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RESEARCH AND EDUCATION

Effect of polyvinylphosphonic acid on resin-dentin bonds and the cytotoxicity of mouse dental papilla cell-23

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ABSTRACT

Statement of problem. Polyvinylphosphonic acid (PVPA) could be used as a biomimetic remineralization analog and a matrix metalloproteinases (MMPs) inhibitor. However, studies are lacking regarding the performance of PVPA in dental bonding systems for maintaining the durability of the resin-dentin bond.

Purpose. The purpose of this in vitro study was to investigate the effect of PVPA on the durability of resin-dentin bonds and the viability of mouse dental papilla cell-23 (MDPC-23). The mechanical properties of resin-dentin interfaces during long-term storage were analyzed, and the potential application of PVPA as a biomimetic remineralization analog in adhesive dentistry was evaluated.

Material and methods. Seventy-five extracted noncarious human third molars were collected and randomly divided into 5 groups, and then the microtensile bond strength (μTBS) data and scanning electron microscope (SEM) images were used to evaluate the preservation condition of resin-dentin bonds after 1 day, 6 months, and 1 year of storage. The cytotoxicity of PVPA was detected by cell proliferation assay and cell apoptosis assay.

Results. Compared with the control and chlorhexidine (CHX) groups, the combined group (treated with both 200-μg/mL PVPA and biomimetic remineralization) had excellent bond durability. The exposed collagen fibril from the PVPA-treated groups (included 200-μg/mL and 500-μg/mL PVPA groups and a combined group) still showed integrity after 1 year of storage when compared with the control group. PVPA up to 500 μg/mL showed no cytotoxicity to MDPC-23 and did not inhibit cell growth.

Conclusions. This study offered evidence that PVPA did not result in cytotoxicity at low concentrations as an MMP inhibitor and a biomimetic remineralization analog. In addition, the application of PVPA improved bond strength and preserved collagen integrity after 1 year of in vitro storage. (J Prosthet Dent 2019;122:492.e1-e6)

The goal of adhesive dentistry is to increase the durability of bonded restorations, but whether bond strength is reduced by the loss of exposed collagen within the hybrid layer is unclear. The endogenous proteases bound to the dentin matrix are vulnerable to potential degradation of the unprotected collagen fibrils and the hybrid layer[1] as the majority of matrix metalloproteinases (MMPs) in dentin, MMP-2, MMP-8, and MMP-9, are collectively capable of endogenously degrading dentin collagen. Previous studies reported that human dentin also contained collagenase stromelysin-1, MMP-3, and MMP-20.[2] Recent studies have stated that the enzymatic degradation of collagen fibrils, which was activated by etch-and-rinse and self-etch adhesives, was one of the main mechanisms of hybrid layer degradation.[2,3] MMP inhibition offered an effective approach to preserving the mechanical properties of composite resin-dentin bonding.[4,5] Biomimetic dentin remineralization, a valuable tool for maintaining the mechanical properties and biostability of collagen fibrils, is an emerging biomedical technology for improving the durability of the bond strength.[6-8]

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Clinical Implications

PVPA as a biomimetic remineralization analog and an MMP inhibitor in dental bonding systems could maintain resin-dentin bond durability.
PVPA-containing adhesives can be recommended for clinical application.

Remineralization of demineralized dentin may reduce collagen degradation,[9-11] and specific agents have been used for this purpose.

Chlorhexidine (CHX) has been found to protect against the degradation of the adhesive interface with a broad-spectrum inhibitory property for MMPs 2, 8, and 9.[12-15] However, CHX exerts toxic effects on human osteoblastic cells and fibroblastic cells in a dose-dependent and time-dependent manner.[16-20]

Inhibition of collagenolytic enzymes and biomimetic remineralization are both experimental strategies to protect collagen matrix from biodegradation.[21-23] Polyvinylphosphonic acids (PVPAs), a protease inhibitor of MMPs, contribute to the formation of nanocrystals that account for intrafibrillar and interfibrillar remineralization of resin-dentin interfaces.[24-26] It remains to be seen whether PVPA not only inhibits MMPs in dentin but also induces remineralization of demineralized dentin matrix to protect the hybrid layer, increase the resin-dentin bond strength, and eventually promote longevity of clinical restorations.

As the biosecurity of PVPA was important for its application in clinical dentistry, evaluating the effects of PVPA on oral cells is essential. Thus, the purpose of this in vitro study was to examine whether PVPA could affect the integrity of the tooth restoration after storage, promote remineralization, and affect the viability of mouse dental papilla cell-23 (MDPC-23). The null hypotheses were that the application of the PVPA would have no effect on the bonding durability and biomimetic remineralization and that the application of PVPA would have no cytotoxic effect on MDPC-23.

MATERIAL AND METHODS

With the patients' consent, 75 extracted noncarious human third molars were obtained and stored in 0.5% chloramine T at 4 °C under a protocol approved by the Sun Yat-sen University Research Ethics Committee. Two specimens were collected from a series of 1×1×8-mm beams produced by each tooth by using a low-speed diamond sectioning saw (IsoMet; Buehler Ltd).

All 150 specimens were pretreated with 35% phosphoric acid and randomly divided into 5 groups (sample size, 30 per group). Each group was then subdivided into 3 groups according to the storage time (sample size, 10 per group). The application of PVPA (Sigma-Aldrich) or CHX (Sigma-Aldrich) on etched substrates for 30 seconds followed before an adhesive (Single Bond 2; 3M ESPE) was applied. After the placement of composite resin (Z350; 3M ESPE), the specimens were stored in distilled water at 37 °C for 24 hours. The combined group was stored in biomimetic remineralized fluid (composed of simulated body fluid [SBF], 500 μg/mL of polyacrylic acid, and 200 μg/mL of PVPA) with Portland cement (Lehigh Cement Co), while the other groups were stored in SBF, which was replaced once a week.[27,28] After storage for 1 day, 6 months, and 1 year, microtensile bond strengths (μTBS) were determined by using a microtensile tester (BISCO, Inc) at a crosshead speed of 1 mm/min. The resin-dentin bonds were examined by using a scanning electron microscope (SEM) (Quanta 200; FEI). The destruction of the mixed layers was observed by using an SEM at 20 kV.

MDPC-23 cells were cultured as previously described.[18] Cell proliferation was evaluated in triplicate by colorimetric assay. Cells were incubated in 5 mg/mL of 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) solution for 4 hours. The supernatants were then discarded, and 150 μL of dimethyl sulfoxide was added to all wells. Optical density (OD) values were recorded at 490 nm by using a microplate reader (BioTek).

Cell apoptosis was detected in triplicate by using a PE Annexin V Apoptosis Detection Kit (BD Biosciences). After exposure to different concentrations of CHX or PVPA for 30 minutes and 3 days and after culture in the fresh medium as the negative control, the cells were stained following the manufacturer's instructions and analyzed by using a flow cytometer (CytoFLEX; Beckman Coulter).

Data were analyzed by using a statistical software program (IBM SPSS Statistics, v20.0; IBM Corp) with a 2-

Table 1. Microtensile bond strength values (means ±standard deviation)

Treatment	Time	Microtensile Bond Strength (MPa)	Bond Strength Reduction (%)
Control	1 d	27.60 ±4.74[a]	0
	6 mo	19.08 ±1.70[c]	30.87
	1 y	13.59 ±2.53[d]	50.76
200 μg/mL of PVPA	1 d	24.64 ±6.80[a]	0
	6 mo	24.04 ±4.92[a]	2.44
	1 y	21.65 ±4.55[a]	12.13
500 μg/mL of PVPA	1 d	25.23 ±3.67[a]	0
	6 mo	24.46 ±3.69[a]	3.05
	1 y	21.37 ±6.46[a]	15.30
Combined	1 d	24.08 ±4.77[a]	0
	6 mo	27.68 ±3.98[a]	-14.95
	1 y	23.97 ±3.34[a]	0.46
200 mg/mL of CHX	1 d	24.54 ±4.88[a]	0
	6 mo	24.47 ±3.30[a]	0.29
	1 y	19.33 ±5.24[e]	21.23

Values with different letters indicate statistically significant differences ($P<.05$).

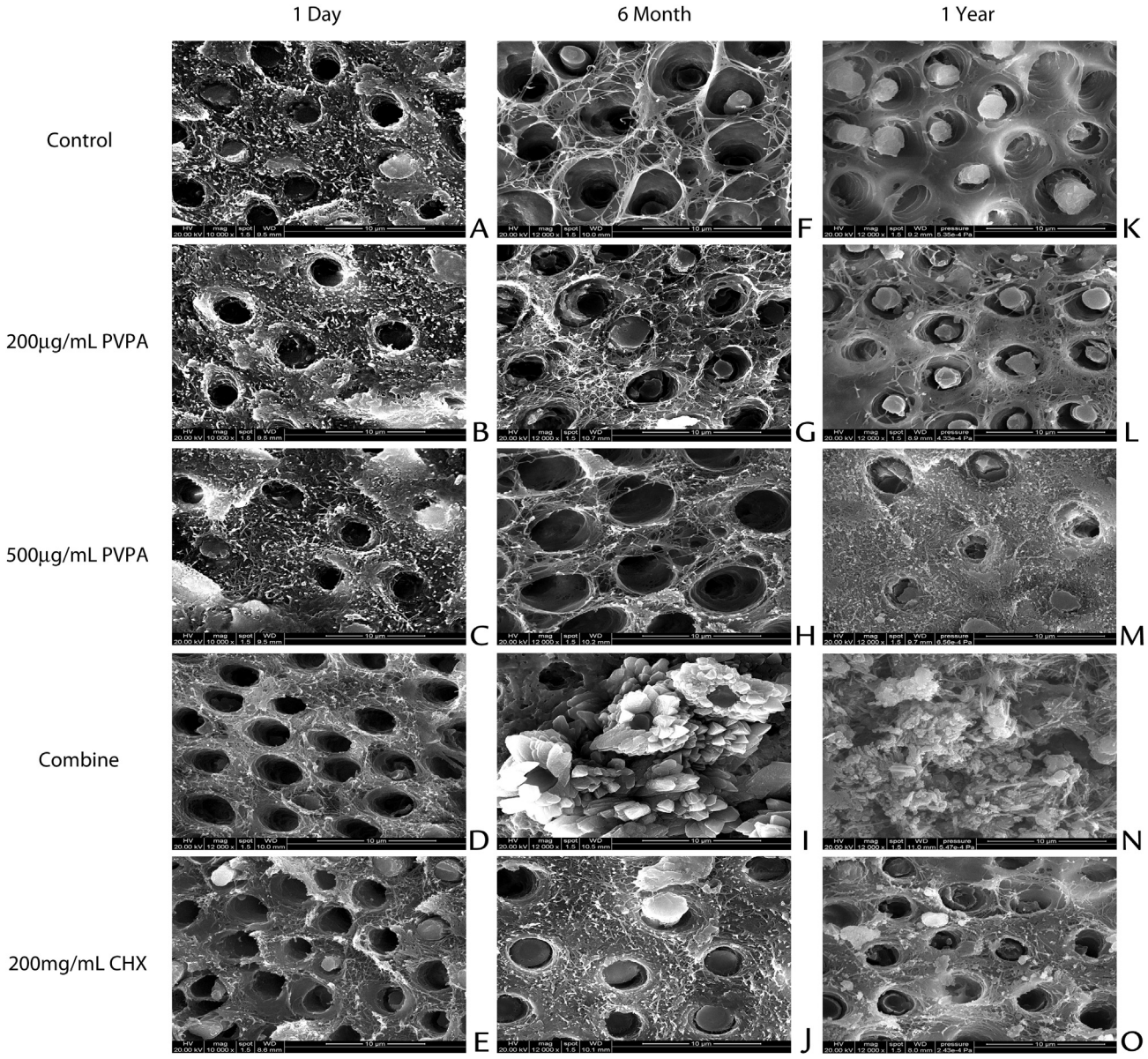

Figure 1. Scanning electron micrographs of fracture surface of 5 groups. A-E, Groups tested in 1 day. F-J, Groups tested at 6 months. K-O, Groups tested at 1 year. Scar bar 10 μm. Compared with chlorhexidine-treated groups and control group, collagen fibrils of fracture surface in polyvinylphosphonic acid groups still integrated with little fibril breakage after 1 year.

way ANOVA to analyze the effects of different treatments and storage times on μTBS. All other data analyses were performed by using 1-way ANOVA (α=.05).

RESULTS

The values of mean bond strength with different treatments are presented in Table 1. The μTBS of both the control and 200-mg/mL CHX groups had significant bond strength reduction after 1 year of in vitro storage (P<.05). However, the combined group decreased in bond strength by 0.46% at the same time, and the reduction of the 200-μg/mL PVPA group was 12.1% and that of the 500-μg/mL PVPA group was 15.30%. The remaining values of microtensile bond strength in PVPA-treated groups were higher than those observed in CHX-treated groups (P<.05).

Representative SEM images of the remineralization effect after 1 day, 6 months, and 1 year are presented in Figure 1. At 1 day, SEM micrographs from all the groups showed integrated and intercrossed collagen fibrils. The fractured surface of specimens aged for 6 months in the control group exhibited a more porous collagen fibril pattern than that at 1 day. At the same time point, the exposed collagen fibrils still showed integrity in the PVPA and CHX groups compared with the control group. Moreover, progressive remineralization of hybrid layers was observed in the combined group. The apatite

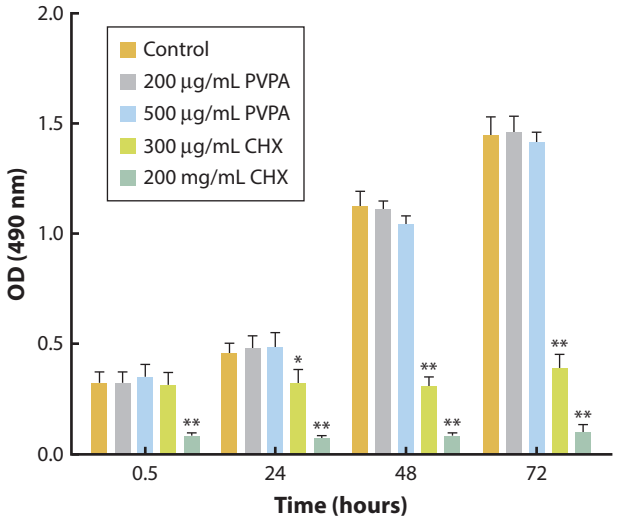

Figure 2. Effects of CHX and PVPA on cell proliferation. MTT assays showing growth curves of MDPC-23 cells after incubation with different concentrations of PVPA and CHX. *P<.05, **P<.01 as determined by the 1-way ANOVA test. CHX, chlorhexidine; MDPC-23, mouse dental papilla cell-23; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide; PVPA, polyvinylphosphonic acid.

deposited along the resin tags and gradually replaced the interfibrillar space. After 1 year of storage in the combined group, the remineralization along the resin-dentin interfaces appeared to be complete. The extensive remineralization was identified in the combined group, and the collagen fibrils were wrapped by apatite deposition, while other groups showed obvious degradation of the collagen meshwork.

For the MTT assay, incubation of MDPC-23 cells with 200 mg/mL of CHX induced a decrease in cell proliferation at each time point (Fig. 2). After 24 hours, the 300-µg/mL CHX treatment induced cytotoxicity, while the 200-µg/mL and 500-µg/mL PVPA treatment did not inhibit the cell viability of MDPC-23 when compared with the control group. The data indicated that PVPA up to 500 µg/mL was safer than CHX.

As the MTT assay confirmed that 200 mg/mL of CHX inhibited cell proliferation, the percentage of apoptotic cells was further detected by exposure to 300 µg/mL of CHX. Compared with the control group, the results showed that cell apoptosis was effectively induced by 300 µg/mL of CHX at 3 days (Fig. 3), while 200 µg/mL and 500 µg/mL of PVPA did not induce apoptosis. Taken together, these results suggested that PVPA at low concentrations displayed no cytotoxicity.

DISCUSSION

As the application of PVPA on resin-dentin bonds resulted in less reduction in bond strength after long-term storage, the first null hypothesis was rejected. However, the addition of PVPA to MDPC-23 did not have an obvious effect on cell proliferation, resulting in acceptance of the second null hypothesis.

The application of MMP inhibitors on the dentin surface as an additional step for dentin treatment could result in the reduction of bond strength loss and the improvement of restoration stability.[5] CHX has been widely used as a broad-spectrum MMP inhibitor to improve the bond strength to dentin. Nevertheless, CHX exhibited cytotoxicity to oral cells, which was consistent with the findings that CHX inhibited cell growth of MDPC-23.[19,20] Previous studies revealed that CHX pretreatment might be useful for the preservation of dentin bond strength, but the poorly resin-infiltrated hybrid layer still degraded after 1 year of aging.[14,15] In this study, 200 mg/mL of CHX, which is equal to 0.2% CHX, negatively affected the adhesive strength after long-term storage as well. Therefore, other MMP inhibitors should be used to further improve the durability of the adhesive interface over time.

PVPA inhibited endogenous MMP activities in demineralized dentin and prevented collagen degradation within hybrid layers, suggesting PVPA is an alternative to CHX to preserve the resin-dentin interfaces[25]; this is consistent with the current findings that PVPA is associated with improvement in the durability of dental bond strength (Table 1). These findings might be explained by the polychelatogenic property of PVPA, which means that PVPA interacts with Zn^{2+} ions and binds to demineralized dentin collagen by electrostatic attraction.[25,29] Bisphosphates are effective synthetic MMP inhibitors from the competitive inhibition of MMPs by chelating divalent cations.[30] PVPA, which is a long-chain polymer with multiple phosphonate groups, has the same basic structure as bisphosphates.[31] These results indicated that PVPA prevents long-term bond strength degradation mainly by inhibiting MMPs through chelation with metal ions.

Biomimetic remineralization, an approach that mimics natural biomineralization, has been reported to play a crucial role in inducing amorphous calcium phosphate nanoparticles to remineralize type I collagen and improve adhesive procedures.[7,8] The evidence suggests that the application of the protease inhibitors might be less effective in promoting dentin remineralization.[23] In a previous study, the use of biomimetic remineralized materials provided durable resin-dentin bonds.[10,11] The remineralization of hybrid layers with size-exclusion characteristics protected against molecules larger than a 40-kDa protein entering the fibril.[32,33] These prevented activated collagenase from penetrating collagen fibers and contributed to the formation of a nanoscale dentin structure.[34,35]

The present study investigated the biomimetic remineralization effect of a treatment containing PVPA on the mechanical properties of interface for a year (Fig. 1). For microtensile bond strength, the µTBS of specimens treated with biomimetic remineralized fluid was higher

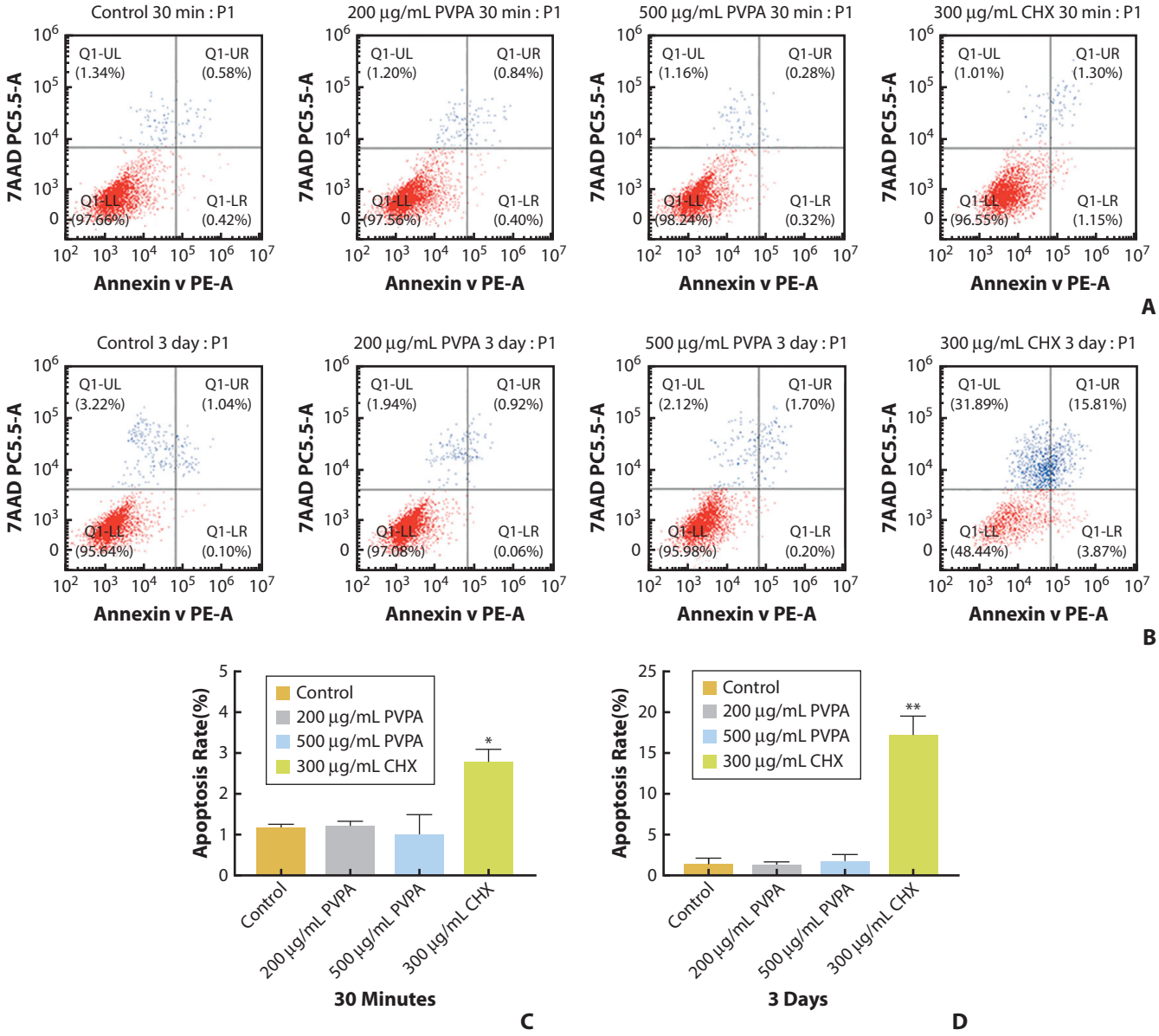

Figure 3. Effects of PVPA and CHX exposure on MDPC-23 cell apoptosis. Cells incubated with 200 μg/mL and 500 μg/mL of PVPA and 300 μg/mL of CHX. A, Incubated for 30 minutes. B, Incubated for 3 days. C, D, Percentage of apoptotic cells. *$P<.05$, **$P<.01$ as determined by 1-way ANOVA test. CHX, chlorhexidine; MDPC-23, mouse dental papilla cell-23; PVPA, polyvinylphosphonic acid.

than that observed in other groups. Identified by SEM, there was a clear presence of minerals along the resin-dentin interfaces in the combined group, while those exposed fibrils of control and CHX groups were still not mineralized and may have degraded because PVPA participated in the recruitment of amorphous calcium phosphate nanoprecursors as templates after binding electrostatically to collagen fibrils, thereby inducing the deposition of apatite nanocrystals.[26]

The cytotoxicity of CHX has restricted its application in adhesive dentistry. CHX decreased cell viability markedly within 30 minutes when the concentration was higher than 0.0003%,[18] which is equal to 300 μg/mL. However, the present study reveals a potential inhibitor of MMP and biomimetic remineralized materials without obvious cytotoxicity to MDPC-23 at a low concentration (Fig. 2). An MTT assay was used to assess cell viability with the treatment of PVPA, and the results showed that PVPA up to 500 μg/mL neither inhibits cell proliferation nor encourages apoptosis in dentin osteoblastic cells, which suggests that PVPA at that concentration has no side effect on the cell proliferation of MDPC-23 (Fig. 3).

The present study provided evidence that PVPA is a potent material in adhesive systems to improve the durability of resin-dentin bonds. However, considering the limitation that bond strength represented the

effectiveness of dentin adhesion, clinical and in vitro studies on PVPA are needed to provide additional information and determine clinical performance. Moreover, the performance of PVPA in the remineralization of caries-infected dentin and the improvement of drug-delivery strategy will be an important topic for further research.

CONCLUSIONS

Based on the findings of this in vitro study, the following conclusions were drawn:

1. PVPA at low concentrations as an MMP inhibitor and a biomimetic remineralization analog did not result in cytotoxicity.
2. The application of PVPA improved bond strength and preserved collagen integrity after 1 year of in vitro storage.
3. PVPA is a promising candidate for incorporation into dental adhesives and for clinical application.

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