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Research article

Effects of calcium-phosphate, laser and adhesive on dentin permeability and bond strength

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ABSTRACT

Objectives: The present study examined a technique for reducing dentin permeability through the application of a calcium phosphate (CaP)-based desensitiser with a laser-assisted process and evaluated adhesive-dentin bond strength. **Methods**: Thirty dentin discs were divided into two groups according to whether the selected desensitiser (TeethMate; Kuraray Noritake) was used prior to dentin bonding. Each group was subdivided into three subgroups (n = 5): A- Adhesive (Single Bond Universal, 3M ESPE), AL- Adhesive + Laser (Nd:YAG 60 mJ) and LAL- Laser + Adhesive + Laser. Dentin permeability values (%) were recorded before and after desensitiser application. Resin composites were placed over the bonded specimens; the latter were aged prior to microtensile bond strength evaluation. Gelatinolytic activity within the hybrid layers was examined with *in-situ* zymography using confocal laser scanning microscopy. Data were analysed with ANOVA and Tukey test ($\alpha = 0.05$).
Results: Significant differences in dentin permeability were identified for all groups ($p = 0.00$). Both laser treatment ($p = 0.182$) and desensitiser application ($p = 0.687$) did not significantly improve dentin bond strength. Ultrastructure of the resin-dentin interface identified presence of calcium phosphate within dentinal tubules. Laser treatment did not affect hybrid layer ultrastructure. Both treatment modalities (intratubular CaP occlusion and laser) had no influence on gelatinolytic activity within hybrid layers.
Conclusion: Although intratubular CaP occlusion and laser treatment were effective in reducing dentin permeability, they did not affect bond strength, interfacial ultrastructure and gelatinolytic activity within hybrid layers.
Clinical relevance: Treatment of etched dentin with Nd:YAG Laser at 60 mJ does not adversely affect collagen ultrastructure and gelatinolytic activity within the hybrid layer. The application of a calcium phosphate-based desensitiser to etch dentin does not affect dentin bond strength.

1. Introduction

During the last couple of decades, adhesive systems have continued to evolve because of the overall perception that bonding to dentin is not as durable as bonding to acid-etched enamel [1]. The principle of contemporary dentin bonding involves partial demineralization of dentin and interaction of the adhesive resin monomers with the partially- or fully-exposed hydrophilic collagen matrix, in the presence of moisture derived from the underlying dental pulp. To date, a combination of hydrophilic and hydrophobic monomers is used to facilitate adhesion of tooth-coloured resinous restorations to wet dentin [1, 2, 3, 4].

Hydrolytic degradation remains one of the biggest challenges in terms of resin-dentin bond durability, with significant decreases in dentin bond strength over time [1, 5, 6, 7, 8, 9]. Although water keeps the collagen matrix expanded in a demineralized collagen matrix for infiltration of adhesive resin monomers during bonding, water entrapment or diffusion into the polymerized resin matrix also promotes degradation of the collagenous and adhesive components of the hybrid layer. This produces

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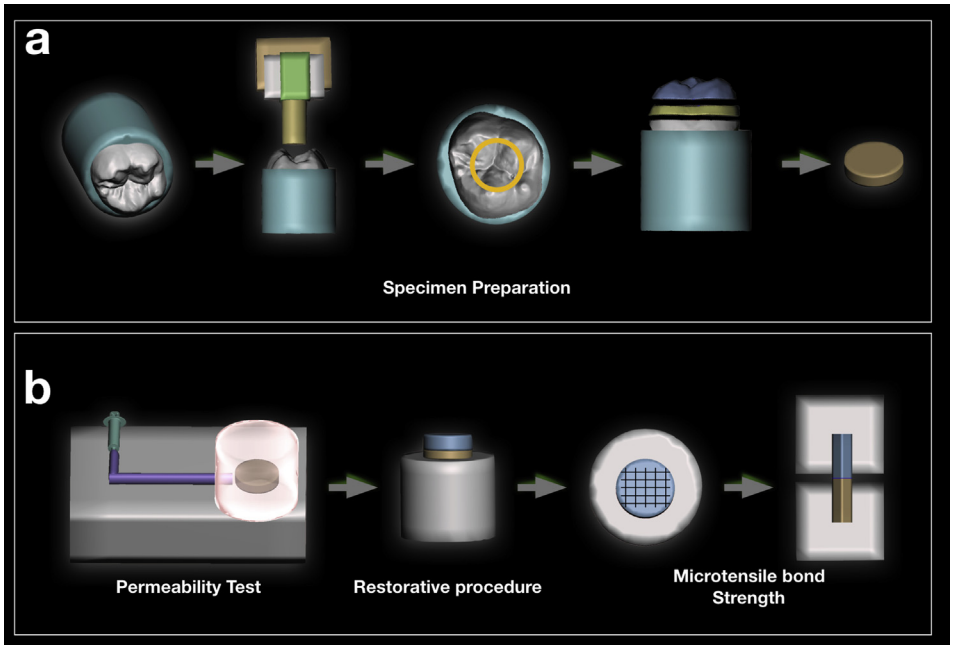

Figure 1. a. Schematic of the specimen preparation. **b**. Schematic of permeability testing, restorative procedure and bond strength testing.

microleakage and compromises the longevity of dentin bonding [4, 10, 11, 12, 13, 14].

To circumvent the aforementioned problem, different strategies have been proposed to reduce the entrapment of water within the resin-dentin interface. For example, water displacement by ethanol has been used to maintain continuity of the interfibrillar spaces when water is removed from acid-etched dentin, and to avoid phase separation of the "oily" resin monomers in the presence of water [15]. Dentin desensitisers have also been used to decrease dentin permeability in acid-etched dentin prior to the application of bonding agents [16, 17]. These desensitisers work by occluding patent dentinal tubules, thereby reducing dentin sensitivity that may arise after the use of etch-&-rinse adhesives. Dentin desensitisers that contain calcium phosphate have been reported to have no adverse effect on dentin bond strength, when compared to calcium oxalate-based desensitisers [18].

Another strategy used to reduce dentin permeability is irradiation of the dentin surface with a neodymium-doped yttrium aluminium garnet (Nd:YAG) laser [19]. Laser irradiation causes melting and recrystallization of the dentin (and enamel) surface. This process produces physical, morphological and chemical changes that generates an irregular surface with evaporation and/or fusion of the smear layer with the underlying intact [20]. The use of Nd:YAG laser to improve dentin adhesion has been reported in previous studies [21, 22, 23]. Acid-etched dentin that is irradiated by Nd:YAG lasers contain melted and occluded dentinal tubules [24, 25, 26, 27, 28], which is responsible for lower dentin permeability [29]. Hence, it is speculated that calcium phosphate formed by a dentin desensitiser may be incorporated in the laser treatment process to enhance occlusion of the dentinal tubules and further reduce dentin permeability prior to the application of etch-&-rinse adhesives.

Accordingly, the objective of the present study was to evaluate the association between calcium phosphate deposition in dentinal tubules, and laser irradiation of dentin, with dentin permeability, as well as the effects of these two attributes on dentin bond strength. The null hypothesis tested was that the use of a calcium phosphate desensitiser alone, or in combination with Nd:YAG laser irradiation, has no effect on dentin permeability and dentin bond strength.

2. Materials and methods

2.1. Specimen preparation

Thirty freshly extracted non-carious human third molars were collected based on a protocol approved by the Human Assurance Committee of the Institute of Science and Technology – Sao Paulo State University, Brazil, with informed consent obtained from the donating subjects with respect to the use of human tissues (Protocol number: 2.022.356, CAAE: 66097117300000077). The research described has been carried out in accordance with the Code of Ethics of the World Medical Association Declaration of Helsinki for experiments involving humans. The extracted teeth were stored at 4 °C in distilled water that was supplemented with 0.9% sodium azide to prevent bacteria growth for no longer than 6 months.

Dentin discs (6 mm in diameter, 3 mm thick) were initially obtained from the extracted teeth, as previously described [30]. Each dentin disc had its occlusal surface polished successively with 300-grit and 600-grit silicon carbide papers under water irrigation until it was 1.6 mm thick, with no traces of enamel remaining. Subsequent polishing was conducted successively on both sides of the specimen with 2400-grit and 4000-grit silicon carbide papers under water irrigation until the specimen was exactly 1.5 mm thick (Figure 1a).

2.2. Dentin permeability

Permeability was quantified using the split-chamber hydraulic conductivity system (THD-02b apparatus, Odeme Ltda, Brazil) reported by Pashley *et al.* [31] (Figure 1b). Initial hydraulic conductance (Lp*ini*) was defined by calculating the mean flow/min as previously described [32, 33, 34]. After defining the Lp*ini*, the exposed dentin from each specimen was treated with 0.3% citric acid for 30 s to remove the smear layer and simulate surfaces with patent dentinal tubules [31]. This permeability measurement was taken to be the maximum hydraulic conductance (Lp*max*). Each specimen was then sonicated in ultrapure water for 10 min prior to further experiments.

Table 1. Composition and protocol of use of the materials tested.

Material	Composition	Application protocol
Adhesive system: Single Bond Universal (3M ESPE, St Paul, USA)	MDP phosphate monomer, HEMA, dimethacrylate resins, Vitrebond copolymer, filler, ethanol, water, initiators, silane	The adhesive was actively applied with a microbrush for 20 s, followed by air blast for 5 s with distance of 10 cm, and cure for 10 s (LED light, 1200 mW/cm^2 - Radii-cal, Australia).
Nd: YAG laser (Pulse Master 600 IQ, Texas, USA)	Wavelength of 1.096 μm, power of 60 mJ	It was used a quartz optical fiber with a diameter of 320 μm, 1 mm from the surface, with irradiance for 60 s. The pulse length was 0.1 ms, and energy density was 74.64 J/cm^2
Calcium Phosphate: TeethMate Desensitizer (Kuraray Noritake Dental Inc.)	Dicalcium phosphate anhydrate, Tetracalcium phosphate, water, preservative	Active application over occlusal dentin surface with a microbrush for 30 s, followed by water/air spray and dry with absorbent paper. This application was performed previously adhesive and/or laser irradiance.

2.3. Dentin treatments

After calculating initial and maximum Lp, the specimens were divided in 2 groups (n = 15) according to whether a calcium phosphate-based dentin desensitiser (Teethmate™ Desensitizer, Kuraray Noritake Dental Inc., Tokyo, Japan) was used prior to dentin bonding. Each group was subdivided in three subgroups (n = 5) according to the treatment performed: adhesive (A), adhesive + Nd:YAG Laser (AL) and adhesive + Nd:YAG Laser + adhesive (ALA). The materials used and the application protocol are depicted in Table 1. Groups treated initially with the desensitiser were designated as FA, FAL and FALA, respectively. Hydraulic conductance was re-measured following a designated treatment (Lp*treat*), using the same parameters described for the initial measurement. Dentin permeability was calculated as percentages, using the formula (Lp*ini*/Lp*max*)*100% for permeability before treatment and (Lp*treat*/Lp*max*)*100% after treatment [32, 33, 34].

After obtaining the post-treatment hydraulic conductance, composite build-up was performed over the bonded dentin (Filtek Supreme, 3M ESPE, St. Paul. MN, USA) using a Teflon matrix (6 mm diameter, 3 mm high). Two increments of the resin composite were placed successively inside the matrix, with each increment light-activated individually for 20 s (Figure 1b). The composite-dentin specimens were subsequently aged in a thermocycling machine (10,000 cycles, 5/55 °C, with 25 s dwell time and 5 s transfer time) corresponding to the 6-month aging in the oral cavity [35].

2.4. Bond strength evaluation

After artificial ageing, each specimen was sectioned into sticks using a cutting machine (Isomet, Buehler Ltd., Lake Bluff, IL, USA) equipped with a diamond saw with water cooling. The specimens had approximate cross-sectional dimensions of 1.0 mm × 1.0 mm (Figure 1b) which was checked with a digital caliper. Eight sticks were produced from each tooth. The adhesive area was measured using a digital calliper and used to calculate the bond strength value in MPa. Each stick was individually fixed on a metal jig using cyanoacrylate glue (Zapit, Dental Ventures of America, Corona, CA, USA). The assembly was tested in a universal testing machine (DL-1000, EMIC, São José dos Pinhais, PR, Brazil) with a 10 kg load cell, at a crosshead speed of 1.0 mm/min (Figure 1b). The bond strength was calculated in MPa dividing the force at failure (N) by the bonding area (mm^2). All specimen that undergo pre-test failures were discarded [36]. After failure, each stick was examined at 40x magnification to determine the failure mode. Failure was classified as adhesive, cohesive in composite, cohesive in dentin or mixed failure.

2.5. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM)

Specimens with adhesive and mixed failures were selected for SEM analysis. The specimens were placed in aluminium stubs, sputter-coated with gold/palladium and examined at 2,000x and 5,000x with a SEM (JSM-5310, JEOL Ltd., Akishima, Tokyo, Japan) operating at 20 kV.

Specimens designated for TEM were prepared as previously described [37]. Each treated tooth specimen was restored with a flowable composite (Clearfil Protect Liner F, Kuraray Medical Inc., Tokyo, Japan), from which a 0.9 mm thick central slice containing the resin-dentin interface was retrieved by sectioning. The slices were individually demineralised in 0.1 mol/L ethylenediaminetetraacetic acid, followed by application of Karnovsky's fixative and post-fixed in 1% osmium tetroxide. Then, they were dehydrated in ethanol in ascending concentration from 50% up to 100%, immersed in propylene oxide and embedded in epoxy resin as previously described [37]. Finally, sections with ninety nanometre thickness were cut with help of an ultramicrotome, stained with 2% aqueous uranyl acetate and 1% Reynold's lead citrate, and examined with a Transmission Electron Microscope (JEM-1230, JEOL).

2.6. Confocal laser scanning microscopy (CLSM)

In-situ zymography of the resin-dentin interface was performed using the method reported by Gou *et al.* [37], which uses gelatin conjugated to quenched fluorescein as the substrate and aims to identify the location and activity of endogenous enzymes within the resin-dentin interface.

Table 2. Results in percentage (%) of dentin permeability before and after treatments.

Groups	Dentin permeability		
	Initial (%)	Final (%)	Permeability Reduction %
A	45.9 (±4.3)	27.2 (±2.7)	40.6 (±4.4) ab
AL	59.9 (±6.5)	36.9 (±5.0)	38.3 (±6.4) ab
LAL	59.2 (±14.9)	44.8 (±13.5)	23.8 (±12.7) a
FA	52.1 (±19.6)	40.3 (±13.9)	21.2 (±11.9) a
FAL	49.4 (±18.6)	28.6 (±6.2)	37.3 (±18.8) ab
FLAL	60.4 (±21.24)	30.2 (±13.6)	49.0 (±14.1) b
RM Anova:	a	b	One-way Anova: p = 0.013

Table 3. Repeated measures ANOVA for dentin permeability.

	SS	Degr. of	MS	F	p
Intercept	119231,0	1	119231,0	395,6953	0,000
Treatment	1682,0	5	336,4	1,1164	0,378
Error	7231,7	24	301,3		
TIME	5889,9	1	5889,9	119,4992	0,000
TIME*Treatment	538,8	5	107,8	2,1865	0,089

Table 4. Mean and standard deviation for the bond strength evaluation.

Groups	MPa	
	Without Phosphate	With Phosphate
A	24.5 (±6.6) ^{Aa}	30.7 (±1.0) ^{Ab}
AL	29.3 (±2.0) ^{Ba}	24.8 (±4.4) ^{Bb}
LAL	30.2 (±2.3) ^{Ba}	30.1 (±3.7) ^{Aa}

*Lowercase letters shows differences within the lines for presence and absence of phosphate. Uppercase letters show differences within the columns for the treatment groups (adhesive and laser).

Table 5. Bond strength results for the Two-way ANOVA.

	SS	Degr. of Freedom	MS	F	p
Intercept	23976,92	1	23976,92	1652,911	0,000
Treatment	52,97	2	26,48	1,826	0,183
Phosphate	2,41	1	2,41	0,166	0,687
Treatment*Phosphate	141,57	2	70,79	4,880	0,0167
Error	348,14	24	14,51		

Briefly, it was obtained from the centre of the tooth a 1-mm thick slab containing the resin-dentin interface. The slab was polished to approximately 50 μm thick and zymography was performed with the Enz-Chek™ Gelatinase/Collagenase Assay Kit (E-12055, Molecular Probes, Eugene, OR, USA). On the top of each slab it was placed a 50 μL aliquot of the quenched fluorescein-conjugated gelatin mixture, followed by protection from light, and incubated in a 100% relative humidity chamber at 37 °C for 48 h. The release of the fluorescein is resultant from the hydrolysis of the quenched fluorescein-conjugated gelatin, which was detectable with a multi-photon CLSM (LSM 780, Carl Zeiss, Oberkochen, Germany) at the excitation/emission wavelength of 458/540 nm. Optical sections of ten micrometre thick were obtained from different focal planes. The stacked images were analysed using the ZEN 2010 software (Carl Zeiss).

2.7. Statistical analysis

The dentin permeability and bond strength data were analysed separately. Data series were examined for their normality (Shapiro-Wilk test) and equal variance assumptions (modified Levene test) prior to the use of parametric statistical methods. Significance differences among the groups for dentin permeability were analysed using repeated measures two-factor analysis of variance (RM-ANOVA) and for bond strength, it was used two-factor ANOVA. For both analysis post-hoc Tukey's tests were performed. Statistical significance was pre-set at $\alpha = 0.05$.

3. Results

The mean results of dentin permeability are presented in Table 2 and the RM ANOVA is presented in Table 3 which shows that there was

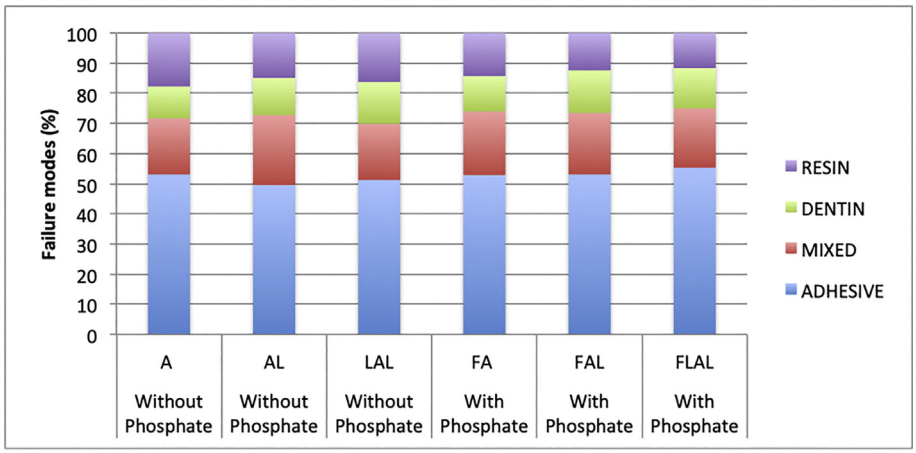

Figure 2. Failure modes of resin-dentin bonds in different specimen groups that had been stressed to failure in tension.

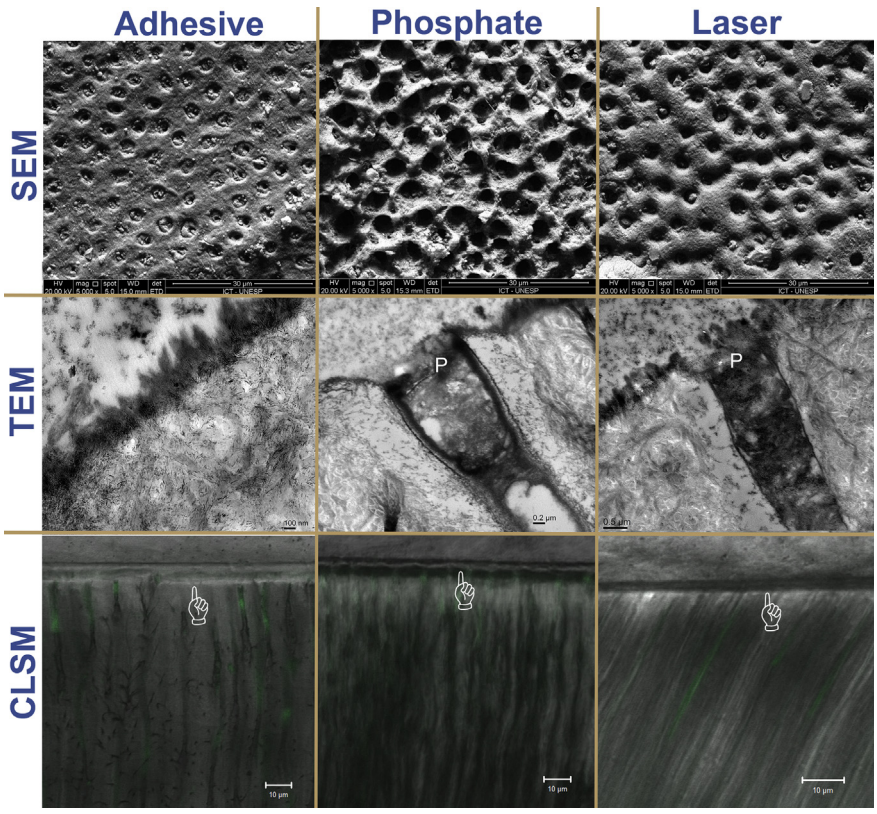

Figure 3. Top row: representative SEM images of the dentin side of fractured composite-dentin sticks showing mineralized dentin and fractured resin tags within the dentinal tubules. Bars = 50 μm. Middle row: Representative TEM images of resin-dentin interfaces created by different treatments. Adhesive: adhesive only; Phosphate: acid-etched dentin treated with calcium phosphate desensitiser prior to adhesive application. Laser: acid-etched dentin treated with calcium phosphate desensitiser and laser irradiation prior to adhesive application. There is no difference in the collagen fibrils within the hybrid layers. P: calcium phosphate deposits within a hybridized smear plug. Bars = 500 nm. Bottom row: Representative CLSM images of *in-situ* zymography of dentin hybrid layers that were placed in contact with quenched fluorescein conjugated gelatine. Several small dots of green fluorescence appeared along the hybrid layer. Pointers: areas in which gelatinolytic activities were absent within the hybrid layer.

significant differences only for the time factor, with dentin permeability before treatment being higher than that after treatment ($p < 0.001$). There was no difference in the effect of different treatment modalities ($p = 0.378$). Likewise, no significant difference was identified in the interaction between time (before and after treatment) and treatment modalities ($p = 0.089$).

The results of bond strength are presented in Table 4 and for the two-factor ANOVA is presented in Table 5. The use of the calcium phosphate desensitiser had no significant effect on bond strength results ($p = 0.687$). Likewise, the use of laser had no significant effect on bond strength results ($p = 0.182$). For the interaction between factors Fisher LSD tests showed that the groups A and FAL presented lower values than the other groups.

Analysis of the failure modes indicated that there was a strong prevalence of adhesive failure in all groups (Figure 2). A weak Pearson product-moment correlation coefficient ($r = 0.375$) was identified between dentin permeability and bond strength.

Representative SEM and TEM images are illustrated in Figure 3. Tubular occlusion with calcium phosphate with or without laser treatment did not appear to affect the dentin hybridization. Representative CLSM images of *in-situ* zymography are also shown in Figure 3. In all treatment groups, no intense fluorescence band was observed within the hybrid layer that is indicative of extensive hydrolysis of the fluorescein-conjugated gelatine that was placed on top of the polished composite-tooth section.

4. Discussion

Water is responsible for the degradation of resin-dentin interface [7, 38, 39, 40]. Techniques to decrease dentin surface moisture and adhesives with lower water absorbance are required for longer adhesion stability [7]. The present work attempted to modify the dentin surface by occluding the dentinal tubules with calcium phosphate deposits and sealing the tubular orifices with laser treatment. The null hypothesis that "the use of a calcium phosphate desensitiser alone, or in combination with Nd:YAG laser irradiation, has no effect on dentin permeability and dentin bond strength" was rejected because application of the calcium phosphate desensitiser, with or without the adjunctive use of laser irradiation, decreased permeability and increased bond strength in some groups. Since this study has not evaluated the initial bond strength, we cannot conclude that the application of treatments would increase the bond strength despite the permeability has decreased.

The dentin permeability reduction was identified in all treatment groups and it can be attributed to the use of the etch-and-rinse adhesive system, associated or not with the dentin desensitizer (Table 2). It was expected that the use of desensitizer agents would cause a reduction in permeability due to tubular occlusion and, consequently, the amount of water trapped within the adhesive layer [19]. Although dentin permeability decreased after use of the Teethmate Desensitizer, the decrease cannot be solely attributed to the use of this product, once it decreased for all groups. Therefore, the adhesive also played an important role in this. Our analysis showed that only the groups LAL and FA presented lower reduction of permeability than the FLAL group, while all others presented similar outcomes. The association of the phosphate desensitizer with the laser irradiation (FLAL) might have created a favourable environment to improve the laser absorption into the dentin tissue.

Although reduction in dentin permeability after laser irradiation has been reported [41, 42] and suggested as a treatment modality for elimination of dentin hypersensitivity [42, 43], tubular occlusion was not observed by TEM in the present work (Figure 3). This may be explained by the laser parameters used in the present study (60 mJ, energy density 74.64 J/cm), which might not be high enough to change the resin-dentin interfacial ultrastructure. Even in groups where the laser was applied before and after adhesive application, the reduction in dentin permeability was not evident. These laser parameters are considered safe for clinical application; further increase in laser energy may result in inadvertent pulpal temperature rise that may endanger pulpal health [44]. A 5.5 °C increase in intrapulpal temperature has been reported to cause irreversible pulpal damage in 15% of vital teeth [45].

Desensitisers have been advocated for the treatment of dentin hypersensitivity, and it has been suggested that the use of calcium phosphate-based desensitisers is preferable over calcium oxalate-based desensitisers when employed prior to the use of etch-&-rinse adhesives [18]. Results of the present study indicate that the use of the Teethmate Desensitizer imporved bonding when only the adhesive was applied (Table 4), possibly due the increase of calcium and phosphate favoring the action of the MDP component from the adhesive. However, when we analyzed the presence or absence of phophate in all groups (Table 5), there was no significant differences, and also for the laser application, indicating that, overall, the application of the Teethmate Desensitizer prior to the use of the etch-&-rinse adhesive does not jeopardize the bond strength of a restotarion if a compatible adhesive system is used. This is in line with the outcome of another previous *in vitro* study [46].

These results are in accordance with the qualitative analysis made with *in-situ* zymography, which is a simple and fast laboratory technique to localize protease activities in tissues [47]. The technique has been adopted for screening of gelatinolytic activities within dentin hybrid layers [48, 49]. In the present study, only weak fluorescence was detected from the base of hybrid layers formed after the application of the desensitiser, with or without adjunctive laser treatment; these results were similar to that of the control group (Figure 3). This indicates that there the use of dentin desensitizer or laser treatment of acid-etched dentin does not adversely increase the gelatinolytic activity within hybrid layers created in dentin.

5. Conclusion

Within the limits of the present *in vitro* study it may be concluded that application of a calcium phosphate-based desensitiser or the use of mild laser irradiation of the acid-etched dentin prior to adhesive application does not compromise resin composite bond strength to dentin. The different treatments do not adversely affect resin-dentin interfacial ultrastructure or increase gelatinolytic activities within the hybrid layer.

Declarations

Author contribution statement

Pablo L. B. Sellan: Conceived and designed the experiments; Performed the experiments.

Rayssa F. Zanatta: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Carlos R. G. Torres, Brian E. Bergeron: Conceived and designed the experiments; Analyzed and interpreted the data.

Fu-cong Tian: Analyzed and interpreted the data.

Li-na Niu: Conceived and designed the experiments; Wrote the paper.

Cesar R. Pucci: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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