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ORIGINAL ARTICLE

Pulpal responses after application of current adhesive systems to deep cavities

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Abstract The aim of the present study was to evaluate comparatively the pulpal tissue reactions of four adhesive systems placed in experimental cavities of healthy dog's teeth. Class V cavities with a mean value of remaining dentin thickness (RDT) ranging between 0.55±0.30–0.68± 0.38 mm were prepared. The cavities were treated with the following adhesive systems: Etch and Prime 3.0 (EP), Single Bond (SB), Clearfil SE Bond (CSE), and Prompt L-Pop (PLP). The pulpal tissue responses to dentin adhesives were assessed histopathologically at postoperative periods of 7, 21, and 65 days, and the results were subjected to statistical analysis. A significantly greater adverse inflammatory response was observed with the materials EP and PLP, while a significantly lesser degree of disorganization in the odontoblastic zone was found with the materials SB and CSE, in the postoperative period of 65 days. In addition, a thicker predentin zone was observed where SB material was applied. Application of the selected adhesive systems to non-exposed cavities, with an RDT, which ranged between the above-mentioned rates, was correlated with slight to moderate inflammation and odontoblast reduction depending on the materials used as well as upon the RDT.

Keywords Adhesive systems · Pulpal responses · Inflammation · Odontoblast differentiation · Reparative dentin · Tertiary dentin

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Introduction

Adhesive dentistry is continuing to develop rapidly through the introduction of new adhesive systems. Improvements in adhesive resins and the development of one-application resin bonding systems have meant that recent bonding systems are not only reliable in conservative treatment but have also resulted in reducing the sensitivity of the technique [7, 24, 25]. Self-etch adhesives do not require a separate "etch and rinse" phase, as they contain acidic monomers that simultaneously condition and prime enamel and dentin [24, 25]. Recently, "all-in-one" adhesives that combine etching, priming, and conditioning into one solution have been brought onto the market [3, 8], thus reducing the number of steps required. Different generations of systems have been reported as producing a variable morphology of hybrid layers and shear bond strengths [5, 8, 14]. Resin tags may be formed when adhesive systems are applied after etching. The resin tag length seems to increase as the remaining dentin thickness (RDT) between the cavity floor and the pulp tissue thins out [28].

It has become generally accepted that iatrogenic injuries are sustained after cavity preparation and restoration with dental materials. Particular attention has been drawn to the chemical activity of resin-based restorative materials and the related adhesive systems. Components, which have been characterized as cytotoxic in many in vitro testing protocols,

are released from adhesive systems, and their diffusing through dentinal tubules can cause pulpal irritation [4, 10]. Thus, placement of these resin-based restorative materials can cause a wide spectrum of pulp-dentinal reactions [17]; however, it is known that pulpal hydrostatic pressure can limit the amount of diffusion of resin components through the dentin [2]. It is also worth noting that restorative materials with a three-step adhesive system have been reported to exert minimal pulp injury [12].

The aim of the present study was to evaluate comparatively the tissue reactions of the pulp-dentinal complex to four adhesive systems placed in experimental cavities of healthy dog's teeth.

Materials and methods

Three healthy 3-year-old male dogs, all from the same litter and with intact dentitions, were used for the present experimental work. The experimental protocol was conducted according to the ethical guidelines for animal care of the Research Committee, Aristotle University of Thessaloniki and approved by the Ethical Committee of the School of Dentistry. Each animal was sedated with an intramuscular injection of 1 mg/kg xylazine. General anesthesia was induced with an intramuscular injection of 6 mg/kg theopentone. The trachea was intubated, and general anesthesia was maintained using halothane (1.5–2.5%) in oxygen, delivered through a semi-closed breathing circuit, before the beginning of all experimental procedures.

Experimental procedures

Permanent maxillary and mandibulary molars, two rooted premolars, canines, and third incisors were selected for experimentation. All teeth were scaled and polished with a rubber cup on the day of the operative procedure. Quadrants of teeth were isolated using sterile cotton rolls, and saliva was controlled through high-speed evacuation.

Eighty-one class V cavities (approximately 2.50 mm wide and 3.00 mm long) were prepared on the buccal surface of teeth. A tungsten carbide pear-shaped bur was used (ISO #330 L SS. White, Lakewood, NJ, USA) at ultra-high speed with a copious water spray. A new bur was employed on every fourth cavity to avoid excessive heating [6]. Cavities were prepared according to the following protocols:

(1) The preparations were cut 0.5–1 mm above the free gingiva, parallel to the cemento–enamel junction (CEJ).
(2) Cavity preparations were cut into the dentin at a depth of 2.00 mm from the outer tooth surface.
(3) The floor of the cavity preparations was maintained curved and parallel to the outer buccal surface of the tooth.
(4) The cavities were washed with sterile saline and dried with air.

The class V cavities were randomly assigned to four experimental groups representing the four different adhesive systems tested. The cavities of the groups were restored using corresponding composite resins:

– Etch and Prime 3.0/Definite (EP; Degussa AG, Hanau Germany)
– Single Bond/Z-250 (SB; ESPE Dental AG, D-82229 Seefeld)
– Clearfil SE Bond/Clearfil AP-X (CSE; Kuraray Europe GmbH, Düsseldorf, Germany) and
– Prompt L-Pop/Z-250 (PLP; ESPE Dental AG)

In all cases, the manufacturers' instructions for adhesive and restorative procedures were strictly followed. The adhesive systems as well as their chemical composition are shown in Table 1. The materials were cured with a visible light source (Astralis 5 Vivadent Ets, Bendererstrasse2, Schaan/Liechtenstein) in accordance with the manufacturers' recommended times. The intensity of the light curing unit (approximately 600 mW/cm^2) was measured using a Cure Rite light intensity meter (Efos, Mississauga, Ontario Canada). Experimental procedures used on the three dogs were conducted in the same way. In the first operation, eight cavities were made in each animal (a total of 24 teeth) and were treated in pairs using the four different adhesive systems. Cavities were restored and remained in place for 65 days. After a period of 44 days had elapsed, a second operation was performed on each animal using the same procedure in eight different teeth (a total of 24 teeth). Finally, 58 days after the initial operative procedure, a third operation was conducted following the same protocol in eight different teeth in each animal (a total of 24 teeth).

In addition, another group was used as control. This group was divided into two categories. The first category of nine teeth comprised of cavities treated with the $Ca(OH)_2$-based material Dycal (DY; Caulk Lab, Milford, DE, USA) and filled with amalgam; the second category included three intact teeth.

The pulpal tissue responses to dentin adhesives were assessed at consecutive postoperative periods of 7, 21, and 65 days. On termination of the experimental periods, the animals were killed using an overdose of pentobarbital sodium. The teeth were then extracted and their roots immediately sectioned midway between the CEJ and the apex. The teeth were fixed in 10% neutral-buffered formalin solution for 2 weeks. The mesial and distal proximal surfaces of the teeth were reduced with a high-speed diamond bur under spray coolant. The specimens were then demineralized in Morse's solution (50% formic

Table 1 Composition of the adhesive systems used in this study according to information provided by the manufacturers

Material	Brand name	Manufacturer	Composition
One-step self-etch adhesive	Etch and Prime 3.0 (EP)	Degussa (Hanau,Germany)	Universal: water; ethanol; HEMA; photoinitiators; stabilizers Catalyst: pyrophosphate; HEMA; photoinitiators; stabilizers
Two-step etch-and- rinse adhesive	Single Bond (SB)	ESPE Dental AG (Seefeld, Germany)	HEMA; Bis-GMA; dimethacrylate; methacrylate functional copolymer of polyacrylic and polyitaconic acids; ethanol; water; photoinitiators
Two-step self-etch adhesive	Clearfil SE (CSE)	Kuraray (Europe Düsseldorf Germany))	SE primer liquid: HEMA; hydrophilic dimethacrylate; MDP; camphorquinone; diethanol-p-toluidine; water SE bond liquid: Bis-GMA; HEMA; hydrophobic dimethacrylate; MDP; camphorquinone; diethanol-*p*-toluidine; silanated colloidal silica
One-step self-etch adhesive	Prompt L-Pop (PLP)	ESPE Dental AG (Seefeld, Germany)	Methacrylated phosphoric acid esters; fluoride complex; stabilizer; parabenes; water; photoinitiator (BAPO)

BAPO bis-acyl phosphine oxide; *Bis-GMA* bisphenol A glycidyl methacrylate; *HEMA* 2 hydroxyethyl methacrylate; *MDP* 10-methacryloyloxydecyl dihydrogen phosphate

acid + 20% sodium citrate) for 2 months. Finally, the teeth were embedded in paraffin and serially sectioned at 7-μm thick. All sections were either stained with Mayer's hematoxylin–eosin stain to assess soft tissue organization and tertiary dentin formation or were subjected to modified Brown–Brenn's technique to detect the presence of Gram-positive and Gram-negative microorganisms.

All stained sections were evaluated, and the RDT was measured by means of a graticule between the cavity floor and the line of interface from the preoperative circumpulpal dentin to the postoperatively formed matrix [22]. The minimum RDT was estimated for every specimen. The 20 adjacent sections were analyzed.

Histological assessment

All sections were evaluated blindly for four histological features. The criteria used to assess the connective tissue reactions were as follows:

Inflammatory cell infiltration Inflammatory cell infiltration of the pulp tissue was classified as: *none*, absence of inflammatory cells; *slight to moderate*, a few scattered to numerous inflammatory cells; and *severe* masses of inflammatory cells or abscess formation.

Change in odontoblast layer The changes in odontoblast layer related to the cut dentinal tubules were classified as: *none*, no change in odontoblast layer; *slight to moderate*, mean reduction of odontoblasts of less than 50%; and *severe*, complete disorganization of the odontoblast layer.

Change in predentin zone The changes in the predentin zone related to the cut dentinal tubules were evaluated and classified as: *none*, unchanged morphology of dentin–predentin–odontoblast layer; *increase in thickness*, the presence of an inhomogeneous zone of postoperatively formed tertiary dentin matrix beneath the axial wall; *decrease in thickness*, a continuous thin zone of postoperatively formed matrix; and *absence*, absence of postoperatively formed matrix.

Bacterial Infiltration Presence of bacteria along the cavity walls or within the cut dentinal tubules was characterized as dentin-positive bacterial infiltration.

The data were submitted to Kruskal Wallis test and Mann–Whitney *U* tests (using a Bonferoni adjustment) and a two-way analysis of variance (ANOVA).

Results

The minimum RDT was compared between the groups. Means and standard deviations of the RDT in each group of teeth are given in Table 2. The minimum RDT ranged between 0.20 to 1.2 mm. Levene's method was used to test the null hypothesis that the error variance of the dependent variable (RDT) is equal across the groups. The tested hypothesis was not rejected ($p=0.254$). In addition, a two-way ANOVA was conducted to compare the mean values of the RDT between the group of teeth ($p=0.729$), the mean values between the three levels of time ($p=0.829$), and any

Table 2 Mean value in μm of remaining dentin thickness and standard deviation in specimens treated with the adhesives systems for the three observation periods

Groups of teeth	*N*			Mean value of RDT	Standard deviation
	7 days	21 days	65 days		
EP	6	6	6	0.55	0.30
SB	6	6	6	0.6	0.32
CSE	6	6	6	0.68	0.38
PLP	6	6	6	0.58	0.34
Dycal	2	2	2	0.52	0.23
Intact teeth	1	1	1		

interaction between them (p=0.955). No main effect or interaction between these factors was found to be significant.

In bacterial infiltration assessment, neither Gram-positive nor Gram-negative bacteria were identified on the cavity floor, along the cavity walls, or within the cut dentinal tubules in any of the teeth operated on. The results regarding inflammatory cell infiltration, changes in odontoblast layer, and predentin zone are given in Table 3 and illustrated in Figs. 1, 2, 3, and 4.

Inflammatory cell infiltration

In general, severe inflammatory cell infiltration was found in only two specimens. Within the total number of teeth used with the tested materials, significant differences were observed between the materials SB and PLP (p=0.008, SB was found more acceptable). Significant statistical differences concerning the severity of inflammatory cell infiltration were only seen in the postoperative period of 65 days for the materials EP and SB or CSE (p=0.001, SB and CSE were found more acceptable). No inflammatory cell response was found in any of the specimens treated with the $Ca(OH)_2$-based material DY.

Changes in odontoblast layer

In general, severe disorganization of the odontoblast layer was only seen in a number of specimens using the strong adhesive materials EP and PLP. A significantly smaller degree of disorganization in the odontoblastic zone was found for the materials SB and CSE. Within the total number of teeth used, significant differences were found between the materials EP and SB (p=0.011), EP and CSE (p=0.001), and CSE and PLP (p=0.004). Significant statistical differences concerning the changes in odontoblast layer were also found in the postoperative period of 65 days between the materials EP and SB (p=0.009) and CSE (p=0.002). In addition, results showed that there were no differences in the control group teeth treated with the DY as far as tissue disorganization in the observation periods was concerned.

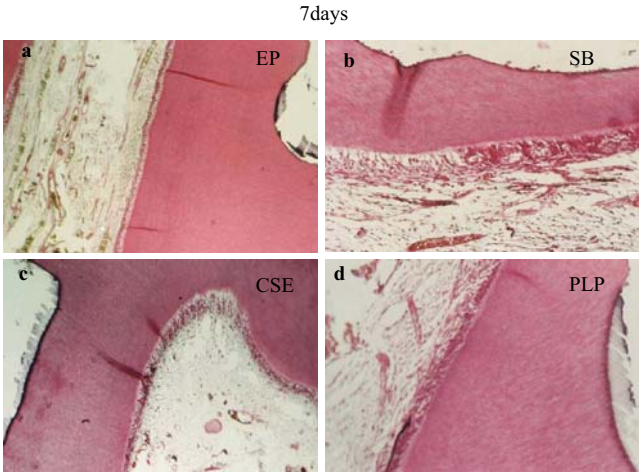

Fig. 1 Observation period 7 days. **a** Treatment of the cavity with Etch and Prime 3.0; RDT 650 μm. Absence of inflammatory cell infiltration and unchanged morphology of the dentin–predentine–odontoblast layer zone (H/E, ×40). **b** Treatment of the cavity with Single Bond; RDT 240 μm. Severe disorganization of the odontoblast layer. Absence of inflammatory cell infiltration (H/E, ×40). **c** Treatment of the cavity with Clearfil SE Bond; RDT 700 μm. Absence of inflammatory cell infiltration and slight reduction of odontoblasts (H/E, ×40). **d** Treatment of the cavity with Prompt L-Pop; RDT 500 μm. Absence of inflammatory cell infiltration and slight to moderate reduction of odontoblasts (H/E, ×40)

Changes in predentin zone

In general, most of the specimens exhibited unchanged morphology of the predentin zone. Increased thickness of the predentin zone was seen in most teeth treated with the material SB for 65 days and with the DY for 21 and 65 days. Within the total number of teeth used, no significant differences were found concerning the predentin zone for the four materials used (p=0.120).

Discussion

The most important variables in the development of pulpal injury after cavity preparation and restoration in animal testing concern the type of restoration material used

Table 3 Frequency of scores for each group of teeth 7/21/65 days after treatment of cavities with the test materials

Histopathological findings									
Groups of teeth	Inflammatory cell infiltration			Change in the odontoblast layer			Change in predentine zone		
	None	Slight/moderate	Severe	None	Slight/moderate	Severe	None	Increase	Decrease/absence
EP	5/5/0	1/1/6	0/0/0	1/2/0	3/2/4	2/2/2	5/5/3	0/0/0	1/1/3
SB	4/5/6	2/1/0	0/0/0	2/3/5	3/3/1	1/0/0	2/5/0	0/1/5	4/0/1
CSE	5/4/6	1/1/0	0/1/0	2/4/6	4/2/0	0/0/0	3/5/3	1/0/0	2/1/3
PLP	3/1/2	2/5/4	1/0/0	0/2/2	4/2/2	2/2/2	5/1/2	0/1/0	1/4/4
DY	2/2/2	0/0/0	0/0/0	2/2/2	0/0/0	0/0/0	2/0/0	0/2/2	0/0/0

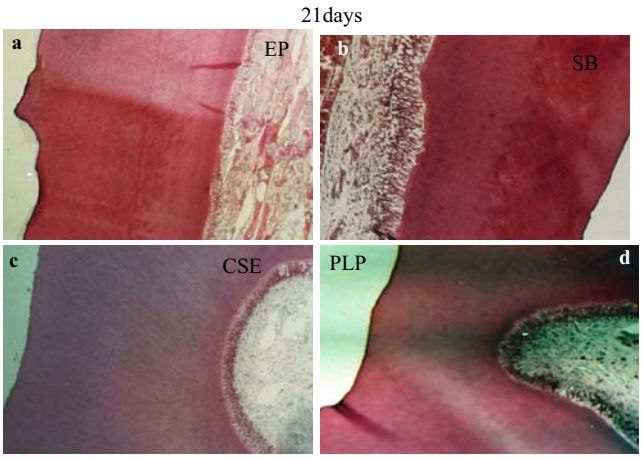

Fig. 2 Observation period 21 days. **a** Treatment of the cavity with Etch and Prime 3.0; RDT 1,000 μm. Hyperemic pulpal vessels and slight to moderate reduction of odontoblasts. Absence of postoperatively formed matrix (H/E, ×40). **b** Treatment of the cavity with Single Bond; RDT 800 μm. Absence of inflammatory cell infiltration and unchanged morphology of the dentin–predentin–odontoblast layer zone(H/E, ×40). **c** Treatment of the cavity with Clearfil SE Bond; RDT 1,200 μm. Absence of inflammatory cell infiltration and unchanged morphology of the dentin–predentin–odontoblast layer zone (H/E, ×40). **d** Treatment of the cavity with Prompt L-Pop; RDT 1,100 μm. Absence of inflammatory cell infiltration and unchanged morphology of the dentin–predentin–odontoblast layer zone (H/E, ×16)

(chemical composition and physical properties-sealing capacity) as well as the RDT of the cavity [1, 19, 20].

The present experiments confirmed the results of previous animal studies on the relation between the specific chemical

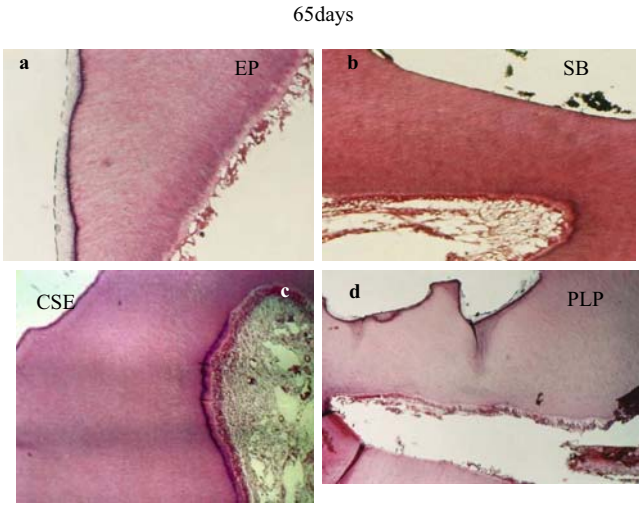

Fig. 3 Observation period 65 days. **a** Treatment of the cavity with Etch and Prime 3.0; RDT 200 μm. Total pulpal necrosis. (H/E, ×40). **b** Treatment of the cavity with Single Bond; RDT 600 μm. Absence of inflammatory cell infiltration with moderate disorganization of odontoblast layer. A thin zone of postoperatively formed predentin. (H/E, ×16) **c** Treatment of the cavity with Clearfil SE Bond RDT 300 μm. Tertiary dentin formation beneath the cavity (H/E, ×40). **d** Treatment of the cavity with Prompt L-Pop; RDT 450 μm. Total pulpal necrosis (H/E, ×16)

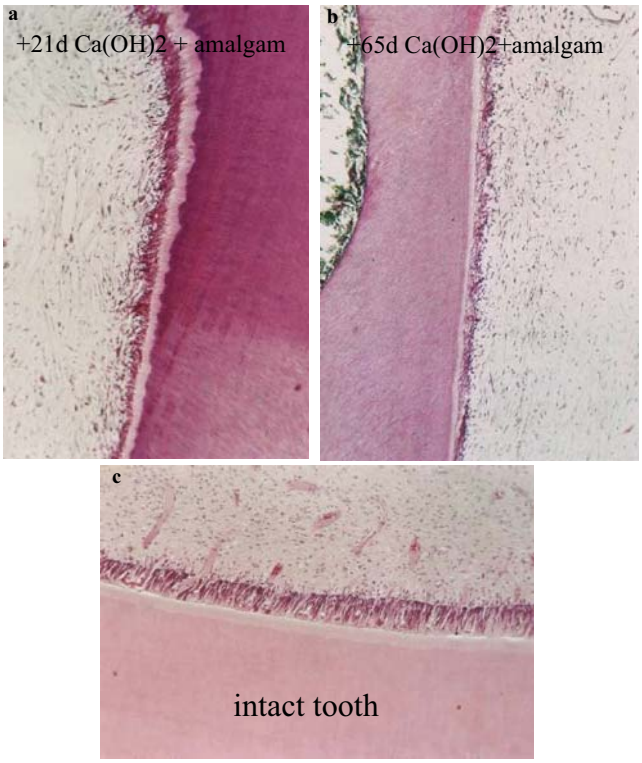

Fig. 4 Control groups **a**, **b** Treatment of the cavity with Dycal for 21 and 65 days, respectively; RDT ~500 μm. Absence of inflammatory cell infiltration and unchanged morphology of the odontoblast layer zone. A thin zone of postoperatively formed predentin (H/E, ×40). **c** Non-operated dog tooth. The typical morphology of dentin–predentin–odontoblast layer zone (H/E, ×40)

composition of the adhesive system and the severity of pulp-dentinal injury [1, 12, 18, 26]. The first goal in the present study was to evaluate comparatively the four adhesive systems to determine the most aggressive one.

Placement of these testing materials in non-exposed cavities of healthy dog's teeth was correlated with slight/moderate pulp inflammation when the strong self-etching adhesive systems were used. In addition, testing materials EP and PLP showed severe odontoblast reduction of the treated cavities in all three postoperative periods.

The adhesive bonding system PLP created the greatest inflammatory cell infiltration and severe odontoblast changes with statistical significant differences from pulps that were treated with SB. Differences between these two materials may be attributed to their pH, to their chemical composition, and to their different polymerization rate. The strong self-etch adhesives have been documented as having an interfacial ultra-morphology of dentin resembling that produced typically by total-etch adhesives [28]. This study does not confirm that. PLP, with a pH of 0.4, belongs to the category of strong self-etch adhesives and its high acidity results in rather deep demineralization effects [28, 30]. At the dentin level, collagen is exposed, and nearly all of the hydroxyap-

atite is dissolved [29]. As with the self-etch adhesive PLP, the total-etch adhesive SB causes deep demineralization of the dentin. Then, as previously demonstrated in the literature [5, 9, 12], the application of bonding agents, including SB [6], after dentin conditioning allows inward diffusion of residual adhesive components to reach the pulpal space. Acidic monomers in PLP consist of methacrylated phosphoric acid mono and diesters (in which the phosphoric acid and methacrylate group are combined into one molecule) that etches and primes simultaneously. There is a possibility that the more highly acidic and hydrophilic resin monomers may deeply penetrate not only intertubular dentin but also water-filled tubules. The water may interfere with acidic monomer polymerization. Nevertheless, unpolymerized acidic monomers in the water-filled tubules would retain their acidity and continue to etch the surrounding dentin [31]. The continued demineralization of the dentin tubules then causes the dentin portion to lose its structural integrity. This hypothesis has been affirmed by the study of Wang and Spencer [30, 31]. Thus, the more unpolymerized acidic monomers related to, the more residual adhesive components manage to reach the pulpal space.

It may also be that differences in chemical composition result in the different polymerization rate. The self-etch adhesive PLP is a unique light-curable adhesive in that it utilizes bisacylphosphine oxide (BAPO) as a photoinitiator that does not require a tertiary amine accelerator as in conventional camphorquinone-containing systems. Results reported by Pashley et al. [20] illustrated that PLP was not completely polymerized on dentin in their study. Incomplete polymerization of the PLP results in the presence of a high amount of uncured free monomer that can easily diffuse across the dentinal tubules to cause pulpal damage. All these mechanisms exacerbate diffusion across the dentin and reach the pulp, triggering a local inflammatory reaction, or exacerbating existing inflammation due to cavity preparation trauma and help to explain the greatest inflammatory cell infiltration that the adhesive bonding system PLP created from pulps that were treated with SB. The aggressive reactions observed with PLP and, in a few cases, with EP seem to be related to long resin tag formation and the diffusion of resin components along the dentinal tubules to reach the pulp space. The good results that were revealed in the dentin/pulp complex when the dentin was treated with CSE could be attributed to the characteristics of the mild self-etch systems [28, 30]. Mild self-etch adhesives demineralize dentin only in a very shallow manner, leaving hydroxyapatite crystals around the collagen fibrils. Usually, the smear plug is not completely removed from the dentin tubule. As a result, a shallow hybrid layer is formed with submicron measurements. CSE, with an acidity pH 1.9, is classified as a ‘mild’ two-step self-etching adhesive, and it produces a hybrid layer only to a depth of a 1-μm thick [13, 28], a depth which is associated with the formation of short resin tags. This superficial demineralization occurs only partially, keeping residual hydroxyapatite still attached to the collagen [29]. The preservation of hydroxyapatite may serve as a receptor for additional chemical bonding [27]. In addition, the hydroxyapatite crystals, which remain around the collagen, are considered to be particularly advantageous for a more intimate chemical interaction with the functional monomers on a molecular level [28]. Keeping hydroxyapatite around the collagen may also offer better protection for the collagen against hydrolysis and, thus, early degradation of the bond [11, 21]. In addition, within the limitations of this study, it is possible that the mild self-etch systems may serve as a trigger to stimulate biological processes, which could lead to specific odontoblast activity and reactionary dentin formation (Fig. 3c).

It is known that the sealing capacity of adhesive restorative systems is postulated to be among the most important variable in mediating pulp inflammation. However, such low-pH self-etch adhesives have often been documented as having rather low bond-strength values, especially at the dentin level [11, 15], thus offering poor dentinal sealing. Besides the initial high acidity, which appears to weaken the bonding performance dramatically, another concern is the residual water that remains within the adhesive interface, which cannot be completely removed [23]. Although EP is classified as a strong pH (0.6) self-etch adhesive, it contains high vapor–pressure solvent (ethanol) to remove residual water, and this probably enhances bonding. Concerning the link with PLP mentioned previously, results reported by Pashley et al. [20] illustrated that PLP was not completely polymerized on dentin in their study. Incomplete polymerization of the hybrid layer probably hastens the leaching of resin components, challenging the durability of the rather low resin–dentin bond.

It has been observed in human teeth that pulpal injury (reduction in the number of odontoblasts and suppression of their biosynthetic activity) increases as the RDT decreases [18]. The protocol used in the present study for cavity preparation did not allow us to standardize the RDT, which ranged between 0.2 and 1.2 mm. For this purpose, the data were submitted for statistical analysis to test whether the variance in this crucial parameter was equally distributed across the four materials tested.

Histological assessment of the total number of specimens showed that when the RDT was less than ~500 μm, postoperative time had an adverse effect on healing regarding the inflammatory cell infiltration and changes in odontoblast layer. The opposite was noted when the RDT ranged between 600 to 1,200 μm.

In addition, in the control group treated with the Dycal, no differences were detected regarding the inflammatory cell response and tissue disorganization. Further, with

regard to predentin, a thin zone of postoperatively formed predentin was observed on the 21- and 65-day postoperation periods. As it was expected, the pulp vitality and odontoblastic function appeared to be maintained after dentine treatment with the $Ca(OH)_2$-based material.

Reactionary dentine is laid down by primary odontoblast cells, in response to specific stimuli, but it is not enough to kill those primary pulp cells [16]. Consequently, the reactionary dentine deposition seems to be the result of slight to moderate irritation and can be inhibited when severe irritation is present. The presence of this tissue is an indication of the variable degrees of irritation promoted by the dentine adhesive system tested, which is capable of causing synthesis as well as deposition of a reactionary dentine matrix. Thus, in the present study, this tissue appeared only with the dentine adhesive system CSE, which is classified as a mild two-step self-etch adhesive and with SB, which is classified as a one-bottle total-etch adhesive. As mentioned previously, the other dentine adhesive systems EP and PLP are classified as strong one-step self-etch adhesives.

Conclusions

1. The application of contemporary adhesives in deep non-exposed cavities is not correlated with severe pulp inflammation or tissue necrosis.
2. The mild self-etch approach may be most promising in terms of biocompatible bonding to dental hard tissue.

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