Adverse effects of human pulps after direct pulp capping with the different components from a total-etch, three-step adhesive system

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Biocompatibility; Pulp therapy; Adhesive system; Calcium hydroxide; Resin monomer; Primer; Cytotoxicity

Summary  Objectives. The objective was to evaluate the response of human pulps capped with different components from a total-etch three-step adhesive system.

Methods. Direct pulp capping was performed in 25 caries-free human premolars scheduled for extraction due to orthodontic treatment. The teeth were randomly divided in five groups, and capped with the following materials: Group 1—acid primer + adhesive were used as recommended; Group 2—only primer was applied; Group 3—only bonding resin (light-cured for 10 s); Group 4—only composite resin (light-cured for 40 s); Group 5—calcium hydroxide. After capping, all teeth were restored with ScotchBond Multi Purpose Plus and Z-100 was placed incrementally. After 60 days, the teeth were extracted and processed for light microscopic examination (H/E) according to a histological score system. These were subjected to non-parametric tests ($\alpha <0.05$).

Results. Overall, the histological features showed that groups 1-4 were quite similar and inferior to group 5. In groups 1-4 the pulp response varied from acute inflammatory cell infiltrate with varying degrees to necrosis. The groups 3 and 4 showed a trend towards better pulp response, since a normal connective tissue could be observed in more than half of the sample. All teeth from group 5 showed normal connective tissue below an amorphous dentin bridge.

Significance. Adhesive components (primer or adhesive) as well as a composite should be avoided for pulp capping. Ca(OH)$_2$ should be the first choice for pulp capping.

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**Introduction**

The advent of adhesive dentistry has broadened the range of possibilities of using resin-based materials for a wide variety of clinical applications in dentistry. Amongst them, the possibility of direct pulp capping with bonding agents has increased. Measuring the biocompatibility of a material is not simple, and the methods of measurement are evolving rapidly as more is known about the interaction between dental materials and oral tissues and as technologies for testing improve [1]. The American Dental Association and American National Standards Institute (ADA/ANSI) have recommended a sequence of biocompatibility tests of dental materials. This sequence consists of 'initial', 'secondary', and 'usage' tests [2] in that order. However, usage tests in humans, as the present investigation, are the most important ones since they provide direct information about the biocompatibility of dental materials.

It is likely that resin components produce more detrimental effects to human pulps than they do in monkeys and other animals. When bonding agents are applied over human pulps, no sign of any mineralized tissue formation and odontoblast-like cell differentiation is observed [3]. The observation of chronic inflammation with the presence of macrophages and giant cells adjacent to the pulp exposure site is common, and also necrosis of connective tissue. Foreign body reaction is also evident around particles of resin found dispersed within the pulp [4-6]. This chronic inflammatory response seems to preclude dentin bridge formation [4-8].

A chronic inflammatory response may be directly related to the cytotoxic effects of the bonding agent’s components. Acid products, from cements or even etchants, were blamed for moderate to severe pulp inflammation observed in human pulps when applied on dentin [9-12]. These studies were reviewed [13] and the role of bacteria in detrimental pulpal responses started being emphasized. Also, acid etching is a short procedure that is completely rinsed off by water and buffered by surrounding dentin and/or dentinal fluids [14,15]. Therefore, the rationale behind the use of dentin bonding materials instead of the commonly used calcium hydroxide compounds has been that prevention of leakage by micromechanical interlocking of adhesive systems to cavity walls is more critical for the healing processes than the material used for capping [16,17].

Besides the acid etchants, the total-etch, three-step adhesive systems contain other components, which are shared between the primer and adhesive bottles. After acid etching, a significant increase in dentin permeability due to smear layer removal and opening of dentinal tubules can facilitate the ingress of resin monomers towards pulp, mainly in deep dentin [17-20].

Monomers like Bis-GMA, HEMA, UDMA, TEGDMA, initiators and solvents were already associated to cytotoxic effects on fibroblast and odontoblast-like cells [21-27]. All these components individually or in combination ultimately compromise the pulp healing as reported by several studies [4-8]. To date, no study has addressed the individual role of each bottle (primer and adhesive) on the pulp response of human teeth. It is likely that if one of these components were more of a problem, pulp capping with adhesive systems could be successfully performed by simply excluding that component from the capping procedure. Therefore the aim of this study was to address the individual role of each adhesive component (bottles) on the pulp response of human teeth.

**Material and methods**

Twenty-five human premolars scheduled to be extracted for orthodontic reasons were selected from patients ranging from 15 to 25 years old. All teeth were clinically and radiographically examined to assure absence of proximal caries and periapical lesions. The patients and their parents signed consent forms after receiving a thorough explanation about the experimental rationale, clinical procedures and possible risks. The parents and adult volunteers were asked to read and sign a consent form allowing the clinical procedure. Both, the consent form and the research protocol were performed according to the Human Subject Review Committee from the University of São Paulo, Brazil.

For the thermal testing, ENDO-ICE frozen gas (Cöltène/Whaledent Inc., Mahwah, NJ) was applied for 5 s on the buccal surface of the teeth scheduled for the pulp therapy, and adjacent teeth. After local anesthesia (Citanest 3%; Merrel Lepetit, São Paulo, Brazil) rubber dam isolation was installed and each tooth was pumiced with rubber cup at low speed. Mesio-occlusal cavities, with 0.5 mm beyond the cementum–enamel junction, were prepared by means of sterile diamond burs (# 1095, KG Sorensen, Barueri, São Paulo, Brazil) at high speed under water/spray coolant. The dimensions of the cavity were approximately: occlusal depth, 3.0 ± 0.2 mm; axial depth, 4.0 ± 0.5 mm; proximal faciolingual width, 3.0 ± 0.2 mm. Pulp exposure...
was performed in the center of the pulpal floor by means of a round diamond bur under water cooling (#1014, ϕ 1.2, KG Sorensen, Barueri, São Paulo, Brazil). One bur was used for each cavity. The teeth were then divided into five experimental groups (n=5) as shown in Fig. 1. All materials employed are described in Table 1.

The pulp hemorrhage was controlled by abundant irrigation with saline solution followed by the application of a damp cotton pellet embedded in saline solution and held in place for 1 min.

In group 1, a metal matrix band was installed and enamel, dentin and pulp were conditioned with phosphoric acid for 20 s. The acidic agent was rinsed off, slightly dried in such way that the dentin stayed visibly moist with a shiny surface. Two coats of the primer were applied and air-dried for 20 s. The adhesive was subsequently applied and light-cured for 20 s at 450 mW/cm² (Ultralux electronic, Dabi Atlante, Ribeirão Preto, SP, Brazil). Incre- ments of Z-100 (3M ESPE, St. Paul, MN, USA) were used to restore the cavities. Each increment was light-cured for 40 s. A radiometer (Model 100P—Demetron Research Corp., Kerr, Danbury, CT, USA) was used to check the light intensity immediately before each clinical section. When necessary, the material excesses were removed using an ultra-fine diamond bur at high speed and water cooling (KG Sorensen, Barueri, SP, Brazil).

In group 2, one coat of the primer solution from ScotchBond Multi Purpose Plus system was applied and air-dried for 10 s. Immediately after, a 2 mm increment of Z100 was placed over the primer and light-activated for 40 s. In group 3, only the adhesive from the ScotchBond Multi Purpose Plus system was applied, followed by light curing for 10 s. Over this layer, a 2 mm thick composite resin increment was placed and light-cured for 40 s. In group 4, over the exposure site, one increment of Z100 was applied (liner), followed by light-curing for 40 s. Care was taken to avoid composite resin insertion in the pulp chamber in all groups. Then, the cavities from groups 2-4 were restored as already described for group 1 (Fig. 1).

In group 5, calcium hydroxide powder was applied on pulp exposure by means of an amalgam carrier after homestasis (group 1). Calcium hydroxide cement (Dycal, Dentsply, Petropólis, RJ, Brazil)

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**Table 1  Products, commercial name and composition.**

<table>
<thead>
<tr>
<th>Product/commercial name</th>
<th>Composition</th>
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| ScotchBond Multi Purpose Plus (3M ESPE, St. Paul, MN, USA) | 1. Etchant: 35% phosphoric acid  
2. Primer: water (40%), HEMA (47%) and polialkenoic acid copolymer (13%)  
3. Adhesive: Bis-GMA (65%), HEMA (34%) and initiators/accelerators (1%) |
| Z-100 (3M ESPE, St. Paul, MN, USA) | Bis-GMA, TEGDMA and silica/zirconium filler |
| Calcium hydroxide powder Labrynth Prod., Diadema, SP, Brazil | Ca(OH)₂ |
| Saline solution Labrynth Prod., Diadema, SP, Brazil | 2% NaCl |
was applied in the occlusal cavity floor. Then the restorative procedure was conducted as described in group 1 (Fig. 1).

The observation period was adapted to the orthodontics treatment plans. During 60 days, the patients were frequently asked about the presence of sensitivity. After this period the extraction of each tooth was performed under local anesthesia. Although 60 days is not usual in biocompatibility tests, several studies have demonstrated a great percentage of dentin bridge formation in human teeth capped before 60 days [6,8,28].

The teeth had their roots sectioned in about 2 mm in order to facilitate fixation in 10% buffered formalin solution for 72 h. They were decalcified in 20% formic acid for 6-8 weeks, prepared according to normal histological techniques and embedded in paraffin. Six-micron thick sections were cut with a microtome parallel to the main vertical axis of the tooth. The sections, mounted on glass slides were stained with hematoxylin and eosin (H/E). The sections were blindly evaluated by an experienced pathologist according to the criteria described in Table 2. The scores attributed to each group were subjected to non-parametric Kruskal-Wallis. This test is considered very powerful for several independent samples [29]. Means were considered significantly different when \( \alpha < 0.05 \) [29]. The correlation between the histological features (scores 1-4) and the frequencies of pain for each group was performed by a Spearman test \( (\alpha < 0.05) \). This test was performed considering the histological features ranks (1-5) for the five groups and the corresponding ranks of the frequencies of pain.

### Results

The percentage of scores observed for each group is shown in Table 3. Overall, the histological features from groups 1-4 were quite similar and inferior to group 5 (Fig. 2(a)-(e)). No correlation between the histological scores and the frequencies of pain was observed \( (p \geq 0.05) \).

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Scores used during the histological exams.</th>
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<tbody>
<tr>
<td>Scores</td>
<td>Inflammatory cell response</td>
</tr>
<tr>
<td>1</td>
<td>None or a few scattered inflammatory cells</td>
</tr>
<tr>
<td></td>
<td>present in the pulp beneath the exposure</td>
</tr>
<tr>
<td></td>
<td>site</td>
</tr>
<tr>
<td>2</td>
<td>Polymorphonuclear leukocytes (acute) or</td>
</tr>
<tr>
<td></td>
<td>mononuclear lymphocytes (chronic) in an</td>
</tr>
<tr>
<td></td>
<td>inflammatory lesion</td>
</tr>
<tr>
<td>3</td>
<td>Severe inflammatory lesion appearing as an</td>
</tr>
<tr>
<td></td>
<td>abscess or dense infiltrate involving one</td>
</tr>
<tr>
<td></td>
<td>third or more of the coronal pulp</td>
</tr>
<tr>
<td>4</td>
<td>Completely necrotic pulp</td>
</tr>
<tr>
<td>Scores</td>
<td>Soft tissue organization</td>
</tr>
<tr>
<td>1</td>
<td>Normal or almost normal tissue morphology</td>
</tr>
<tr>
<td></td>
<td>below the exposure site and throughout the</td>
</tr>
<tr>
<td></td>
<td>pulp</td>
</tr>
<tr>
<td>2</td>
<td>Lack of normal tissue morphology below the</td>
</tr>
<tr>
<td></td>
<td>exposure site, with deeper pulp tissue</td>
</tr>
<tr>
<td></td>
<td>appearing normal</td>
</tr>
<tr>
<td>3</td>
<td>Loss of general pulp morphology and</td>
</tr>
<tr>
<td></td>
<td>cellular organization below the exposure</td>
</tr>
<tr>
<td></td>
<td>site</td>
</tr>
<tr>
<td>4</td>
<td>Necrosis in at least the coronal third of</td>
</tr>
<tr>
<td></td>
<td>the pulp</td>
</tr>
<tr>
<td>Scores</td>
<td>Dentin bridge formation</td>
</tr>
<tr>
<td>1</td>
<td>New barrier tissue directly adjacent to</td>
</tr>
<tr>
<td></td>
<td>some portion of the restorative material</td>
</tr>
<tr>
<td>2</td>
<td>New dentin bridge some distance from the</td>
</tr>
<tr>
<td></td>
<td>material interface</td>
</tr>
<tr>
<td>3</td>
<td>No evidence of any dentin tissue formation</td>
</tr>
<tr>
<td></td>
<td>in any of the tissue sections</td>
</tr>
</tbody>
</table>

(1) **Inflammatory infiltrate**: An intense and chronic inflammatory response was observed in groups 1 (80%) and 2 (100%). In 60% of the cases from groups 3 and 4 and 100% from group 5, none, or a few scattered inflammatory cells were presented in the pulp beneath the exposure site. Completely necrotic pulp was observed in 40% of the cases from group 3 and 20% from group 4.

(2) **Soft tissue response**: A connective tissue with no signs of normality, below the exposure site, was seen in most cases from group 1 (80%) and group 2 (100%). The majority of the teeth from groups 3 and 4 (60%) showed normal or quite normal connective tissue morphology below the exposure site and throughout the pulp. In group 3, the formation of a 'hybrid layer' was noticed between the adhesive and pulp tissue. Pulp necrosis occurred in 40 and 20% of
specimens from group 3 and 4, respectively. In group 5, a loose connective tissue with several blood vessels delineated by an odontoblastic layer in the pre-dentin, was seen in all specimens.

3) **Dentin bridge formation**: There was no evidence of any dentin tissue formation in any of the tissue sections in specimens from groups 1–4. In group 5, an amorphous dentin bridge formation with irregular contour and varied mineralization degree was observed in all specimens. Along with the dentin bridge, a similar odontoblastic layer was also observed.

4) **Pain**: Pain occurred in 40% of the cases in group 1, 40% from group 3 and 20% of specimens from group 4. In group 3, two teeth had to be avulsed before the 30-day period due to severe pain.

### Discussion

The present investigation did not aim to stain bacteria because they cannot be stained very well with the Brown and Brenn technique. Added to this, many bacteria are lost during tissue processing due to the histologic procedure: with fixation and decalcification, the staining ability of bacteria may be reduced [30] or they may adhere to the restoration that is removed during the process [31].

A large number of studies have reported that as long as bacteria are not present, bonding agents can be successfully used for pulp capping [32–35]. It is interesting to note, however, that most of the articles reporting acceptable biocompatibility of adhesive agents were conducted either in monkeys or rodents [36]. Although animal studies, in general, are necessary and their results have generated much valuable information, a certain caution must be exercised if results from usage tests in animals are extrapolated to humans [37].

Therefore, the results of the present investigation confirm that all components presented in the adhesive solutions as well as the composite resin, individually or in combination may result in degenerative pulp alterations when placed directly over pulp exposure sites. This is in agreement with several studies where pulp capping was performed with adhesive systems over human pulps [4–8].

As seen in Table 1, the main constituents of primer and adhesive solution are HEMA and Bis-GMA, respectively. The composite resin used has a mixture of Bis-GMA and TEGDMA. In a ranking of toxicity HEMA, which is the main component of primer, is the least toxic substance in comparison with other monomers such as Bis-GMA, UDMA and...
TEGDMA after 24 and 72 h exposures [22]. Based on such findings, one could expect worse results from groups 3 and 4, where moieties with high molecular weight are present. However, contrary to the expected, groups 1 and 2 showed a trend towards worse pulp response than groups 3 and 4. The high percentage of HEMA in the primer, its lack of polymer conversion due to the absence of photo-initiators, its low molecular weight and hydrophilic features, facilitate its diffusion through pulp tissue and consequently avoid pulp healing [38].

When monomers are polymerized, as occurred in groups 3 and 4, the amount of unreacted product leached out is reduced and thus, their cytotoxic action over cells is dramatically diminished [24,27,28]. An evaluation of the cytotoxic effect of three current total-etch, two-step adhesive systems showed that both the acidic and non-acidic components of un-polymerized adhesive resins were responsible for the high cytotoxic effects on odontoblast-like cells [24]. When the experimental materials were light-cured and rinsed off in order to remove acidic agents as well as residual unreacted monomers, the cytotoxic effects of the adhesive resins decreased [24]. However, when primers were applied to pulp wounds and the residual monomers not rinsed out, as occurs clinically, the cytotoxic effects of resinous monomers promote pulp damage.

When the biocompatibility of the ScotchBond Multi Purpose was compared to an aqueous solution of HEMA (50%) on connecting tissue of a rat, the HEMA solution showed the worst results and this was attributed to the fact that the adhesive system was light-cured, which gave rise to a decreased number of resin particulates when compared with HEMA [39].

Actually, the in vivo diffusion of spherical resin globules into dentinal tubules of human teeth has been previously observed [40]. These diffusing
resinous materials reached the pulp space and were observed among odontoblast cells [40]. It seems that the presence of resin particulates can trigger a foreign body response characterized, by the presence of mononuclear inflammatory infiltrate as well as multinuclear giant cells [7].

It is reported that as low as 3.6 mmol/L of HEMA can reduce cell metabolism to 50% after 24 h exposure [22]. The concentration of HEMA in the Scotchprep dentin primer, Scotchbond 2 dental adhesive, and Gluma sealer is 4.09, 2.75, and 2.6 mmol/L, respectively [41]; figures much higher than those reported to reduce cell metabolism to 50% [41]. Consequently, these high concentrations of HEMA may impart remarkable cytotoxic effects on cultured pulp cells [24].

However, another issue, also important to address, is the duration of exposure. This feature has a strong effect on the toxicity of dentin bonding agents. For instance, the same depression in cell metabolism (about 50%) can occur in pulp cells with lower amounts of HEMA (1.0 mmol/L) when this substance is maintained in place for 72 h [22]. This is particularly relevant if we consider that there is clinical degradation of hydrophilic bonding agents due to water sorption and exposure to oral fluids that not only compromise the mechanical properties of the adhesive layer [42,43], but also favor the release of increasing amounts of monomers and byproducts to pulp tissues. This is likely to be responsible for chronic inflammatory response of pulps and also to necrosis that occurs after longer periods of evaluation [4,6,7,44]. The adhesive resin might promote more intense pulp damage over time [22,24,27,45] since these substances are not degraded over time by macrophages and giant cells. A persistent inflammatory pulp response has also been reported after application of adhesive resins to the human pulp wound [4,6]. In addition, Costa et al. [6] demonstrated a delayed pulp healing associated to the lack of dentin bridge formation, even at 300 days long-term evaluation. Adhesive components inhibit proliferation of immunocomponent cells causing a chemical immunosuppression which favors the development of pulp pathologies [46].

As discussed earlier, when the bonding resin or the composite were applied over pulp exposures (groups 3 and 4, respectively), a trend towards better pulpal response was observed; however, no dentin bridging was found in these groups. Although these components were light-cured prior to restoration of the cavity, it is known that the conversion of monomers to polymers is never complete [47]. All the organic components of the adhesive systems, photo-initiators and constituents generated during the setting process, are released, in particular shortly after setting [22,48,49].

It is known that oxygen acts as an inhibitor of resin polymerization [50]. It has been previously reported that an unfilled resin cured, in room atmosphere had a significantly greater thickness of polymerization-inhibited material than did resin cured in argon atmosphere [50]. Other studies have demonstrated that the wet environment (as occurs to monomers when in contact with the pulp tissue) may preclude the adequate polymerization of resinous materials [49,51]. An addition of approximately 20 μL of water per mL of adhesive solution reduces the conversion degree of a water-free bonding resin from 53% to about 25% [52]. The residual water, present in the pulp tissue, may dilute the priming components and preclude the production of high-quality polymers, compromising the integrity of a dentin interface over time [53].

In spite of the relatively high number of inflammatory pulps or necrosis without bridge formation in groups 1-4, no correlation between histological features and postoperative sensitivity was observed. This finding is coincident with the published literature, which indicates that, postoperative symptoms can be absent despite various inflammatory reactions [5,6]. In fact, this is not a new finding, since an old study has already demonstrated this [54].

Calcium hydroxide and its compounds are the materials against which new candidates for capping are tested because of a long record of success from clinical and histologic studies. The ability of calcium hydroxide to stimulate reparative dentin formation, shown in the present investigation, has already been observed in many other clinical studies [4–8]. In addition its bacterial effect may be attributed to effects on induction and up-regulation of odontoblast-like cell differentiation for new matrix deposition and especially its effect on growth factors from the dentin matrix [55].

It must be emphasized that this study was performed on sound teeth in a clinical situation. Pulp exposure frequently occurs by a carious process in which the level of inflammation is much higher and more difficult to predict than in the clinical evaluation of pulp therapies. Had inflamed pulps been capped in this study the use of adhesive systems for capping could have caused disastrous effects in vital pulp. This fact, however, merits further evaluation.

Based on the experimental conditions, it was concluded that none of the adhesive constituents are capable of inducing pulp healing. Thus, under
clinical conditions, exposed pulps should be capped with calcium hydroxide.

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