



Influence of grape seed extract in adhesion on dentin surfaces conditioned with Er,Cr:YSGG laser

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Abstract

The proanthocyanidin (PA)-rich grape seed extract (GSE) is a collagen cross-linking agent that can perform a chemical bond with the dentin's collagen. The objective of this study was to evaluate the influence on shear bond strength (SBS) of the pre-conditioning of GSE, on human dentin surfaces conditioned with Er,Cr:YSGG laser. The sample consisted of 64 non-carious human teeth, divided into eight groups, four groups conditioned with Er,Cr:YSGG laser (4.5 W, 50 Hz, 50 μ s, 70% air, 90% water) and four prepared with conventional methods (control). In both groups, a GSE solution was applied before using the two adhesives tested: ClearfilTM SE Bond (CSE) and ScotchbondTM Universal (SU). Subsequently, a SBS test, a scanning electron microscopy, and a statistical analysis were performed. In the laser groups, the best SBS mean (20.08 ± 4.01 MPa) was achieved in the group treated with GSE and CSE. The control group with the application of CSE showed the highest SBS mean (24.27 ± 10.28 MPa), and the group treated with laser and SU showed the lowest SBS mean (12.94 ± 6.51 MPa). Between these two groups there was a statistically significant difference ($p = 0.05$). However, this was not observed among the laser or control groups. The type of dentin surface preparation can influence the SBS. The CSE showed better SBS in laser and control groups. The presence of GSE did not improve the adhesion on surfaces conditioned with laser, but more studies should be carried out in the future to confirm this conclusion.

Keywords Lasers · Adhesives · Matrix metalloproteinases · Collagen · Grape seed extract · Proanthocyanidin

Introduction

Erbium lasers show several advantages in tooth cavity preparations, when compared to conventional drilling, as they promote the selective ablation of more hydrated tissues, like the carious dentin, and reduce the dental tissue loss, and the need for anesthesia. They also have a bactericidal effect and a low vibration level, which prevents microfractures [1–4].

The ablation on dentin performed by the Er,Cr:YSGG laser (2.79 μ m) occurs as a result of its absorption by the water present in the dentin, with its expansion, resulting in the water evaporation and the increase of the internal pressure, with a consequent explosion and destruction of this tissue [5, 6].

Erbium lasers can be used to prepare the tooth cavity or to condition the surface that will be submitted to bonding procedures, depending on the parameters of the laser.

During the restorative procedures, the formation of the hybrid layer in situ is believed to be essential for dentin bonding. Therefore, the stability and maintenance of dentin collagen is critical for an effective bonding [7].

Despite all the efforts to develop simpler, less technique-sensitive, and more lasting adhesives, bonding to dentin and

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the complete sealing of the dentinal surfaces are still a challenge.

The type of dentin surface after its preparation or conditioning plays an important role in the bonding performance of the restorative materials to tooth structure [8].

The preparation or conditioning of dentin with erbium lasers results in the absence of smear layer, opened dentinal tubules, and microirregularities due to the preferential removal of the intertubular dentin. Although the effect of laser on collagen network is still not completely clear, it is known that laser irradiation is able to develop microstructural changes as well as microrupture of collagen fibers [9].

The matrix metalloproteinases (MMPs) have a central and important role in several biological and pathological processes, and can hydrolyze extracellular matrix components [10]. MMPs are present in the calcified dentin, in active or proenzyme forms [11, 12], that can be activated by an acidic environment [12, 13]. The application of mild acids, etch-and-rinse, and self-etch adhesives can activate the MMPs on the dentin [14].

The MMPs induce an enzymatic degradation that interferes with the resin-dentin bonding stability [15]. In order to avoid this kind of event, a research is ongoing to find procedures that can improve dentin's collagen resistance, such as MMP inhibitors and collagen cross-linking agents [16].

The proanthocyanidin (PA)-rich grape seed extract (GSE) is a collagen cross-linking agent that can perform a chemical bond with the dentin's collagen, increasing its resistance to demineralization and decreasing the dentin bond to MMPs, without cytotoxicity [16].

It is known that PA is not affected by low pH due to its cross-links with the collagen fibers that are not covalent, forming hydrogen bonds and hydrophobic interactions which enhance the mechanical properties of the demineralized dentin matrix, improving the resistance to the MMPs [17].

Objective

The objective of this study was to evaluate how the shear bond strength (SBS) is influenced by the application of a grape seed extract solution on dentin surfaces conditioned with Er,Cr:YSGG laser.

The null hypothesis is that the application of a GSE solution on dentin would not affect the bond strength of the adhesives applied to dentin conditioned with Er,Cr:YSGG laser or prepared with conventional methods.

Material and methods

The sample consisted of 64 human non-carious molars or premolars extracted for periodontal or orthodontic reasons. After

extraction, the teeth were cleaned and stored in 0.5% chloramine-T at a temperature of about 4 °C. Afterward teeth were randomly divided into eight groups, four groups for conditioning with laser and four with conventional methods (control). This study project was approved by the Ethics Committee of the Faculty of Dental Medicine of Oporto University.

For the SBS test, five teeth from each group were longitudinally sectioned into two parts, where the proximal enamel was removed by a waterproof silicon carbide paper disk grit 800, 230 mm, with copious supply of water, to expose the dentin.

For the scanning electron microscopy (SEM), three teeth from each group had their coronal occlusal third and the roots removed, by bisecting the tooth transversely with a low-speed diamond saw and copious supply of water. It resulted in dentin disks of approximately 2 mm thick.

Shear bonding samples were then submitted to a surface treatment that included an adhesive technique using two different adhesives (Table 1).

Groups 1, 2, 3, and 4 were submitted to an Er,Cr:YSGG laser (Waterlase iPlus – Biolase Technology Inc., Irvine, CA), with the settings 4.5 W, 50 Hz, 50 μ s, 70% air, 90% water (20 ml/min), 0.09 J of Pulse Energy, and the MZ8 tip with 1–3 mm of tip-to-tissue distance (quartz, 6 mm length, 0.80-mm diameter, round shape, and 0.50 mm² of area, spot area of irradiation at tissue level (1 mm/3 mm distance [taking in consideration the 45° angle] = 0.0159 cm²/0.0389 cm²). The beam was applied to the surface with an angle of 45°, because it is an angle often used clinically, with smooth horizontal and vertical movements, for 12 s in the SBS dentin disks samples (area of 16 mm², energy density 1–3 mm distance = 212–86.76 J/cm², total energy per sample 1–3 mm distance = 33.92–13.88 J), and 8 s in the SEM samples, where the area was smaller than the disks (area of 12 mm², energy density 1–3 mm distance = 186.79–76.34 J/cm², total energy per sample 1–3 mm distance = 22.44–9.12 J).

The samples of the Groups 5, 6, 7, and 8 were not submitted to any laser treatment, working as control groups.

In Groups 1, 4, 6, and 8, a Solution of GSE (Pure Series™ Grape Seed Extract, Bulk Powders®, Colchester, United Kingdom) (2 g/ml, in 20% ethanol solution) was also applied before the adhesive. The GSE solution was applied for 10 s and dried with mild air for 5 s.

In Groups 3, 4, 7, and 8, Clearfil™ SE Bond (Kuraray, Medical Inc., Tokyo, Japan) (CSE) 2 bottle, two-step was applied as follows: application of primer for 20 s, drying with mild air for 5 s, application of the adhesive making a uniform bond film using gentle air flow, and light curing (DouroLed X-Lite3; 1100 mW/cm²) for 10 s.

In Groups 1, 2, 5, and 6, the Scotchbond™ Universal (3M ESPE, Seefeld, Germany) (SU) was applied as follows: application with a microbrush for 20 s, drying with mild air for 5 s, and light curing for 10 s.

Table 1 Surface treatment performed in the laser and control groups

| Groups | Surface treatment | Pre-conditioning | Adhesive |
|---------|-------------------|------------------|--|
| Group 1 | Er,Cr:YSGG laser | – | Scotchbond™ Universal Adhesive 3M |
| Group 2 | Er,Cr:YSGG laser | GSE | Scotchbond™ Universal Adhesive 3M ESPE |
| Group 3 | Er,Cr:YSGG laser | – | Clearfil™ SE Bond |
| Group 4 | Er,Cr:YSGG laser | GSE | Clearfil™ SE Bond |
| Group 5 | – | – | Scotchbond™ Universal Adhesive 3M ESPE |
| Group 6 | – | GSE | Scotchbond™ Universal Adhesive 3M ESPE |
| Group 7 | – | – | Clearfil™ SE Bond |
| Group 8 | – | GSE | Clearfil™ SE Bond |

To perform the SBS test, the Grandio® (VOCO, Germany) resin was applied on the dentin surface of each sample, using a tape of polyester (Mylar) and a silicone mold with 3 mm of diameter and 4 mm of height, and all layers were light-cured for 20 s with a LED curing light (DouroLed X-Lite3; 1100 mW/cm²). The same resin was directly applied on the dentin disks used in the SEM.

All the samples were placed in an incubator at 37 °C with 100% humidity for 24 h. Next, they were thermocycled 500 times in baths of 5 °C and 55 °C for 20 s at each temperature to simulate the in vivo conditions in the oral cavity, as the International Organization for Standardization (ISO) TR 11450 standard (1994) indicates that a thermocycling regimen comprising 500 cycles in water between 5 and 55 °C is an appropriate artificial aging test [18]. At the end of thermocycling, the samples returned to the incubator for 24 h.

The SBS was performed using the Instron® (Norwood, MA, USA) with cell specification of 1-KN carda cell. SBS was tested at 1 mm/min.

Regarding the SEM, the samples were split in half (MD) with a diamond blade mounted on the handpiece with copious water, which resulted in restored hemi-disks.

The scanning electron microscopy–energy dispersive spectroscopy (SEM/EDS) evaluation was performed using a High resolution (Schottky) Environmental Scanning Electron Microscope with X-Ray Microanalysis and Electron Backscattered Diffraction analysis: Quanta 400 FEG ESEM/EDAX Genesis X4M. The electron microscopy micrographs were taken between the variation of $\times 300$ and $\times 5000$ magnifications. The photomicrographs were evaluated according to the following criteria: formation of hybrid layer, formation of resin tags, and their length.

All statistical analyses were performed using SPSS version 22.0 (SPSS Inc., Chicago, IL, USA).

The SBS values were analyzed using the Kolmogorov-Smirnov test to evaluate the normal distribution of the variable. The homogeneity assumptions were controlled by the Levene test. The one-way ANOVA was applied and the Tukey post hoc test was performed to compare pairwise, with a confidence interval of 95%.

Results

Regarding the SBS test, in the laser groups, Group 4 (20.08 ± 4.01 MPa) had the highest shear bond strength value, and Group 1 (12.94 ± 6.50 MPa) had the lowest. There was no statistically significant difference between the laser groups.

In the control groups, Group 7 (24.27 ± 10.28 MPa) had the highest value of SBS, and Group 6 (14.80 ± 8.85 MPa) had the lowest. Nevertheless, there was no statistically significant difference between any of the groups.

The results of mean and standard deviation of the SBS (MPa) for all groups are presented in Table 2 and Graph 1.

Group 7 had the highest SBS mean (24.27 ± 10.28 MPa), even though it also had the highest standard deviation. By contrast, Group 1 showed the lowest SBS mean (12.94 ± 6.50 MPa) (Graph 1). Between these two groups there was a statistically significant difference ($p = 0.05$) (Table 2 and Graph 1).

Comparing the two self-etch adhesives, the groups where CSE was applied had better SBS results than the groups with SU overall ($p = 0.032$).

The microphotos of the scanning electron microscopy in Group 1 and Group 2 show a gap between the dentin substrate and the hybrid layer, some tags penetrating the dentin tubules, and a thin hybrid layer (Fig. 1a, b).

In Groups 3 and 4, it is possible to observe a homogeneous and thick adhesive layer, with penetration of resin tags in the dentin tubules. The microphotos in Group 4 show a higher penetration of substrate tags in the dentin tubules (Fig. 1c, d).

There are a few resin tags penetrating in the dentin tubules, in Groups 5 (Fig. 2a), 7 (Fig. 2c), and 8 (Fig. 2d), being longer in Group 7 (Fig. 2c) and more numerous on Group 8 (Fig. 2d). Group 6 (Fig. 2b) shows almost an absence of resin tags (Fig. 2).

Discussion

Resin adhesion to dentin has always been considered a challenge due to the complex structure of dentin, with collagen fibers and high content of proteins and water [19].

Table 2 Mean and standard deviation of the shear bond strength (MPa) in the eight groups

| | Laser groups | | | | Control groups | | | |
|-----------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|-----------------------|-----------------------|
| | Group 1 | Group 2 | Group 3 | Group 4 | Group 5 | Group 6 | Group 7 | Group 8 |
| SBS (MPa) | 12.94 (± 6.50) | 15.32 (± 6.34) | 19.43 (± 6.84) | 20.08 (± 4.01) | 20.04 (± 9.71) | 14.80 (± 8.85) | 24.27 (± 10.28) | 22.52 (± 10.37) |

The introduction of new technologies in dental field such as the erbium lasers brings up well-known advantages to the dentist and patient. In that sense, there was a necessity to study and understand how the restorative materials used in the cavities, as resin and adhesives, prepared by conventional methods, would behave in the surfaces prepared with laser [7, 20].

The Er,Cr:YSGG laser is highly absorbed by the water, and when the steam pressure within the tissue exceeds the structural strength, microexplosions occur, with the ejection of particles from the fractured material removing effectively the hard tissue [21]. The ablation occurs by a thermo-mechanical mechanism.

Despite the theoretical advantages related to surface preparation with erbium lasers, such as the absence of smear layer and presence of open dentinal tubules, still several studies show a low bond strength in surfaces prepared with Er,Cr:YSGG laser, when compared to conventional methods [9, 22–24].

To achieve a comparable SBS on dentin surfaces treated with laser and dentin surfaces prepared with conventional methods, it is necessary to improve the existing bonding systems or change the surface conditions for those bonding agents [24].

It is known that the adhesion between dentin and resin is more micromechanical than chemical [25]. To ensure a good adhesion, it is necessary to have tags and a uniform hybrid layer that is achieved by removing the smear layer and

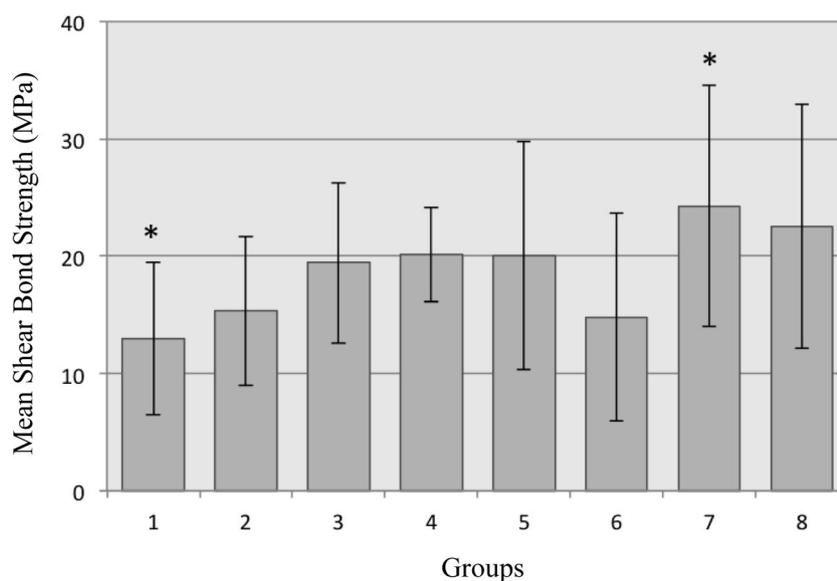
demineralizing the surface of the dentin in order to expose the collagen fiber network, and allow the infiltration of the bonding agents, when using etch-and-rinse adhesives [25].

Several studies showed that the hybrid layer could suffer deterioration due to physical and chemical factors, namely, the hydrolysis and enzymatic degradation of the exposed collagen and adhesive [26–28]. Other studies claim that the host derived MMPs play a central role in this process [29], and that the use of acid etching or self-etching can activate and increase the expression of MMPs [30, 31].

A possible solution to inactivate the MMPs is to use inhibitors with the adhesive or before the application of the adhesive [26–30]. The PAs present in the GSE act as inhibitors of MMPs by cross-linking with collagen [17, 32]. The GSE interacts with the proteins inducing the cross-linking by covalent interaction, ionic interaction, hydrogen bonding interaction, or hydrophobic interaction [17, 33].

The purpose of this study was to evaluate the influence of the grape seed extract in adhesion to dentin surfaces conditioned with Er,Cr:YSGG laser and to compare it to control groups.

The SBS values showed that the null hypothesis cannot be rejected, since there was no statistically significant difference between the laser groups or control groups with or without GSE pre-conditioning the dentin.

**Graph 1** Mean and standard deviation of the shear bond strength (MPa) in the eight groups (* $p < 0.05$)

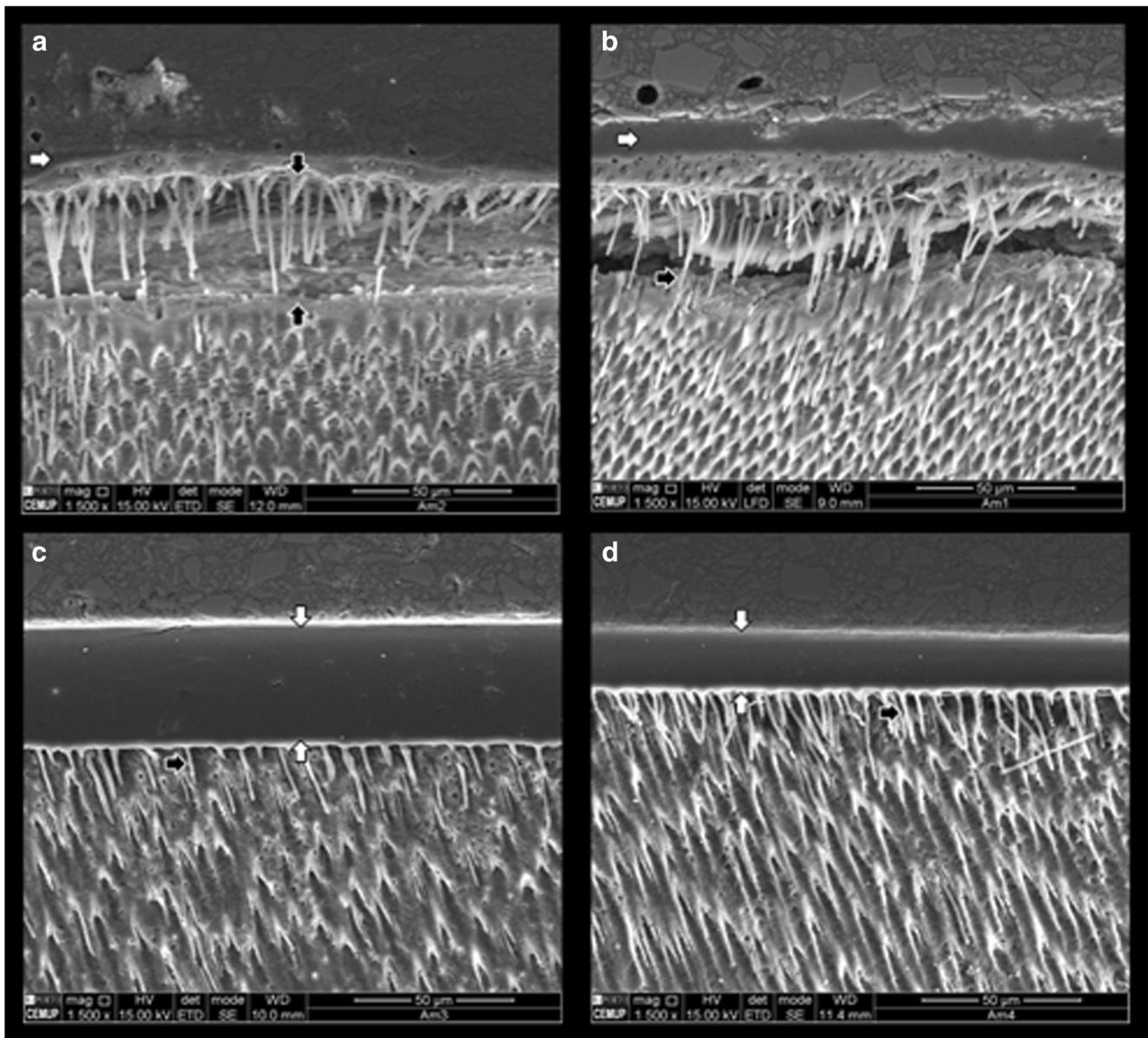


Fig. 1 **a** Micrograph of SEM of the dentin surface pre-treated with Er,Cr:YSGG laser from Group 1, showing the formation of a gap along the interface (▸) and almost absence of adhesive layer (◁) ($\times 1500$). **b** Micrograph of SEM of the dentin surface pre-treated with Er,Cr:YSGG laser from Group 2, showing some resin tags inside the dentinal tubule and a non-homogeneous adhesive layer (◁) ($\times 1500$). **c** Micrograph of

SEM of the dentin surface pre-treated with Er,Cr:YSGG laser from Group 3, showing the resin tag penetration on the dentine tubules (▸) and a homogeneous and thick adhesive layer (◁) ($\times 1500$). **d** Micrograph of SEM of the dentin surface pre-treated with Er,Cr:YSGG laser from Group 4, showing the resin tag penetration on the dentine tubules (▸) and homogeneous adhesive layer (◁) ($\times 1500$)

To test the adhesion in this study, two different self-etch adhesive agents—the Clearfil™ SE Bond, two-step self-etching and primer, and the one-step Scotchbond™ Universal Adhesive 3M ESPE—were used. The SBS results between these two adhesives showed a significant difference ($p = 0.032$).

Regarding the control groups, Group 7 showed the best SBS (24.27 ± 10.28 MPa), followed by Group 8. In both groups, the CSE adhesive was used without applying laser treatment to the surface. In Group 8, the GSE solution

was also applied. By analyzing the SEM microphotos (Fig. 2c, d), it is possible to observe that both groups present a thick adhesive layer and homogeneous hybrid layer and long resin tags. Nonetheless, the resin tags are longer in Group 7.

Within the laser groups, Group 4 (GSE + CSE) showed the best SBS. The results also show an increasing SBS in the groups where the GSE was applied (G4 and G2) when compared to those without GSE, but with no statistical significance.

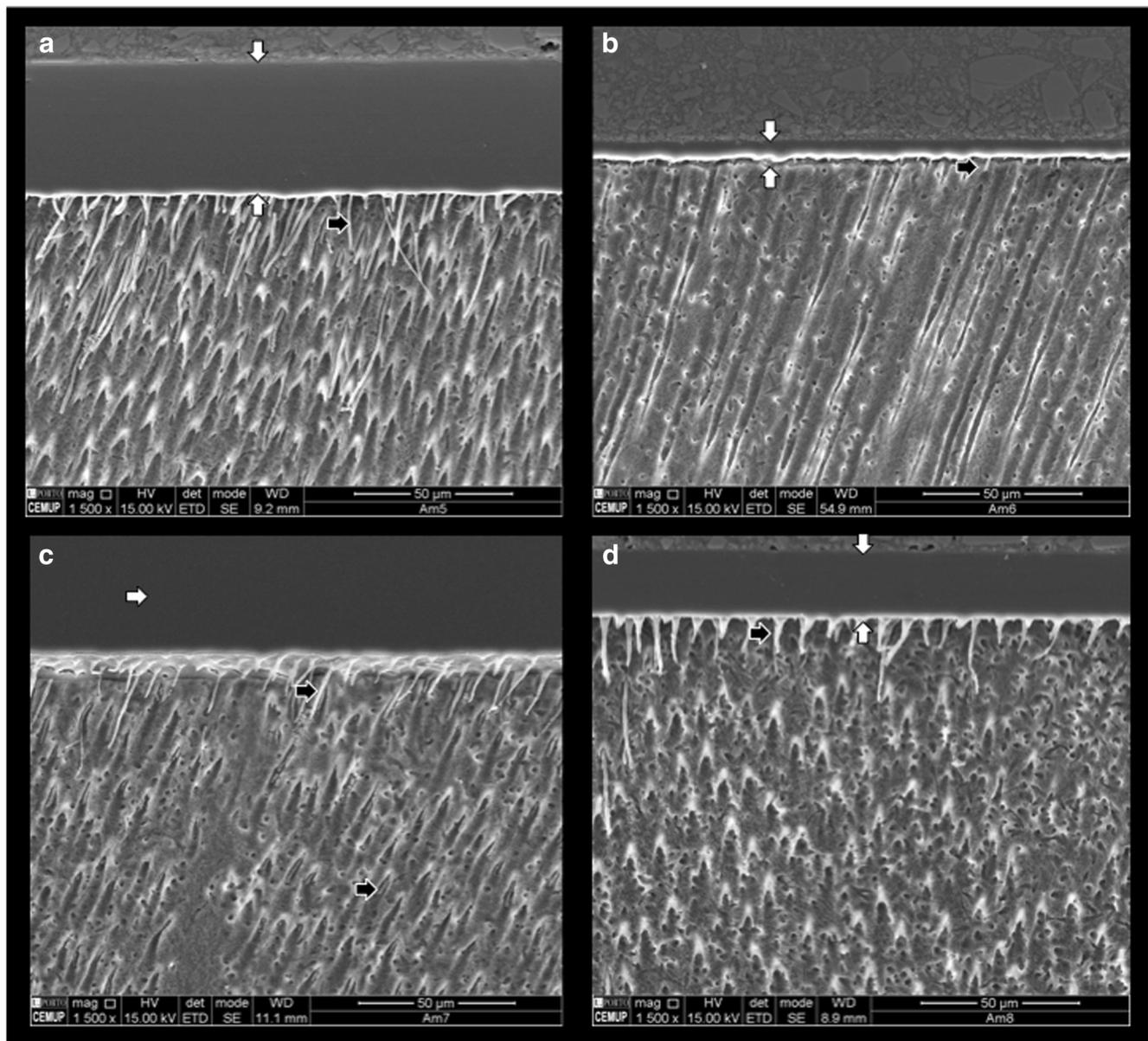


Fig. 2 **a** Micrograph of SEM of the dentin surface from Group 5 (control), showing the resin tag penetration on the dentine tubules (➡) and homogeneous hybrid and adhesive layer (↔) ($\times 1500$). **b** Micrograph of SEM of the dentin surface from Group 6 (control), showing almost the absence of resin tags penetrating the dentine tubules (➡) and a thin adhesive layer (↔) ($\times 1500$). **c** Micrograph of SEM of the dentin

surface from Group 7 (control), showing the resin tag penetration on the dentine tubules (➡) and adhesive layer (↔) ($\times 1500$). **d** Micrograph of SEM of the dentin surface from Group 8 (control), showing resin tag penetration on the dentine tubules (➡) and the adhesive and hybrid layer (↔) ($\times 1500$)

The CSE has a primer with a weak acid ($\text{pH} = 2$), causing little demineralization of the dentin, and allowing the deep penetration of the resin tags in the tubules [9]. The bonding procedure depends on the infiltration of the hydrophilic monomers into the exposed dentin collagen [34], so the exposition and integrity of the collagen fiber network is essential [9]. It is also known that the self-etch adhesives were developed to use the smear layer as a bonding substrate [35].

In the control groups, the presence of the smear layer could have prevented the proper action of the GSE, since the

collagen was not exposed. Additionally, the groups with the application of GSE before the adhesive avoided its interaction with the smear layer, which could interfere with the SBS.

On the other hand, the dentin surfaces prepared with Er,Cr:YSGG laser present a scaly, irregular appearance, with no smear layer and exposed dentinal tubules [22].

In the laser groups, G1–G4, the SBS increased with the application of the GSE solution before the adhesive, as in this situation there was not a smear layer, and the collagen was partially exposed. Even though the difference of SBS is not

considerable, it could lead to other studies [22]. On the laser groups with the application of CSE, it is possible to observe more and longer resin tags in the SEM microphoto of Group 4 (Fig. 1d) than on Group 3 (Fig. 1c), probably due to the action of the GSE solution, making changes in the dentin collagen, and increasing the mechanical properties of the bond interface [36].

Additionally, with the application of SU, it is possible to observe the formation of a gap along the adhesive and dentin interface, with some resin tags penetrating the dentinal tubules on Group 2 (Fig. 1b), comparing to Group 1 (Fig. 1a) that presents a gap along the adhesive and dentin interface, with almost absence of the adhesive layer.

These results cannot be compared because there are no other studies on the effect of GSE on laser-treated dentin surfaces. The existing studies focus only on surfaces without laser treatment [36–38]. In these studies, the SBS improved with the application of GSE, but the time of immersion in GSE was longer, and this study tried to simulate a clinical protocol [36–38].

In laser-irradiated dentin, it has been demonstrated that there are some zones with loss of water, protein, carbonate, and hydroxyapatite, showing chemical composition changes that influence the capacity of adhesion in this surface [39].

According to some studies, the laser irradiation can develop microstructural alterations and microrupture of the collagen fibers [9]. Moreover, if the collagen collapses or alters, the penetration of primers or monomers will not be complete [35]. Furthermore, the study performed by Lee et al. concludes that the irregularities present on laser-treated dentin can avoid uniform stress distribution in the interface adhesive-dentin, and reduce the bonding strength, without a uniform adhesive thickness [22]. This might help explain why the laser groups had lower SBS overall.

Comparing the two self-etch adhesives, the groups where CSE was applied had better SBS results than the groups with SU overall ($p = 0.032$) (Table 2). In the composition of the adhesives, one important characteristic is the level of acidity, which in the two tested adhesives is provided by the 10-methacryloyloxydecyl dihydrogen phosphate monomer (MDP) [40]. The dentin surface is demineralized and the smear layer dissolved by this acid property [41]. The adhesives can be classified according to their pH as mild ($\text{pH} \geq 2$), intermediate ($\text{pH} = 1.5$), and strong ($\text{pH} \leq 1$) [42], and this provides the adhesive's ability to dissolve the smear layer and demineralize the dentin [43].

The CSE has a pH of 2 and the SU has a pH of 2.7. Consequently, the CSE has higher acidity, providing more dentin demineralization and dissolving more effectively the smear layer, which allows a better monomer infiltration [43], maybe explaining why the CSE had better SBS results on the control groups and laser groups.

The settings tested in this study, 4.5 W, 50 Hz, 50 μs , 70% air, and 90% water, were selected following a previous study [44], which had the best SBS, comparing with other settings. The manufacturer's pre-set parameter, for the pulse width in

the device used (Waterlase iPlus – Biolase Technology Inc., Irvine, CA) is 60 μs , but in this study it was decreased to 50 μs , following a previous study to check its relevance in the dentin's adhesion. The use of different settings on the dentin surface influences the SBS, which could mean that the settings used in this study influenced the low SBS results, and other settings should be tested.

The study performed by Takada et al. showed that the application of phosphoric acid on dentin after laser preparation resulted in widely open tubules and intertubular dentin protruded [45]. Also, Lee et al. mentioned that the Er,Cr:YSGG laser could not remove the hydroxyapatite crystals selectively, without causing any harmful effects on the collagen fiber network, and that additional acid etch was necessary to completely expose the collagen fiber [22]. This could explain why the SBS was higher on the laser groups with the application of CSE ($\text{pH} = 2$) than with the SU ($\text{pH} = 2.7$). This could also explain the gap between the hybrid layer and the dentin surface present on the laser groups with the application of the SU, Group 1 and Group 2 (Fig. 1a, b). These gaps probably resulted from the collagen alteration [9] without a proper exposure of the collagen fibers. The incomplete protection of the collagen fibrils in the hybrid layer can result in gaps and sites where collagen hydrolysis can occur [29, 46].

Knowing that the PA present in the GSE act as inhibitors of MMPs by cross-linking with collagen [26–30], there is a chance of increasing the adhesive performance not only on the dentine surfaces conditioned with laser but also in those with conventional preparation. As these adhesives were produced with the conventional methods in mind, better results could be expected in conventional dentine preparation than in laser.

The use of GSE could be a tool to improve the adhesion on surfaces prepared with laser, considering its effect on collagen, but more studies should be performed using different settings, different concentrations of GSE, and different duration of the application of the GSE solution.

Conclusion

The type of dentin surface preparation can influence the SBS, and there was a significant statistical difference of SBS between the groups with SU and CSE.

It was not found a statistically significant difference between the laser groups or control groups with or without a GSE pre-conditioning the dentin.

At the conditions and parameters of the present study, the presence of GSE did not improve the adhesion on surfaces conditioned with laser, but more studies should be carried out in the future.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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