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Original Article

Effect of a novel pretreatment on the microtensile bond strength of universal adhesives with dentin

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KEYWORDS

Pretreatment agent;
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Abstract *Background: /purpose:* The quality of the hybrid layer is of great importance for dentin bonding. The purpose of this study was to develop a novel copper-based pretreatment and investigate the effect of the pretreatment combined with universal adhesives on the dentin bond strength.

Materials and methods: Etch-and-rinse adhesive Single Bond 2 (SB2) and two universal adhesives Prime Bond Universal (PBU) and Single Bond Universal (SBU) were selected. The dentin surfaces were pretreated with CuSO₄ solution and K₂HPO₄ solution in turn (Cu–P pretreatment), and the adhesive was applied following the manufacturer's instructions. There were four groups of Cu–P pretreatment: HH-Cu (1.5 mol/L CuSO₄ + 1.0 mol/L K₂HPO₄); H–Cu (0.15 mol/L CuSO₄ + 0.1 mol/L K₂HPO₄); L-Cu (0.015 mol/L CuSO₄ + 0.01 mol/L K₂HPO₄); and LL-Cu (0.0015 mol/L CuSO₄ + 0.001 mol/L K₂HPO₄). The microtensile bond strength (μ-TBS) and fracture mode were determined. The dentin surface after pretreatment and the antimicrobial properties of the pretreatment agent were also evaluated.

Results: The minimum inhibitory concentration and minimum bactericidal concentration of Cu–P pretreatment were 0.012 mol/L CuSO₄ + 0.008 mol/L K₂HPO₄. Combined with SB2, the H–Cu and L-Cu groups showed a higher μ-TBS ($P < 0.01$), while the HH-Cu group showed a lower

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μ -TBS ($P < 0.001$), and the LL-Cu group showed a similar μ -TBS with the control group without Cu–P pretreatment. Combined with universal adhesives PBU and SBU, the H–Cu and L-Cu groups also showed significantly increased μ -TBS ($P < 0.01$).

Conclusion: The copper-based pretreatment in combination with universal adhesives improved the dentin microtensile bond strength.

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Introduction

Adhesive dentistry has developed rapidly in recent decades. Dentin bonding is currently a key concern of adhesive restorations.¹ The hybrid layer is an important structure in dentin bonding and its properties affect the bond strength and durability.²

The main problems associated with dentin bonding are the poor infiltration of resin monomers into the demineralized dentin matrix and the subsequent degradation of collagen and resin monomers, which affect the quality of the hybrid layer.^{3,4} Various methods have been explored to improve the permeability of adhesive monomers in demineralized dentin,⁵ including ethanol wet bonding,^{6,7} using electric current to promote resin infiltration,⁸ applying crosslinking agents,^{9,10} and removing proteoglycans from the demineralized dentin.¹¹ However, no strategy can completely solve the fundamental problems in dentin bonding and further research is required.

Universal adhesives have the advantage of fast application and the potential of wide-ranging applications with further research.¹² As a single-bottle system, universal adhesives contain acidic functional monomers, such as 10-methacryloyloxydecyl dihydrogen phosphate (10-MDP), dipentaerythritol penta acrylate monophosphate (PENTA), polyalkenoic acid copolymer (PAC), adhesive monomers, accessory hydrophilic and hydrophobic monomers, solvents, and other components.^{13,14} The composition requires good chemical stability while ensuring the functionality of the various chemical components, which may be challenging in terms of hybrid layer formation.¹⁵

In previous studies, we found that calcium and fluoride ions can rapidly generate CaF₂ nanoparticles in situ in a demineralized dentin matrix.¹⁶ The use of metallic elements may create a more hydrophobic environment for dentin bonding. Copper is a bivalent metallic element and can inhibit matrix metalloproteinase MMP-2,¹⁷ thus reducing collagen degradation. Copper has an indirect role as a cross-linking agent and thus can increase the strength of the collagen network.^{18,19} Copper also has efficient antibacterial activity against both Gram-positive and Gram-negative bacteria.²⁰ These advantages endow copper with high application value and development prospects in dentin bonding. Thus far, there are few studies on the use of copper for dentin bonding pretreatment.

In this study, we developed a novel dentin bonding pretreatment using copper sulfate (CuSO₄) and dipotassium hydrogen phosphate (K₂HPO₄). The appropriate concerns for using CuSO₄ and K₂HPO₄ were screened and the effect of

the pretreatment on the bonding of universal adhesives to dentin was investigated. The null hypothesis was that this novel pretreatment agent did not affect the bonding of universal adhesives to dentin.

Materials and methods

Material selection and grouping

The novel pretreatment consisted of CuSO₄ (Macklin Biochemical Co., Shanghai, China) and K₂HPO₄ (Macklin Biochemical Co.), which was divided into four groups according to concentration: HH-Cu (1.5 mol/L CuSO₄ + 1.0 mol/L K₂HPO₄), H–Cu (0.15 mol/L CuSO₄ + 0.1 mol/L K₂HPO₄), L-Cu (0.015 mol/L CuSO₄ + 0.01 mol/L K₂HPO₄) and LL-Cu (0.0015 mol/L CuSO₄ + 0.001 mol/L K₂HPO₄). The process of pretreating the dentin surface with CuSO₄ and K₂HPO₄ solution was called Cu–P pretreatment.

One etch-and-rinse adhesive and two universal adhesives were selected, which were used in combination with Cu–P pretreatment. Single bond 2 (SB2) (3M ESPE, St. Paul, MN, USA) contained bisphenol-A-glycidyl dimethacrylate (Bis-GMA) as the functional monomer as well as ethanol and water as solvents. Prime Bond Universal (PBU) (Dentsply Sirona, Konstanz, Germany) contained PENTA as the functional monomer as well as isopropanol and water as solvents. Single Bond Universal (SBU) (3M ESPE) contained 10-MDP and PAC as functional monomers as well as ethanol and water as solvents. These three bonding adhesives with different compositions are representative adhesives of dentin bonding.

Observation of dentin interface after Cu–P pretreatment

The color and properties of the CuSO₄ and K₂HPO₄ solutions before and after mixing were recorded using a camera (Canon, Tokyo, Japan). The morphology of the precipitate formed immediately after mixing the CuSO₄ solution (0.15 mol/L) and an equal volume of the K₂HPO₄ solution (0.1 mol/L) were observed using a Talos F200C cryo-transmission electron microscope (cryo-TEM) (FEI, Hillsboro, OR, USA).

Ninety freshly extracted non-carious human molars were used. The crown enamel was removed with a slow-speed diamond saw (SYJ-150; Kejing Auto-Instrument Co., Shenyang, China) to expose the dentin surface, which was then

wet-polished with 600-grit silicon carbide sandpaper to simulate a smear layer. The polished dentin surface was acid etched with 37% phosphoric acid (Aladdin Holding Group Co., Beijing, China) for 20 s and then rinsed with deionized water for 20 s. The acid-etched samples were randomly divided into five groups.

For the control group, the dentin surface was kept moist using a small brush to remove excess water. For the experimental groups (HH-Cu, H-Cu, L-Cu, LL-Cu), the dentin surface was first rubbed with 20 μL of CuSO_4 solution for 20 s, and the excess solution was absorbed with a small dry brush. The dentin surface was then rubbed with 20 μL of K_2HPO_4 solution for 20 s. After rinsing with deionized water for 20 s, the small brush was used to remove excess water and to keep the dentin surface moist. The operation mentioned above was called Cu-P pretreatment. The morphology of the dentin interface before, during, and after Cu-P pretreatment was observed with a camera.

Cu-P pretreated dentin discs were dehydrated by successive ethanol baths (30, 50, 70, 90 and 100%) and dried with hexamethyldisilazane. The cross-sectional surface morphology was observed using a field emission scanning electron microscope (SEM, Thermo FEI, Brno, Czech Republic) at an accelerating voltage of 5 kV.

Minimum inhibitory concentration and minimum bactericidal concentration

Streptococcus mutans (UA159) was used to examine the antimicrobial activity of the Cu-P pretreatment agent. The inoculum was grown in brain heart infusion (BHI) broth at 37 °C for 24 h, and the concentration was adjusted to 10^8 CFU/mL.

The CuSO_4 and K_2HPO_4 solutions were diluted using the twofold dilution method. The specific numbers and corresponding concentrations are shown in Fig. 3A. Briefly, 100 μL of 10^8 CFU/mL bacterial solution was added and incubated for 24 h (37 °C, 5% CO_2). Then, the absorbance at 600 nm (OD600) of the solutions was measured with an enzyme marker (BioTek Instruments, Winooski, VT, USA) to determine the turbidity of the solution. The minimum inhibitory concentration (MIC) was determined jointly from the concentration at which the solution changed from clarity to turbidity and the inflection point at which the absorbance changed.

After MIC determination, samples containing the lowest concentration of visible growth and all samples without visible growth were incubated in Petri dishes containing BHI agar for 48 h (37 °C, 5% CO_2). The minimum bactericidal concentration (MBC) was determined by the lowest concentration of the test substance at which there was no microbial growth.

Microtensile bond strength test

After Cu-P pretreatment, adhesive samples were prepared using adhesive and composite resin (Ceram; Dentsply, Tulsa, OK, USA) following the manufacturer's instructions. Dentin-resin strips (Fig. 4A) with a cross-section of 1×1 mm were then prepared using a slow-speed diamond

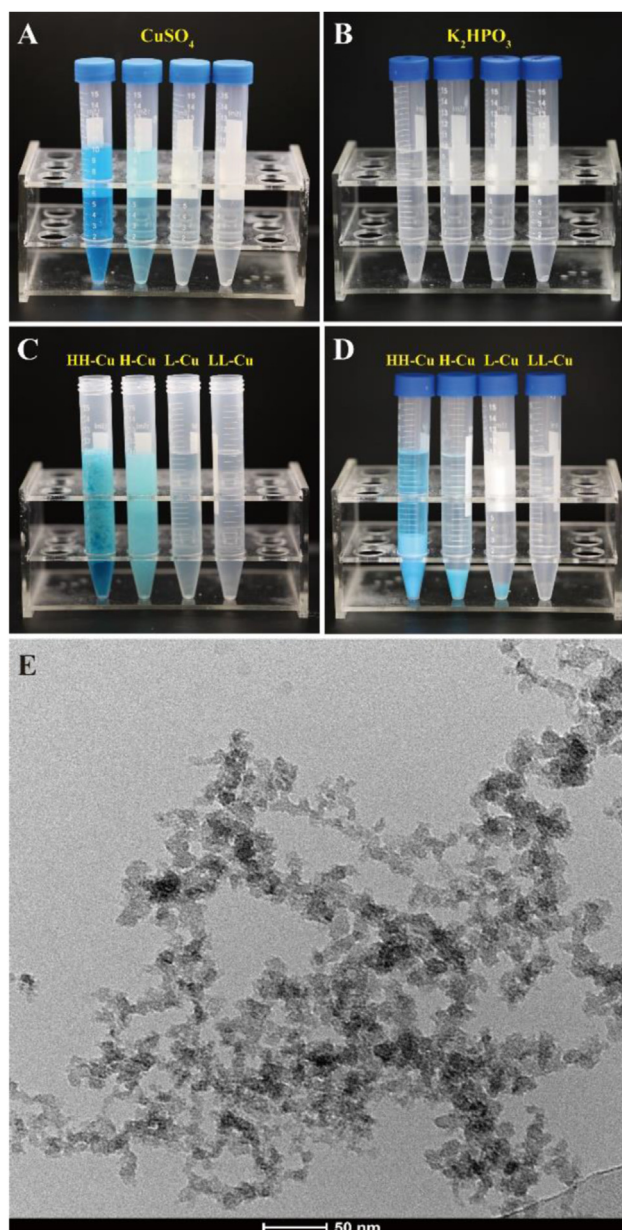


Figure 1 (A) Different concentrations of CuSO_4 solutions. From left to right are 1.5 mol/L, 0.15 mol/L, 0.015 mol/L, and 0.0015 mol/L. (B) Different concentrations of K_2HPO_4 solutions. From left to right are 1.0 mol/L, 0.1 mol/L, 0.01 mol/L, and 0.001 mol/L. CuSO_4 and K_2HPO_4 solutions (C) mixed immediately and (D) after centrifugation for 10 min. The concentration decreases from left to right. (E) Characterization of cupric phosphate nanoparticles in water by cryo-transmission electron microscopy. Abbreviations: HH-Cu: 1.5 mol/L CuSO_4 + 1.0 mol/L K_2HPO_4 ; H-Cu: 0.15 mol/L CuSO_4 + 0.1 mol/L K_2HPO_4 ; L-Cu: 0.015 mol/L CuSO_4 + 0.01 mol/L K_2HPO_4 ; LL-Cu: 0.0015 mol/L CuSO_4 + 0.001 mol/L K_2HPO_4 .

saw and stored in artificial saliva (pH = 6.8) at 37 °C for 1 day (10 teeth per group, 10 samples per tooth). The microtensile bond strength ($\mu\text{-TBS}$) was tested using a microtensile tester (Bisco Inc., Schaumburg, IL, USA) at a loading rate of 1 mm/min.

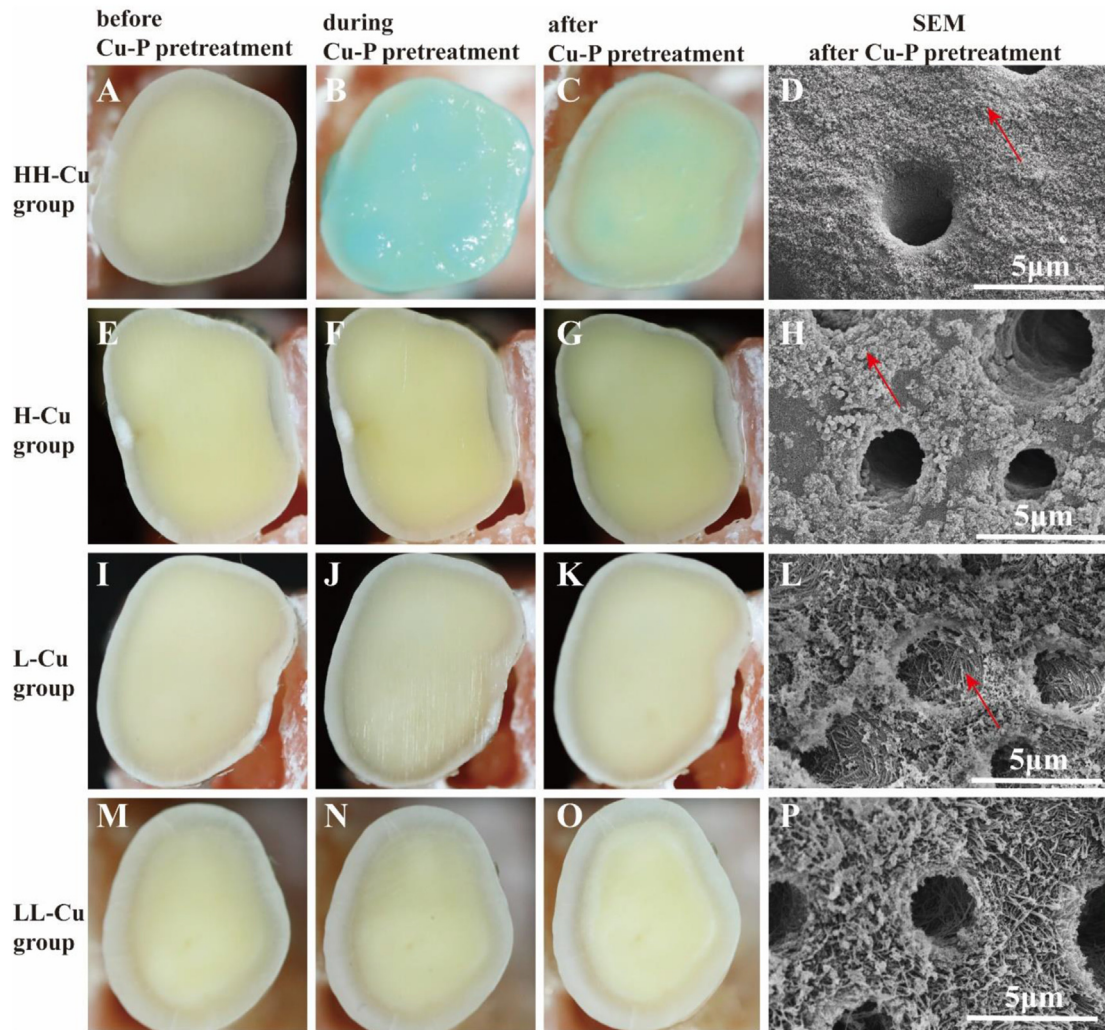


Figure 2 The dentin interface after Cu–P pretreatment. A, B, and C show the morphology of the dentin interface before, during, and after Cu–P pretreatment with HH–Cu. E, F, and G show the morphology of the dentin interface before, during, and after Cu–P pretreatment with H–Cu. I, J, and K show the morphology of the dentin interface before, during, and after Cu–P pretreatment with L–Cu. M, N, and O show the morphology of the dentin interface before, during, and after Cu–P pretreatment with LL–Cu. D, H, L and P show the SEM image of the dentin interface after Cu–P pretreatment for the HH–Cu, H–Cu, L–Cu and LL–Cu groups, respectively. The red arrow shows the nanoparticle-like material. Abbreviations: Cu–P pretreatment: $\text{CuSO}_4 + \text{K}_2\text{HPO}_4$; HH–Cu: 1.5 mol/L $\text{CuSO}_4 + 1.0$ mol/L K_2HPO_4 ; H–Cu: 0.15 mol/L $\text{CuSO}_4 + 0.1$ mol/L K_2HPO_4 ; L–Cu: 0.015 mol/L $\text{CuSO}_4 + 0.01$ mol/L K_2HPO_4 ; LL–Cu: 0.0015 mol/L $\text{CuSO}_4 + 0.001$ mol/L K_2HPO_4 ; SEM: scanning electron microscopy.

The fracture mode (Fig. 4B) was examined using a scanning electron microscope (SEM) (Hitachi Ltd., Tokyo, Japan) and classified: adhesive fracture, mixed fracture, and cohesive fracture (failure within the resin composite or dentin).

Statistical analysis

Data of microtensile strength are presented as mean \pm standard deviation (Mean \pm SD). Statistical analysis was performed using the SPSS software (version 27.0, SPSS Inc., Chicago, IL, USA). Microtensile bond strength data were compared by two-way analysis of variance (ANOVA) with post hoc comparisons using the Tukey test. The distribution of failure mode was analyzed using the chi-square

test. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ were considered statistically significant.

Results

CuSO_4 solutions were blue liquids, and the intensity of the color was positively correlated with the concentration (Fig. 1A). K_2HPO_4 solutions of different concentrations were colorless liquids (Fig. 1B).

When CuSO_4 and K_2HPO_4 solutions in the molar ratio of 3:2 were mixed, precipitation was observed immediately in the HH–Cu group (Fig. 1C). As the concentration decreased, the amount of precipitate also decreased, and no precipitation was observed in the LL–Cu group (Fig. 1D).

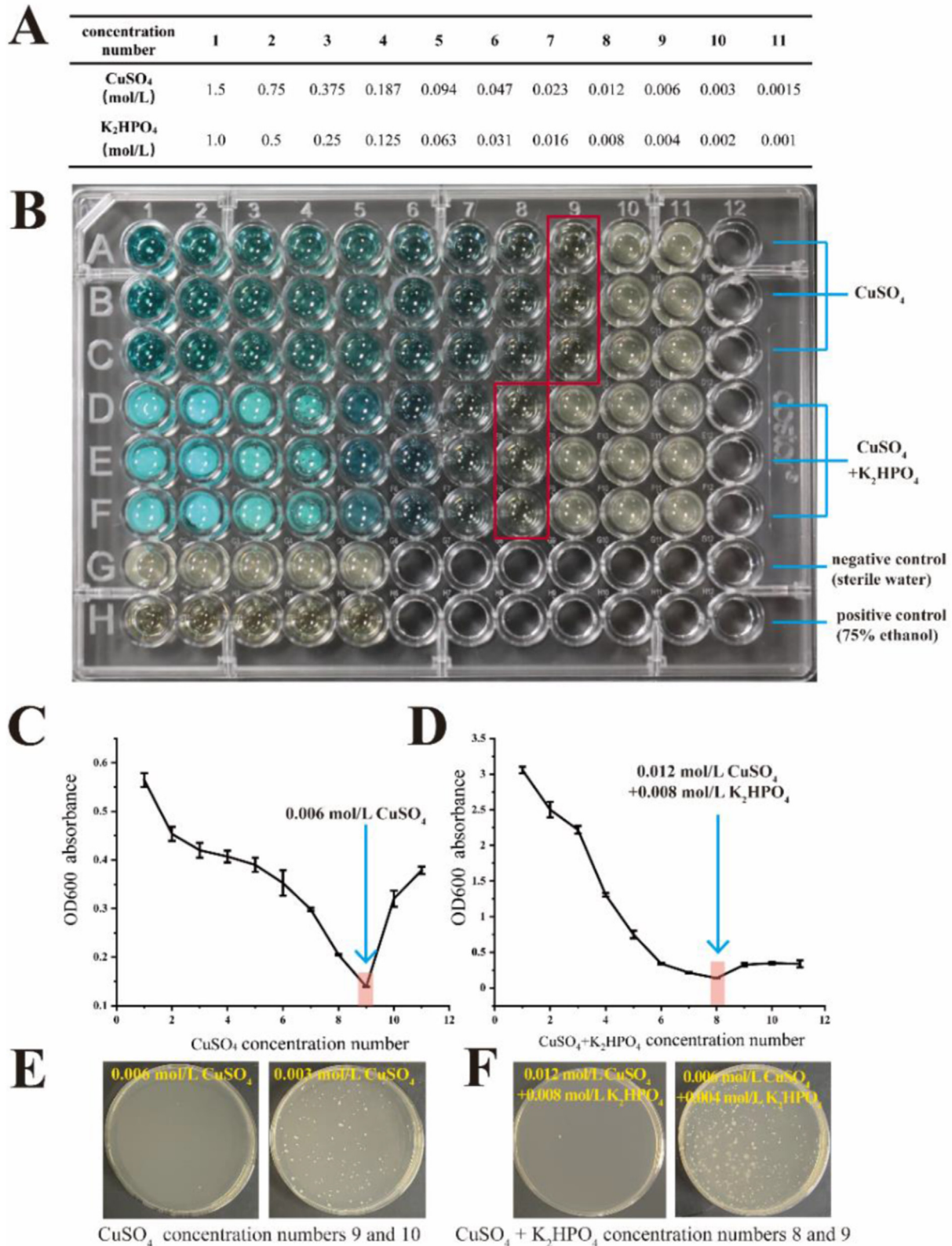


Figure 3 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). (A) Corresponding concentrations of CuSO_4 and K_2HPO_4 solutions by twofold dilution method. (B) MIC experiments for Cu–P pretreatment agent. Rows A–C are CuSO_4 concentrations from 1.5 mol/L to 0.0015 mol/L, rows D–F are CuSO_4 concentrations from 1.5 mol/L to 0.0015 mol/L + K_2HPO_4 concentrations from 1.0 mol/L to 0.001 mol/L, row G is negative control (sterile water), and row H is positive control (75% ethanol). OD600 absorbance values correspond to (C) different concentrations of CuSO_4 and (D) different concentrations of CuSO_4 + K_2HPO_4 . MBC results for (E) CuSO_4 concentration numbers 9 and 10 and (F) CuSO_4 + K_2HPO_4 concentration numbers 8 and 9.

Under cryo-TEM, $\text{Cu}_3(\text{PO}_4)_2$ was in the form of nanoparticles with a particle size of 5–10 nm, and the particles aggregated into a cluster-like distribution (Fig. 1E).

Dentin interface after Cu–P pretreatment

In the HH-Cu group, a large amount of blue precipitates was generated on the dentin surface during Cu–P pretreatment (Fig. 2). After rinsing with deionized water, blue granular precipitates were still visible on the dentin surface. In the other groups, there was no color change on the dentin surface during and after the Cu–P pretreatment.

SEM results (Fig. 2) showed that a large amount of nanoparticle-like material was observed within and between dentin tubules in the HH-Cu group, and collagen fiber structures were almost not visible, while a medium amount of nanoparticle-like material was observed in the H–Cu group. Nanoparticle-like material deposition was observed around collagen fibers in the L-Cu group, and almost no nanoparticle-like material was deposited in the LL-Cu group.

MIC and MBC of Cu–P pretreatment agent

The results of in vitro bacterial inhibitory ability of Cu–P pretreatment agent assays are shown in Fig. 3. The MIC (Fig. 3B–D) and MBC (Fig. 3E and F) of the Cu–P pretreatment agent were both 0.012 M CuSO_4 + 0.008 M K_2HPO_4 . This indicated that the concentration of the HH-Cu, H–Cu, and L-Cu groups had bacterial inhibitory effects. Because the concentration of the LL-Cu group was lower than MIC and MBC, the LL-Cu group had no bacterial inhibitory effect.

Effect of Cu–P pretreatment on dentin bonding

Combined with etch-and-rinse adhesive SB2, universal adhesives PBU and SBU, the H–Cu and L-Cu groups showed a higher μ -TBS ($P < 0.01$), while the HH-Cu group showed a lower μ -TBS ($P < 0.001$) than the control group without Cu–P pretreatment. There was no significant difference between the LL-Cu and control groups (Fig. 4C).

The main fracture mode of all groups (Fig. 4D) was mixed fracture, and there was no significant difference among all groups.

Discussion

The quality of the hybrid layer is critical to high-performance dentin bonding. In this study, a novel pretreatment aimed to create a bond-friendly surface for dentin bonding was developed. On the basis of the results, the null hypothesis that the novel pretreatment did not affect the strength of dentin bonding was rejected.

In this study, the microtensile bond strength was significantly increased when Cu–P pretreatment with the H–Cu and L-Cu groups was combined with the etch-and-rinse adhesive SB2 and universal adhesives PBU and SBU. Previous studies by our group showed that after a facile sequential treatment of CaCl_2 and NaF solutions,¹⁶

endogenous negatively charged groups of non-collagenous origin rapidly form amorphous CaF_2 nanoparticles in situ in the demineralized dentin matrix, thus increasing the bonding efficiency of various adhesives. Previous studies also showed that pretreatment of CaCl_2 and K_2HPO_4 solutions formed nanoparticles that similarly improved the dentin bonding properties.²¹ In this study, the role of $\text{Cu}_3(\text{PO}_4)_2$ nanoparticles, formed during Cu–P pretreatment, in improving the microtensile bond strength may be similar to that of the nanoparticles mentioned above. It was postulated that Cu–P pretreatment may be able to reduce the hydrophilicity of demineralized dentin, and the formed copper phosphate nanoparticles may have a supporting effect on the demineralized collagen network. This promotes the entry of adhesive monomers into the interstices of the demineralized collagen fibril network and improves the quality of the hybrid layer. However, the exact mechanism after Cu–P pretreatment needs to be systematically verified by contact angle test, water absorption property test, ion staining experiment, solid surface potential experiment, and atomic force microscopy in the future.

Copper has advantageous properties for dentin bonding. Because the collagen cross-linking enzyme, lysyl oxidase (LOX), is dependent on copper.²² As a cross-linking agent, the strength of the collagen network increases in the presence of copper, which is one of the components of the hybrid layer. Other studies have shown that oral tissue matrix metalloproteinase (MMP) activity may be regulated by copper ions,²³ which can decrease MMP-2 secretion and show MMP inhibitory properties.²⁴ The effect of Cu–P on the degree of collagen cross-linking and MMP inhibition will be verified subsequently.

In this study, two universal dental adhesives were selected. The type and characteristics of the solvent of the adhesive are critical to the diffusion of water and resin in the dentin matrix.^{25,26} The solvent of PBU is isopropyl alcohol and water,²⁷ and the solvent of SBU is ethanol and water.²⁸ The Cu–P pretreatment agent used in combination with these two universal bonding adhesives significantly improved the bond strength, which further indicated the value and wide applicability of Cu–P pretreatment.

However, the effectiveness of Cu–P pretreatment was concentration-dependent. When the concentration was too high, as in the HH-Cu group, the large amount of precipitates produced immediately after mixing completely covered the dentin interface. The agglomerated precipitates blocked the dentin tubules at the demineralized dentin interface and affected the penetration of the bonding monomer. When used in combination with SB2, the results showed a significant decrease in bond strength. Dentin staining in the proximal pulp angle after deionized water rinsing was presumed to be too high a concentration, which may affect the viability of the pulp cells. When the concentration was too low, as in LL-Cu group, the pretreatment was unable to modify the dentin surface and improve the bond strength. Therefore, it is crucial that the copper concentration of the novel pretreatment is cautiously selected.

In addition to adhesive durability, secondary caries is one of the main reasons for the failure of adhesive restorations.²⁹ Therefore, imparting adhesive agents with antimicrobial properties to inhibit secondary caries is one of

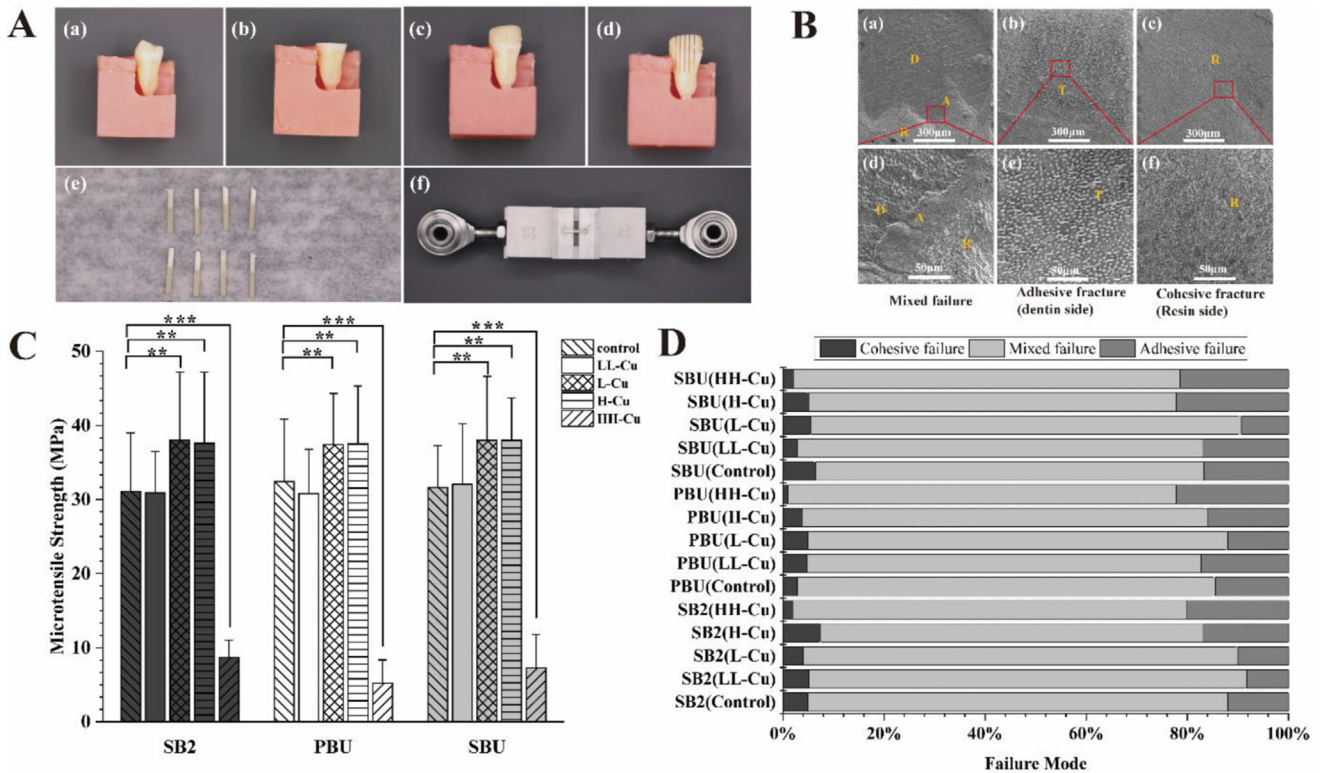


Figure 4 Effect of Cu–P pretreatment in combination with different adhesives on dentin bonding. (A) Preparation of specimens for testing microtensile bond strength. (B) Schematic diagram of microtensile fracture mode: (a) and (d) are mixed fracture, (b) and (e) are dentin side of adhesive fracture, (c) and (f) are cohesive fracture (resin cohesive fracture). Abbreviations: A (adhesive); D (dentin); R (composite resin); T (resin tag). (C) Microtensile bond strength and (D) fracture mode statistics for Cu–P pretreatment with different concentrations in combination with different adhesives. (** $P < 0.01$, *** $P < 0.001$). Abbreviations: Cu–P pretreatment: $\text{CuSO}_4 + \text{K}_2\text{HPO}_4$; HH-Cu: 1.5 mol/L $\text{CuSO}_4 + 1.0$ mol/L K_2HPO_4 ; H-Cu: 0.15 mol/L $\text{CuSO}_4 + 0.1$ mol/L K_2HPO_4 ; L-Cu: 0.015 mol/L $\text{CuSO}_4 + 0.01$ mol/L K_2HPO_4 ; LL-Cu: 0.0015 mol/L $\text{CuSO}_4 + 0.001$ mol/L K_2HPO_4 ; PBU: Prime Bond Universal; SB2: Single Bond 2; SBU: Single Bond Universal.

the main directions presently adopted to improve adhesive agents.³⁰ Copper exerts bactericidal or bacteriostatic effects on different forms of microorganisms.³¹ The two main mechanisms by which copper exerts antimicrobial effects are membrane depolarization and the production of reactive oxygen species (ROS).³² It has been shown that copper nanoparticles added to a two-step etch-and-rinse adhesive can exert antibacterial effects against both Gram-positive and Gram-negative bacteria without increasing the inherent cytotoxicity of the adhesive.³³ However, whether there is a difference in the principle of bacterial inhibition and cytotoxicity between the in vitro synthesized copper nanoparticles and the copper solution issued in Cu–P pretreatment agent is still unknown and needs to be further investigated subsequently. In this study, we initially confirmed the antibacterial properties of the Cu–P pretreatment agent by determining MIC and MBC. The results showed that the Cu–P pretreatment agent had antibacterial effects, and the antibacterial effect was positively correlated with the concentration.

In conclusion, a simple pretreatment using CuSO_4 and K_2HPO_4 solutions increased the strength of dentin bonding with universal adhesives and showed antibacterial effects. However, future studies on the durability of the bonding as well as the possible mechanisms are needed. This study will

provide new ideas and strategies for further improvement of the dentin-adhesive interface.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

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