Histological evaluation of direct pulp capping with all-in-one adhesives in rat teeth

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The aim of this study was to histologically evaluate direct pulp capping using different all-in-one adhesives in rat teeth. Five all-in-one adhesives and a control material (MTA) were used. Each material was applied on the exposed pulp, and each cavity was subsequently restored with the resin composite. Rats were sacrificed 14 days after the surgical procedure. Serial stained sections were histologically evaluated for examining pulp tissue disorganization (PTD), inflammatory cell infiltration (ICI), dentin bridge formation (DBF), and bacterial penetration (BP). We found that rat pulps, which were direct capped with all-in-one adhesives, showed various degrees of PTD, ICI, and DBF depending on the material, and that there were no complete dentin bridges. In contrast, rat pulps capped with MTA showed no PTD and ICI, and there were complete dentin bridges in all, but one specimen. No BP was observed in any specimen.

Keywords: Histological evaluation, Direct pulp capping, All-in-one adhesive, Rat pulp, Biocompatibility
15 rats (Sprague-Dawley male rats; 8–9 weeks old; and approximately 300–400 g in weight) were treated with direct pulp capping. Five teeth were assigned to each experimental group. This study was approved by the Laboratory Animal Committee of The Nippon Dental University School of Life Dentistry at Niigata (receipt and permission number: 126).

**Specimen preparation**

The rats were sedated with ether (diethyl ether, Wako Pure Chemical Industries, Osaka, Japan), and general anesthesia was subsequently achieved by an intraperitoneal injection of a medetomidine (Domitor®, Nippon Zenyaku Kogyo, Fukushima, Japan), midazolam (Sandoz®, Sandoz, Tokyo, Japan), and butorphanol (Domitor®, Meiji Seika Pharma, Tokyo, Japan) mixture at a dose of 0.15, 2.0, and 2.5 mg/kg (body weight/rat), respectively. Each rat was fixed on an operating board, and the mouth was kept in an open position using a jaw prop. The teeth were cleaned using 3% hydrogen peroxide (Oxydol®, Yoshida Pharmaceutical, Tokyo, Japan), rinsed using physiological saline (Physisalz® PL-D, Fuso Pharmaceutical Industries, Osaka, Japan), and disinfected using diluted iodine tincture (Yoshida Pharmaceutical).

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 using an FG #440SS regular-cut diamond point (Shofu, Kyoto, Japan) in a high-speed hand piece (Air-turbine hand piece, Super Load 9000, Yoshida, Tokyo, Japan) under a water coolant. The pulps were subsequently carefully exposed using a CA #1/2 steel round bur (Hager & Meisinger, Neuss, Germany) in a low-speed hand piece (Micromotor hand piece, Micro-Mega, Yoshida) under a distilled water coolant without penetrating into the pulp space. Hemorrhage from the exposed pulp was stopped with the application of 10% sodium hypochlorite (NaOCl) gel (AD Gel, Kuraray Noritake Dental, Tokyo, Japan) for 5 min. The AD gel was reapplied if hemorrhage continued. Alternate irrigations using 3% H₂O₂ and 6% NaOCl solution (Purelox, Oyalox, Tokyo, Japan) were performed thrice to remove the AD gel and dentin chips followed by rinsing with physiological saline. The excess water was removed using sterilized, small cotton pellets, and the cavity was gently air-blown to dry.

Each all-in-one adhesive (Table 1) was applied to the cavities, according to each manufacturer’s instruction, to achieve direct pulp capping. The teeth capped with MTA were used as a control group. After the direct capping procedures, all cavities were filled with a hybrid resin composite (Clearfil Majesty A3, Kuraray Noritake Dental) and photo-polymerized using a curing light (Candelux, Morita, Tokyo, Japan) for 40 s.

**Perfusion fixation and tissue preparation**

The rats were sacrificed with an intraperitoneal overdose injection of the general anesthetic solution mentioned above after an observation period of 14 days. Each pulp was fixed through transcardial perfusion with a 4% paraformaldehyde phosphate buffer solution (pH 7.4; Wako Pure Chemical Industries). The maxillary bone specimens containing the experimental teeth were carefully removed and immersed in the same fixative at 4°C for an additional 24 h. After fixation, the specimens were trimmed of excess tissue and decalcified using 10% EDTA-2Na solution (pH 7.4) at room temperature for 4 weeks; next, the filled material was carefully removed from the cavity and rinsed with running water for 24 h. The specimens were subsequently dehydrated in increasing concentrations of ethanol, dealcoholized with xylene, and finally embedded in paraffin.

**Table 1  Composition of the materials used in this study**

<table>
<thead>
<tr>
<th>Material</th>
<th>Abbrev.</th>
<th>Lot #</th>
<th>Composition</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clearfil Tri-S</td>
<td>CTB</td>
<td>00009A</td>
<td>Bis-GMA, MDP, HEMA, Photoinitiators, Ethanol, Water, Silanated colloidal silica, CQ</td>
<td>Kuraray Noritake Dental</td>
</tr>
<tr>
<td>Bond ND</td>
<td></td>
<td></td>
<td>Phosphonic acid monomer, 4-MET, Dimethacrylate, Acetone, Water, Nano silica filler, Initiator, CQ</td>
<td></td>
</tr>
<tr>
<td>G Bond Plus</td>
<td>GBP</td>
<td>1009101</td>
<td>Phosphoric acid monomer, Bis-GMA, HEMA, TEGDMA, Alcohol, Water, CQ</td>
<td>GC</td>
</tr>
<tr>
<td>Bond Force</td>
<td>BF</td>
<td>086</td>
<td>HEMA, Bis-GMA, Methacrylated phosphoric esters, 1,6 Hexanediol dimethacrylate, Polyalkenoic acid, Silica filler, Ethanol, Water, Stabilizers, CQ</td>
<td>Tokuyama Dental</td>
</tr>
<tr>
<td>Adper Easy Bond</td>
<td>AEB</td>
<td>407565</td>
<td>Bifunctional acrylic amides, Acryloamido acylsulfonic acid, Functionalized phosphoric acid ester, Acrylic acid, Butylated benzenediol, Water, Tertiary butanol, Initiators, CQ</td>
<td>3M ESPE Dental</td>
</tr>
<tr>
<td>Pro root MTA</td>
<td>MTA</td>
<td>09003850</td>
<td>CaO, SiO₂, Al₂O₃, Fe₂O₃, Bi₂O₃, CaSO₄•2H₂O</td>
<td>Dentsply Sankin</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Dentsply Tulsa Dental</td>
</tr>
</tbody>
</table>
Serial sectioning and staining

Serial sections of 6 μm thick were longitudinally cut through the center of each tooth using a sliding microtome (Jung Histoslide 2000R, Leica Microsystems Vertrieb, Wetzlar, Germany) and stained using Mayer’s hematoxylin-eosin (HE) staining, modified NF-Watanabe’s (NF) silver impregnation staining, and Hucker-Conn (HC) bacterial staining methods. DMP1 staining method was used for immunohistochemical evaluations.

Observation items and evaluation criteria

The stained sections were observed under a light microscope (Eclipse E1000; Nikon, Tokyo, Japan). Four histological features, such as pulp tissue disorganization (PTD), inflammatory cell infiltration (ICI), dentin bridge formation (DBF), and bacterial penetration (BP), were evaluated, according to the criteria listed in Table 2. Two observers determined the score of each specimen; in cases of disagreement between the two observers, a third observer participated to determine the score.

Measurement of the diameter of exposed pulp area

The diameter of the exposed area was measured using a stereomicroscope (Measuring Microscope MM-40, Nikon), and the widest dimension was recorded as the size of the pulp exposure for the specimen.

Statistical analysis

The exposure size data were analyzed using the one-way analysis of variance and Tukey’s post hoc test. Results of the histopathological evaluation were statistically analyzed using the Kruskal-Wallis test to determine the differences between the experimental groups. Statistical procedures were performed at a significance level of 0.05 using the statistical software (Microsoft Excel 2010 for windows, SSRI, Tokyo Japan).

We wished to establish the smallest number of rats scarified in this study, because of the spirits of animal protection. We calculated the power of the Kruskal-Wallis H-test at an effect size of 0.5 (Cohen’s large effect size), alfa error probability of 0.05, total sample size of 30, and number of groups of 6 using Power analysis software (G Power 3.0.1.0). As the results, the power of the Kruskal-Wallis H-test performed in this study was 0.43.

RESULTS

Diameter of exposed pulp area

The diameter (mean±SD) of the exposed pulp area in each group ranged from 0.197±0.043 to 0.303±0.077 mm. There were no significant differences between the groups in the pulp exposure size (p=0.080).

Histopathological evaluation (statistical assessment)

Figure 1 presents a summary of the results of the histopathological evaluation. Representative histopathological images of the materials are shown in Figs. 2–7. The results of the Kruskal-Wallis test for

<table>
<thead>
<tr>
<th>Pulp tissue disorganization</th>
<th>None</th>
<th>Normal or almost normal tissue morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mild</td>
<td>Odontoblast layer disorganization, but the deep part of the pulp appears normal</td>
<td></td>
</tr>
<tr>
<td>Moderate</td>
<td>Loss of general tissue morphology</td>
<td></td>
</tr>
<tr>
<td>Severe</td>
<td>Necrosis in the coronal one-third or more of the pulp</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Inflammatory cell infiltration</th>
<th>None</th>
<th>Absence or presence of a few scattered inflammatory cells in the pulp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mild</td>
<td>Mild acute/chronic cell lesions</td>
<td></td>
</tr>
<tr>
<td>Moderate</td>
<td>Moderate inflammatory cell lesions seen as abscesses or densely stained infiltrates of polymorphonuclear leucocytes, histiocytes, and lymphocytes in one-third or more of the coronal pulp and/or the mid-pulp</td>
<td></td>
</tr>
<tr>
<td>Severe</td>
<td>Pulp necrosis due to severe degree of infection or lack of tissue in one-half or more of the pulp</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Reparative dentin formation</th>
<th>None</th>
<th>No dentin bridge formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>Initial dentin bridge formation extending to not more than one-half of the exposure site</td>
<td></td>
</tr>
<tr>
<td>Partial</td>
<td>Partial/incomplete dentin bridge formation extending to more than one-half of the exposure site but not completely closing the exposure site</td>
<td></td>
</tr>
<tr>
<td>Complete</td>
<td>Complete dentin bridge formation</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Bacterial penetration</th>
<th>None</th>
<th>Absence of stained bacterial profiles in any parts of the sections</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mild</td>
<td>Presence of stained bacterial profiles along the coronal or apical walls of the cavity</td>
<td></td>
</tr>
<tr>
<td>Moderate</td>
<td>Presence of stained bacterial profiles within the cut dentinal tubules or axial walls of the cavity</td>
<td></td>
</tr>
<tr>
<td>Severe</td>
<td>Presence of stained bacterial profiles within the dental pulp</td>
<td></td>
</tr>
</tbody>
</table>
the histopathological evaluation showed no significant differences among all materials for PTD parameter ($p=0.072$) and ICI parameter ($p=0.082$), and among the five adhesive materials (CTB, GBP, BF, AEB, and XV) for the DBF parameter ($p=0.858$). In contrast, the results of the Kruskal-Wallis test showed significant differences among all materials for the DBF parameter ($p=0.014$), and Steel post-hoc test showed significant differences between MTA and each adhesive material ($p<0.05$).

PTD and ICI findings
Normal pulpal tissue morphology was observed in all specimens capped with CTB, GBP, and MTA, whereas mild to moderate PTD and ICI were observed in some specimens capped with BF, AEB, and XV at 14 days postoperatively. Severe PTD and ICI were observed in one XV specimen. As shown in Figs. 2 (the specimen capped with CTB) and 5 (the specimen capped with AEB), mild PTD and no ICI were observed at the exposure site, but the surface of the exposed pulp was not covered by hard tissue.
**DBF findings**

Four of the five specimens in the control group (capped with MTA) showed a complete DBF; the remaining one specimen showed an incomplete DBF. In contrast, all specimens in the all-in-one adhesive groups showed an incomplete DBF; however, some specimens showed some initial to partial DBF. As shown in Fig. 3 (the specimen capped with GBP), an initial DBF was observed at the exposed pulp surface.
Fig. 5 Representative histologic images of AEB (×100). (a) H-E staining, (b) NF staining, (c) DMP1 staining. The surface of the exposed pulp was not covered by hard tissue.

Fig. 6 Representative histologic images of XV (×100). (a) H-E staining, (b) NF staining, (c) DMP1 staining. There was an incomplete DBF at a comparatively deeper position from the pulpal exposed site. Reticular collagenous fibers were observed at the exposed pulp surface.

Exposed pulp surface. A partial DBF was observed at the exposed pulp surface in the specimens capped with BF (Fig. 4) and those capped with XV (Fig. 6).

**BP findings** At 14 days postoperatively, none of the specimens stained positively for bacteria.
DISCUSSION

The results of statistical analysis for the histopathological evaluation showed no significant differences among all materials for PTD and ICI parameters ($p>0.05$). However, those for PTD and ICI parameters showed a tendency of dependence on the materials. No PTD and ICI was observed in the specimens capped with CTB, GBP or MTA; however, BF, AEB and XV showed mild to severe PTD and ICI. This discrepancy may be due to the differences in the chemical composition of the adhesives. Some adhesive components might chemically irritate the dental pulp and cause inflammatory cell invasions beneath the exposed pulp surface. A previous study, which had evaluated the cytotoxic effects of conventional and resin-modified glass-ionomer cements on an odontoblast cell line (MDPC-23), showed that the conventional types were the least cytotoxic, whereas the resin-modified types were the most cytopathic. Furthermore, the authors suggested that unreacted HEMA significantly contributes to the cytotoxicity of the resin-modified types used in pulp cell cultures. Becher et al. reported that TEGDMA was more cytotoxic than HEMA; HEMA caused a larger accumulation of apoptotic cells using the MTT assay and fluorescence microscopy. Kurata et al. reported that the fibroblast growth after exposure to acrylic acid decreased with an increase in acid concentration and was lesser than that after exposure to methacrylic acid. Thus, the cytotoxicity of these chemical components has been recognized in many studies, and the cytotoxic effects varied among the studies. The inflammatory changes that remained after 14 days in the three XV specimens might have been caused by chemical irritation from the acrylic acid contained in the components. Those in the one BF specimen and two AEB specimens might have been caused by chemical irritation from the HEMA or TEGDMA; however, all specimens capped with CTB showed no inflammatory changes in spite of containing HEMA. Thus, from the results of this study, the correlation between chemical composition of adhesives and dental pulp irritation remained unclear. The permeability of the adhesive monomers into pulp tissue and the polymerization degree of the adhesives might also correlate to the irritation of the pulp tissue.

In the direct pulp capping treatment, bacterial microleakage has been identified as an important factor for pulpal irritation. Several studies have described that the response of the dentin-pulp complex does not depend on the dental materials used for pulp capping, but on their ability to prevent bacterial microleakage. Our previous study revealed that some of the self-etching adhesives induced moderate to severe inflammatory pulp responses with positive bacterial staining. Cui et al. also reported that most specimens with positive bacterial staining showed diverse pulpal responses. Scarano et al. emphasized that bacterial microleakage critically affected pulpal repair and that the direct pulp capping with a self-etch adhesive system did not damage the dental pulp. Conversely, Silva et al. reported that pulp necrosis occurred in all specimens capped with Adper Prompt even without bacterial staining, and
they speculated that this pulp necrosis was primarily induced by the adhesive chemical component toxicity to the exposed pulp tissue. The results of the present study showed negative bacterial staining for all specimens, whereas some specimens capped with an all-in-one adhesive exhibited mild to severe PTD or ICI after 14 days. We speculate that some components contained in the all-in-one adhesives might chemically irritate the dental pulp. This might have caused the pulpal responses that remained after 14 days.

The slow DBF with all-in-one adhesives used in this study corresponded to that of a two-step self-etch adhesive system, which was investigated in our previous studies and other studies. Some specimens with all-in-one adhesives exhibited an initial to partial DBF after 14 days, whereas the MTA controls formed complete DBFs in five of the six specimens. Furthermore, several studies found that the adhesive systems used for direct pulp capping exhibited incomplete DBF with varying degrees of mineralization, and none of the adhesive systems exhibited complete DBF. Costa et al. speculated that some resin components were toxic to exposed pulp over a short period and that the resin components released into the pulp may delay pulpal healing.

Several investigations have shown that non-human dental pulps capped with adhesives exhibited a slight initial inflammatory reaction; however, pulp repair with DBF was observed even after the short-term evaluation period. A previous study involving human teeth reported that the histological reactions of the exposed dental pulp to total-etch and self-etch systems were generally comparable to those to calcium hydroxide after 15 days, and active odontoblasts were observed at the periphery of the exposed pulp beneath the composite resins in all specimens.

As shown in Figs. 2–6, the positive staining of DMP1 at the odontoblastic layer indicated the reparative dentin formation in the present study. Therefore, it will be necessary to investigate the long-term pulpal responses to direct pulp capping with self-etch adhesive systems in a future study.

From the results of this study, MTA controls demonstrated excellent efficacy for hard tissue formation, as shown in several previous studies. Min et al. reported that MTA was superior to calcium hydroxide in terms of DBF during the early wound healing process and that the superiority of MTA was associated with the expression of a cytoprotective molecule, hemoglobin (Hb)-1, in human dental pulp. Kuratate et al. clarified that the reparative process in exposed pulps after the use of MTA involved the initial deposition of osteopontin in the superficial pulp tissue followed by the appearance of newly-differentiated nestin-immunoreactive odontoblast-like cells. In our study, the odontoblasts beneath the dentin bridge formed by the MTA application showed positive staining for DMP1. This finding also supported the speculation that DBF in response to MTA involved the proliferation of stem cells, their migration into the exposed pulp surface, and their differentiation into odontoblast-like cells.

Furthermore, it has been reported that MTA induced dose-dependent contractions in the rat thoracic aorta; this property of MTA may allow appropriate control of hemorrhage, which is important in the success of direct pulp capping.

In our study of a series of direct pulp capping procedures, we applied the AD gel (10% NaOCl gel) to the exposed pulp surface to control hemorrhage, following three alternating irrigations using both 3% H2O2 and 6% NaOCl solutions to remove the AD gel and dentin chips. Some studies have reported that the application of NaOCl to exposed pulp surface is an important procedure because this solution can control infection and hemorrhage and prevent blood clot formation. On the other hand, several studies have demonstrated the cytotoxicity of NaOCl even at low concentrations. Costa et al. demonstrated that 0.12% NaOCl was toxic to a cultured odontoblast cell line (MDPC-23). Heggars et al. has reported that 0.025% NaOCl solution is therapeutically effective because it retains its bactericidal properties and eliminates any harmful impacts on wound healing. For these reasons, some researchers have controlled hemorrhage using sterile water instead of NaOCl solution. Based on our experience during animal studies, it is very difficult to stop bleeding using only sterile water irrigation; however, application of 10% NaOCl gel on the exposed pulp is very effective. Although, 10% of NaOCl gel is a very high concentration its cytotoxic effect may be limited on the superficial pulp tissues because of its low viscosity. Our previous studies had not demonstrated any harm from the direct application of 10% NaOCl gel on the exposed dental pulp. Therefore, we used 10% NaOCl gel for controlling hemorrhage from exposed pulp tissue in our present study. We also experienced that re-bleeding from exposed pulp occurred after the self-etching primer application to the cavity for direct pulp capping using two-step self-etching adhesives. However, in the present study, re-bleeding did not occur after the direct application of all-in-one adhesives. From this result, all-in-one adhesives are advantageous in direct pulp capping compared with other multi-step adhesives.

Numerous concerns should be pointed out while evaluating the results of direct pulp capping. For instance, there is little correlation between the results of non-human and human studies because of the differences in pulp healing capacities. The effect of direct pulp capping on pulp that was affected by caries in clinical condition cannot be extrapolated from the effects on sound pulp tissue. Moreover, the formation of the hard tissue between the capping materials and pulp has been a controversial issue because it could be produced by either pulpal healing or a reaction to irritation. Therefore, within the limitations of the results of the present study and allowing for DBF as a pulpal healing, the pulpal healing ability of all-in-one adhesives was inferior to that of the control material when they were used as a direct pulp capping material on rat teeth.
REFERENCES


