In Vitro Effects of 2.5% Titanium Tetrafluoride on Streptococcus Mutans and Lactobacillus Casei in Dentin Followed by Self-Etching Adhesive Systems

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ABSTRACT

The aim was to evaluate the effect of a 2.5% titanium tetrafluoride (TiF_4) solution followed by self-etching adhesives against Streptococcus mutans/Sm and Lactobacillus casei/Lc. Four cylindrical-shaped cavities were performed on each dentin surface of 40 third molars and contaminated with Sm or Lc. Each one of the four cavities received one of the following treatments (n=10): 1) control; 2) TiF_4; 3) Clearfil SE Bond/CSE or Adper EasyOne/AEO; 4) TiF_4 followed by CSE or AEO. ANOVA was applied to data. The TiF_4 solution showed an antimicrobial effect, although the TiF_4 used for dentin pretreatment before CSE or AEO showed no influence on antimicrobial effect.

INTRODUCTION

The advances in adhesive restorative materials and the knowledge gained in caries lesion progression have enabled the development of a minimal intervention dentistry concept, which lays claim to the conservative removal of caries lesions in dentin.[1] This is a conceivable option because caries lesions may occur on two dentin levels: on the outer layer, where there is a higher level of infection — infected dentin is degraded to a point where it cannot be remineralized,[2] and on the inner layer, where there is a lower level of infection — affected dentin[3] is capable of being remineralized, and must be preserved.[1,2]

The efficacy of a caries removal procedure depends on operator ability.[4] That is to say, ensuring removal of only infected tissue is a clinically difficult procedure.[4] Poor removal may lead to the permanence of residual infected dentin, specifically cariogenic-related infections, such as Streptococcus mutans[5,6] and Lactobacillus casei.[7,8] In theory, incomplete removal of carious dentin and subsequent tooth sealing will result in lesion arrest, because the residual bacteria technically have no substrate to ferment and produce lactic acid. However, any adhesion to carious dentin is more susceptible to bond degradation, which may affect bond stability in the long term[9] and may compromise restoration longevity.

For the purpose of diminishing or eliminating cariogenic microorganisms, some antimicrobial agents have been incorporated into restorative materials, like adhesive systems, resin composites, or glass ionomer cements.[10-12] Among these antimicrobial substances, MDPB monomer (12-methacryloyloxydodecylpyridinium bromide) added to adhesive systems has proved effective in eliminating cariogenic microorganisms, while not influencing the bond strength of self-etching adhesives to dental substrates.[11,13-15]

Fluorized compounds have also been incorporated into adhesive systems, with the aim of preventing and interfering with caries lesion development. The rationale behind this addition is to diminish dental substrate solubility in an acid environment, by forming fluorapatite.[16,17] Moreover, fluoride promotes antibacterial activity against Streptococcus mutans, by reducing its metabolism and growth.[18-20]

Titanium tetrafluoride (TiF_4) has been applied to enamel and dentin surfaces topically to prevent the formation of erosion and abrasion lesions.[21,22] Its use as a dentin pretreatment was initially proposed by Dündar *et al.*,[23] and subsequently evaluated by Devabhaktuni & Manjunath[24] and Bridi *et al.*[25] The application of TiF_4 to dentin enables the formation of a vitreous layer that enhances the resistance of the dental substrate against demineralization,[26-28] while not influencing the bond strength of self-etching to dentin[24,25] or interfering with hybrid layer formation.[25] Because a TiF_4 solution has a low pH (around 1), one could speculate that it has antimicrobial potential and can be used as a cavity decontaminant.[12,17] Despite this rationale, Skartveit *et al.*[29] showed that the presence of a Ti-rich coating did not influence bacterial growth of Streptococcus mutans and Bacteroides gingivalis on topically treated enamel and dentin in an in situ study.

Thus, dentin pretreatment with a TiF_4 solution followed by the application of a primer or a self-etching adhesive with antimicrobial properties could be an important tool during routine practice, for the decontamination of dentin after caries removal. Then, the aim of the present study was to evaluate the *in vitro* effect of 2.5% titanium tetrafluoride followed by the application or non-application of self-etching adhesive systems against Streptococcus mutans and Lactobacillus casei to dentin. The null hypotheses to be tested were that: a) TiF_4 solution used as a dentin pretreatment has no antimicrobial effect, and b) TiF_4 solution application followed by application or non-application of self-etching adhesive systems has no influence on S. mutans and L. casei in dentin.

MATERIALS AND METHODS

ETHICAL ASPECTS

This study was approved by the Research Ethics Committee of the São Leopoldo Mandic School of Dentistry and Research Institute, Campinas, São Paulo, Brazil (Protocol no. 2011/0105).

TOOTH SELECTION AND CAVITY PREPARATION

Forty sound human third molars recently extracted (no more than 6 months prior and stored in a 0.1% aqueous solution of thymol) were used in this study. The occlusal enamel portion was removed with a diamond disk mounted in a precision electric cutter 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. Dentin surfaces were flattened with a water-cooled polishing machine using decreasing granulations (400, 600 and 1200) of water abrasive paper in order to obtain a standardized and smooth dentin surface. The roots were sealed in the apical portion with resin composite, using a two-step conventional adhesive system.

Four cylindrical-shaped cavities (2mm in diameter and 2mm in depth) were prepared on the flattened dentin surface of each tooth without pulpar exposure, midway between the enamel and the pulp chamber, using a diamond tip #2292 with a stop, at high speed under water cooling. The teeth were sterilized in steam for 15 minutes at 121ºC.

CAVITY INFECTION

The teeth were separated into two groups, according the specific microbial contamination (Streptococcus mutans or Lactobacillus casei). Lactobacillus casei (ATCC 393) and Streptococcus mutans (ATCC 25175) strands were then activated.

Each cavity of the sterile teeth was dried with sterilized pointed paper tips and filled with 10µl of 1.5 x 10^8 CFU/ml Streptococcus mutans suspension or 10µl of 1.4 x 10^7 CFU/ml Lactobacillus casei suspension, and was not agitated for 3 minutes to provide microorganism penetration into the cavities and in the dentin. The teeth were then immersed in the respective media for either microorganism, and incubated in an atmosphere of 5% CO_2 incubator for 48 h at 36°C ± 1ºC to promote cavity infection.

DENTIN PRETREATMENTS - TiF_4 AND ADHESIVES

The teeth were removed from the media and dried with sterile gauze. One cavity of each tooth was randomly selected and used as a positive control cavity (C), to which no treatment was applied. This procedure was undertaken to substantiate that the dentin cavities from that tooth were infected. The other three cavities received each specific agent according to the respective group *(Table 1)*. For the TiF_4 groups, a 2.5% (wet/v) TiF_4 solution of pH 1.1 was prepared as suggested by Dündar *et al.*[23] and used by Bridi *et al.*[25] After the solution was weighed, 2.5 g of titanium tetrafluoride powder was mixed in 100 ml ultra pure water. The pH of the solution was measured by a pH electrode. The TiF_4 solution was applied actively to the dentin surfaces with a disposable brush for 60 seconds[25,30] followed by air-drying for 5 seconds.

The manufacturer's instructions were followed for the application of the self-etching adhesive systems (CSE and AEO) to each cavity, followed by dentin pretreatment with TiF_4 or no pretreatment *(Table 2)*. Photocuring was performed by

Table 1. Treatments applied to each cavity according to each microorganism.

Microorganism	Group/ Acronym	Cavity	Treatment
Streptococcus mutans	Control/ C	1	No treatment
	Titanium tetrafluoride/ TiF_4	2	Dentin pretreatment with titanium tetrafluoride
	Clearfil SE Bond/ CSE	3	Two-step self-etching adhesive application
	Titanium tetrafluoride + Clearfil SE Bond/ TiF_4CSE	4	Dentin pretreatment with titanium tetrafluoride + Two-step self-etching adhesive application
	Control/ C	1	No treatment
	Titanium tetrafluoride/ TiF_4	2	Dentin pretreatment with titanium tetrafluoride
	Adper EasyOne/AEO	3	One-step self-etching adhesive application
	Titanium tetrafluoride + Adper EasyOne/ TiF_4AEO	4	Dentin pretreatment with titanium tetrafluoride + One-step self-etching adhesive application
Lactobacillus casei	Control/ C	1	No treatment
	Titanium tetrafluoride/ TiF_4	2	Dentin pretreatment with titanium tetrafluoride
	Clearfil SE Bond/ CSE	3	Two-step self-etching adhesive application
	Titanium tetrafluoride + Clearfil SE Bond/ TiF_4CSE	4	Dentin pretreatment with titanium tetrafluoride + Two-step self-etching adhesive application
	Control/ C	1	No treatment
	Titanium tetrafluoride/ TiF_4	2	Dentin pretreatment with titanium tetrafluoride
	Adper EasyOne/ AEO	3	One-step self-etching adhesive application
	Titanium tetrafluoride + Adper EasyOne/ TiF_4 AEO	4	Dentin pretreatment with titanium tetrafluoride + One-step self-etching adhesive application

Table 2. Materials used, composition, lot number, application protocol and manufacturer.

Material/ pH (lot number)	Composition	Application protocol	Manufacturer (city, state, country)
Clearfil SE Bond / 2 (Primer:01090A Bond:01628A)	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.	– Dry the cavity; – Apply primer for 20 s; – Apply light air jet; – Apply bond; – Apply light air jet; – Light activate for 10 s.	Kuraray Medical Inc. (1621 Sakazu, Kurashiki, Okayama, Japan)
Adper EasyOne / 2.3 (436286)	HEMA, Bis-GMA, methacrylated phosphoric esters, 1.6 hexanediol dimethacrylate, methacrylate functionalized polyalkenoic acid, finely dispersed bonded silica filler with 7 nm primary particle size, ethanol, water, initiators based on camphorquinone, stabilizers	Apply the product actively with a microbrush for 20 seconds; Apply a gentle air flow for 5 seconds; Light activate for 10 s.	3M ESPE (St. Paul, MN, USA)

Bis-GMA Bisphenol Glycidyl methacrylate; HEMA: 2-hydroxyethyl methacrylate; MDP: 10-methacryloyloxydecyl dihydrogen phosphate

a halogen light unit with a mean irradiance of 451 mW/cm^2 (minimum of 382 mW/cm^2 and maximum of 533 mW/cm^2) measured with a radiometer.

After applying the treatments, each cavity was covered with a sterile absorbent paper disc 2 mm in diameter, placed on the most superficial area of the cavity.[31] The discs were sealed temporarily with resin composite, and care was taken to place the sealer only 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 incubator for 72 h at 36ºC ± 1ºC.

The temporary sealing with resin composite was removed with sterile diamond bur #9713FF, attached to a high-speed turbine, with no water spray. Care was taken not to let the diamond bur touch the cavity dentin walls. The paper discs were then removed with sterile tweezers. Dentin samples were collected and microbial cultivation was performed.

The teeth were removed from the media and dried with sterile gauze. Dentin samples were collected from the walls (pulpar and surrounding walls) of each tooth. A previous pilot study was developed to standardize the amount (in weight) of dentin sample to be collected for the microbial analysis.[32] The mean weight of dentin to be collected from each cavity was determined as 7mg.

Dentin samples from each cavity were obtained in two collections. The samples were collected with a # 2 sterile bur on a handpiece for 5 s at low rotation. The bur was previously immersed in sterile saline solution to provide better removal and impregnation of the dentin samples. The bur was positioned inside an Eppendorf tube containing 2ml sterile saline solution. After the second collection, the bur with the dentin samples was left inside the Eppendorf tube. The flasks containing the dentin samples with the bur were shaken in a tube shaker for 30 seconds to disperse the bacterial aggregates. The solution was shaken for an additional 20 seconds to ensure homogeneousness. Decimal dilutions from 10-1 to 103 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 supplemented with 20% sucrose, 0.2 units/ml bacitracin and 1% potassium tellurite (MSB) for Streptococcus mutans, and MRS (Man, Rogosa, Sharpe) agar for Lactobacillus casei. The MSB and MRS agar plates were incubated in an atmosphere of 5% CO_2 incubator for 48 h at 36ºC ± 1ºC.

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. A value of 999 CFU of bacteria was used for statistical purposes for the cavities that had <103 CFU microorganisms, so that the mean values could be calculated.[32] Only the positive control cavities (C) from teeth that showed higher values than 104 CFU g-1, infected with Streptococcus mutans and Lactobacillus casei, were counted in this study. An illustrated flowchart of the experiment procedures is presented in Figure 1.

The exploratory statistical analysis of the data indicated a logarithmic conversion to meet the assumptions of parametric analysis. Split-plot Analysis of Variance (ANOVA) was applied (α=5%). Data analysis was performed with the SAS statistical program (SAS Institute Inc., Cary, NC, USA, Release 8.2, 2001).

RESULTS

There was a statistically significant difference (p≤0.05) between the control cavity and the cavities that received the treatments after infection with Streptococcus mutans *(Table 3)* or Lactobacillus casei *(Table 4)*.

There were no statistical differences between the microbial counts for the groups of cavities that received or not titanium tetrafluoride, followed by one of the two adhesive systems (p>0.05) *(Tables 3&4)*. Titanium tetrafluoride did not influence the antimicrobial potential of the one or two-step self-etching adhesive systems. Furthermore, there were no statistical differences between the cavities treated with titanium tetrafluoride alone and those restored with either Clearfil SE Bond or Adper EasyOne self-etching adhesives.

Table 3. Microbial count (CFU/mg dentin) of Streptococcus mutans

	CONTROL		TiF_4		Adhesive System		TiF_4+Adhesive System		Tukey
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
AEO	1.7X104 X104 X104	4.1X104	6.0X102	2.3X103	4.9X102	1.4X103	3.0X102	6.3X102	a
CSE	3.0X104	1.1 X105	7.2X102	3.4X103	9.5	2.9X10 X104	2.4X102	6.8X102	a
Tukey	A		B		C		C		

Means followed by different letter types (uppercase horizontally and lowercase vertically) differ among each other (p≤0.05). For statistical analysis purposes, the data were turned into a logarithmic count+0.5.
AEO: Adper Easy One; CSE: Clearfil SE Bond; TiF_4: titanium tetrafluoride

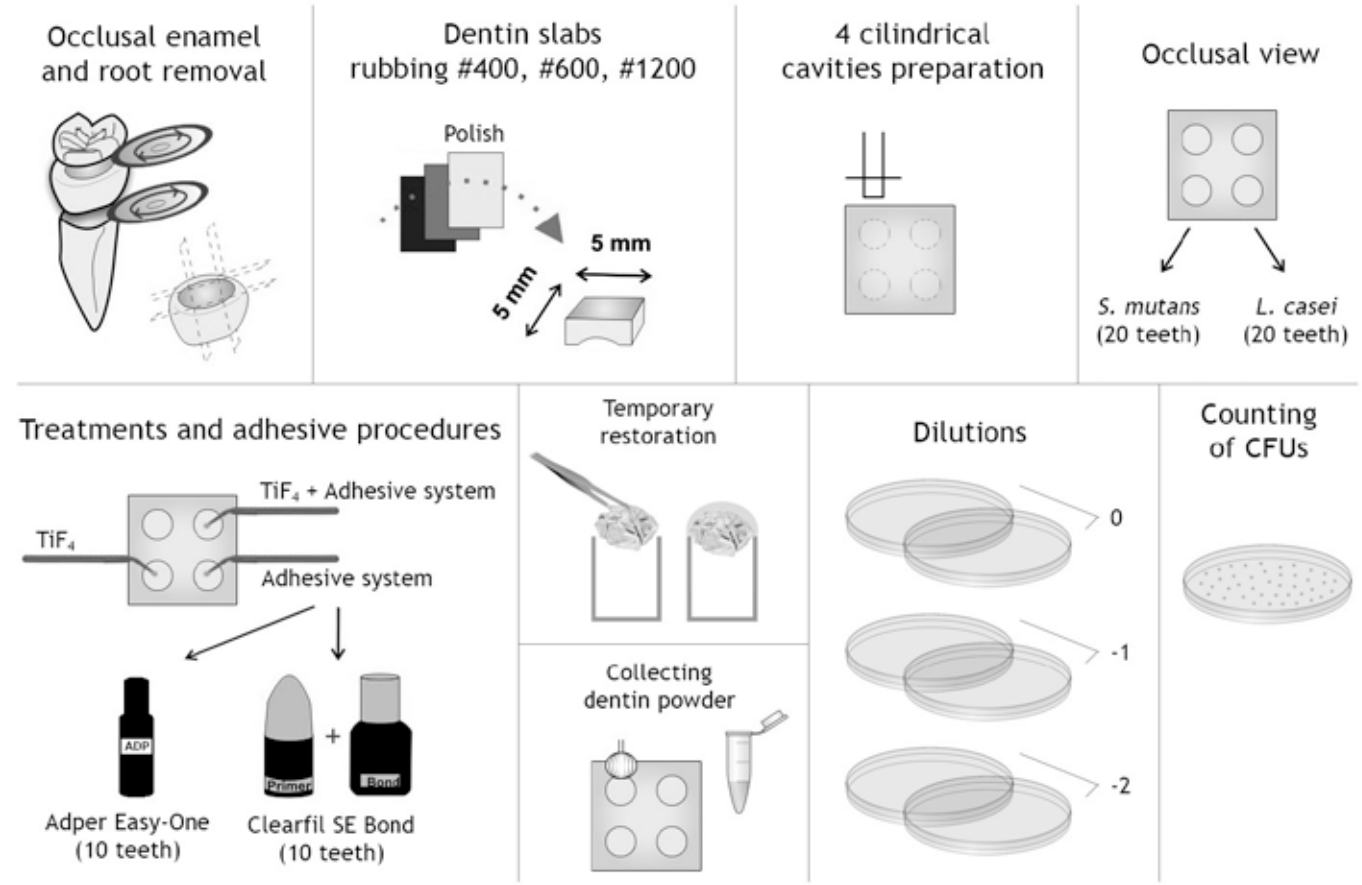

Figure 1: Illustrated flowchart of the experiment. After removal of the enamel portion, dentin surfaces were flattened. Four cylindrical-shaped cavities on the flattened dentin surface of each tooth were prepared. After separating teeth into two groups, according the specific microbial contamination (S. mutans or L. casei), they were immersed in the respective media for either microorganism. One cavity of each tooth was randomly selected and used as a positive control cavity. The other three cavities received each specific agent according to the respective group. After applying the treatments, each cavity was covered with a sterile absorbent paper disc placed on the most superficial area of the cavity, and sealed temporarily with resin composite. After incubation, the temporary sealing was removed, dentin samples were collected, microbial cultivation was performed and microbial count was undertaken.

Table 4. Microbial count (CFU/mg dentin) of Lactobacillus casei

	CONTROL		TiF_4		Adhesive System		TiF_4+Adhesive System		Tukey
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
AEO	3.90E+05	7.22E+05	9.09E+04	5.37E+04	2.87E+04	8.44E+03	2.09E+03	2.96E+03	a
CSE	5.35E+04	8.36E+04	7.10E+04	1.53E+05	1.18E+05	2.88E+05	6.98E+03	1.11E+04	a
Tukey	A		AB		AB		B		

Means followed by different letter types (uppercase horizontally and lowercase vertically) differ among each other ($p \leq 0.05$). For statistical analysis purposes, the data were turned into a logarithmic count+0.5.
AEO: Adper Easy One; CSE: Clearfil SE Bond; TiF_4: titanium tetrafluoride

DISCUSSION

Although this was not a clinical study due to difficulties in standardizing some inclusion criteria, such as depth of cavities and initial microbial counts,[33] the methodology used in this study was the same as that described by Polydorou *et al*.[32] and Türkün *et al*.[31] Furthermore, a clinical study using carious dentin that would be partially removed and then restored with a TiF_4 solution as a dentin pretreatment and self-etching adhesive could not yet be performed. This is because all important properties of this TiF_4 solution and its effects on dentin cavities have not been evaluated, such as biocompatibility, physical and mechanical characteristics and longevity. Moreover, Polydorou *et al*.[32] designed a model with a dental cavity to evaluate the antimicrobial potential of papain gel applied before using self-etching adhesives. Although our methodology was more complex than using agar wells or the diffusion method with paper or dentin discs,[34,35] it proved effective in evaluating the antimicrobial potential of the adhesive systems and of the titanium tetrafluoride.

The present study showed that the application of TiF_4 as a dentin pretreatment promoted an antimicrobial effect, as compared to the control cavities. Therefore, the first null hypothesis was rejected. However, no differences were detected in the inhibiting colony-forming units that were grown, when these units were used either in association with groups TiF_4CSE or TiF_4AEO, or only with self-etching adhesives (CSE or AEO). Thus, the second null hypothesis was accepted, probably due to the antimicrobial potential of the composition of the adhesive systems.

Ionization of fluoride has been responsible for the anticariogenic activity of substances containing fluoride. Ionized fluoride acts, among other mechanisms, by reducing the metabolism and bacteria growth of the main microorganisms involved in dental caries: Streptococcus mutans and Lactobacillus casei.[18-20] Although fluoride has been recognized for its antimicrobial properties,[20,36,37] little is known about the effect of fluoride on cells, or about the defense organism mechanisms against fluoride toxicity.[20] Fluoride is capable of penetrating into bacteria cells in an acidic pH. When fluoride encounters a neutral pH inside an intracellular medium, it dissociates.[38] In the Fform, and at high concentrations, fluoride is capable of inhibiting enolase, a glycolytic enzyme that participates in the metabolism of bacteria, and that acts in the bacteria acid-production process.[39,40] Even at lower concentrations, fluoride can reduce bacterial growth and acid production *in vitro*, and modify biofilm composition.[23]

In regard to the application of TiF_4, micromorphological images of enamel and dentin surfaces treated with 1% TiF_4 evaluated by Skartveit *et al*.[29] showed great variation in bacterial growth between subjects, but no systematic difference between fluoride-treated and -untreated specimens.

Adhesive systems can have an antimicrobial potential, insofar as their effect is attributed particularly to their pH, viscosity, diffusion capacity and the antimicrobial components in their composition, and is further related to dentin permeability and thickness.[10,15,31] The acid nature of the primer composing some self-etching adhesives has been considered one of the main factors related to microorganism inhibition.[15] The acidic monomers incorporated in self-etching adhesives are Phenyl-P, 4-META and MDP, although only MDP is present in the two-step adhesive system tested in the present study.

The MDP monomer in the two-step adhesive system has shown effective microorganism inhibition.[11,35] However, the one step adhesive used in the present study does not have MDP monomer in its composition, and there was no difference in microbial count between the two-step and the one-step adhesives tested. It is speculated that the pH of adhesives was responsible for finding no difference in the antimicrobial potential of the two adhesive systems tested. These adhesives are classified as materials of strong acidity, and previous studies have reported that low pH adhesives have antimicrobial activity.[10]

When the TiF_4 solution was used alone (not combined with an adhesive), its low pH (pH=1.1) may have been responsible for yielding no statistical differences, in comparison to when the solution was used in association with the adhesive systems, since the adhesive systems used in this study also had low pH (Clearfil SE Bond pH=2; Adper Easy One – pH=2.3).[10]

Considering that dentin pretreatment with the TiF_4 solution showed an antimicrobial effect on S. mutans and L. casei microorganisms, in comparison with the control cavities, the use of this agent before applying self-etching adhesive systems should be considered. Although no commercial TiF_4-based products are available on the market, recent reports have observed that pretreatment with TiF_4 did not influence the bond strength of adhesive systems to dentin[24,25] or hybrid layer formation,[25] and provided bio-modified smear layered dentin. Solutions of TiF_4 alone are not stable enough to be used for long periods, since TiF_4 forms deposits at the bottom of test tubes, and does not have a stable pH. Nevertheless, this agent forms a vitreous layer on dentin, which significantly enhances its nanohardness *in vitro* and is resistant to erosive challenges.[28] Self-etching adhesives are able to infiltrate the vitreous biomodified layer.[25]

Despite the use of TiF_4 as a dentin pretreatment before application of a self-etching adhesive system, it showed no antimicrobial effect against Streptococcus mutans and Lactobacillus casei. The clinical applicability of this result is that the potential benefits promoted by the fluoride agent are enhanced, such as a decrease in dentin permeability and the remineralization of carious dentin.[17] However, subsequent research is necessary to evaluate dentin pretreatment with 2.5% titanium tetrafluoride, as well as the anticariogenic potential of this pretreatment *in vitro* and in situ, and its antimicrobial potential in situ and in vivo.

CONCLUSION

It can be concluded that the 2.5% TiF_4 solution showed an antimicrobial effect; however, the use of TiF_4 as a dentin pre-treatment before application of a self-etching adhesive system showed no antimicrobial effect against Streptococcus mutans and Lactobacillus casei.

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MANUFACTURERS' DETAILS

- precision electric cutter (Isomet 1000 Precision Diamond Saw, Buehler Ltd., Lake Bluff, IL, USA)
- water-cooled polishing machine (Politriz Aropol 2V, Arotec, São Paulo, SP, Brazil)
- water abrasive paper (Imperial Wetordry, 3M, USA)
- resin composite (Filtek Z350/ 3M ESPE, St. Paul, MN, USA)
- two-step conventional adhesive system (Adper Single Bond 2/ 3M ESPE, St. Paul, MN, USA)
- diamond tip #2292 with a stop (KG Sorensen, Barueri, SP, Brazil)
- steam (Vertical Steam 30L, CS 078, Prismatec, Itu, SP, Brazil)
- CO_2 incubator (TE 399 CO_2 incubator, Tecnal, Piracicaba, SP, Brazil)
- pH electrode (Seven Multi, Mettler Toledo GmbH, Schwerzenbach, Switzerland)
- halogen light unit (Demetron, LC Kerr Corporation, Orange, CA, USA)
- radiometer (Newdent, Ribeirão Preto, SP, Brazil)
- absorbent paper disc (Melitta, Guaiba, RS, Brazil)
- diamond bur #9713FF (JET Carbide Burs, Beavers Dental Div. Syborn, Morrisburg, Ontario, Canada)
- high-speed turbine (Kavo do Brasil Ind. Com. Ltda, Joinvile, Santa Catarina, Brazil)
- bur # 2 (JET Carbide Burs, Beavers Dental Div. Syborn, Morrisburg, Ontario, Canada)
- handpiece (Kavo do Brasil Ind. Com. Ltda, Joinvile, Santa Catarina, Brazil)
- tube shaker (AP56, Phoenix Luferco, Araraquara, SP, Brazil)
- mitis salivarius agar (Difco-BD, Sparks, MD, USA)
- MRS agar (Difco-BD, Sparks, MD, USA)

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