Influence of polar solvents on permeability, stiffness and collagen dissociation of demineralized dentin

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ARTICLE INFO

ABSTRACT

The purpose of this in vitro study was to evaluate and compare the effect of dimethyl sulfoxide (DMSO) or ethanol on the permeability, stiffness and collagen dissociation of demineralized dentin.

Dentin cubes ($2 \times 2 \times 2$ mm) were demineralized in EDTA and incubated in DMSO or ethanol (0.01, 0.1, 1, 5, 10, 20, 50 and 100%) (n = 10/group) for 30 s, followed by 100% HEMA incubation. Extracted HEMA was quantified. For elastic modulus (E), demineralized dentin beams ($6 \times 2 \times 1$ mm) were incubated in DMSO or ethanol (1, 10, 20, 50 and 100%) for 10, 30 or 60 min at 3-point bending. Additional demineralized dentin discs (1 mm) were incubated in DMSO (1, 10, 50 and 100%) for 10, 30 and 60 min and the optical clearing effect was observed. The data were analyzed using ANOVA and Tukey's test ($\alpha = 0.05$) using SigmaPlot (Systat Software Inc., San Jose, CA).

Compared to controls, HEMA uptake was significantly higher with all DMSO concentrations, and with 0.1% or higher ethanol concentrations (p < 0.05). HEMA uptake in DMSO-incubated specimens (0.01, 5 and 10%) was significantly higher than with the ethanol incubation. Significant increase in elastic moduli was observed with 50–100% DMSO- and only with 100% ethanol after 10 min incubation. The optical clearing effect of 50–100% DMSO-incubated dentin disks was observed starting from 10 min.

The pretreatment of dentin surfaces with low concentrations of DMSO resulted in significant improvement of the penetration of monomers to demineralized dentin matrices. Increase in penetration of monomers combined with a reversible stiffening of dentin collagenous matrix may explain the previously shown increase in durability of wet- or dry-bonded adhesive interfaces with DMSO treatment.

1. Introduction

Despite the substantial improvements in formulations and adhesive technologies over the last decades, achieving a durable dentin bonding is still the main challenge in adhesive dentistry [1,2]. Resin-dentin bonding relies on the effective penetration of solvated adhesive resins into demineralized collagen matrix to create a durable hybrid layer [2]. However, replacement of residual water in acid-etched dentin is the main challenge [3] resulting in incomplete infiltration of the adhesive resin monomers into the demineralized collagen network [1,2]. Imperfect hybrid layers are prone to degradation of the resin component [4], as well as host-derived enzymatic degradation of the incompletely impregnated collagen fibrils in the presence of water [5].

Solvents are essential components of dental adhesive resins to solvate the resin monomer mixtures, decrease viscosity and increase molecular mobility [6]. Solvated adhesive application to dentin enhances the penetration of resin monomer into inter-fibrillar spaces and simultaneously facilitates the displacement of water from dentin [6,7]. Furthermore, some solvents contribute to the inhibition of the host-derived endogenous enzymatic activity in dentin [8]. Ethanol is the most commonly used solvent in commercial dental adhesives, either alone or incorporated to water [6].

During the bonding procedures, dentin surface is modified by acid-etching resulting in a thin apatite-depleted surface layer to create micromechanical spaces for retention of the restorations [9]. Moist dentin surface is required to maintain demineralized collagen matrix in expanded state for proper resin infiltration by relatively hydrophilic monomers such as hydroxyethyl methacrylate (HEMA) [9,10]. However, the diffusion of the comonomer mixtures are not without problems: it has been shown that mixtures of monomers diffuse into expanded inter-fibrillar spaces at different rates, resulting in a gradient of different monomers at the hybrid layer [11].

Dimethyl sulfoxide (DMSO) is a polar, aprotic solvent that has the ability to dissolve polar and non-polar solvents and compounds [7]. It possesses the polarity needed to break the self-association tendency of water, and displace water molecules [12]. It has been widely used in medicine because of its ability of penetrating biological surfaces [13] and in the treatment of several inflammatory diseases, interstitial cystitis even in pediatric patients [13–15].

Although DMSO was originally proposed as a potential solvent to simplify the reaction of polymerization in adhesive applications [16], only recently several *in vitro* studies have shown that DMSO pretreatment of dentin can improve the immediate and long-term dentin bond strength and lower long term nanoleakage from relatively low (0004%) to high concentrations (50%) [17–24]. Enhanced monomer penetration into the collagen matrix [18,21] has been suggested as a possible mechanism but no systematic information is available regarding the effect of DMSO on adhesive monomer penetration.

Therefore, the aim of this study was to compare the penetration of HEMA (the most widely used adhesive hydrophilic monomer) to DMSO- or ethanol-treated dentin, and to evaluate the effect of DMSO and ethanol on the stiffness of demineralized dentin. Finally, the concentration-dependence of DMSO on dentin collagen dissociation was analyzed. The null hypothesis was that DMSO or ethanol pretreatment does not affect the uptake of HEMA, the stiffness of dentin or the clearing effect on demineralized dentin differently.

2. Materials and methods

2.1. Materials

Sound human third molars extracted as a part of patient's routine dental treatment from anonymous donors and exempt from notification to the Ethics Committee according to the Finnish law (Tissue act, section 20) were used in this study. Teeth were stored in 0.9% NaCl supplemented with 0.02% sodium azide, and used within three months after extraction.

2.2. Methods

2.2.1. Effect of solvent on HEMA permeability

Fifty-five teeth were used to evaluate the HEMA (2-hydroxyethyl methacrylate, Sigma-Aldrich, St. Louis, MO, USA) uptake of dentin pretreated with either DMSO (Merck KGaA, Frankfurt, Germany) or ethanol (Berner OY, Helsinki, Finland). Dentin discs of 2 mm thickness were prepared by removing the occlusal enamel and superficial dentin from the crown and further sectioned into $2 \times 2 \times 2$ mm dentin cubes using a precision saw with a cut-off diamond blade under continuous water-cooling (Isomet, Buehler, Lake Bluff, Il, USA). Three to four dentin cubes were obtained from each tooth, to produce total of 180 dentin cubes which were randomly divided into two main groups after measuring their dimensions under the microscope. The cubes were incubated in 0.5 M EDTA (ethylene diamine tetra-acetic acid; pH 7.2) for 20 days for demineralization, washed in distilled water four times for 6 h each to remove the remnants of EDTA and the dimensions were remeasured under the microscope. Digital radiography [26] was used to confirm the absence of residual minerals.

After demineralization, dentin cubes were incubated in eight

different concentrations of DMSO or ethanol (0.01, 0.1, 1, 5, 10, 20, 50 and 100%) (n = 10/group) in shaking incubator at room temperature for 30 min. The control group cubes were incubated in distilled water. After incubation, each dentin cube was gently blot-dried and transferred into glass vials containing 2 ml of 100% HEMA to allow the HEMA diffusion for 100 min at room temperature. After HEMA diffusion, dentin cubes were blot-dried to remove the excess adherent HEMA on the surface. Each cube was then transferred into a test tube containing 2 ml of fresh distilled water to extract the HEMA in the shaking incubator. After 1 h extraction, each cube transferred to another test tube containing 2 ml of distilled water to extract the remaining HEMA. The first and second extracts were combined for HEMA analysis [28].

Spectral scan of HEMA in water was performed using a UV-spectrophotometer (Shimadzu model UV-1601, Kyoto, Japan) with UV-cuvettes (UV-Cuvettes semi-micro, Wertheim, Germany). Several spectral scans were first performed with only HEMA, and the best strength of absorption was observed at wavelength 222 nm. Therefore, this wavelength was used as a reference (λ_{max}) for all absorbances. Standard HEMA/water solutions were used to create a calibration curve to convert absorbance readings to concentrations. Extracts of HEMA in distilled water were scanned using the spectrometer and the absorption values were used to calculate the amount of HEMA in the extracts.

2.2.2. Effects of solvents on modulus of elasticity (E)

Forty-five non-carious human third molars were used to prepare dentin disks of 1 mm thickness using the precision saw (Isomet) under continuous water cooling. Disks were then fixed on a glass slab and sectioned ($6 \times 2 \times 1$ mm) to obtain a total of one hundred and twenty dentin beams. Dentin beams were demineralized following the standard protocol as described above. The initial modulus of elasticity of the beams was measured by 3-point bending at 15% strain that has been shown not to cause plastic deformation of demineralized dentin beams [29]. Digital radiography [26] and modulus of elasticity [27] were used to confirm the absence of residual minerals. Beams with an initial modulus of elasticity > 5 MPa were accepted as demineralized [27].

After initial modulus of elasticity measurements, the beams were distributed to two main groups according to the incubation solvent (DMSO or ethanol). Various concentrations of DMSO and ethanol (1, 10, 20, 50 and 100%) (n = 10/group) were used for incubation of beams for 10, 30 or 60 min prior to reevaluation. Demineralized dentin beams were placed on a three-point bending fixture with 2.5 mm distance between the lower supports while immersed in incubation solutions. The specimens were loaded using a universal test frame (AGS-10, Shimadzu Corporation, Kyoto, Japan) with a 5 N load cell (Shimadzu Corporation, Kyoto, Japan) at rate of 0.5 mm min^{-1} until a pre-set 15% strain was obtained and the load was returned immediately to zero to prevent permanent deformation of the beam by creep [29]. Calculation of E from each dentin beam was performed using the modification of the method described before [27], using the equation:

$$E = \frac{mL^3}{4bh^3}.$$

Where m is the slope of the linear portion of the load-displacement curve, L is the span length, b is the width of the test specimen and h is the beam thickness. Beams were evaluated after each incubation period, and further reassessment was performed after 24 h water incubation, to evaluate the reversibility effect of DMSO or ethanol incubation on dentin stiffness.

2.2.3. Effect of DMSO concentration on dentin collagen dissociation

The optical clearance method to analyze dentin collagen dissociation after incubation in 100% DMSO was adapted from a previous work [17]. Five sound third molars used to prepare dentin disks of 1 mm thickness from the coronal and deep dentin and demineralized as described above. Two dentin discs from different teeth (one from the coronal and one from the pulpal side) were assigned randomly per
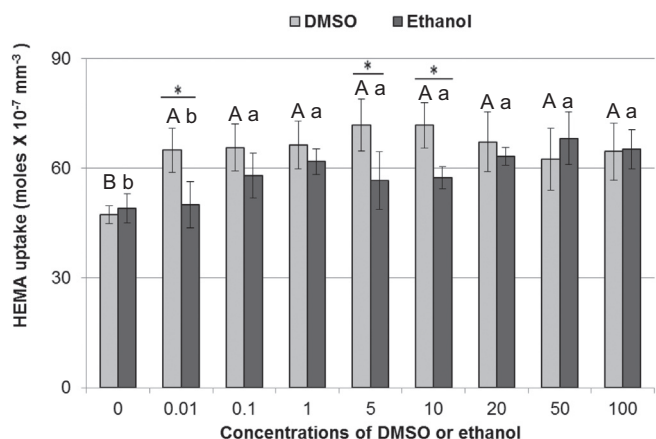
Fig. 1. HEMA uptake by demineralized dentin pretreated with different concentrations of DMSO or ethanol. Groups identified by different upper case letters for DMSO or lower case letters for ethanol indicate statistically significant differences between the concentrations within the solvent. Asterisks indicate statistically significant difference between DMSO or ethanol treated specimens at the same concentrations ($p < 0.05$).

group according to the concentration of DMSO used for incubation (1, 10, 50 and 100%) for 10, 30 and 60 min. Optical clearance of the disks was observed against a ruler.

2.2.4. Statistical analysis

The monomer uptake was evaluated using two-way analysis of variance (ANOVA). Variables were "solvent type", and "concentrations of the solvent". Modulus of elasticity data was evaluated using repeated-measures ANOVA with "pretreatment" condition and solvent type as the group variables and "time-point" as the repeated factor. Holm-Sidak test was used as a post-hoc test to evaluate the interaction and differences between the groups at $\alpha = 0.05$. All the statistical calculations were carried out using SigmaPlot Version 13.0 (Systat Software Inc. San Jose, CA).

3. Results

3.1. Effect of solvent on HEMA permeability

The results of the HEMA uptake by demineralized dentin are shown in Fig. 1. Uptake of HEMA was significantly higher with all the DMSO concentrations (mean: 62.45×10^{-7}- 71.8×10^{-7} moles mm^{-3}), compared to the control group (47.28×10^{-7} moles mm^{-3}). Ethanol pretreated groups in concentration of 0.1% or higher also showed significantly higher HEMA uptake compared to the controls. When both solvents were compared, HEMA uptake after DMSO-pretreatment was significantly increased with 0.01, 5%, and 10% solvent concentrations (Fig. 1).

3.2. Effects of solvents on modulus of elasticity

The changes in the elastic moduli (E) of the beams in time are shown in Fig. 2. The baseline mean E values ranged between 2.02 – 2.6 MPa, which was not significantly different among the test groups ($p > 0.05$). The solvent concentration and the incubation time showed significant effects on E ($p < 0.001$) and also the interaction between factors was significant ($p < 0.001$), so all the concentrations were compared to each other for the same solvent (Fig. 2) and the same was performed for each time point (Fig. 2).

An increase in E was observed starting from 20% DMSO, but the significant increase was only observed with 50–100% DMSO-treated beams from the first 10 min of incubation. Specimens treated with lower DMSO concentrations did not show significant change in E
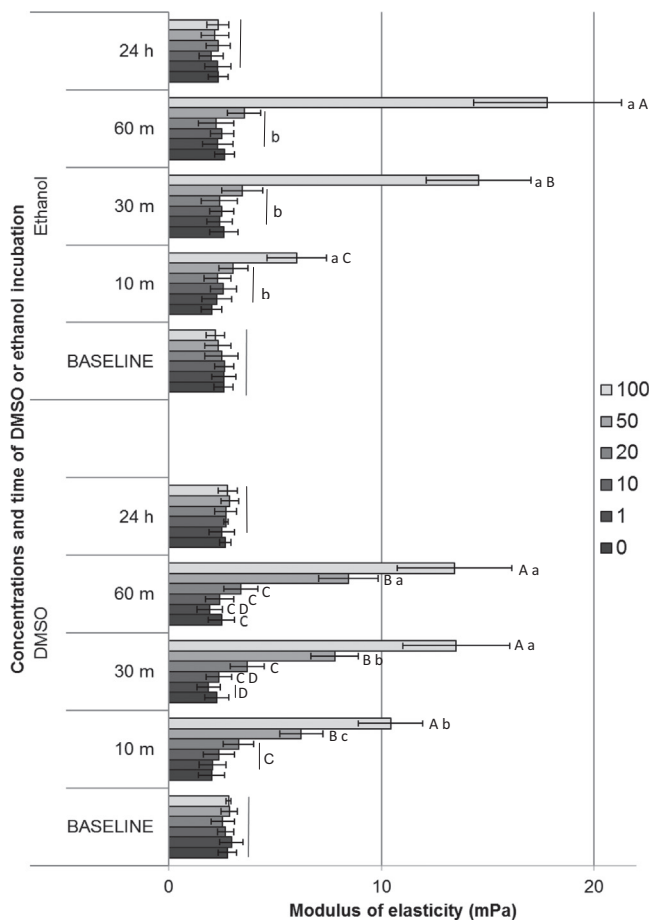

Fig. 2. Modulus of elasticity (E) of dentin beams treated with DMSO or ethanol. In DMSO-treated beams: Upper case letters indicate the significant difference between DMSO concentrations of the same time point. Lower case letters indicate the significant difference between the same concentrations in different time points within the solvent ($p < 0.05$).

(Fig. 2). Similar trend was observed also in ethanol groups, but significant increase in E was observed only with beams incubated in 100% ethanol, starting from the first 10 min of incubation. When DMSO was compared to ethanol, beams incubated in 50% DMSO showed significantly higher E values than respective ethanol concentration after 10 min incubation. However, the highest values were observed with 100% ethanol-treated beams after 60 min incubation. The effect between DMSO and ethanol was time-dependent: at 10 min, E of DMSO was significantly higher, at 30 min there were no difference between the groups, and after 60 min the ethanol-treated group had significantly higher in E compared to DMSO-treated group (Fig. 2). However, the increase in stiffness was reversible for both groups after 24 h water incubation.

3.3. Optical clearing effect

The optical clearing effect of 50–100% DMSO incubated dentin discs was observed from the first incubation time (10 min), while the effect was not clearly observed with dentin discs incubated in lower concentrations of DMSO (1 or 10%). Similar effect was also confirmed after 30, or 60 min incubation both for the discs incubated in 50 and 100% DMSO (Fig. 3).

4. Discussion

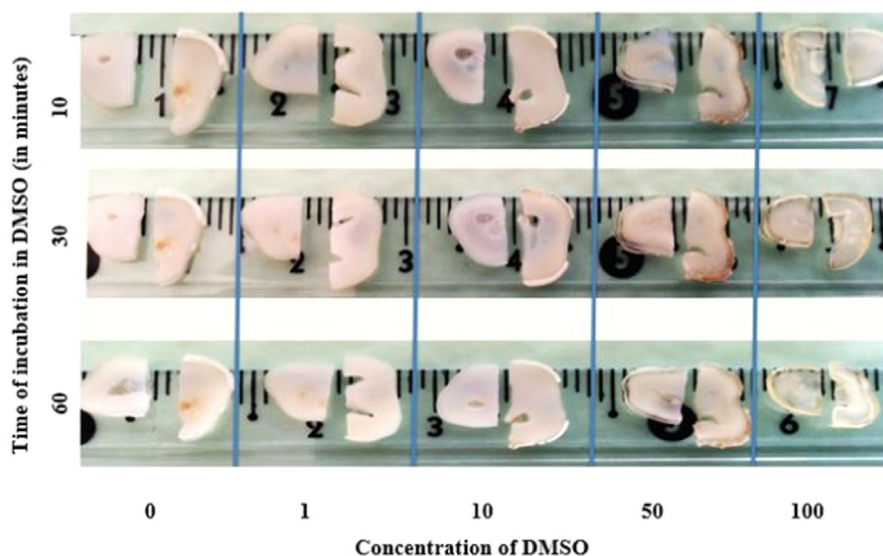The quality and efficiency of adhesive bonding to dentin is

Fig. 3. DMSO optical clearing effect on demineralized dentin discs, indicating dentin collagen dissociation. Appearance of clearing effect appeared especially with 50–100% DMSO incubated discs. No effect seen with 10% DMSO incubated discs or less.

determined by the infiltration of the adhesive monomers [1]. HEMA (2-hydroxymethyl methacrylate) is a low molecular weight hydrophilic monomer widely used in dental adhesives as an adhesion-promoter to ensure the wetting of demineralized collagen network and also as a solvent to stabilize the hydrophilic and hydrophobic monomers in the adhesive mixtures. [6,28,30]. Furthermore, HEMA does not bind to collagen [28], and for that reason it was selected as a model resin monomer to evaluate the amount of diffusion in dentin after pretreatment with different solvents. The uptake of HEMA after dentin modification by solvents is strongly dependent on the interaction between the solvents and dentin collagen [28,31–34] and indirectly reflects the volume of open dental tubules and surface absorption after surface treatment in corresponding solvent concentration [10,35].

Pretreatment of dentin cubes with different concentrations of DMSO or ethanol prior to HEMA incubation showed a significant increase in HEMA uptake compared to water-saturated demineralized dentin (Fig. 1). The increase in HEMA uptake was obvious with both solvents. Therefore, these findings allowed the rejection of the first null hypothesis. The increase in HEMA uptake ranged between 32–55% for DMSO-pretreatment and between 2–39% for ethanol pretreatment compared to only water-saturated dentin cubes. The increase in HEMA uptake with the lowest concentration of DMSO (0.01%) was 32% and a further increase up to 55% was observed with 10% DMSO treatment. DMSO's ability to increase spaces between collagen fibrils by suppressing the interpeptide hydrogen bonds within collagen fibrils [36] as well as improving dentin wettability due to lower surface tension, high dielectric constant and the equilibrium between polar and surface tension properties [25] plays a significant role in increasing the HEMA uptake in DMSO-saturated demineralized dentin. Furthermore, DMSO can lower the surface tension of water by breaking down the self-associative tendencies of water, which in turn further increases the wettability properties [12,25]. Similar to DMSO, ethanol treatment also showed an increase in HEMA uptake (Fig. 1). However, this increase was rather slow, showing only 2% increase with lowest concentration of ethanol (0.01%), which further increased up to 39% with 50% ethanol (Fig. 1). The slow increase in HEMA uptake with ethanol (0.1 -100%; Fig. 1) might be related to the dose-dependent enhancement of dentin wettability in ethanol saturated dentin environment [37].

Quantifying the changes in stiffness of demineralized dentin matrix after different treatments is important as it reflects the degree of intermolecular changes occurred. Mineralized dentin contains about 50% mineral by volume, but during acid etching for restorative procedures,

dentin surface is demineralized and the space freed from minerals are simultaneously replaced by water to maintain the interfibrillar spaces for adhesive monomers [37]. However, excess water in demineralized dentin during restorative procedures is problematic, compromising the quality of the interface [2,10]. Conversely, if water is removed from the demineralized dentin, there is rapid, spontaneous development of interpeptide hydrogen bonds between collagen peptides, resulting in the stiffening of the collagen matrix in a collapsed state and reduction of the interfibrillar spaces that serve as diffusion path for adhesive resin penetration [37]. Adhesive monomers cannot break those interpeptide hydrogen bonds, so penetration and infiltration will be compromised. This study showed that both the solvent concentration and the incubation time have significant effects on E, requiring the rejection of the second null hypothesis. Exposing the demineralized dentin samples to ethanol has previously been shown to cause stiffening in dentin due to the spontaneous formation of interpeptide hydrogen bonding [35,38,39]. In line with the previous work, current study also confirmed significant changes in elastic modulus of the demineralized dentin beams only with high concentrations of ethanol (100%) or DMSO (50–100%). Water has a very strong hydrogen bonding capacity and can hydrogen bond to carbonyl oxygen and amide nitrogen moieties in collagen peptides and prevents interpeptide hydrogen bonding between collagen peptides [37]. Ethanol can hydrogen bond less strongly to loosely-bound water around demineralized collagen matrices [3]. Therefore, low concentrations of ethanol/water solutions do not necessarily cause significant dehydration that would result with spontaneous interpeptide bonds between the collagen peptides. On the other hand, incubation with 100% ethanol can result in dehydration that increase the collagen peptide bonding and stiffness of the demineralized dentin matrix, as observed in this study. Similarly, addition of DMSO into water breaks down water self-association and could compete for hydrogen bond sites within dentin organic matrix. This was confirmed with the slight increase in stiffness as well as a slight reduction in HEMA uptake with DMSO concentrations higher than 10%. These results are also in line with a previous work showing less expansion of dentin matrix when higher concentration of DMSO was used [25].

The effect of high concentration of DMSO on the stiffness and dissociation of dentin collagen can be observed from the first treatment time (10 min) (Fig. 2 and Fig. 3). Previous work has demonstrated that the dissociation of dentin disks incubated in 100% DMSO for 30 min is reversible [17]. This effect may result from the ability of DMSO to

change the way of interaction between water molecules within the demineralized dentin [17].

Ethanol vapor pressure (43.7 mmHg at 20°C) is higher than water (17.5 mmHg) and especially of DMSO (0.417 mmHg), allowing it to evaporate easier and faster than DMSO [6]. Traditionally, evaporation of dental adhesive solvents has been regarded essential for an optimal penetration of adhesive monomers [6,7] and durability of the hybrid layer [10,30]. However, relatively low vapor pressure of DMSO might be an advantage. The presence of DMSO in collagen matrix may result in further displacement of adhesive water molecules and preservation of interfibrillar spaces for monomers even deeper inside the collagen fibrils [17,20,25] and even improve the chemical bond of functional monomers into collagen [20]. On the other hand, high vapor pressure of ethanol may induce the reversible dehydration of dentin and result in alteration of some mechanical properties such as increases in stiffness, strength and toughness [38]. The limited, provisional improvement of mechanical properties after ethanol exposure may associate with the enhancement of cross-linking between dentin collagen and inter-molecular hydrogen bonds [38,39].

The DMSO or ethanol immersion or HEMA incubation times used in this study are not clinically applicable. Unfortunately, it is not possible to quantitatively analyze the monomer penetration in 10–15 μm collagen matrix layer exposed after acid etching in a clinical situation. The size of the dentin cubes ($2 \times 2 \times 2$ mm) requires significantly longer immersion than the collagen matrix exposed in a clinical situation, since the passive diffusion of HEMA across the demineralized dentin is a slow process [28]. Respective "macro-model" setup has been successfully used in other studies where surface demineralization does not allow the planned measurements, e.g. to study the solvation and expansion of demineralized dentin by different solvents [40–42], mechanical properties of demineralized dentin with and without adhesive monomers [29,31,43] and dimensional changes of demineralized dentin after monomer penetration [44]. Further studies are needed to investigate the potential effect of DMSO- or ethanol- dentin pretreatment on the penetration of other bulkier monomers (*i e* BisGMA) into demineralized dentin cubes. Results of such study may guide toward better understanding of DMSO or ethanol interaction to dentin.

5. Conclusion

Within the limitation of this study, it can be concluded that biomodification of demineralized dentin collagen with DMSO may improve the diffusion of small-molecule hydrophilic monomers to dentin collagen. The enhancement occurs due to the ability of DMSO to displace/replace the residual water from dentin collagen, allowing dentin collagen interfibrillar spaces to be occupied by monomers.

Conflict of interest

The authors declare that they have no conflict of interest.

Funding

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