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Pulpal responses to bacterial contamination following dentin bridging beneath hard-setting calcium hydroxide and self-etching adhesive resin system

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Abstract – To evaluate the pulp healing to bacterial contamination beneath a hard-setting calcium hydroxide (DY: Dycal, L.D. Caulk Co.) and a self-etching adhesive resin (2V: Clearfil Liner Bond 2V, Kuraray Medical Inc.) following dentin bridge formation. Class V cavities were prepared on 30 monkey teeth, and the pulps were exposed with a carbide bur through the cavity floor. Each exposed pulp was capped with either DY or 2V. The cavities were restored with a hybrid resin composite. The resin composite was removed at 180 days after capping, and then cavities were left open to the oral environment for 2 weeks to obtain bacteria contamination DY (BDY) and 2V (B2V; $n = 10$). A non-bacterial-contaminated group capped with DY was used as control. After bacterial challenges, inflammatory cell infiltration, incidence and differentiation of dentin bridges were evaluated histologically. There were significant differences in the presence of inflammatory cell infiltration among all groups ($P < 0.05$). No moderate or severe inflammatory reaction was found in Group DY. Group BDY showed moderate or severe inflammatory cell infiltration in 50%, and showed four necrotic specimens. Although no statistically significant difference was found in the formation and differentiation of dentin bridges among all groups, tunnel defects in dentin bridges were detected in 70% (DY), 80% (BDY), and 50% (B2V). Group B2V showed a significantly lower presence of inflammatory cell infiltration than Group BDY ($P < 0.05$). Bonding agent is supposed to seal the exposure site, and the remaining bonding agent on the cavities was effective as the barrier in the dentin bridges after bacterial challenges.

Numerous histologic studies have demonstrated the ability of hard-setting calcium hydroxide [$Ca(OH)_2$] to induce dentin bridge formation following direct pulp capping (1, 2). Although dentin bridges are often perforated by tunnels and cell inclusions (3, 4), its presence is considered to better protect the pulp against bacterial penetration than no hard-tissue formation (5, 6). However, some researchers claim that a long-term seal cannot be provided with hard-setting $Ca(OH)_2$ because it dissolves with time and this incomplete seal may allow bacterial leakage (4, 7, 8).

Recent developments in adhesive resin systems have also increased the potential of reducing pulpal complications by providing restorations with minimal tooth–restoration, therefore lessening the risk for detrimental bacterial leakage. In order to overcome the disadvantages of hard-setting $Ca(OH)_2$, *in vivo* studies have demonstrated the possibility of adhesive resin materials as alternative direct pulp capping materials (9–11). These authors demonstrated that the main cause of pulpal inflammation was leakage of bacterial components through faults along the tooth/restoration interface, and not the biocompatibility of the materials used. However, *in vitro* studies have shown that acid etchants, monomers, and other ingredients in the composition of various adhesive resins can be cytotoxic to cultured cell lines (12). Moreover, pulp capping with adhesive resins is technique-sensitive, as bleeding can cause failures in the sealing of the exposed pulp (13).

There are many commercially available adhesive resin systems with different chemical components and procedural steps. Sübay & Demirci (14) showed that the three-step resin bonding system, Scotchbond Multi-Purpose Plus (3M, ESPE Dental Products, St. Paul, MN, USA), etched with 37% phosphoric acid may cause inflammation in human pulp tissue. In the three-step resin bonding system, acid-etching is first performed to demineralize the dentin surface, followed by a priming step and the application of adhesive resin. Most recent developments have focused on simplification of the

multistep bonding process (15). The two-step, self-etching primer system is one of the bonding systems developed to simplify and shorten the bonding procedures by combining the dentin conditioning and priming steps (16). Demarco et al. (17) demonstrated that the two-step self-etching adhesive system, Clearfil Liner Bond 2, exhibited dentin bridge formation in 38% of human specimens. Similar pulp-healing responses beneath self-etching systems were observed in animal studies (10, 11, 18). Because those different chemical components and steps in procedures may have a significant influence on the final results of direct pulp capping (19), the results with adhesive resin capping seem to be quite controversial.

Bacterial leakage at the margins of composite restorations and capping agents is still a major concern. The polymerization shrinkage of the resin composite can cause separation of $Ca(OH)_2$ from the dentin surface forming internal gaps (8). Though there are many comparative histopathologic studies with a hard-setting $Ca(OH)_2$ and adhesive resin as a direct pulp capping material (9, 10), there are no data suggesting that either capping material might be adequate against bacterial contamination following dentin bridge formation. Moreover, the little information available about the significance of the self-etching primer system on pulpal healing is insufficient to support their use as a direct pulp capping agent. The aim of this study was to evaluate the pulp healing to bacterial contamination beneath a hard-setting $Ca(OH)_2$ and self-etching adhesive resin following dentin bridge formation.

Materials and methods

Animals

Two monkeys (*Macaca fuscata*), aged 7 years, showing no evidence of dental or periodontal disease, were housed in the research facilities following approval by the Tokyo Medical and Dental University. The animal use protocol form was reviewed and approved by the Screening Committee for Animal Research of the Tokyo Medical and Dental University in accordance with the US National Institutes of Health (NIH) Office of Laboratory Animal Welfare Guidelines regarding the care and use of animals for experimental procedures. Because a non-bacterial-contaminated group capped with Dycal (Dentsply/Caulk Div, Milford, DA, USA) was a control group (Group DY), two bacterial-contaminated groups capped with one of two capping materials [hard-setting $Ca(OH)_2$: Dycal as Group BDY or self-etching adhesive resin system: Clearfil Liner Bond 2V (Kuraray Medical Inc., Tokyo, Japan) as Group B2V] were used in this study (Fig. 1), samples (n = 10) of one group were obtained from two animals (15 cavities per animal, total 30 samples). To maintain the clear operation filed in monkey mouth, both upper and lower anterior teeth including upper premolar teeth were used in this study.

Experimental procedures

After anesthesia with 20 mg kg^{-1} ketamine (Ketaral, Sankyo Co., Tokyo, Japan) and 10 mg kg^{-1} pentobarbital sodium (Nembutal, Abbott Laboratories, Abbott Park, IL, USA), infiltration anesthesia was performed into the gingiva adjacent to each buccal surface with Xylocaine (Fujisawa Co., Osaka, Japan) containing 1:80 000 epinephrine to control hemorrhage and exudate from the exposure site. Teeth were cleaned with a rubber cup and prophylaxis paste prior to operative procedures and the surrounding field was cleaned with 70% alcohol. Because the anesthetized monkey breathes via a mouth mainly, the sterilized cotton pellet has been used for isolation instead of the rubberdam. Class V buccal cavities were prepared using cylindrical diamond burs (ISO #109, GC Corp., Tokyo, Japan) with an air turbine under a saline spray, extending as deep as 3.0–3.5 mm without exposing the pulp tissue. Pulps were then exposed using a 0.8 mm diameter round carbide bur (ISO #001, GC Corp.). New burs were used for each tooth. After repeated and alternate irrigation with 3% hydrogen peroxide and 6% solution of sodium hypochlorite three times to remove cutting debris, hemostasis, and drying of the pulp exposure was performed with saline and sterilized cotton pellets. The exposed pulps were capped with one of the two capping materials.

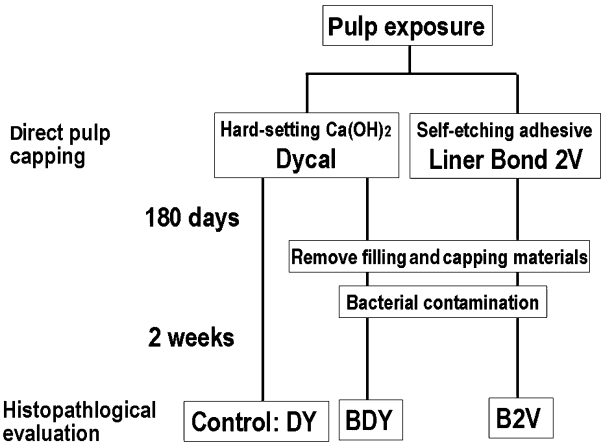

Fig. 1. Experimental procedure.

Self-etching adhesive resin system: Clearfil Liner Bond 2V
The cavity and exposed pulp were conditioned with a mixture of LB Primer A and B for 30 s, gently air dried, and then coated with a mixture of Bond A and B, which was light-cured for 20 s. A low viscosity resin composite, Protect Liner F (Kuraray Medical Inc.), was applied to the cavity walls and light-cured for 40 s. All cavities were restored to the cavosurface margin with a hybrid resin composite (Clearfil AP-X, shade A3; Kuraray Medical Inc.) and photo-cured for 40 s.

Hard-setting $Ca(OH)_2$: Dycal
Base and catalyst were mixed and directly applied on the exposed pulp. Effort was made to leave the cavity walls uncovered by the cement as far as possible. After the cement had set, the cavities were restored with Clearfil Liner Bond 2V and Clearfil AP-X in the manner described.

Bacterial challenge

At 180 days after capping, all samples had an X-ray photograph taken to check the dentin bridge formation and remaining dentin thickness. The resin composite was removed from both bacterial-contaminated groups using cylindrical diamond burs (ISO #109, GC Corp.) with an air turbine under a saline spray, extending as deep as 3.5–4.0 mm without new exposing the pulp tissue. After checking that there were no new exposures during removal of the resin composite, the cavities were left open to the oral environment for 2 weeks to obtain bacterial-contaminated samples.

Tissue preparation

At 14 days after cavity opening to the oral environment, the monkeys were killed by intravenous injection of 250 mg kg^{-1} thiopental sodium (Ravonal, Tanabe Pharmaceutical Co., Osaka, Japan). The teeth were extracted and immersed in 10% neutral-buffered formalin solution for 2 weeks. Before immersion, the mesial and distal approximal surfaces of the teeth were reduced with a high-speed diamond bur under spray coolant until the pulp became almost visible through the remaining dentin in order to facilitate the penetration of the fixative. After application of Plank-Rychlo's decalcifying solution at 4°C for 5 days, the teeth were embedded in paraffin. Serial sections 5 μm thick were cut through the cavities and pulp, obtaining approximately 80–100 sections per cavity. Sections were stained with hematoxylin and eosin for routine histopathologic evaluation or with Taylor's modification of Brown and Brenn technique (Gram's staining) for detecting microorganisms (20).

Inflammatory cell infiltration

It was classified into four grades: none, slight, moderate, and severe (21). No reaction was characterized by the absence of inflammatory cells. Slight reaction was characterized by scattering of a small number of inflammatory cells. Moderate reaction was characterized by a distinct presence of inflammatory cells. Severe reaction was characterized by small abscess formation or necrotic pulp.

Dentin bridge formation

All dentin bridging was evaluated by measuring the rate of dentin bridge formation in relation to the diameter of the exposed area. All dentin bridges were divided into four different types (no dentin bridging, partial dentin bridging, almost complete dentin bridging, complete dentin bridging).

Tunnel defects within dentin bridge

The tunnel defects represent the opening from the capping material interface to the underlying pulp (4). This was classified into two grades: absent or present.

Statistical analysis

One-way analysis of variance and Fisher's PLSD test were used to determine significant differences of the diameter of the exposed area among the three groups. The results of pulpal responses, presence and differentiation of dentin bridging were statistically analyzed by the Mann–Whitney *U*-test with Bonferroni's correction for difference between control and experimental groups. The rate of presence of tunnel defects in dentin bridges was tested by the chi-squared test. All statistical calculations were performed using SPSS statistical software program 10.01 (Chicago, IL, USA).

Results

For all experimental groups, there was no re-exposure case during removal of the resin composite at 180 days after the pulp capping. Findings on the histologic sections are summarized in Table 1. The diameter of the exposed areas generated in this study ranged between 0.3 and 1.3 mm with the mean values for the groups ranging from 0.68 to 0.72 mm in diameter. No significant differences were found among the diameters of the three experimental groups ($F = 0.33$; d.f. = 24,223; $P = 0.99$). Fig. 2 showed the representive bacterial-contaminated sample. The modified Brown–Brenn technique showed Gram-positive microorganisms inside the dentinal tubules.

No statistically significant difference was found in the formation of dentin bridges among all groups. Tunnel defects within the dentin bridge were detected in seven of 10 bridges in Group DY, eight of 10 bridges in Group BDY, and five of 10 bridges in Group B2V. However, there was no statistically significant difference in the presence of tunnel defects among all groups.

Group DY: non-bacterial-contaminated pulp capped with Dycal

Bacterial penetration along the cavity walls and pulp tissue was not detected in any case. No moderate or

Table 1. Results of the histopathologic findings

Code (n = 10)	Diameter of exposed areas (mm; mean ± SD)	Inflammatory cell infiltration				Dentin bridging				Tunnel defect (%)
		NO	SL	MO	SE	IDB	PDB	ACDB	CDB	
DY	0.68 ± 0.19	8	2	0	0	0	0	5	5	70
BDY	0.72 ± 0.20	3	2	1	4	0	0	6	4	80
B2V	0.69 ± 0.15	4	4	2	0	0	2	4	4	50

DY, Dycal as a control; BDY, bacterial-contaminated Dycal; B2V, bacterial-contaminated Liner Bond 2V; NO, none; SL, slight; MO, moderate; SE, severe; IDB, initial dentin bridging; PDB, partial dentin bridging; ACDB, almost complete dentin bridging; CDB, complete dentin bridging.

Fig. 2. Group BDY: the remaining hard-setting $Ca(OH)_2$ (DY) and bacterial contamination (BC) were detected on the cavity floor. The modified Brown–Brenn technique showed Gram-positive microorganisms inside the dentinal tubules (arrows) modified Brown–Brenn (200×).

severe inflammatory reaction of the exposed pulps was found for all specimens. A slight inflammatory cell infiltration was the main inflammatory reaction. Group DY showed a significantly lower presence of inflammatory cell infiltration than Groups BDY and B2V ($P < 0.05$).

Group BDY: bacterial-contaminated pulp capped with Dycal

Moderate or severe inflammatory cell infiltration was most commonly detected, and necrosis formation in four of 10 cases (Fig. 3a–c). Group BDY showed a significantly higher presence of inflammatory cell infiltration than Groups DY and B2V ($P < 0.05$). The modified Brown–Brenn technique showed Gram-positive microorganisms inside the tunnel defects (Fig. 3d). Accumulations of neutrophils were observed below the tunnel defect in the subjacent pulp tissue.

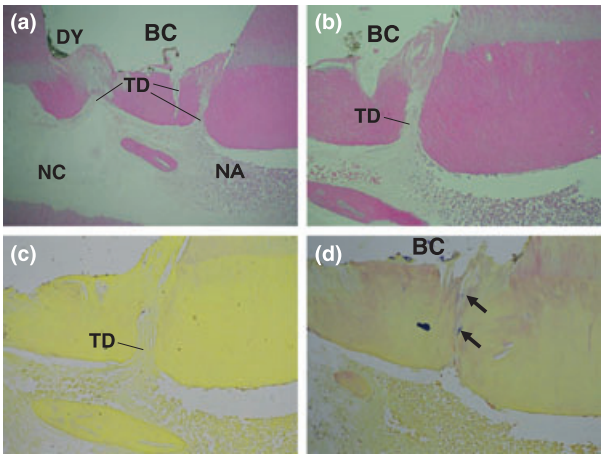

Fig. 3. Group BDY: (a) almost complete dentin bridging with multitunnel defects (TD). Necrosis formation (NC) and neutrophil accumulation (NA) were observed below the tunnels. (b and c) Another section of the same case of Fig. 4a. (d) The modified Brown–Brenn technique showed Gram-positive microorganisms inside the tunnel defects (arrows). (a) H&E (64×), (b) H&E (160×) and (c and d) modified Brown–Brenn (160×).

Group B2V: bacterial-contaminated pulp capped with Clearfil Liner Bond 2V

Group B2V showed a significantly lower presence of inflammatory cell infiltration than Group BDY ($P < 0.05$). Moderate inflammatory cell infiltration was detected below the tunnel defects (Fig. 4a,b). When the bonding agent remained, no moderate or severe inflammatory cell infiltration was observed in eight of 10 bridged specimens. Tubular dentin-rich hard tissue was found in six of 10 cases in B2V group (Fig. 4a,c).

Discussion

The dentin/pulp complex exhibits a variety of interrelated defensive reactions, such as reparative dentin formation and pulpal inflammation. Cox et al. (4) reported that 89% of dentinal bridges beneath a hard-setting $Ca(OH)_2$ contained multiple tunnel defects, which may serve as a pathway for bacterial contamination leading to 'slow' infection or become necrotic because of microleakage. In Group BDY, eight of 10 specimens (80%) showed tunnel defects. Group BDY showed moderate or severe inflammatory cell infiltration in 50%, and showed four necrotic specimens (40%). The untreated pulpal exposures created in laboratory animals showed evidence of tissue breakdown in response to the bacterial challenge (22). This pulpal breakdown might occur particularly if food debris and bacterial masses accumulated on the exposure site and blocked the potential for drainage (23). In the present study, a severe inflammatory response including pulpal breakdown occurred only in Group BDY. These findings supported the concept that bacterial microleakage from cavity margins and its products, is responsible for eventual pulpal inflammation (24).

From the results of this study, the presence of moderate or severe inflammatory cell infiltration is likely to be associated with the formation of tunnel defects. On the other hand, in all bacterial-contaminated cases in Group BDY, the extensive accumulations of neutrophils was crucial for resistance to bacterial invasion (18). In this position, neutrophils below the tunnels, are likely to block both diffusion of bacterial substances and invasion of bacterial organisms (25). The destructive effects of the phagocytic activity and the death of neutrophils must be regarded as crucial in the process of pulpal necrosis (6). The four of 10 cases in BDY group exhibited necrosis of the whole or partial subjacent pulp, suggesting that tissue necrosis was a direct effect of the bacterially induced acute inflammatory response.

Cleafil Liner Bond 2V (CLB 2V) system does not use phosphoric acid to etch the tooth surface, but uses the self-etching primer (pH 2.1) to dissolve the smear layer and allow hybridization of the tooth substrate. The low pH of the primers allows mineralized tissue to be

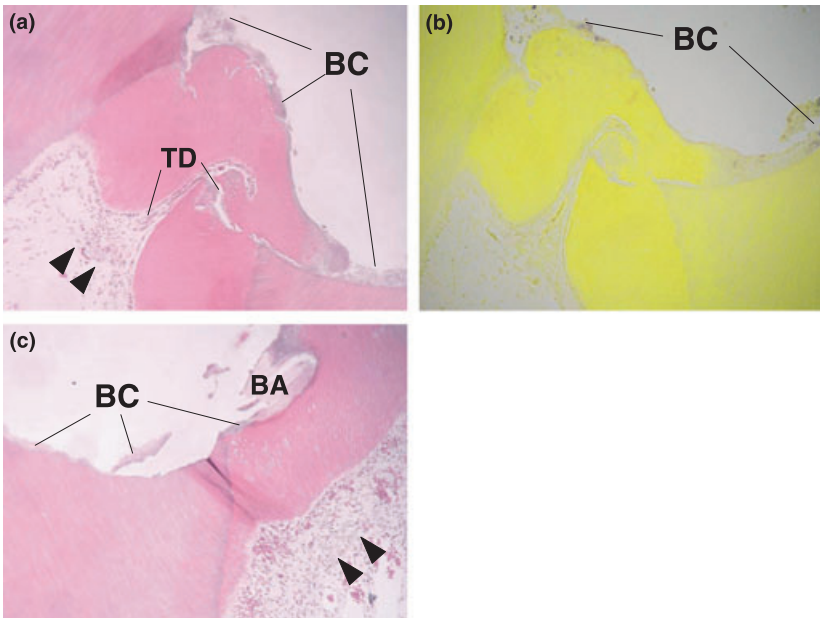

Fig. 4. Group B2V: (a and b) almost complete dentin bridging with tunnel defects (TD). Moderate inflammatory cell infiltration (arrowheads) with no remaining bonding agent was detected below the tunnel defect (TD). (c) Moderate inflammatory cell infiltration (arrowheads) with remaining bonding agent (BA) was detected. Bacterial contamination (BC). (a) H&E (64×), (b) modified Brown–Brenn (64×) and (c) H&E (64×).

conditioned and etched in a single treatment step, and all these primers are classified as mild self-etch systems based on relatively high pH values (15). Costa et al. (19) assessed the response of human pulps capped with CLB 2V bonding agent and demonstrated that this self-etching bonding agent released only small amounts of its components into the pulp space. Using the self-etching primer, the superficial tissue of the exposed pulp seemed to become 'fixed' and that prevented pulpal bleeding (11, 17). At the exposure site, hard-tissue repair developed, suggesting a low degree of tissue irritation from CLB 2V. It is important to point out that this result cannot be related to other adhesive system, as each adhesive system differs in its chemistry and complexity of application (26). Further studies are required to optimize the application protocols of adhesive systems to reduce the penetration of potentially cytotoxic monomers into pulpal tissue.

Although a hard-setting $Ca(OH)_2$ showed higher presence of dentin bridge formation than the self-etching adhesive system, 70–80% cases in Groups DY and BDY showed a tunnel defect in newly developed dentin bridges. These defects connected and permitted bacterial invasions to reach the vital pulp. Half of Group BDY showed moderate or severe inflammatory cell infiltration, meaning that hard-setting $Ca(OH)_2$ that is exposed to the oral environment could not seal the exposed pulp and the tunnel defects served as pathways for bacterial contamination. On the other hand, even though tunnel defects occur in dentinal bridges capped with the resin bonding, the underlying pulp showed no severe inflammation when the exposed pulp was completely sealed with the adhesive material. From a clinical perspective, the statistical difference between Groups DY and BDY is significant for the following reasons. Newly formed dentin bridges beneath a hard-setting $Ca(OH)_2$ are usually covered with a permanent restoration using acid etch/bonding agent/composite resin restoration. When the marginal sealing of that composite restoration is successful, the underling vital pulp beneath a hard-setting $Ca(OH)_2$ is equally well protected compared with those cases that are not covered with a hard-setting $Ca(OH)_2$. From the results of Group DY, bacterial penetration along the cavity walls and into pulp tissue was not detected in any case at 180 days after operation. However, this result should not be extrapolated to other self-etching adhesive systems. Using another self-etching system or the so-called 'all-in-one' adhesive systems as a direct capping and restoration material, one recent *in vivo* study reported that bacteria were detected in several cases (three of 72 cases) and in the superficial zone of the lateral cavity walls at 65 days after operation (27). Even under a hard-setting $Ca(OH)_2$, the success rate of the direct pulp capping depends on the quality and durability of the marginal seal of the exposed pulp and on the quality of the dentin bridge that has been formed. The direct capping with the two-step self-etching adhesive system may possibly provide a longer lasting bacterial barrier than a hard-setting $Ca(OH)_2$ even after dentin bridging. Although the Dycal and bonding agent were not removed from the cavities completely according to this experimental condition, the Dycal was easily washed out and dissolved in the BDY group (4, 7, 8) and this incomplete seal might allow bacterial leakage.

In conclusion, no significant histopathologic differences were observed on formation of dentin bridges between the hard-setting $Ca(OH)_2$ and the self-etching adhesive resin system. The self-etching adhesive resin system showed a significantly lower presence of inflammatory cell infiltration than the hard-setting $Ca(OH)_2$ against bacterial contamination following dentin bridging. When calcium hydroxide agent was used as pulp capping agents, this material was not supposed to seal the exposure site but to induce hard-tissue formation to induce hard-tissue formation. Bonding agent is supposed to seal the exposure site physically, and the remaining bonding agent on the cavities was effective as the barrier in the dentin bridges after bacterial challenges.

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