

## Original Article

# Effect of Er:YAG laser cavity preparation on the bond strength of 2-hydroxyethyl methacrylate-free and 2-hydroxyethyl methacrylate-rich self-etch adhesive systems: An *in vitro* study

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## ABSTRACT

**Background:** Despite many advantages of lasers and reduction of the risk of surface bonding errors with newer self-etch systems, they have not been thoroughly researched. This study was done to evaluate the effect of Er:YAG laser cavity preparation on the microtensile bond strength of 2-hydroxyethyl methacrylate (HEMA)-rich and HEMA-free one-step self-etch adhesive systems. **Materials and Methods:** In this *in vitro* study, eighty freshly extracted human premolars were collected. Cavities were prepared in 40 teeth with carbide bur (Group 1) and in other 40 teeth with Er:YAG LASER (490 mJ and 15 Hz) (Group 2). Subgroups of twenty teeth each were made according to the adhesive systems used. After placement of restoration, the mean values of the bond strength were calculated using universal testing machine. Data were then tabulated and analyzed using descriptive statistics (Significant at  $P < 0.05$ ).

**Results:** The overall microtensile bonding strength was higher when the cavities were prepared with bur compared to those with Er:YAG laser. Mean bond strengths of single-bottle self-etching seventh-generation dentin bonding agents to bur-prepared cavities were higher than those to laser-prepared cavities irrespective of the adhesive system ( $P = 0.01$ ). No statistically significant difference was observed between HEMA-free and HEMA-rich self-etch adhesive systems.

**Conclusion:** The effect of Er:YAG laser for cavity preparation did not show improved performance when evaluated using microtensile bond strength with seventh-generation bonding agents, Adper Easy One and G-Bond. More studies are required to assess the effect of lasers.

**Key Words:** Adhesives, dentin bonding agent, Er:YAG laser

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## INTRODUCTION

Dental caries is an infectious disease which leads to pain, tooth loss, localized soft- and hard-tissue infection, and in severe cases, generalized infection and death. It is one of the most common diseases worldwide. Incipient carious lesions regress by true

reprecipitation of minerals (remineralization), which are dependent on the control of dental caries as a disease.<sup>[1]</sup> Meanwhile, cavitation requires surgical means of carious lesions removal. There is a quest to

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find a method to remove diseased and healthy dental hard tissue without the negative stimuli associated with dental handpieces.<sup>[2]</sup>

Lasers have been investigated as a potential tool for selective dental caries and hard tissue removal since the 1960s. However, early dental laser investigations reported damage and excessive temperature increase in the dental pulp and surrounding tissues.<sup>[3]</sup> In early 1990s, researchers found that mid-infrared lasers (Er:YAG and Er, Cr: YSGG) with the use of water cooling promote effective caries<sup>[2]</sup> and hard tissue removal, patient comfort, while minimizing noise and vibration, and maintaining the intrapulpal temperature within the safe limit.<sup>[3,4]</sup> These lasers have gradually gained publicity, especially in treating anxious patients, and in recent years, have been established as a viable alternative for caries removal. Er:YAG laser with appropriate parameters proposed to can selectively remove enamel hydroxyapatite crystals resulting in irregular surface that would enhance the micromechanical retention.<sup>[2]</sup> Pulsed lasers whose wavelengths are strongly absorbed by dental hard tissues and hydroxyapatite, for example, erbium lasers (Er:YAG and Er, Cr: YSGG), can successfully be used for dental hard-tissue procedures including conditioning or etching without any side effects.<sup>[2]</sup>

Different cavity preparation techniques might lead to differences in the qualities of dentin after preparation. These differences might be clinically significant when considering the surface bonding ability with different adhesive systems.<sup>[5]</sup> During the past few years, the trend has been to move one step further by combining the etching, priming, and bonding with an attempt to develop fifth-, sixth-, and seventh-generation adhesives. The seventh-generation adhesives are the acidic primers, and the rationale behind these systems is to superficially demineralize dentin and to simultaneously penetrate it to the depth of demineralization with monomers that can be polymerized *in situ*. Newer self-etch system combines the etchant, primer, and adhesive in one container. As no separate etching or rinsing is required in these systems, the risk of errors during application is low. The simultaneous occurrence of demineralization and resin infiltration is another benefit of self-etch adhesive systems. However, their ability to appropriately etch the mineralized tooth structure as well as the bonding to such substrates has been questioned. Assessments of the bonding performance of adhesive systems have been generally conducted on flat tooth surfaces

without maintaining the cavity configuration factor, whereas in clinical conditions, complex cavity designs are prepared. Therefore, it might be more clinically relevant to measure bond strength to prepare cavities. Moreover, the bonding performance of current self-etch systems to cavities prepared with a laser has not been thoroughly researched.<sup>[5]</sup>

The hydrophilicity of 2-hydroxyethyl methacrylate (HEMA) makes it an excellent adhesion promoting monomer and by enhancing wetting of dentin, HEMA significantly improves bond strength. To attain high bonding strengths to dentin, it is essential for the dentin substrates to have good penetrability and diffusibility. In addition, the resin penetration into tubules can effectively seal the tubules and intertubular dentin and the resin infiltration can only occur if the mineral phase of dentin is removed by acidic conditioners. However, acidity of conditioner not only removes mineral from the dentinal matrix but may also protonate collagen, changing the charges on the peptides and extracting noncollagenous proteins. However, once collapsed, it can be reexpanded with hydrophilic monomers such as HEMA. The higher bond strength following HEMA may be due to the fact that the demineralized collagen is kept wet and does not collapse as much as dentin that is dried with air blast. Furthermore, the moist dentin may permit a more porous collagen network, which permits greater infiltration of adhesive monomers than do surfaces that are air-dried and hence collapse.<sup>[6]</sup>

Several studies and research papers have been published comparing the above two which have shown consistently the better bonding strengths and performance of HEMA-rich bonding agents.<sup>[6]</sup> However, the difference in bonding strength between HEMA-rich and HEMA-free bonding agents on surfaces prepared with Er:YAG laser and carbide bur has not been evaluated. This study was done to evaluate the effect of carbide bur and Er:YAG laser cavity preparation on the microtensile bond strength of two one-step self-etch adhesive systems (HEMA-rich and HEMA-free) using universal testing machine.

## MATERIALS AND METHODS

For this *in vitro* study, 80 freshly extracted human premolar teeth between the age group of 20–30 years were collected randomly from the Department of Oral Surgery, Rajasthan Dental College and Hospital, Jaipur, India, over a period of 6 months from July

2015 to December 2015. Inclusion criteria comprised intact premolar teeth indicated for extraction due to orthodontic or periodontal reasons. The teeth were then cleaned with the ultrasonic cleaner to remove the tissue remnants, calculus, or any other debris. Then, the teeth were randomly assigned to two groups of 40 teeth according to the cavity preparation technique and then these groups were further divided into two subgroups of twenty teeth each according to the different adhesive systems used. All teeth were mounted in mold with plaster of Paris. Cavities were then prepared in 40 teeth (size = 3-mm length × 2-mm width × 2-mm depth) with occlusally diverging walls. Forty cavities were prepared in 40 teeth with the help of carbide bur (Group 1) and 40 cavities in other 40 teeth with the help of Er:YAG laser (490 mJ and 15 Hz, Lightwalker dental Lasers, QSP technology [Quantum Square Pulse, Fotona d. o. o, Stegne 7, 1000 Ljubljana, Slovenia, EU]) (Group 2) [Tables 1 and 2].

Group I – Box-shape Class-I cavities were prepared at the occlusal crown center of the 40 study teeth samples with the pulp floor ending at the mid-coronal dentin, with a carbide bur in high-speed turbine handpiece under constant water cooling.

- Subgroup A1 – One-step self-etch (HEMA-free) adhesive system was used on 20 study samples of the teeth [Figure 1]
- Subgroup B1 – One-step self-etch (HEMA-rich) adhesive system was used on 20 study samples of the teeth [Figure 2]

Group II – The cavities were prepared with the same dimensions on the 40 study teeth samples as in Group I using an Er:YAG laser [Figure 3]

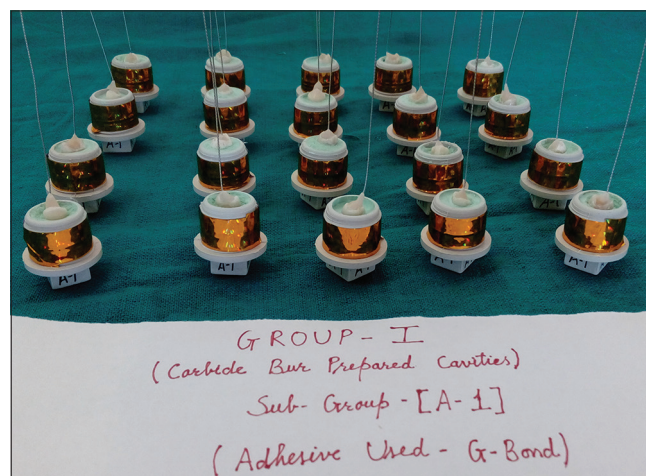
- Subgroup A2 – One-step self-etch (HEMA-free) adhesive system was used on 20 study samples of the teeth [Figure 4].
- Subgroup B2 – One-step self-etch (HEMA-rich) adhesive system was used on 20 study samples of the teeth [Figure 5 and Table 1].

### Bonding procedure

After sample preparation, all self-etch adhesives were applied strictly according to the manufacturer's instructions on the prepared cavity walls of the teeth.

#### Subgroup A1 and Subgroup A2

G-Bond (GC America Inc., GC Corporation, 4300, 123<sup>rd</sup> St, Alsip, IL, USA) [Table 3] adhesive was applied one coat on the dentinal surface, left undisturbed for 10 s, dried thoroughly under maximum air pressure for 5 s, and light cured for 20 s using SmartLite Focus



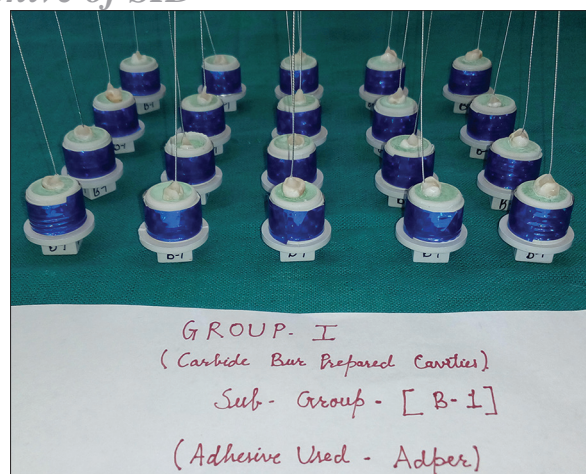
**Figure 1:** Photograph showing cavities prepared in Subgroup A1 – One-step self-etch (2-hydroxyethyl methacrylate-rich) adhesive system used on 20 teeth.

**Table 1: Distribution of the study sample into groups and subgroups**

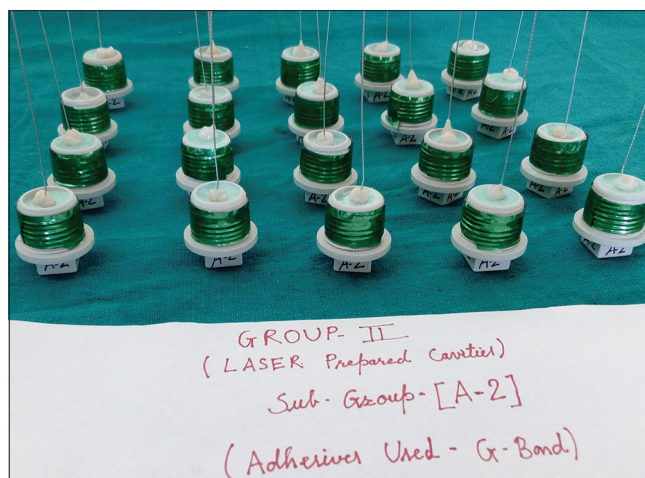
Group	Subgroup	Material Used
Group 1 (carbide bur-prepared cavity)	Subgroup A1 (G-Bond)	One-step self-etch - HEMA-free - 20 samples
	Subgroup B1 (Adper Easy One)	One-step self-etch - HEMA-rich - 20 samples
Group 2 (Laser-prepared cavity)	Subgroup A2 (G-Bond)	One-step self-etch - HEMA-free - 20 samples
	Subgroup B2 (Adper Easy One)	One-step self-etch - HEMA-rich - 20 samples

**Table 2: Details of the devices used in the study**

Names of materials and devices	Manufacturer name and address
Devices	
Er:YAG Laser (490 mJ and 15 Hz)	Fotona d.o.o, Stegne 7, 1000 Ljubljana, Slovenia, EU
Lightwalker dental Lasers, QSP technology (Quantum Square Pulse)	
SmartLite Focus Pen-Style LED Curing Light	DENTSPLY IH Ltd - Building 3, The Heights, Weybridge, Surrey, KT13 0NY, United Kingdom
Universal testing machine	Tinius Olsen India Pvt Ltd., J3 SDF, NSEZ Noida Phase 2, UP 201305, India



**Figure 2:** Photograph showing cavities prepared in Subgroup B1 – One-step self-etch (2-hydroxyethyl-methacrylate-free) adhesive system used on 20 teeth.



**Figure 4:** Photograph showing cavities prepared in Subgroup A2 – One-step self-etch (2-hydroxyethyl-methacrylate-rich) adhesive system used on 20 teeth.

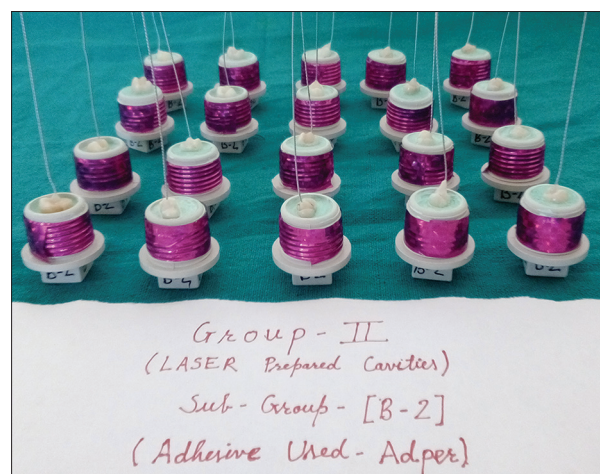


**Figure 3:** Photograph showing ER:YAG laser machine.

Pen-Style LED Curing Light (DENTSPLY IH Ltd., Building 3, The Heights, Weybridge, Surrey, KT13 0NY, United Kingdom) [Table 2 and Figure 6].

#### Subgroup B1 and Subgroup B2

Adper Easy One (3M ESPE, St. Paul, Minnesota,



**Figure 5:** Photograph showing cavities prepared in Subgroup B2 – One-step self-etch (2-hydroxyethyl-methacrylate-free) adhesive system used on 20 teeth.

USA) [Table 3] adhesive was applied one coat on the dentinal surface, gently agitated with applicator for 20 s, air thinned the adhesive until there was no movement (at least for 5 s), and light cured for 20 s [Figure 6].

About 1-mm thickness of universal restorative composite (SOLARE-X, GC Asia Dental Pvt. Ltd) [Table 3] was placed over the bonded dentinal surface and light cured it for 40 s. Then, a stainless steel ligature wire was placed over the bonded composite and second layer of 1-mm thickness of composite was placed and light cured.

The specimens were stored in distilled water and placed in incubator at 37°C temperature. After 24 h, the specimens were removed from the incubator and

**Table 3: Manufacturer details of materials and devices used in the study**

Names of materials and devices	Manufacturer name and address
Materials	
G-Bond (Composition 4-META Urethane dimethacrylate, Dimethacrylate component, Phosphoric ester monomers, Acetone, water Photo initiators, stabilizers)	GC America Inc., GC Corporation, 4300, 123 <sup>rd</sup> St, Alsip, IL, USA
Adper Easy one (Composition - 2-hydroxyethyl-methacrylate (HEMA), Bis-GMA, Methacrylated phosphoric esters, 1,6 hexanediol dimethacrylate, Methacrylate functionalized Polyalkenoic acid, Finely dispersed silica filler with 7-nm primary particle size, Ethanol, Water, Initiators based on Camphorquinone, Stabilizers)	3M ESPE, St. Paul, Minnesota, USA
Universal restorative composite	SOLARE-X, GC Asia, 11 Tampines Concourse, #03-05, Singapore 528729

tested tensile mode in universal testing machine (Tinius Olsen India Pvt Ltd., J3 SDF, NSEZ Noida Phase 2, UP 201305, India) [Table 2]. The equipment was adjusted to operate at 1 mm/min, as different crosshead speeds may influence the bond strength values.

For the tensile bond strength measurement, the wire protruding out of the mold was gripped into the superior crosshead and mold was held in the inferior crosshead of the universal testing machine [Figure 6]. Tensile loading was done until the dislodgment of the restoration from the dentinal surface occurred. The breaking load was measured, and the results of the debonding force were tabulated in the values of the force (MPa). The tensile bond strength was calculated by dividing force (N) by the debonding area (mm<sup>2</sup>).

### Statistical analysis

The mean values of the bond strength were calculated for each experimental group. Data were tabulated and were statistically analyzed. Mean and standard deviation were used for descriptive statistics.

## RESULTS

The present study revealed that the overall microtensile bonding strength was higher when the cavities were prepared with carbide bur when compared to cavities prepared with Er:YAG hard-tissue laser. The mean bond strengths to bur-prepared cavities that used single-bottle self-etching seventh-generation dentin bonding agents were higher than those used single-bottle self-etching seventh-generation dentin bonding agents and laser-prepared cavities irrespective of the adhesive system ( $P = 0.01$ ) [Table 4].

The highest value of microtensile bond strength was present with cavities prepared with carbide bur and adhesive was used Adper Easy One (Subgroup B1) = 107.3 N, while the lowest value

**Table 4: Microtensile bond strength values in different groups as measured by universal testing machine**

Groups	N <sub>o</sub>	Mean (N)	SD	SE
Subgroup A1	20	106.5000	21.69526	4.85121
Subgroup A2	20	91.9500	29.57858	6.61397
Subgroup B1	20	107.3000	22.98306	5.13917
Subgroup B2	20	82.5000	27.44468	6.13682
Total	80	97.0625	27.22496	3.04384

N<sub>o</sub>: Number; N: Newton; SD: Standard deviation; SE: Standard error

**Figure 6:** Photograph showing G-Bond (GC CORP) adhesive and Adper Easy One (3M ESPE) adhesive.

of microtensile bond strength was presented by cavities prepared with LASER and adhesive was used Adper Easy One (Subgroup B2) = 82.5 N. The values of microtensile bond strength for cavities prepared with carbide bur and adhesive G-Bond (Subgroup A1) was 106.5 and for the cavities prepared with LASER and adhesive G-Bond (Subgroup A2) was 91.95 N [Table 3]. Thus, the values of microtensile

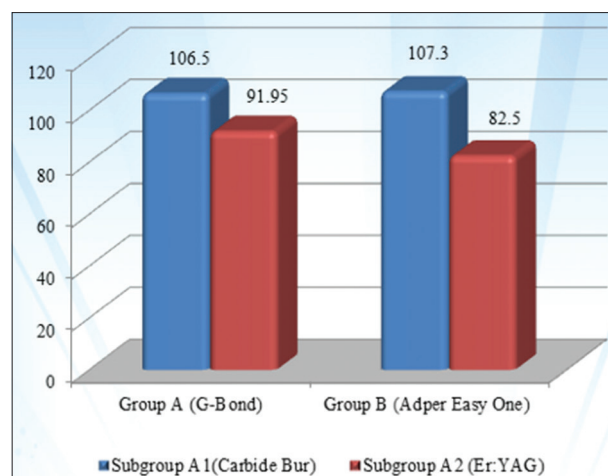
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bond strengths, when arranged in descending order, are as ( $P = 0.01$ ): Subgroup B1 (Carbide bur + Adper Easy One) = 107.3 N < Subgroup A1 (LASER + Adper Easy One) = 106.5 N < Subgroup A2 (LASER + G-Bond) = 91.95 N < Subgroup B2 (LASER + Adper Easy One) = 82.5 N. When comparison was done between the groups, the values obtained for the Subgroup A1 (Carbide bur + G-Bond) = 106.5 N and Subgroup A2 (LASER + G-Bond) = 91.95 N showed significant statistical difference. Similarly, the comparison between the values of Subgroup B1 (Carbide bur + Adper Easy One) = 107.3 N and Subgroup B2 (LASER + Adper Easy One) = 82.5 N also revealed significant statistical differences [Figure 7]. When comparison was done between carbide bur and LASER preparation, despite the values of Subgroup B1 (Carbide bur + Adper Easy One) = 107.3 N being slightly higher than Subgroup A1 (Carbide bur + G-Bond) = 106.5 N, statistical evaluation revealed no significant difference. Similarly, not much statistical significant difference was noted between values of Subgroup A2 (LASER + G-Bond) = 91.95 N and B2 (LASER + Adper Easy One) = 82.5 N. One-way ANOVA revealed no significant difference between the groups ( $P > 0.05$ ) [Figure 8].

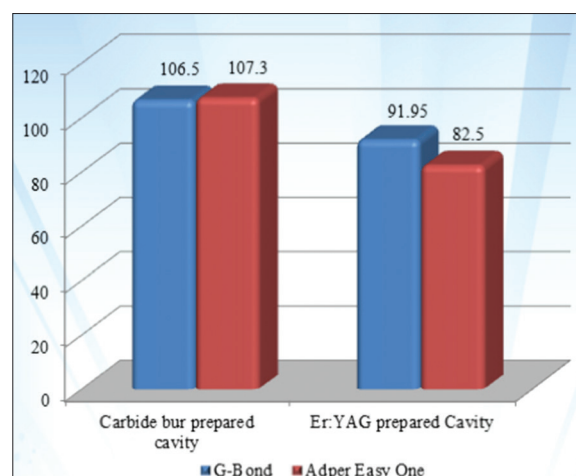
## DISCUSSION

Treatment of carious lesion indicates removal of damaged enamel and dentin and the tooth be restored. The use of rotating instruments (conventional drilling) is the most common method for removing caries. The method is efficient and fast, and teeth treated with this technique have a good prognosis. However, there are also disadvantages with the method: risk of overpreparation, even sound dentine is easily removed, the pulp could be adversely affected by vibrations, and heat from the bur and drilling is painful. These negative consequences have been the main reasons for seeking alternative ways to remove dental caries. Examples of such methods are air abrasion, sono-abrasion, chemomechanical methods, and lasers.<sup>[7]</sup>

Recently, an Er:YAG laser apparatus was developed for cavity preparation. Cavity preparation with Er:YAG laser has advantages such as minimal pulp injury while removing dental hard tissues, creation of a rough and irregular surface, less side effects due to severe heat produced by conventional methods, for example, cracking, melting, or charring in the remaining tissue, lowering the pain and vibration



**Figure 7:** Graph showing comparison between different groups.



**Figure 8:** Graph showing comparison between carbide bur and LASER cavity preparation.

during preparation, which thereby increases the patient's comfort.<sup>[8]</sup>

However, the literature reporting the bond strength of adhesive resin to the Er:YAG laser irradiated dentin is limited. All the adhesive materials that include surface treatment before bonding were developed for bonding to dentin cut by rotary instruments. It is thus important to determine the appropriate bonding system for laser-irradiated dental hard tissue because the Er:YAG laser irradiated surfaces have been confirmed to have different morphology and acid resistance from cut surfaces.

The two major simplified approaches are total-etching adhesive systems and self-etching adhesive systems. These are characterized by demineralization and infiltration of resin monomers simultaneously. This technique is attractive because of the reduced sensitivity associated with retaining the smear layer

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and smear plugs, thereby, minimizing the possibility of inadvertent contamination of the bonding surface with dentinal surface through dentinal fluid transudate; this avoided the occurrence of several disadvantages from total-etching systems. The hybridized complexes of self-etching systems comprise a surface zone of hybridized smear layer and a subsurface zone of hybridized intertubular dentine.<sup>[9]</sup> To achieve this goal, the self-etching primer should penetrate beyond the smear layer into intact mineralized dentin. Contemporary self-etching systems have been developed by increasing the concentration of acidic resin monomers and combining them with HEMA. A great variety of self-etching systems are available in the market. They differ in number of the bottles, steps, acidity of the primer solution, and resinous monomers.<sup>[9]</sup> Self-etch adhesives used in the present study; Adper Easy One is HEMA-rich and ethanol used as solvent, whereas G-Bond is HEMA-free and acetone used as a solvent.

The restorative material used in the present study after the application of dentine adhesive was visible light-activated direct restorative nanocomposite designed for anterior and posterior restorations SOLARE-X (GC Asia Dental Pvt. Ltd). SOLARE-X contains silica nanoparticles, prepolymerized fillers containing silica nanoparticles, and fluoroaluminosilicate glass fillers.<sup>[10]</sup> The results obtained from this study revealed that HEMA-rich self-etch adhesives showed marginally better results than the HEMA-free self-etch adhesive when evaluated for tensile bond strength in cavities prepared with carbide bur; however, this difference in value was found to be statistically insignificant.

Conversely, the values obtained for the tensile bond strength for both the bonding agents for cavities prepared with Er:YAG laser at 490 mJ and 15 Hz showed that the bond strength with HEMA-rich bonding agent (Adper) was better when compared to that of HEMA-free (G-bond), and this value was statistically significant. The minute inferior bond strength values obtained for G-Bond in comparison with Adper Easy one (HEMA-rich) for cavities prepared by carbide bur can be attributed to the absence of HEMA. The hydrophilicity of HEMA makes it an excellent adhesion-promoting monomer and by enhancing wetting of dentin, HEMA improves bond strength. To attain high bonding strengths to dentin, it is essential for the dentin substrates to have good penetrability and diffusibility.

The acidity of conditioner not only removes mineral from the dentinal matrix, but may also protonate collagen, changing the charges on the peptides and extracting noncollagenous proteins. Therefore, once collapsed, it can be re-expanded with hydrophilic monomers such as HEMA. The better bond strength using HEMA may be due to the fact that the demineralized collagen is kept wet and does not collapse as much as dentin that is dried with air blast. Furthermore, the moist dentin may permit a more porous collagen network, which permits greater infiltration of adhesive monomers than do surfaces that are air dried and hence collapse.<sup>[6]</sup> Furthermore, Adper Easy one contains ethanol as a co-solvent as compared with acetone present in case of G-Bond. Ethanol is a polar solvent that will form hydrogen bonds with its solutes. Ethanol removes water from these spaces causing the hydrogel to collapse, thus enlarging the interfibrillar spaces and allowing more resin infiltration.<sup>[6]</sup>

Acetone has a high dipole moment and forms much lesser hydrogen bonds due to which it is unable to expand the shrunken demineralized collagen. It has a high vapor pressure of 184 mmHg at 20°C as compared with that of ethanol (43.9 mmHg at 20°C). As the solvent evaporates, the viscosity reduces the ability of the bonding system to penetrate around the exposed collagen fibers and the opened dentinal tubules producing poor and incomplete hybrid layers.<sup>[6]</sup> The potential impact of the Er:YAG laser on collagen network has not been clearly disclosed yet. It remains unclear if the microstructural alteration and microrupture of collagen fibers caused by laser irradiation could actually compromise the interaction of adhesive systems with laser dentin substrate, which would inherently affect the resulting bond strength. This speculation is based on the fact that the major mechanism of bonding to dentin surface relies directly upon the entanglement of hydrophilic monomers to the exposed collagen web and thereby depends upon the availability and integrity of the fiber mesh. Therefore, if the structure of the collagen net somehow collapses or is altered, the penetration of primer monomers and hence the adhesive protocol is hampered and incomplete.<sup>[11]</sup> Thus, it may be due to the disruptive changes in the collagen matrix due to the LASER irradiation which may be the cause of significantly inferior performance of the both HEMA-rich (Adper – 82.5 N) and HEMA-free (G-Bond – 91.95 N) bonding agent when evaluated by comparing the tensile bond strength.

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Furthermore, it is also important to highlight the action of laser irradiation on the mineral components of dentinal substrate. Although scanning electron microscope observations have revealed that dentin surface treated by Er:YAG laser shows very little or absent smear layer and open dentinal tubules, the laser does not effectively act on peritubular dentin and hence is not able to enlarge tubules' openings. There have been reports on various patterns of surface microirregularities (often accompanied by microfissure propagation), besides few fusion and recrystallization areas. That peculiar morphology of lased surface, resulting from the thermomechanical ablation process, could interfere negatively with the acid reactivity of dentin substrate, as the etchant agents or acidic monomers may not be as efficient at dissolving the mineral component of the superficial layer of lased dentin.<sup>[11]</sup>

Studies have shown that the surfaces prepared with Er:YAG laser were mostly devoid of smear layer and hence the efficacy of the etchant on such surfaces may also play an important role in determining the quality of bond formed between the bonding agent and composite restorative material.<sup>[12]</sup> It can thus be postulated that the acidic monomer responsible for etching of the surface present in G-Bond which is phosphoric ester monomer was more effective when compared to the monomer of Adper Easy One which is methacrylate-functionalized polyalkenoic acid (Vitrebond™ Copolymer) when used on the surfaces prepared with Er:YAG laser.

In this context, the findings of the conducted research seem to corroborate the forementioned assumption that the Er:YAG laser may exert an adverse effect on both the mineral and collagen components of dentin and would thereby impair or affect to some degree the optimal interaction of adhesive systems to laser-etched substrate.<sup>[11]</sup> A possible explanation for such results would be that Er:YAG laser irradiation provides a quite rough appearance compared to margins produced by high-speed cutting, thus marginal contouring could result in increased microspacing and greater leakage.

These morphological changes on tooth structure caused by laser irradiation might affect the degree of performance of restorative materials, especially adhesives systems.<sup>[13]</sup>

## CONCLUSION

The effect of Er:YAG laser intended for the use for

cavity preparation did not show improved performance when evaluated using microtensile bond strength and that in combination with seventh-generation bonding agents, Adper Easy One and G-Bond bonding agents. The values obtained were significantly inferior to those obtained with cavities prepared with carbide bur, where in the performance of both Adper Easy One and G-Bond were very similar. More studies are required to assess the effect of laser cavity preparation on the bond strength with different self-etch adhesive systems to validate the results through both *in vitro* and *in vivo* methods.

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## Conflicts of interest

The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or non-financial in this article.

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