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ORIGINAL ARTICLE

The Effect of Casein Phosphopeptide-Amorphous Calcium Phosphate Containing Bonding Agents on Dentin Shear Bond Strength and Remineralization Potential: An *in Vitro* Study

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ABSTRACT

Objective: To assess the effect of Casein Phosphopeptide-Amorphous Calcium Phosphate (ACP) containing bonding agents on dentin shear bond strength and remineralization potential. **Material and Methods:** This *in vitro* study evaluated 45 extracted human premolars. The teeth were decoronated, and the tooth crown was split into buccal and lingual halves. The specimens were then flat-grounded by a 180-grit abrasive. The specimens were then randomized into three groups (n=15). Adper Scotchbond Multi-Purpose (SBMP) primer and adhesive were used for bonding in the control group. ACP in 10wt% and 20wt% concentrations was added to SBMP adhesive and used in groups 2 and 3, respectively. After the application of primer and adhesive and light-curing them for 10 s, a transparent silicon cylinder was placed on a dentin surface and cured for 10 s; then, the cylinder was filled with composite resin and was cured for the 40s from each side. The specimens underwent 3000 thermal cycles, and a universal testing machine measured the SBS. To assess the remineralization quality, a total of 6 dentin samples (2 specimens for group) were prepared and underwent X-ray diffraction, attenuated total reflection Fourier-transform infrared spectroscopy, and scanning electron microscopy-energy dispersive X-ray analysis. One-way analysis of variance was used to analyze the data. The level of $p<0.05$ was considered significant. **Results:** No significant difference in dentin shear bond strength was noted between the groups ($p>0.05$) — the addition of ACP to SBMP adhesive enhanced dentin remineralization. Increasing the ACP concentration from 10% to 20% increased the formation of hydroxyapatite. **Conclusion:** Adding amorphous calcium phosphate confers remineralizing property to SBMP adhesive without compromising its shear bond strength to dentin.

Keywords: Dental Cement; Tooth Remineralization; Dental Bonding.

Introduction

At present, improvement of the esthetic appearance of the teeth is a significant demand of many patients seeking dental treatment. Thus, tooth-colored restorative materials, particularly composite resins, are increasingly used in contemporary dentistry [1]. Nonetheless, composite restorations increase biofilm accumulation, leading to acid production and the development of dental caries [2]. Secondary caries are the leading cause of failure and replacement of dental restorations [3]. Therefore, the manufacturers attempt to synthesize tooth-colored dental restorative materials with remineralizing properties to repair the affected dentin [4].

In recent decades, researchers have tried synthesizing bioactive materials to reverse the process of caries development and induce remineralization of affected tooth structure. One of the bioactive inorganic fillers proposed to augment dental adhesive properties is Amorphous calcium phosphate (ACP). Once ACP particles are exposed to aqueous solutions, such as saliva with physiological pH and saturation of Ca and P ions favoring mineral formation, they alter to hydroxyapatite (HA) and release calcium and phosphate ions [5]. For this reason, dental composite resins and dental adhesives have been synthesized using ACP particles, which are proven to release higher amounts of calcium and phosphate ions, and by providing a supersaturated source of ions, they decrease demineralization and increase remineralization [6].

In vivo, demineralization of the tooth structure occurs following the dissolution of calcium and phosphate ions and their release into saliva. On the other hand, remineralization of the tooth structure occurs by mineral deposition into the tooth structure and increasing its mineral content. Although saliva contains calcium and phosphorous ions, dental adhesives containing ACP can elevate the concentration of calcium and phosphorous ions to enhance the remineralization process and prevent demineralization [2]. On the other hand, any improvement in the mechanical properties of the tooth-bonding agent interface can result in a more durable bond and improve the clinical efficacy of bonding. Adding fillers to dental adhesives may improve the mechanical properties of the adhesive layer [7,8].

This study aimed to assess the effect of the addition of ACP in different weight percentages on the remineralizing property and shear bond strength (SBS) of a dental adhesive to dentin. The study's null hypothesis was that adding ACP to SBMP adhesive would not significantly affect its remineralizing property or SBS to dentin.

Material and Methods

Study Design and Ethical Clearance

This *in vitro* experimental study evaluated 45 human maxillary first premolars extracted for orthodontic purposes. The ethics committee of Qazvin University of Medical Sciences (IR.QUMS.REC.1394.389) approved the study protocol.

Sample Size Calculation

The sample size for this study was calculated based on a clinically relevant difference in bond strength (Delta) of 5 MPa and a standard deviation (SD) of 4.8 MPa, which was observed in a previous study with a similar testing method. Using a power of 0.8 and a significance level of 0.05, we calculated a required sample size of 15 for each group using the following formula:

$$n = \frac{\left(Z_{1-\frac{\alpha}{2}} + Z_{1-\beta}\right)^2 (2\sigma^2)}{(\delta)^2}$$

Where:

- n is the sample size for each group;
- $Z_{1-\frac{\alpha}{2}}$ is the critical value of the standard normal distribution (we used $\alpha = 0.05$, so $Z_{1-\frac{\alpha}{2}} = 1.96$);
- $Z_{1-\beta}$ is the critical value of the standard normal distribution (we used $\beta = 0.2$, so $Z_{1-\beta} = 0.84$);
- σ is the standard deviation;
- δ is the minimal clinically expected difference (delta).

Based on this calculation, we enrolled 45 participants in our study to ensure sufficient power to detect a clinically relevant difference in bond strength.

ACP Synthesis

Water-soluble calcium and phosphate compounds, namely calcium nitrate (Merck & Co., Rahway, NJ, USA) and di-ammonium hydrogen phosphate (Merck & Co., Rahway, NJ, USA), were used to synthesize ACP. Calcium and phosphate solutions with the desired Ca/P molar ratio and concentration of $(PO4)^{3-}$ were prepared using deionized water and refrigerated at 3°C. After reaching the pH of the phosphate solution to 10.5 by adding sodium hydroxide (Merck, USA), the solution was stirred in a magnetic stirrer (Medpip, Tehran, Iran) at 400 rpm at room temperature. The calcium solution was added to the phosphate solution such that the pH of the mixture reached 8.5 after 30 seconds of mixing; to prevent crystallization, the deposit on the filter was immersed in ethanol after rinsing with water. A concentrating system was used at 45°C for 30 minutes to evaporate residual water and alcohol. The obtained material was frozen at -80°C and then desiccated at -40°C and 2 x 10^{-2} torr pressure for eight hours [9,10]. For structural and elemental analysis of the ACP powder and to ensure its amorphous structure, X-ray diffraction (XRD) was performed by an X-ray diffractometer (Xpert PRO; Bureau Veritas, Australia) (Figure 1).

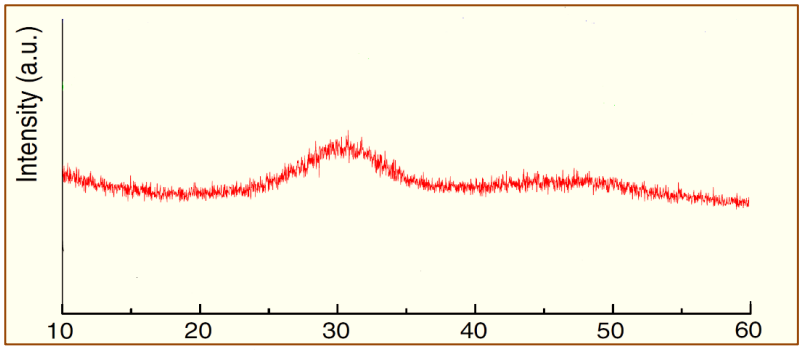

Figure 1. ACP XRD pattern.

The ACP powder was then added to SBMP adhesive (3M ESPE, St. Paul, MN, USA) based on a weight/volume ratio. To add 10wt% ACP to the SBMP adhesive, 0.8 g of the ACP powder was sufficiently mixed with 8 mL of SBMP (one bottle). To add 20wt% ACP to SBMP adhesive, 0.16 g of the ACP powder was sufficiently mixed with 8 mL of SBMP (one bottle).

SBS Test

The extracted human premolars were rinsed under running water and stored in 12% formaldehyde for one week. They were then stored in 0.9% NaCl solution. The teeth were sectioned at the dentinoenamel junction and flat-ground by a 180-grit abrasive disc to prepare smear layer-coated dentin surfaces. Next, the teeth were decoronated with a high-speed handpiece under water spray. Each crown was split into buccal and lingual halves. Subsequently, the specimens were mounted in putty and etched with 37% phosphoric acid for 15 s, followed by 15 seconds of rinsing and drying [11-13].

The specimens were randomized into three groups (n=15). SBMP was used in group 1 as the control group. In groups 2 and 3, 10wt% and 20wt% ACP were added to SBMP adhesive, as explained earlier. According to the manufacturer's instructions, the SBMP primer was rubbed on dentin surfaces by a micro brush for 15 seconds; the surface was air-dried for five seconds for the solvent to evaporate. The adhesive was then applied and was light-cured for 10 seconds (Optilux VLC; Demetron Kerr, Danbury, CT, USA).

A transparent silicone tube with an internal diameter of 0.7 mm and 1 mm height was then placed on the dentin surface, and after 10 s of curing, the tube was filled with A2 shade of Z250 composite resin (3M ESPE, St. Paul, MN, USA) and cured from the top for 40 s. The tube surrounding the composite cylinder was then removed by a surgical scalpel, and the composite cylinder was cured again for 40 seconds from the top, 40 seconds from the left side, and 40 seconds from the right side to complete polymerization. Next, the specimens were stored in deionized water at 37°C for 24 hours. Then, they were immersed in water baths at 5 °C and 55 °C for 10 seconds at each temperature under 3000 thermal cycles using a thermocycling machine (TC-300, Vafaei Industrial, Tehran, Iran). Each specimen was then fixed to the universal testing machine (Dillon; Quantral TM, USA) with cyanoacrylate glue [11]. The SBS was measured by the wire and loop technique [14,15]. A thin wire with a 0.2 mm diameter was tied around each composite cylinder such that it was in contact with the lower half of the cylinder and the tooth surface. The other end of the wire was tied around the rods designed for this purpose so that the wire connecting the three components was in the exact alignment to ensure the shear load was applied to the interface. Before loading, it was assured that the wire loop was as close to the interface as possible (Figure 3). The specimens were then subjected to shear force at a 0.5 mm/min crosshead speed until failure (debonding). The load at debonding was recorded in Newtons (N). By dividing the load (N) by the cross-sectional area of the composite cylinder (with 0.7 mm diameter), the SBS was calculated in Megapascals (MPa). The reliability of SBS testing was assessed in a pilot study with a sample size of 20. The test involved evaluating the bond strength of dental restorative materials using a standardized testing procedure. The SBS test was repeated on the same specimens after a time interval of one week to assess test-retest reliability.

The pilot study results showed that the SBS testing method had good reliability. The mean bond strength was 28.2 MPa, with a standard deviation of 4.8 MPa. The intra-class correlation coefficient (ICC) for the test-retest reliability was 0.89, indicating a high level of agreement between the two test sessions. Based on these findings, the SBS testing method was deemed reliable for evaluating the bond strength of dental restorative materials. A more extensive study with a sample size of 45 was subsequently conducted to validate these results further.

Determination of the Mode of Failure

To determine the failure mode, the debonded specimens were inspected under a stereomicroscope (SZX9; Olympus, Tokyo, Japan) at 20x magnification. The mode of failure was categorized as adhesive (failure at the dentin-adhesive or adhesive-composite interface), cohesive (failure in composite), or mixed (both adhesive and cohesive failures).

Remineralization Tests

A total of 6 dentin samples were prepared for remineralization tests (2 specimens for each of the three groups). One specimen was assessed after seven days and the other after 14 days. Dentin slices were stored in a demineralizing solution composed of 12% lactic acid with a pH of 4 (Merck, USA) for two weeks to simulate the demineralization-remineralization cycle. The solution was refreshed daily every five days before the pH changes. After demineralization, the specimens were rinsed with distilled water for 2 minutes and dried [16]. These six dentin slices were then randomized into three groups (n=2). All six specimens were etched with 37% phosphoric acid for 15 seconds, followed by 15 seconds of rinsing and drying. SBMP then was used in group 1 as the control group. In groups 2 and 3, 10wt% and 20wt% ACP were added to SBMP, as explained earlier.

According to the manufacturer's instructions, the SBMP primer was rubbed on dentin surfaces by a micro brush for 15 seconds; the surface was air-dried for 5 seconds for the solvent to evaporate. The adhesive was then applied and cured for 10 seconds. Next, the specimens were immersed in the remineralizing solution containing 0.86 g sodium chloride, 0.30 g potassium chloride, and 0.33 g calcium chloride dihydrate (saline; Darou Pakhsh Pharmaceutical Co., Tehran, Iran) with a pH of 7.4 at 37°C for 7 and 14 days. The remineralizing solution was refreshed every 24 h [17].

XRD Analysis

One specimen from each group underwent XRD at seven days and the other specimen at 14 days using an X-ray diffractometer (Xpert PRO, Bureau Veritas, Australia) with a generator operating at 40 kV with 40 mA with an angle range of 20-55° and scanning accuracy of 1 cps/degree (intensity/degree). In addition, SBMP bonding was added to one specimen and cured without further placement in the mineralization solution. This specimen was considered as the control zero and underwent XRD analysis.

Attenuated Total Reflection Fourier-Transform Infrared Spectroscopy (ATR-FTIR)

The specimens were rinsed with distilled water and thoroughly dried. Two specimens from each group underwent ATR-FTIR (Nicolet IS10; Thermo Scientific, USA) at 350-7800 cm^{-1} with 10.4 cm^{-1} resolution at 7 and 14 days, and the HA bands were identified and analyzed using Omnic 8 software.

Scanning Electron Microscopy-Energy Dispersive X-ray Spectroscopy (SEM-EDX)

Two specimens from 10% ACP and 20% ACP groups were rinsed with distilled water, completely dried, gold sputter-coated, and underwent SEM-EDX after 7 and 14 days (one at each time point). HA crystals formed in dentin were observed under SEM (XL30, Philips, USA) with 20 kV at x10,000 magnification. SEM-EDX was used for elemental analysis of the dentin surface after treatment.

Statistical Analysis

Data were analyzed using IBM SPSS Statistics for Windows version 20 (IBM Corp., Armonk, NY, USA). The Kolmogorov-Smirnov test confirmed the normal distribution of SBS data. Therefore, the groups were compared regarding SBS using one-way ANOVA. Paired comparisons were carried out using the Bonferroni post hoc test, and the Chi-square test was used to compare groups in failure modes. $P<0.05$ was considered statistically significant.

Results

Table 1 presents the SBS test results using the universal testing machine in the three groups and then the one-way ANOVA test. The highest average bond strength was seen in the control group (28.50 Mpa), followed by the ACP %10 + SBMP (28.05 Mpa) and ACP %20 + SBMP (27.45 Mpa). Moreover, according to ANOVA, the mean SBS was not significantly different among the three groups (p=0.799).

Table 1. Distribution of groups according to shear bond strength values (MPa).

Group	Mean	Std. Deviation	Minimum	Maximum	F	p-value*
Control	28.50	4.55	18	34.30	0.225	0.799
10% ACP	28.05	4.80	17	35.00		
20% ACP	27.45	3.45	19	31.30		

*One-way ANOVA test

Table 2 presents the failure modes in the three groups using a stereomicroscope and then the chi-square test. Most failures were adhesive in all three groups, followed by cohesive and mixed.

Table 2. Percent distribution of failure modes according to the group.

Group	Adhesive	Cohesive	Mixed	$\chi 2$	p-value
Control	80.0%	20.0%	0.0	1.38	0.84
10% ACP	66.7%	26.7%	6.7%		
20% ACP	73.3%	20.0%	6.7%		

With the help of an X-ray diffractometer, a peak at 2θ=20-35' was seen in all three groups, indicating remineralization and formation of HA. The intensity of the apatite peak increased with an increase in the concentration of ACP fillers from 10% to 20% (Figures 2A and 2B).

A band at 990 (800-1800 cm^{-1}) belonging to PO4 was seen in 10% ACP and 20% ACP groups at seven days. The intensity of this band was more significant in the 20% ACP group (Figure 2C). At 14 days, A peak at 3692 belonging to the OH of HA was seen with higher intensity in the 20% ACP group (Figure 2D).

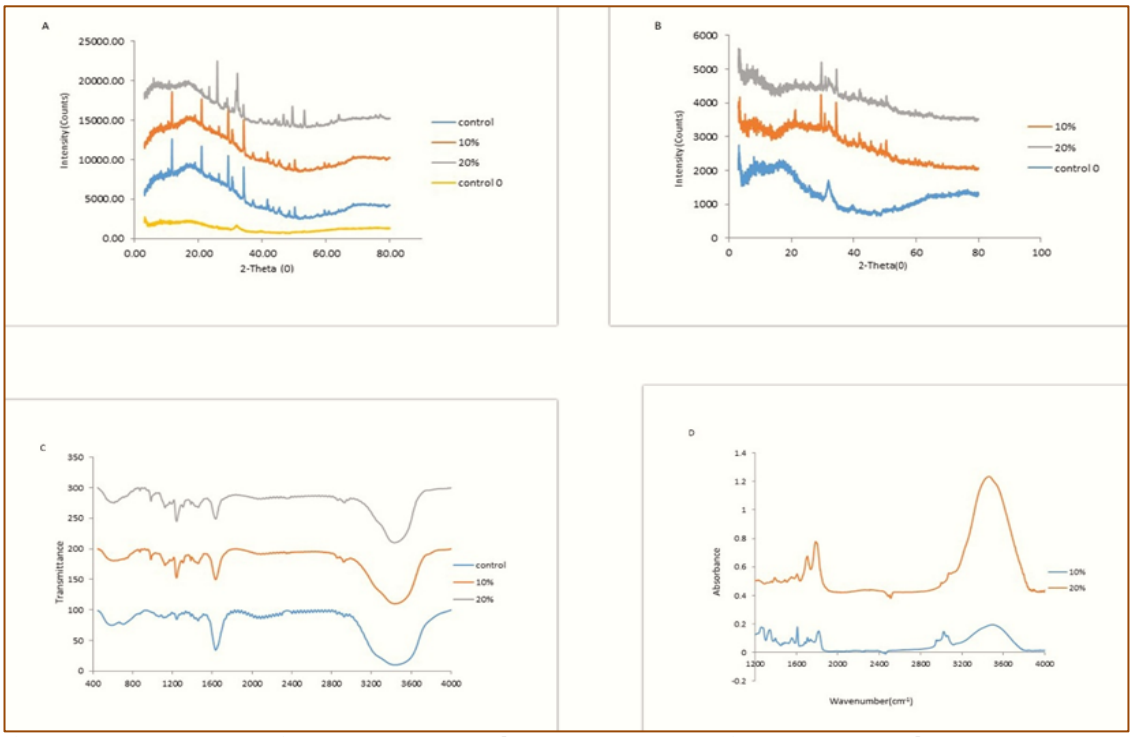

Figure 2. A: XRD analysis results at seven days; B: XRD analysis results at 14 days; C: ATR-FTIR results at seven days; and D: ATR-FTIR results at 14 days.

SEM assessment at x10,000 magnification revealed agglomerated rods on the bonding surface in 10% and 20% ACP groups at 7 and 14 days due to HA's crystallization on the surface. At 14 days, the number of HA crystals on the bonding surface of 10% and 20% ACP groups was higher than that at seven days (Figure 3).

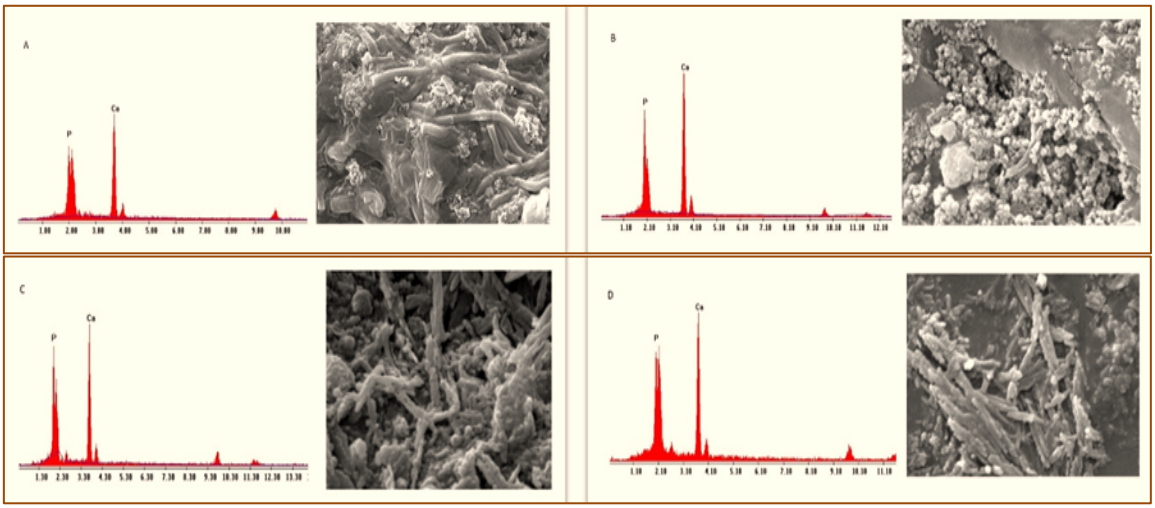

Figure 3. A: SEM-EDX micrograph of 10% ACP specimen at seven days; B: SEM-EDX micrograph of 20% ACP specimen at seven days; C: SEM-EDX micrograph of 10% ACP specimen at 14 days, and D: SEM-EDX micrograph of 20% ACP specimen at 14 days.

EDX analysis identified calcium and phosphorous ions, with a more significant percentage in the 20% ACP group than in the 10% ACP group. Also, the rate of these ions at 14 days was more substantial than the corresponding values at seven days (Figure 4).

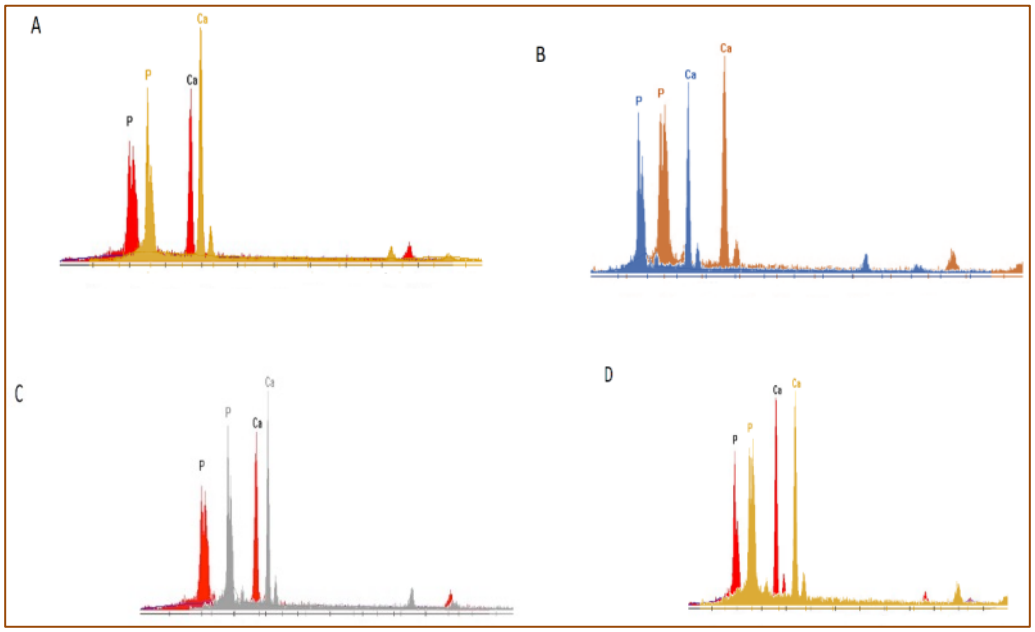

Figure 4. A: Comparison of SEM-EDX micrograph of 10% ACP specimen at seven days (red graph) with SEM-EDX micrograph of 10% ACP specimen at 14 days (yellow graph); B: Comparison of SEM-EDX micrograph of 20% ACP specimen at seven days (blue graph) with SEM-EDX micrograph of 20% ACP specimen at 14 days (brown graph); C: Comparison of SEM-EDX micrograph of 10% ACP specimen at seven days (red graph) with SEM-EDX micrograph of 20% ACP specimen at seven days (gray graph), and D: Comparison of SEM-EDX micrograph of 10% ACP specimen at 14 days (red graph) with SEM-EDX micrograph of 20% ACP specimen at 14 days (yellow graph).

Discussion

This study assessed the effect of the addition of ACP in different weight percentages on the remineralizing property and SBS of SBMP to dentin. Evidence shows that adding ACP to the adhesive component of SBMP does not compromise the bond strength, while its addition to SBMP primer decreases the bond strength to dentin [2]. Thus, we added the ACP to the adhesive component of SBMP and assessed its effect on SBS to dentin and dentin remineralization. Moreover, since ACP concentrations lower than 10% were not used in any previous study [2,3] and also the greater viscosity in case of using higher concentrations, ACP in 10% and 20% concentrations was added to SBMP adhesive in the present study. An adhesive containing ACP can increase the concentration of calcium and phosphorous ions to enhance remineralization and prevent demineralization, which is an advantage in a marginal gap at the tooth-restoration interface [2].

In the present study, the SBS was not significantly different among the three groups ($p>0.05$). Thus, the first part of the null hypothesis regarding no significant effect of ACP on SBS was accepted. This finding was in agreement with the results of Chen et al. [2], Melo et al. [3], and Gao et al. [8]. In the present study, the SBS in the 10% ACP group was slightly, but not significantly, higher than the SBS in the 20% ACP group. The reason may be the viscosity of the bonding agent. Adhesives with high viscosity cannot adequately flow to fill the gaps in the surface [18]. Melo et al. [3], Gao et al. [8] and Chen et al. [19] used the spray-drying technique and ACP nanoparticles. The technique used for ACP synthesis in the present study is more straightforward and cost-effective than the spray-drying technique and can synthesize high amounts of ACP. The amorphous structure of the synthesized calcium phosphate compound was also confirmed by XRD analysis.

The failure mode was dominantly adhesive followed by cohesive and then mixed in the present study, and the failure mode did not correlate with the SBS to dentin. This finding was in agreement with the results of O'Keefe et al. [20] and Torkani et al. [21] and different from the findings of Gateva and Dikov [22] and Perdigao et al. [23]. In the present study, SBMP three-step etch-and-rinse adhesive was used. In contrast, Gateva and Dikov [22] used a self-etch adhesive, and Perdigao et al. [23] and Sayahpour et al. [24] used a two-step etch-and-rinse adhesive system, which may explain the variability in the results. In the present study, XRD, FTIR, and SEM-EDX analyses were used to assess the effect of adding ACP to the bonding agent, which revealed the formation of HA crystals and consequent remineralization. This finding differed from the results of Melo et al. [3], who added ACP nanoparticles to the bonding agent, and the spray-drying technique synthesized the ACP nanoparticles. The bonding interface in each group underwent SEM analysis after applying the bonding agent. Several ACP nanoparticles were identified in the resin tags within the dentinal tubules and the hybrid layer. However, SEM analysis was unable to reveal remineralization in dentin areas containing resin tags with ACP nanoparticles. Thus, in the current study, the formation of HA crystals at 7 and 14 days was evaluated by SEM-EDX, XRD, and FTIR analyses.

In the present study, the ACP groups showed a significant increase in calcium and phosphorous elements on the surface of specimens. The formation of HA crystals was also noted. Therefore, the second part of the null hypothesis regarding no significant effect of ACP on the remineralizing property of the adhesive was rejected. Chen et al. [25] used a carboxy methyl chitosan/ACP scaffold to remineralize demineralized dentin. The SEM-EDX revealed that treatment with carboxy methyl chitosan/ACP significantly increased the calcium and phosphorous ions on the specimen surface, which was in agreement with the current study's findings.

Weir et al. [26] reported significant enamel remineralization using ACP nanoparticles determined by microradiography. Longhorst et al. [27] indicated that ACP-containing composites caused more significant mineral recovery of the enamel compared with fluoride-releasing types of cement using microradiography. In a

study by Choudhary et al. [28], both groups of ACP-containing sealants and fluoride-containing sealants caused enamel remineralization at the sealant-tooth interface on SEM assessment. The three studies mentioned above agreed with the present results, confirming that the presence of ACP leads to enamel remineralization. However, the present study showed dentin remineralization and used XRD, FTIR, and SEM-EDX analyses to confirm the formation of HA.

In the present study, an increase in the concentration of ACP filler from 10% to 20% increased the formation of HA crystals. This result was in agreement with the findings of Chen et al. [2], Marovic et al. [4] and Xu et al. [29]. Additionally, Xu et al. [29] assessed the caries prevention efficacy of nanocomposites containing ACP nanoparticles. They showed that an increase in ACP nanoparticles enhanced ion release. The release of calcium at a pH of 4 on day 28 was higher in the 20% nano-ACP group than in the 10% nano-ACP. In the study by Chen et al. [2], increasing the filler content in the adhesive from 10% to 40% enhanced the release of calcium and phosphorous ions and subsequently improved remineralization. Also, Marovic et al. [4] showed that the release of calcium and phosphate ions from composite resins containing ACP was maximum at 28 days and minimum at one day, which was in agreement with the current results that showed a higher rate of formation of HA crystals on day 14 compared with day seven by SEM analysis, which can be due to higher deposition of calcium and phosphorous ions during the time interval between 7 and 14 days. The increase in phosphate is probably related to the formation of HA and indicates that the addition of ACP to the adhesive component of SBMP leads to the formation of HA. The intensity of the PO4 peak was higher in the 20% ACP group, indicating that increasing the concentration of ACP from 10% to 20% provides a higher number of seeding areas for crystallization.

In the present study, qualitative tests were used to assess the remineralization, and two specimens from each group were considered for this purpose. The control specimen failed after 14 days due to its separation into multiple pieces, which was a limitation of this study. Future studies are needed to assess more samples for this purpose. As most studies showed anterior teeth to be the most commonly affected site of demineralization [30], anterior teeth can be assessed in future studies. Furthermore, studies are required to evaluate the properties of bonding agents containing ACP in the long term. Clinical studies are also recommended to assess the remineralizing efficacy of ACP-containing bonding agents *in vivo*. Moreover, incorporating mechanically milled ACP nanoparticles could improve further studies, as ACP's aggregation and excess water sorption are a concern [6].

Conclusion

The addition of amorphous calcium phosphate confers remineralizing property to SBMP adhesive without compromising its shear bond strength to dentin.

Authors' Contributions.

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HT	https://orcid.org/0000-0001-8875-1578	Methodology, Validation, Formal Analysis and Supervision.
BRO	https://orcid.org/0000-0002-3711-8841	Conceptualization, Visualization and Project Administration.
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All authors declare that they contributed to a critical review of intellectual content and approval of the final version to be published.

Financial Support

None.

Conflict of Interest

The authors declare no conflicts of interest.

Data Availability

The data used to support the findings of this study can be made available upon request to the corresponding author.

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