Antimicrobial Potential of Papain Chemomechanical Agent on *Streptococcus Mutans* and *Lactobacillus Casei* Followed by the Use of Self-Etching Adhesive Systems

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Objective: *This study evaluated the antimicrobial effect of papain-based gel (Papacárie/ P) followed by self-etching adhesive systems with MDPB monomer (Clearfil Protect Bond/ PB) and without (Clearfil SE Bond/ Cl) on Streptococcus mutans (Sm) and Lactobacillus casei (Lc).* ***Study design:*** *The dentin of twenty human teeth was exposed to prepare four cylindrical cavities in each tooth. The cavities were incubated with Sm or Lc. One cavity from each tooth served as contamination control (positive control group); the other three were treated with P, P+CL and P+PB. The cavities were sealed and after 72 h, dentin samples were collected and microbial cultivation was performed. Microbial count was undertaken (CFUs/mg) according to the morphological characteristics for Sm and Lc. Analysis of variance and Tukey's test were applied (α=5%).* ***Results:*** *For Sm, groups P+Cl and P+PB had lower microbial count than group P (no statistical differences between P+Cl and P+PB). For Lc, group P+Cl had microbial count similar to group P. There was statistical difference between cavities treated with P and P+PB but none between groups P+Cl and P+PB. After using P, both self-etching adhesives showed antimicrobial potential, although Clearfil Protect Bond proved better against Lc.* ***Conclusions:*** *Both self-etching adhesives used after application of Papacárie showed antimicrobial potential, although Clearfil Protect Bond proved more effective against Lactobacillus casei.*

Key words: *antimicrobial effect ; Lactobacillus casei ; papain-based gel; self-etching adhesive systems; Streptococcus mutans*

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INTRODUCTION

The traditional concept of dentin caries lesion removal is based on removing all the carious dentin, including both infected and affected regions. However, carious lesions advance in an irregular manner, initially affecting the more superficial intertubular (less mineralized) dentin rather than the deeper intertubular dentin. After dentin demineralization by the action of bacterial proteolytic enzymes, the collagen begins to progressively disintegrate.[1] Nonetheless, the possibility of remineralizing the deeper layer, containing the affected or demineralized dentin, has already been studied.[2] This affected dentin is partially demineralized, characterized by intact collagen fibers, peritubular dentin, odontoblastic processes and tubules, and by a lower level of infection by microorganisms.[3] It has been suggested that this deeper layer should not be removed because of its remineralizing potential and because the risk of pulp exposure may be reduced by maintaining the demineralized dentin.[2,4]

Among the methods that can used to remove carious tissue, manual instruments have been shown to perform with good results. These instruments include dentin excavators and rotary instruments using burs adaptable to a low speed motor.[5,6] Other methods have also been developed, including chemical agents, able to preserve tooth structure health optimally, maintain dentin demineralized, prepare the dental remainder to receive restorative materials and increase the acceptability of these materials by patients.[7]

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In 2003, a new chemomechanical system was introduced, containing papain (an endoprotein of the family of cysteine protease of broad proteolytic activity), which has antimicrobial and anti-inflammatory potential,[8] toluidine, which acts as a dye and has an antimicrobial effect, thus reducing the total amount of microbiota in demineralized dentin, and chloramine, which has been used as a root canal irrigant due to its bactericide and disinfectant properties.[8,9] This system consists of applying the papain-gel to the infected dentin. It has been suggested that it acts by breaking down the partially degraded collagen molecules and contributes to degrading and eliminating the fibrin "mantle" formed by the carious process, without damaging intact collagen fibrils.[8] This selective interaction of the enzyme with the affected components of the carious dentin has been attributed to the lack of an antiprotease α-1-anti-trypsin, which inhibits protein digestion in sound collagen-based tissues,[10] although Bertassoni and Marshall[11] showed that intact non mineralized type I collagen fibrils are partially degraded by papain-gel.

It has been reported that microorganisms may remain in the dentin cavity after caries removal, insofar as mechanical procedures or microorganism association with chemomechanical agents for cavity preparation are unable to ensure complete elimination of cariogenic bacteria.[12-14] Furthermore, the use of self-etching adhesive systems, which have a low pH,[15] may prevent the development of residual microorganisms.[16-20] There is one such system (Clearfil Protect Bond/ Kuraray) that seems to have an antibacterial effect attributed to the addition of an MDPB (12-methacryloyloxydodecylpyridinium bromide) monomer to an adhesive system.[17,21] MDPB may provide the primer with antimicrobial activity before and after the material sets,[16,17] thus providing a post-cure antibacterial effect against bacterial development. Imazato *et al* [19] showed that Clearfil Protect Bond was effective for inhibiting bacterial penetration through the gaps at the bond interface after the restoration was inserted, leading to the inhibition of secondary caries.

Thus, pretreatment use of a chemomechanical agent to facilitate caries removal, such as a papain-based gel, may reduce the microbial count[9,22] of *Streptococcus mutans*—mainly involved in the initiation of enamel caries—and *Lactobacillus casei*—the predominant cultivable organism in dentin caries lesions without endodontic infections.[23] Since viable bacteria may persist in the cavities after caries removal, the aim of this study was to evaluate the antimicrobial effect of a papain-based gel followed by self-etching adhesive systems with or without MDPB monomer on *Streptococcus mutans* and *Lactobacillus casei*.

MATERIALS AND METHODS

This study was approved by the Research Ethics Committee of the São Leopoldo Mandic Research Institute School of Dentistry at Campinas, São Paulo, Brazil (Protocol no. 2009/0287). The experimental units consisted of four cylindrical dentin cavities made in each of 20 human teeth. The cavities were contaminated with *Streptococcus mutans* (10 teeth, 40 cavities) or *Lactobacillus casei* (10 teeth, 40 cavities) that received the application of a papain-based gel for chemomechanical caries removal (Papacárie), followed by either the use or nonuse of a self-etching adhesive system.

The factor under study was the "treatment" on four levels (n=10): (1) *control/ C* (absence of any treatment; the cavities were used to control the presence of dentin contamination - positive control); (2) papain-based gel agent *Papacárie/ P* (application of only this chemomechanical agent in the cavity); (3) papain-based gel agent followed by the two-step self-etching adhesive system, *Clearfil SE Bond/ P+Cl*; (4) papain-based gel agent followed by the two-step self-etching adhesive system containing MDPB, *Clearfil Protect Bond/ P+PB*.

The response variable was the colony forming unit (CFU) count of *Streptococcus mutans* and *Lactobacillus casei* incubated in selective media. The materials used, the composition and the protocol for use are presented in Table 1.

Twenty sound human third molars recently extracted (no longer than 6 months) were used in this study. The teeth were cleaned with a Robinson brush and water for 25 seconds and stored in a 0.1% aqueous solution of thymol. The occlusal enamel portion was removed to obtain a dentin substrate for cavities preparation, and an exposed dentin surface was obtained on the occlusal third, perpendicular to the long axis of the tooth. The enamel surface was removed with a diamond disk mounted in a precision electric cutter (Isomet 1000 Precision Diamond Saw, Buehler Ltd., Lake Bluff, IL, USA). The roots were sealed in the apical portion with resin composite (Z350/ 3M Espe, St. Paul, MN, USA), using a two-step conventional adhesive system (Single Bond/ 3M Espe, St. Paul, MN, USA).

Four cylindrical cavities were prepared without pulpar exposition (24) on the planned dentin surface of each tooth, using a diamond tip with stop #2292 (KG Sorensen, Barueri, SP, Brazil), water-cooled at high rotation (cavity dimension with 2mm in diameter and 2mm depth). The teeth were sterilized in steam (Vertical Steam 30L, CS 078, Prismatec, Itu, SP, Brazil) for 15 minutes at 121ºC.

Lactobacillus casei (ATCC 393) and *Streptococcus mutans* (ATCC 25175) were activated. The sterile teeth were immersed inside the respective media for either microorganism and incubated in an atmosphere of 5% CO_2 for 48 h (TE399 CO_2 incubator, Tecnal, Piracicaba, SP, Brazil) at 36°C ± 1ºC to promote cavity infection. The treatments for each type of microorganism, applied to each dental cavity, were administered randomly, according to Table 2.

The teeth were removed from the media and dried with sterile gauze. Dentin samples were collected from the walls (pulpar and surrounding walls) of the positive control cavity (C) of each tooth before applying the treatments to other cavities. This procedure was undertaken to substantiate that all the dentin cavities were infected.[24]

A previous pilot study was developed to standardize the amount (in weight) of dentin sample to be collected for the microbial analysis.[24] It was ascertained that the mean weight of dentin to be collected from each cavity would be 7mg.

Dentin samples from each cavity were collected in two steps in a similar manner, i.e.: the samples were collected with a no. 2 sterile bur (JET Carbide Burs, Beavers Dental Div. Syborn, Morrisburg, Ontario, Canada) for 10 s at a very-low rotation enabled by using a dental contra angle 20:1 reduction (975 AE, W & H, Bürmoos, Áustria) mounted on a handpiece (LB 100, Beltec, Araraquara, SP, Brazil). The bur was positioned inside an *eppendorf* containing 500µl sterile saline solution, and the contra angle was operated for 10 s. After the second collection, the bur with the dentin samples was left inside the *eppendorf*. The flasks containing the dentin samples with the bur were shaken in a tube shaker (AP56, Phoenix Luferco, Araraquara, SP, Brazil) for 1min to disperse bacterial aggregates. The solution was shaken an additional 20 s to ensure

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homogeneousness[25,26] and decimal dilutions from 10^{-1} a 10^{-2} were then prepared in sterile saline solution.

Next, 10µL aliquots of each dilution were spread in duplicate on the following solid media: mitis salivarius agar (Difco-BD, Sparks, Md., USA) supplemented with 20% sucrose, 0.2 units/ml bacitracin and 1% potassium tellurite (MSB) for *Streptococcus mutans*, and MRS agar (Man, Rogosa, Sharpe) for *Lactobacillus casei*. The MSB and MRS agar plates were incubated in an atmosphere of 5% CO_2 for 48 h at 36ºC ± 1ºC.

For treatments that received the chemomechanical agent (P), the manufacturer's instructions were followed (Table 1). After applying for 30 seconds, the chemical agent and the softened dentin residues were removed with the cutting portion of a dentin excavator of a size compatible with that of the cavity. This process was repeated twice more, for a total of 3 applications.

Manufacturer's instructions were also followed for applying the self-etching adhesive systems (P+Cl and P+PB) after the chemomechanical agent (Table 1). Photocuring was performed by a halogen light unit (Demetron, LC Kerr Corporation, Orange, CA, USA) with a minimum irradiance of 450mW/cm^2, measured with a radiometer (Newdent, Ribeirão Preto, SP, Brazil) in each of the three restored cavities.

After applying the treatments, each cavity was filled with a sterile absorbent paper disc (Coffee filter, Melitta, Guaiba, RS, Brazil) (27) with 2 mm in diameter placed on the most superficial area of the cavity. The discs received temporary sealing with resin composite (P60/ 3M ESPE, Seefeld, Germany) and care was taken to place the sealer just on the surface of the dentin (not inside the cavity). The resin composite was photocured for 20s. The teeth were kept separately according to each incubated media, in an atmosphere of 5% CO_2 for 72 h at 36ºC ± 1ºC.

The temporary sealing with resin composite was removed with sterile curettes no. 15/16 (Maillefer, Dentisply, Tulsa, Oklahoma, USA) exerting light pressure. Dentin samples were collected and microbial cultivation was performed as described previously for all the remaining three cavities receiving the treatments (P, P+Cl and P+PB).

Microbial count and statistical analysis

Microbial count was undertaken by counting the colony-forming units (CFUs)/mg of dentin according to the morphological characteristics for *Streptococcus mutans* and *Lactobacillus casei*. For the cavities that had <10^3 CFU microorganisms, a value of 999 CFU of bacteria was used for statistical reasons, in order to calculate the mean values.[24] Only the positive control cavities (C) from teeth that showed higher values than 10^4 CFU g^{-1} infected with *Streptococcus mutans* and *Lactobacillus casei* were counted in this study.

The exploratory statistical analysis of the data indicated a logarithmic transformation to meet the assumptions of analysis of variance (ANOVA). ANOVA and Tukey's test were applied. The significance level was set at 5%. Data analysis was performed with the Bioestat 5.0 statistical program (Mamirauá Maintainable Development Institute, Belém, Pará, Brazil, 2008).

Table 1: Materials used, composition, lot number, protocol for use and manufacturer

Material (lot number)	Manufacturer (city, state, country)	Composition and pH	Protocol for use
Papacárie (8201)	Fórmula e Ação, (São Paulo, SP, Brazil)	Papain, chloramine, toluidine blue, salts, preservatives, thickening agents, vehicle qsp. pH= 6.97	– Apply the product; – Wait 30 s; – Remove softened dentin with the non-cutting portion of a curette; – Reapply as many times as required.
Clearfil SE Bond/ (Primer: 00969[a] Bond: 01440)	Kuraray Medical Inc. (Sakazu, Kurashiki, Okayama, Japan)	Primer: MDP, HEMA, hydrophilic dimethacrylate, camphorquinone, N,N-Diethanol p-toluidine, water. Bond: MDP, Bis-GMA, HEMA, hydrophobic dimethacrylate, camphorquinone, N,N-Diethanol p-toluidine, silanized colloidal silica. pH = 2.0	– Dry cavity; – Apply primer for 20 s; – Apply light jet of air; – Apply bond; – Apply light jet of air; – Light activation for 10 s.
Clearfil Protect Bond (lot number 061127)	Kuraray Medical Inc. (Sakazu, Kurashiki, Okayama, Japan)	Primer: MDP, MDPB, HEMA, hydrophilic dimethacrylate, water, Bond: MDP, bis-GMA, HEMA hydrophobic dimethacrylate, DL-camphoroquinone, N,Ndiethanol p-toluidine, silanized colloidal silica, surface-treated sodium fluoride pH= 2.1	Dry cavity; Apply primer for 20 s Apply light jet of air for 20 s Apply bond; Light activation for 10 s.

Bis-GMA Bisphenol Glycidyl methacrylate; HEMA: 2-hydroxyethyl methacrylate; MDP: 10-methacryloyloxydecyl dihydrogen phosphate; MDPB, 12-methacryloyloxydodecylpyridinium bromide

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Table 2: Treatments applied to each cavity according to each microorganism

Cavity per tooth	Group	Treatment	
		Streptococcus mutans	*Lactobacillus casei*
1	C	Positive control	Positive control
2	P	Papain-based gel agent	Papain-based gel agent
3	P+Cl	Papain-based gel agent followed by the use of a two-step self-etching adhesive system (*Clearfil SE Bond)*	Papain-based gel agent followed by the use of a two-step self-etching adhesive system (*Clearfil SE Bond*)
4	P+PB	Papain-based gel agent followed by the use of a two-step self-etching adhesive system containing MDPB *(Clearfil Protect Bond)*	Papain-based gel agent followed by the use of a two-step self-etching adhesive system containing MDPB (*Clearfil Protect Bond)*

Table 3. Mean and standard deviation of microbial count for *Streptococcus mutans* according to treatment group

Treatment	Microbial count (CFU/mg dentin)	
	Mean	Standard deviation
C	1.2×10^5	4.8×10^4
P	2.3×10^4 A	2.6×10^4
P+Cl	9.8×10^2 B	1.0×10^3
P+PB	2.5×10^2 B	3.3×10^2

Means followed by the same letters do not differ among themselves according to ANOVA and Tukey test ($p<0.05$)

Table 4. Mean and standard deviation of microbial count for *Lactobacillus casei* according to treatment groups

Treatment	Microbial count (CFU/mg dentin)	
	Mean	Standard deviation
C	$1.2x\ 10^5$	$8.1x\ 10^4$
P	3.9×10^4 A	2.8×10^4
P+Cl	1.2×10^4 AB	2.5×10^4
P+PB	1.7×10^3 B	2.5×10^3

Means followed by equal letters do not differ among themselves according to ANOVA and Tukey test ($p<0.05$)

RESULTS

Table 3 shows that using papain-based gel for chemomechanical caries removal followed by the application of the two self-etching adhesive systems used in the study presented a lower microbial count for *Streptococcus mutans* than using papain-based gel only. There were no statistical differences between microbial counts for groups of cavities restored with both adhesive systems.

For *Lactobacillus casei*, Table 4 shows that using papain-based gel for chemomechanical caries removal followed by the application of the two-step self-etching Clearfil SE Bond presented a microbial count similar to that of papain-based gel only. There were statistical differences between cavities treated with papain-based gel and those restored with the Clearfil Protect Bond self-etching adhesive. There were no statistical differences between microbial counts for cavities restored with both adhesive systems.

Control groups for *Streptococcus mutans* and *Lactobacillus casei* showed a higher microbial count. This confirmed that the cavities were infected; however, the control groups were not compared to the other groups because no treatment was applied to the cavity.

DISCUSSION

Carisolv was the first chemomechanical agent introduced on the market.[28,29] However, its short shelf life, required application with specialized instruments and high cost prompted the formulation of an alternative biomaterial for caries removal, leading to the development of Papacárie by Bussadori *et al.*[8] This composition has the advantage of providing bactericidal, bacteriostatic and anti-inflammatory action at a low cost.[9,22]

Studies have shown that papain-based gel does not influence the bond strength of total-etch and self-etching adhesive systems to demineralized dentin *in vitro*[30,31] or *in situ*.[32] Furthermore, papain gel does not affect the longevity *in vitro* of self-etching adhesives to dentin after 180 days.[33] The influence on bond strength depends on the type of adhesive system, according to evaluations performed using extracted teeth with pre-existent carious lesions.[34] In a micromorphological study, it was observed that the papain chemomechanical agent formed an amorphous layer similar to the smear layer and few exposed dentinal tubules. In comparison, a rotatory instrument produced a smooth and regular dentinal surface, with a typical smear layer and exposed dentinal tubules, in spite of a similar tag formation when a conventional total etching adhesive system was used.[35]

Papain has been shown to have bactericidal properties.[36,37] Chloramine is a compound of chloride and ammonia, and also offered bactericidal as well as disinfectant action, as ascertained when toluidine blue was added to Papacárie as a coloring agent. Toluidine also has an antimicrobial effect.[8] In the present study, there was a reduction in the microbial count of *Streptococcus mutans* and *Lactobacillus casei* after papain-based gel was applied. Although the mechanical removal with a curette may also have contributed to reducing the microorganisms in the cavity, Papacárie may also have played a role in this bacterial reduction, but the absence of bacteria is not achieved after chemomechanical caries removal, as showed by El-Tekeya *et al* [9] and Almeida *et al* [22] Moreover, the consistency of Papacárie presented in gel form limits the in-depth penetration of the product; consequently, the microorganisms present in demineralized dentin are not totally eliminated during the excariation process.[22]

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It should be considered that microorganisms always remain in the cavity after total excavation of a carious lesion,[38] and it is important to reduce or prevent microorganism proliferation by cavity sealing, in which case, a glass ionomer is the preferred sealant (to provide an anticariogenic effect)[39,40] or an adhesive material (to seal the interface between tooth and composite resin).[41,42]

Although this was not a clinical study due to difficulties in standardizing some inclusion criteria, such as depth of cavities and initial microbial counts,[22] the methodology used was the same as that described by Polydorou *et al* [24] and Türkün *et al.*[27] Moreover, a model with a dental cavity was designed for this study, to evaluate the antimicrobial potential of papain gel applied before using self-etching adhesives. Although this is a more complex methodology than using agar wells or the diffusion method with paper or dentin discs,[27,43] it proved effective in evaluating the antimicrobial potential of the adhesive systems and the papain-based gel.

Some studies have evaluated the antimicrobial potential of self-etching adhesive systems,[19,20,24,43,49] as those evaluated in this study. So, the antimicrobial potential of self-etching adhesives without Papacárie were not evaluates in this research. The antibacterial properties of self-etching adhesives may be related to adding MDPB to their composition[18-20] or to the low pH of these adhesives.[44,46] If tooth conditioners, such as primers of the two-step self-etching adhesives, had antibacterial activity, bacteria could be eliminated or reduced, thereby preventing secondary caries. However, the antibacterial potential presented by the low pH of adhesive systems may be compromised by the acidophilic properties of microorganisms, such as the *Lactobacillus* casei.[20,44] This could explain why the association of the self-etching adhesive Clearfil SE Bond to a papain-based gel resulted in a lower microbial count, as compared to using papain alone for *Lactobacillus casei*, but resulted in no difference regarding *Streptococcus mutans*.

Accordingly, the use of self-etching adhesives containing antimicrobial agents such as MDPB could compensate the absence of Papacárie's antimicrobial potential. It was ascertained that Clearfil Protect Bond – containing MDPB – applied after Papacárie provided less significantly values of colony forming units for *Lactobacillus casei*, although no differences were observed for *Streptococcus mutans* when using Clearfil SE Bond. The antimicrobial potential of this adhesive system was observed separately by several authors [19,27,44] .

The polymerized MDPB monomer is a bactericidal agent against oral microorganisms.[17,18] MDP is synthesized with an antimicrobial agent, and MDPB is co-polymerized with other monomers. The antibacterial agent is immobilized in the polymeric matrix of the resinous materials (primer and adhesive) after polymerization.[17,18] MDPB-containing primer has been shown to provide antimicrobial activity against *Streptococcus mutans*.[16,18,19]

A cavity is judged to be clinically caries-free according to tactile, acoustic and optical criteria. Several investigations could show that a low number of residual microorganisms (10^1–10^3 CFU) often remain in clinically sound hard dentine despite a significant reduction in the bacterial count; however, this number of bacteria is considered clinically acceptable by several authors.[50-53] Although this was not a clinical study, the number of *Streptococcus mutans* and *Lactobacillus casei* was significantly reduced after using a chemomechanical agent based on papain gel followed by both self-etching adhesive systems applied, especially when an adhesive containing MDBP was used.

CONCLUSION

According to our results, it can be concluded that the selection of the adhesive system to be used is solely a question of professional choice, since both self-etching adhesives used after application of Papacárie showed antimicrobial potential, although Clearfil Protect Bond proved more effective against *Lactobacillus casei*.

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