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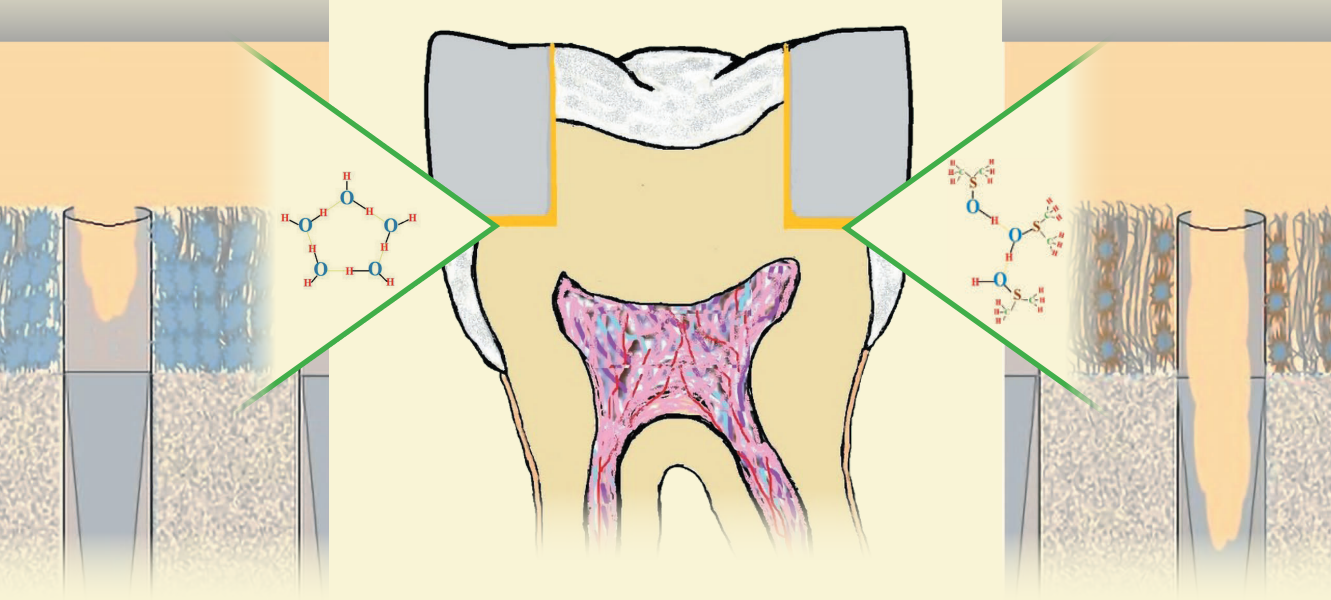

TOWARDS ENHANCING THE DURABILITY AND STRENGTH OF DENTIN-RESIN BONDS: THE ROLE OF DIMETHYL SULFOXIDE (DMSO) AS AN ALTERNATIVE SOLVENT IN DENTAL ADHESIVES

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"In the name of God, the Most Gracious, the Most Merciful"

﴿الحَمْدُ لِلَّهِ الَّذِي لَهُ مَا فِي السَّمَاوَاتِ وَمَا فِي الأَرْضِ وَلَهُ الْحَمْدُ فِي الآخِرَةِ وَهُوَ الْحَكِيمُ الْخَبِيرُ﴾

الحمد لله الذي ما تـم جهد ولا ختـم سعي الا بكرمه ، وما تخطى العبد من عقبات الا بتوفيقه ومعونتـه
لك الحمد يارب بعدد ما سبح الملائكة الحافين حول عرشك وبعدد ما سبح من شيء بحمدك..
ولك الحمد كما ينبغي لجلال وجهك وعظيم سلطانك .. سبحانك لا نحصي ثناء عليك انت كما اثنيت على نفسك...
فلك الحمد في الاولى والاخرة ولك الحمد حتى ترضى ولك الحمد اذا رضيت ولك الحمد بعد الرضى ولا حول ولا قوة الا بك.

"All praise is to Allah, to whom belongs whatever is in the heavens and whatever is in the earth, and to Him belongs all praise in the Hereafter. And He is the Wise, the Acquainted."

.....

If roses grow in heaven,
Lord please pick a bunch for me,
Place them in my Mother's arms
and tell her they're from me.

Tell her I love her and miss her,
and when she turns to smile,
place a kiss upon her cheek
and hold her for awhile.
Because remembering her is easy,
I do it every day,
but there's an ache within my heart
that will never go away.

Dolores M. Garcia

To my late mother,
Father,
And lovely family

UNIVERSITY OF TURKU
Faculty of Medicine
Institute of Dentistry
Cariology and Restorative Dentistry
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ABSTRACT

One of the main goals in adhesive dentistry is the preservation of the hybrid layer, a unique biological composite layer, formed by the impregnation of collagen fibrils in the dentin structure with adhesive resin. Different adhesive strategies have been used to achieve this.

One strategy focuses on the inhibition of endogenous protease activity, and the other strategy on improving the penetration and impregnation of the adhesive monomers in demineralized dentin.

Dimethyl sulfoxide (DMSO; $(CH3)_2SO$) is a polar aprotic solvent which dissolves polar and nonpolar compounds. It has the ability to penetrate biological tissues and has been used to solvate dental resin monomers. It has recently been suggested to improve the durability and longevity of bonding, by enhancing the penetration of resin monomers in dentin.

Four studies were designed to evaluate the impact of DMSO on the durability of resin-dentin bonding, to evaluate the effects of incorporating DMSO into experimental adhesives with different hydrophilicities on mechanical and physical properties, as well as the biological effects on cells. The aim of this series of studies is to evaluate the effect and mechanism of action of DMSO on resin-dentin bonding, to find one optimal concentration or range of concentrations of DMSO that can be safely incorporated into resin adhesive systems to improve the integrity and stability of bonding to dentin.

Results of these studies showed that pre-treating dentin with low DMSO concentrations (1–5 vol. %) preserve the integrity of adhesion and enhance the permeability of small-molecule monomers in dentin. Results also showed that incorporation of 1 w/w % or less DMSO to adhesive did not impair the mechanical and physical properties of hydrophobic and hydrophilic adhesives. Results also showed that incorporation of DMSO into hydrophobic adhesive did not increase the cytotoxicity, while 1 w/w % and more DMSO incorporation into hydrophilic adhesive showed an increase of cytotoxic effects.

These results suggested that when DMSO (1–5 vol. %) used as dentin- pretreatment, it improves the durability and quality of resin-dentin bonding. Results also suggested that the addition of DMSO to hydrophobic and hydrophilic adhesives (up to 1 % w/w), did not negatively affect their physical or mechanical properties. Addition of DMSO (up to 10 % w/w) to hydrophobic or hydrophilic adhesives did not increase the cytotoxicity from eluates, while the addition of DMSO (1 w/w %) to hydrophilic resin caused an increase in the transdentinal cytotoxic effects.

KEYWORDS: dentin collagen, dimethyl sulfoxide, ethanol, water, degradation, adhesive resin, resin monomers, hydrophilicity, mechanical/physical properties, cytotoxicity.

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ANAS AAQEL SALIM, SALIM AL-ANI: Dentiinisidoksen kestävyyden ja sidoslujuuden lisäys: dimetyylisulfoksidin (DMSO) merkitys vaihtoehtoisena liuottimena sidosaineissa
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TIIVISTELMÄ

Yksi liimahammashoidon päätavoitteista on hybridikerroksen, ainutlaatuisen biologisen yhdistelmäkerroksen, säilyttäminen. Kerros muodostuu, kun dentiinirakenteen kollageenisäikeet kyllästetään liimah

artsilla. Tämän saavuttamiseksi on käytetty erilaisia liimausmenetelmiä. Tämän ongelman ratkaisemiseksi tutkijat ovat päätyneet kahteen erilaiseen strategiaan tarttumisen kestävyyden parantamiseksi. Yksi strategia keskittyy endogeenisen proteaasin aktiivisuuden estämiseen, ja toinen adhesiivisten monomeerien parempaan tunkeutumiseen demineralisoituun dentiiniin ja sen kyllästämiseen.

Dimetyylisulfoksidi (DMSO; $(CH3)_2SO$) on polaarinen aproottinen liuotin, joka liuottaa polaarisia ja ei-polaarisia yhdisteitä. DMSO:lla on kyky tunkeutua biologisiin pintoihin. DMSO: ta on käytetty myös erilaisten hartsimonomeerien liuottamiseen. DMSO:n on äskettäin ehdotettu parantavan sidoksen kestävyyttä ja pitkäikäisyyttä edistämällä hartsimonomeerien tunkeutumista dentiiniin.

Neljä tutkimusta suunniteltiin selvittämään DMSO:n vaikutusta hartsin ja dentiinin välisen sidoksen kestävyyteen. DMSO lisättiin kokeellisiin liimoihin, joilla oli erilaiset hydrofiilisyydet, ja tutkinttiin lisäyksen vaikutusta liimojen mekaanisiin ja fysikaalisiin ominaisuuksiin ja sytotoksisuuteen.

Tämän tutkimussarjan tarkoituksena oli arvioida DMSO:n vaikutusta ja vaikutusmekanismeja hartsi-dentiini-sidokseen, löytää optimaalinen DMSO-konsentraatio, joka voidaan sisällyttää turvallisesti hartsiliimajärjestelmiin parantamaan sitoutumista dentiiniin.

Näiden tutkimusten tulokset osoittivat, että dentiinin esikäsittely matalilla DMSO-konsentraatioilla (1–5%) säilyttää tarttumisen kestävyyden ja parantaa dentiinin läpäisevyyttä. Tulokset osoittivat myös, että 1 paino-% tai vähemmän DMSO:ta liimoissa ei heikentänyt hydrofobisten ja hydrofiilisten liimojen laatua. Tulokset osoittivat myös, että DMSO: n sisällyttäminen hydrofobiseen liimaan ei lisännyt sen sytotoksisuutta, kun taas 1 paino-% ja enemmän DMSO:ta hydrofiilisessä liimassa lisäsi sytotoksisia vaikutuksia.

Nämä tulokset viittaavat siihen, että kun 1–5% DMSO:ta käytetään dentiinin esikäsittelyyn, se parantaa hartsin ja dentiinin sitoutumisen kestävyyttä ja laatua. Tulokset viittaavat myös siihen, että DMSO:n lisääminen liimoihin (korkeintaan 1 paino-%) ei vaikuttanut negatiivisesti niiden fysikaalisiin tai mekaanisiin ominaisuuksiin. DMSO:n lisääminen hydrofobiseen liima-aineeseen ei lisännyt sytotoksisuutta, kun taas 1 paino-%:n ja enemmän lisäminen sytotoksisuutta.

AVAINSANAT: dentaalinen kollageeni, dimetyylisulfoksidi, etanoli, vesi, hajoaminen, tarttuva hartsi, hartsimonomeerit, hydrofiilisyydet, mekaaniset / fysikaaliset ominaisuudet, sytotoksisuus.

Table of Contents

Abbreviations

µg	Micro gram
µL	Micro liter
µm	Micrometer
µTBS	Microtensile bond strength
2MP	Bis [2-(methacryloyloxy) ethyl] phosphate
$AgNO_3$	Silver nitrate
AL	Adhesive layer
ANOVA	Analysis of variance
BisGMA	Bisphenol A glycidyl methacrylate
C	Carbon
CCs	Cysteine cathepsins
CD	Cohesive failure in dentine
CHX	Chlorhexidine
cm	Centimeter
CQ	Camphorquinone
CR	Cohesive failure in composite resin
DC	Degree of conversion
DDM	Demineralized dentin matrix
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
E & R	Etch-and-rinse
E	Elastic moduli
ECM	Extra cellular matrix
EDMAB	Ethyl N, N-dimethyl-4-aminobenzoate
EDTA	Ethylenediaminetetraaceticacid
FTIR	Fourier transform infrared spectroscopy
g mol^{-1}	Gram per mole
h	Hours
H_2O	Water
HEMA	2-hydroxy ethyl methacrylate
HL	Hybrid layer

ISO	International standards organization
J/cm^3	Joules per cubic centimeter
M	Molarity
MEHQ	Monomethyl-ether-hydroquinone
MF	Mixed failure
mm	Millimeter
mm^2	Square millimeter
mmHg	Millimeter of mercury
MMPs	Matrix metalloproteinase
MPa	Mega Pascals
n	Number
N	Newton
pH	Power of hydrogen
SEM	Scanning electron microscope
SiC	Silicon carbide
ß	Beta
TEGDMA	Triethylene-glycol dimethacrylate
UV	Ultraviolet
v/v %	Volume per volume percentage
Vol. %	Volume percentage
w/w %	Weight per weight percent
Wsp	Water sorption
Wsu	Water solubility
Wt. %	Weight %
α	Alpha

List of Original Publications

This thesis is based on the following original publications, which is referred to in the text by the Roman numerals I to IV.

I. **Anas Aaqel Salim, Salim Al-Ani**, Murat Mutluay, Thiago Stape, Leo Tjäderhane, Arzu Tezvergil-Mutluay (2018). Effect of various dimethyl sulfoxide concentrations on the durability of dentin bonding and hybrid layer quality. *Dental Materials Journal*, *37*, 501–505.

II. **Anas Aaqel Salim, Salim Al-Ani,** Murat Mutluay, Leo Tjäderhane, Arzu Tezvergil-Mutluay, (2019). Influence of polar solvents on permeability, stiffness and collagen dissociation of demineralized dentin. *International Journal of Adhesion and Adhesives*, *89*, 148–153.

III. **Anas Aaqel Salim, Salim Al-Ani,** Thiago Stape, Murat Mutluay, Leo Tjäderhane, Arzu Tezvergil-Mutluay (2019). Incorporation of dimethyl sulfoxide to model adhesive resins with different hydrophilicities: Physico/mechanical properties. *Journal of the Mechanical Behavior of Biomedical Materials*, *93*, 143–150.

IV. **Anas Aaqel Salim, Salim Al-Ani,** Ikram Aqel Salim, Roda Seseogullari Dirihan, Murat Mutluay, Leo Tjäderhane, Arzu Tezvergil-Mutluay. Incorporation of dimethyl sulfoxide into model adhesive resins: evaluation of cytotoxic activities. *Manuscript submitted to Journal of Dentistry.*

1 Introduction

Dental caries remains one of the most widespread chronic infectious diseases in the world, despite significant advances in prevention over past decades (Kassebaum *et al.*, 2015). In the last decades, depending on the extension of the carious lesion, tooth-coloured dental composites along with the adhesive techniques have formed the standard treatment for the replacement of tissue loss resulting from carious lesions (Shenoy *et al.,* 2008; Perdigão *et al.,* 2009). The use of dental composites is still expected to increase due to the toxicity of mercury released from amalgam (Maserejian *et al.*, 2012; Rodríguez-Farre *et al.*, 2016), and legislation restricting the manufacture and disposal of mercury-containing materials (Fisher *et al.*, 2018).

The ultimate goal of adhesive procedures is to achieve a good, long-lasting seal between restoration and tooth structure, through surface modification and micromechanical retention (Söderholm, 2007; Tezvergil-Mutluay *et al.,* 2015b). This goal has been successfully achieved in enamel (Van Meerbeek *et al.,* 2008), as confirmed by *in vitro* and short-term studies (Walls *et al.,* 2001; De Munck *et al.,* 2005; Van Meerbeek *et al.,* 2008; Kimmes *et al.,* 2010), as well as long-term studies (Loguercio *et al.,* 2008; Reis *et al.,* 2009). As opposed to successful enamel bonding, the breakdown of dentin-resin bonds is a well-known issue (Dahl & Stenhagen, 2018; Spencer *et al.,* 2010).

In addition to the complexity of dentin as a bonding substrate, significant progress has been made in understanding the additional mechanisms that lead to failure of resin-dentin bonds over time. These include the degrading effect of water (Tjäderhane *et al.*, 2013b; Breschi *et al.*, 2018), and salivary esterases (Bourbia *et al.*, 2013; Huang *et al.*, 2018) on the adhesive resin part of the interface. This also includes host-derived degradation of dentin matrix collagen due to host-derived enzymatic activity of matrix metalloproteinase (MMPs) and cysteine cathepsins (CCs) in demineralized dentin matrices (Pashley *et al.*, 2004; Mazzoni *et al.*, 2006; Tjäderhane *et al.,* 2015; Nascimento *et al.,* 2011).

Different approaches to improving the durability of adhesives have been presented, including host-derived enzyme inhibition (Frassetto *et al.*, 2016), different resin chemistry (Bedran-Russo *et al.*, 2014), and switching from the commonly used hydrophilic monomers toward more hydrophobic ones, or by step-

wise dehydration of dental tissues using ethanol-wet bonding techniques (Sadek *et al.*, 2010). Even though these approaches proved to enhance durability in *in vitro* studies, most of the strategies were not well accepted by clinicians because of the additional steps and time required for these applications (Bedran-Russo *et al.*, 2017). Therefore, contemporary dental adhesive systems are still under development to optimize bonding to dentin (Jandt *et al.,* 2009; Peumans *et al.*, 2014).

The use of dimethyl sulfoxide DMSO as pre-treatment for dentin surfaces was recently suggested as a new strategy for increasing the bond strength to dentin (Stape *et al.,* 2016a; Tjäderhane *et al.,* 2013c). However, little is known at this point about the interaction of DMSO with adhesive components or biological tissues. Therefore, in this series of studies, the aim was to systematically evaluate the interaction of DMSO with dentin, or experimental adhesive systems with hydrophobic and hydrophilic properties. An additional aim was to evaluate the biocompatibility of DMSO-modified adhesives in biological systems.

2 Review of the Literature

2.1 Microstructure of enamel and dentin

Enamel and dentin are the outer tooth surfaces, resisting and encountering bacterial invasion. They work as the protective layers of the pulp. Enamel microstructure is homogenous compared to that of dentin and composed mainly of inorganic components (around 94–96 wt. %), while organic components, and water make up 1–5 wt. % (Hueb De Menezes Oliveira *et al.*, 2010). Dentin is the second layer of the tooth, below the enamel layer. It is formed during tooth formation by odontoblast cells, presented in the pulp. It has a complex inhomogeneous microstructure. It is composed of extracellular organic matrix (20–33 weight %), inorganic minerals (≥ 70 weight %), and water (≥ 10 mass %) (Tjäderhane *et al.*, 2009). Most of the organic components of dentin consist of collagen (≥ 90 weight %), while the remainder are non-collagenous proteins (Tjäderhane *et al.*, 2009). Dentin contains millions of dentinal tubules containing dentinal fluid that freely move and diffuse between dentin and pulp tissue (Pashley *et al.,* 1996). Dentinal fluid is basically free, unbound water located within the dentinal tubules of dentin. It moves freely from dentin to pulp as a physiological response to thermal, osmotic stimuli across dentin (Pashley *et al.,* 1996; Tjäderhane *et al.*, 2009). Dentin is considered either as a barrier to external irritants, or as permeable structure, depending on its thickness and age (Pashley *et al.,* 1996; Tjäderhane *et al.*, 2009).

Progressive demineralization during dentin caries dramatically changes the mechanical properties of dentin, increases porosity, and results in changes in collagen structure (Marshall *et al.*, 2001; Zavgorodniy *et al.,* 2008). Bonding to caries dentin is also difficult to achieve, and the immediate bond strengths are usually 20–50% lower than bond strength to sound dentin (Perdigão *et al.,* 2010; Cardoso *et al.*, 2011; Tjäderhane *et al.,* 2015; Ekambaram *et al.,* 2015b).

2.2 Contemporary dental adhesives

Resin-based adhesives are “one-bottle” or “multi-bottle” system low-viscosity materials whose formulations contain a complex mixture of hydrophobic and hydrophilic monomers, as well as solvents, initiators, and inhibitors (Vaidyanathan

et al., 2009; Manuja *et al.,* 2012). They are used as an intermediate adherent layer between tooth structure and restorative materials (Van Landuyt *et al.*, 2007).

Generally, adhesive systems are classified into two main systems as etch-and-rinse or self-etch adhesives, according to the steps of application, and the presence or absence of an acid-etching step (De Munck *et al.,* 2005; Van Landuyt *et al.,* 2007). In the etch-and-rinse system, adhesives are applied after demineralization of the superficial layer of exposed dentin to reveal collagen fibrils and the opening of dentinal tubules, using 35–37% phosphoric acid (H_3PO_4) (Pashley *et al.*, 2011). To simplify the clinical procedure, researchers successfully divided the restorative procedures into two subgroups, either three- step or two- step etch- and -rinse systems. These subgroups differ in the number of consecutive steps of application and the number of bottles containing primer and adhesives (Pashley *et al.*, 2011; De Munck *et al.*, 2005; Van Landuyt *et al.*, 2007).

Self-etch adhesives, also known as etch-and-dry systems, do not have a separate acid-etching step, and dentin surface modification for micromechanical retention is achieved by the acidic resin monomers presented as active components in self-etch adhesives (Van Meerbeek *et al.,* 2011). Self-etch adhesive systems are also further classified into two subgroups, according to the number of consecutive steps of application, as two-step self-etch adhesive or one-step self-etch adhesive systems (Van Meerbeek *et al.,* 2011).

The smear layer is a thin layer (1–2 μm) of loosely attached cutting debris composed of hydroxyapatites, denatured collagen, and bacteria on the tooth surface, produced during the cavity preparation step of restorative procedures (Pashley *et al.,* 1981; Pashley *et al.,* 1993). The smear layer constitutes an unstable barrier for adhesive bonding and is either removed during the acid-etching step of etch-and rinse adhesives (Pashley *et al.,* 1981; Grégoire *et al.,* 2003; Van Meerbeek *et al.,* 2003), or modified and impregnated with resin when using self-etch adhesive systems (Aguilar-Mendoza *et al.,* 2008; Thanatvarakorn *et al.,* 2018).

Currently, the trend is toward simpler and fewer-step systems, as in the two-step etch-and-rinse system, or even one-step self-etch systems. However, in theory, several aspects need to be considered when selecting the proper adhesive system, especially in terms of accuracy and durability of bonding (Frankenberger and Tay, 2005; Van Landuyt *et al.,* 2009; Masarwa *et al.,* 2016). Despite continued developments, satisfactory clinical outcomes of resin-based restorative procedures were well-maintained with three-step etch-and-rinse or two-step-self etch adhesive systems, rather than with more simplified systems (Cardoso *et al.,* 2011).

2.3 Composition of dental adhesives

The main components of dental adhesives are monomers, initiator system, solvents, fillers, and inhibitors (Van Landuyt *et al.,* 2007).

2.3.1 Resin monomers

Resin monomers are a main component of adhesive systems and resin-based composites (Van Landuyt *et al.,* 2007). Monomers are usually in a liquid form when placed in the mixture of adhesives and hardened after photo-polymerization (Peutzfeldt *et al.,* 1997). They are classified into two main categories, functional monomers and cross-linker monomers (Van Landuyt *et al.,* 2007). Functional monomers typically have one polymerizable group, whereas cross-linker have two polymerizable groups. Functional monomers are typically hydrophilic in nature and contain a functional group that may enhance the wetting or demineralization of dentin. Common functional groups are phosphate, carboxylic acid, and alcohol groups. Crosslinkers will form crosslinked polymers whereas functional monomers will form linear polymers that show lower mechanical properties and are prone to faster hydrolytic degradation compared to crosslinked polymers (Van Landuyt *et al.,* 2007).

Many different monomers have been used in dentin adhesives. In three-step etch-and-rinse or two-step self-etch adhesives, hydrophilic monomers such as hydroxyethylmethacrylate (HEMA) are added to the primers, while hydrophobic cross-linkers such as BISGMA, UDMA, TEGDMA monomers, are added to adhesive systems (Van Landuyt *et al.,* 2007).

Monomers are incorporated into adhesive systems in specific concentrations. Their properties differ, especially their molecular weight (100–580 g/mol), as well as their molar concentration (0.3–5 mol/L) (Nishitani *et al.,* 2006). This reflects the behavior and hydrophilicity of the final resin mixture (Park *et al.,* 2011). Following the acid-etching step of the restorative procedure, water usually replaces the empty spaces of removed minerals (Pashley *et al.*, 2011). Therefore, hydrophilic monomers are needed to optimize the interaction with the water-saturated collagen fibrils (Nishitani *et al.*, 2006). However, differences in the molecular weight and molar concentrations of resin monomers make a complete replacement of water in dentin difficult (Nishitani *et al.,* 2006).

2.3.2 Fillers

Fillers are not always a part of adhesive systems, but in low amounts can be used to increase the mechanical properties of the adhesive layer (Van Landuyt *et al.,* 2007; Kiran *et al.*, 2018). They are also important in preventing the over-thinning of the adhesive layer (Miyazaki *et al.*, 1995; Nunes *et al.,* 2001). In addition, they help to

reduce the shrinkage stresses produced during curing and provide radio-opacity (Van Landuyt *et al.*, 2007). The filler in most adhesive resins consists of silicon dioxide glass particles manufactured in different sizes (Van Landuyt *et al.*, 2007). Furthermore, reactive silicate glasses are added with the intention of releasing ions. Their beneficial effects, however, are not well established. These fillers are usually silanized to improve adhesion between the filler particles and resin matrix (Van Landuyt *et al.*, 2007).

2.3.3 Initiators and initiator systems

Initiators are also an essential part of each adhesive system, because all adhesive materials should be efficiently cured prior to the application of resin composite. It is important to achieve an acceptable degree of conversion and mechanical stability in the adhesive layer (Yoshida *et al.,* 1994; Van Landuyt *et al.*, 2007). There are two types of initiators: the photo-initiator system and the chemical initiator system (Van Landuyt *et al.,* 2007). Photo-initiators are the most commonly used initiator system in adhesive dentistry. They are incorporated into adhesives at low percentage (0.1–1 % w/w) to initiate polymerization of resin adhesive monomers together through the absorption of light for the appropriate time and at a specific, sufficient intensity of wavelength (Van Landuyt *et al.,* 2007). They should be light-activated before the application of resin composite, for two reasons; first, to obtain proper mechanical properties of adhesive (Yoshida *et al.,* 1994; Van Landuyt *et al.*, 2007), and second, to ensure the production of a thin layer of adhesive prior to composite application (Van Landuyt *et al.,* 2007, Bae *et al.,* 2005). Polymerization occurs when the free radicals of initiator molecules initiate the polymerization reaction, under light stimulation in the case of photo-polymerization (Van Landuyt *et al.,* 2007).

Camphorquinone (CQ) is the most popular photo-initiator used in adhesives, either alone or in combination with a co-initiator (*i.e.* amine) (Van Landuyt *et al.,* 2007). Absorption of light by CQ at wavelengths of 400–550 nm causes activation of amine co-initiators to produce the free radicals needed for polymerization. This process is very fast and enough to form polymerization of adhesive resin components (Talungchit *et al.,* 2012). Other photo-initiators include diketone 1-phenyl-1,2 propanedione (PPD) and acylphosphine oxides (Van Landuyt *et al.,* 2007). PPD has two advantages over CQ, in that it is a yellow and viscous fluid at room temperature, which allows better compatibility with resin mixture (Park *et al.,* 1999). In addition, the presence of PPD in the polymer resulted in higher mechanical strength and better polymerization efficiency (Park *et al.,* 1999; Van Landuyt *et al.*, 2007; Park *et al.*, 2011). Acylphosphine oxides as photo-initiators on the other hand, are less suitable for water-containing adhesives (Moszner *et al.,* 2005; Van Landuyt *et al.*, 2007).

2.3.4 Solvents in dental adhesives

Solvents are essential component in dental adhesive systems. Their function in hydrated dentin is to eliminate water molecules prior to curing of resin adhesive, without collapse of collagen fibrils (Van Landuyt *et al.,* 2007). Solvents are also needed to facilitate the penetration of hydrophilic, small-molecule resin monomers into the collagen meshwork of demineralized dentin (Ekambaram *et al.,* 2015a). Furthermore, solvents are included in adhesives systems to dissolve and reduce the viscosity of monomers, which result in simplifying transportation of monomers into demineralized collagen fibrils (Van Landuyt *et al.,* 2007).

The polarity of solvents is an important chemical property, because it determines a solvent's chemical interaction with surrounding molecules (Nalla *et al.*, 2005; Armstrong *et al.*, 2008). Accordingly, solvents are classified into three categories, according to polarity: polar protic, dipolar aprotic, and apolar (Van Landuyt *et al.,* 2007).

Currently, commercial dental adhesives contain one solvent or two co-solvents in different percentages (Perdigão *et al.,* 2001; Ekambaram *et al.,* 2015a). Most commonly used solvents in dental adhesives include ethanol, water, and acetone (Van Landuyt *et al.,* 2007; Ekambaram *et al.,* 2015a). Other less common solvents are also incorporated into dental adhesive systems (Van Landuyt *et al.,* 2007).

In order to simplify the application steps of adhesive, manufacturers combine more than one solvent with adhesive resin monomers (Cardoso *et al.,* 2011). The appropriate storage and handling of the solvent/resin homogenous composition is a very important issue to consider. Improper handling and storage may influence the stability of mixture and result in mixtures with improper properties that may lead to failures of the restorative procedures (Perdigao *et al.,* 1999; Abate *et al.,* 2000; Lima *et al.*, 2005).

2.3.4.1 Water

Water (H_2O) is a strong polar solvent that can dissolve many other polar solvents (Van Landuyt *et al.,* 2007). It is able to form strong H-bonding; however, it is not efficient by itself at dissolving monomers. In dental materials, it is therefore, combined with another solvent (co-solvent) (Van Landuyt *et al.,* 2007; Manso *et al.,* 2008; Talungchit *et al.,* 2012). Two main chemical properties of water as a solvent control its behavior in the collagen of dentin, namely vapor pressure and boiling temperature (**Table 1**). The low vapor pressure of water makes it almost impossible to remove from hydrated dentin, which may negatively affect the polymerization and quality of the resulting hybrid layer (Jacobsen et al, 1995; Tay, Spencer *et al.,* 2002).

2.3.4.2 Ethanol

Ethanol (C_2H_6O) is a commonly incorporated solvent in dental adhesives (Ekambaram *et al.,* 2015a). It is an example of a polar protic solvent that efficiently forms a bond to water, since it has a hydroxyl-group needed to produce strong hydrogen bonds (Van Landuyt *et al.,* 2007). Its vapor pressure is 40 mmHg, which is higher than that of water (17 mmHg). It therefore evaporates more easily when air pressure is applied **(Table 1)**.

Ethanol is incorporated into dental adhesives either by itself or with water (Van Landuyt *et al.*, 2007; Ekambaram *et al.,* 2015a). The addition of ethanol to adhesive systems is performed to enhance monomer infiltration into collagen fibrils, enhance the free movement of radicals within the polymer chain of resin adhesive, and reduce the viscosity of adhesive mixtures (Cadenaro *et al.*, 2009a; Faria-E-Silva *et al.*, 2013; Ekambaram *et al.,* 2015a; Jee *et al.*, 2016).

2.3.4.3 Acetone

Acetone is a polar aprotic solvent and does not contain the hydroxyl-group needed to produce a hydrogen bond (**Table 1**). It has only large a dipole group (Van Landuyt *et al.,* 2007). Two main problems are associated with acetone: high vapor pressure and weak H-bond to water in dentin (Van Landuyt *et al.*, 2007; Ekambaram *et al.,* 2015a). As a result, rapid acetone evaporation frequently occurs following the application of adhesive, and there is a high chance of shrinkage of collagen fibrils prior to polymerization of the adhesive layer (Cho *et al.,* 2004; Ekambaram *et al.,* 2015a; Sousa Júnior *et al.*, 2015).

2.3.4.4 Other less common solvents used in adhesive systems

There are also a number of other solvents used in adhesive formulations. Examples include 2-propanol (Ekambaram *et al.,* 2015a), tert-butanol (Ekambaram *et al.,* 2015a), tetrahydrofuran (THF) (Van Landuyt *et al.*, 2007; Fontes *et al.*, 2009; Ekambaram *et al.,* 2015a), and certain other alcohols (Van Landuyt *et al.*, 2007; Ekambaram *et al.,* 2015a; Tezvergil-Mutluay *et al.,* 2011a). Each of these solvents can be used either alone or with a co-solvent in adhesive systems (Ekambaram *et al.,* 2015a). Alternative solvents were investigated in order to overcome the disadvantages of commonly used solvents incorporated into adhesives. One example of a recent *in vitro* investigation of an alternative solvent to replace currently used solvent systems is tetrahydrofuran (Fontes *et al.*, 2009, 2013). It is a polar aprotic solvent with a high vapor pressure (173 mmHg) able to dissolve many other components (Ekambaram *et al.,* 2015a). It showed improvement of bonding both

immediately and after 1 year of storage (Fontes *et al.*, 2009, 2013). However, concerns were reported related to intermediate cytotoxicity (Fontes *et al.*, 2013), as well as the high vapor pressure of tetrahydrofuran (173 mmHg) which is very close to acetone (178 mmHg) (Ekambaram *et al.,* 2015a). The high vapor pressure might result in a fast, uncontrolled rate of solvent evaporation from collagen when applied as a primer, leading to shrinkage of collagen fibrils prior to adhesive application. Furthermore, when incorporating tetrahydrofuran into adhesive resin, the content of the adhesive bottle may be potentially unstable, especially with multiple usage times of the adhesive bottle. This may result in an inhomogeneous mixture of tetrahydrofuran/resin with compromised physical and mechanical properties, which in turn may negatively affect the integrity and stability of resin-dentin bonding (Perdigao *et al.,* 1999).

2.4 Dimethyl sulfoxide (DMSO)

Dimethyl sulfoxide (DMSO; $(CH_3)_2SO$) is a organosulfur, colorless, dipolar aprotic solvent, derived from wood pulp as by-product. It has a small amphiphilic molecule chemically composed of a hydrophilic sulfoxide group and two hydrophobic methyl groups (**Table 1**) (Guillory *et al.,* 2007). The efficiency of DMSO as a solvent for water-insoluble compounds and its capability to dissolve most of other solvents, including polar and non-polar compounds, are due to its physicochemical properties (Ruiz-Delgado *et al.*, 2009). Details about this solvent are described subsequently.

2.4.1 Pharmacological effects of DMSO

There are several documented pharmacological effects of the chemical solvent DMSO. They include: a) penetration of different biological membranes; b) anti-inflammatory effect; c) analgesic effect; d) enhancement of delivery of specific drugs; e) bacteriostatic effect; f) diuretic effect; g) solvation of collagen; h) enhancement of infection resistance; and i) vasodilation (Jacob *et al.,* 1967; Brayton *et al.,* 1986; Santos *et al.*, 2003).

The slow and reversible penetration- ability through different permeable or semi-permeable biological membranes is a unique characteristic of DMSO (David *et al.,* 1972; Anchordoguy *et al.*, 1992; Santos *et al.*, 2003). This also includes cells, without destroying the structural contents (Greve *et al.,* 2008; Marren *et al.,* 2011). Furthermore, DMSO is an accepted solvent in medicine because of its known capability to penetrate biological tissues rapidly and efficiently (Swanson *et al.,* 1985). Moreover, in up to 50% concentration is safe to use in clinical practice to treat certain inflammatory diseases, interstitial cystitis (Parkin *et al.,* 1997) and knee osteoarthritis (Rosenstein *et al.,* 1999; Simon *et al.*, 2009). DMSO also has numerous

applications in cell biology, cell fusion (Ahkong *et al.*, 1975), and differentiation (Lyman *et al.,* 1976).

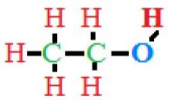
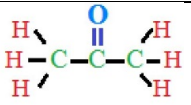
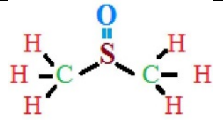
2.4.2 Effects of DMSO in dentistry

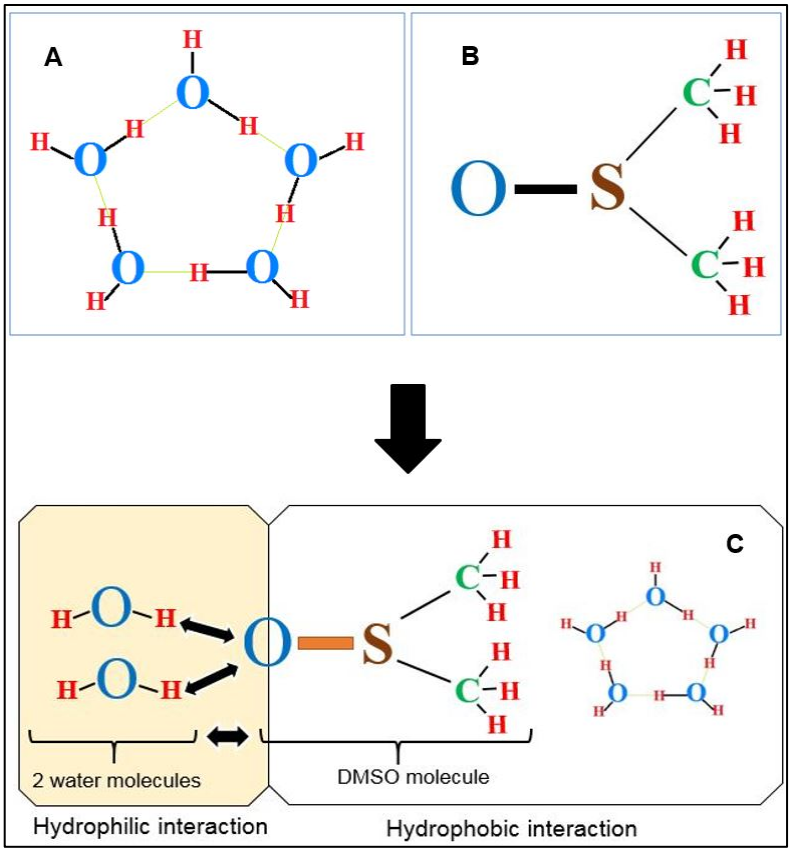
DMSO has been previously used in dental adhesives to solvate resin monomers during cytotoxicity testing (Geurtsen *et al.*, 1998). Recently, DMSO was also used in low concentrations (0.04%) (Tjäderhane *et al.*, 2013c), as well as relatively high (50%) concentration (Stape *et al.*, 2015). It has been shown to improve short-term and long-term dentin-resin bond stability and successfully preserve the hybrid layer. The enhancement of monomers' penetration, especially small-molecule, hydrophilic monomer, was suggested as one possible reason for bond stability. Furthermore, the inhibitory effect of DMSO, especially to matrix metalloproteinase (MMPs), was suggested as a reason for bond stability (Tjäderhane *et al.*, 2013c).

The mechanism of DMSO's action in dentin is not clearly understood. However, DMSO-water interaction is key to understanding the action of DMSO on the free and bound water present in collagen fibrils (Mehtälä, Pashley and Tjäderhane, 2017). DMSO has two endings when it interacts with water, a hydrophobic end and a hydrophilic end. The hydrophilic end (oxygen atom) has a strong affinity to two hydrogen atoms of water molecules (Luzar *et al.,* 1993). On the other end, the hydrophobic end of each DMSO molecule breaks the water self-association, because the strength of the bond between the DMSO molecule and the water molecule is stronger than that of water to water (Vishnyakov *et al.,* 2001) (**Fig. 1**). Therefore, preliminary studies suggest that pretreating collagen fibrils with DMSO may improve the polarity needed to break down the self-association tendency of water, leading to displacement of water molecules within collagen fibrils (Tjäderhane *et al.,* 2013c).

Previous studies have shown that each molecule of DMSO is attracted to two or three water molecules (Luzar *et al.,* 1993; Catalán, Díaz *et al.,* 2001). Moreover, DMSO improves the wettability of collagen, since it strongly binds to the water molecules available between collagen meshwork (Tjäderhane *et al.*, 2013c; Mehtälä, Pashley and Tjäderhane, 2017).

Table. 1. Properties of organic solvents discussed in the thesis, modified from (Smallwood *et al.,* 1966; Ekambaram *et al.,* 2015a).

	Water	Ethanol	Acetone	DMSO
Structure				
Chemical formula	H_2O	C_2H_6O	C_3H_6O	C_2H_6OS
Density (g/mL)	0.998	0.789	0.786	1.092
relative polarity	1.000	0.654	0.355	0.444
Boiling point (˚C)	100.00	78.5	56.20	189.00
Melting point (˚C)	0.00	-114.1	-94.3	18.4
Vapor pressure 20ºC (hPa)	17.5	59	240	0.61 (at 25 ºC)
Molecular weight (mol^{-1})	18.02	46.07	58.08	78.13
Dipole movement (D)	1.85	1.7	2.85	3.9
Dielectric constant	80.1	24	21	46.7
Viscosity 10^{-3} Pa s	0.89	1.08	0.30	2.00
Solubility in water	Miscible	Miscible	Miscible	Miscible



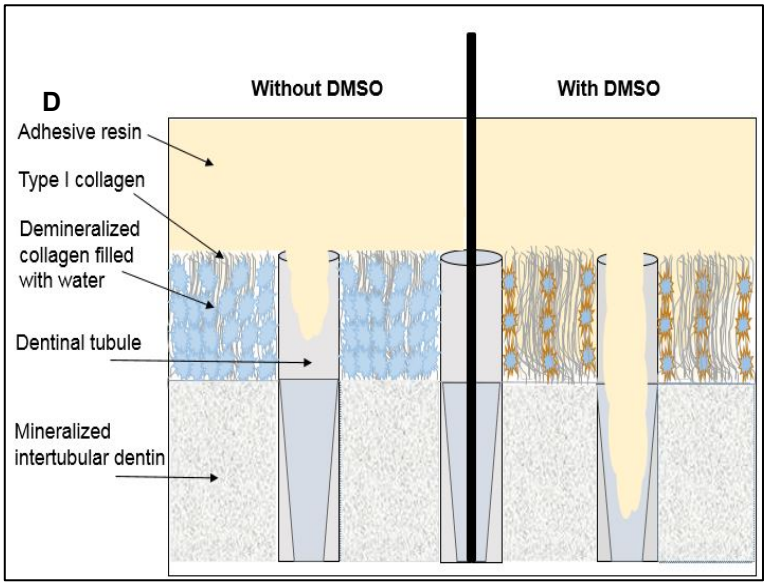


Figure 1. Possible mechanism of DMSO's action on the water molecules accumulated in demineralized dentin. A) water-cluster composed of multiple water molecules and accumulated inside the demineralized dentin. B) DMSO molecule binding to two water molecules, the oxygen atom of each DMSO molecule forms a strong bond with hydrogen bonds of two water molecules. C) DMSO molecule has two ends and therefore it causes hydrophilic and hydrophobic changes to water molecules, first by breakdown self-association of water (between the hydrophobic end of DMSO, since DMSO-water molecules bonding is stronger than water-water molecules bonding. Secondly by interaction with two water molecules (each oxygen atom of DMSO interact with hydrogen bonds of two water molecules). D) addition of DMSO to dentin as primer or incorporation into adhesive resin cause displacement of water molecules and alteration in their arrangement, by breaking the water-self association from one side and interaction with two water molecules from the other side, leading to increase of spaces occupied by resin monomer during the restorative procedure. Therefore, enhancement of hybrid layer integrity as well as improvement of restoration durability and strength.

2.5 Other components of dental adhesives

The remaining components of resin adhesive systems are inhibitors. These are basically antioxidants added to extract and remove the prematurely reacted initiators from unreacted initiators (Van Landuyt *et al.*, 2007). Therefore, they enhance the shelf life of an adhesive bottle and prevent accumulation of decomposed or incompletely reacted initiators (Van Landuyt *et al.*, 2007). Two main types of inhibitors are used in dental adhesives: butylhydroxytoluene (BHT) and monomethyl-ether-hydroquinone (MEHQ). BHT is always incorporated into hydrophobic resin adhesive systems, while MEHQ is always incorporated into hydrophilic adhesive resins (Van Landuyt *et al.*, 2007).

2.6 Challenges in resin-dentin bonding

Preservation of bond integrity and stability has been the main goal in adhesive dentistry (Tjäderhane, *et al.*, 2013b; Tjäderhane, 2015). Despite developments in adhesive formulations and techniques, progressive loss of resin-dentin bond integrity and reduction in bond strength have been extensively reported (Salz *et al.*, 2005; Carvalho *et al.*, 2012; Moretto *et al.*, 2013; Opdam *et al.*, 2018). Generally, many factors were investigated as potential causes of bonding failure. According to the origin of failure initiation, the reasons for failures were attributed to the hydrophilic nature of the contemporary adhesives systems causing unwanted water absorption, phase separation, and resin leaching (Yoshiyama *et al.,* 2002; De Munck *et al.*, 2005; Spencer *et al.*, 2010). Failures were also attributed to degradation of dentin collagen by proteases such as matrix metalloproteinases and cysteine cathepsins (Pashley *et al.*, 2004; Tersariol *et al.,* 2010; Liu *et al.*, 2011; Tjäderhane, 2015) that are activated during the acid-etching step of restorative treatments. Hydrophilic monomers such as HEMA that are included in adhesive resins increase the water sorption of polymerized adhesive layers over time, resulting in progressive degradation of mechanical properties (Ito *et al.*, 2005). Furthermore, in addition to hydrophilicity, the retained solvents (ethanol/ acetone) and water within the hybrid layer can hinder the polymerization of monomers and compromise the integrity of the hybrid layer (Ikeda *et al.*, 2008).

2.7 Currently applied strategies to limit degradation

The durability of resin-dentin bonds has been extensively tested for two reasons: optimization of the effectiveness of bonding, also, enhancement of the clinical outcomes (Peumans *et al.,* 2005; Liu *et al.*, 2011; Breschi *et al.*, 2018). It is understood that laboratory studies evaluating the effectiveness of resin-based restorations are needed to modify the manufactural recommendations, toward enhancement of performance of dental adhesives clinically (Van Meerbeek *et al.*, 2003; Carvalho *et al.*, 2012; Carvalho *et al.,* 2016).

Currently available resin adhesive systems are mostly hydrophilic in nature, and therefore, exhibit hydrolytic degradation and reduction in the stability of restorations over time (Tjäderhane, *et al.*, 2013b). Several methods can be applied to overcome the hydrolytic degradation problem, including the use of ethanol wet-bonding technique (Sadek *et al.*, 2010; Liu *et al.*, 2011; Talungchit *et al.,* 2012), and dry-bonding procedures (Pashley *et al.*, 2007; Manso *et al.*, 2008).

Inhibition of dentin enzyme activity has been extensively evaluated during the last two decades (De Munck *et al.*, 2009; Liu *et al.*, 2013; Perdigão *et al.,* 2013; Tjäderhane *et al.*, 2013a; Sabatini *et al.*, 2014; Tezvergil-Mutluay *et al.*, 2015a; Seseogullari-Dirihan *et al.*, 2016). Several approaches were proposed to inhibit these

enzymes associated with initiation of the degradation process (Sabatini *et al.,* 2014). One example of such approaches includes use of the antimicrobial agent chlorhexidine to inhibit matrix metalloproteases (Gendron *et al.*, 1999), as well as cysteine cathepsins (Scaffa *et al.*, 2012), with the aim of preserving hybrid layer integrity (Hebling *et al.*, 2005; Carrilho *et al.*, 2007). Thus, chlorhexidine was involved in some clinical investigations as a pretreatment of acid-etched dentin, followed by primer and resin application. On the other hand, due to its electrostatic nature, chlorhexidine can leach out from dentin within a short period, the leaching from the hybrid layer resulted in the loss of its inhibitory effect (Blackburn *et al.,* 2007).

Other approaches include the use of antimicrobial-quaternary ammonium compounds (*i.e.,* benzalkonium chloride) (Tezvergil-Mutluay *et al.*, 2011b; Tezvergil-Mutluay *et al.,* 2011c; Cheng *et al.*, 2013; Sabatini *et al.,* 2015); or synthetic (*i.e.,* glutaraldehyde and carbodiimide) (Bedran-Russo *et al.*, 2011; Mazzoni *et al.*, 2013; Sabatini *et al.*, 2014; Scheffel *et al.*, 2014); or natural crosslinkers (*i.e.,* proanthocyanins) (Fang *et al.*, 2012; Bedran-Russo *et al.*, 2014; Balalaie *et al.,* 2018), for enzyme inhibition.

2.8 Biocompatibility of dental adhesives

The biological compatibility of the dental materials used in clinical dentistry is very important for both patients and dental practitioners. Developing novel dental materials with no or minimal cytotoxic effects inside the oral cavity (De Souza Costa *et al.,* 2014; da Silva *et al.*, 2014; Schmalz *et al.,* 2017; Dahl *et al.,* 2018) is therefore of utmost importance. The issue is even more critical in the use of resin-based restorative materials in deep cavities, where unbound or free toxic monomers are released inside pulp tissue (Hebling *et al.,* 1999; De Souza Costa *et al.,* 2007; Koliniotou-Koumpia *et al.,* 2007; Rathke *et al.*, 2007). To reduce cytotoxicity at the early stage of the adhesive mixture, several factors must be considered, including the type, concentration, and duration of the restorative procedure (da Silva *et al.*, 2014; Kerezoudi *et al.*, 2016). Local or systemic adverse effects might be observed with the use of dental adhesives (Schmalz *et al.,* 2009). Local adverse effects initiated at the exposure site (*i.e.,* dental pulp or gingival tissues), appeared as inflammation after application of specific types of resin-based materials, while systemic reactions appeared far away from the exposure site (Stanley *et al.,* 1993).

Generally, effects depend on the eluted substances or released particles from the resin adhesive mixture (Kaga *et al.*, 2001; Schweikl *et al.,* 2006; Dahl *et al.,* 2007; Polydorou *et al.*, 2007; Van Landuyt *et al.*, 2011; Reichl *et al.*, 2012; Schmalz *et al.,* 2017). Several substances or particles eluted from dental adhesives may cause an adverse reaction in oral tissues (*i.e.,* monomers, fillers) (Söderholm *et al.,* 1996;

Kaga *et al.*, 2001; Schweikl *et al.,* 2006; Polydorou *et al.*, 2007). Free, unbound monomers are an example of components in resin adhesive that immediately leach deeper inside the dentin, gingival tissue, pulp tissue, or other living tissues inside the oral cavity (Goldberg *et al.,* 2008). These residual monomers vary in their level of cytotoxicity (Geurtsen *et al.,* 1998).

The importance and role of solvents in dental adhesives was explained in section 2.3.4. The toxicity of the most commonly used solvents in dental adhesives (*i.e.,* ethanol, acetone) is listed as Class 3 (solvents with low toxic potential) (International Council for Harmonisation, ICH, 2016). However, the presence of solvents in high concentrations may negatively affect the mechanical and physical properties of the adhesive, leading to improper polymerization of the adhesive layer and an increase in the quantity of unreacted free monomers that cause the initiation of hybrid layer degradation (Dickens *et al.,* 2005; Holmes *et al.*, 2007; Ye *et al.*, 2007).

During the restorative procedures of deep cavities, the possible cytotoxic effect of incorporation of DMSO in high concentrations should be considered (Tjäderhane *et al.*, 2013c). However, this issue is not related to the cytotoxicity of DMSO itself (Hebling *et al.*, 2015), but to the potential enhancement of the penetration of monomers and bacterial toxins from dentin to pulp. Therefore, in clinical scenarios, where resin-based material is applied to a deep cavity, risk of cytotoxicity of the resin components must be carefully considered (Bouillaguet *et al.,* 2004).

3 Aims of the Thesis

The purpose of this series of studies was to evaluate the role of dimethyl sulfoxide as a solvent for dentin bonding. To accomplish that, DMSO was directly applied to the primer or adhesive, and several concentrations of DMSO were used in two different applications: either as a dentin- pretreatment agent (dentin primer) prior to adhesive application (**Study I**) or incorporated into experimental hydrophobic and hydrophilic resins (**Studies III and IV**). Furthermore, comparison between DMSO and ethanol effects on the demineralized dentin was also investigated (**Study II**). The overall aim of the thesis was to find an optimal, biocompatible concentration or range of DMSO concentrations, that can be used improve the durability of resin-dentin bonding, without impairing the properties of adhesive resin, or showing cytotoxic effects.

4 Specific Objectives of the Thesis

The specific aims of these studies were:

1. To evaluate the effect of various concentrations of DMSO pretreatment on bond stability to demineralized dentin (**Study I**). The hypothesis was that pretreatment of dentin with several DMSO concentrations does not affect the bond strength or nanoleakage (short-term and long-term effect).
2. To investigate and compare collagen changes in terms of stiffness, monomer diffusion and dissociation when dentin incubated in DMSO or ethanol (**Study II**). The hypothesis was that dentin pretreatment with DMSO or ethanol does not affect the uptake of HEMA, stiffness of dentin, as well as collagen dissociation.
3. To evaluate certain mechanical and physical properties of adhesive resins when incorporating DMSO (**Study III**). The hypothesis was that incorporation of DMSO in various concentrations into hydrophobic (R2) or hydrophilic (R5) experimental adhesives does not affect the degree of conversion, crosslinking density of polymers, water sorption/solubility, and mechanical properties.
4. To investigate the potential trasndentinal and eluates cytotoxic effects of hydrophobic (R2) and hydrophilic (R5) methacrylate-based experimental adhesives, containing various concentrations of DMSO (**study IV**). The hypothesis was that pretreating dentin with experimental DMSO-incorporated resins does not decrease cell viability.

5 Materials and Methods

5.1 Materials

The materials used in this series of studies are listed in **Table 1**. Sound third molars were extracted during routine extraction procedures from anonymous donors. Patients' informed consents were obtained and approved by the Ethical Committee of the Faculty of Medicine, University of Oulu (Register #23-2003) (**Study I**). The teeth collected for **studies II and IV** were exempt from notification to the Ethics Committee, in accordance with Finnish law (Tissue Act, Section 20. All teeth used in this project were stored in a solution containing sodium azide (0.02%) to prevent bacterial growth and NaCl (0.9%) at 4 °C and used within three months after extraction (**study I, II,** and **IV**).

Table 2. Materials used in these studies.

Trade name	Type	Manufacturer	Lot No	Study
DMSO	100% concentration of Dimethyl Sulfoxide	Merck KGaA, Frankfurt, Germany	41629833	I, II, III, IV
Ethanol	100% concentration of Ethanol	Berner OY, Helsinki, Finland	64-17-5	II
Resin adhesive system	Adper Single Bond Plus Adhesive	3M ESPE, USA	N468093	I
Restorative composite	Filtek Supreme XTE	3M ESPE, USA	N470314	I
Etching Gel	Scotchbond™ Universal Etchant 37% phosphoric acid	3M ESPE, USA	505995	I
HEMA	100% 2-Hydroxyethylmethacrylate, resin monomer.	Sigma-Aldrich, St. Louis, MO, USA	081M1110 V	II
EDTA	Ethylenediaminetetraacetic acid	Merck KGaA, Darmstadt, Germany	6381-92-6	II
Artificial saliva	50 mM HEPES ($C_8H_{18}N_2O_4S$), 25 mM $CaCl_2.H_2O$, 3mM NaN_3, 0.2 mM $ZnCl_2$	Sigma-Aldrich St. Louis, MO, USA	-	I
Light curing unit	light-emitting diode (LED)	Elipar, 3M ESPE, Seefeld, Germany	-	I, III, IV
R2: Hydrophobic resin	70 wt.% BisGMA, 28.75 wt.% TEGDMA	Experimental resins produced by Bisco	728-93B	III, IV
R5: Hydrophilic resin	40 wt.% BisGMA, 30 wt.% BisMP, 28.75 wt.% HEMA	Experimental resins produced by Bisco	724-195B	III, IV

Abbreviations: Bis-GMA: bisphenol A diglycidyl ether dimethacrylate; TEGDMA: triethylene-glycol dimethacrylate; CQ: camphorquinone; EDMAB: ethyl N, N-dimethyl-4-aminobenzoate; HEMA: 2-hydroxyethyl methacrylate; 2MP: Bis [2-(methacryloyloxy) ethyl] phosphate; DMSO: dimethyl sulfoxide. HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (buffering agent);

5.1.1 Specimens preparation in dentin bonding study (Study I)

Flat dentin surfaces of 48 teeth were prepared by removing the occlusal enamel and superficial dentin perpendicularly to the long access of the tooth, using a diamond saw (Isomet, Buehler, Lake Bluff, IL, USA) under water cooling. Teeth were randomly distributed among the eight experimental groups according to the DMSO concentrations used (0.001, 0.01, 0.1, 1, 10, 20 vol.%), and assigned as six teeth per group. Non-DMSO pretreated dentin surfaces were assigned as control. Abrasive paper (600-grit SiC) was used to standardize the smear layer. Teeth were then stored at 4 °C until use.

Phosphoric acid (37%) was used to acid-etch the dentin surfaces for 15 s to remove the inorganic components and expose the collagen fibrils. The dentin surface was then washed and dried using an air-water syringe, to remove the remnants phosphoric acid. Each tooth was pretreated with specific concentration of DMSO actively for 30 s using a micro-brush. A cotton pellet and air syringe were used to remove the excess pretreatment solution of DMSO/water. Adhesive resin was applied on dentin for 15 s and agitated gently, followed by using the air syringe carefully for 5 s to remove adhesive solvents. Resin was then light-activated for 10 s (Elipar S10, 3M ESPE) at 1,200 mW/cm^2. Incremental build-up of composite (Filtek Supreme XTE, 3M ESPE) was performed, in a thickness of 1-1.5 mm for each increment that light polymerized separately for 20 s in a total of 4 to 5 mm.

After preparation of the artificial saliva (AS), which was composed of 50 mM HEPES, 25 mM $CaCl_2.H_2O$, 3 mM NaN_3, and 0.2 mM $ZnCl_2$, teeth were incubated in artificial saliva for 24 h. At the end of incubation period, the restored teeth were sectioned mesio-distally and bucco-lingually to produce resin-dentin sticks of cross-section 0.9 x 0.9 mm. Half of the sticks were tested after 24 h of incubation, and the second half were stored for 6 m in AS at 37 °C.

5.1.2 Preparation of dentin beams, cubes, and slices (Study II)

Occlusal enamel surfaces and superficial dentin were removed from all teeth assigned to **Study II**. Teeth were then randomly distributed to produce dentin beams, cubes, and slices (**Fig. 2**). Preparation of dentin disks (1 mm in thickness), perpendicularly to the long axis of the tooth, was performed using Isomet saw blades. Disks were glued onto a histology glass slabs and sectioned mesio-distally to produce dentin beams (length 6 mm, width 2 mm, thickness 1 mm). In total, 45 teeth were assigned to prepare the 120 beams used to evaluate the modulus of elasticity of dentin beams pretreated with DMSO or ethanol. Beams pretreated with water only were assigned for control group.

Other teeth (n=55) were used to prepare dentin disks of 2 mm thickness, perpendicularly to the long axis of the teeth. Discs were sectioned mesio-distally and

bucco-lingually to produce dentin cubes of 2 x 2 x 2 mm in dimension (in total, 180 dentin cubes were produced). After measuring the dimensions of the cubes under light microscope, cubes were demineralized by incubation in 0.5 M of EDTA for 20 days. Cubes were immersed in several concentrations of DMSO or ethanol prior to HEMA immersion. Cubes incubated in water only prior to HEMA were assigned for control.

Other teeth (n=5) were sectioned perpendicularly to the long axis to produce dentin slices (half discs) of 1 mm thickness from coronal and deep dentin (close to pulp). For each DMSO incubation medium used, one coronal and one pupal slice were assigned. Dentin slices were incubated in several ascending concentrations of DMSO (1, 10, 50, 100 vol. %) for different ascending time intervals (10, 30, 60 min). Non-DMSO incubated slices were assigned for control.

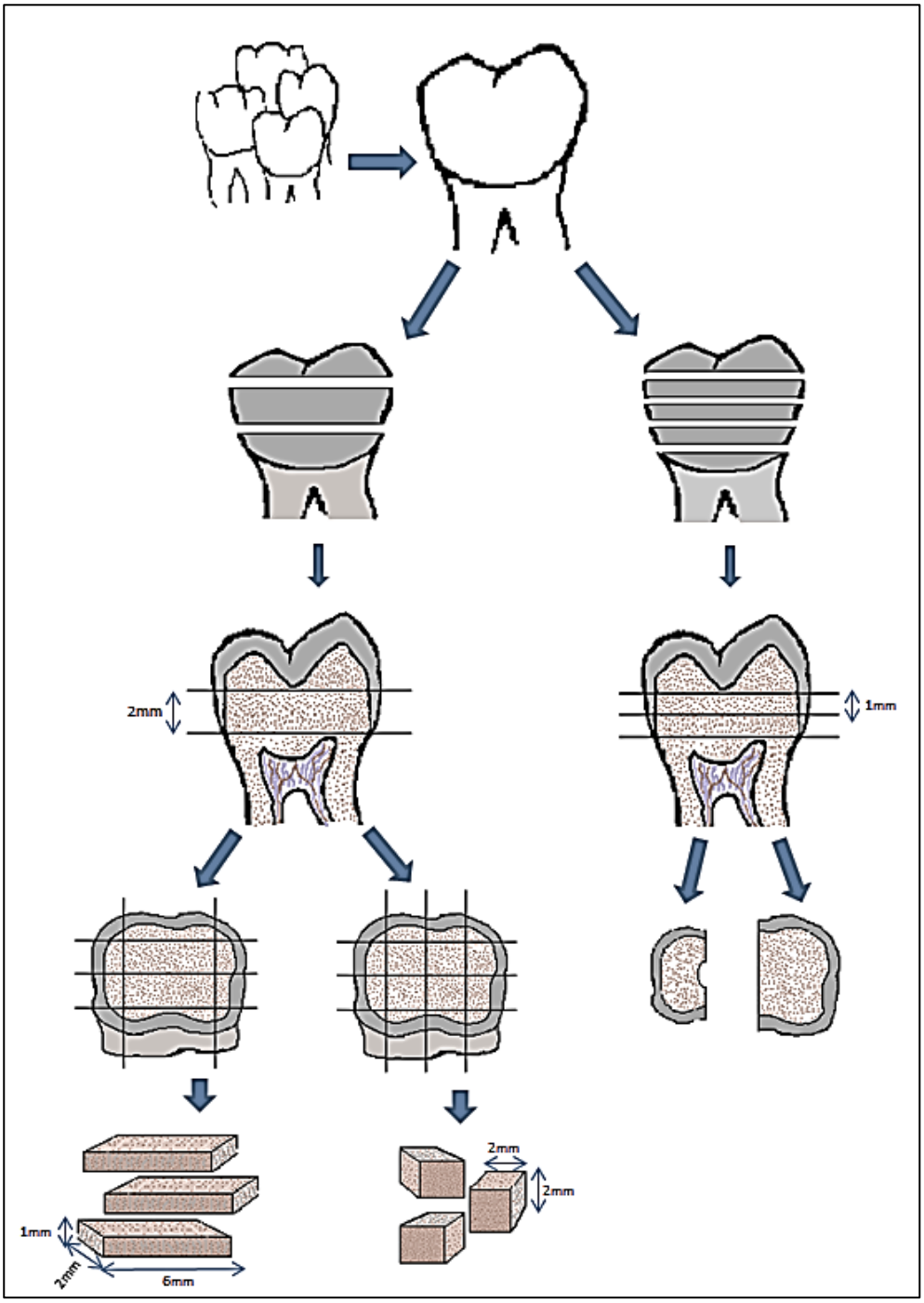


Figure 2. Illustration of dentin preparation for **Study II**. Dentin beams (2x1x6 mm), or dentin cubes (2x2x2 mm), or dentin slices (1 mm) were used to evaluate dentin permeability, stiffness, and dissociation, respectively (**study II**, section 5.1.2).

5.1.3 Preparation of resin discs of two experimental resins (Study III)

Two experimental resins, R2 (relatively hydrophobic) and R5 (relatively hydrophilic) (Bisco Dental Products, Schaumburg, IL, USA), were used for **Study III**. Several ascending concentrations of DMSO (0.01, 0.1, 1, 5, 10 w/w %) were incorporated into each resin to produce homogenous mixtures of DMSO- modified resin adhesives (w/w %). All mixtures were magnetic stirred (VWR International Ltd, Lutterworth, UK). Controls were non-DMSO-containing resins (neat resins). All the DMSO modified resin mixtures were used to produce disc shaped resin specimens (thickness 0.5 ± 0.02 mm, diameter 6 ± 0.1 mm), using a custom-made stainless-steel mold. To ensure the flatness of the specimens, a Mylar strip was placed on a glass slide. A drop of 25 µL of each resin/DMSO mixture was dropped inside the mold. After that, another Mylar strip and glass slide were added to prevent formation of a void and oxygen inhibition layer. To polymerize resin/DMSO drops, a curing light unit (LED; Elipar, 3M ESPE, Seefeld, Germany) at 1,200 mW/cm^2 was used for 20 s on each side at 1 mm distance.

Discs were incubated in a humidified atmosphere for 24 h at 37 ˚C, to allow complete polymerization. The dimensions of each specimen were measured using a digital micrometer (Mettler Toledo, Columbus, OH, USA).

Table 3. Composition of the experimental bonding resins solvated in DMSO, used in study III and IV (sections 5.1.3, 5.1.5).

	Resin	Composition	% (w/w%)
Neat resin ***Hydrophobic resin*** **Batch# 727-206-2**	R2	BisGMA TEGDMA CQ EDMAB	70.00 28.75 0.25 1.00
Neat resin ***Hydrophilic resin*** **Batch# 727-206-5**	R5	BisGMA HEMA 2MP CQ EDMAB	40.00 28.75 30.00 0.25 1.00
Solvated resins	0.01% DMSO / R2 or R5	DMSO + Neat R2 or R5	0.01 / 90.99
	0.1% DMSO / R2 or R5	DMSO + Neat R2 or R5	0.1 / 99.90
	1% DMSO / R2 or R5	DMSO + Neat R2 or R5	1 / 99.00
	5% DMSO / R2 or R5	DMSO + Neat R2 or R5	5 / 95.00
	10% DMSO / R2 or R5	DMSO + Neat R2 or R5	10 / 90.00

Abbreviations: BisGMA: bisphenol A diglycidyl ether dimethacrylate; TEGDMA: triethylene-glycol dimethacrylate; CQ: camphorquinone; EDMAB: ethyl N, N-dimethyl-4-aminobenzoate; HEMA: 2-

hydroxyethyl methacrylate; 2MP: Bis [2-(methacryloyloxy) ethyl] phosphate; DMSO: dimethyl sulfoxide.

Table 4. Chemical formula and molecular weight of monomers and photo-initiators used in this project.

Abbreviation	Scientific name	Chemical formula	Molecular weight (g/mol)
BisGMA	bisphenol A diglycidyl ether dimethacrylate	$C_{29}H_{36}O_8$	512.599
TEGDMA	triethylene-glycol dimethacrylate	$C_{14}H_{22}O_6$	286.324
EDMAB	ethyl N, N-dimethyl-4-aminobenzoate	$C_{11}H_{16}N_2O_2$	208.261
HEMA	2-hydroxyethyl methacrylate	$C_6H_{10}O_3$	130.14
2MP	Bis [2-(methacryloyloxy) ethyl] phosphate	$C_{12}H_{19}O_8P$	322.25
CQ	Camphorquinone	$C_{10}H_{14}O_2$	166.22

5.1.4 Preparation of dentin disks and measurement of dentin permeability (study IV)

Intact third molars were used in **Study IV** to prepare 128 dentin slices. Teeth were transversally sectioned above the level of cemento-enamel junction (CEJ), using an Isomet saw. Dentin discs were extracted from the deep dentin, directly above the pulp horns. Dentin slices of 0.5-0.6 mm were prepared, then polished with abrasive papers (600-grit SiC) to get a final thickness of 0.40 ±0.02 mm. The thickness of each disc was measured by digital micrometer (Mettler Toledo, Columbus, OH, USA). Light microscope (FM-700, Future-Tech, Tokyo, Japan) was used to confirm the absence of pulp horn or perforation (at 50x). Citric acid (50%) was used to remove the smear layer from the pulpal side of each dentin disc for 30 s, as described in ISO 7405 (2018).

Permeability measurement of dentin discs was performed with SLI 1000 Liquid Flow Meter (Sensirion AG, Staefa ZH, Switzerland) to ensure homogenous distribution of dentin discs between groups. Six concentrations of DMSO (0, 0.01, 0.1, 1, 5, 10 w/w %) were incorporated into R2 and R5. Two controls, a positive control (an experimental glass-ionomer cement) and negative control (polyvinylsiloxane impression material; Imprint 4 Super Quick Ultra-Light; 3M ESPE, Neuss, Germany) were also prepared. After distributing dentin discs, depending on their individual permeability, to different groups, eight dentin discs

were used each time. Discs were bonded with the six DMSO/resin concentrations and two controls to evaluate transdentinal cytotoxicity

5.1.5 Preparation of resin discs (Study IV)

Several ascending concentrations of DMSO/resin (w/w %) were used to produce homogenous mixtures of DMSO/R2 or R5 resins that were used to fabricate round discs (0.5 mm in thickness, 6 mm in diameter). A stainless-steel mold was placed on a Mylar strip, 25 µL from each DMSO/resin mixture was applied inside the stainless-steel mold, covered gently with Mylar strip and then a glass slide to ensure the flatness of the final resin after polymerization. A photo polymerization unit (LED; Elipar, 3M ESPE, Seefeld, Germany) at 1,200 mW/cm^2 was used for 20 s on each side to produce discs of 0.5 mm thickness. Discs of DMSO/resin were then incubated in DMEM (Sigma-Aldrich, New Road, Gillingham, UK) for 24 h at 37 ˚C in a shaking bath.

5.2 Research methods

5.2.1 Evaluation of microtensile bond strength (short-term and long-term µTBS) (Study I)

Sticks of each group were used to evaluate the microtensile bond strength (µTBS) at the speed of 0.5 mm/min using Bisco Micro Tensile Tester (Bisco, Schaumburg, IL, USA). To calculate the bond strength values, the force (Ns) needed to separate resin composite from dentin was recorded for each resin-dentin stick as well as for the interface surface (mm^2). The microtensile bond strength of each stick was calculated by dividing the force by the interface area (in MPa).

5.2.2 Assessment of failure mode (Study I)

After measuring the microtensile bond strength of each resin-dentin stick, a light microscope (FM 700, Future-Tech, Tokyo, Japan) was used to investigate failure location at 50x magnification. Four types of failures were observed and recorded. The failures were either cohesive in resin composite (CR), cohesive in dentin (CD), mixed failure (MF), or failures that occurred before testing (pretesting failure; PF).

5.2.3 Evaluation of nanoleakage (short-term and long-term nanoleakage) (study I)

Evaluation of nanoleakage was performed for 6 sticks randomly selected from each group. Three of them were tested after 24 h (short-term evaluation and the rest after 6 m of storage (long-term evaluation) in AS at 37 °C. Nail varnish was used to coat the sticks, except for 1 mm around the resin-dentin interfaces. After rehydration, specimens were immersed in ammoniac silver nitrate (50 w/v %) for 24 h in the dark. The next day, the sticks were removed from the solution, rinsed with water, and placed for 8 h in a photo-developing solution (Kodak Professional D-76 Developer, Birmingham, UK), under fluorescent light. This step was needed to transform silver ions into metallic silver particles, to be visualized under SEM later.

Wet polishing with 1000-grit SiC paper of each stick was performed to remove the remaining nail varnish, followed by insertion in epoxy resin (EpoFix Resin, Struers, Ballerup, Denmark). After 24 h, blocks of epoxy-containing sticks from each group were polished with a series of wet-polishing sand papers (1000-, 2000- and 4000-grit SiC), followed by another, smoother polishing with 1, 0.1, and 0.05 μm diamond paste (Buehler). Blocks were then coated with a thin layer of carbon immediately before scanning under scanning electron microscope (SEM; Phenom Pro, Phenom-World B.V., Eindhoven, Netherlands). Three images were systematically recorded for each stick. In total, nine images were recorded for each group from different areas of resin-dentin interfaces. The extent and percentage of silver precipitation within the hybrid layer was measured, first by measuring the length of hybrid layer, and then the extension of silver particles precipitation within the hybrid layer, using digital image-analysis software (ImageJ; National Institute of Health, Bethesda, Maryland, USA).

5.2.4 Effect of solvents on HEMA uptake (Study II)

After complete demineralization of dentin cubes in EDTA, cubes were randomly distributed into groups (10 cubes/group), immersed in plastic vials, incubated for 30 min in ascending concentrations of DMSO or ethanol. After pretreatment with a specific concentration of DMSO or ethanol (0.01, 0.1, 1, 5, 10, 20, 50, 100%), each dentin cube was dipped in 100% HEMA (2-hydroxyethylmethacrylate) for 100 minutes at room temperature, to allow maximum HEMA uptake into collagen, as described above (Pashley *et al.,* 2000).

To evaluate the degree of HEMA uptake of each DMSO- or ethanol- pretreated, demineralized dentin cube, plastic vials containing 2 ml of fresh distilled water were used for the first extraction of HEMA uptake through each cube for 1 h, followed by other vials containing 2 ml of distilled water to extract the remaining HEMA from each cube. After combining the extracts, 1 ml of the total extracts was placed in UV-

cuvettes (UV-Cuvettes Semi-micro, BrandTech Scientific, Inc., Wertheim, Germany) to evaluate the spectral scan of HEMA in water, using UV-spectrophotometer (model UV-1601, Shimadzu Corp., Kyoto, Japan). A standard curve of absorption based on known concentrations of HEMA was obtained. The standard curve of absorption was used to convert the absorption values into the amount of extracted HEMA from each dentin cube. The reference wavelength was assigned to 222 nm, since the pilot analysis demonstrated it had the best strength of absorption.

5.2.5 Evaluation of the effect of solvents on elastic moduli (Study II)

Demineralized dentin beams (6 x 2 x 1 mm) were used evaluate the effect of various concentrations of DMSO or ethanol (1, 10, 20, 50, 100%) incubation on the stiffness of beams after 10, 30 and 60 min of incubation. To evaluate this, beams were loaded into a universal test machine (AGS-10, Shimadzu Corp., Kyoto, Japan), using a three-point bending fixture with a distance between lower supports at 2.5 mm. The test was performed using 5 N load cell (Shimadzu Corp., Kyoto, Japan) at speed of 0.5 mm min^{-1} and 15% strain.

The following equation was used to evaluate elastic moduli (E) of each beam in each different solvent incubation time:

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

m: slope of the linear portion of the load-displacement curve; L: length of the span; b: width of the test specimen; and h: thickness of the beam. All the beams were initially assessed (before starting incubation in solvents), then assessed again after 10, 30 and 60 min of incubation in each concentration of solvent; finally, each beam was re-assessed after 24 h in distilled water to investigate the reversibility of solvent immersion.

5.2.6 Evaluation of collagen dissociation (Study II)

Collagen dissociation of demineralized dentin slices was performed through visual examination of 1 mm dentin slices (one from coronal superficial dentin and one from deep dentin) treated with several concentrations of DMSO (1, 10, 50, 100%) for three incubation times (10, 30, 60 m). After each incubation period, dentin slices were placed against a ruler to visualize the potential effect of DMSO concentration and time of DMSO incubation.

5.2.7 Evaluation of physical and mechanical properties of adhesive resins (Study III)

Several physical and mechanical properties were evaluated for DMSO- solvated R2 and R5 resin mixtures. Mixtures of DMSO incorporated into resins were prepared by addition of DMSO in several concentrations (0.01, 0.1, 1, 5, 10 w/w %). These mixtures were used to produce DMSO/resin discs. The mechanical and physical properties evaluated in **Study III** were degree of conversion, polymer crosslink density, biaxial flexural strength, and water sorption and water solubility.

5.2.7.1 Biaxial flexural strength (Study III)

Two sets of R2 and R5 resin discs containing several concentrations of DMSO (w/w %) were assessed either after 24 h of water incubation or after 30 d of distilled water immersion at 37 °C. A custom-made jig fabricated for this purpose was used to hold resin discs during a flexural strength test, using a universal testing machine (Shimadzu, Shimadzu Corp., Kyoto, Japan) at the speed of 1 mm/min until fracture of the resin disc. The force (N) needed to fracture each specimen was recorded and used to calculate flexural strength through this equation:

$$\sigma = -0.2387\ P(X - Y)/\ d^2$$

σ: maximum center tensile stress (MPs); P: total load causing fracture (N); and d: thickness of the specimen (mm). X and Y were calculated through these equations:

$$\boldsymbol{X = (1+\nu)In(r_2/r_3)^2 + (r_2/r_3)^2} \qquad \boldsymbol{Y = (1+\nu)[1+In(r_1/r_3)^2] + (1-\nu)(r_1/r_3)^2}$$

ν: Poisson's ratio (ν used as fixed number=0.25); r_1: radius of support circle (mm); r_2: radius of loaded area (mm); and r_3: radius of the specimen (mm).

5.2.7.2 Degree of monomer conversion (Study III)

Uncured and cured experimental adhesives were used to obtain the absorption spectra, using a Fourier transform infrared spectroscopy device (FTIR; Spectrum One, Perkin Elmer, Beacons field, Bucks, UK). The FTIR is equipped with a universal attenuated total reflectance (ATR) accessory (Rueggeberg *et al.,* 1990).

Drops of DMSO/resin mixtures were used to analyze the degree of monomer conversion, by calculating the ratio of the aliphatic carbon-to-carbon (C=C) absorption at 1,640 cm^{-1} to the aromatic absorption at 1,608 cm^{-1} as internal standards (Rueggeberg *et al.,* 1990). A silicon mold (diameter 6 mm, thickness 0.6 mm) was placed over the ATR crystal surface. A drop of each experimental adhesive (5 µL) was placed inside the mold and contacted with the ATR crystal. A Mylar strip was

placed on the adhesive with continuous collection of infrared spectra before polymerization. After polymerization, using LED light-curing unit for 30 s at a distance of 1 mm, the absorption spectrum of each specimen was recorded for 300 s. These spectrums were used to calculate the degree of conversion. The degree of conversion was calculated by calculating the changes between the aliphatic and aromatic peaks of the experimental adhesives in both conditions (cured and uncured conditions). The following equation was used to calculate the degree of conversion:

$$DC(\%) = \left(1 - \frac{R^{(Cured)}}{R^{(Uncured)}}\right) X\ 100$$

R: ratio of aliphatic and aromatic peak intensities at 1640 cm^{-1} and 1608 cm^{-1} in cured and uncured adhesives.

5.2.7.3 Water sorption and water solubility (Study III)

To evaluate the water sorption and solubility of the specimens, following the ISO 4049 standard, they were gradually dried in a desiccator at 37 °C with regular follow-up of their weights to obtain constant weight, when the difference was not more than 0.01 mg between weight measurements. After recording the initial constant weights of the specimens (M1), resin discs were then individually immersed in plastic vials containing 5 ml of fresh distilled water and incubated at 37 °C for several ascending day- intervals within 28 days (1, 2, 3, 4, 5, 6, 7, 14, 21, 28 days). After each day-interval, each specimen was gently dried to remove access water from both sides and weighed. At the end of the incubation period (28 d), measurements of specimen mass were performed after removing the excessive water on both sides. These masses were considered as (M2). Specimens were then returned to desiccator at 37 °C to obtain a constant final weight (M3). The recorded masses were used to calculate the amount of water sorption (*Wsp*) and solubility (*Wsu*), using the following equations:

$$\boldsymbol{Wsp} = \frac{(\mathbf{M2} - \mathbf{M3})}{\boldsymbol{V}} \qquad \boldsymbol{Wsu} = \frac{(\mathbf{M1} - \mathbf{M3})}{\mathbf{V}}$$

M1: constant initial mass (μg) of the specimen prior to water incubation; *M2*: mass (μg) after immersion in water; *M3*: constant dehydrated mass (μg) after the second desiccation process until constant mass was obtained; and *V*: volume (mm^3) of a specimen.

5.2.7.4 Microhardness testing of DMSO-containing experimental resins (Study III)

Ethanol- and water- solvation technique of resin discs containing several concentrations of DMSO was modified from the standard softening test (Schneider *et al.*, 2008; Leitune *et al.*, 2013), and used to indirectly evaluate the polymer crosslinking effect of DMSO. Digital Knoop microhardness tester (HMV 2, Shimadzu Corporation, Tokyo, Japan) was used to register first the initial microhardness numbers (KHN1) through three indentations. Blocks of epoxy-containing resin discs were then immersed in distilled water for 24 h at 37 °C to obtain the second microhardness measurements (KHN2). After that, 100% pure ethanol was used to soften the discs for 4 h at 37 °C, to obtain the third microhardness measurements from each disc (KHN3). Thus, Knoop microhardness was measured, and the reduction in microhardness was calculated (ΔKNH%) from each measurement in relation to baseline. These measurements were performed to estimate the effect of DMSO concentration on polymer crosslinking density.

5.2.8 Evaluation of DMSO-resin biocompatibility (Study IV)

5.2.8.1 Preparation of transfected bovine pulp-derived cell culture

Clonal large T-antigen transfected bovine pulp-derived cells (SV40) were received as a kind donation from Regensburg University. Cells were maintained in growth medium and cultured in Eagle's minimum essential medium (α MEM) (Sigma-Aldrich, Gillingham, UK), and supplemented with 10% fetal calf serum (Gibco, Thermo Fisher, Boston, USA), 2% L-glutamine, penicillin (100 U/ml), and streptomycin (100 µg/ml) (Sigma-Aldrich, Gillingham, UK), at 37 °C, 100% humidity, and 5% CO_2. Polyamide nylon meshes of 150 µm pore size and 8 mm in diameter were prepared. The nylon meshes were cleaned with 0.1 M acetic acid for 30 min, washed 3 times with sterile water, and coated with 0.03 mg/ml fibronectin (fibronectin bovine plasma, Sigma-Aldrich, St. Louis, MO, USA). A 6-well tissue culture plate was filled with 1.25 ml of MEM α (Minimum Essential Media; Gibco, NY, USA) supplemented with 20% fetal serum. Four meshes were then inserted in each cell culture insert (Greiner bio-one, Nurtigen, Germany), under sufficient nutritional medium for 48 h to allow proper cell growth over the polyamide nylon mesh and incubated for 48 h, at 37°C, 5% CO_2 and 100% humidity. After incubation, each polyamide nylon mesh was separately placed in 24-well tissue plate. In each well, medium of 1 ml of MEM α and 10% FBS was added to feed cells. The medium

was changed every day for 14 d in the incubator, to produce cells on mesh in a three-dimensional form.

5.2.8.2 Preparation of human gingival fibroblast (HGF) cell culture

Primary human gingival fibroblast cells were extracted from stocks stored in liquid nitrogen. The cells were cultured in DMEM, supplied with 100U/ml penicillin, and 100µg/ml streptomycin (Sigma-Aldrich, Gillingham, UK). The cells were then incubated at 37° C in 100% humidity and 5% CO_2. Cultures were incubated in 5% CO_2 and 100% humidity at 37 °C until usage.

5.2.8.3 Evaluation of cell viability by MTT assay

Methyltetra-zolium assay (MTT) test is one of the most commonly used tests to evaluate cytotoxicity precisely and quickly. It is based on a quantitative measurement of cell viability from their metabolic activity, through reduction of yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) which reflects the amount of viable cells, and can be analyzed spectrophotometrically by a plate reader (Mosmann, 1983). In this test, the viability of living cells is detected as a reflection of the succinate dehydrogenase enzyme (SDH) action that reduces MTT reagent to formazan crystals, which can be analyzed after their dissolution by solubilizing solution.

5.2.8.3.1 Using MTT assay to evaluate the transdentinal cytotoxicity to SV40 cells

The flow of medium within the tightly closed perfusion culture system was assured several times. In each container, a polyamide nylon mesh containing SV40 cells under continuous freshly nutritional medium was placed at the bottom of the gradient perfusion culture container for 24 h. After that, each dentin disc was placed above each polyamide nylon mesh, in which the pulpal side was attached to the polyamide mesh and its contents during the whole experiment and fixed by a stainless-steel holder inside the perfusion chamber.

Each time, eight perfusion culture containers of nutritional polyamide meshes, and the dentin discs were placed together in a closed system for 24 h to ensure the flow of the nutritional medium to the cells. After that, one drop of each DMSO/R2 or R5 adhesive was carefully applied on the occlusal side of the dentin disc, polymerized for 30 s, after which the perfusion chamber was tightly closed. After 24 h, each mesh was gently removed from the stainless-steel holder, then incubated in

1 ml of freshly prepared MTT solution (5 ml) in a 48-well tissue culture plate for 2 h. The incubation in MTT solution was performed to allow the conversion of the yellow water-soluble tetrazolium salt 3-(4 5-dimethylthiazol-2-yl)-2 5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO, USA) into dark-blue formazan crystals stored in the cytoplasm of cells (Vajrabhaya *et al.*, 2009). After removal of the remaining MTT solution, DMSO in a 100% concentration was added (250 µL) to each well to allow dissolution of MTT formazan from the cells for 30 min in shaker, followed by taking 200 µl of the cell solution into a new 96-well tissue culture plate. The solution extracted from each well was analyzed spectrophotometrically at the wavelength of 570 nm. Positive control (experimental glass-ionomer cement) and negative control (polyvinylsiloxane impression material; Imprint 4 Super Quick Ultra-Light dental impression material; 3M ESPE, Neuss, Germany) groups were assigned. In total, eight dentin discs were used each time to assess the eight groups (n=eight discs/group) for each experimental resin mixture, neat resins, and two controls.

5.2.8.3.2 Using MTT assay to evaluate the cytotoxicity of eluates of resins containing DMSO on HGF cell culture

At the end of 24 h incubation in DMEM, DMSO/resin discs were removed from the glass vials containing DMEM and eluted materials from each disc. Moreover, HGF cells were cultured in a 96-well plate. Eluates from each disc (150 µL) were added to each well for 24 h in a humid atmosphere, at 37 °C. The HGF cell viability was spectrophotometrically analyzed using MTT assay at a wavelength of 570 nm.

6 Statistical Analysis

The data used in all studies conducted as a part of this project were subjected to statistical analysis using either SPSS (SPSS Inc., Armonk, NY, USA), or Sigma Plot version 13.0 **(**Systat Software Inc., San Jose, CA, USA). All the data were subjected to the Shapiro-Wilk test to confirm the normality of data distribution and modified Levene's test to confirm the homoscedasticity.

In **Study I**, the data on microtensile bond strength (μTBS) and nanoleakage (NL) were subjected to two-way ANOVA. The variables were storage time and concentrations of DMSO. *Post-hoc* multiple comparisons were performed with Tukey's HSD test. The statistical significance was set to $\alpha = 0.05$.

In **study II**, the data on HEMA diffusion in collagen after DMSO or ethanol incubation was subjected to two-way ANOVA. The variables were type of solvent and the solvent's concentration. To determine the interaction between solvents, Holm-Sidak test as a *post-hoc* was performed at $\alpha = 0.05$. The data on elastic moduli (M) was evaluated by repeated-measures ANOVA. Variables were solvent type and pretreatment condition, while the time-point assigned as the repeated factor. The Holm-Sidak test was also used to evaluate the interaction and differences between the tested groups and assigned at $\alpha = 0.05$.

In **study III**, two-way ANOVA was performed to evaluate the data of monomer conversion (DC %), biaxial flexural strength, and ΔKNH%. Data of water sorption (Wsp) and water solubility (Wsu) were performed using three-way ANOVA. *Post hoc* analyses were performed with Tukey's test ($\alpha = 0.05$), using SPSS statistics.

In **study IV**, transdentinal and eluates cytotoxicity were analyzed using two-way ANOVA. Variables were resin type and concentration of DMSO. Tukey's multiple comparison test was used to compare the data and assigned at $\alpha = 0.05$, using SPSS statistics, version 23 (SPSS Inc., Armonk, NY, USA).

7 Results

7.1 Microtensile bond strength (Study I)

The short-term microtensile bond strength results (after 24 h of incubation at 37 °C), as well as results of stored specimens, are presented in **Fig. 3**. The short-term μTBS did not show significant changes between DMSO- pretreated specimens compared to control (no dentin pretreatment with DMSO) ($p>0.05$). On the other hand, after 6 months of incubation in AS, there was a significant 36% reduction in bond strength of control specimens ($p<0.05$), compared to a 5–16% reduction in bond strength for DMSO-pretreated specimens, which did not significantly differ from the short-term μTBS results ($p>0.05$). The lowest reduction in bond strength after storage was observed with the 5% DMSO-pretreated group (**Fig.3**).

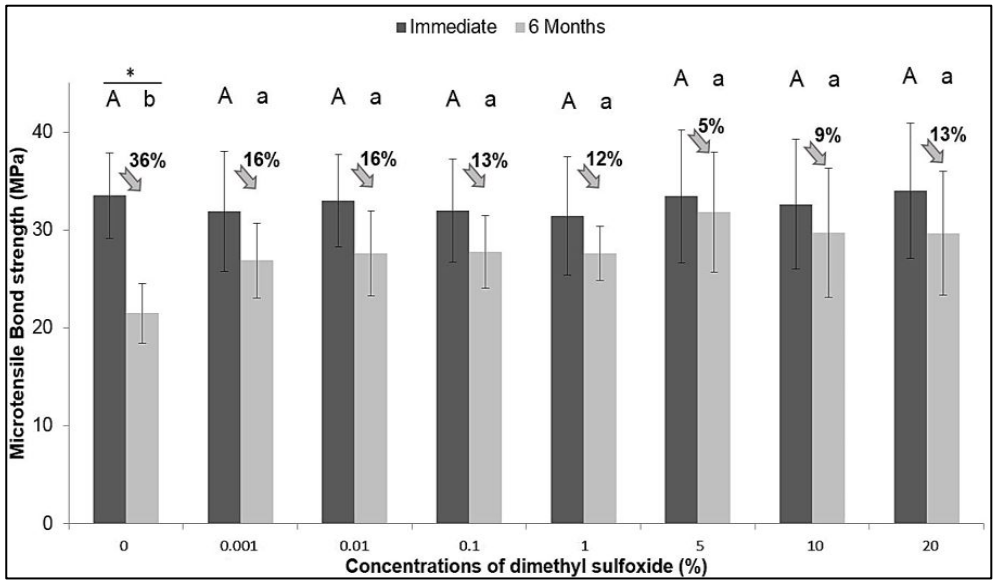


Figure 3. Results of microtensile bond strength (MPa) of control or several DMSO concentrations used (n=6 teeth/group) after 24-h or after 6-m storage. Upper and lower case letters show the statistical significance between short-term and aged specimens, respectively. Asterisks indicate the significant difference between the time points within the same tested group ($p<0.05$). Modified from Salim Al-Ani *et al.*, 2018, study I, with permission.

7.2 Failure mode (Study I)

Results of failure mode are presented in **Fig. 4**. For both incubation times, most of the failure types were mixed fractures. Premature failures were observed with aged specimens, especially with control and 0.001% DMSO-pretreated dentin. Two pretesting failures were noticed with short-term control specimens, compared to 2 and 12 specimens with aged 0.001% DMSO-pretreated and aged control specimens, respectively.

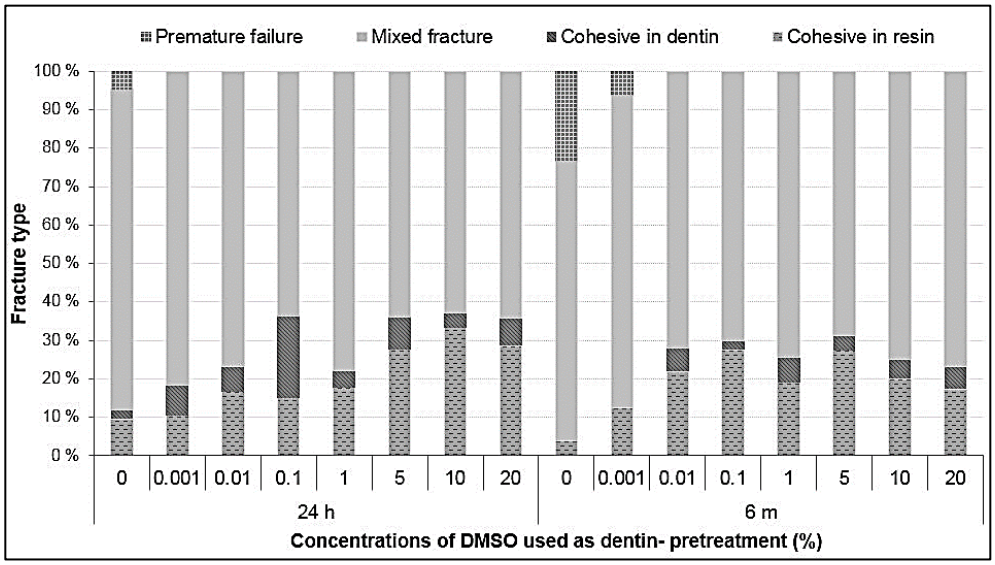


Figure 4. Distribution of failure mode of the DMSO-pretreated dentin in several concentrations (%), after 24-h or after 6-m storage. Modified from Salim Al-Ani *et al.*, 2018, study I, with permission.

7.3 Nanoleakage (Study I)

Results of silver particles accumulated at the hybrid layer of resin-dentin bonded sticks are shown in **Fig. 5**. Nanoleakage of short-term-evaluated specimens was significantly lower with the 5–10% DMSO-pretreated specimens, compared to other DMSO-pretreated specimens and control (no DMSO) ($p<0.05$). Aged specimens showed an overall increase in silver accumulation. However, compared to the short-term specimens, significant increase in the percentage of silver precipitation was observed only with control and 0.001–0.1 vol. % DMSO pretreated specimens ($p<0.05$). The 5 vol. % DMSO-pretreated specimens showed the lowest insignificant increase in nanoleakge (with 6% increase in silver percentage).

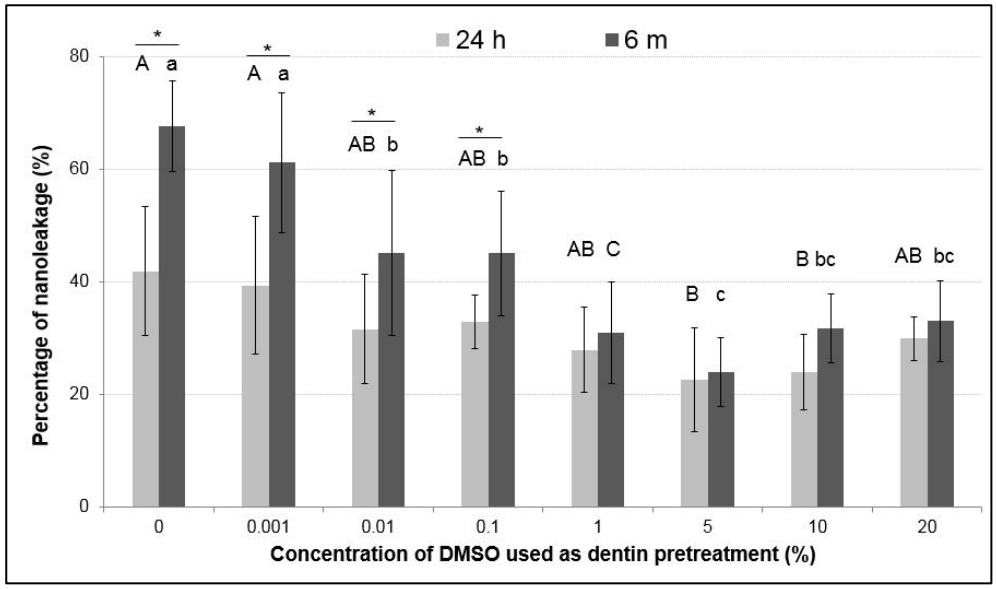


Figure 5. Percentage of silver particles accumulated within the hybrid layer (%). Mean values and standard deviation (n=6 sticks/group). Different upper case and lower-case letters indicate statistical significance between short-term and aged groups, respectively. Asterisks indicate significant difference between shot-term and after 6 months aging ($p<0.05$). Salim Al-Ani *et al.*, 2018, study I, with permission.

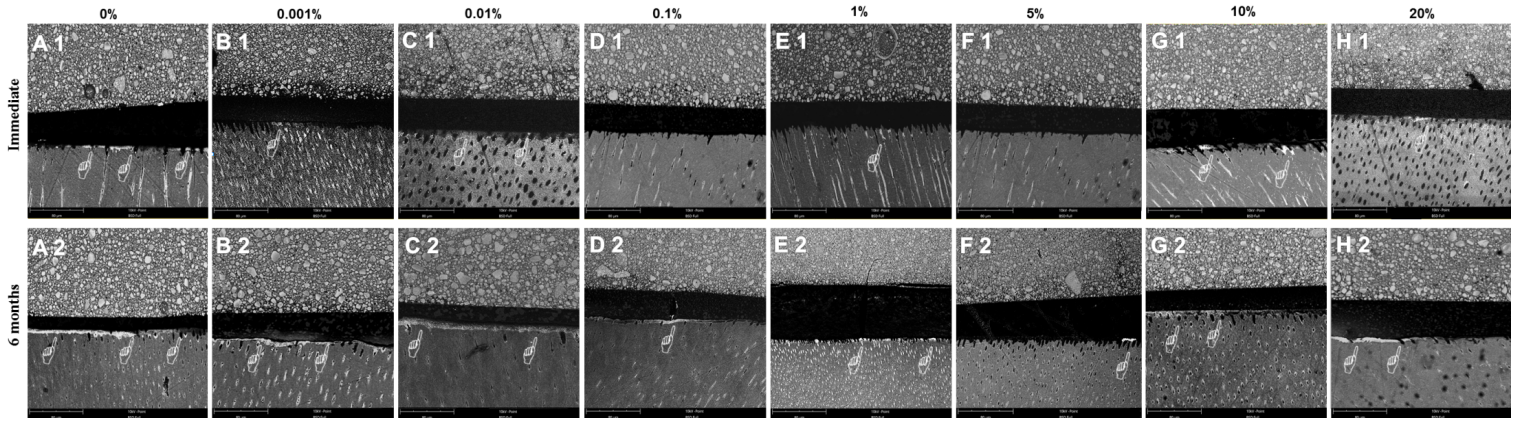


Figure 6. Representative backscattered SEM micrographs at 1000X presenting accumulated silver within the hybrid layer (HL) after several concentrations of DMSO's pretreatment and control (0, 0.001, 0.01, 0.1, 1, 5, 10 and 20 vol. % DMSO respectively). Immediately DMSO-pretreated groups (A1, B1, C1, D1, E1, F1, G1, and H1), and 6-months stored specimens in AS (A2, B2, C2, D2, E2, F2, G2 and H2). Silver particles were accumulated more clearly at the HA with control, 0.001, 0.01, 0.1% DMSO-pretreated stored specimens in AS (A2, B2, C2 and D2). More silver also observed with stored specimens compared to immediately DMSO-pretreated specimens for all groups, especially with the control, 0.001, 0.01% DMSO-pretreated specimens. Specimens treated with 5% DMSO showed the lowest impregnation of silver particles within hybrid layer compared to control. (Salim Al-Ani et al.2018, study I, with permission).

7.4 HEMA uptake of demineralized dentin (Study II)

Results of HEMA uptake are presented in **Fig. 7**. When DMSO was used as dentin pretreatment, there was a significant increase in HEMA uptake with all DMSO-pretreated dentin cubes, compared to control (no DMSO-pretreatment). On the other hand, 0.1% and higher ethanol-pretreated dentin cubes showed significant increase in HEMA uptake compared to lower concentration (0.01% ethanol) and control. By comparing the results of both polar solvents, significant increase in HEMA uptake was observed with 0.01%, 5%, and 10% DMSO-pretreatment compared to similar concentrations of ethanol-pretreatment specimens.

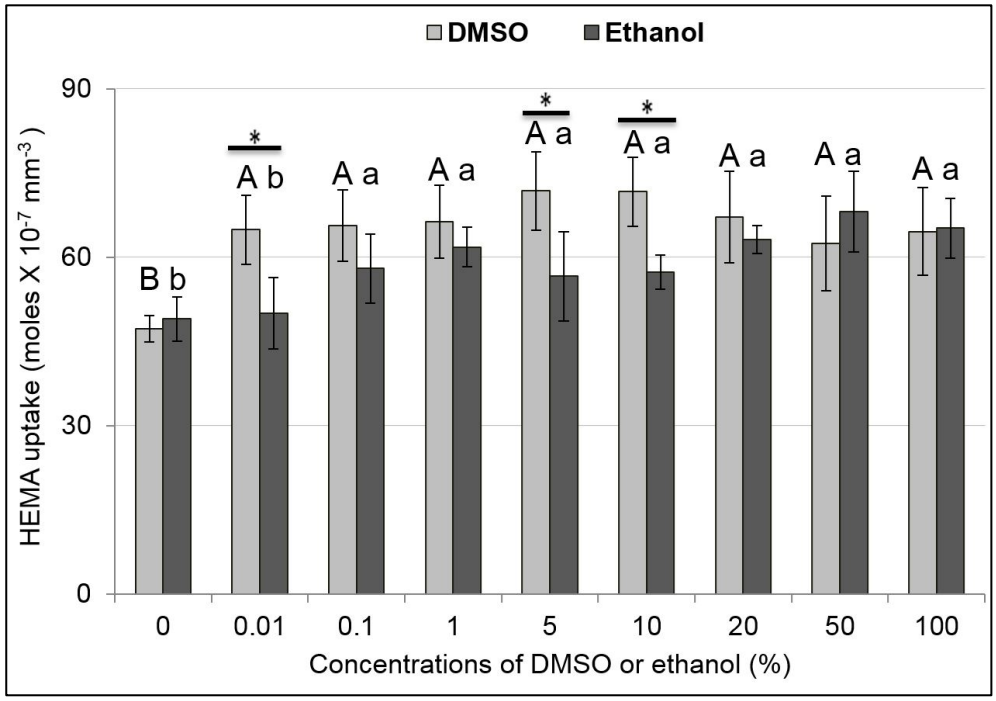


Figure 7. HEMA uptake by demineralized dentin pretreated with DMSO or ethanol. Different upper case and lower case letters indicate statistical significance of DMSO or ethanol treated dentin, respectively. Asterisks show statistical significance between DMSO or ethanol treated specimens at the same concentrations (%) ($p < 0.05$). Salim Al-Ani *et al.*, 2019a, study II, with permission.

7.5 Modulus of elasticity (Study II)

Results of elastic moduli of dentin beams incubated in DMSO or ethanol for 10, 30, 60, and 24 h are shown in **Fig. 8**. The baseline readings of all beams before DMSO or ethanol immersion (evaluated after water incubation, before DMSO or ethanol immersion) were less than 3 MPa. The baseline readings were not significantly

different between test groups ($p>0.05$). Both variables (solvent concentration and incubation time) as well as their interaction were significantly different ($p<0.001$). Therefore, each solvent effect was statistically analyzed with respect to its concentrations and time of incubation. Dentin beam pretreatment with DMSO in a concentration of 50% or more showed significant elevation in elastic moduli (in MPa) after the first time point of incubation (10 min), compared to beams immersed in lower DMSO concentrations and control (incubated in water) ($p<0.05$). On the other hand, E of dentin after 100% ethanol treatment was significantly higher than other lower percentages of ethanol and control after 10 min of ethanol incubation ($p<0.05$). Comparing the data of ethanol and DMSO-incubated beams after the first incubation time (10 min), the stiffness values of 50–100% DMSO-pretreated beams were significantly higher than for similar concentrations of ethanol-pretreated beams. After 24 h of immersion in water, all the beams treated with DMSO or ethanol returned back to the initial stiffness.

The highest values of E (in MPa) was observed from dentin beams treated with 100% ethanol for 60 min, compared to similar or lower ethanol or DMSO pretreatments. The effect of both solvents was time- and concentration-dependent. E of dentin beams when using high concentrations of DMSO (50–100%) was significantly increased from the first period of DMSO incubation (10 min). Furthermore, only 100% ethanol pretreatment showed a significant increase from the first incubation period (10 min).

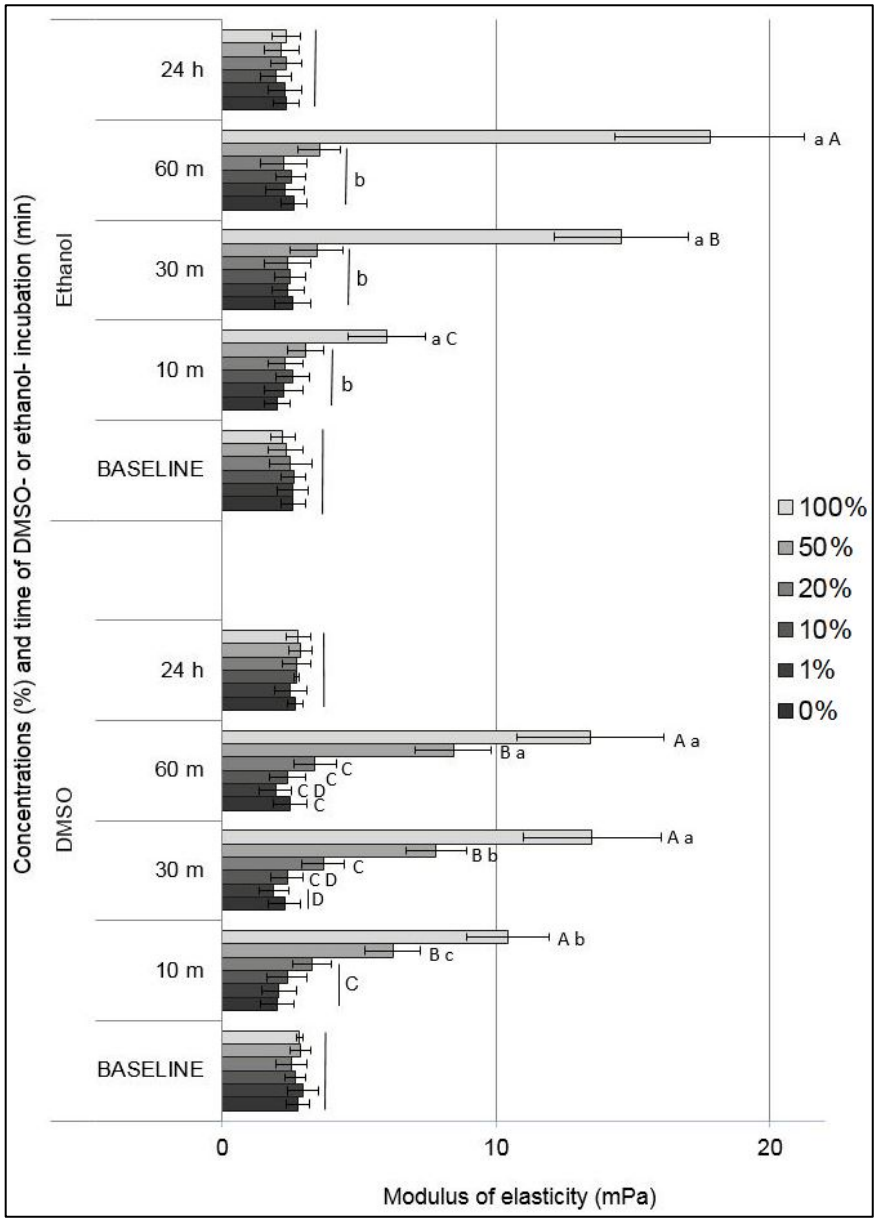


Figure 8. lastic moduli (E) of dentin beams pretreated with several concentrations of DMSO or ethanol (%), for different time points. In DMSO- treated beams: Different upper-case letters indicate the statistical significance between DMSO concentrations of the same time point. Different lower case- letters indicate the statistical significance between the same concentrations in different time points. In ethanol-treated beams: Lower case letters indicate the statistical significance between ethanol concentrations of the same time point. Upper case letters indicate the statistical significance between the same concentrations in different time points ($p<0.05$). Salim Al-Ani *et al.*, 2019a, study II, with permission.

7.6 Dissociation of dentinal collagen (Study II)

Dentin dissociation occurred in dentin slices incubated in several DMSO concentrations (1, 10, 50, 100%) for 10, 30, 60 min. Visual observation of the specimens showed that dentin slices pretreated with 50 and 100% DMSO were different, showing increased transparency when compared to the lower DMSO concentrations used from the first 10 min of incubation. Other lower DMSO concentrations (1, 10%) did not show the dissociation effect on dentin slices (**Fig. 9**).

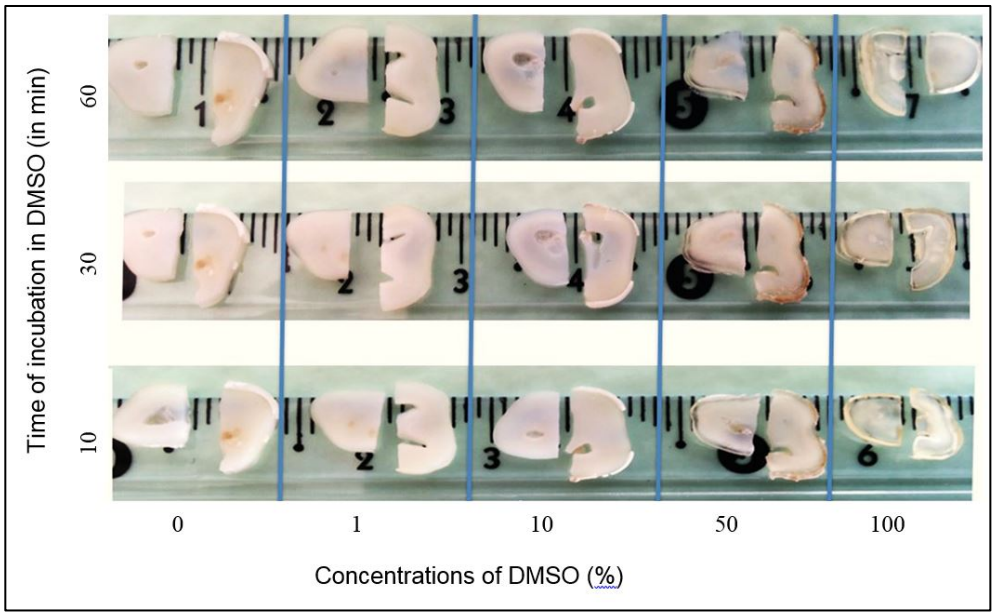


Figure 9. Effect of concentration of DMSO (%) on dentin dissociation appears as clearing effect on dentin slices after DMSO incubation for specific time (10, 30 and 60 min). The effects were observed when high concentration of DMSO (50–100%) was used to incubate dentin beams. Lower DMS concentrations did not show dissociation of dentin. Salim Al-Ani *et al.*, 2019a, study II, with permission.

7.7 Biaxial flexural strength (Study III)

The results of biaxial flexural strength of both DMSO/R2 and R5 are shown in **Fig. 10**. Generally, DMSO/R2 showed significantly higher flexural strength values compared to DMSO/R5. A statistically significant decrease in flexural strength was observed with 5–10% DMSO/R2, in the range of 30 to 50%, compared to control and other lower DMSO concentrations used after 24 h of water storage, respectively. After 30 d of water storage, the significant reduction in flexural strength was between 65–80% for the 5–10% DMSO/R2, compared to control and other lower DMSO

concentrations used, respectively. DMSO/R5 up to 1% did not cause reduction in flexural strength; higher concentrations of DMSO (5–10%) caused significant reduction in flexural strength after 24 h of water storage. After 30 d of storage, 5–10% DMSO/R5 caused around 30% reduction in flexural strength.

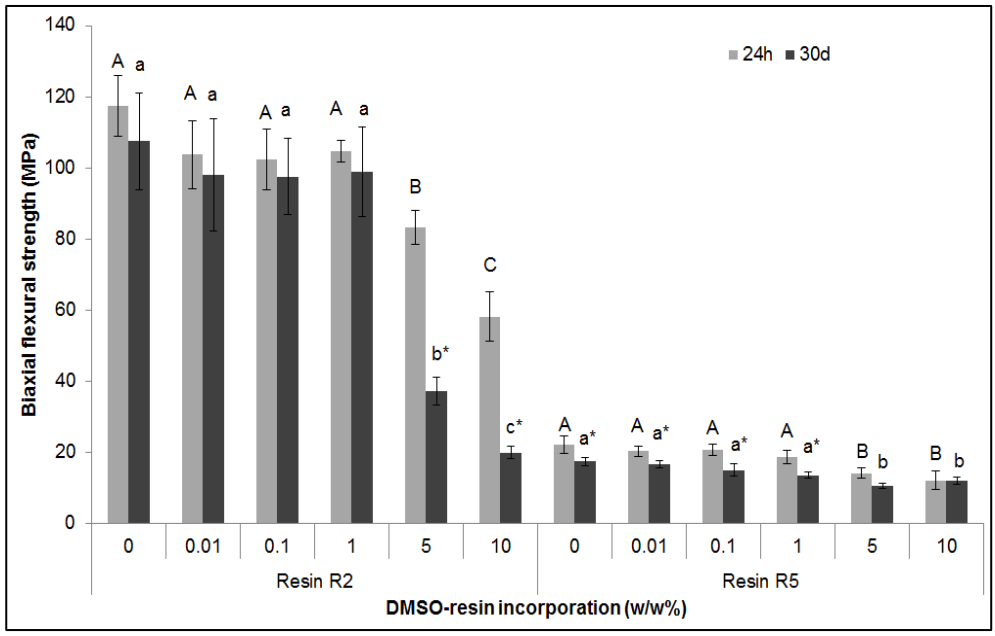


Figure 10. Results of biaxial flexural strength (n=6) of R2 and R5 resins containing several concentrations of DMSO and evaluated after 24 h or 30 days of water storage at 37 ℃. Different upper-and lower-case letters indicate statistical significance between DMSO concentrations at 24 h and 30 days, respectively. Asterisks indicate significant differences between specific concentrations at different incubation times. Salim Al-Ani *et al.*, 2019b, study III, with permission.

7.8 Degree of conversion (Study III)

The results for degree of conversion are shown in **Fig. 11**. DMSO incorporation into hydrophilic resin (R5) showed a significantly higher degree of conversion compared to hydrophobic resin in all DMSO percentages used for incorporation ($p<0.05$). DMSO incorporated into R2 in a concentration of 1% or less showed no significant effects on conversion compared to neat R2 (control). Similarly, DMSO incorporated into R5 in lower concentrations (≤ 1%) was not significantly different from the neat resin (control) ($p>0.05$). However, 5–10% incorporation into R2 or R5 was significantly higher than lower DMSO concentrations and control ($p<0.05$). The increase observed with 5% and 10% DMSO/R2 ranged between 12% and 22%,

respectively. Furthermore, 5% and 10% DMSO/R5 showed 4% and 14% increases in degree of conversion, respectively.

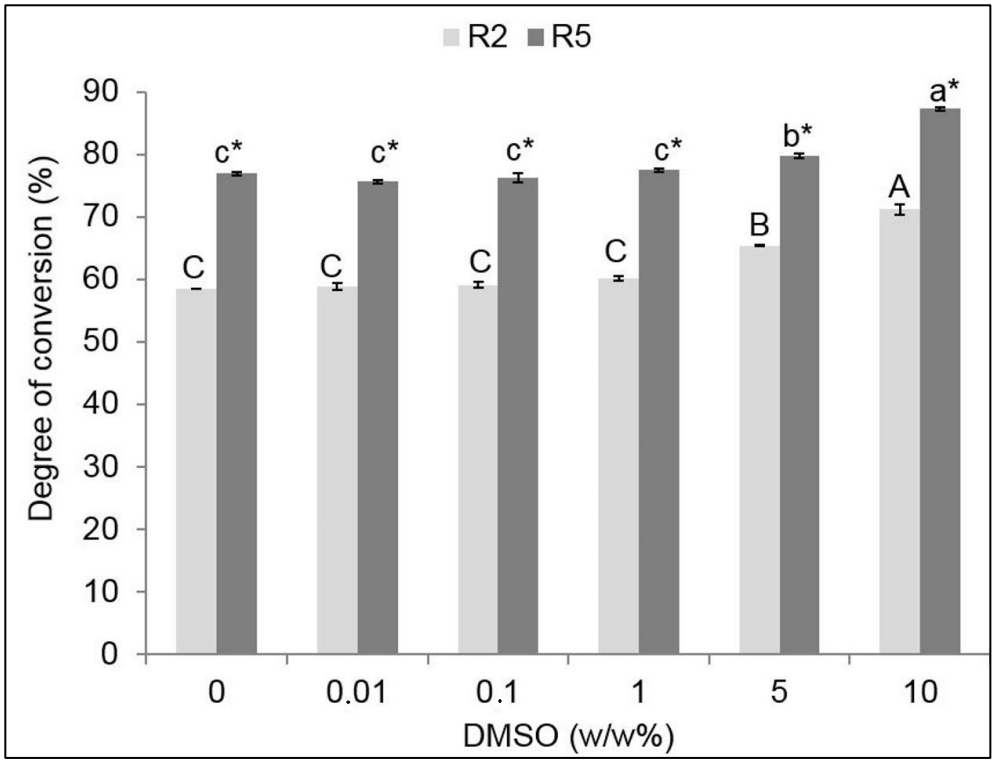


Figure 11. Results of degree of conversion (n=5) of R2 and R5 resins containing several concentrations of DMSO. Different upper- and lower- case letters indicate significant differences between DMSO concentrations for R2 and R5, respectively (p<0.05). Asterisks indicates significantly higher conversion degrees considering the corresponding DMSO concentration between R2 and R5 (p<0.05). Salim Al-Ani *et al.*, 2019b, study III, with permission.

7.9 Water sorption/solubility (Study III)

Results of DMSO/R2 or R5 are presented in **Fig. 12**. Neither water sorption nor solubility was significantly affected with up to 1% DMSO incorporation in either resin used (R2 and R5) compared to the neat resins (p>0.05). Higher DMSO incorporation into R2 and R5 (5% and 10%) caused a significant increase in water sorption and solubility (p<0.05).

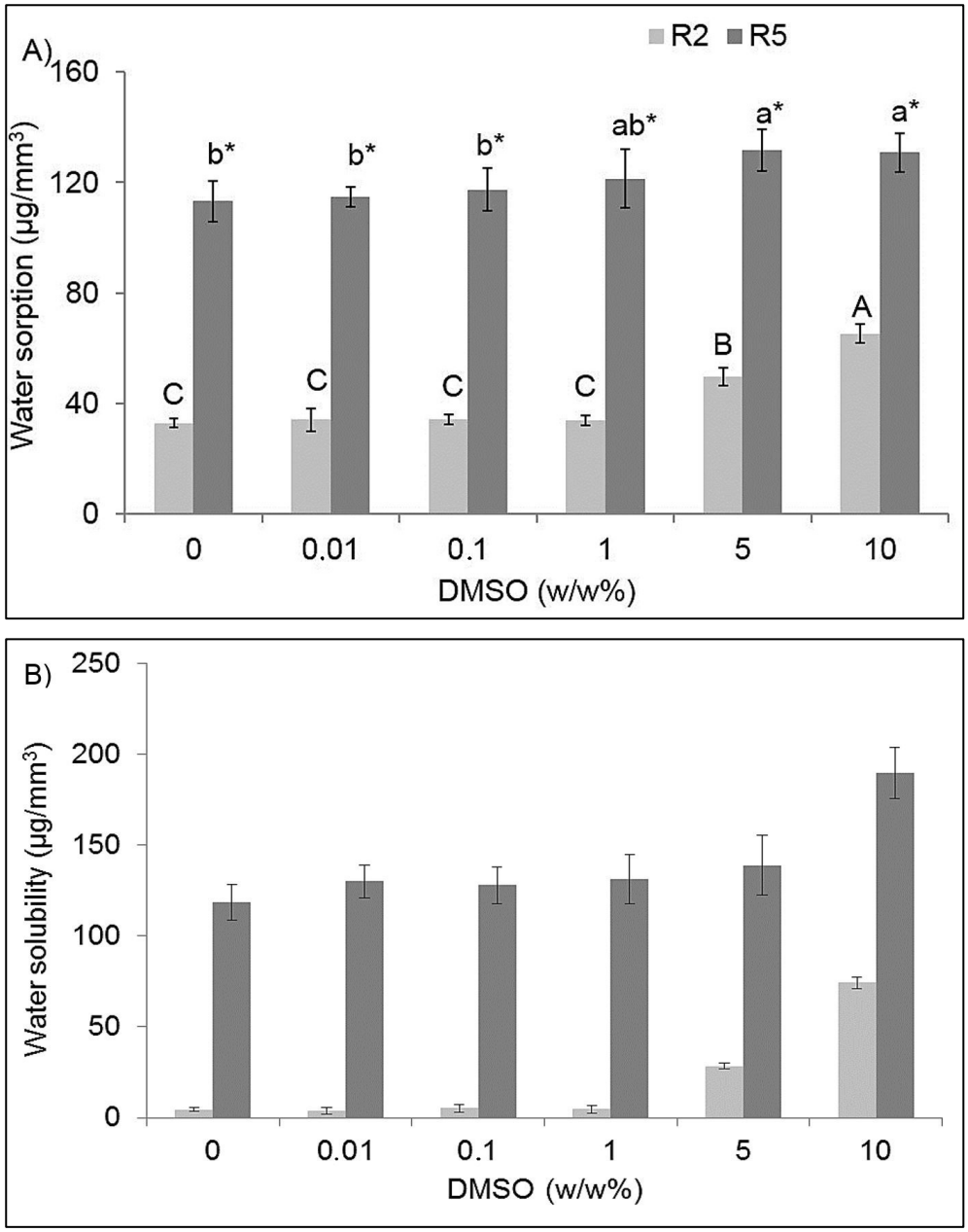


Figure 12. Results of water sorption (A) and water solubility (B) (n=7) of R2 and R5 resins containing several concentrations of DMSO, after 28 days of water storage at 37℃. Upper and lower case- letters indicate significant differences between DMSO concentrations for resin R2 or R5, respectively (p<0.05). Asterisks indicate statistical significance between R5 and R2 for each DMSO concentration (p<0.05). Salim Al-Ani *et al.*, 2019b, study III, with permission.

The variations in mass changes are presented in **Fig. 13**. Changes in discs masses (as percentages) as a result of water incubation for 10 ascending time intervals within 28 days were evaluated to obtain the kinetics of water uptake from each DMSO-resin disc. The highest water uptake percentages were observed from the first or second day of water incubation for both DMSO/R2 and R5. However, low concentrations of DMSO/R5 (≤ 1%) showed more water uptake compared to similar concentrations of DMSO/R2. Incorporation of 1% or less of DMSO into R2 or R5 did not affect water sorption, compared to neat R2 or R5, respectively. Higher concentrations of DMSO- incorporated into resin discs (5–10%) showed more water uptake in the first 48 h of water incubation, but then loss of mass was also higher. The opposite occurred with DMSO/R5. High concentrations of DMSO (5–10%) showed less water uptake in the first 48 h of water immersion, compared to lower DMSO/R5.

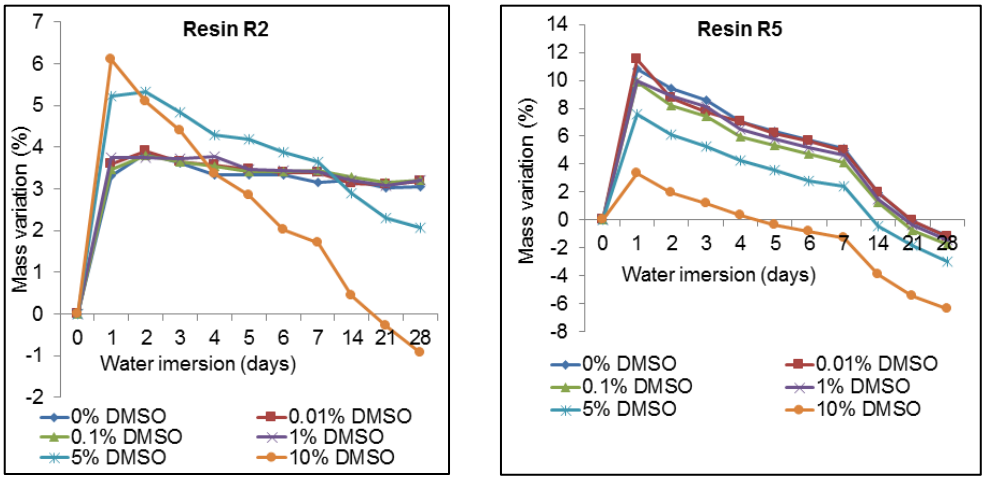


Figure 13. Changes of masses over time (in %) for R2 and R5 containing several concentrations of DMSO during 28 days of water storage at 37℃. Salim Al-Ani *et al.*, 2019b, study III, with permission.

7.10 Microhardness (Study III)

The Knoop microhardness measurement of dry, 24 h H_2O storage and 4 h ethanol storage samples are presented in **Fig. 14**. In general, ΔKNH means were significantly higher with DMSO/R2 resins compared to DMSO/R5 with regard to the storage condition. At the first dry stage, DMSO incorporation up to 1% to both resins did not cause significant change in microhardness, while 5 and 10% DMSO incorporation caused a significant increase. The reduction of microhardness with 5 and 10% DMSO/R2 ranged between 33% and 45%, respectively. When ethanol storage was applied, a reduction of 55–70% was observed with 5 and 10%

DMSO/R2, while water storage did not show significant reduction in microhardness compared to the dry stage. Furthermore, water storage of DMSO/R5 caused extensive reduction in microhardness (approximately 70%), compared to the dry stage. Ethanol storage caused more reduction in microhardness for DMSO/R5.

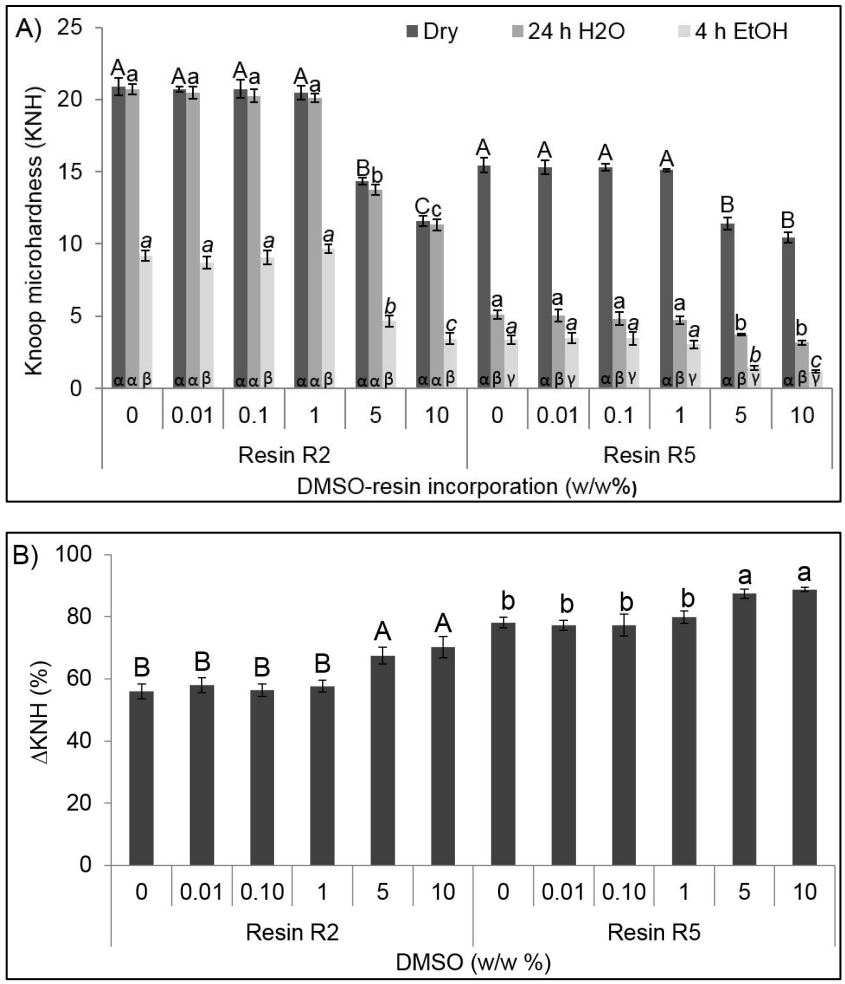


Figure 14. Means and standard deviation of: (A) Knoop microhardness and (B) ΔKNH % reduction of hydrophobic or hydrophilic resins containing several percentages of DMSO after 24 h of water incubation or pure ethanol incubation. In Fig. A, for neat and all DMSO-solvated hydrophobic (R2) or hydrophilic (R5) resins: Different upper-case capital letters indicate statistical significance when specimens stored in dry state (no treatment). Different lower-case letters indicate statistical significance after 24 h of storage in distilled water. Italic lowercase letters indicate statistical significance after incubation in 100% ethanol for 4 h Greek letters indicate the statistical significance between all storage mediums used according to Tukey test (p<0.05). Fig. B (ΔKNH %), Different capital letters indicate the statistical significance for the hydrophobic resins groups (R2), different lowercase letters indicate the statistical significance between groups of the hydrophilic resin (R5). Salim Al-Ani *et al.*, 2019b, study III, with permission.

7.11 Dentin barrier test (Study IV)

Results of SV40 cell viability after dentin pretreatment with DMSO incorporated into the hydrophobic or hydrophilic resins are presented in **Fig. 15**. The cell viability of dentin discs treated with all DMSO/R2 did not change significantly, compared to negative control and neat R2 ($p>0.05$). Furthermore, the cell viability of dentin discs treated with 1 w/w % and more of DMSO/R5 were significantly lower than all DMSO/R2 and negative control ($p<0.05$). Discs treated with 1 w/w % and more of DMSO/R5 showed no significant difference compared to positive control.

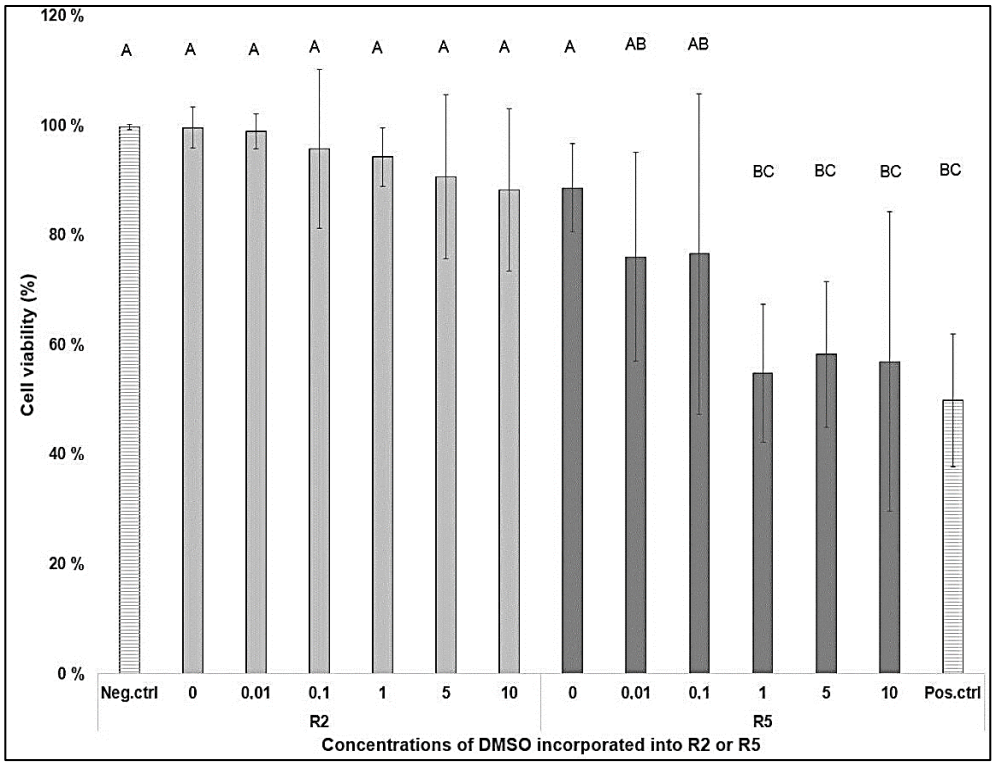


Figure 15. he percentage of cell viability (mean and standard deviations) of test groups at dentin barrier test. The groups marked with similar capital letters are not significantly different ($p>0.05$).

7.12 Cytotoxicity of DMSO- resin elutes (Study IV)

Results of HGF-1 cell viability after 24 h exposure to eluates from DMSO-incorporated resins are shown in **Fig. 16**. All the resins were significantly lower than negative control. Eluates from all DMSO/R2 had significantly higher percentages of cell viability than DMSO/R5 ($p<0.05$). Slight, non-significant variation in the cell

viability is more obvious with 1–5 w/w % DMSO/R2, compared to other DMSO/R2 concentrations (p>0.05). No significant difference in the percentages of cell viability was observed among all groups treated with eluates of DMSO/R5 (p>0.05).

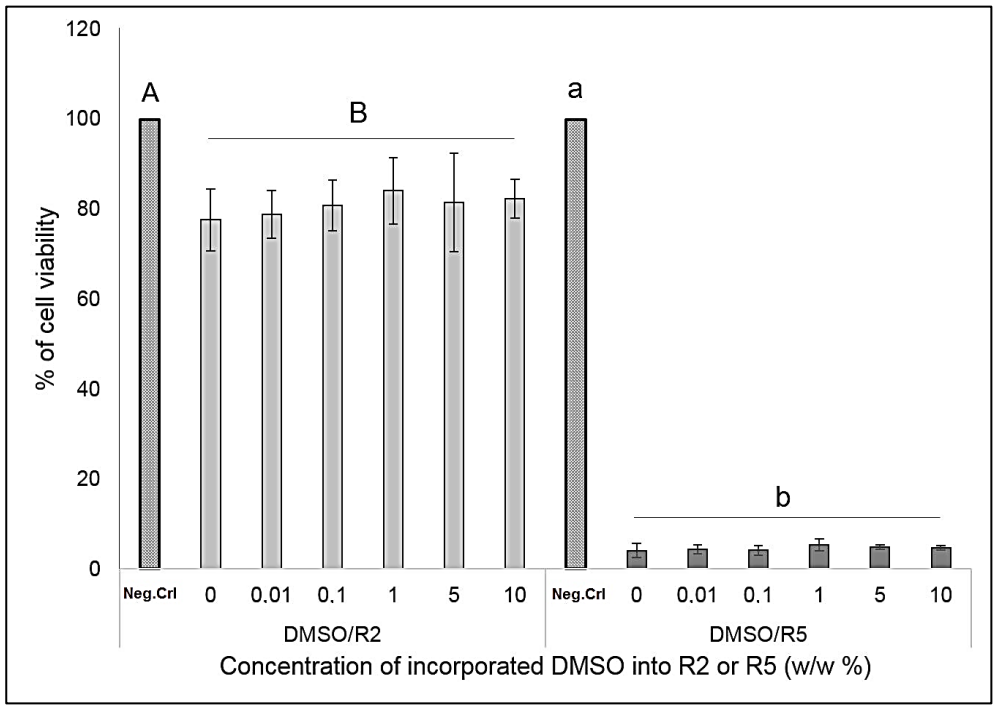


Figure 16. Percentage of the viable cells after exposure to eluates materials from each mixture of R2 or R5 containing several concentrations of DMSO and negative control (n=10/group; p<0.05). Different upper-case letters indicate the statistical significance for R2-DMSO groups. Different lower-case letters indicate the statistical significance for R5-DMSO groups.

8 Discussion

The aim of the present series of studies was to investigate the possibility of incorporating DMSO into dental adhesive systems, to optimize the long-term stability of resin-dentin bonding. More specifically, the aim is to find the optimal concentration or concentrations of DMSO that can be safely included in adhesive system, and a way of incorporation, either directly onto the adhesive, or as a dentin-pretreatment agent. Thus, several concentrations of DMSO were evaluated in two ways. The first way was to use DMSO directly on demineralized dentin surfaces as pretreatment and evaluating the bond strength and nanoleakage **(Study I)**, or evaluating the effects on dentin stiffness, permeability, and dissociation **(Study II).** The second way was to incorporate DMSO into adhesive resins, by evaluating the effects on mechanic-physical properties **(Study III)** and evaluating the effects on direct and indirect cytotoxicity **(Study IV)**.

Microtensile bond strength is a well-established and reliable method for investigating the stability of adhesive bonding to dentin (Armstrong *et al.*, 2010). The prediction of clinical outcomes is depending on not only the initial bond-strength results, but also on long-term results as well as other factors (Van Meerbeek *et al.*, 2010; Heintze *et al.,* 2015). Nevertheless, obtaining long-term microtensile bond strength results and performing other laboratory investigations can help predicting the clinical outcomes (Van Meerbeek *et al.*, 2010; Heintze *et al.,* 2015). Therefore, evaluation of microtensile bond strength and nanoleakage was performed after 24 h of storage (initial μTBS) and after 6 m of storage (aged μTBS). The stored specimens were evaluated after 6 months, since that time was long enough to undergo a rapid degradation process (Hebling *et al.*, 2005), and sufficient to demonstrate the loss of bond strength (Tjäderhane *et al.,* 2015).

Nanoleakage is another scientifically accepted method for investigating the quality of resin-dentin bonds after dentin bio-modification, when combined with other laboratory methods (Okuda *et al.*, 2002). This method has been widely used in several studies after pretreatment of dentin with solvents, compounds, crosslinkers and enzyme inhibitors (Hashimoto *et al.*, 2004; Stanislawczuk *et al.,* 2011; Almahdy *et al.,* 2012; Tjäderhane *et al.*, 2013c; Sabatini *et al.,* 2015; Hass *et al.*, 2016). It is important to observe the leakage that occurs at the hybrid layer (Sano *et al.*, 1995).

Specimens were evaluated under SEM, according to the protocol described above (Tay *et al.,* 2003; Klein-Júnior *et al.*, 2008). The test was performed after 24 h or after 6 months, which was sufficient to show changes in leakage at the hybrid layer (Hebling *et al.*, 2005).

The ability of solvents to facilitate diffusion of monomers through dentin is an important criterion to investigate. It allows better understanding of the mechanism of monomer penetration in dentin (Van Landuyt *et al.*, 2007). Dentin uptake of HEMA, a small-molecule hydrophilic monomer widely used in adhesives has been used to reflect the result of dentin pretreatment with solvents (Pashley *et al.*, 2000).

Therefore, HEMA uptake through demineralized dentin cubes after ethanol or DMSO pretreatment was used to highlight the role of both solvents on monomer uptake in demineralized dentin matrix (**Study II**). Modulus of elasticity of demineralized dentin beams after pretreatment with a specific agent/solution/crosslinker is an established and reliable method used to understand the impact on dentin after treatment (Maciel *et al.*, 1996; Nalla *et al.*, 2006; Agee *et al.,* 2006; Bedran-Russo *et al.*, 2008; Cadenaro *et al.*, 2009c; Tezvergil-Mutluay *et al.*, 2010). Ethanol and methanol are examples of volatile solvents that are used to solvate different small- or large- molecule monomers. These solvents were also used as dentin pretreatment, to investigate their role on the stiffness of dentin (Carvalho *et al.,* 2003; Becker *et al.*, 2007). Since DMSO is a non-volatile solvent, it is important to evaluate and compare its effect and that of ethanol on the stiffness of dentin (**Study II**).

The optical clearing effect of DMSO, indicating collage fibrils dissociation, has previously been demonstrated on skin (Bui *et al.*, 2009; Zimmerley *et al.*, 2009) and dentin (Tjäderhane *et al.*, 2013c). However, only absolute DMSO has been used to pretreat dentin for 30 min (Tjäderhane *et al.*, 2013c). Therefore, the effect of other lower concentrations of DMSO on collagen, as well as the effect of DMSO incubation time, was evaluated to demonstrate the potential time- and concentration-dependence of its action in dentin (**Study II**).

Degree of monomer conversion (DC) (Rueggeberg *et al.,* 1990) is a well-established and scientifically accepted method. It is one of the methods used to detect unreacted residual monomers in resin-based adhesives and composites (Yoshida *et al.,* 1994; Gauthier *et al.*, 2005). It determines the amount of carbon double bonds (C = C) converted into single carbon bonds, which reflects the efficiency of polymerization (Peutzfeldt *et al.,* 1994). Evaluation of DC is necessary to present the mechanical properties of adhesive systems (Peutzfeldt *et al.,* 1997). An improper degree of conversion reflects the hydrolytic degradation of monomer, and low quality of monomers interaction (Peutzfeldt *et al.,* 2000). To evaluate the role of DMSO in the degree of monomer conversion of adhesive systems, DMSO was incorporated in several concentrations into R2 and R5 resin adhesives (**Study III**).

Water sorption and water solubility are also methods used to evaluate the mechanical properties of dental adhesives with different hydrophilicities (Malacarne *et al.*, 2006). There is a strong correlation between the degree of hydrophilicity and the amount of absorbed water, which diffuses inside the resin and causes changes in solubility (Malacarne *et al.*, 2006; Malacarne-Zanon *et al.*, 2009). Furthermore, penetration of water within the polymer network causes reduction of H-bonding efficiency. This may lead to an increased chance of plasticization and degradation of resin components (Musto *et al.*, 2002; Ito *et al.*, 2005; Manso *et al.*, 2008).

However, hydrophilicity is not the only factor that determines water sorption. The presence of residual solvent within the intermolecular polymer network is another factor that can affect the amount of absorbed water (Yiu *et al.*, 2006; Malacarne-Zanon *et al.*, 2009). Incubation of resin discs of neat and DMSO-incorporated R2 and R5 was performed for 28 d in water to allow water to fully penetrate resin discs. Disc mass was measured every 24 h for the first week to investigate the gradual changes in mass variation (Michelsen *et al.,* 2003). In total, the masses were measured 10 times within 28 d to investigate the maximum increase/decrease in mass, reflecting the absorption and diffusion rate of water inside resin discs (Malacarne *et al.*, 2006) (**Study III**).

The biaxial flexural strength test is used to investigate the flexural strength of resin-based composites. It is a more accurate and accepted method compared to the uniaxial flexural strength test for use with resin-based materials (Chung *et al.*, 2004; Pick *et al.*, 2010). In this method, the stress is distributed at the disc center throughout the disc thickness (Huang *et al.,* 2011). It is one method used to evaluate the mechanical properties of resin adhesives, since it circumscribes different types of stresses, including tension, compression, and shear stress under load (Sauro *et al.*, 2018). It was reported that aging in water for 30 d reduces the flexural strength of dental composites up to 25% (Ferracane *et al.,* 1995). Therefore, this method was addressed here as well, to investigate the effect of water- aging of DMSO-incorporated resin discs (**Study III**).

Softening of resin specimens was used as an indirect method to investigate the degree of polymer crosslinking, determined by comparing the hardness of discs prior to and after water or ethanol immersion (Benetti *et al.,* 2009; de Moraes *et al.,* 2007; Soh *et al.*, 2004). Thus, DMSO/R2 and DMSO/R5 discs were prepared and solvated in water, then ethanol, to evaluate the degree of polymer crosslinking, using Knoop microhardness (**Study III**). ΔKNH % was analyzed to investigate the possible gradual effect of DMSO incorporation into R2 and R5, since the possible changes in ΔKNH% appear with the increase in uptake of solvents (Schneider *et al.*, 2008).

The cytotoxic reaction of pulp cells and tissues after direct or indirect exposure to resin-based materials is a widely used method to simulate pulpal response to dentin bonding agents (Stanley, 1993; Hebling *et al.,* 1999; Kaga *et al.*, 2001; Chen

et al., 2003; Soheili *et al.,* 2003). Monolayer cultures of odontoblast-like cells or fibroblast cells are used to evaluate biocompatibility of bonding materials (Moharamzadeh *et al.,* 2009; Schmalz *et al.,* 2009). The pulp-derived bovine cells were used for *in vitro* investigation of pulp chamber methodology when used in 3-D model (Schmalz *et al.*, 2001; Thonemann *et al.*, 2002). They demonstrate the phenotypic characteristics of the odontoblast-like cells and show higher sensitivity toward tested materials (Thonemann *et al.,* 2000). On the other hand, human gingival fibroblasts were selected to receive eluted materials because their response comes closer to the clinical scenario in the event of contact of eluted materials with the gingival epithelium (Moharamzadeh *et al.,* 2009).

Transdentinal cytotoxicity is a method to simulate the clinical response of biological reaction of pulp tissue to resin-based materials. Dentin disks act as barriers between cell culture (pulp tissue in clinical scenarios) and the tested resin-based material (Schmalz *et al.,* 1996; Schmalz *et al.,* 2001; Lanza *et al.*, 2009; Rosetti Lessa *et al.*, 2010; Bianchi *et al.*, 2013; Scheffel *et al.*, 2015a; Scheffel, *et al.*, 2015b; da Fonseca Roberti Garcia *et al.*, 2016). Another technique was used to investigate the cytotoxicity of eluted substances from resin-based materials, after incubation in medium for 24 h (Kaga *et al.*, 2001; Huang *et al.,* 2002; Szep *et al.*, 2002) (**Study IV**). MTT assay was used to evaluate the proliferation rate of cells after direct or indirect contact with resin discs and their corresponding DMSO. This assay is one of the most commonly used methods to investigate the cytotoxicity of resin-based materials (Mosmann, 1983). It is a fast, simple, and inexpensive method to evaluate cell proliferation (Moharamzadeh *et al.,* 2009).

8.1 The effect of DMSO on dentin (Studies I and II, part of Study IV)

After 24-h storage, microtensile bond strength was not affected by pretreatment with DMSO. However, the bond strength of stored specimens pretreated with DMSO was stabilized during 6 months of incubation, compared to control, in which 36% reduction in bond strength was observed (**Study I**). The percentage of silver precipitation at the hybrid layer was observed with almost all 24- h or 6 m control and DMSO- treated groups. However, it was significantly higher with 0.1% and less DMSO-pretreated specimens, including control. On the other hand, only slight increase appeared with 1% and more DMSO- pretreated specimens after aging for 6 m (**Fig. 5)**. Interestingly, 5% and 10% DMSO- pretreated specimens showed the lowest percentage of silver precipitation compared to control and 0.001% DMSO-pretreated specimens. It might be also related to the ability of DMSO to improve both dentin wettability and resin penetration (Tjäderhane *et al.*, 2013c; Mehtälä *et al.,* 2010; Mehtälä, Pashley and Tjäderhane, 2017).

The stability of bond strength after 6 m of storage might be related to the potential ability of DMSO to penetrate the exposed collagen network and to improve the wettability of demineralized dentin (Mehtälä, Pashley and Tjäderhane, 2017), leading to the enhancement of monomers infiltration into wet demineralized dentin (Stape *et al.*, 2015). Furthermore, DMSO molecules can strongly bind to free, unbound water molecules within collagen fibrils, after breaking their self-association and suppressing their H-bonding capacity (Luzar *et al.,* 1993). As a result, more spaces within the fibrils may appear, allowing more monomers to occupy the spaces in the presence of DMSO (Stape *et al.*, 2016a).

Complete removal of organic solvents and free water from the resin-dentin interface is almost impossible (Liu *et al.*, 2011). Similarly, solvent DMSO, due to its low vapor pressure, is most likely impossible to evaporate from wet dentin (Ekambaram *et al.,* 2015a).

In **Study II**, dentin was used as a macro model to evaluate the effect of solvent treatment on dentin. HEMA was used as a model monomer to evaluate the diffusion ability of demineralized dentin in different states (dry or wet) (Pashley *et al.*, 2000). HEMA has a low molecular weight (**Table 4**) and relatively hydrophilic nature (Van Landuyt *et al.*, 2007), and has been used as promoter to enhance adhesion, improve the hydrophilic nature of demineralized dentin, and as a solvent to stabilize monomers presented in adhesive systems (Pashley *et al.*, 2000; Van Landuyt *et al.*, 2007). Therefore, HEMA is present in most dental adhesive systems to promote adhesion to hydrated interfibrillar spaces of demineralized dentin (Rathke *et al.*, 2007; Van Landuyt *et al.*, 2008; Pashley *et al.*, 2011; Van Meerbeek *et al.*, 2011). The effectiveness of adhesive bonding is partially determined by the infiltration of monomers in dentin (Liu *et al.*, 2011; Tjäderhane *et al.,* 2015). Therefore, measuring the quantity of HEMA uptake through demineralized dentin after pretreatment with solvents may help understand the effectiveness of bonding. Two solvents (DMSO and ethanol) with different chemical properties were used in **Study II**. Immersion of the demineralized dentin cubes in different concentrations of DMSO or ethanol prior to HEMA incubation showed improvement of HEMA uptake. However, even the lowest DMSO concentration significantly enhanced HEMA uptake (**Fig. 7**).

The dissociative effect of DMSO on demineralized dentin collagen was previously investigated, when demineralized dentin discs were incubated in 100% DMSO for 30 min (Tjäderhane *et al.*, 2013c). In addition, the reversible effect of DMSO on the discs was investigated by the incubation in distilled water, which showed complete disappearance of the clearing effect (Tjäderhane *et al.*, 2013c). The reversibility of DMSO's action on demineralized dentin was observed previously by incubating dentin discs in 100% DMSO for 30 min (Tjäderhane *et al.*, 2013c). Dentin was then reversibly returned to its original appearance before DMSO immersion for 24 h in distilled water. That supports the reversible nature of DMSO effect on

collagen (Tjäderhane *et al.*, 2013c). Solvents used to evaluate infiltration of HEMA through demineralized dentin are different in their properties, especially vapor pressure, which ranges between 43.7 and 0.417 mmHg for ethanol and DMSO, respectively (Ekambaram *et al.,* 2015a). Because of that, ethanol can easily be evaporated from demineralized dentin. DMSO remains in dentin, because it has low vapor pressure, and therefore cannot evaporate from dentin. It is hypothesized that the continuous existence of DMSO within the interfibrillar spaces of demineralized dentin may produce a positive impact on the durability of resin-dentin bonding, because DMSO enhances the wettability within interfibrillar spaces (**Fig. 1**). In addition, DMSO facilitates penetration of resin monomers to occupy water spaces deeper inside the interfibrillar spaces of demineralized dentin (Tjäderhane, Mehtälä, *et al.*, 2013; Stape *et al.*, 2015; Stape *et al.*, 2016b; Mehtälä, Pashley and Tjäderhane, 2017).

Among different solvents (ethanol, acetone, methanol, and propanol), ethanol and acetone in another study caused the highest stiffness, demonstrating that dentin stiffness is dependent on type of solvent and duration of immersion (Garcia *et al.*, 2005). Similarly, in **Study II**, an increase in the stiffness of dentin beams was observed with an increase in time and solvent concentration (**Fig. 8**). Moreover, ethanol also showed enhancement of HEMA uptake in dentin. This enhancement might relate to the improvement of ethanol-saturated dentin wettability (Cadenaro *et al.*, 2009b; Sartori *et al.*, 2015).

The finding of this thesis report that incubation of demineralized dentin in high DMSO concentrations (50–100%) clearly changed collagen dissociation (**Fig. 9**). It has been reported that DMSO could destabilize collagen structure of the skin, thus reducing the optical scattering degree and enhancement of visibility (Bui *et al.*, 2009). Similarly, in the collagen of dentin, when dentin disc was immersed in 100% DMSO reversible changes in the collagen dissociation were seen (Tjäderhane *et al.*, 2013c). The reversible change in dentin collagen might related to the ability of DMSO to break down the self-associative tendency of water (Vishnyakov *et al.,* 2001). Therefore, presence of DMSO in collagen may replace or displace the residual water, leaving empty spaces within interfibrillar spaces occupied by monomers.

8.2 The effect of DMSO on adhesive resins (study III and part of study IV)

Dental adhesives are complex mixtures of several components in homogenous mixtures (Van Landuyt *et al.*, 2007). Solvent incorporation into dental adhesives is essential to optimize the integrity of final resin-based restoration (Malacarne-Zanon *et al.*, 2009). However, only solvents with high vapor pressure are incorporated into

contemporary adhesives and investigated in preclinical and clinical studies (Pashley *et al.*, 2007; Ekambaram *et al.,* 2015a).

Incorporation of a new solvent require proper evaluation of several properties in order to understand the interaction with hydrophobic or hydrophilic resin types, towards the optimization of integrity and stability of resin adhesive (Carrilho *et al.*, 2005; Liu *et al.*, 2011; Carvalho *et al.*, 2012). That can be performed first by the addition of solvent in several concentrations to resin adhesives with different hydrophilicities, followed by evaluation of main mechanical and physical properties of the resulted discs as well as the biological effect of the resulted resin. Therefore, several concentrations of DMSO were incorporated into a relatively hydrophobic (R2) or relatively hydrophilic (R5) methacrylate-based experimental adhesives. The hydrophilicity of resins was determined by percentages of HEMA and BisGMA available in each of them. R2 resin contains 70% BisGMA and 28.75% TEGDMA, compared to 40% BisGMA and 28.75% HEMA in R5 (**Table 3**).

8.2.1 Effects of DMSO-resins on physico/mechanical properties

A significant increase in the degree of conversion (DC) was observed only with high DMSO concentrations (5–10 w/w %) incorporated into both resins (**Fig. 11**). The hydrophobic resin (R2) used is rich in BisGMA that has high molecular weight and a rigid structure, presenting strong intermolecular hydrogen bonding interactions within the neat and low DMSO-hydrophobic resin (R2) (**Table 3**). Therefore, the overall DC of the hydrophobic resin was lower than with the hydrophilic resin. In addition, due to the presence of a high percentage of BisGMA (70%), monomers mobility is compromised during polymerization process (Sideridou *et al.,* 2002; Cadenaro *et al.*, 2009b). The significant increase in conversion with high concentrations of DMSO (5–10 w/w %) in R2 and R5 resins indicated that incorporation of high DMSO concentrations (5 w/w % or more) reduces the viscosity of the final DMSO/resin mixture. Moreover, DMSO facilitates the free movement of mixture composition during photo-polymerization (Dickens *et al.*, 2003; Holmes *et al.*, 2007; Cadenaro *et al.*, 2008; Cadenaro *et al.*, 2009a). Furthermore, DMSO in high concentrations (5 w/w % or more) may cause impairment in the photo-initiators and the accuracy of polymerization, similar to the ethanol effect on polymerization (Cadenaro *et al.*, 2010). The presence of high concentrations of DMSO (5 w/w % or more) in the adhesive mixtures may also be beneficial and may explain the acceleration in the rate of conversion. It may be explained that DMSO slow the chain termination reaction of the methacrylate free radicals prior to polymerization and during conversion (Gupta *et al.,* 1970). However, the significant acceleration of the

conversion does not necessarily mean enhancement of polymer structure quality (Ye *et al.*, 2007).

Water and ethanol were used to solvate different resins containing several percentages of DMSO (**Table 3**). Solvation of DMSO/resin discs first in water and then ethanol (two-step softening protocol) was performed in order to understand the effect of water immersion on polymer networks containing DMSO. The presence of linear polymers (as in hydrophilic resin) facilitates the diffusion of solvent molecules within polymer structures (Malacarne *et al.*, 2006). Furthermore, hydrophobic resins resist degradation and water diffusion between polymer networks, compared with hydrophilic resin, which has weaker crosslinking between its polymer networks and therefore allows more solvent to diffuse inside the polymer structures (Malacarne *et al.*, 2006).

The significant reduction in microhardness appeared with 5–10 w/w % DMSO in resins. Lower DMSO concentrations (up to 1 w/w %) did not significantly reduce microhardness. Effect of water- softening on neat R2 and low-DMSO-incorporated discs (up to 1 w/w %) was not observed, compared to higher DMSO/R2 (5–10 w/w %). On the other hand, significant reduction in the means of microhardness was observed with the neat and all DMSO/R5 discs after softening with water. The reason for this is related to the significantly higher percentage of BisGMA in R2 that did not allow water to break the intermolecular interaction, compared to almost 30% HEMA in R5 (Malacarne *et al.*, 2006).

Furthermore, ethanol was used for further solvation of the polymers of DMSO-R2 and DMSO-R5 discs for 4 h. A significant reduction in the results of microhardness was observed with all neat and DMSO/R2 discs, which points to the role of the softening effect of ethanol, by breaking down the intermolecular interactions between components of DMSO/R2. DMSO/R5 resin discs also showed significant reduction, but less than DMSO/R2, which is relates to the changes in solubility parameters of ethanol and water, and their effects on both resins with different hydrophilicities (Ferracane *et al.,* 2006; Cadenaro *et al.*, 2009b).

Results of ΔKNH% showed that R5 was more linear polymeric chains than R2. Incorporation of low concentrations of DMSO into both resins (up to 1 w/w %) did not significantly change crosslinking density. Higher DMSO incorporation changed the crosslinking density of both resins, since these percentages (5–10%) may not allow proper polymerization of monomers, and as a result, improper and incomplete polymer crosslinking appeared with lower mechanical properties (Ye *et al.*, 2007; Park *et al.*, 2009, 2010).

Two sets of resin discs were prepared to evaluate biaxial flexural strength. The first set was evaluated after 24 h and the second after 30 d at 37 °C of water incubation. In line with the crosslink density results, biaxial flexural strength results showed the same trend. The strong polymer crosslinking of R2 appeared as more

stable mechanical properties than the weaker linear polymer crosslinking of R5 (Sideridou *et al.,* 2003). That explains the generalized increase of flexural strength of DMSO/R2 compared to DMSO/R5. After 24 h or water incubation, low DMSO incorporation into R2 did not affect flexural strength. However, a significant difference was observed with 5 and 10 w/w % DMSO/R2 (**Fig. 10**).

Effect of water immersion was not significant for R2 with low concentrations of DMSO incorporation (up to 1 w/w %), since the polymeric crosslinking of R2 did not allow water molecules to penetrate polymer networks. On the other hand, higher DMSO (5–10 w/w%) appeared to negatively affect flexural strength after 24 h and 30 d of water incubation; 5–10 w/w % incorporation into R2 was able to break- down the strong polymer network, leading to a significant reduction in flexural strength.

The effect of water incubation on hydrophilic resin (R5) followed the same trend. No significant differences were observed in the flexural strength of R5 discs with low DMSO concentrations (up to 1 w/w %) after 24 h or 30 d water storage, whereas higher DMSO (5–10 w/w %) showed reduction in flexural strength. All the 30- d water-stored discs showed significant reduction in flexural strength compared to 24 h water immersion, perhaps because water penetrated the intermolecular polymer network of R5, and the presence of high DMSO concentrations in resin (5–10 w/w %) made the situation even worse (Lemon *et al.,* 2007). Furthermore, specimens containing 5–10% DMSO in R5 did not show statistical differences between initial and post-30 d of water storage. This may indicate that the maximum level of saturation between water molecules and DMSO/R5 was reached with 5% DMSO.

Generally, the results of water sorption and solubility of DMSO/R2 were significantly lower than DMSO/R5 because of the high percentage of BisGMA and the strong crosslinking between the polymer networks of R2. The lower crosslinking between the polymer networks of R5 causes higher values of water sorption and solubility (Ajithkumar *et al.,* 2000; Yiu *et al.*, 2006). Water sorption and solubility of R2 and R5 resins with higher (5–10 w/w %) concentrations of DMSO were significantly higher, possibly because of a similar effect as observed with the presence of residual solvent (Yiu *et al.*, 2004). Therefore, high DMSO concentration in adhesive could attract more water molecules to infiltrate inside the polymer network and cause expansion of resin discs, especially with hydrophilic adhesives (Ito *et al.*, 2005; Malacarne *et al.*, 2006; Yiu *et al.*, 2006).

The interaction of resin polarity and water sorption has been studied previously (Ito *et al.*, 2005; Malacarne *et al.*, 2006). The addition of DMSO in concentrations of 5–10 w/w% to R2 and R5 caused increases in resin polarity, resulting in significantly higher sorption and solubility levels. During the first 24–48 h of water incubation, since R5 allows more diffusion of water, mass changes were much higher than R2. Furthermore, the presence of 5% and 10% DMSO incorporation increased the absorbed water within 24–48 h in R2, since incorporation of high percentages of

solvent may lead to improper polymerization and allows more water to penetrate between intermolecular polymer chains. Similar results were observed with R5, presence of 5–10 w/w % DMSO in the hydrophilic resin increased amount of absorbed water. On the other hand, 1% and less of DMSO incorporation into R2 and R5 did not change water sorption and solubility, or the amount of diffused water.

8.2.2 Biological effects of resins containing DMSO

In order to investigate the safety and possibility of incorporating DMSO into dental adhesives, evaluation of resins containing several ascending concentrations of DMSO was performed on two types of cells (monolayer cultures of fibroblast or odontoblast-like cells). DMSO- resins were used to evaluate the biological effect in two ways, either by evaluating transdentinal cytotoxicity, or by evaluating the eluates from resins containing DMSO.

Results of the dentin barrier test of the DMSO-incorporated- hydrophobic resin (R2) showed slight reduction in the percentage of cell viability. The effect was not statistically different compared to negative control (**Fig. 15**). On the other hand, the impact of DMSO on hydrophilic resin (R5) was significantly different. Incorporation of 1 w/w % and more DMSO into R5 caused significant reduction in the percentage of viable cells, along the same levels as the positive control group, but significantly lower than the negative control group and DMSO/R2 (**Fig. 15**). Therefore, incorporation of DMSO in concentrations of 1 w/w % and more into hydrophilic adhesives may increase transdentinal cytotoxicity when used in clinical scenarios to pretreat deep dentin. The contents of each experimental resin used in this study differ in term of their properties, composition, and molecular weight, as well as their hydrophilicities (**Tables 3 and 4**). The increased cytotoxicity might be related to the different chemical composition of the resin used (Malacarne *et al.*, 2006). Hydrophilic resin (R5; **Table 3**) contains a high percentage of HEMA, which is a highly toxic monomer (Schweikl *et al.,* 2006; Van Landuyt *et al.*, 2011).

The other factor might be related to the diffusion rate of monomers from adhesives (Putzeys *et al.*, 2018), meaning that the presence of DMSO in concentrations of 1 w/w % and more might facilitate the diffusion of small molecules through the thin dentin disc toward cells causing a decrease in the percentage of cell viability.

It was demonstrated that the degree of conversion and composition of resins is responsible for the level of released monomers (Bianchi *et al.*, 2013; Van Landuyt *et al.*, 2015). The significant increase in the degree of monomer conversion occurred with the increase of DMSO incorporation into hydrophobic resin (Stape *et al.*, 2016b), due to the higher fractions of crosslinking monomers (*i.e.,* 70 % BisGMA in neat R2). Similar ascending results were observed with the neat and DMSO-

modified hydrophilic resin, the increase of degree of conversion was observed with the increase of DMSO concentration, especially 5–10 w/w % (Salim Al-Ani *et al.*, 2019b). Moreover, the significant increase in the degree of conversion of the neat and DMSO-modified hydrophilic resin might be related to the reduction of resin viscosity, especially when high DMSO concentrations (5–10 w/w %) were incorporated into the hydrophilic resin. The significant reduction in the percentages of cell viability with DMSO/R5 might be related to presence of strong polymer crosslinks in R2, compared to linear weak polymer crosslinks in R5 that allow water to diffuse and extract more cytotoxic components. Therefore, the presence of DMSO in R5 resulted in reduction of cross-linked density and enhancement of water sorption and solubility, which can partially explain the significant cytotoxicity with R5 containing 1 w/w % and more of DMSO.

Moreover, all the tested DMSO/R5 showed significant reduction in the percentage of cell viability compared to DMSO/R2 (**Fig. 16**). This might be related to the amounts of monomers released from the hydrophilic resin (R5), especially when incorporated into high percentages of DMSO (1 w/w % and more).

It was concluded that 500-µm thickness of dentin is sufficient to protect pulp tissue from potential toxicity of unreacted monomers during or after the restorative procedure with resin-based restoratives (Hanks *et al.*, 1988; Lanza *et al.*, 2009). Our findings in **study IV** indicate that even 400-µm dentin was also sufficient to protect pulp tissue from the eluted unbound monomers and other components as a result of DMSO addition. This means that the presence of DMSO in adhesives (up to 10 w/w %) was not problematic when incorporated into hydrophobic resin. This is in spite of the fact that incorporation of 5 w/w % or more DMSO into R2 or R5 resins impairs the mechanical and physical properties of final resin mixtures (Salim Al-Ani *et al.*, 2019b). The explanation may be that DMSO has a limited effect on the depth of penetration within collagen (few µm inside dentin) (Mehtälä, Pashley and Tjäderhane, 2017). However, when DMSO was incorporated into hydrophilic resin, higher toxicity levels were observed with 1 w/w % and more (**Fig. 15**), which was expected from the unbound, highly toxic monomers from the hydrophilic resin containing high percentage of DMSO.

Finally, DMSO is classified as a Class III solvent, in the same level of ethanol and acetone (International Council for Harmonization, ICH, 2016). Thus, the main biological concern with DMSO was related to the possibility of transferring monomers having small molecular weight and the bacterial toxins deeper inside dentin toward pulp tissue (Tjäderhane *et al.*, 2013c).

9 Future Perspectives and Further Studies

The studies presented as part of this project focus on the possibility of using DMSO in two forms, either as dentin pretreatment in etch-and-rinse dental adhesives or incorporated into experimental resins (w/w incorporation), aiming to preserve of the stability and strength of resin-dentin bonds.

Pretreatment of demineralized dentin with DMSO in concentrations of (0.001–20%) prior to adhesive application enhanced the durability and quality of restoration, at least for 6 months during aging in AS, especially 5% DMSO-pretreated dentin. Further studies are needed to investigate the effect of similar DMSO concentrations for longer incubation times in AS (1 or 2 y). However, other studies showed that relatively low concentration of DMSO (Tjäderhane *et al.*, 2013c), or relatively high concentration of DMSO (Stape *et al.*, 2015) used as dentin pretreatment caused improvement and stability in bond strength of adhesives to dentin, even after two years of storage. Here, other factors related to DMSO must be considered, especially the potential inhibitory effect of DMSO on dentin proteases. Since 5% and higher DMSO concentrations showed significant inhibition of gelatinases (Tjäderhane *et al.*, 2013c), further studies are also needed to confirm the enzymatic effect of lower (less than 5%) DMSO concentrations.

Another critical issue related to incorporation of DMSO in high concentrations into adhesive systems is the potential cytotoxicity of monomers having small molecular weight in deep cavities. The problem here is not related to the cytotoxicity of DMSO itself, since it is classified at the same level of ethanol and acetone (Class 3). The problem here is that DMSO proved to enhance penetration of hydrophilic monomers having small molecular weight (*i.e.,* HEMA). Nevertheless, our findings regarding the effect of DMSO on the mechanical and physical properties of resins clearly showed that incorporation of up to 1 w/w % DMSO into R2 or R5 resins did not impair the mechanical and physical properties of resins.

10 Summary/Conclusions

Based on the series of studies described in this PhD project, the following conclusions were found:

1. Dentin pretreatment with DMSO in a concentration of 1–5% enhances the durability and improves the quality of resin-based restoration bonding to dentin.
2. Presence of DMSO in the demineralized dentin collagen improves the infiltration of small-molecule hydrophilic monomers (*i.e.* HEMA). Presence of DMSO also enhances the stiffness of the collagen matrix. The reason for the enhancement may be related to DMSO's capability to replace/displace water clusters within the collagen matrix, to allow penetration of monomers more efficiently.
3. DMSO incorporation into resin at a concentration of 5 w/w % or more causes impairment of the quality of polymers networks of resins and negatively affects the physical and mechanical properties of methacrylate hydrophobic and hydrophilic resins. Furthermore, incorporation of low concentration of DMSO into resin (≥1 w/w %) had no negative effects on the mechanical and physical properties. Therefore, addition of 1 w/w % DMSO or less may be a successful step toward formulation of hydrophobic or hydrophilic resin contains DMSO.
4. Pretreatment of dentin with hydrophobic resins containing DMSO does not cause transdentinal cytotoxicity on transfected bovine pulp-derived cells. In contrast, 1 w/w % and more DMSO incorporation into hydrophilic may cause cytotoxic reaction to cells. In the hydrophobic resins, the biocompatibility is not influenced by percentage of DMSO incorporated into hydrophobic resins. While in the hydrophilic resin, high percentages of DMSO are negatively affecting the cytotoxicity. In general, the biocompatibility of resins containing DMSO is depending on the hydrophilicity, chemical composition of resin adhesive, and partially on the concentration of DMSO used.
5. The overall conclusion based on the series of studies is that DMSO can be incorporated into dental adhesives, either directly in concentrations 1–5% as

dentin pretreatment agent or added into dental adhesives mixtures with different hydrophilicities (up to 1 w/w %), without impairing the physical and mechanical properties. The biocompatibility is not affected by the addition of 1 w/w % DMSO or less into hydrophobic adhesive, or 0.1 w/w % or less into hydrophilic adhesive.

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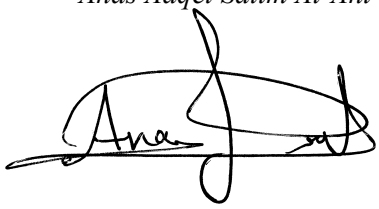
Turku, November 2019
Anas Aaqel Salim Al-Ani



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