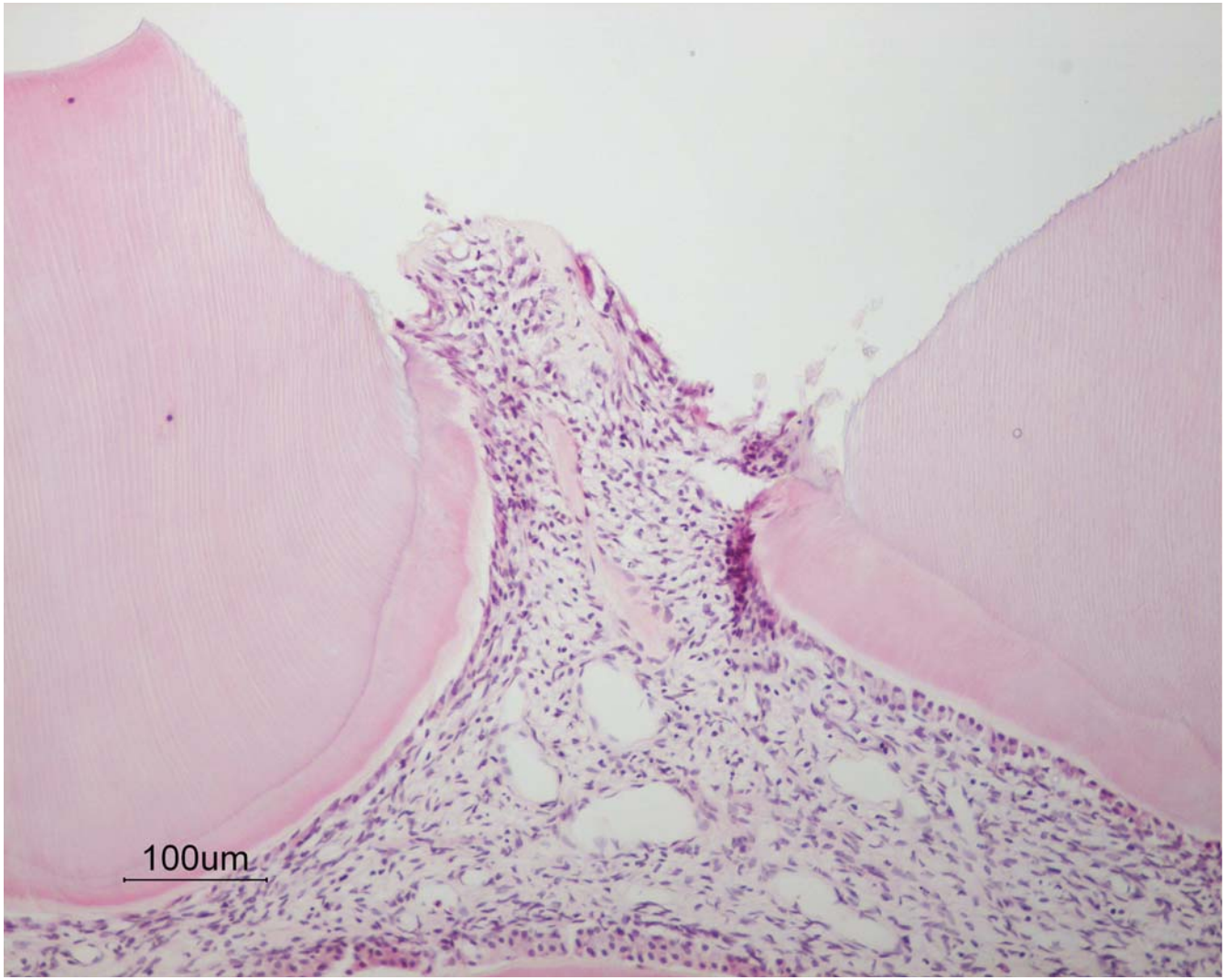
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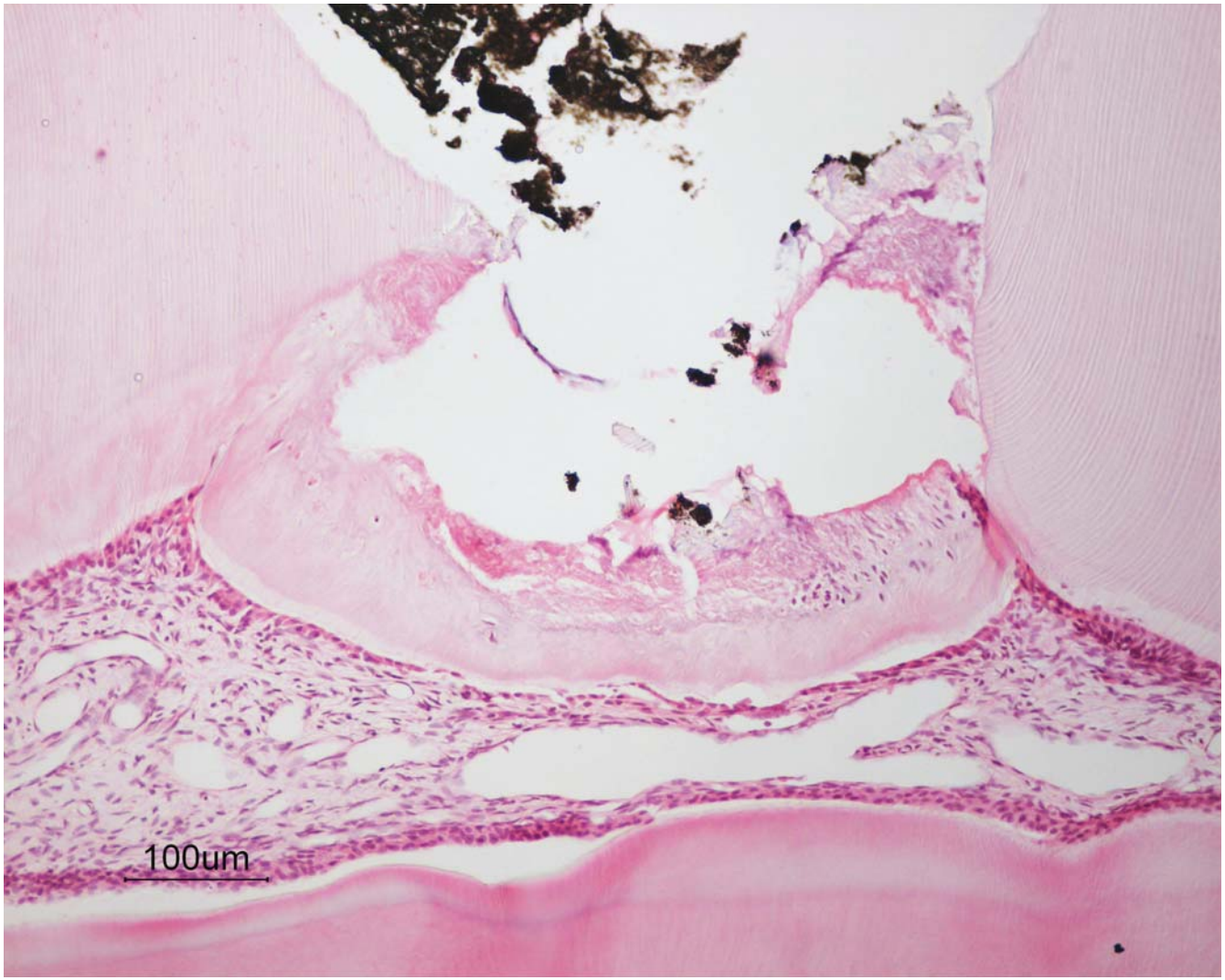
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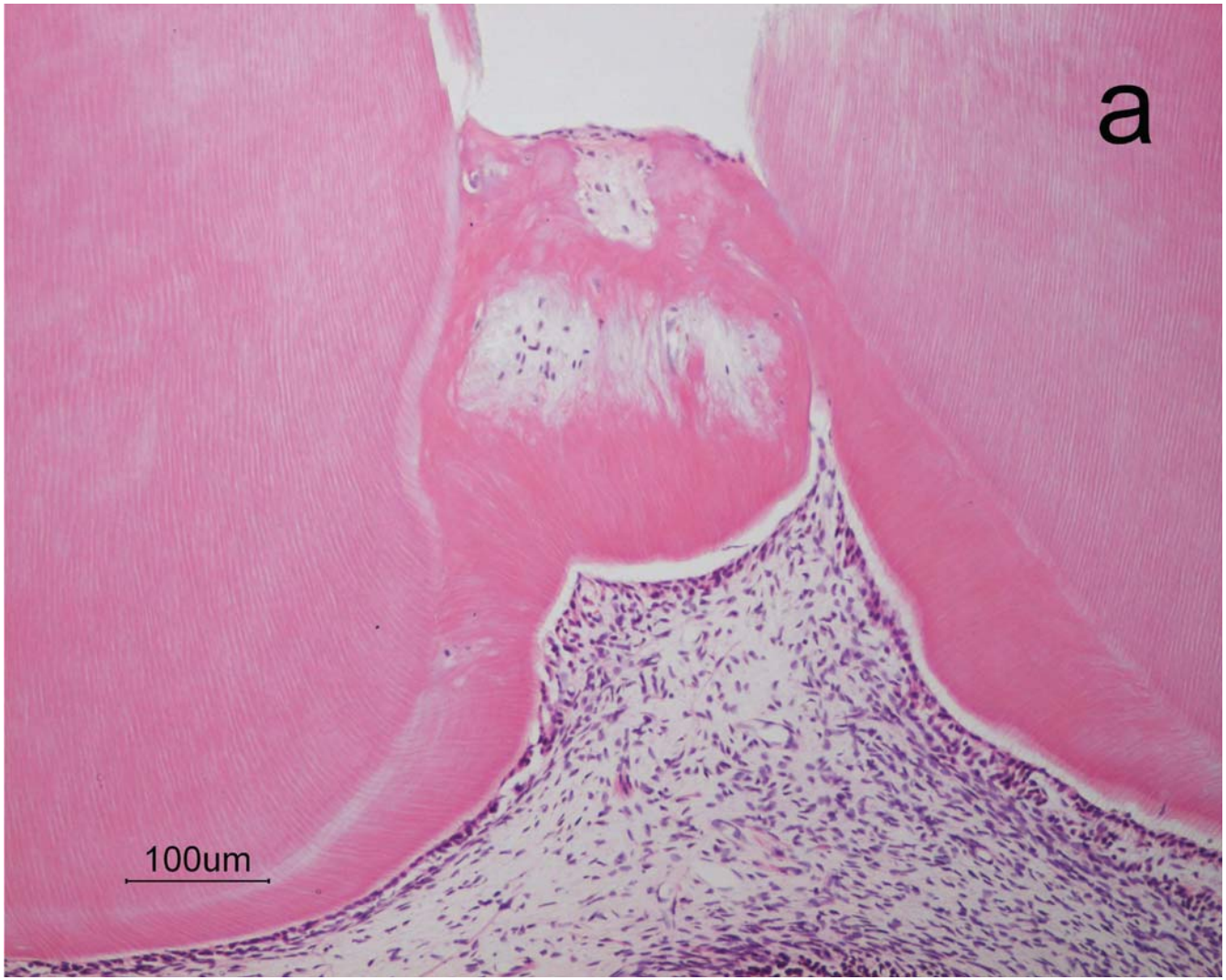
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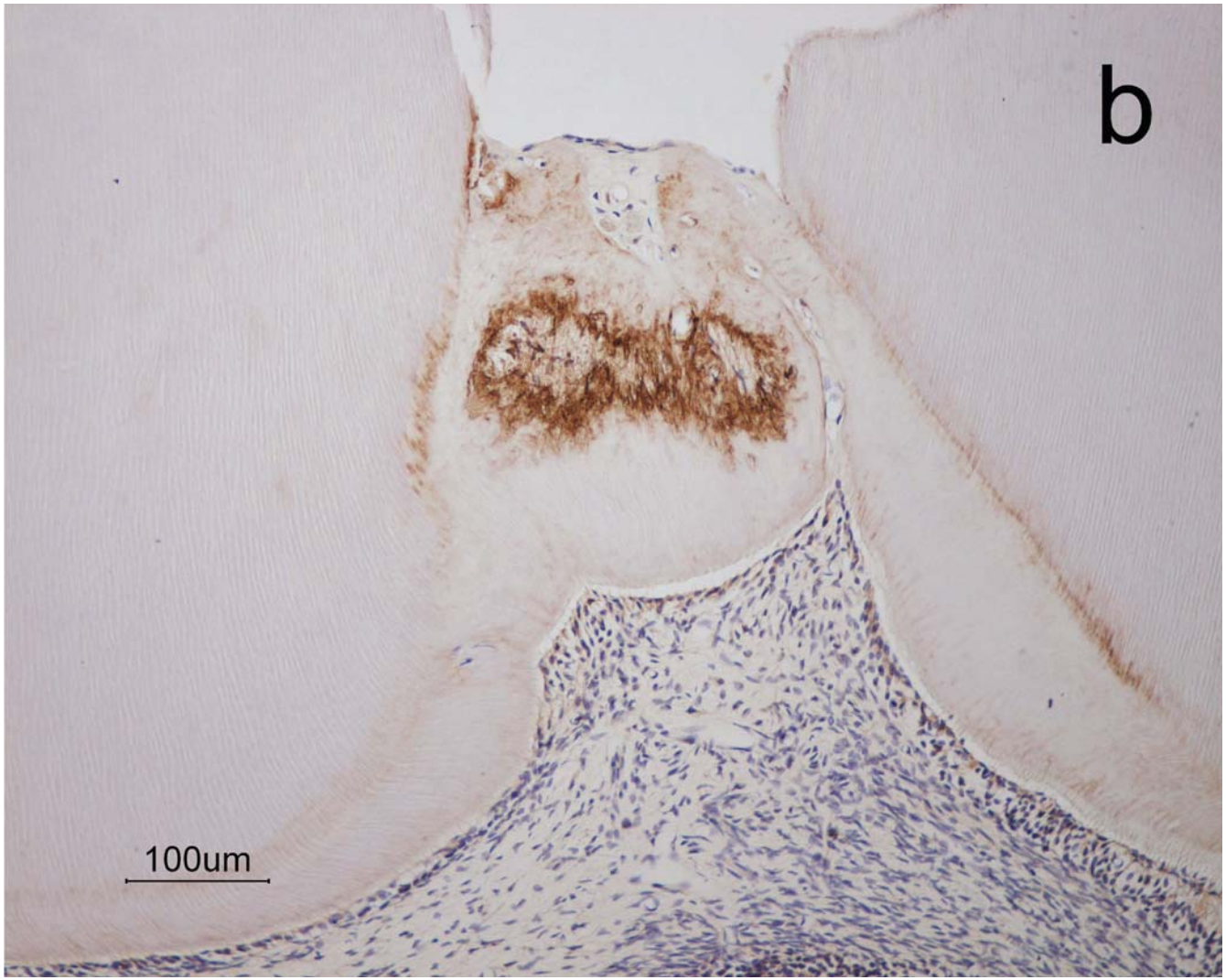
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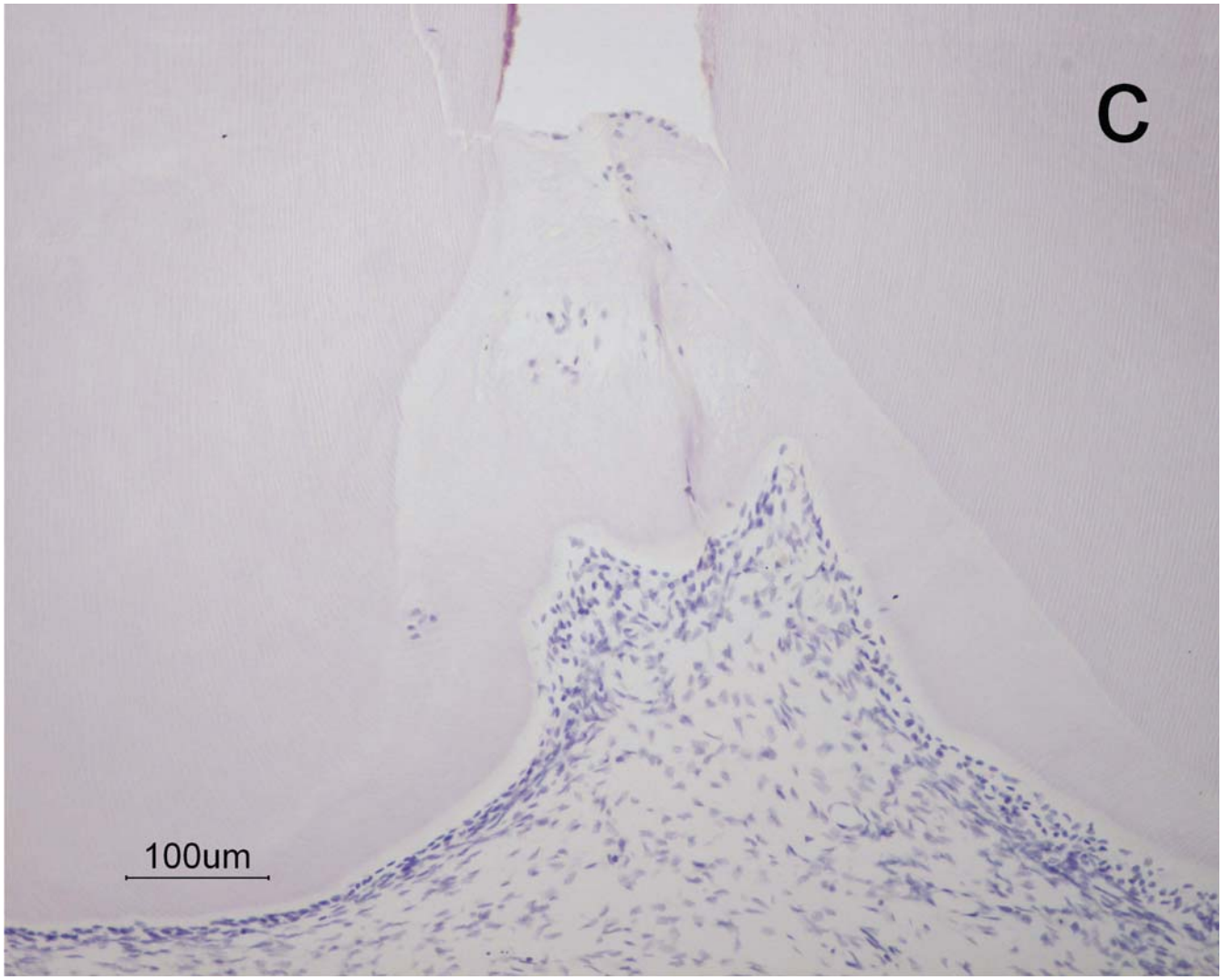
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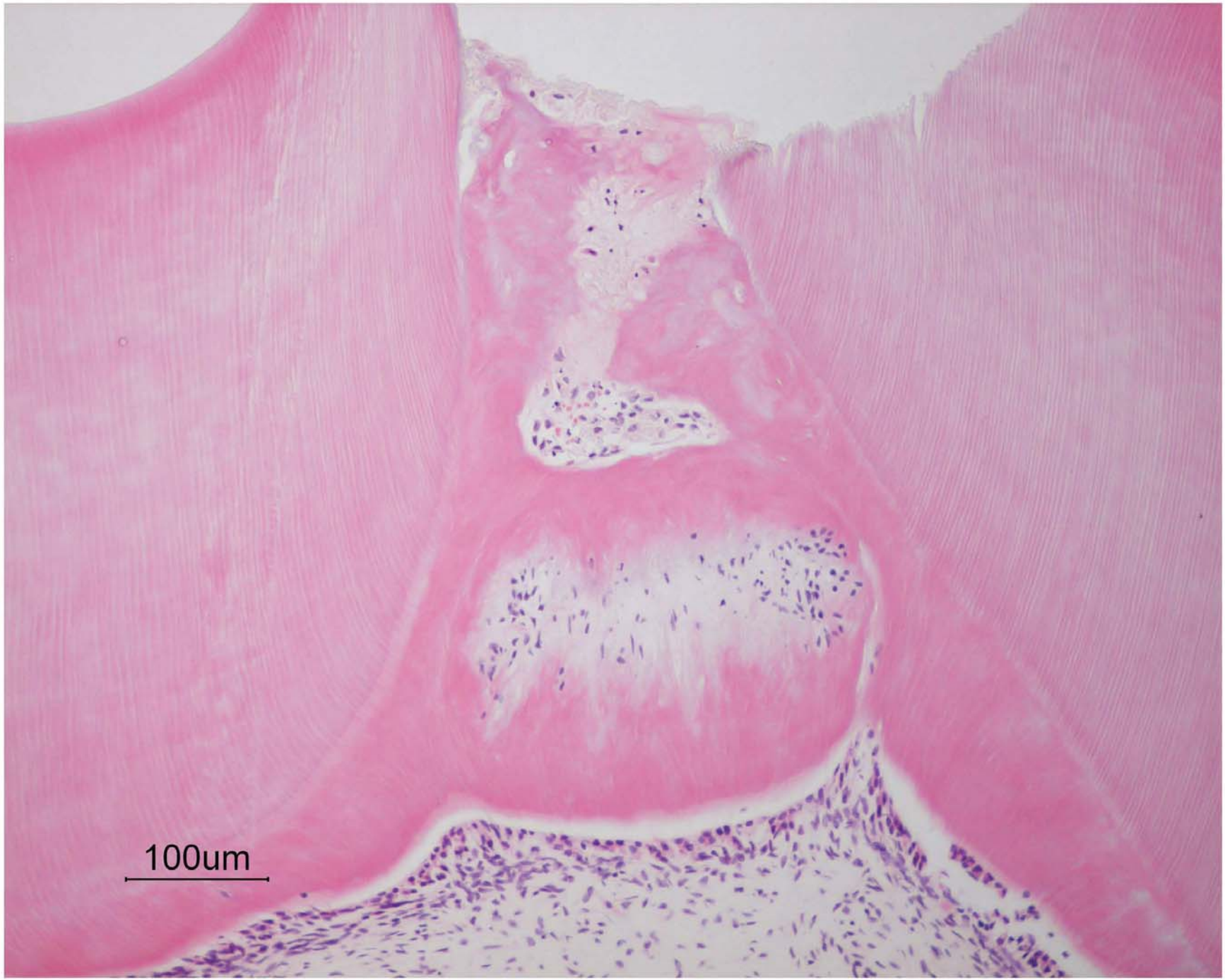
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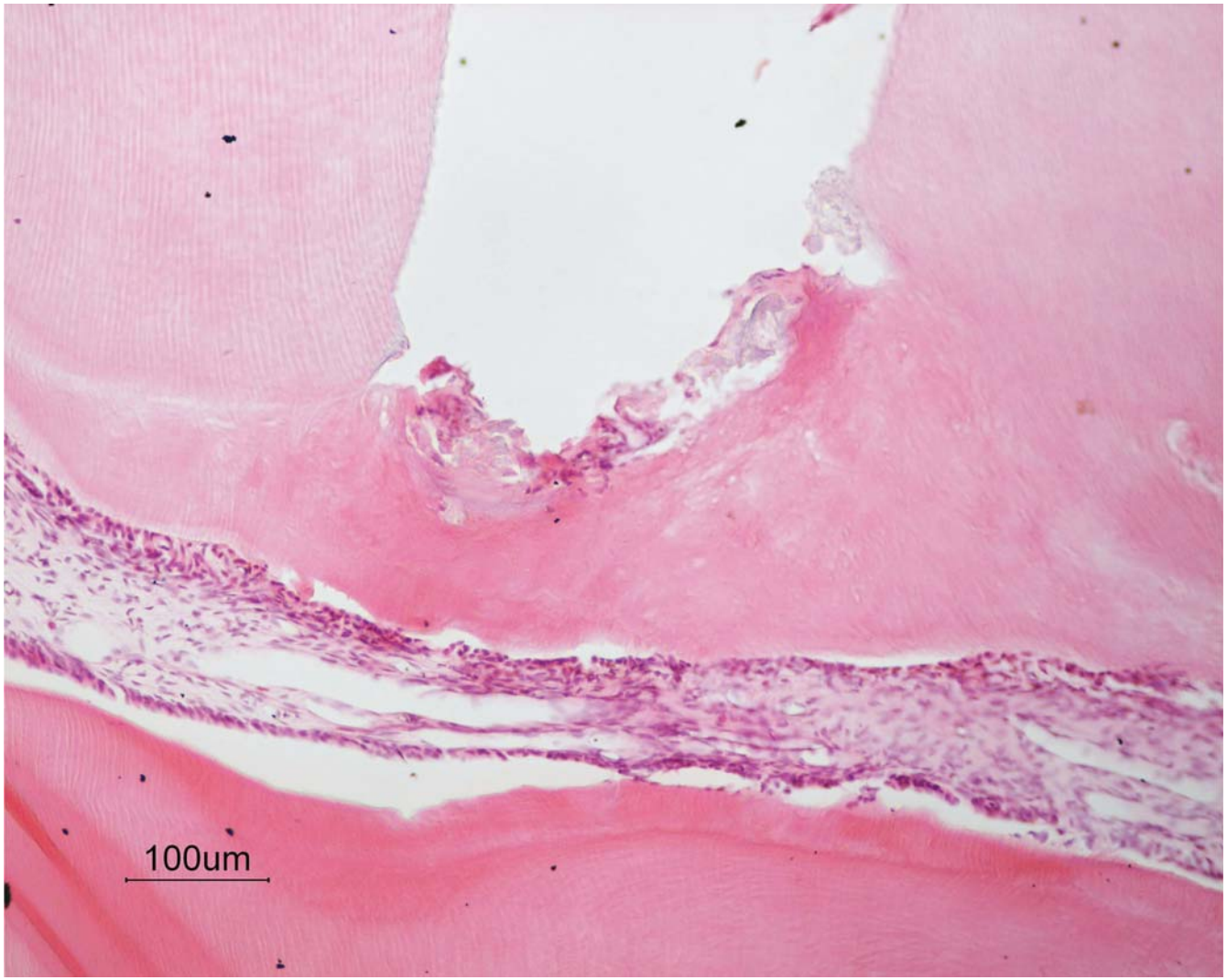
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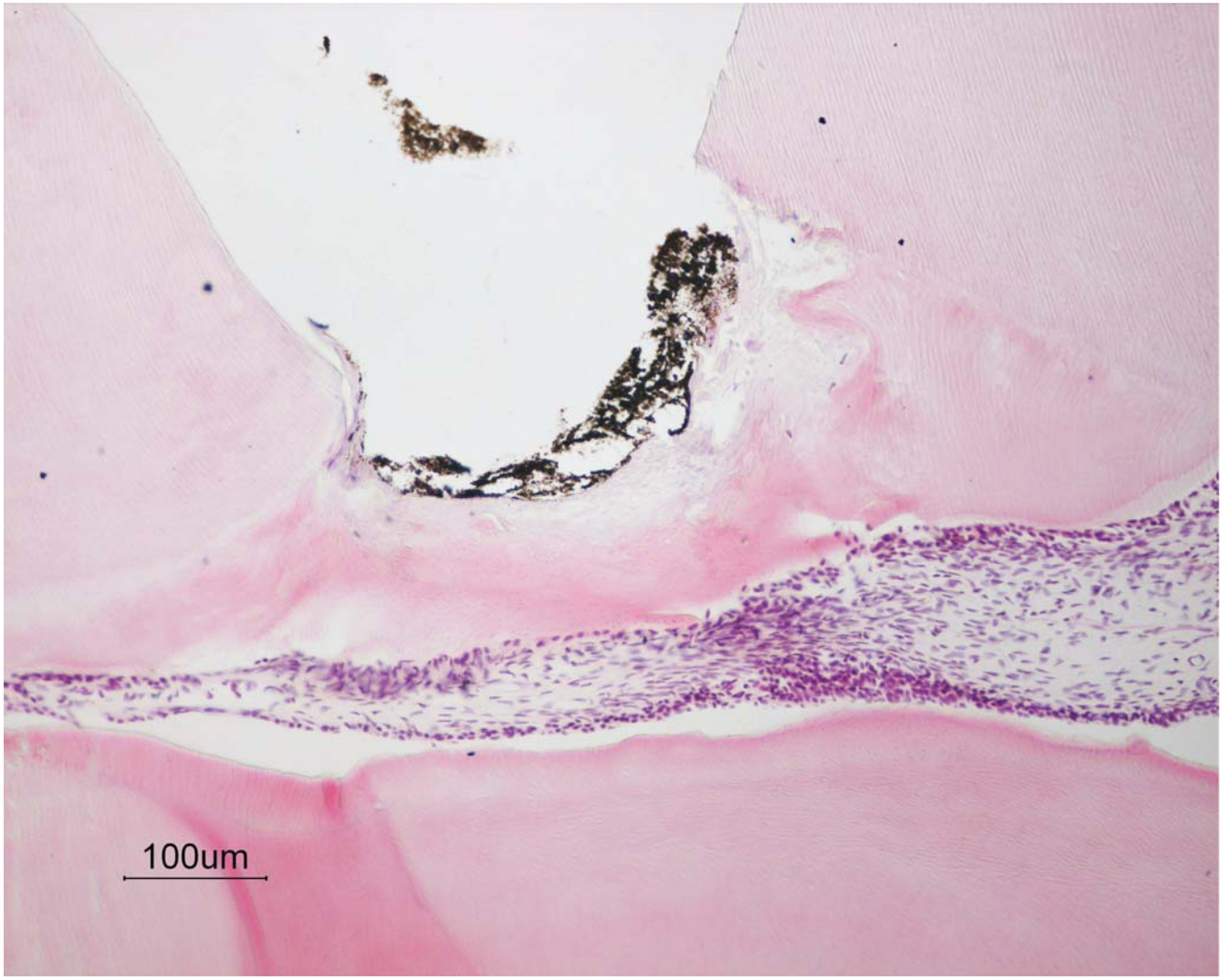
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