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Original Article

Comparison of Microleakage in Human and Bovine Substrates Using Confocal Microscopy

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Abstract

Microleakage in human and bovine teeth was compared. Cavities were prepared on the buccal surface in 20 human and 20 bovine teeth (3 mm × 2 mm in depth). The teeth were divided into 4 groups (n = 10) according to the substrate and adhesive (CLEARFIL SE Bond-CF or Scotchbond 1-SB1). Resin composite (Wave) was applied in two increments, each cured for 30 sec. Specimens were stored in 100% relative humidity at 37°C for 24 hr and submitted to 1,000 thermal cycles, followed by immersion in 0.6% aqueous rhodamine for 48 hr. Specimens were rinsed and sectioned at the center. Microleakage length was measured and the score recorded using the following scale: 0-none, 1-up to enamel junction, 2-up to pulp wall, 3-in pulp wall, 4-beneath pulp wall. Percentage of leakage penetration into the cavity was submitted to an ANOVA and Tukey's test (5%) and the scores submitted to the Kruskal-Wallis and Dunn's multiple comparison tests (5%). When bovine teeth were used, SB1 (87.76%) showed a statistically higher penetration mean than CF (66.22%). When human teeth were used, no difference was found between SB1 (47.35%) and CF (36.01%). When scores were analyzed, SB1 showed no difference to CF. The differences found should be taken into consideration when evaluating adhesive microleakage using bovine teeth.

Key words: Microleakage—Substrates—Human teeth—Bovine teeth

Introduction

With advances in dentistry, a better understanding of dentin substrate characteristics has provided a significant decrease in marginal leakage of restorations²³⁾. However, resin com-

posites still present polymerization shrinkage and linear thermal expansion coefficients that differ from those of the natural tooth structure. The linear thermal expansion coefficient of a material, thermal and occlusal stresses, and polymerization shrinkage have been noted as

factors influencing microleakage²⁰.

Clinical failure of resin composite restorations is often the result of incomplete sealing at the tooth/restoration interface¹¹. Several studies^{6,27} have identified resin composite polymerization stress as a major cause of marginal integrity loss and consequent post-operative occurrences such as hypersensitivity, marginal staining and secondary caries.

In order to predict the clinical performance of enamel and dentin bonding systems, three different methods of *in vitro* evaluation are usually performed: morphological observations, microleakage studies and bond strength tests. Class V restorations have been shown to be useful models in dye penetration studies when studying the efficacy of the marginal seal due to bonding created under clinical conditions⁷. Microleakage tests, which have been defined as the clinically undetectable passage of fluids and bacteria between a restorative material and the prepared tooth¹², are the most frequently used laboratory tests to study the mechanisms that may minimize, or eliminate, leakage around restorations²³. Microleakage is commonly assessed with *in vitro* dye penetration studies to detect bond failure at the enamel-sealant interface⁴.

To evaluate adhesive bond strength, a great deal of *in vitro* research has been conducted using extracted human teeth, which are increasingly difficult to obtain, due to advances in preventive dentistry¹³. Therefore, it has become necessary to look for an alternative substrate. As mammalian teeth are histologically and morphologically similar³⁰, investigators have turned to using bovine, ovine, equine, or swine teeth to provide standardized material for studies. Bovine teeth have been used as substitutes for human teeth because of their availability and larger size¹.

Confocal laser scanning microscopy (CLSM) is a non-destructive technique for visualizing subsurface tissue features and can be used to detect fluorescence deep within tissues²⁹. One of its advantages is the clear indication of leakage limits, due to a lens focus that can occur some microns beneath the observed surface. This eliminates the

stain spread¹⁶ caused by specimen sectioning and also avoids polishing artifacts that exaggerate dye penetration²⁸. Subsurface observation of CLSM is made possible by elimination of scattered, reflected and fluorescent light from planes other than the plane from which the image is created, the focal plane¹⁸. The laser scanning microscope scans the sample sequentially point by point and line by line and assembles the pixel information into one image. By moving the focus plane, single images (optical slices) can be put combined to build up a three-dimensional stack that can be digitally processed afterwards.

The hypothesis tested was that bovine teeth presented a leakage pattern similar to that of human teeth.

Materials and Methods

Twenty human teeth extracted for orthodontic reasons and 20 bovine teeth were used. The teeth were stored in 0.5% aqueous chloramine for a maximum of 2 months prior to use. Cavities were prepared at the center of the buccal surface using a handpiece in a special device to produce standardized square cavities (3 mm × 2 mm in depth). The teeth were divided into 4 groups of 10 according to the substrate and adhesive applied: CLEARFIL SE Bond (Kuraray Co., Ltd, Tokyo, Japan; also known as CLEARFIL Megabond inside Japan) or Scotchbond 1 (3M Espe, Saint Paul, MN, USA; named CLEARFIL Megabond inside JAPAN). The adhesives were applied and polymerized for 20 sec. Resin composite (Wave, SDI Limited, Bayswater, Australia) was applied in two increments, each polymerized for 30 sec. All materials were applied according to the manufacturers' instructions.

The specimens were stored at 100% relative humidity at 37°C for 24 hr and were then submitted to 1,000 thermal cycles at 5°C and 55°C with a dwelling time of 1 min at each temperature. The specimens were covered with 2 layers of nail varnish, except the resin composite restoration and 1-mm area around it, followed by immersion in 0.6% aqueous

rhodamine for 48 hr. The specimens were rinsed and sectioned with a saw (model 650, South Bay Technology Inc., San Clemente, CA, USA) at the center of the restoration. The specimens were polished using alumina paste in decreasing order of granulation (5, 3 and 1 μm), followed by an ultrasonic bath.

Microleakage was measured using confocal microscopy (LSM-510 Duo Scan, Carl Zeiss Microimaging GmbH, Jena, Germany) at $25\times$ magnification and in the fluorescent mode. Approximately two photographs were taken of each specimen to obtain the full perimeter of the restoration (Fig. 1), with the leakage measured using software (UTHSCSA Image-tool for Windows, v.3.0). The total length of the internal restoration margins and the leakage length were measured and the percentage of leakage length over total length was obtained. The score was recorded using the following scale: 0-none, 1-up to enamel junction, 2-up

to pulp wall, 3-in pulp wall, 4-beneath pulp wall. Penetration values were submitted to an ANOVA and Tukey's test (5%) and the microleakage scores were submitted to the Kruskal-Wallis and Dunn's multiple comparison tests (5%).

Results

When bovine teeth were used, Scotchbond 1 showed statistically higher mean penetration than CLEARFIL SE Bond. When human teeth were used, no difference was found. Human teeth showed a significantly lower percentage of leakage than bovine teeth. When scores were analyzed, no difference was shown between Scotchbond 1 and CLEARFIL SE Bond (Table 1).

Discussion

Resin-dentin interface sealing is a desirable property of dentin bonding systems in preventing the pulp-dentin complex from being exposed to bacteria and their toxins²⁴. A variety of factors can affect bond formation, such as the density and orientation of dentin tubules¹⁰, cavity depth²², type of dentin surface¹⁹, presence of sclerotic dentin³² and different *in vivo* conditions. The current study measured microleakage using confocal microscopy at low magnification ($25\times$), differing from other microleakage studies⁹. As the evaluation was made to verify and measure the perimeter of the restoration and the perimeter of total leakage, without evaluating

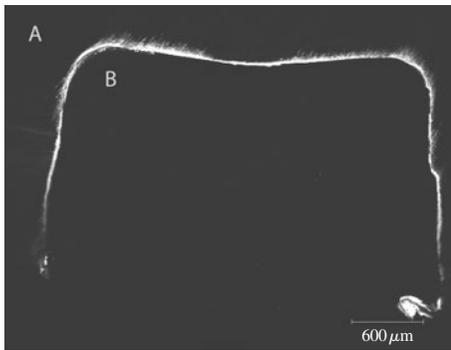


Fig. 1 Microleakage in bovine tooth using Scotchbond 1 by confocal microscopy
Visible line represents leakage. A shows dentine and B resin composite.

Table 1 Means of penetration in percentage (SD) and median of scores

		CLEARFIL	Scotchbond
Human	Mean (SD)	36.01% (15.55) a	47.35% (9.21) a
	Median	2A	3A
Bovine	Mean (SD)	66.22% (6.02) b	87.76% (8.14) c
	Median	3A	3A

Means followed by different small letters and median followed by different capital letters indicate statistical difference.

the topography of the cracks in detail, there was no reason for greater magnification.

The clinical relevance of dye leakage studies has been questioned³¹. No correlation has ever been established between the results of microleakage studies for restorative materials and the prevalence of secondary caries when the same materials were tested under clinical conditions¹⁵. In the current study, all experimental groups showed extensive leakage and, although significant differences were found among them, the clinical relevance of these differences remains questionable. On the other hand, microleakage tests can evaluate the ability of restorative materials to prevent fluid penetration at the interfaces between dentin and restorative materials¹⁵.

The main problem and important limitation of microleakage studies is that microleakage only reveals a minor aspect of adhesion, emphasized in the current study by the fact that no differences were found for the human substrate between an etch-and-rinse two-step adhesive versus a self-etch two-step adhesive. It is known that Scotchbond I and CLEARFIL SE Bond show differences in terms of bond strength on enamel and dentin¹³, bond stability over time⁵, nanoleakage expression²⁶ (*i.e.* extensive nanoleakage was described for Scotchbond I, while CLEARFIL SE Bond is known to show minimal nanoleakage throughout the adhesive interface), and clinical effectiveness³. However, the aim of microleakage studies is to verify how much an agent can penetrate the interface, simulating clinical leakage. The mechanism of how this leakage occurs is not the overall objective, as others studies evaluate such mechanisms. Therefore, this study contributes to the literature by comparing microleakage between human and bovine teeth, while verifying that differences exist between the two and that the use of bovine teeth for this kind of study must be carefully evaluated.

From the clinical relevance perspective, human teeth must be considered as the most appropriate hard tissue substrate for *in situ* studies³³, although human teeth are of a highly variable composition due to genetic

influences, environmental conditions and age. The concern is that bovine enamel has a more uniform composition than human enamel, providing a less variable substrate for research purposes. However, bovine enamel is more porous and demineralizes faster than human enamel^{17,33}. Furthermore, Fonseca *et al.*⁸ found that bovine and swine teeth had lower radio density values when compared with human teeth, confirming the difference in mineral composition between human and bovine teeth.

In the current study, microleakage between human and bovine teeth was compared. Bovine teeth showed statistically significant higher mean leakage values when compared with human teeth for all groups. In a previous study, it was observed that bovine dentinal tubules were wider and in greater quantity per area than human dentinal tubules¹⁴. Because of their larger size and quantity, these tubules may have more dentinal fluid, while the intertubular dentin is probably thinner than human dentin. When incomplete marginal sealing occurs, the external fluids and bacteria may spread more easily into the bovine tooth. Oesterle *et al.*²¹ also verified that the enamel bond to bovine teeth is 21% to 44% weaker than the bond to human enamel. The difference in size between human and bovine teeth and, consequently, lower ratio of cavity size (size of filling material) to tooth size for bovine teeth may also play a role in the higher microleakage seen in bovine tooth. According to Brown *et al.*², due to their size, bovine teeth are more affected by thermocycling than human teeth, with a significant enamel crack propagation after 650 cycles compared to 2,000 cycles for human. These factors together may contribute to the reason for greater microleakage values in bovine teeth. Abuabara *et al.*¹ also found a greater leakage for bovine teeth when compared with human teeth.

A different pattern of results between human and bovine specimens was also noticed. When Scotchbond I was used, statistical differences were found when using bovine teeth. However, the same did not happen when human

teeth were used. When scores were analyzed, CLEARFIL SE Bond showed no statistical difference when bovine teeth were used, but the score was lower than when human teeth were used, indicating a lower leakage, agreeing with the percentage measurements. In a microleakage test, Reeves *et al.*²⁵⁾ and Abuabara *et al.*¹⁾ also found differences in the material ranking with different substrates. The hypothesis of this study, that bovine teeth present a leakage pattern similar to that of human teeth, is rejected.

Conclusions

1. When bovine teeth were used, Scotchbond 1 showed statistically higher mean penetration than CLEARFIL SE Bond. When human teeth were used, no difference was found.
2. When scores were analyzed, no difference was shown between Scotchbond 1 and CLEARFIL SE Bond.
3. Human teeth presented a significantly lower percentage of leakage than bovine teeth.
4. The differences found should be taken into consideration when evaluating adhesive microleakage using bovine teeth.

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