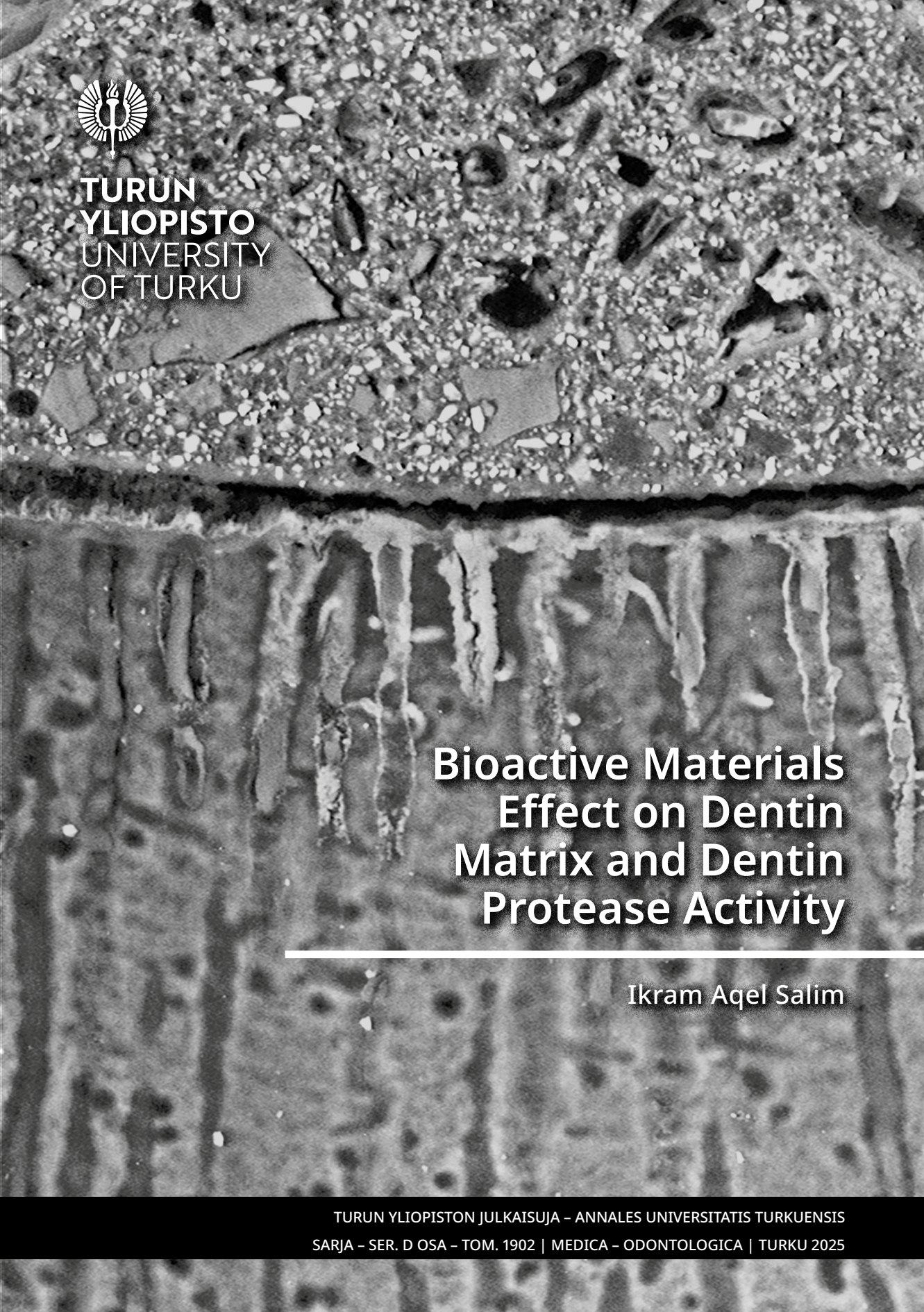

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Bioactive Materials Effect on Dentin Matrix and Dentin Protease Activity

Ikram Aqel Salim

TURUN YLIOPISTON JULKAISUJA – ANNALES UNIVERSITATIS TURKUENSIS
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وَقَالُوا الْحَمْدُ لِلَّهِ الَّذِي هَدَانَا لِهَـٰذَا وَمَا كُنَّا لِنَهْتَدِيَ لَوْلَا أَنْ هَدَانَا اللَّه

وتدعو أن لا يكون طريقك وعراً لتنجو، فيكونُ وعراً وتنجو لتعلم أن النجاة من الله لا من الطريق

"You pray for a smooth path to survive, yet the path is rugged—and still you survive. Then you understand: salvation comes from God, not from the road".

To my present-absent mother,
Dear father, brothers and sisters,
For your unconditional love and support through this journey.

UNIVERSITY OF TURKU
Faculty of Medicine
Institute of Dentistry
Department of Cariology and Restorative Dentistry
IKRAM AQEL SALIM: Bioactive materials effect on dentin matrix and dentin protease activity
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ABSTRACT

Material selection for endodontic use in cases of deep cavities, apexification, or root canal sealing must be carefully performed due to undesirable possible outcomes related to the type and effect of the materials on biological activity and dental hard tissues. Different bioactive materials have been used for dental applications, and the fast development and modifications to the formula of those materials require investigations. This series of studies aimed to evaluate the effect of different bioactive calcium silicate-based materials, and an experimental ion-releasing surface pre-reacted glass (S-PRG) cement or released ions on dentin matrix integrity and dentin protease activity. Additionally, characterize the Ca-leaching profile, pH changes of these materials over time. The use of solvent solutions containing high concentrations of dimethyl sulfoxide (DMSO) and /or ethanol was suggested to increase the penetration of materials into dentin. Dentin pretreatments containing DMSO in either ethanolic or aqueous solutions were utilized to evaluate the effect of those solvents at different high concentrations on the cytotoxicity and bond strength of bioactive materials to root dentin. The ion-releasing material seemed to be a safe option in terms of cytotoxicity and effect on enzymatic activity but had low bond strength to radicular dentin. It has a good ability to prevent any cytotoxic effects, due to its high biocompatibility and ability to release different ions; some of the ions released by the S-PRG cement, as well as some of the tested materials, could inhibit the matrix metalloproteinases (MMPs) and cysteine cathepsins (CCs) activity. Although TheraCal had a better bond strength to dentin compared to MTA and S-PRG and seemed not to affect the dentin protease activity, it showed cytotoxic reaction toward odontoblast-like cells. In addition, both tested MTAs had caused dentin degradation over time and showed moderate cytotoxicity and lower bond strength compared to TheraCal. Results of these studies showed that the selection of the material could change depending on the use of the material.

KEYWORDS: bond strength, calcium silicate cement, cysteine cathepsins, cytotoxicity, dentin collagen, dimethyl sulfoxide, ethanol, matrix metalloproteinase, mechanical properties, mineral trioxide aggregate, Surface pre-reacted glass.

TURUN YLIOPISTO
Lääketieteellinen tiedekunta
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TIIVISTELMÄ

Materiaalin valinta eri endodonttisten toimenpiteiden yhteydessä, kuten syviä kaviteetteja korjattaessa, apeksifikaatiota tehtäessä tai juurikanavan tiivistämisessä, on tehtävä huolellisesti, sillä materiaalien tyyppi ja vaikutus biologiseen aktiivisuuteen ja hampaan kovakudoksiin voivat johtaa epätoivottuihin lopputuloksiin. Erilaisia bioaktiivisia materiaaleja on käytetty hammaslääketieteellisissä sovelluksissa, ja näiden materiaalien nopea kehitys sekä koostumuksen muutokset vaativat jatkuvaa tutkimusta. Tämä tutkimussarja pyrki karakterisoimaan ajan myötä tapahtuvaa kalsiumin liukenemisprofiilia ja pH- muutoksia eri bioaktiivisten kalsium-silikaattipohjaisten materiaalien sekä kokeellisen, ioneja vapauttavan lasi-ionomeerisementin (surface-pre-reacted glass ionomer, SPRG) käytön yhteydessä. SPRG:ssa happo-emäs reaktio tapahtuu ennen materiaalin komponenttien sekoittamista keskenään. Lisäksi arvioitiin näiden materiaalien ja vapautuneiden ionien vaikutusta dentiinimatriksin eheyteen ja dentiinin proteaasiaktiivisuuteen. Liuottimia, joissa on suuri dimetyylisulfoksidi (DMSO)- ja/tai etanolipitoisuus, käytettiin parantamaan materiaalien tunkeutumista dentiiniin. Liuottimien vaikutusta sytotoksisuuteen ja bioaktiivisten materiaalien kiinnitysvoimaan juuridentiiniin selvitettiin dentiinin esikäsittelyllä, jossa käytettiin DMSO:ta joko etanolipohjaisena tai vesiliuoksena. Ioneja vapauttava materiaali vaikutti olevan turvallinen vaihtoehto sytotoksisuuden ja entsyymiaktiivisuuden suhteen, mutta sen kiinnitysvoima juuridentiiniin oli heikko. Materiaalilla on hyvä kyky estää sytotoksisia vaikutuksia sen korkean biologisen yhteensopivuuden ja eri ionien vapautumisen vuoksi; osa SPRG-sementin sekä joidenkin testattujen materiaalien vapauttamista ioneista saattoi estää matriisin metalloproteinaasien (MMP) ja kysteiinikatepsiinien (CC) aktiivisuutta. Vaikka TheraCalilla oli hyvä kiinnitysvoima dentiiniin eikä se näyttänyt vaikuttavan dentiinin proteaasiaktiivisuuteen, TheraCal osoitti sytotoksista reaktiota odontoblastin kaltaisia soluja kohtaan. Lisäksi molemmat testatut mineraalikalsiumaggregaatit (MTA:t) aiheuttivat dentiinin hajoamista ajan myötä, ja osoittivat kohtalaista sytotoksisuutta sekä heikompaa kiinnitysvoimaa verrattuna TheraCaliin. Näiden tutkimusten tulokset osoittivat, että materiaalivalinta voi muuttua riippuen materiaalin käyttötarkoituksesta.

AVAINSANAT: dentiinin kollageeni, dimetyylisulfoksidi, etanoli, matriksin metalloproteinaasi, mekaaniset ominaisuudet, mineraalitrikalsiumaggregaatti, kalsium-silikaattisementti, kysteiini-katepsiini, SPRG, sytotoksisuus.

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Abbreviations

ACP	Amorphous calcium phosphate
ANOVA	Analysis of variance
AS	Artificial saliva
$Ca(OH)_2$	Calcium hydroxide
CC	Cysteine cathepsins
CM	Calcium-containing media
CSCs	Calcium-silicate cements
CTX	C-terminal crosslinked telopeptide of type I collagen
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
$DMSO/H_2O$	50 % (v/v) DMSO solution in water
DMSO/EtOH	50 % (v/v) DMSO solution in ethanol
E	Elastic modulus
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EtOH	Ethanol
$EtOH/H_2O$	50 % (v/v) ethanol solution in water
H_3PO_4	Phosphoric acid
HAP	Hydroxyapatite
HV	Vickers hardness number
HYP	Hydroxyproline
ICTP	Crosslinked carboxy-terminal telopeptide of type I collagen
IRM	Intermediate Restorative Material
ISO	International Standards Organization
µg	Microgram
µl	Microliter
µm	Micrometer
mm	Millimeter
ml	Milliliter
MMP	Matrix metalloproteinase

MPa	Mega Pascals
MTA	Mineral trioxide aggregate
MTT	Colorimetric assay based on the reduction of: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N	Newton
NaN_3	Sodium azide
ng	Nanogram
pg	Picogram
pH	Power of hydrogen
ppm	Part per million
SEM	Scanning electron microscope
SiC	Silicon carbide
S-PRG	Surface pre-reacted glass
v/v %	Volume per volume percentage
Vol %	Volume percentage

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I. **Ikram Aqel Salim,** Roda Seseogullari-Dirihan, Satoshi Imazato, Arzu Tezvergil-Mutluay. The inhibitory effects of various ions released from surface-active fillers on dentin protease activity. *Dental Materials Journal*, 2023; 42(1): 99–104

II. **Ikram Aqel Salim**, Thiago Henrique Scarabello Stape, Roda Seseogullari-Dirihan, Mustafa Murat Mutluay, Arzu Tezvergil-Mutluay. Effect of solvent pretreatments on trans-dentinal cytotoxicity of silicate-based biomaterials. Revised version, *Dental Materials Journal*.

III. **Ikram Aqel Salim**, Anas Aaqel Salim, Thiago Henrique Scarabello Stape, Murat Mutluay, Arzu Tezvergil-Mutluay. Effect of solvent pretreatments on intraradicular bond strength of bioactive cements. *International Journal of Adhesion and Adhesives*, 2025; 138, 103952

IV. **Ikram Aqel Salim** and Marcelo Capitanio, Roda Seseogullari-Dirihan, Mustafa Murat Mutluay, Arzu Tezvergil-Mutluay. The direct inhibitory effect of various bioactive materials on dentin matrix and dentin protease activity. Manuscript.

1 Introduction

The development of bioactive and biocompatible materials has gained significant attention in recent years, particularly for their potential to preserve tooth vitality or improve the strength and longevity of endodontic restorations (Duncan *et al.* 2019). The term bioactive refers to materials capable of interacting with biological tissues through a biomineralized interface, promoting tissue integration and regeneration (Ferracane *et al.* 2023). The term bioactivity generally describes the ability of the materials to have a biological effect and form a bond between the tissues and the material (Hench *et al.* 1971). For many decades, research in dental materials has focused on different approaches to remineralize dentin in contact with a restoration to biomimetically reinforce the integrity of dentin (Sinclair-Hall *et al.* 1964). Dentin is a complex histologic tissue primarily composed of a collagen network (≥ 90 wt%), with type I collagen being the most abundant type present in dentin, with some trace amounts of type III and V collagen, forming a dynamic and heterogeneous matrix with varying degrees of mineralization. The remaining portion consists of non-collagenous proteins, including proteoglycans, phospholipids, and enzymes (Tjäderhane *et al.* 2009). The collagen contains proteins such as growth factors and matrix proteases, mainly matrix metalloproteinase (MMPs) and cysteine cathepsins (CCs), which are responsible for dentin collagen matrix degradation when activated; therefore, compromising the matrix integrity.

An ideal endodontic treatment includes placing a material intended to seal the contents of the root canal from the surrounding peri-radicular tissues and to repair any defects in the root (Gawkrodger *et al.* 2004). It must form a reliable seal with dental tissues, maintain dimensional stability, be biocompatible, and non-resorbable (Johnson *et al.* 1999; Kratchman *et al.* 2004) and interact favorably with the periodontal tissues (Lee *et al.* 2004). Historically, materials such as amalgam, zinc oxide eugenol, composite resin, and glass ionomer cement (Bryan *et al.* 1999; Johnson *et al.* 1999) have been used for perforation repair, retrograde fillings, and root canal sealers. However, none of these materials have successfully met all the necessary criteria for an ideal endodontic material (Johnson *et al.* 1999; Kratchman *et al.* 2004).

Dental materials derived from Portland cement, which is used in the construction industry, have gained popularity in dentistry due to its hydraulic properties. Mineral trioxide aggregate (MTA) has gained popularity due to its favorable properties (Torabinejad *et al.* 1993). Derived from Portland cement, MTA includes bismuth oxide for radiopacity (Funteas *et al.* 2003). Despite its clinical success, MTA has limitations such as poor handling, long setting time (Torabinejad *et al.* 1995; Storm *et al.* 2008; Gandolfi *et al.* 2011), difficult retrieval from the operation area, and potential for tooth discoloration after the treatment (Lenherr *et al.* 2012; Felman *et al.* 2013). These drawbacks have led to the development of modified calcium silicate-based materials, including Biodentine™, Bioaggregate™, Endosequence™, TheraCal™, and others (Grech *et al.* 2013; Dawood *et al.* 2017). Those modifications included changes of the compositions and chemistry of the MTA and have been suggested to overcome certain drawbacks of the MTA, including discoloration, long setting time, and handling properties. For example, Biodentine™ can release higher calcium amounts and at greater depth, promoting odontoblastic differentiation. Other types of MTA, like Biodentine™ and MTA Angelus™ had no bismuth oxide in their composition, which can reduce the cytotoxicity of the material and the possibility of discoloration. In addition, light-curable resins have been added to certain types of MTA, like the TheraCal™ to overcome the long setting time. Surface pre-reacted glass fillers, known for their ion release and recharge capabilities, were suggested as bioactive materials, and have been incorporated into resin composites (Saku *et al.* 2010; Hosoya *et al.* 2012), root canal sealers (Han *et al.* 2011), and pits and fissure sealants (Shimazu *et al.* 2011). However, the interaction between some of these newer bioactive materials and dentin remains questionable.

The connection between the materials and the underlying mineralized dentin occurs through the collagen fibrils extending from the mineralized matrix to the hybrid layer. Collagen fibrils are composed of non-collagenous proteins such as growth factors and matrix proteases, which are primarily inactive and stable in mineralized tissue. However, the high alkalinity nature of the calcium silicate-based materials can activate those enzymes, resulting in progressive degradation of the collagen fibrils, which therefore leads to the solubilization of dentin collagen and loss of retention of the restoration.

Recently, the use of solvents such as dimethyl sulfoxide (DMSO) has been proposed to enhance the bond strength to dentin (Tjäderhane *et al.* 2013; Stape *et al.* 2016). DMSO improves dentin wettability (Stape *et al.* 2021) and facilitates the penetration of high molecular weight molecules (Stape *et al.* 2015), potentially improving the seal between restorative materials and dentin (Stape *et al.* 2021; Salim Al-Ani *et al.* 2021; Ismail *et al.* 2023). However, the effects of DMSO and ethanol,

particularly in various dilutions, on the interaction between bioactive materials and dentin are not yet fully understood.

This thesis investigated the effect of various bioactive materials on the dentin matrix integrity and protease activity. The main aim of this study series was to evaluate and systematically analyze the effects of different bioactive materials on the collagen matrix integrity, cytotoxicity, and bond strength of demineralized dentin. Additionally, it explores the effect of solvent pretreatments (DMSO, ethanol, and their aqueous dilutions) influence biocompatibility and bonding performance.

2 Review of the Literature

2.1 Introduction to tooth structure

2.1.1 Enamel

Enamel is an aesthetic and durable tissue that constitutes the coronal outer portion of the teeth and lacks the ability to repair itself. It is the stiffest and most mineralized mammal tissue. The enamel microstructure is relatively uniform compared to dentin and is primarily composed of inorganic components (approximately 94–96 wt%, or 85% by volume), primarily carbonated hydroxyapatite, along with trace elements such as sodium, magnesium, potassium, and fluoride (Oliveira *et al.* 2010).

The organic matrix is composed of proteins (3% vol, or 1 wt%) and loosely bound water molecules (12% vol, or 3% wt). During enamel maturation, most of the organic content is removed, leaving behind residual proteins such as ameloblastin and remnants of amelogenin. These proteins, along with collagen, help bind hydroxyapatite crystals, thereby maintaining the enamel's hierarchical structure and contributing to its mechanical strength and optical properties (Bachmann *et al.*, 2004). Enamel also exhibits selective ion permeability, allowing it to incorporate calcium and fluoride ions from dietary and oral hygiene sources. As a result, its physicochemical properties can vary among individuals (Lacruz *et al.* 2017).

2.1.2 Dentin

Dentin is a complex hydrated structure that forms the bulk of the tooth beneath enamel and cementum. Depending on its thickness and age, dentin is considered either a barrier to external irritants or a permeable structure (Pashley *et al.* 1996; Tjäderhane *et al*. 2009). Dentin is composed of approximately 70% minerals, 20% organic components, which are mainly type I collagen, and 10% water by weight and by volume. This translates to 50% minerals, 30% organic components, and 20% water (Nakabayashi *et al*. 1998).

The mineral phase is primarily hydroxyapatite; intrafibrillar minerals are found in the inner part of the regularly spaced gap zones in the collagen fibril, while extrafibrillar minerals are situated in the spaces between fibrils. The majority of

the mineral content, around 70 to 75%, is believed to be situated outside the fibrils (Pidaparti *et al.* 1996). Most of the organic components of dentin are primarily composed of collagen (≥ 90 wt%), with type I collagen being the most abundant type present in dentin, with some trace amounts of type III and V collagen, with the remaining portion consisting of non-collagenous proteins (Tjäderhane *et al.* 2009). Dentin has millions of dentinal tubules that diffuse between dentin and pulp tissue, those dentinal tubules contain the dentinal fluids which are free unbound water located in the dentinal tubules and move freely from dentin to pulp as a physiological response to thermal, osmotic stimuli across dentin (Pashley *et al.* 1996; Tjäderhane *et al.* 2009). The gradual demineralization during dentin caries significantly alters the mechanical characteristics of dentin, raises its porosity, and leads to modifications in collagen structure (Marshall *et al.* 2001; Zavgorodniy *et al.* 2008).

2.1.3 Dentin collagen structure

Dentin is a mineralized tissue primarily composed of inorganic apatite crystals embedded within an extracellular matrix (ECM) rich in collagen. Approximately 90% of the organic matrix consists of type I collagen (Linde 1989), while the remaining 10% comprises non-collagenous proteins, including proteoglycans, phospholipids, and enzymes. During tooth development, odontoblast cells secrete type I collagen fibrils to form predentin, which subsequently undergoes crosslinking and mineralization. Non-collagenous proteins, such as glycosaminoglycans, SIBLINGs (Small Integrin Binding Ligand N-Linked Glycoproteins), Fetuin-A, and non-phosphorylated proteins like osteonectin and osteocalcin, play a key role in regulating hydroxyapatite crystal growth during these processes. Structurally, each collagen molecule forms a left-handed triple helical structure, composed of two α1(I) chains and one α2(I) chain, with 3.3 residues per turn and an 8.7 Å pitch, equivalent to 18 amino acids per turn (Hofmann *et al*. 1978; Yamauchi *et al*. 2002). Glycine, the smallest and most abundant amino acid in collagen, occupies every third position, allowing the tight packing of the triple helix (Germann *et al*. 1988). It is uniquely positioned at the centre of the triple helix, while other amino acids are located on the exterior (Fraser *et al.* 1979; Germann *et al.* 1988).

2.1.4 Endogenous dentin enzymes

Endogenous enzymes in dentin, particularly matrix metalloproteinases (MMPs) and cysteine cathepsins, have gained significant attention due to their possible roles in both physiological and pathological processes within the dentin-pulp complex.

2.1.4.1 Matrix metalloproteinases

Matrix metalloproteinases are zinc and calcium-dependent endopeptidases involved in the remodelling and degradation of the extracellular matrix. To date, 23 members of the MMP family have been identified in humans, capable of degrading a wide range of ECM components, including fibrillar and nonfibrillar collagens, such as type I collagen (Tallant *et al.* 2010). MMPs can be classified: i According to their substrate specificity into 6 groups, including collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs, and others (Tallant *et al.* 2010). ii According to their structural properties into 4 types: basic-structure MMPs, MMPs with fibronectin-domain inserts, membrane-bound MMPs, and minimal-domain MMPs (Page-McCaw *et al.* 2007). The other classification is based on the ability of MMPs to degrade several substrate types under in vitro conditions. Structurally, MMPs consist of four main domains: a prodomain with a cysteine residue, a catalytic domain (containing catalytic and structural zinc ions and calcium ions), a hinge region, and a hemopexin-like domain for substrate binding (Bode *et al.* 1999; Visse *et al.* 2003; Tallant *et al.* 2010). MMPs are secreted as inactive zymogens and activated via the 'cysteine switch' mechanism, which exposes the catalytic site. Activation can occur spontaneously or be triggered by other proteases, chemical agents, or environmental changes such as pH or temperature changes (Tjäderhane *et al.* 2013; Christensen *et al.* 2015).

2.1.4.2 MMPs in dentin and pulp

Collagenolytic activity in carious dentin was first demonstrated in the 1980s (Dayan *et al.* 1983). Odontoblasts are known to express several MMPs, including MMP-1, -2, -3, -8, -9, -13, -14, and - 20, as well as membrane- type matrix metalloproteinase-1 and -2 (MT1-MMP and MT2-MMP) (Sulkala *et al.* 2002; Palosaari *et al.* 2003). While the exact physiological functions of MMPs in dentin are not fully understood, MMPs are believed to contribute to peritubular and tertiary dentin formation and the release of dentinal growth factors. MMP-13, for instance, is expressed in both sound and carious pulp tissue (Sulkala *et al.* 2004). Elevated levels of MMP- 1, -3, and -9 have been observed in pulpitis, suggesting their involvement in inflammation and tissue repair (Gusman *et al.* 2002; Shin *et al.* 2002). Interestingly, MMP-3 may also exhibit anti-inflammatory properties during pulpitis (Eba *et al.* 2012).

2.1.4.3 Cysteine cathepsins (CCs)

Cysteine cathepsins (CCs) are papain-like proteases belonging to the C1 subfamily. They play a critical role in ECM degradation and MMP activation. Eleven types of CCs have been identified (B, C, F, H, K, L, O, S, V, X, and W) in human tissues.

Others, like cathepsin K (mainly expressed by osteoclasts), S (by antigen-presenting cells), and W (CD8+ lymphocytes and natural killer cells), are tissue-specific (V. Turk *et al.* 2012). These enzymes are produced as inactive zymogens and activated in endosomes via acidification or proteolytic cleavage (Sundararaj *et al.* 2012).

2.1.4.4 Cysteine cathepsins in dentin and pulp

Odontoblasts and pulp cells express various CCs (Tersariol *et al.* 2010). Active and precursor forms of cathepsins B, K, and L have been isolated from dentin matrices. (Scaffa *et al.* 2012; Vidal *et al.* 2014). These enzymes are believed to become entrapped during dentin mineralization and can migrate through dentinal tubules when released from the pulp (Nascimento *et al.* 2011). Their presence and activity levels vary depending on the degree of mineralization level and the localization and severity of caries lesions (Nascimento *et al.* 2011; Vidal *et al.* 2014; Scaffa *et al.* 2017).

2.2 Strategies to prevent the degradation of dentin collagen matrices

As mentioned, MMPs and CCs play essential roles in dentinogenesis and extracellular matrix remodelling (Tersariol *et al.* 2010; Chaussain *et al.* 2013). Although these enzymes become inactive and entrapped during dentin mineralization, they can be reactivated in demineralized dentin, leading to collagen degradation (Kato *et al.* 2012; Tezvergil-Mutluay *et al.* 2013; Vidal *et al.* 2014; Zarella *et al.* 2015). Since these proteases are hydrolases, they require free, unbound water to function; strategies that reduce water availability or inhibit enzymatic activity are key to preserving dentin integrity.

2.2.1 Inhibition of enzymatic activity

The inhibition of endogenous enzymes can prevent collagen degradation in demineralized dentin, enhancing remineralization potential and the longevity of the restorations (Kato *et al.* 2014; Giacomini *et al*. 2017). Several inhibitors have been explored, including chemical inhibitors such as chlorhexidine, benzalkonium chloride (Tezvergil-Mutluay *et al.* 2011), as well as cationic metal ions such as zinc and iron (Kato *et al.* 2012), MMP inhibitor monomers and solvents designated to integrate into adhesive systems were also explored (Tezvergil-Mutluay *et al.* 2015). Additionally, natural or synthetic crosslinking agents such as curcumin, epigallocatechin gallate, riboflavin, or glutaraldehyde to enhance the resistance of collagen fibril degradation have been investigated (Hiraishi *et al*. 2013; Frassetto *et*

al. 2016; Seseogullari-Dirihan *et al.* 2016). Furthermore, the biomineralization of collagen fibrils to protect them from enzymatic attacks has been proposed as a promising approach (Niu *et al.* 2014; Xie *et al.* 2016).

Matrix metalloproteinases rely on calcium for structural integrity and zinc for catalytic activity (Nagase *et al*. 2006; Tezvergil-Mutluay *et al*. 2010). Chelating these ions disrupts enzyme function, offering a therapeutic approach to limit tissue degradation. Pashley *et al*. (2004) first demonstrated collagenolytic activity in acid-etched dentin, independent of bacterial presence. Subsequent studies confirmed that MMPs are the primary enzymes responsible for collagen degradation, with activity levels up to ten times higher than those of CCs (Hu *et al.* 2015).

2.2.2 Remineralization

Remineralization is another mechanism suggested for protecting the exposed unprotected collagen from the protease attack and reinforcing dentin collagen under occlusal forces (Toledano *et al.* 2013; 2014). This process involves the deposition of inorganic minerals, often mediated by organic molecules, onto demineralized dentin surfaces (He *et al.* 2003; Zhu *et al.* 2020). Biomimetic remineralization uses ion-releasing materials to promote apatite infiltration into collagen fibrils, restoring the hybrid layer and enhancing bond durability (Kim *et al.* 2010). Common agents include fluoride (Neel *et al.* 2016), bioactive glasses (Kim *et al.* 2021), and amorphous calcium phosphate (ACP) as a precursor of the hydroxyapatite (HAP) (Fontaine *et al.* 2016). While ACP-based systems can mineralize collagen fibrils and inhibit MMP activity, current models often fail to achieve complete intrafibrillar remineralization, limiting their ability to fully replicate natural tooth structure (Boonrungsiman *et al.* 2012; Kerschnitzki *et al.* 2016).

2.3 Bioactive Materials

The term bioactivity was used to describe the ability to cause reactions, including cellular stimulation or antimicrobial activity. Therefore, a bioactive material is a material able to induce specific biological activity and stimulate a beneficial response from the bonding material to the host tissue. (Hench *et al.* 1993; Williams 2005). In other definitions, bioactivity is the ability to form mineral depositions under specific biological conditions (Hill and Brauer 2011). In dentistry, the term bioactivity refers to a material that elicits a beneficial biological response, such as stimulating mineral deposition or cellular activity (Hench *et al.* 1993; Williams *et al.* 2005). Bioactivity can involve hydroxyapatite reprecipitation, ion release, or antimicrobial effects, depending on clinical application (Hill *et al.* 2011). Although definitions vary, Vallittu *et al.* (2018) proposed that only materials scientifically

validated to release ions and promote biomineralization in clinical settings should be classified as bioactive. These materials are increasingly used in restorative dentistry to enhance tissue integration, promote remineralization, and inhibit enzymatic degradation (Ibrahim *et al.* 2020; Ferracane *et al.* 2023).

2.3.1 Calcium Silicate -based Biomaterials

Calcium silicate-based cements (CSCs) are self-setting hydraulic materials primarily composed of dicalcium and tricalcium silicates (Darvell *et al.* 2011). Upon hydration, these compounds react to form calcium hydroxide and calcium silicate are produced primarily, and a sticky colloidal gel forms (calcium silicate hydrate gel) which hardens into a solid matrix (Camilleri 2008; 2014). The initial setting involves hydrolysis and ion exchange (Parirokh *et al.* 2010). The interaction of CSCs with their environment underpins their biocompatibility, dentinogenic activity, and sealing ability.

An ideal filling material should have clinically adequate physical characteristics, including good sealing, dimensional stability (Camilleri *et al.* 2011) and color stability (Marciano *et al.* 2014), radiopacity (Islam *et al.* 2006), ease of manipulation and insertion (Hungaro *et al.* 2012), and should be insoluble in contact with fluids (Fridland *et al.* 2003). Moreover, it should also have biological and physical properties, including the alkaline pH, calcium ion release (Hungaro *et al.* 2003), bioactivity (Gandolfi *et al.* 2010) and biocompatibility (Camilleri *et al.* 2004). CSCs meet many of these criteria and are widely used in procedures such as pulp capping (Bhavana *et al.* 2015), pulpotomy, apexogenesis, and apexification (Camilleri *et al.* 2013), perforation repair, and root-end filling (Parirokh *et al.* 2010).

Tricalcium silicate -based sealers, enhanced with radiopacifying agents, exhibit surface bioactivity due to calcium hydroxide release, which reacts with phosphate ions to form hydroxyapatite are employed as sealers. (Camilleri *et al.* 2011; 2015) Among various root-end filling materials, mineral trioxide aggregate (MTA) is recognized for its superior bioactivity (Enkel *et al.* 2008). However, CSCs had several disadvantages, including poor handling properties, long setting time, and weak adhesion to the tooth structure (Torabinejad *et al.* 1995; Duarte *et al.* 2005; Monteiro Bramante *et al.* 2008; Boutsioukis *et al.* 2011). Therefore, various products of CSCs with different components and properties have been developed, and some studies have evaluated their clinical performance (Camilleri 2011; Grech *et al.* 2013; Kunert *et al.* 2020).

2.3.1.1 Classification of Calcium Silicate-based Cement

Proper classification of the materials is necessary due to the differences in chemistry and clinical applications (Dong *et al.* 2023). Different classification systems have

been used to identify the various products proposed as calcium silicate cements. Overall, CSCs are classified according to their basic components (Dong *et al.* 2023), the presence of additives and whether supplied as powder and liquid or directly suspended from a non- aqueous vehicle (Camilleri *et al.* 2020). Camilleri *et al.* (2020) proposed two classifications for hydraulic calcium cements according to i: constitution, based on chemistry and presentation, and ii: the clinical context, based on environment and specific use. The clinical context classification depends on the changes the material will undergo based on its location and has three subdivisions: intracoronal, for pulp protection and regenerative endodontic procedures; intraradicular, for root canal sealing and apical plug; and extraradicular, for root end fillings and perforation repair. The classification according to the constitution is based on the behaviour and properties, especially of the hydration process, which depends on the cement chemistry, the modifiers used, and whether the material is mixed with water or not. **Table 1**. represents the classification of the CSCs based on the characteristics and history of modification, which was developed by Tsuchiya *et al.* 2025 classifying the CSCs into six generations.

Table 1. Classification of silicate cements based on their history of modification and characteristics.

Generation	Characteristics	Example product and manufacturer	Year
Generation I	The base powder is mainly composed of PC.	ProRoot™ MTA (Grey version and white version) (Dentsply, Tulsa, OK, USA)	1998 and 2002
Generation II	Incorporation of additives or removal of some components from the base powder used in Generation I.	MTA Angelus™ (Angelus, Londrina, PR, Brazil)	2001
Generation III	The base powder is primarily composed of laboratory-synthesized CSC, not derived from PC.	BioAggregate™ (Innovative Bioceramix, BC, Canada)	2007
Generation IV	The base powder is mixed with a special liquid form instead of only distilled water.	-Biodentine™ (Septodont, St. Maur de Fosses, France) -MTA Repair HP™ (Angelus, Londrina, PR, Brazil)	2009 2016
Generation V	Premixed type	- EndoSequence™ (Brasseler USA, Savannah, GA, USA) - Bio-C Repair™ (Angelus, Londrina, PR, Brazil)	2009 2020
Generation VI	Resin-modified CSCs	TheraCal™ LC (Bisco, Schaumburg, USA)	2011

2.3.1.2 Effect of the materials' properties on clinical applications

It is important to understand the properties of each material for the material's choice in different clinical scenarios. Materials with short setting times might be used in cases where it is difficult to control bleeding, like retrograde fillings and perforation repairs. In addition, CSCs with good handling properties might be useful in small, deep, and complicated cavities where it is difficult to access with instruments. Also, CSCs in direct or indirect contact with pulp tissue in cases of pulp capping or pulpotomy should be biocompatible and have an optimal pH. Moreover, CSCs containing bismuth oxide might cause tooth discoloration; therefore, can't be used in aesthetic areas.

2.3.1.3 Physical and Biological properties of the different generations

The physical and biological properties of the materials vary depending on their composition. Both generation I (G-I) and generation III (G-III) have a long setting time, with almost 4 hours (Saraswat *et al.* 2023). Generation IV(G-IV) and Generation VI (G-VI) have shorter setting times (Kaur *et al.* 2017; Gasperi *et al.* 2020). The setting time for generation II (G-II) and generation V (G-V) is almost 2 hours (Mahgoub *et al.* 2019). The handling properties also vary depending on the composition. G-V and G-VI have better handling properties since they are ready-to-use premixed materials that come in syringe paste or putty form (Chen *et al.* 2015; Kunert *et al.* 2020). Similarly, G-IV have relatively good handling properties since it comes in capsules that automatically mix the powder and the liquid (Rajasekharan *et al.* 2018). In contrast, the first three generations require hand mixing of the powder and liquid, which makes it difficult to handle those materials (Kunert *et al.* 2020).

Aesthetically, G-I and G-II are not preferred to be used in aesthetic areas due to the bismuth oxide present in their composition, that have a high risk of causing discoloration (Camilleri *et al.* 2005; Kunert *et al.* 2020). In regard to the compressive strength, G-IV and G-VI have higher compressive strength and shorter setting time compared to G-I, G-II, and G-III (Kaur *et al.* 2017; Gasperi *et al.* 2020). However, the compressive strength of G-I is higher than both G-II and G-III (Basturk *et al.* 2013). The compressive strength of G-IV and G-VI makes them useful to be used as liner materials, especially for premolars and molars (Tsuchiya *et al.* 2025). However, G-VI is not suitable to be used for pulp capping or pulpotomy since previous studies showed lower biocompatibility and pH of RMCSCs (Bhavana *et al.* 2015; Manaspon *et al.* 2021). Moreover, it might be difficult to use G-VI for retrograde fillings due to the difficulty in exposing filled areas to the light-cure.

In terms of bioactivity, G-I and G-II, and G-III showed better dentin bridge formation compared to G-VI (Jia Kim *et al.* 2016). Forming hydroxyapatite (HAp) is essential for enhancing the bioactivity of calcium silicate cements (CSCs). This

improvement increases the sealing ability between the material and the dentin wall, which is crucial for preventing microleakage. Additionally, this characteristic can positively influence the remineralization of dentin, helping to restore its structural integrity and function. Therefore, incorporating HAP into CSC formulations can significantly enhance their performance in dental applications, leading to better clinical outcomes in restorative dentistry (Jia Kim *et al.* 2016). In this regard, G-I, G-II, and G-III can form the hydroxyapatite layer (Parirokh *et al.* 2010). Although G-IV has the potential to induce mineralization on the material surface, G-V lacks this ability, and G-VI has a lower remineralization ability compared to G-I (Yamamoto *et al.* 2017). The high pH and calcium release are considered the main reasons for the antibacterial properties of CSCs (Kunert *et al.* 2020), G-I, G-II, G-III, G-V, and G-VI had similar amounts of the released calcium ions to G- IV, which had superior antibacterial properties (Mahgoub *et al.* 2019).

2.3.1.4 Physical and biological properties of the CSCs

Upon setting, MTA-based materials form calcium hydroxide $Ca(OH)_2$, creating an alkaline environment that promotes antimicrobial activity and hard tissue formation (Holland *et al.* 2002, Sarkar *et al.* 2005). Released calcium ions enhance osteoblastic activity, while hydroxide ions increase pH, inhibiting bacterial growth (Maeno *et al.* 2005; Estrela *et al.* 2000). Biodentine is rich in calcium compounds and can release calcium ions in higher amounts and at a greater depth than MTA (Han *et al.* 2011; Matsumoto *et al.* 2013), promoting odontoblastic differentiation and reparative dentin formation (Zanini *et al.* 2012; Jung *et al.* 2015). Like most CSCs, TheraCal can stimulate hard tissue repair due to its ability to release high amounts of calcium ions and its initial high alkalinity (Gandolfi *et al.* 2012; Camilleri *et al.* 2014). The released calcium ions act as bioactive signalling molecules that promote pulp tissue repair (Tada *et al.* 2010). A study showed that after 28 days, TheraCal caused the formation of a complete dentin bridge and mild inflammation when employed for direct pulp capping in primate teeth (Cannon *et al.* 2014).

Different types of MTA have a relatively long and variable setting time. For example, ProRoot MTA sets in approximately 2.5 h, while MTA Angelus sets in under 24 minutes (Vivan *et al.* 2010). MTA demonstrates good dimensional stability, with less than 3% weight loss after 24 hours in water (Fridland *et al.* 2003). It is compressive strength increased from 40 MPa at 24 hours to 67 MPa over 24 h, though still lower than amalgam and IRM (Torabinejad *et al.* 1995; Chedella *et al.* 2010). The compressive strength of Angelus MTA was 46.4 MPa after 24 hours and increased to 65.1 MPa after 4 days of storage in an aqueous environment at 37 °C (Basturk *et al.* 2013; Dawood *et al.* 2015). The surface microhardness of ProRoot MTA and Angelus MTA was measured at 37.5 and 32.7 HV, respectively, after 24

hours of storage (Rhim *et al.* 2012; Dawood *et al.* 2015). The compressive strength (67.2-78.5 MPa) and surface hardness (45.4-48.4 HV) of Biodentine are higher than MTA. This might be related to the presence of water-soluble polymer in the liquid, which leads to a low water/powder ratio (Grech *et al.* 2013; Jang *et al.* 2014; Dawood *et al.* 2015). In addition, the flexural strength and elastic modulus of the Biodentine are also higher than the MTA and very similar to dentin (Camilleri *et al.* 2013; Rajasekharan *et al.* 2018). Although TheraCal is well tolerated by the immortalized odontoblast-like cells, cellular proliferation produced by TheraCal is lower than the MTA and Biodentine. It also has antibacterial activity higher than Biodentine and lower than MTA (Hebling *et al.* 2009; Poggio *et al.* 2014). TheraCal has lower solubility when compared to MTA Angelus, Pro Root MTA, and Biodentine (Gandolfi *et al.* 2012; 2015). The water sorption and porosity of TheraCal are similar to ProRoot MTA and Biodentine but lower than Angelus MTA (Gandolfi *et al.* 2015).

2.3.1.5 Sealing ability and bond strength of the CSCs

The sealing ability of the MTA is influenced by its bond strength to dentin, setting expansion, and mechanical interlocking (Hachmeister *et al.* 2002; Storm *et al.* 2008; Reyes-Carmona *et al.* 2010). A previous study reported that accelerated setting with calcium chloride ($CaCl_2$) enhances bond strength, likely due to increased calcium ion release and subsequent formation of hydroxyapatite-like precipitates (Bortoluzzi *et al.* 2006; Hong *et al.* 2010). These precipitates form within the dentin collagen fibrils, establishing both chemical and mechanical bonds that improve sealing and push-out bond strength (Reyes-Carmona *et al.* 2010). TheraCal has good sealing ability (Gandolfi *et al.* 2012). In neutral or acidic environments, the push-out bond strength of TheraCal is higher than Biodentine and MTA but lower than GIC. However, in an alkaline environment, the push-out bond strength of TheraCal becomes lower than Biodentine and higher than MTA and GIC (Makkar *et al.* 2015). Biodentine has a good sealing ability, comparable to resin-modified glass ionomer cement (S. Koubi *et al.* 2012). Biodentine forms a mineralized interfacial layer with tag-like structures penetrating dentinal tubules (Atmeh *et al.* 2012; Kim *et al.* 2015). Like other CSCs, Biodentine can release Ca^{+2} ions that form apatite-like crystals when in contact with phosphate (Colon P *et al.* 2012). Its high calcium ion release enhances biomineralization (Han *et al.* 2011; Gandolfi *et al.* 2013; Dawood *et al.* 2015). The pushout bond strength of (Guneser *et al.* 2013).

2.3.1.6 Limitations and drawbacks of the CSCs

Despite its clinical advantages, MTA-based materials have several limitations, including poor handling properties, long setting time, and presence of toxic components in the cement composition, difficult retrieval from the treated area, post-treatment tooth discoloration, and high cost (Torabinejad *et al.* 1995; Duarte *et al.* 2005; Bramante *et al.* 2008; Boutsioukis *et al.* 2008; Belobrov *et al.* 2011). However, although Biodentine has a shorter setting time and lower cost when compared to MTA, it is still considered to have a high cost and long setting time compared to the conventional calcium hydroxide (Kusum *et al.* 2015). Although it can be used as a dentine substitute under composite restorations or as a temporary filling for up to 6 months, it can't be used as an enamel substitute due to its susceptibility to abrasion (Koubi *et al.* 2013). Biodentine should be kept for 2 weeks before the placement of the composite restoration to allow for adequate setting and maturation (Hashem *et al.* 2014). The setting of the calcium silicate phase in TheraCal is slower when compared to Biodentine and is triggered by the moisture from the environment. This could raise doubts about the real impact of this stage on pulpal repair, as there may be inadequate moisture diffusion from the pulp-dentin complex to the cement. It is advisable to apply a thin layer to prevent aesthetic issues and discoloration under resin composite restorations due to its off-white colour (Qureshi *et al.* 2014). Moreover, as TheraCal is a resin-based cement could depend on the micromechanical bonding, which requires acid etching and bonding, which is not recommended in pulp capping procedures. In addition, the shrinkage of the resin-based material, which leads to bond failure, might be another issue with this material (Loguercio *et al.* 2004). In addition, the mechanism of inducing hard tissue formation is known to cause inflammatory and necrotic changes in the pulp tissue (Kuratate *et al.* 2008) and prolonged exposure to $Ca(OH)_2$ can weaken dentin structure (White *et al.* 2002; Parirokh *et al.* 2010). Moreover, arsenic is released in low amounts from the MTA-based materials. However, the amount of arsenic released is not a contraindication of the use of MTA (De-Deus *et al.* 2009). Another issue related to the MTA is the difficulty in retrieval and retreatments, as there is no known solvent to ease the retrieval (Boutsioukis *et al.* 2008). Another drawback is the tooth discoloration caused by the bismuth oxide, which is a component of the MTA (Naik *et al.* 2005; Maroto *et al.* 2006; Felman *et al.* 2013).

2.4 Ion-releasing materials

The use of ion-releasing dental materials-such as liners, bioactive adhesives, and restoration materials, has been shown to support the remineralization of sealed carious dentin. This process complements the natural remineralization mechanisms mediated by osteoblasts and mineral-rich pulpal fluid (Marchi *et al.* 2008; Corralo

et al. 2013). Among these materials, glass-ionomers were the first to actively release fluoride ions, thereby promoting remineralization while also chemically bonding to tooth structure without the need for additional adhesive agents. However, their clinical application has been limited due to their sensitivity to dehydration (Sidhu *et al.*1997), and susceptibility to excessive water uptake, which can lead to a rapid deterioration in mechanical properties (Feilzer *et al.* 1995). To address these limitations, a variety of ion-releasing materials have since been developed, incorporating fluoro-aluminosilicate as a filler component. A notable advancement in this area is the development of Surface Pre-Reacted Glass-ionomer (S-PRG) filler technology, initially introduced by Shofu Inc. Resin composites containing S-PRG fillers were commercialized in 2000, offering bifunctional benefits through their ion release and recharge capabilities (Saku *et al.* 2010).

S-PRG fillers are formulated as a fine powder capable of releasing multiple ions (Imazato *et al.* 2023). Structurally, S-PRG consists of a three-layered architecture: a fluoro-boro-aluminosilicate glass core, an intermediate glass ionomer phase, and an outer layer of silicone oxide (SiO_2) **(Figure 1).** The glass ionomer phase is responsible for the release of six distinct ions, including strontium (Sr^{2+}), borate (BO_3^{3-}), fluoride (F^-), sodium (Na^+), silicate (SiO_3^{2-}), and aluminium (Al^{3+}) ions. Notably, relatively high concentrations of borate and strontium ions are released into aqueous environments (Ito *et al.* 2011). These ions are released individually into solution and do not form salts, thereby enhancing their bioavailability.

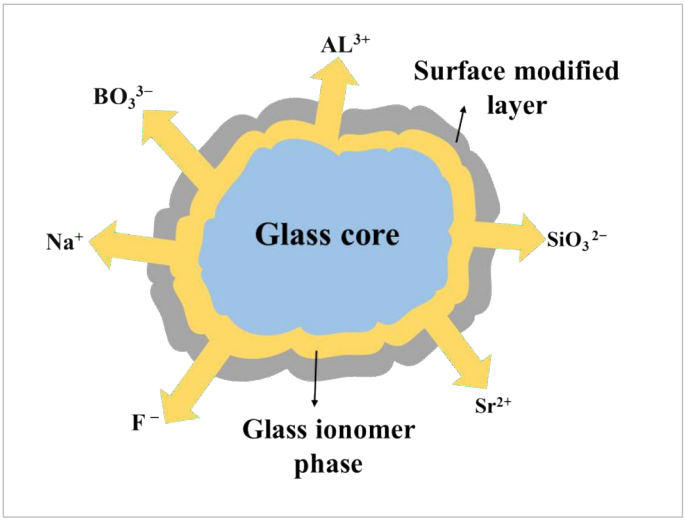


Figure 1. Surface pre-reacted glass filler particle, an outer SiO2 coating layer, a pre-reacted glass-ionomer phase, and an inner functional glass core. The S-PRG filler releases various ions, including strontium (Sr^{2+}), borate (BO_3^{3-}), fluoride (F^-), sodium (Na^+), silicate (SiO_3^{2-}), and aluminium (Al^{3+}) ions.

2.4.1 Bioactive functions exhibited by the S-PRG filler

Due to their ability to release multiple ions, S-PRG fillers demonstrate a range of bioactive functions, including tooth reinforcement, acid neutralization, promotion of mineralization, antibacterial and antifungal activity, inhibition of enzymatic degradation, and enhancement of cell viability (Imazato *et al.* 2023).

2.4.1.1 Tooth reinforcement

The tooth reinforcement effect of S-PRG fillers is attributed to the ions released from the fillers. Fluoride ions, released in high concentrations, contribute to the formation of fluorapatite, thereby enhancing the resistance of the tooth structure. Uo et al (2017) reported a 100-fold increase in strontium content in enamel after just one hour of immersion in S-PRG filler eluate (Uo *et al.*2017). Furthermore, Ogawa et al (2019) observed an increased uptake of aluminium, boron, and strontium ions, with strontium incorporation reaching up to 7900 ppm after 28 days. Strontium is effective in enhancing the acid resistance of teeth by converting hydroxyapatite to strontium apatite (Dedhiya *et al.* 1973; Featherstone *et al.* 1983). Additionally, borate ions released from S-PRG fillers exhibit acid buffering capacity and promote remineralization by absorbing into enamel and dentin (Hiraishi *et al.* 2021).

2.4.1.2 Antimicrobial Properties

Several previous studies have demonstrated the antibacterial effect of the S-PRG fillers against oral pathogens found in saliva and dental plaque, including *Enterococcus faecalis*, *Actinomyces*, *Propionibacterium acnes*, *Porphyromonas gingivalis*, and *Fusobacterium nucleatum* (Nao *et al.* 2015). The inhibition of *Streptococcus* mutans was shown to be dose-dependent, with the stationary phase delayed in the presence of S-PRG filler eluate (Nomura *et al.* 2018). Borate and fluoride ions are primarily responsible for these effects, even at low concentrations (Miki *et al.* 2016; Kitagawa *et al.* 2018). Miki *et al.* (2016) further demonstrated that borate and fluoride ions significantly inhibited *S. mutants'* growth when released from experimental resin composites containing 55.9 vol% S-PRG fillers. Additionally, S-PRG eluates have been shown to inhibit the growth of *P. gingivalis*, *F. nucleatum*, and *Aggregatibacter actinomycetemcomitans* (Yoneda *et al.* 2012; Kono *et al.* 2021).

2.4.2 Incorporation of the S-PRG fillers in dental treatments

S-PRG fillers have been incorporated into a wide range of dental materials, including resin composites, adhesives, resin cements, coating resins, fissure sealants, polishing

pastes, and temporary fillings. Experimental applications have extended to inorganic cements, root canal sealers, denture bases, tissue conditioners (Takakusaki *et al.* 2018), denture adhesives (Hatano *et al.* 2021), toothpaste (Amaechi *et al.* 2018), varnishes (Fernandes *et al.* 2024), CAD/CAM composites (Nakase *et al.* 2022), and even toothbrush filaments. Although the S-PRG fillers are incorporated into the resin-based matrices through salinization, the material can still release multiple ions at high concentrations. The capability of releasing the different ions grants various therapeutic effects, which are important for restorative treatment (Miki *et al.* 2016; Saku *et al.* 2010), endodontic treatment and perforation repair (Hirata-Tsuchiya *et al.* 2020), treatment of periodontal diseases (Yoneda *et al.* 2012; Kono *et al.* 2021), and prevention of denture stomatitis (Sudbery *et al.* 2004; Tsutsumi *et al.* 2016).

2.4.3 Limitations of the Surface pre-reacted glass containing materials

The bioactive properties of S-PRG fillers and S-PRG filler-containing materials have been extensively studied in both in vitro and in vivo (animal) models. These studies consistently demonstrate the potential of S-PRG fillers to enhance various aspects of dental treatment and oral care. However, despite promising experimental and preclinical findings, clinical evidence supporting the therapeutic benefits of S-PRG fillers remains limited (Imazato *et al.* 2023). Most available data are derived from laboratory or animal studies, and the translation of these findings into clinical practice remains a significant challenge. Long-term clinical trials are needed to validate the efficacy and safety of S-PRG-based materials in clinical applications.

2.5 Solvents in dentistry

A solvent is defined as any substance capable of dissolving another substance. In dentistry, solvents play a significant role, particularly in adhesive systems, where they are used to dissolve and reduce the viscosity of the monomers. This facilitates the infiltration of the monomers into demineralized collagen fibrils (Landuyt *et al*. 2007). The most commonly used solvents in adhesive dentistry are ethanol, acetone, and water (Landuyt *et al*. 2007; Ekambaram *et al*. 2015).

In endodontics, solvents are essential for dissolving root canal filling materials, especially in anatomically complex areas and dentinal tubules. Their use enhances cleaning efficacy and improves the success rate of retreatment procedures (Kaplowitz *et al*.1990; Oyama *et al*. 2002). In addition, it is proven to have the ability to remove gutta-percha by softening it (Taşdemir *et al*. 2008). For years, chloroform has been used in endodontics for retreatments, until it was banned in 1976 by the U.S. FDA due to its potential carcinogenicity and cytotoxicity. Other solvents that

were used in endodontics included eucalyptus essential oil, xylol, orange oil, tee tree oils have also been used (Karlović *et al*. 2000).

The selection of an appropriate solvent depends on its physical properties, including vapour pressure, viscosity, surface tension, solubility parameters, and hydrogen bonding capacity. These properties influence the diffusion of solvent-monomer mixtures into the collagen matrix, the subsequent removal of the solvent through evaporation post-application, and the ability to re-expand collapsed collagen. While water has a high hydrogen bonding capacity and low toxicity, it is not efficient at dissolving monomers on its own. Therefore, it is often combined with organic solvents to enhance performance. Proper storage and handling of the solvent/resin homogeneous composition are crucial considerations, as mishandling and improper storage can affect the stability of the mixture, leading to properties that may result in failures during restorative procedures (Abate *et al*. 2000; Lima *et al*. 2005).

2.5.1 Water

Water is a polar solvent that is naturally present on the dentinal surface and throughout the entire depth of demineralized collagen. Etching dentin with H_3PO_4, followed by water rinsing, is estimated to create a superficially demineralized layer consisting of 30% (v/v) collagen and 70% (v/v) water (Pashley *et al.* 2011). Although water forms strong hydrogen bonds, it is not effective in dissolving monomers and is therefore used in combination with co-solvents (Landuyt *et al.* 2007).

The strong hydrogen bonding ability of water helps to break the collagen interpeptide bonds after drying (Pashley *et al.* 2007). Water is known for its low vapour pressure compared to ethanol and acetone; this could be beneficial in slowing the evaporation from the adhesives, contributing to the longer self-life of commercial adhesives (Ekambaram *et al.* 2015). However, this same property makes evaporation from bonding surfaces more difficult (Jacobsen *et al.* 1998). Residual water in the hybrid layer can interfere with polymerization and contribute to the hydrolytic degradation of the bonded interface (Cadenaro *et al.* 2009; Malacarne-Zanon *et al.* 2009).

2.5.2 Ethanol

Ethanol (C_2H_6O) is the most commonly used solvent in adhesive dentistry. Due to higher molecular weight and vapour pressure compared to water, ethanol evaporates faster under air pressure and is more effective at dissolving monomers (Ekambaram at al. 2015). Ethanol is incorporated into dental adhesives alone or with water (Landuyt *et al.* 2007; Ekambaram *et al.* 2015).

Ethanol incorporation into adhesives improves the degree of conversion but may reduce the rate of polymerization compared to solvent-free resin formulations (Cadenaro *et al.* 2009). Adhesives containing ethanol pose the risk of solvent evaporation from containers due to improper storage and frequent opening, which can shorten shelf life. Additionally, the negative effects of residual ethanol on water sorption and solubility are more pronounced as the hydrophilicity of the resin blends increases (Malacarne-Zanon *et al.* 2009). In endodontics, several studies have evaluated the use of alcoholic solutions for the final rinse of the root canal treatment, and the results show that it enhances the penetration of the sealer (Stevens *et al.* 2006), and an increased wettability of root canal dentin after irrigation with ethanol (Pantoja *et al.* 2018).

2.5.3 Dimethyl sulfoxide (DMSO)

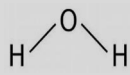
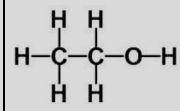
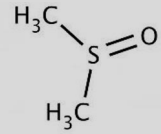
Dimethyl sulfoxide (DMSO; $(CH_3)_2SO$) is a colourless, transparent dipolar aprotic solvent, derived from wood pulp as a by-product with high solvent capacity for a wide range of inorganic and organic compounds (Martin *et al.* 1967). Due to its physicochemical properties, DMSO is effective as a solvent for water-insoluble compounds, and it has the ability to dissolve most other solvents, including polar and non-polar compounds (Ruiz- Delgado *et al.* 2009). DMSO-water interactions are 1.3-fold stronger than water-water complexes; therefore, DMSO has the proper polarity to break down water's self-associative tendencies and to form stable complexes with water (Mehtälä *et al.* 2017). It also allows the DMSO to replace water (Kirchner *et al.* 2002).

Water plays an important role in maintaining the structure of the collagen triple helix (Bertassoni *et al.* 2012 and DMSO can replace water in this role. Several medical and pharmaceutical effects of DMSO have been reported previously, including the analgesic effects, anti-inflammatory effects, enhancement of the delivery of specific drugs, diuretic effect, and their ability to penetrate different biological membranes (Jacob *et al.* 1967; Santos *et al.* 2003). DMSO is widely accepted as a solvent in medicine due to its well-established ability to quickly and effectively penetrate biological tissues (Swanson *et al.* 1985). It also has wide applications in cell biology (Ahkong *et al.* 1975), cell fusion and differentiation (Lyman *et al.* 1976).

Recently, the use of DMSO has extended to several dental applications, it has been used to solvate resin monomers during cytotoxicity testing, in addition, it has been demonstrated to enhance the stability of dentin resin bonds in both the short term and long term, effectively preserving the hybrid layer when used in low concentrations (0.04%) (Tjäderhane *et al.* 2013), and in relatively high

concentrations (50%) (Stape *et al.* 2015). **Table 2** represents the physical and chemical properties of the different solvents used in this series of studies.

Table 2. Physical/chemical properties of the different solvents used in this study series. Modified from (Smallwood 2012; Ekambaram, *et al*. 2015).

	Water	**Ethanol**	**DMSO**
Structure			
Chemical formula	H_2O	C_2H_6O	C_2H_6OS
Molecular weight	18.02	46.07	78.13
Vapour pressure at 20℃ (mmHg)	17.54	44	0.61 at 25° C
Boiling point (℃)	100.00	78.5	189.00
Melting point (℃)	0.00	-94.3	18.4
Density (g/ml)	0.998	0.789	1.092
H-bonding capacity (J/cm^3)	42.3	19.3	13.1
Surface tension at 20℃ (dyn/cm)	72.8	22.27	43.53

3 Aims

With the emergence of the new commercially available bioactive materials and concerns about their potential for adverse effects on tooth integrity, this Ph.D. thesis aimed to systematically analyze the direct and indirect effects of different bioactive materials on the collagen matrix integrity of mineralized and partially demineralized dentin, cytotoxicity to pulp, and bond strength to intraradicular dentin.

The specific aims of these studies were to:

- Investigate the effect of different ions released from a bioactive filler on the dentin protease activity. The tested hypothesis was that pretreatment with ion solutions would not inactivate matrix-bond cysteine cathepsins and MMPs.
- Evaluate the trans-dentinal cytotoxicity of dentin pretreatments containing different solvents (DMSO, ethanol, and their aqueous dilutions) in combination with bioactive materials on trans-dentinal cell viability in simulated deep clinical cavities. The hypothesis tested was that neither the composition of the pretreatment nor the bioactive materials have a significant effect on the trans-dentinal cytotoxicity.
- Assess the effect of solvent-containing pretreatments (DMSO, ethanol, and their aqueous dilutions) on the push-out bond strength of different bioactive cements. The tested hypotheses were that neither the composition of bioactive cement nor dentin pretreatments would significantly influence intraradicular bond strengths or interfacial integrity.
- To investigate the direct effect of bioactive materials on dentin protease activity when in direct contact with the dentin. The hypothesis is that treatment with bioactive materials would not inactivate matrix-bond cathepsin K and MMPs nor induce changes in the mechanical properties of dentin.

4 Materials and Methods

4.1 Materials

4.1.1 Preparation of dentin specimens (Studies I–IV)

Five hundred and thirty-two extracted sound human teeth were used in these series of studies. The teeth were extracted for routine dentin treatments and were exempt from ethical approval according to local regulations (Tissue Act 20). Teeth were stored in 0.9 % NaCl containing 0.02% NaN_3 at 4 ℃ and used no later than 3 months after extractions.

4.1.1.1 Preparation of dentin beams (Studies I, IV)

One hundred and thirty specimens (Eighty specimens for study I and 50 specimens for study IV) were obtained by sectioning the mid-coronal dentin (with dimensions of 6 mm x 2 mm x 1 mm for study I, and 0.3 mm x 3 mm x 7 mm for study III) of human third molar teeth after removing the enamel and superficial dentin with a low-speed saw (Isomet, Buehler Ltd., Lake Bluff, IL, USA) under water-cooling. Beams were treated with 10 wt% H_3PO_4 for 24 h at 25 ºC for complete demineralization and then rinsed in distilled water at 4 ºC for 1 h (study I). The absence of residual minerals was confirmed using digital radiography. In study III, Dentin beams were demineralized with 0.5 M ethylenediaminetetraacetic acid (EDTA) and rinsed with distilled water, both for 2 hours at 4 °C under constant stirring. After demineralization, beams were placed into individually labelled 96-well plates and dried in a vacuum desiccator containing dry silica beads for 72 h.

4.1.1.2 Preparation of Dentin discs and measurement of dentin permeability (Study II)

Dentin discs were sectioned, immediately above the pulp horns of each tooth (500 μm in thickness using a precision diamond saw (Isomet 1000 Precision Saw, Buehler, and Lake Bluff, IL, USA) under water-cooling. Discs with perforations, indicating pulpal exposures, were discarded and replaced. The occlusal surface of

dentin discs was polished with 320-grit SiC abrasive paper (CarbiMet, Buehler Ltd; USA) to achieve a final thickness of 300 μm (±15 μm). To remove the smear layer, 50% citric acid was applied for 30 s and rinsed for 30 s. Dentin permeability was measured using an SLI-1000 Liquid Flow Meter (Sensirion, Switzerland) within a modified split-chamber unit, connected to a deionized water container, simulating a hydrostatic pressure of 20 cm. After measurement, dentin discs were rinsed with distilled water for 15 s, autoclaved in 0.9% sodium chloride at 121 °C for 25 min, and then evenly distributed among 23 balanced groups based on dentin permeability.

4.1.1.3 Preparation of root specimens (Study III)

Sound bicuspid teeth with completely formed apexes were horizontally decoronated at the level of 0.5 mm radicular to the cemento-enamel (CE) junction using a low-speed saw (Buehler Ltd., Lake Bluff, IL, USA), generating 16 ± 1 mm long roots. Apical patency was verified using an ISO size-10 K-file (Dentsply Maillefer, Ballaigues, Switzerland). The working length of each root was determined. Then canals were instrumented using Protaper rotary nickel-titanium instruments (SX, S1, S2, F1, F2, F3, F4; Dentsply Maillefer) using a low-speed rotary endodontic handpiece (X-smart; Dentsply Maillefer). The master apical instrument was the Protaper F4 with a tip size of 40. Fiber post-preparation burs (3M ESPE RelyX, St. Paul, USA) were used up to size blue #3 with a tip size of 0,9 mm to standardize the canal diameter. Irrigation was performed using 27-G Endo-Eze irrigator tips (Ultradent Products Inc, South Jordan, UT) alternating 2 ml 2.5% sodium hypochlorite and 17% ethylenediamine tetra acetic acid (EDTA) solution (Dentsply Maillefer, Ballaigues, Switzerland) between the use of each instrument and at the end of instrumentation with 2 ml 17% EDTA (Inter-Med Inc/Vista Dental Products, Racine, WI) for 1 minute, 2 ml 2.5% sodium hypochlorite, and 2 ml normal saline. The root canals were dried with 2 paper points size F4 (Dentsply, York, PA, USA).

4.1.2 Preparation of ion solutions (Study I)

Standard solutions containing 100 ppm of boron, fluoride, sodium, silicon, strontium, and a 10-ppm solution of aluminium were prepared in distilled water to simulate the range of ions potentially released from S-PRG fillers. To prepare the S-PRG filler eluate, distilled water was mixed with S-PRG fillers (1 μm) at a ratio of 1:1 by weight (1L: 1000 g). The mixture was stirred for 24 h at 23 °C and centrifuged to precipitate the solid phase, and the supernatant S-PRG filler eluate was collected. The supernatant was filtered (pore size 0.45 μm) to remove any residual insoluble material, and the resulting filtrate was used as S-PRG filler eluate. The concentrations of various ions in the S-PRG-filler eluate were quantified using an emission spectrophotometer (ICPS-

8000, Shimazu) and an F-electrode (Model 9609BNWP, Orion Research) with an ion-selective electrode meter (Model 720A, Orion Research). Ion concentrations in the S-PRG filler eluate were: Al^{3+} (19.4 ppm), BO_3^{3-} (1480.2 ppm), Na^{+} (549.2 ppm), SiO_2^{3-} (14.5 ppm), Sr^{2+} (151.5 ppm), and F^{-} (135.5 ppm).

4.1.3 Preparation of pretreatment solutions (studies II, III)

Pretreatment solutions were obtained by diluting DMSO in either distilled water (DMSO/H_2O) or ethanol (99.8 % Ethanol, Sigma-Aldrich, St Louis, MO, USA; DMSO/EtOH) using a graduated cylinder. The 50% (v/v) binary solutions were prepared 24 hours before use. DMSO was poured into the graduated cylinder, followed by the respective solvent (water or ethanol) to reach twice the initial volume of DMSO with minimal splashing of the solutions. The content was transferred to a 100 ml glass reagent bottle, which was then closed and swirled in a circular motion for 30 s to mix the binary solutions. Pretreatments were kept from light and at room temperature during use.

4.1.4 Preparation of the silicate-based biomaterials (Studies II-IV)

The different silicate-based materials used in the series of studies, their composition, manufacturer, and application are explained in **Table 3.** Different silicate-based bioactive materials have been used in this series of studies, including two different types of MTA: MTA Orbis (MTA, Orbis Oy, Espoo, Finland), and MTA Angelus, or what is known as the Bio-C (Angelus Indústria de Produtos Odontológicos S/A, Londrina, Brazil), TheraCal L.C (TheraCal LC, Bisco, Chicago, Illinois, USA), and a pre-reacted glass-ionomer filler cement (S-PRG, Shofu Inc, Higashiyama-ku, Kyoto, Japan). Both types of MTA used were mixed according to the manufacturer's instructions. MTA Orbis comes in a powder and liquid form. MTA Angelus comes in a premixed syringe. TheraCal is a light-curable material; application and light-curing were carried out according to the manufacturer's instructions.

4.1.5 Preparation of bioactive materials blocks (study IV)

Silicone molds measuring 1 mm × 3 mm × 7 mm were employed to create ten blocks of the materials explained in Table 3. Bio-C, Orbis, and S-PRG were mixed as per the manufacturer's instructions, loaded into the mold, and kept moist on both sides within a humidified chamber for 2 h until set. TheraCal was placed on the mold and then light-cured for 20 seconds using a light-curing unit (Elipar Deepcure; 3M ESPE) at 1200 mW/cm^2.

Table 3. Different materials used in this series of studies, their composition, manufacturer, and application procedure.

Materials	Study	Composition	Manufacturer	Application procedure
Standard Ions solutions	Study I	A standard solution containing 100 ppm of boron, fluoride, sodium, silicon, strontium, and a 10-ppm solution of aluminium in distilled water. S-PRG filler eluate was prepared by mixing 1000g of S-PRG fillers in 1L of distilled water in a 1:1 ratio.	Shofu Inc., Higashiyama-ku, Kyoto, Japan	Each dentin beam was treated for 5 min with 200 µL of the respective ion or S-PRG filler eluate.
TheraCal L.C	Study II, III, IV	Portland cement type III (20–60%), poly (ethylene glycol) dimethacrylate (10–50%), bis-GMA (5–20%), and barium zirconate (1–10%)	Bisco Inc, Schamburg, IL, USA.	Study II: A 1 mm-thick layer was applied over moist dentin and light-cured for 20 s. Study III: 2 mm thick increments were applied inside the canal and light-cured for 20 s. Study IV: Molds made of silicone were used to prepare ten blocks (1 mm × 3 mm× 7 mm) of TheraCal and light-cured for 20 s.
Mineral Trioxide Aggregate (Orbis)	Study II, III, IV	Tricalcium silicate, Dicalcium silicate, Calcium carbonate filler, Zirconium Oxide (radio-opacifier) (5%), Iron oxide (coloring agent).	MTA; Orbis Oy, Espoo, Finland	Study II: A1 mm-thick layer was applied over moist dentin and the MTA layer was covered with a sterile wet cotton pellet. Study III: Increments of the material were placed inside the canals using an amalgam carrier and a plugger, to assure the filling of the whole canal. A wet cotton plate was placed over the MTA. Study IV: Silicone molds were used to prepare ten blocks (1 mm × 3 mm × 7 mm) of the material. MTA was loaded into the mold and kept wet on both sides inside a humidified chamber for 2 hours until set.
Mineral Trioxide Aggregate (Bio-C)	Study IV	Calcium silicates, calcium aluminate, calcium oxide, zirconium oxide, iron oxide, silicon dioxide, and a dispersing agent	Angelus Indústria de Produtos Odontológicos S/A, Londrina, Brazil	Study IV: Silicone molds were used to prepare ten blocks (1 mm × 3 mm × 7 mm) of the material. The material was mixed according to the manufacturer's instructions, loaded into the mold, and kept wet on both sides inside a humidified chamber for 2 hours until set.

Materials	Study	Composition	Manufacturer	Application procedure
Surface pre-reacted glass (S-PRG)	Study II, III, IV	Powder: Zinc oxide-based inorganic compound filler, S-PRG filler, additives. Liquid: Poly carboxylic acid derived solution, water	Shofu Inc., Higashiyama-ku, Kyoto, Japan	Study II: A 1 mm-thick layer was applied over moist dentin. Study III: Increments of the material were placed inside the canals using an amalgam carrier and a plugger, to assure the filling of the whole canal. Study IV: Silicone molds were used to prepare ten blocks (1 mm × 3 mm × 7 mm) of the material. S-PRG was loaded into the mold and kept wet on both sides inside a humidified chamber for 2 hours until set.

4.2 Research methods

Different research methods were included in this study series to evaluate the activity of different bioactive materials. The effect of the Surface pre-reacted glass, TheraCal, and MTA was assessed directly or indirectly via mechanical, enzymatic, microscopic, and spectroscopic tests.

4.2.1 Measurement of loss of dry dentin mass (Studies I, IV)

The degradation of demineralized dentin matrices was assessed indirectly by measuring the loss of dry dentin mass. This method allowed for an estimation of the amount of solubilized collagen matrix over time (Tezvergil-Mutluay *et al*. 2011). To determine the initial dry mass of demineralized specimens, the beams were demineralized, rinsed, and then stored in a vacuum desiccator for 72 h. After this period, the dry mass of each beam was measured using an analytical microbalance (XP6 Microbalance, Mettler Toledo, Hightstown, NJ, USA). Following the initial measurements, the beams were divided into different groups so that the mean dry mass of each group was statistically similar. Subsequently, the demineralized dentin beams were rehydrated in distilled water at 4 ºC for 1 h before being treated with relevant ion solutions (study I) or placed in direct contact with the occlusal surface of the previously prepared materials blocks (study IV). The initial mass of demineralized dentin beams was used as a reference. After each incubation period, the dentin specimens were rinsed free of buffer salts in distilled water at 4 ºC for 24 hours, and the measurement of dry mass was repeated after each incubation period.

4.2.2 Measurement of direct inactivation effect by generic MMP assay (Studies I, IV)

A Sensolyte Generic MMP assay kit (Sensolyte Generic MMP assay; Anaspec, San Jose, CA, USA) was utilized to evaluate the catalytic activity of MMPs bound to the dentin matrix. This method measures the collective activity of various MMPs, including MMP-1, 2, 3, 7, 8, 9, 12, 13, and 14, providing direct insights into the effectiveness of MMP activation or deactivation. The assay quantifies MMP activity using Ellman's Reagent, a thiol-reactive agent that produces a coloured product, 2-nitro-5-thiobenzoic acid (TNB), which can be detected using a spectrophotometer at around 412 nm. (Riddles *et al.* 1983; Riener *et al.* 2002). The test is capable of detecting active MMPs on dentin at levels of nanograms (Tezvergil-Mutluay *et al.* 2010; 2012). To evaluate the baseline activity of matrix-bound MMPs, demineralized dentin beams of control or experimental groups (1 mm x 2 mm x 6 mm in study I and 0.3 mm x 3 mm x 7 mm in study IV) were incubated with 150 µl chromogenic substrates and 150 µl assay buffer of the kit in 96-well plate for 5 min

at 37 °C in study I, and for 60 minutes at 25 °C in study IV, and 200 µL of incubation solution were read every 15 min using a microplate reader (Synergy HT, BioTek Instruments, Winooski, VT, USA) at 412 nm wavelength until peak value obtained individually. After the baseline measurements of individual MMP activity of dentin, beams were divided into 8 groups in Study I and 5 groups in Study IV (n=10/ group) so that the baseline activity was not significantly different among different treatment groups. Beams were rinsed free of MMP substrate in distilled water, and then dentin beams were treated with 200 µL of the respective ion or S-PRG filler eluate for 5 min in study I, and beams were incubated with the material blocks in study IV for the different tested incubation times. After each incubation period, the beams were rinsed in distilled water for 5 min. Untreated demineralized dentin beams served as controls. The total MMP activity of experimental groups was retested using the generic assay as described above. The generic MMP activity of the groups was expressed as a percentage of the untreated baseline level of each specimen to determine the relative inhibition or activation of the specimens.

4.2.3 Measurement of collagen degradation products (Studies I, IV)

Enzyme-linked immunosorbent assays (ELISAs) were used to measure enzyme-specific degradation products of collagen molecule C-terminal telopeptide fragments in the incubation media. Both cathepsin K and matrix metalloproteinases (MMPs) can cleave specific type I collagen telopeptides. These telopeptide markers have been utilized to identify the enzyme family responsible for type I collagen degradation. MMPs generate type I collagen telopeptides (ICTP) (Garnero *et al*. 1999; 2003; Osorio *et al*. 2011), while cathepsin K generates CTX (Sung *et al*. 2003; Tersariol *et al*. 2010; Tezvergil-Mutluay *et al*. 2013; Takahashi *et al*. 2013). Following the treatment (5 min treatment with corresponding ion solution in Study I. And placing in direct contact with the materials block in Study IV) dentin beams were incubated in individually labelled tubes containing 1 ml of the simulated body fluid containing 5 mM HEPES, 2.5 mM $CaCl_2$•H_2O, 0.05 mM $ZnCl_2$, 0.68 mM KH_2PO_4, 30 mM KCl and 0.3 mM NaN_3 (pH 7.4) for 1 week in study I. and 0.5 ml calcium and zinc containing media at 37 °C for 24 hours, 1 week, 1 month, 2 months, and 6 months in a shaking-water bath (60 cycles/minute). The CM contained 5 mM HEPES, 2.5 mM $CaCl_2 \cdot H_2O$, 0.02 mM $ZnCl_2$, and 0.3 mM NaN_3 (pH 7.2) in Study IV.

4.2.3.1 ICTP assay (Study I, IV)

Evaluation of MMP-mediated type I collagen degradation was conducted using ELISA kits for ICTP. Therefore, the incubation medium of each beam was analyzed

using human serum ICTP assay kits (ICTP RIA, Orion Diagnostica, Espoo, Finland). The amount of the ICTP in the storage solution of the dentine beams was calculated with a 5-point fitting curve with known concentration standards from 1 to 50 ng/ml, with the limit of 0.6 ng/ml by a gamma counter (WIZARD 1470 Wallac, Perkin Elmer Finland). Each measurement was performed in duplicate (n=10/group). After each incubation period, samples of the incubation media were collected and replaced with fresh medium for further incubation (study IV).

4.2.3.2 CTX assay (Study I, IV)

Measurement of cathepsin K-induced type I collagen degradation was performed by assessing the release of CTX (Serum CrossLaps ELISA, Immunodiagnostic System, Farmington, UK) into the storage media. With a plate reader (Synergy HT, BioTek Instruments, Winooski, VT, USA). Ten to fifty µL of the incubation medium was used for the CTX ELISA assay. The amount of CTX release was calculated with a standard curve using standards with known concentrations provided in the kits. The assays were performed in duplicates.

4.2.3.3 Hydroxyproline quantification (Study IV)

The solubilization of collagen was evaluated by hydroxyproline quantification. The amino acid composition of type I collagen includes 9.6 % hydroxyproline. To measure the overall quantity of collagen fragments released by MMPs and CCs into the incubation medium, a hydroxyproline assay was carried out following the protocol described by Tezvergil-Mutluay in 2010 (Tezvergil-Mutluay *et al.* 2010). In Brief, aliquots of HYP standards (2–20 µg) prepared from stock solutions and 25 µl of re-suspended solutions were combined with 25 µl of 4 N NaOH (2 N final concentration) in a total volume of 50 µl in 2 ml Nalgene O-ring tubes. The samples were hydrolysed by autoclaving at 120 °C for 20 minutes. Then, 450 µl of chloramine-T was added to the hydrolysed tubes and mixed gently to allow oxidation for 20 min at room temperature. Following this, 500 µL of Ehrlich's aldehyde reagent was added to each specimen to facilitate chromophore formation by incubating the specimens at 65°C for 20 minutes. Absorbance values were measured using a spectrophotometer (Model UV-A180, Shimadzu, Tokyo, Japan) at 550 nm and plotted against the standard HYP curves to determine the HYP release (ng/mg of dry dentin).

4.2.4 Preparation of pulp-derived three-dimensional cell culture (Study II)

Bovine pulp-derived cells, transfected with clonal large T-antigen (SV40), were received as a kind donation from Regensburg University. These cells were cultured in Eagle's minimum essential medium (α MEM) (Sigma Aldrich, Gillingham, UK), 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. Nylon meshes with a pore size of 150 µm and a diameter of 8 mm were prepared by cleaning with 0.1 M acetic acid for 30 minutes, followed by three washes with sterile water, and then 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 supplemented with 20% fetal serum. Four meshes were placed in each cell culture insert (Greiner bio-one, Nurtigen, Germany) with sufficient nutritional medium for 48 hours to facilitate proper cell growth over the nylon mesh and were then incubated for 48 hours at 37 °C, 5% CO_2, and 100% humidity. Following incubation, each nylon mesh was transferred to a separate well in a 24-well tissue plate. In each well, 1 ml of MEM α and 10% FBS were added to nourish the cells. The medium was changed daily for 14 days in the incubator to produce cells on the mesh in a three-dimensional form.

4.2.5 Evaluation of cell viability by MTT assay (Study II)

One of the most commonly used tests to evaluate cytotoxicity precisely and quickly is the MTT test. It is based on a quantitative measurement of cell viability from their metabolic activity, through the 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. In this test, the viability of living cells is detected as a reflection of the succinate dehydrogenase enzyme (SDH) action that reduces the MTT reagent to formazan crystals, which can be analyzed after their dissolution by solubilizing solution.

4.2.6 Using MTT assay to evaluate the trans-dentinal cytotoxicity (Study II)

The evaluation of trans-dentinal cytotoxicity was conducted following ISO standards (ISO, 2017; Schmalz and Galler, 2017). In this method, the tested materials/pretreatments were applied to dentin discs and allowed to permeate through dentinal tubules to assess cell response in split-chamber compartments. After a 14-day incubation period, polyamide meshes containing three-dimensional

cell cultures were positioned in the lower compartment of commercially available cell culture perfusion chambers (Minucells and Minutissue, Bad Abbach, Germany), in direct contact with the pulpal side of the dentin disc and secured in place by a stainless- steel holder. The pulpal compartment was perfused with assay medium supplemented with 5.96g/L hydroxyethyl piperazineethanesulfonic acid (HEPES) at a rate of 0.3ml/h for 24 hours using a precision pump (Minucells and Minutissue, Bad Abbach, Germany). Perfusion was briefly halted, and treatment solutions (1.5µl) were applied for 10 seconds over the dentin slice inside the chamber, followed by the tested biomaterials according to experimental groups. The test materials (MTA, TheraCal, and SPR-G) were mixed according to the manufacturer's instructions and applied in a 1 mm-thick layer as per the manufacturer's recommendations. MTA was then covered with a wet cotton pellet (sterile NaCl 0.9%), and TheraCal was light-cured for 20 seconds using an LED light-curing unit (Elipar; 3M ESPE) at 1200mW/cm^2. After sealing the split chambers, cells were perfused at a rate of 0.2ml/h for 24 hours. The cell meshes were sectioned by the metallic inserts into 4 mm2 circular pieces, retrieved from the chambers, and stored for 2 hours in a cell incubator at 5% CO_2 and 100% humidity at 37 °C. Each experiment was conducted with two replicates and repeated eight times.

4.2.7 Evaluation of the push-out bond strength (Study III)

The push-out test was carried out using a Universal Testing Machine (Autograph AGS-X, Shimadzu, Japan), where each specimen underwent compressive loading apico-coronally with a 5 KN load cell at a crosshead speed of 1 mm/min until bond failure occurred, and the maximum failure load was measured in newtons (N). Subsequently, images of the specimens were captured using a light microscope at 20x magnification with a stereomicroscope (Leica M165C, Leica Microsystems GmbH, Wetzlar, Germany). The area of each specimen's bonding interface was determined using digital image-analysis software (ImageJ; National Institutes of Health, Bethesda, Maryland, USA). Since the shape of the filling and the bonded interface represents the shape of a trapezium, the area was calculated in a similar way to the calculation of the trapezium area, following the equation:

$$\text{Adhesion surface area (mm}^2\text{)} = \left(\frac{D1+D2}{2}\right) \times \pi \times \text{h}$$

Where D1 and D2 are the largest and smallest canal diameters, respectively, and h is the thickness of the root slice (El-Ma'Aita *et al.* 2013; Yassen *et al.* 2016). To calculate the push-out bond strength in Mega Pascals (MPa), the ultimate load at failure recorded in newtons (N) was divided by the area (mm²) of the filling-dentin interface.

4.2.8 Assessment of failure mode (Study III)

The failure modes were examined using a stereomicroscope at 40x magnification. The failure mode was classified into adhesive (failure at the sealer-dentin or the sealer-core material interface), cohesive (failure within sealer or dentin), or mixed (failure in both the sealer and dentin) (Skidmore *et al.* 2006).

4.2.9 Assessment of adhesion surface (Study III)

Qualitative analysis of the interface between dentin and cement was performed using scanning electron microscopy (SEM) imaging in the backscattering mode at an accelerating voltage of 10 kV (Phenom ProX, Phenom-World). Sequential micrographs captured at a magnification of 2000x were obtained from all experimental groups to analyse the characteristics of the dentin-cement interface.

4.2.10 Total extractable protein (Study IV)

Since the original work of Smith *et al* (Smith *et al*. 1985), the bicinchoninic acid (BCA) assay has been widely utilized for determining protein concentration due to its simplicity, high sensitivity, and ability to tolerate interfering substances (Sapan *et al*. 1999; Walker *et al*. 2002). This method is extensively employed in biochemistry and molecular biology laboratories for quantifying protein concentrations in samples. BCA is a sensitive, stable, and highly specific reagent for Cu^{2+}. The method simply relies on the biuret reaction for the generation of a coloured complex between peptide bonds and copper atoms when protein is placed in an alkaline environment containing Cu^{2+} (Weichselbaum *et al*. 1946; Gornall *et al*. 1949). To quantify the amount of protein in the extracts, the total protein content in the extracts was measured using a Micro BCA Protein Assay Kit (Thermo Scientific, IL, USA). In brief, 150 µl of each standard and storage media of the samples were added to a microplate well along with 150 µl of working reagent, and the mixture was shaken on a plate shaker for 30 seconds. The plate was then covered and incubated at 37°C for 2 hours. After cooling to room temperature, the absorbance was recorded at 562 nm using a plate reader (Synergy HT, BioTek Instruments, Winooski, VT, USA), and the protein concentration of each unknown sample was determined using a standard curve.

4.2.11 PH assessment (Study IV)

The pH measurements of each specimen were carried out immediately after collecting the specimens for dry mass. The pH values were determined with a previously calibrated electrode pH meter (PHM210; Radiometer analytical SAS,

France) with an accuracy of ± 0.01. After each measurement, the electrode was washed with distilled water to avoid subsequent contamination. The mean pH of the groups was calculated for each incubation period. The CM was stored frozen (-80 °C) until the end of the experiment, when the media were analyzed for ICTP, CTX, hydroxyproline, and micro-BCA.

4.2.12 Modulus of elasticity (Study IV)

The 3-point bending test was selected for evaluation of the apparent elastic modulus due to its non-destructive nature, allowing repeated measurements to be performed on the same sample. After the preparation of dentin beams, a dimple was made on the corner of each beam on the occlusal surface to allow repeated measures on the same beam surface. Digital images of the beams were obtained on a stereo microscope (Leica M60, Leica Microsystems) to determine the thickness and width of the demineralized beams. Dimensional measurements were made using an open-source image analysis software (ImageJ, National Institute of Health, Bethesda, MD, USA). Beams were then placed on a 3-point flexure jig, with a 5 mm span length between supports, Using a 5 N load cell (SMT1-5N, Interface, Scottsdale, AZ, USA) mounted on a universal testing machine (Autograph AGS-X, Shimadzu, Japan), the beams were loaded to a 3% strain at a displacement rate of 0.5 mm min–1 and kept fully immersed in distilled water during testing (Bedran-Russo *et al.* 2007). After maximum displacement, the load was immediately returned to 0% stress within 15 s without further holding, to prevent creep of the demineralized collagen matrix. Specimens were tested by a blinded operator. Load-displacement curves were converted to stress-strain curves and the apparent modulus of elastic (E) in MPa was calculated using the following formula:

$$\text{Modulus of elasticity (MPa)} = \frac{ml^3}{4bh^3}$$

where m is the steepest slope of the linear portion of the load- displacement curve (N/mm), L is the span length (5 mm), b is the width of the test specimen and h is the beam thickness. The test was performed in duplicates for each beam initially, after fresh application, and after each incubation time; the average of the two readings was used as the final elastic modulus value.

4.2.13 Assessment of calcium leaching of bioactive materials (Study IV)

For the evaluation of calcium ion leaching, cylindrical specimens of 10 mm diameter and 2 mm thickness were prepared and incubated in 5 ml of distilled water and

incubated for 3,7,14, and 28 days. After incubation periods, calcium ion leaching was assessed using an atomic absorption spectrophotometer (AA6800; Shimadzu, Tokyo, Japan) equipped with a calcium-specific hollow cathode lamp. A blank solution was also tested, and the amount of calcium released from the blank solution was reduced from the tested materials.

4.3 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 relative MMP activity, loss of dry mass (%), the release of ICTP (ng telopeptide/mg dry dentin), and CTX (pg/mg dry dentin) were individually analyzed using the Kolmogorov- Smirnov test for normality and the modified Levine test for homoscedasticity. After confirming the normality and equality of variance assumptions, the data underwent a one-way analysis of variance (ANOVA). Post hoc comparisons were conducted using the Tukey test in SPSS (SPSS Statistics for Mac, Version 27, IBM, NY, USA). Statistical significance was set at α=0.05. In study II, the evaluation of cell damage was also classified as non-cytotoxic, moderately cytotoxic, and severely cytotoxic by ISO 7405 (ISO, 2017). As the trans-dentinal datasets did not follow a normal distribution, the data were assessed using the Kruskal-Wallis test with a significance level of α=0.05. The analysis was conducted using IBM SPSS Statistics for Windows, version 26 (IBM Corp., Armonk, NY, USA). In study III, the push-out bond strength values for all the tested groups were analyzed using IBM SPSS Statistics for Windows, version 26 (IBM Corp., Armonk, NY, USA). The data did not pass the normality and homoscedasticity tests ($P < 0.05$). The Kruskal-Wallis test and ANOVA on ranks for pair-wise comparisons were utilized, with a significance level of $P < 0.05$ considered statistically significant. In study IV, the Kruskal-Wallis one-way analysis of variance on ranks was utilized to determine if there were differences between groups at different time points. In the event of significant differences, pairwise multiple comparisons were conducted using Dunn's method. These analyses were carried out following the validation of normality and equality of variance using Shapiro-Wilk and Brown-Forsythe's tests, respectively.

5 Results

5.1 Evaluation of the loss of dry dentin mass (Study I, IV)

Studies I and IV evaluated the loss of dry mass of demineralized dentin matrices to measure the degradation of dentin after treatment with the ion solutions or the bioactive silicate-based biomaterials. In study I, the result of the loss of dry mass showed that the mass loss of the fluoride-treated group was significantly lower compared to the strontium and the control group *(p>0.05)* **(Figure 2)**; however, strontium showed the highest mass loss among the tested groups.

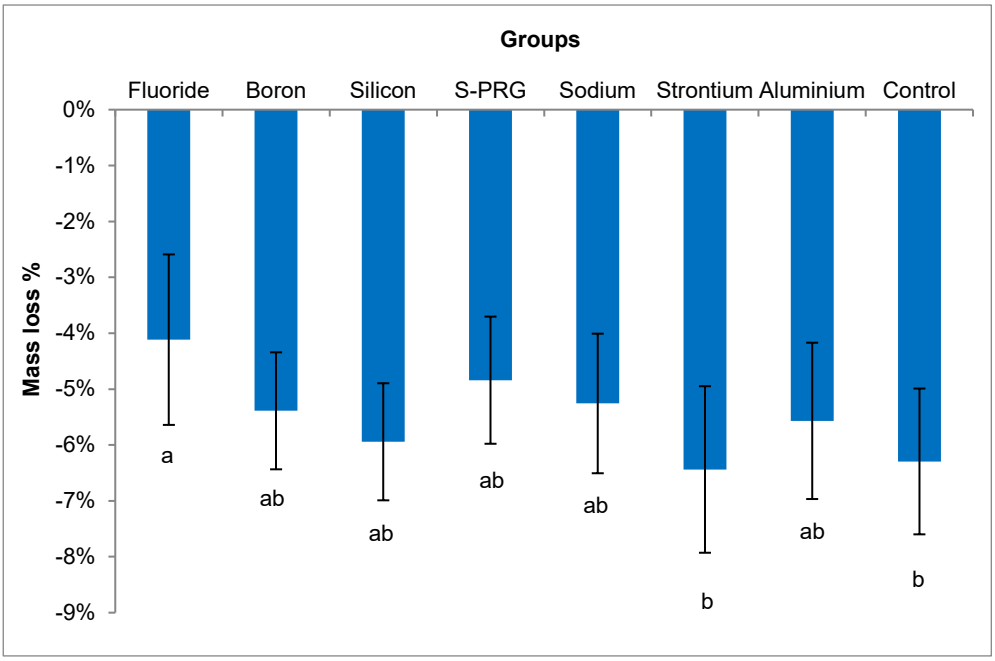


Figure 2. Dry mass loss percentage after the 1-week incubation period. (From the supplementary data published in Study I).

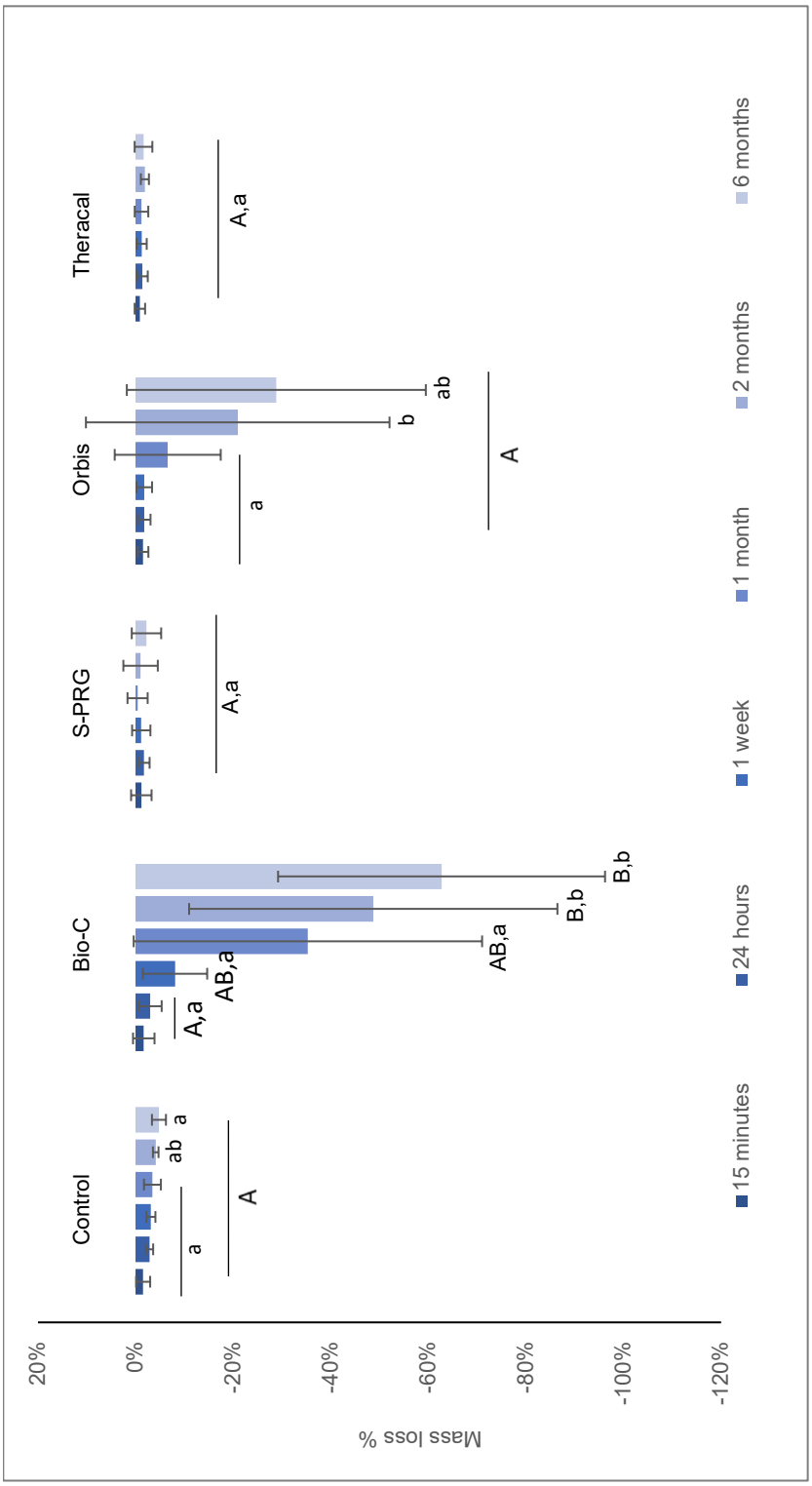


Figure 3. Dry mass loss values by endodontic materials after storage in body-simulated fluid at 37 °C after 24 hours, 1 week, 1 month, 2 months, and 6 months. The chart columns represent the mean loss values, and the error bars represent the standard deviation. Different letters represent statistical differences between groups *(p<0.005)*. Different capital letters represent differences for each material at different time points. Different small letters represent the differences between the values of the different materials at the same time point. (From the supplementary data of Study IV)

Among the different tested bioactive materials, the analysis of dry mass loss revealed changes in the dry mass over time **(Figure 3)**. In particular, the Bio-C group exhibited a progressive loss of dry mass, which was significantly different from the control, S-PRG, and TheraCal groups at the 6-month mark *($p<0.05$).* While Orbis also displayed a similar pattern, Bio-C was the only group that demonstrated a significant difference between its initial and final dry masses ($p=0.005$). By the end of the experiment, Bio-C and Orbis had 48% and 10.6% less mass than the control group, respectively.

5.2 Evaluation of endogenous protease activity (Studies I, IV)

5.2.1 Activity of matrix-bound dentin MMPs (Studies I, IV)

Study I showed that all treatment groups exhibited a significant reduction in total MMP activity compared to the untreated control group *($p<0.05$)* **(Figure 4)**. During the incubation period, the MMP activity of the control group increased by 99.8 ± 13%, while the increase in the S-PRG filler eluate-treated group was limited to 23.2 ± 4.6%. The fluoride-treated group showed the lowest value, reducing its activity to -3.5 ± 2.3% after treatment.

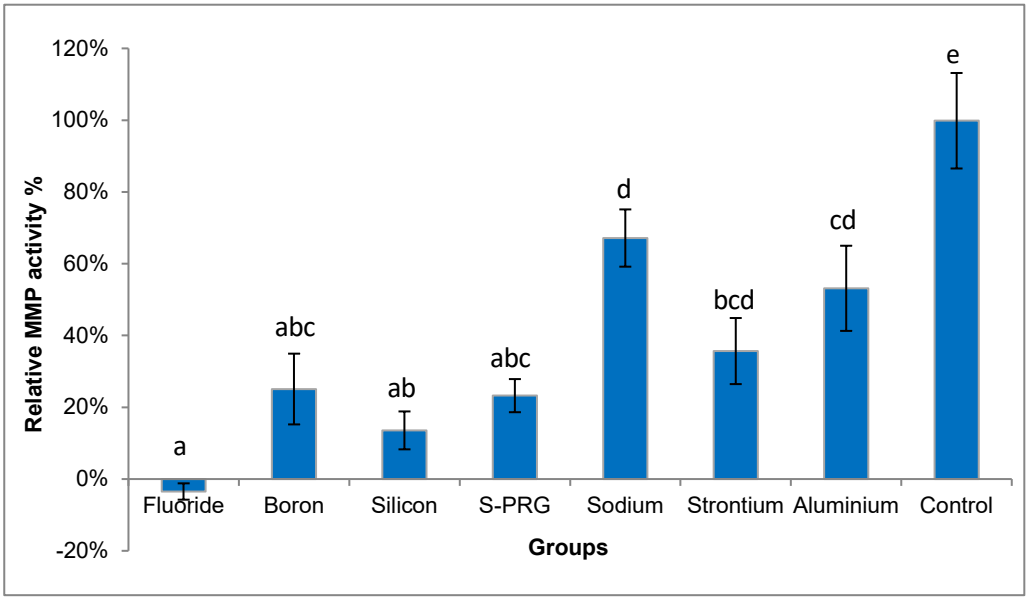


Figure 4. Relative MMP activity percentage after 5 min treatment. (From the supplementary data published in Study I)

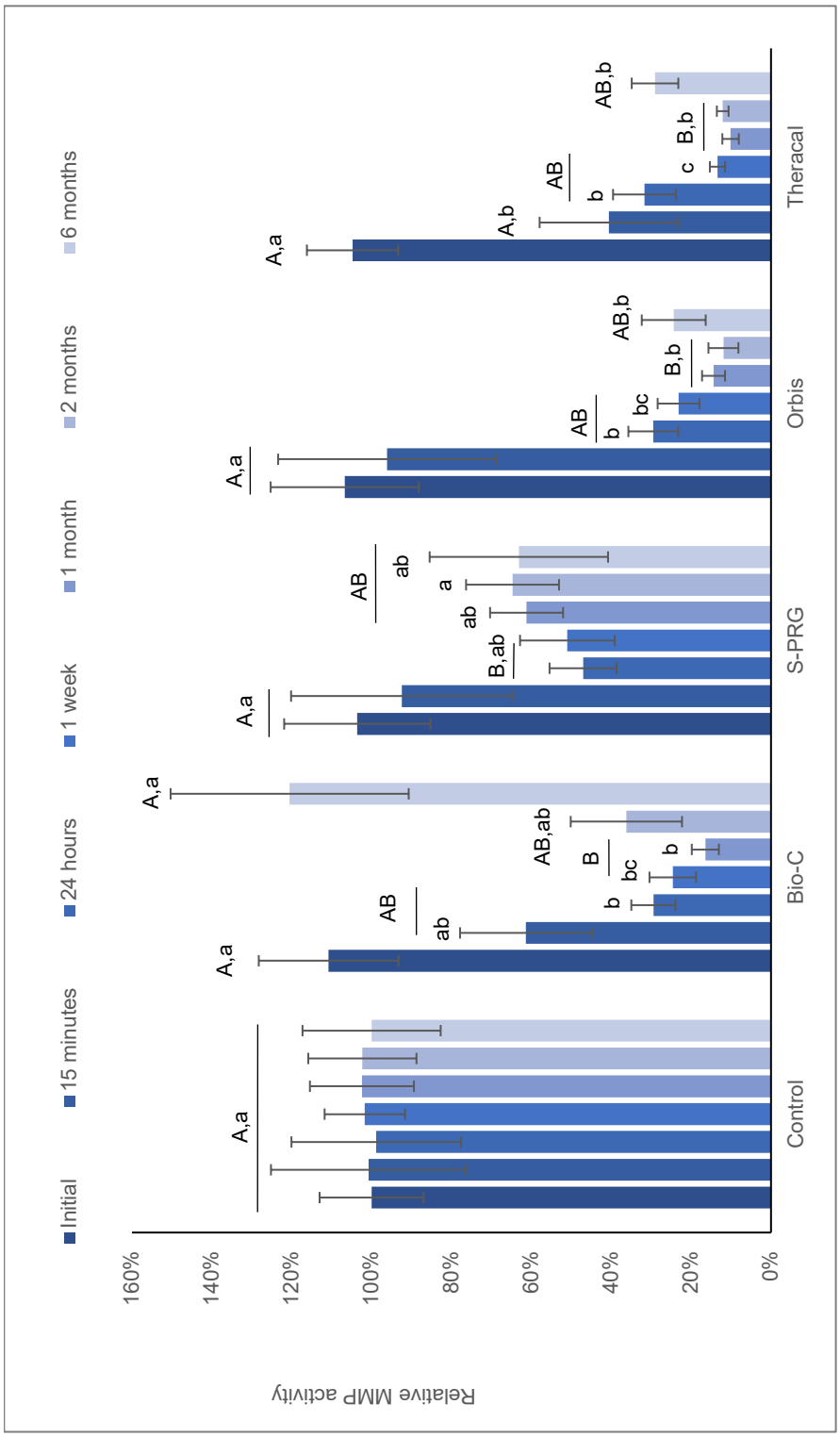


Figure 5. The mean values and the standard deviation of the MMP activity after storage in artificial saliva at 37 °C for different time points. Different capital letters represent differences for each material at different time points. Different small letters represent the differences between the values of the different materials at the same time point. (From the supplementary data of Study IV)

Except for the control group, which maintained constant MMP activity throughout the entire experiment, the other groups exhibited different behaviour (refer to Figure 5). In general, all the materials consistently inhibited MMP activity compared to the corresponding time points in the control group, except for Bio-C, which showed 20.5% activation at 6 months. The minimal MMP activity for the Bio-C and TheraCal groups was observed at 1 month of incubation, and it was statistically different from their highest readings and the control *($p<0.05$)*. Similarly, the minimal MMP activity for S-PRG and Orbis occurred at 24 hours and 1 month, respectively, and was also statistically different from their initial readings *($p<0.05$)*. Furthermore, when compared to the control activity level, all the experimental groups exhibited higher MMP inhibition at 1 month (Bio-C 84%; Orbis 88.6%; TheraCal 90.1%; S-PRG 85.7%), with only S-PRG not showing a statistical difference from the control group at 1 month *($p>0.05$)*.

5.2.2 ICTP assay (Studies I, IV)

The mean (±SD) ICTP levels of the demineralized dentin beams incubated in simulated body fluid (Study I) or calcium and zinc-containing media (Study IV) are demonstrated in Figures 6 and 7. In **Figure 6,** the control group experienced an ICTP release of 53.5 ±14.1 ng ICTP/mg dentin. The fluoride-pre-treated group exhibited the lowest ICTP release at 15.4 ±7.3 ng ICTP/mg dentin, significantly differing from the untreated group, as well as the boron and silicon-treated groups *($p<0.05$)*. The strontium, sodium, and S-PRG filler eluate-treated groups displayed a similar trend in ICTP release and were significantly lower compared to the untreated control *($p<0.05$)*. Among the treatment groups, silicon showed the highest ICTP release at 39.8 ±7.7 ng ICTP/mg dentin, following the control *($p>0.05$)*.

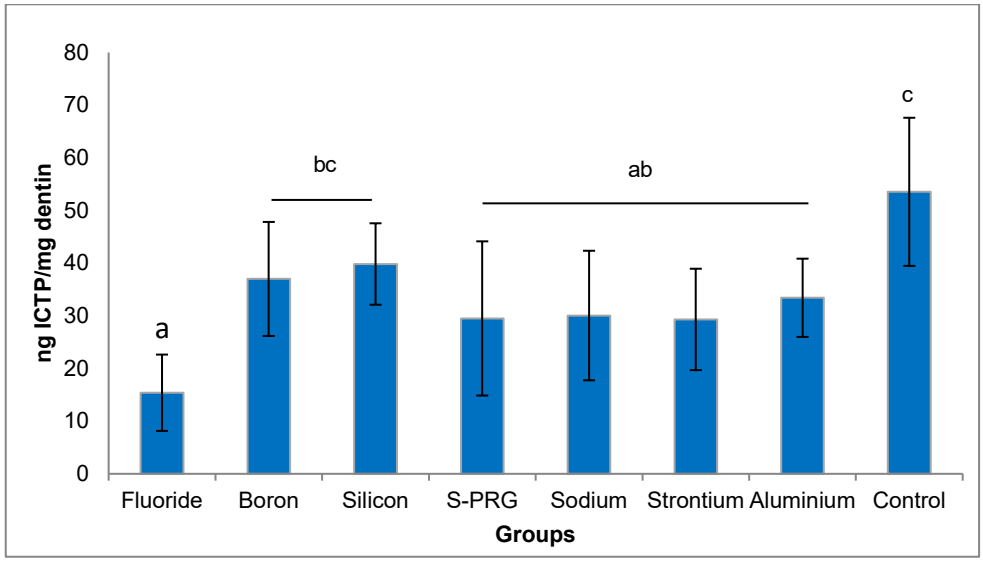


Figure 6. The release of ICTP telopeptide fragments in ng/mg after 1-week incubation (From the supplementary data published in Study I).

Figure 7 represents the average ICTP release (±SD) from the demineralized dentin beams to the incubation medium. The highest cumulative ICTP release was observed in the TheraCal (7471.8 pg/mg dry dentin) and Control (6920.9 pg/mg dry dentin) groups, while the lowest release was observed in the Orbis (1389.5 pg ICTP/mg dry dentin) and S-PRG groups (1478.2 pg ICTP/mg dry dentin). In the control group, the highest ICTP release occurred at 2 months of incubation and was significantly higher than at 1 month *($p<0.05$)*. Bio-C exhibited a different trend, with the highest ICTP fragment release occurring at 24 hours and significantly decreasing to less than 95 pg ICTP/mg dentin dry weight at the 1-month mark *($p<0.05$)*. A similar pattern was observed in the S-PRG, Orbis, and TheraCal groups, where the highest ICTP release occurred at 24 hours and reached a minimum at 6 months *($p<0.05$)*. Additionally, there was a significant difference between TheraCal (2538.2 pg ICTP/mg dry dentin) and Orbis (427.5 pg ICTP/mg dry dentin) at 24 hours *($p<0.007$)*. At 1 week, TheraCal (1843.3 pg ICTP/mg dry dentin) also released significantly more ICTP than S-PRG (382.1 pg ICTP/mg dry dentin) and Orbis (307.1 pg ICTP/mg dry dentin) *($p<0.05$)*. At 1 month, both the control (2611.1 pg ICTP/mg dry dentin) and TheraCal (1282.1 pg ICTP/mg dry dentin) groups showed significantly higher release compared to the Bio-C group (93.5 pg ICTP/mg dry dentin) *($p<0.05$)*. The highest ICTP release at 6 months was observed in the Bio-C group (552.8 pg ICTP/mg dry dentin), which was significantly different from the Control and TheraCal groups, both with zero ICTP release *($p<0.05$)*.

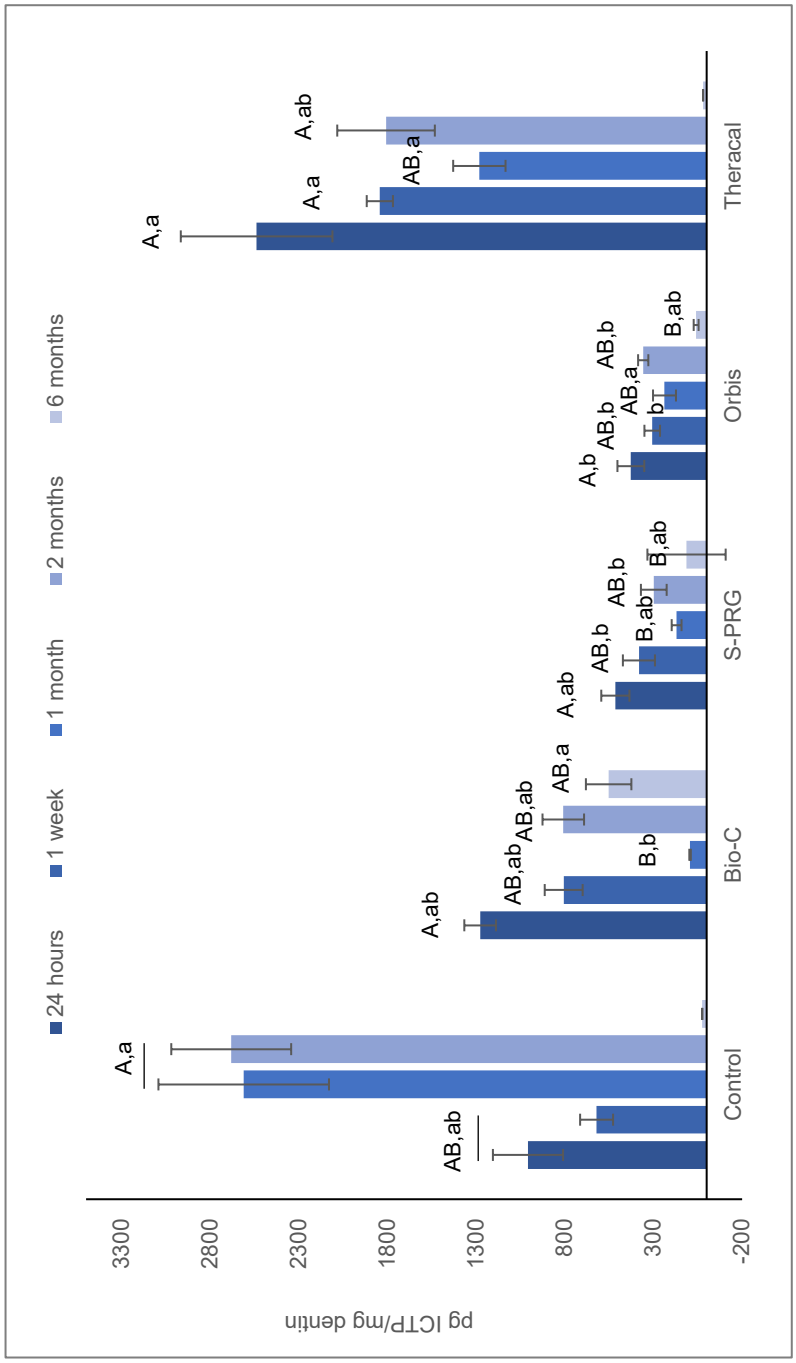


Figure 7. The release of ICTP telopeptide fragments after storage in artificial saliva at 37°C for different time points. Different capital letters represent differences for each material at different time points. Different small letters represent the differences between the values of the different materials at the same time point. (From the supplementary data of Study IV

5.2.3 CTX assay (Studies I, IV)

Figure 8 represents the quantity of CTX peptide released (±SD) during a 1-week incubation is depicted as pg/mg dentin. There were no significant differences between the groups *(p>0.05)*. However, the S-PRG group exhibited the lowest CTX release at 122.6±41.3 pg CTX/mg dentin, while the strontium group showed the highest release of CTX fragments at 186.9±50.1 pg CTX/mg dentin, followed by the control group at 165.5±17.5 pg CTX/mg dentin. The remaining groups displayed similar levels of CTX release.

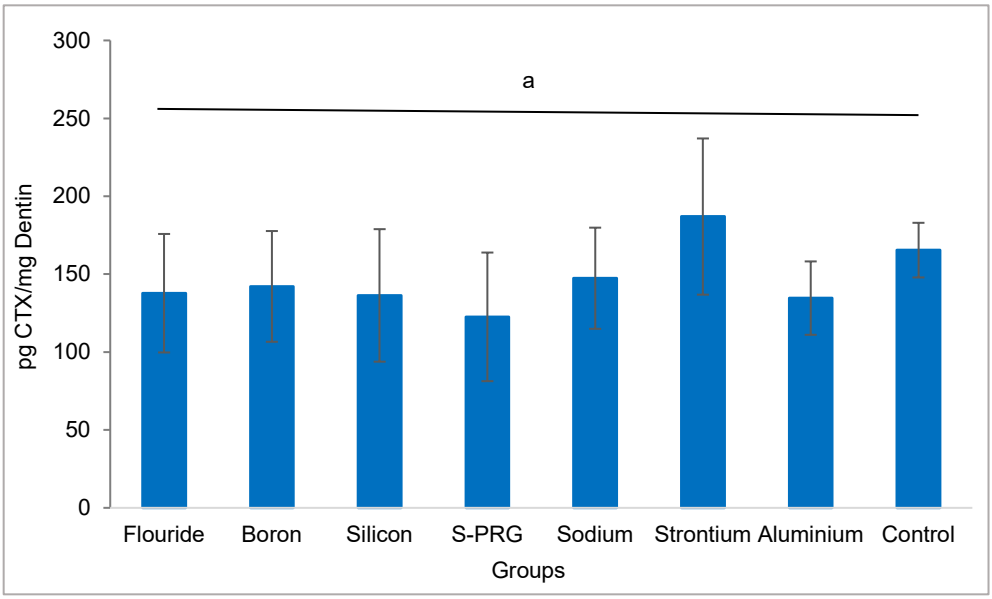


Figure 8. The release of CTX fragments in Pg\mg after 1-week incubation (From the supplementary data published in Study I).

The CTX peptide release (±SD) of the different tested materials is illustrated in **Figure 9**. The control group exhibited the highest cumulative CTX release (122.4 pg CTX/mg dry dentin), followed by TheraCal (86.2 pg CTX/mg dry dentin). In the control group, CTX release progressively increased over the first 1 month of incubation, while the other groups displayed a different pattern, with CTX decreasing after 24 hours of incubation. The released CTX gradually increased, reaching its peak at 1 month of incubation for the control group (57.1 pg CTX/mg dry dentin), and then significantly decreased at 2 months (p=0.02). Following the 24-hour readings, the Bio-C, Orbis, and S-PRG groups exhibited a decline in CTX release until reaching a minimum at 1 month, 2 months, and 6 months of incubation, respectively.

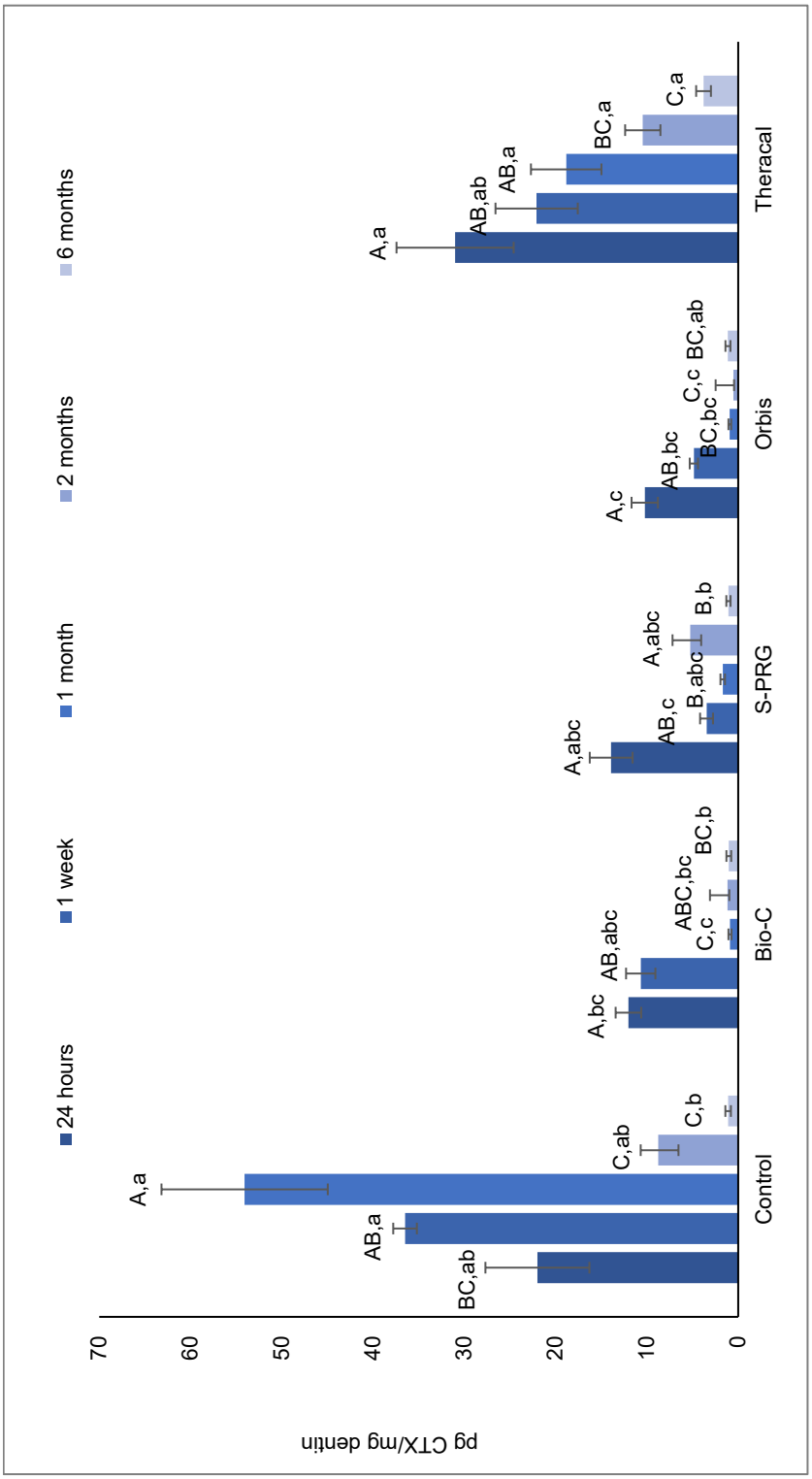


Figure 9. CTX type I collagen fragments were released after storage in artificial saliva at 37 °C after 24 hours, 1 week, 1 month, 2 months, and 6 months. Different capital letters represent differences for each material at different time points. Different small letters represent the differences between the values of the different materials at the same time point. (From the supplementary data of Study IV)

5.2.4 Hydroxyproline assay (Study IV)

The mean (±SD) of the hydroxyproline levels is explained in **Figure 10.** Overall, Bio-C exhibited the highest cumulative HYP release (23.3 µg HYP/mg dry dentin), while the control group showed the lowest HYP release (2.5 µg HYP/mg dry dentin). Except TheraCal, all experimental groups displayed a similar pattern in which HYP levels gradually increased to reach their peak (between 1.3 and 7.6 µg HYP/mg dry dentin) at 1 month of incubation, followed by a subsequent decrease. A significant difference was observed between the initial and 1- month HYP readings for the control, Bio-C, and Orbis groups *($p<0.05$)*. The TheraCal group exhibited the highest HYP release at 6 months, although it was not statistically different from the other groups *($p>0.05$)*.

5.3 Dentin barrier test (Study II)

The trans-dentinal viability of pulp-derived cells after 24 hours is illustrated in **Figure 11**. The Kruskal- Wallis test indicated that bioactive materials, pretreatment solutions, and their combined use had a significant impact on cell viability *($p<0.01$)*. Among the pretreatment solutions, the most substantial reduction was observed for EtOH and EtOH/H_2O *($p<0.05$)*. There were no significant differences between DMSO/H_2O, DMSO/EtOH, or DMSO *($p>0.05$)*. S-PRG was the least cytotoxic silicate-based material, followed by MTA, with no significant differences between them *($p>0.05$)*. TheraCal exhibited the highest reduction in cell viability among the tested silicate-based materials *($p<0.05$)*. Overall, EtOH and EtOH/H_2O resulted in the lowest overall cell viability, in contrast to DMSO/H_2O and DMSO/EtOH, which had higher values regardless of the silicate-based material used *($p<0.05$)*. EtOH and DMSO significantly reduced the cell viability produced by TheraCal *($p<0.05$),* while DMSO/H_2O, DMSO/EtOH, and EtOH/H_2O had no significant effect *($p>0.05$)*. EtOH and EtOH/H_2O significantly reduced the cell viability produced by MTA *($p<0.05$)*, while DMSO/H_2O, DMSO/EtOH, and DMSO had no significant effect *($p>0.05$)*. DMSO/H_2O and DMSO/EtOH had no significant effects on the cell viability produced by S-PRG *($p>0.05$)*, unlike EtOH, EtOH/H_2O, and DMSO, which significantly lowered cell viability *($p<0.05$)*.

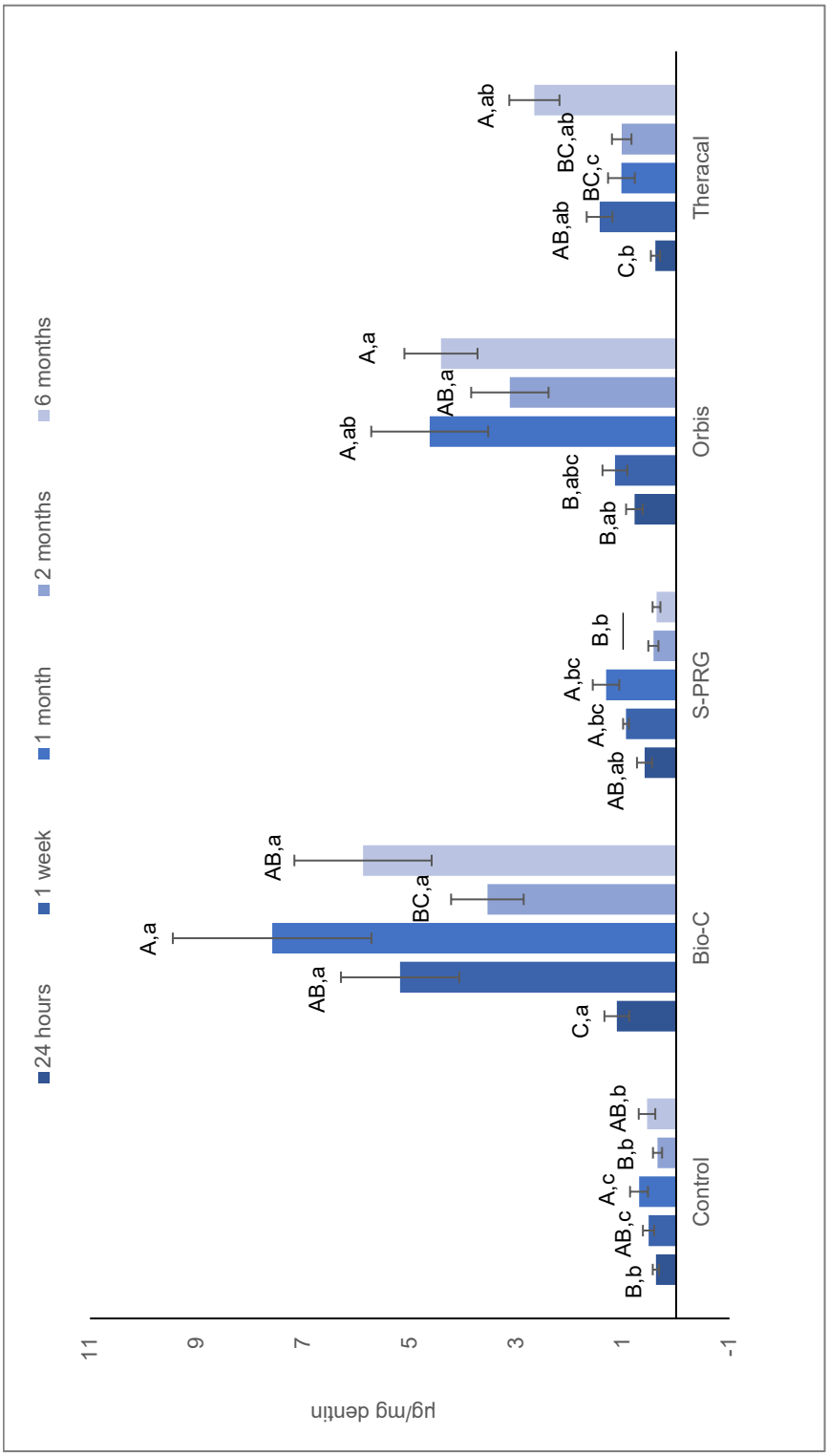


Figure 10. The mean values and the standard deviation of the Hydroxyproline released after storage in artificial saliva at 37 °C for 24 hours, 1 week, 1 month, 2 months, and 6 months. Different capital letters represent differences for each material at different time points. Different small letters represent the differences between the values of the different materials at the same time point. (From the supplementary data in Study IV)

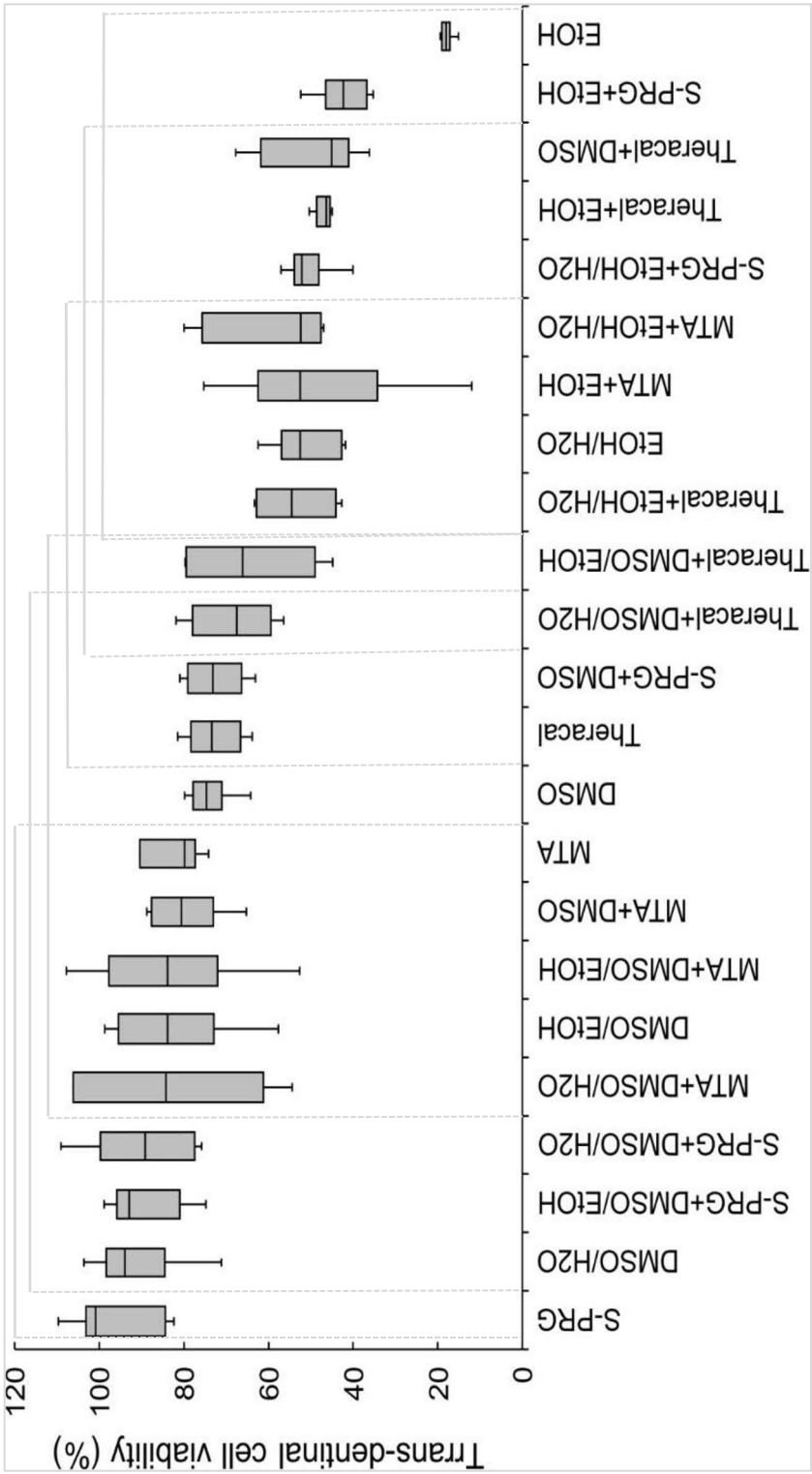


Figure 11. Mean and standard deviation of cell viability of the different tested materials with/out the use of solvent solutions. (From the supplementary data in Study II)

Significant variances were noted between the negative and positive control groups *($p<0.05$)*. The utilization of silicate-based materials, pretreatment solutions, or their combined application had a noteworthy impact on cell damage. The grading evaluations of cell damage, following the guidelines of ISO 7405 (ISO, 2017), are presented in **Table 4.**

Table 4. Assessment of cell damage* (SV40 pulp-derived cells) after different solvent treatments used in combination with silicate-based materials. (From the supplementary data in Study II)

Material	Treatment	Cell damage
-	DMSO/H_2O	Non cytotoxic
	DMSO	Moderately cytotoxic
	DMSO/EtOH	Non cytotoxic
	EtOH/H_2O	Severely cytotoxic
	EtOH	Severely cytotoxic
TheraCal LC	No treatment	Severely cytotoxic
	DMSO/H_2O	Severely cytotoxic
	DMSO	Severely cytotoxic
	DMSO/EtOH	Severely cytotoxic
	EtOH/H_2O	Severely cytotoxic
	EtOH	Severely cytotoxic
MTA	No treatment	Moderately cytotoxic
	DMSO/H_2O	Moderately cytotoxic
	DMSO	Moderately cytotoxic
	DMSO/EtOH	Moderately cytotoxic
	EtOH/H_2O	Severely cytotoxic
	EtOH	Severely cytotoxic
S-PRG	No treatment	Non cytotoxic
	DMSO/H_2O	Non cytotoxic
	DMSO	Moderately cytotoxic
	DMSO/EtOH	Non cytotoxic
	EtOH/H_2O	Severely cytotoxic
	EtOH	Severely cytotoxic

*According to ISO 7405. *Abbreviations:* DMSO = dimethyl sulfoxide; EtOH = ethanol; MTA = mineral trioxide aggregate; S-PRG = pre-reacted glass ionomer fillers. DMSO/H2O, DMSO/EtOH, and EtOH/H2O followed 50% (v/v) dilutions.

5.4 Push-out bond strength testing (study III)

Figure 12. represent the push-out bond strength of the whole roots restored with the different silicate-based biomaterials, with/without the use of the different solvent pretreatment solutions. In general, TheraCal exhibited the highest bond strength among the tested groups, which was significantly different from the MTA and the S-PRG cements. The various pretreatments had differing effects on the bond strength of the materials. However, none of the pretreatments significantly increased or decreased the bond strength compared to the control for each material separately *($p>0.05$)*. The use of ethanol appeared to decrease the bond strength of TheraCal, but it increased the bond strength of MTA to be higher than the control MTA, although the differences were not statistically significant *($p>0.05$)*. The bond strength of MTA was significantly higher in the control group, the DMSO-treated group (DMSO), and the Ethanol-treated group (EtOH) compared to all the S-PRG-treated groups *($p<0.05$).* While not significantly different from the MTA control group, the use of the diluted forms of DMSO and ethanol (DMSO/H_2O, DMSO/EtOH, EtOH/H_2O) seemed to reduce the bond strength of MTA. For the groups where S-PRG was used, none of the pretreatment solutions significantly affected the experimental S-PRG bond strength *($p>0.05$).*

5.5 Push-out bond strength at different root levels (study III)

Table 5. represents the push-out bond strength of the tested materials varied across the different root levels (coronal, middle, and apical). The bond strength was higher in the coronal third compared to the middle and apical thirds for the TheraCal groups; this difference was statistically significant between the coronal and apical thirds across all TheraCal groups *($p<0.05$)*. Additionally, there were significant differences between the coronal and middle thirds in the TheraCal control group and the ethanol-treated (EtOH) group *($p<0.05$)*. Significant differences were also observed between the middle and apical thirds in the control TheraCal group, DMSO diluted in water (DMSO/H_2O) group, and DMSO (DMSO) treated group *($p<0.05$)*. The use of DMSO diluted in water (DMSO/H_2O), DMSO diluted in ethanol (DMSO/EtOH), and ethanol diluted in water (EtOH/H_2O) significantly reduced the TheraCal bond strength in the coronal third compared to the TheraCal control group *($p<0.05$)*. However, no significant effects were seen in the middle and apical thirds with TheraCal. In the groups where MTA was used, the different pretreatments had varying effects on bond strength. There were significant differences between the coronal and middle thirds compared to the apical third in the MTA control group, DMSO diluted in water (DMSO/H_2O) group, and ethanol-treated (EtOH) group *($p<0.05$)*. In contrast, there were no significant differences between the root thirds

in the groups where DMSO pretreatment (DMSO), DMSO diluted in ethanol (DMSO/EtOH), and ethanol diluted in water (EtOH/H_2O) pretreatments were used *($p<0.05$)*. The use of DMSO diluted in ethanol significantly reduced the MTA bond strength in the coronal and middle thirds compared to the control *($p<0.05$)*. The DMSO pretreatment (DMSO) significantly increased the MTA bond strength in the apical third compared to the control, while the diluted DMSO in water significantly decreased the apical third bond strength *($p<0.05$)*. Among the experimental S-PRG cement groups, the S-PRG control group had significantly higher bond strength in the coronal third compared to the apical third *($p<0.05$)*. A similar trend was observed with the DMSO diluted in ethanol (DMSO/EtOH) pretreatment, where the coronal third bond strength was significantly higher than both the middle and apical thirds *($p<0.05$)*. No significant differences were found between the root thirds in the groups where DMSO diluted in water (DMSO/H_2O), DMSO pretreatment (DMSO), and ethanol pretreatment (EtOH) were used *($p<0.05$)*. Coronally, all pretreatments significantly reduced the S-PRG bond strength *($p<0.05$)*. Except for the DMSO diluted in water (DMSO/H_2O) pretreatment, which improved the apical third bond strength compared to the S-PRG control *($p<0.05$)*. In the middle third, the use of DMSO or DMSO diluted in ethanol significantly decreased the S-PRG bond strength *($p<0.05$)*.

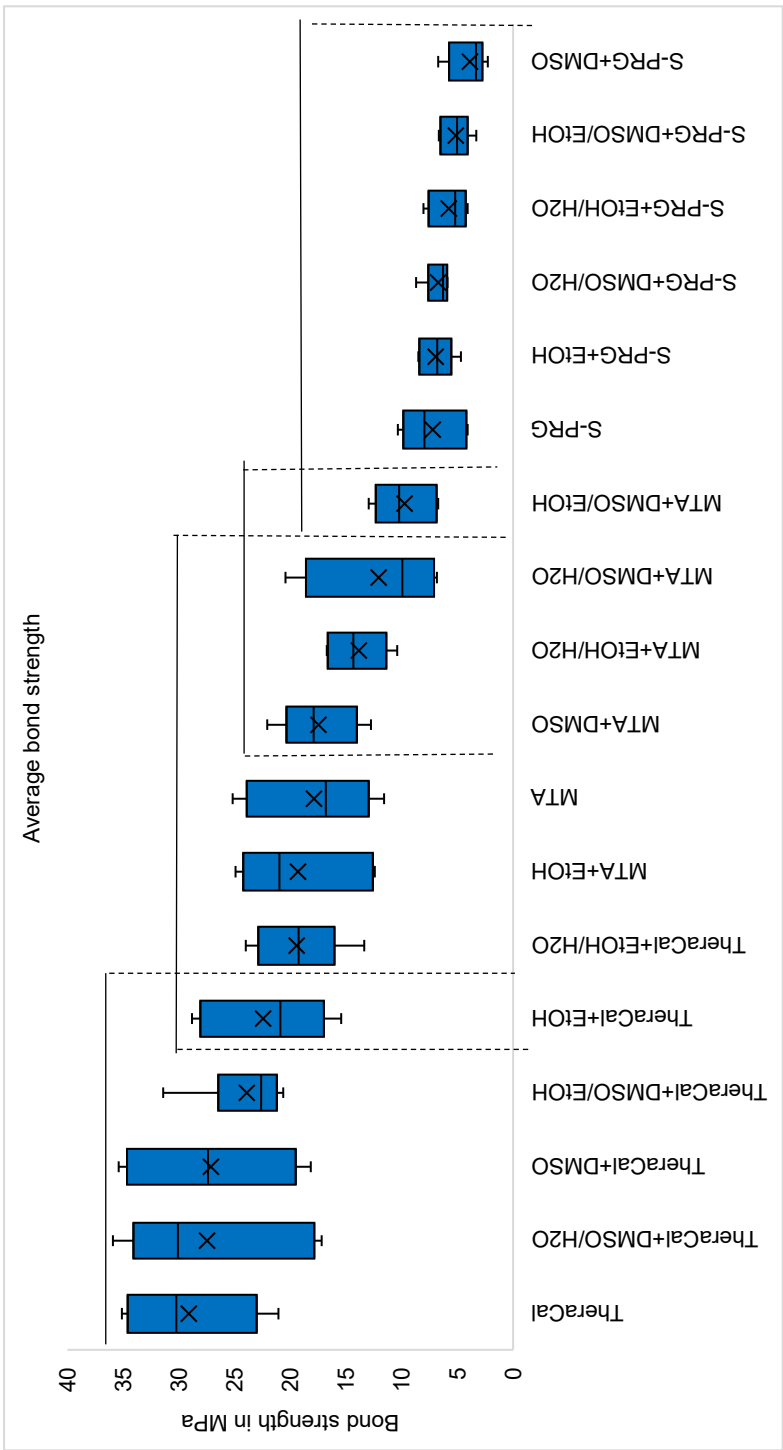


Figure 12. Box-plot diagram representing the distribution of the push-out bond strength of different tested materials and pretreatments in megapascals. (From supplementary data published in study III)

Table 5. Means and standard deviations (±) of push-out bond strengths (MPa) at different root thirds. (From supplementary data published in study III).

	TheraCal			MTA			S-PRG		
	Coronal	**Middle**	**Apical**	**Coronal**	**Middle**	**Apical**	**Coronal**	**Middle**	**Apical**
Control	31.46 ^{Aa} ±10.49	25.45 ^{Aab} ±9.22	20.52 ^{Ab} ±8.97	20.12 ^{Aa} ±11.9	21.88 ^{Aa} ±10.21	12.08 ^{Aa} ±4.81	8.10 ^{Aa} ±3.25	8.59 ^{Aa} ±3.22	4.18 ^{ABb} ±1.98
DMSO/H_2O	27.81 ^{Aa} ±10.32	28.38 ^{Aa} ±11.16	22.27 ^{ABa} ±6.7	12.16 ^{Aa} ±5.82	13.57 ^{Aa} ±7.13	11.61 ^{Aa} ±3.11	7.44 ^{Aa} ±2.01	6.66 ^{ABab} ±3.02	4.96 ^{Ab} ±2.24
DMSO	28.71 ^{Aa} ±8.52	25.54 ^{Aa} ±8.69	14.82 ^{Bb} ±5.12	17.78 ^{Aa} ±4.20	17.62 ^{Aa} ±5.31	17.39 ^{Aa} ±5.57	5.36 ^{Aa} ±3.55	4.33 ^{Bab} ±1.51	2.99 ^{Bb} ±1.07
DMSO/EtOH	25.75 ^{Aa} ±8.91	25.45 ^{Aa} ±12.81	22.14 ^{Aa} ±7.31	11.70 ^{Aa} ±6.81	15.78 ^{Aa} ±7.01	13.80 ^{Aa} ±6.45	7.11 ^{Aa} ±2.84	6.11 ^{Aa} ±1.05	3.82 ^{Ab} ±1.02
EtOH/H_2O	27.92 ^{Aa} ±15.06	26.80 ^{Aa} ±9.58	18.02 ^{ABb} ±6.02	13.73 ^{Aa} ±5.72	15.38 ^{Aa} ±5.20	11.61 ^{Aa} ±5.37	7.71 ^{Aa} ±3.34	6.28 ^{ABa} ±3.21	3.79 ^{Ab} ±0.92
EtOH	30.06 ^{Aa} ±9.29	26.08 ^{Aa} ±4.81	22.39 ^{Ab} ±4.41	18.55 ^{Aa} ±11.34	19.99 ^{Aa} ±9.30	12.39 ^{Ab} ±8.81	8.40 ^{Aa} ±4.31	8.91 ^{Aab} ±1.23	4.56 ^{Ab} ±1.81

Different capital letters represent significant differences between different pretreatments within the root third (column) for each silicate-based material according to ANOVA on ranks *($p<0.05$)*. Different lowercase letters represent significant differences between root thirds within dentin pretreatments (row) for each silicate-based material according to ANOVA on ranks *($p<0.05$)*.

5.6 Analysis of Failure Modes (Study III)

The failure modes observed for all the tested materials are shown in **Figure 13.** Generally, the adhesive failure mode was the least common across all the tested groups. The mixed failure mode was the most prevalent in the TheraCal groups, accounting for around 50% of the failures, except for the ethanol-treated (EtOH) group, where cohesive failure was observed in 95% of the specimens. A different trend was seen in the MTA groups. The most common failure mode was the mixed failure, occurring in more than 60% of the specimens in the groups where ethanol was used at different dilutions. When DMSO was used as a pretreatment, both adhesive and cohesive failures were recorded at a similar percentage of around 48%. The S-PRG groups consistently exhibited failures within the material itself, as evidenced by the high percentage of cohesive failure (more than 40% with all pretreatments). The control group showed a mixture of cohesive and mixed failures, with more than 57% being mixed failures.

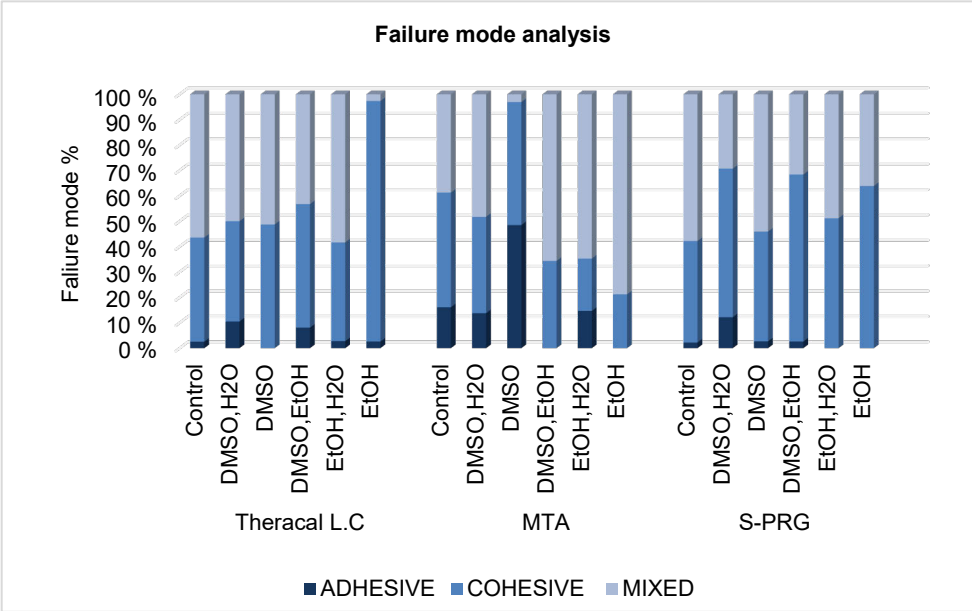


Figure 13. Failure percentage is seen in the different materials and pretreatments. (From the supplementary data published in study III)

5.7 Analysis of adhesion surface (study III)

The scanning electron microscopic images of all the tested materials and pretreatments are shown in **Figure 14.** In the TheraCal groups, a clear penetration of the material into the dentinal tubules was observed in the control group, the DMSO

diluted in water (DMSO/H_2O) group, and the DMSO-treated (DMSO) group. Small material tags were seen at the bonding interface between TheraCal and dentin in the ethanol-treated groups. For the MTA groups, the deepest penetration was seen in the group with ethanol and the control group, where no pretreatment was applied. Small material tags or an accumulation of the material on the dentin-material margin were observed in all the other MTA-treated groups. In the experimental S-PRG groups, little to no penetration was seen, which indicates the low bond strength between the S-PRG cement and dentin. Overall, the scanning electron microscopic analysis revealed varying degrees of material penetration and interfacial characteristics depending on the tested materials and the pretreatment conditions.

5.8 Total Extractable Protein (Study IV)

The materials' effect on the total extractable proteins is depicted in **Figure 15**. Following extraction, there was a noticeable contrast in the cumulative release among the groups, with Bio-C exhibiting an extractable protein level of 38.5 protein µg/mg dentin dry weight, a value higher than that of the control group (6.6 protein µg/mg dry dentin). S-PRG demonstrated similar behaviour to the control group at all time points *($p>0.05$),* with a cumulative release of 5.0 µg protein /mg dry dentin. However, all groups displayed variations in protein release between the different incubation periods. Except for TheraCal, all groups released more proteins in the first and third weeks of incubation. At one and three weeks, Bio-C exhibited higher protein release than the control and S-PRG groups *($p<0.05$).* TheraCal exhibited a completely different pattern, with a progressive decrease in release until the first month.

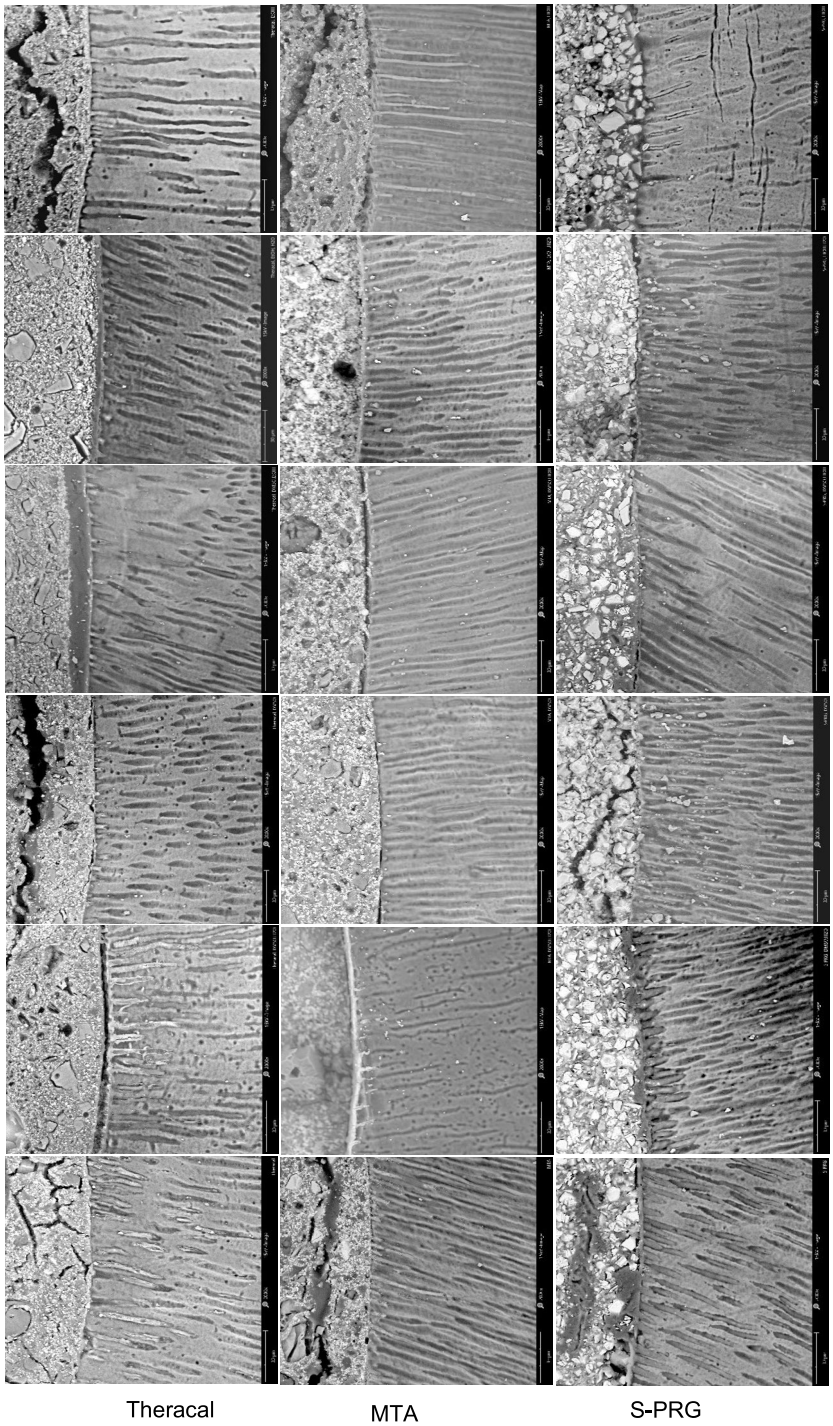


Figure 14. Representative Scanning electron microscopy (SEM) images of the tested materials and pretreatments at 2000X magnification. (From data published in study III)

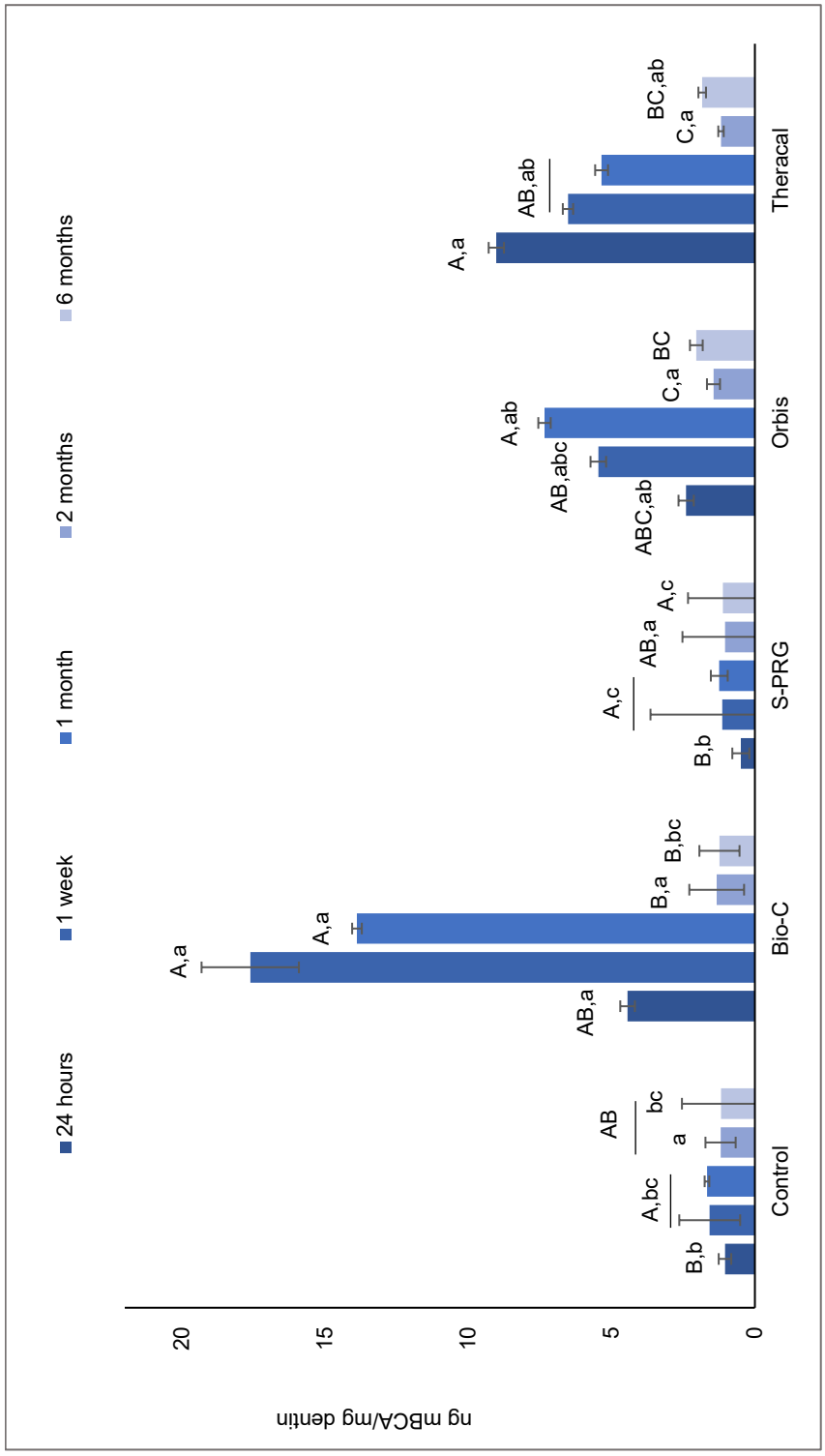


Figure 15. The amount of the extracted protein in ng mBCA/mg dentin for the different silicate-based materials at the different tested time points. Different capital letters represent differences for each material at different time points. Different small letters represent the differences between the values of the different materials at the same time point. (From the supplementary data in Study IV)

5.9 pH assessment (study IV)

The results can be found in **Table 6.** The treatments led to an increase in the average pH, ranging from 2% to 68% compared to the control group with a pH of 7.32. Bio-C exhibited the highest average pH after all storage periods (pH 12.31). This value was statistically similar to Orbis (pH 11.63) *(p>0.05)* but higher than TheraCal (pH 10.93), S-PRG (pH 7.47), and the control *(p<0.05).*

Table 6. pH value of the different tested endodontic materials at the different time points.

	24 hours	1 week	1 month	2 months	6 months
Control	7.33^{ABd} ±0.04	7.28^{Bd} ±0.03	7.39^{Ac} ±0.07	7.26^{Bc} ±0.04	7.36^{Ac} ±0.02
Bio-C	12.50^{Aa} ±0.02	12.19^{Ba} ±0.13	12.28^{Ba} ±0.13	12.26^{Ba} ±0.10	12.32^{ABa} ±0.11
S-PRG	7.78^{Acd} ±0.05	7.52^{ABcd} ±0.20	7.46^{ABCc} ±0.15	7.29^{Cc} ±0.09	7.32^{BCc} ±0.05
Orbis	12.28^{Aab} ±0.07	11.22^{Bab} ±0.45	11.75^{ABab} ±0.19	11.48^{Bab} ±0.13	11.42^{Bab} ±0.16
TheraCal	11.80^{Abc} ±0.04	9.93^{CDbc} ±0.12	11.09^{ABbc} ±0.04	10.87^{CDbc} ±0.04	10.95^{BCbc} ±0.04

Note: values represent the mean and (standard deviation). Different letters represent statistical differences between groups *(p<0.001)*. Different capital letters represent differences between the pH values of the different materials at the same time point. Different small letters represent the difference for each material at different time points. (From the supplementary data in Study IV)

5.10 Modulus of elasticity (study IV)

The elasticity (E) of the tested dentin beams remained constant from the initial to the one-week incubation values *(p>0.05)* **(Figure 16).** However, at three weeks, Bio-C exhibited a significant decrease in E, showing notable differences from the control and TheraCal groups *(p<0.05)*. By the end of one month, the Bio-C group had lost all elasticity, with all dentin beams broken or degraded inside the tubes. Orbis displayed a similar behaviour to Bio-C and lost the majority of its elasticity by the end of the experiment. Both Bio-C and Orbis showed significant differences from their initial and 4-month E values (p=0.002), while the control, S-PRG, and TheraCal maintained the same values *(p>0.05).*

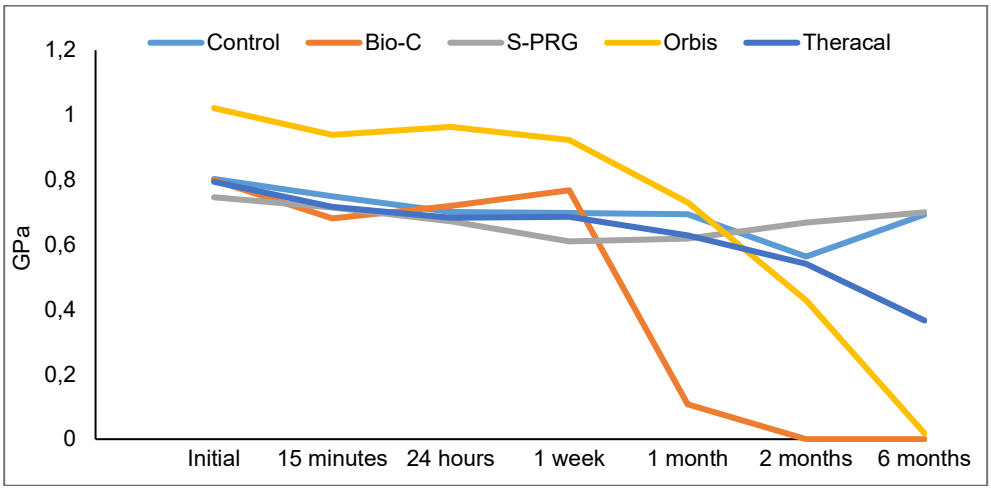


Figure 16. The modulus of elasticity of the different materials at different time points. (From the supplementary data in Study IV)

5.11 Assessment of calcium leaching from bioactive materials (Study IV)

The amount of calcium ion leached from the tested materials are presented in **Table 7**. The amount of calcium leached was significantly affected by the material type and the tested time point *($p<0.05$)*. S-PRG didn't release any calcium to the distilled water; TheraCal had significantly higher calcium release compared to both MTA types, Bio-C and Orbis *($p<0.05$)*. In addition, the time of incubation had an effect on the calcium leach *($p<0.05$)*. At 14 days, TheraCal released more calcium compared to both MTA types; however, the amount of leached calcium of Bio-C increased at 28 days to be significantly higher than MTA Orbis but similar to TheraCal.

Table 7. Leaching of calcium ions at different time points measured in µg/ml. Different capital letters represent differences between materials at the same time point, different small letters represent differences for each material at the different time points. (From supplementary data of study IV).

	Leaching of calcium ions			
	3 days	**7 days**	**14 days**	**28 days**
Bio-C	17.51 ^{Ab} ±3.26	22.99 ^{Ab} ±1.90	34.27 ^{Aba} ±4.85	37.06 ^{Ba} ±5.87
S-PRG	0	0	0	0
Orbis	13.87 ^{Ab} ±3.91	24.08 ^{Aa} ±4.58	30.83 ^{Ba} ±4.02	31.31 ^{Ba} ±4.10
TheraCal	12.93 ^{Ad} ±4.49	29.93 ^{Ac} ±4.32	39.09 ^{Ab} ±5.99	47.10 ^{Aa} ±4.11

6 Discussion

There is an increased focus on bioactive materials due to their favourable characteristics, including good sealing ability, suitable bond strength, and low cytotoxicity (Gandolfi *et al.* 2010; Silva *et al.* 2013). These materials are a good choice of treatment in cases of indirect pulp capping after caries removal, especially in cases of deep cavities and thin remaining dentin. Previous studies have demonstrated the acidic effect of caries on collagen matrix degradation, attributed to the activation of matrix metalloproteinases (MMPs) and cathepsins (CCs). MMPs and cysteine cathepsins could be activated during caries formation (Vidal *et al.* 2014), or even dental procedures (Mazzoni *et al.* 2006). It is well known that the activation of MMPs and cysteine cathepsins causes degradation of dentin matrices (Garnero *et al.* 2003). In addition, MMPs and cathepsin K have the ability to cleave different parts of type I collagen, in specific two fragments of type I collagen on the C-telopeptide had the main focus, ICTP (crosslinked carboxyterminal telopeptide of type I collagen) and CTX (C-terminal crosslinked telopeptide of type I collagen) (P Garnero *et al.* 2003). ICTP is generated by MMP and is considered the final product of type I collagen MMP-dependent degradation (P Garnero *et al.* 2003; Eriksen *et al.* 2004), while CTX is cleaved from cathepsin K and considered the specific marker of CC breakdown (Sassi *et al.* 2000; Garnero *et al.* 2003; Tezvergil-Mutluay *et al.* 2015).

Host-derived enzymatic degradation plays a crucial role in the structural integrity of demineralized dentin matrices; therefore, recent studies have focused on inhibiting or inactivating these enzymes to counteract their destructive impact on dentin matrices. However, the alkalinity effect of the different materials on the dentin matrix remains questionable. Moreover, the effects of different types of silicate-based materials on the dentin matrix remain uncertain. Following this framework, the present series of studies aimed to investigate the effect of the use of surface pre-reacted glass materials and compare it to the conventional bioactive silicate-based materials in terms of ion release and possible effect on dentin matrix and dentin protease activity. And to evaluate the effect of the incorporation of different solvents on the cytotoxicity and pushout bond strength of the tested bioactive materials. The inhibitory effects of the ions released from surface-pre-reacted glass (Study I) on

dentin matrix and dentin protease activity were evaluated. In addition, the effect of bioactive silicate-based cements and an experimental ion-releasing material on the cell viability with/out the use of solvents (Study II), and the direct inhibitory effect of several silicate-based biomaterials when placed in direct contact with dentin (Study III). Also, to evaluate the effect of different solvent treatments on the push-out bond strength of different silicate-based cements and a S-PRG cement (Study IV).

6.1 Study design for the evaluation of dentin matrix

Evaluation of the proteolytic activity in dentin organic matrix was performed using demineralized dentin matrix models through direct and indirect analytical methods. Dentin beams with 2 mm thickness in Study I and 0.3 mm thickness in Study IV were obtained from mid-coronal dentin and assigned to groups based on their relatively homogenous distribution of the enzymes. Beams were completely demineralized in Study I with 10% phosphoric acid, which is effective in activating the catalytic enzymes without denaturation of dentin matrix (Tezvergil-Mutluay *et al.* 2013), and partially demineralized in Study IV with 0.5 M EDTA. Since the MMPs and CCs are distinct enzyme families that cause proteolytic degradation of demineralized dentin matrix, and any potential inhibiting agent can interact with other constituents of dentin, including collagen fibrils or residual calcium ions, the MMPs and CCs were investigated using direct and indirect methods. The direct methods involved the total MMP activity, while the indirect methods included dry mass loss and measurement of the collagen degradation product involving CTX and ICTP, hydroxyproline levels, and micro-BCA. In addition, further evaluation of the bioactive materials, including pH, calcium leach, and the effect of direct placement of the material on dentin was performed.

6.1.1 Direct indicators of enzymatic activity

A generic MMP activity assay was used to evaluate the direct inactivation of the MMPs in demineralized dentin (Liu *et al.* 2011). The total MMP activity was assessed in the first step to assign the beams to homogenously distributed groups. The demineralized dentin beams were immersed in thiopeptide substrate and assay buffer mixture to determine the amount of the total MMP activity (Ozcan *et al.* 2015). The thiopeptide substrate has the ability to diffuse into the demineralized dentin in which will be degraded by the MMPs, causing differences in the absorbance values, which gives general information of the inhibition percentage that would be compared to the control without specification of the inhibited MMP type. In this thesis, all tested bioactive materials reduced MMP activities. Ion release plays

an important role in mineralization and cell differentiation, while pH affects the enzymatic activity and cellular metabolism (Jun *et al.* 2019). The formation of calcium hydroxide is correlated to the release of calcium ions and the alkalinization of the surrounding fluids (Gandolfi *et al.* 2012). The reaction of the tricalcium silicate and dicalcium silicate with water forms calcium silicate hydrate and calcium hydroxide (Camilleri *et al.* 2015). Calcium ions also play an important role in the remineralization of dental tissue since they facilitate the formation of apatite-like crystals (Coleman *et al.* 2009; Taddei *et al.* 2010; Gandolfi *et al.* 2012). MMP requires two Zn^{2+} ions for enzymatic activity and one Ca^{2+} ion for structural stability (D'Armiento *et al.* 2002; Tjäderhane *et al.* 2013), therefore, the released calcium ions have the ability to disrupt the MMP activity by affecting the ionic environment required for optimal Zn^{2+} and Ca^{2+} binding within the enzyme's active site. According to this study, MTA typically showed lower calcium release compared to TheraCal, which goes along with previous studies (Gandolfi *et al.* 2012). In addition, S-PRG didn't show any calcium leaching, which goes along with the previous report (Fujimoto *et al.* 2010). Previous studies showed that calcium ions released from materials have the ability to reduce collagen degradation (Osorio *et al.* 2012; Tezvergil-Mutluay *et al.* 2014). Calcium silicate-based materials are known to release calcium ions during and after the setting reaction in the presence of water (Sarkar *et al.* 2005; Tay *et al.* 2007; Camilleri *et al.* 2014). The setting reaction of tricalcium and dicalcium silicate-based materials is activated by reaction with water, which initiates the formation of calcium silicate hydrate and calcium hydroxide (Camilleri *et al.* 2015). Although materials including TheraCal do not form calcium hydroxide during their setting reaction, however, they still release calcium, which is confirmed by the calcium phosphate deposition on the surface of the dentin (Camilleri *et al.* 2014). Although no release of calcium ions was detected from the S-PRG, it maintained a consistent inhibition of MMPs. This might be related to its ion exchange capacity or bioactive glass composition. Moreover, all ions released by the S-PRG could partially inhibit the MMP activity as shown in the first study, most efficiently the fluoride, which showed the highest inactivation compared to other pretreatments, which goes along with previous findings that showed inactivation of the MMPs by fluoride in saliva (Kato *et al.* 2014), and in dentin (Altinci *et al.* 2016; 2018). Fluoride had the ability to bind with calcium ions of the dentin or the dentin-bound ionized calcium to form calcium fluoride (CaF2), which inactivates MMP in a concentration-dependent manner (Kato *et al.* 2014). In addition, strontium ion, which is also released by the S-PRG fillers, was previously shown to reduce the MMP-1 and MMP-2 production and had an effect on the tissue inhibitors of the MMPs (TIMPs) (Braux *et al.* 2011). Moreover, boron had a partial inhibitory effect on the MMPs, this might be due to its ability to inhibit MMP-9 but not MMP-2 (Chebassier *et al.* 2004). MMP-2 along with MMP-9, are considered the

most abundant MMPs in dentin (Tjäderhane *et al.* 2013). Therefore, the inhibitory effect of the S-PRG can be explained through the effect of the ions released in the solution. Previous studies have shown that S-PRG elutes inhibit MMPs and collagen matrix degradation as effectively as 2% chlorhexidine digluconate (Soares *et al.* 2022).

6.1.2 Indirect indicators of enzymatic activity

The proteolytic degradation of the demineralized dentin matrix was evaluated using indirect methods, including mass loss and measurement of collagen degradation products in the incubation medium, including ICTP, CTX, hydroxyproline, and micro-BCA levels. Dry mass loss measurement of demineralized dentin over time is considered a simple and sensitive analytical method to quantify the enzymatic degradation of the dentin matrix collagen (Carrilho *et al.* 2009; Tezvergil-Mutluay *et al.* 2011). It reflects the total amount of solubilized collagen fragments without specifications of the activity levels of MMPs or CCs (Tezvergil-Mutluay *et al.* 2013). CTX and ICTP are the indirect measurements of the MMP and cathepsin K activity sustainability over time. Enzyme- specific degradation of C-terminal telopeptides in the incubation media was measured using enzyme-linked immunosorbent assays (ELISA). ICTP is the final product of MMP-dependent collagen type I degradation (Garnero *et al.* 2003), while CTX is a specific marker of collagen cysteine cathepsins degradation (Garnero *et al.* 2003; Tezvergil-Mutluay *et al.* 2015).

In Study I, the reduction of ICTP release of all the pretreatments confirmed the inhibition of the MMPs. Among the tested pretreatments, Fluoride had the least ICTP release. This might be related to the zinc and calcium-dependent MMP inactivation caused by fluoride; calcium ions and zinc ions interact in the catalytic side of the MMPs, which results in reversible inactivation of the enzymes at the lower concentrations and irreversible inactivation at high concentrations. Among the tested materials of Study IV, TheraCal had the highest amount of degradation fragments of ICTP and CTX compared to the other materials, although TheraCal partially inhibited MMP activity compared to the control group. This could be explained by the reduced ion release and lower solubility of TheraCal when compared with other types of MTA, including the Proroot MTA, MTA Angelus, and Biodentine. In addition, TheraCal poses similar rates of water sorption and porosity to Proroot MTA and Biodentine but lower than Angelus MTA (Gandolfi *et al.* 2012; 2015). Moreover, there is a limited source of MMPs and cathepsins in the peripheral dentin due to the lack of cellular and odontoblastic activities in the peripheral part (Turco *et al.* 2018). TheraCal acts as a scaffold for the formation of reparative dentin through the release of calcium and formation of an apatite layer, which plays an

important role in dentin remineralization and repair (Yamamoto *et al.* 2017). In addition, TheraCal had the highest initial release of CTX and ICTP.

Storage time and surface area affect the release of ICTP and CTX by the matrix-bound endogenous proteinase; the cleaved collagen fragments near the surface are easier to be released to the incubation medium while the fragments far from the surface remain entrapped in the collagen matrix. However, MMPs can only fragment the substrate within their molecular mobility zone (Turco *et al.* 2018). Previous studies reported that demineralized dentin releases CTX at a lower rate compared to the ICTP (Tezvergil-Mutluay *et al.* 2013; Turco *et al.* 2016). Cathepsin K cleaves collagen at multiple sites. 98% of cathepsin protease activity is related to cathepsin K, since it cleaves helical collagen (Garnero *et al.* 1999), while other cathepsins cleave only the non-helical telopeptides (Brömme *et al.* 2011).

The mass loss of all pretreatments after one week of incubation was similar to the control for all groups except for fluoride, which showed significantly less mass loss compared to the control and strontium-treated group *($p>0.05$)*. While the immediate impact of MMPs and Cathepsin K on dentin mass may be minimal, their effects can become significant with prolonged incubation periods. In the short term, the degradation of dentin by these enzymes is relatively insignificant; however, over extended periods, the cumulative action of MMPs and Cathepsin K can lead to considerable loss of dentin mass according to previous findings (Seseogullari-Dirihan *et al.* 2015; Altinci *et al.* 2016; 2018). Mass loss wasn't much affected by time with both TheraCal and S-PRG; on the other hand, both tested MTA types caused a dramatic reduction of the mass between the initial testing and the 6-month evaluation, especially with the Bio-C. The precipitation of a layer on the surface of TheraCal might be the main reason for the minimum mass loss, as it might mask the possible dry mass loss of the specimens during incubation.

Dissociation of the $Ca(OH)_2$ in the calcium silicate materials into calcium ions and hydroxyl ions raises the pH of the surrounding tissues (Teixeira *et al.* 2005). The ability to maintain high pH in dentin is related to the diffusion capacity of the calcium hydroxide through the dentinal tubules. In the fourth study, both tested MTA types exhibited high pH compared to TheraCal, while S-PRG had a neutral pH similar to the control group. This is expected since calcium hydroxide is not a byproduct of the S-PRG's setting reaction. Calcium hydroxide can contribute to the chemical degradation of dentin through disruption of the bond between the hydroxyapatite crystals and collagenous network due to its alkaline nature (Doyon *et al.* 2005). The pH-related degradation was investigated in the fourth study by quantifying the extractable total protein after material incubation through micro-BCA. Higher cumulative protein levels were seen in alkaline solutions, especially in the Bio-C group.

To further analyze those proteins and detect the presence of collagen, a hydroxyproline assay was performed since 90% of the demineralized dentin dry mass comprises type I collagen, containing 10% hydroxyproline (Butler *et al.* 2000). Similarly, higher hydroxyproline levels in high alkaline media also revealed that a substantial amount of the proteins detected by the micro-BCA were type I collagen fragments, mostly due to the chemical degradation under alkaline conditions. Bio-C had almost 10 times higher release of hydroxyproline compared to the control. In contrast, S-PRG didn't increase collagen solubilization compared to the control group. Moreover, the evaluation of the dry mass measurement showed higher mass loss with the alkaline materials increasing at 1 month and peaking at 6 months, in parallel with the elastic modulus results, giving that the flexural properties of dentin depend on the link between the hydroxyapatite and the collagen matrix of dentin; therefore, chemical degradation of the organic matrix negatively affects the mechanical properties of dentin (Andreasen *et al.* 2002; Doyon *et al.* 2005). Moreover, a previous study showed that incubation of dentin in calcium and zinc-containing media resulted in a relatively rapid and significant decrease in stiffness and increase in mass loss (Tezvergil-Mutluay *et al.* 2010). In addition, decreased dentin fracture resistance and flexural strength following exposure to calcium hydroxide was reported previously (White *et al.* 2002; Grigoratos *et al.* 2001; Andreasen *et al.* 2002; Doyon *et al.* 2005). Although minimal effect was seen on the elastic modulus under short exposure, which might be related to the limited penetration of the of calcium hydroxide affecting the surface but not the bulk of dentin, other reports showed gradual loss of elasticity after 3 months of exposure to calcium hydroxide as a result of protein denaturation within dentin matrix (Twati *et al.* 2009). According to the findings of this thesis, there was a reduction in the elastic modulus of the demineralized dentin matrix over time, especially with the Bio-C and Orbis groups, with complete loss of elasticity at 2 and 6 months, respectively. The variations between the results of the studies might be related to methodological differences. The evaluation of the elastic modulus was used to assess the structural integrity of the dentin beams since the flexural test establishes pure tension on the convex surface of the dentin beam (Berenbaum *et al.* 1959). Moreover, the organic matrix of the dentin, such as the collagen molecules, is usually protected from degradation by intrafibrillar and extrafibrillar apatite crystallites in the mineralized dentin (Trębaz *et al.* 2005; Kim *et al.* 2010). However, the disintegration of dentin beams at the later incubation stages prevented measurement, lowering elastic modulus values. Keeping in mind the relatively thin dentin used and the loss of hydroxyapatite particles due to the demineralization process.

The majority of the endodontic materials tested led to increased degradation of the collagen matrices over time. This could compromise the mechanical integrity of the restorations. Silicate-based biomaterials are still valid materials for clinical use

in dentistry. However, caution is advised when they are used to obturate root canals with thin dentinal walls and in filling the full length of the canal to prevent collagen degradation that might lead to root fracture.

6.2 Biological effects of bioactive materials and pretreatments (Study II)

The reaction of pulp cells tissue simulating pulpal response to different materials through direct or indirect methods is a widely used testing technique to evaluate the cytotoxicity of the materials (Stanley *et al.* 1993; Hebling *et al.* 1999; Kaga *et al.* 2001; Chen *et al.* 2003). For the biocompatibility evaluation of bonding materials, monolayer cultures of fibroblast cells or odontoblast-like cells were used (Moharamzadeh *et al.* 2009; Schmalz *et al.* 2009). In addition, pulp-derived bovine cells were used for the 3-D model used for the in vitro pulp chamber methodology (Schmalz *et al.* 2001; Thonemann *et al.* 2002) since they have higher sensitivity toward tested materials, and they demonstrate the phenotypic characteristics of odontoblast-like cells (Thonemann *et al.* 2000). The transdentinal cytotoxicity method used in this thesis represents the clinical response of the biological reaction of pulp cells to the bioactive material. Dentin discs acted as a barrier between the cell culture that represents pulp tissue in clinical scenarios and the tested bioactive material (Schmalz *et al.* 1996; 2001; Scheffel *et al.* 2015). In addition, MTT assay is a commonly used method to investigate the cytotoxicity of resin-based materials (Mosmann *et al.* 1983), as it is a fast, inexpensive, and simple method for the evaluation of cell proliferation (Moharamzadeh *et al.* 2009). Study II in this thesis was utilized to evaluate the cells' proliferation rate after the indirect contact with the bioactive materials or the dentinal pretreatments. The results of this study showed that the tested bioactive materials (S-PRG, TheraCal, and MTA) affected the trans-dentinal viability of pulp-derived cells. According to the ISO 7405 standards, S-PRG was found to be non- cytotoxic, while TheraCal and MTA were deemed severely and moderately cytotoxic, respectively. The composition of the materials was a decisive factor in their cytotoxicity. A recent study evaluated the effect of short-term and long-term application of the eluates of S-PRG on odontoblast-like cells and found similar results (Soares *et al.* 2024). The cytotoxicity of the S-PRG filler eluates was found to be dose and time-dependent (Nemoto *et al.* 2018; Nishimaki *et al.* 2021; Kashiwagi *et al.* 2021; Ishigure *et al.* 2021). The higher concentration of S-PRG filler eluates is associated with low cell proliferation and metabolism, while diluted forms of the eluates (1:100) or more were shown to be biocompatible with bone marrow mesenchymal stem cells (Nemoto *et al.* 2018), human dental pulp stem cells (Ishigure *et al.* 2021), and human gingival fibroblasts (Kashiwagi *et al.* 2021). The effects of the S-PRG and the S-PRG eluates are mainly related to the released

ions. Previous studies have shown that fluoride at a concentration of 5 ppm can simulate the human pulp cells' proliferation (Thaweboon *et al.* 2003), strontium can also cause the proliferation of human mesenchymal cells from bone marrow and palcenta at certain concentrations (0.1 to 10 mM) (Huang *et al.* 2019). Boron has the ability to increase the synthesis of pro-inflammatory mediators and stimulate the proliferation of lymphocytes (Routray *et al.* 2016). Calcium silicate materials are known for their ions released, including hydroxyl, calcium, and other ions (Koutrouli *et al.* 2024). Mineralization and pulp proliferation are enhanced by the release of calcium ions, which interact with carbonates in the pulp tissue, causing the formation of calcium carbonate. However, relatively high amounts of calcium released could lead to a reduction of cell proliferation and cell death (Natu *et al.* 2015; Miller *et al.* 2017; Phang *et al.* 2024). TheraCal has higher calcium release compared to the MTA (Lopez-Cazaux *et al.* 2006; Camilleri *et al.* 2014; 2015). It also has resin in its composition, and resins are well known for their cytotoxicity (Pedano *et al.* 2020). Uncured monomers cause cell death by increasing the reactive oxygen species production and oxidative DNA damage (Gallorini *et al.* 2014), which affects cell metabolism and protein expression (Bortoluzzi *et al.* 2015). Therefore, the efficiency of the light-curing has a direct impact on the cell viability (Fujioka-Kobayashi *et al.* 2019). The leaching of uncured monomers, causing cell death (Collado-González *et al.* 2017), could be the main factor contributing to TheraCal´s cytotoxicity. Previous studies that evaluated the effect of TheraCal eluates showed similar results, the complex resin-dentin structure might have a direct effect on the monomer diffusion and conversion rates (Lord *et al.* 2006; Stape *et al.* 2021).

There was no significant difference in cell viability between MTA and TheraCal at 24 h. However, MTA was considered moderately cytotoxic according to the ISO standards. MTA is another calcium silicate cement that releases calcium hydroxide upon setting (Camilleri *et al.* 2005) and has bismuth oxide in its composition. Previous studies have shown the formation of calcium silicate bismuth hydrate (C-Si-H-Bi) during the hydration phase, which affects the calcium hydroxide participation in the hydrated material, therefore affecting materials' bioactivity (Formosa *et al.* 2012). In addition, previous studies proved the negative effect of bismuth-containing materials on cell viability. The ability of calcium silicate materials to release the bismuth was recorded for a long period up to 180 days and could be detected systematically in kidneys, liver, blood, and brain, especially accumulated in the kidney (Marciano *et al.* 2023; Pelepenko *et al.* 2024). However, previous studies evaluated the cytotoxic effects of bismuth oxide present in calcium silicates and proved that the cytotoxic effect is concentration dependent (Padrón-Alvarado *et al.* 2023; Marciano *et al.* 2023; Gomes *et al.* 2011). Nonetheless, several studies demonstrated lower cytotoxicity and higher viability when zirconium oxide was used instead of bismuth oxide (Silva *et al.* 2014; Gulzar *et al.* 2021). previous

study evaluated the cytotoxicity of elutes of different calcium silicate materials by the MTT assays showed that the concentrated elutes were cytotoxic while the diluted elutes (50% and 25%) were not cytotoxic (Nashibi *et al.* 2025). another study evaluated the cytotoxicity of MTA materials on mesenchymal stem cells manifested that 100% and 50% were cytotoxic while 25% and 12.5% diluted extracts were not cytotoxic (Kim *et al.* 2019). Another study published in 2023 (Melo *et al.* 2023) also showed a reduction in cell viability with the 1:1 dilutions of silicate-based sealers. Therefore, the biological properties of the calcium-silicate cements are related to their components, the surface structure, different exposure times, and the concentration of the applied material (Kim *et al.* 2019; Melo *et al.* 2023; Nashibi *et al.* 2025). The evaluation of the solvent solutions cytotoxicity was based on the proven effect of solvents on improving the dentin and resin-based materials interaction and enhancing wetting, Therefore, the same concentrations of solvents were evaluated (Stape *et al.* 2018; 2021a; 2021b). DMSO is proven to enhance the penetration of the monomers into the dentinal tubules, which might have negative or positive effects depending on the carried particles (Gallorini *et al.* 2014). The cytotoxic effect of low concentrations of DMSO (0.008%) was previously evaluated and proved to be noncytotoxic (Hebling *et al.* 2015). However, the effect of the higher concentrations of DMSO remains questionable. The tested dentin pretreatments significantly affected the trans- dentinal cytotoxicity; DMSO was moderately cytotoxic while ethanol was severely cytotoxic in its concentrated form. However, 50% v/v dilutions of DMSO with water or ethanol didn't have any cytotoxic effects, differently, the dilution of ethanol in water didn't affect its high cytotoxicity according to the ISO standards (ISO 2008). The cytotoxic effect of the solvents might be related to their effect on cell permeability and diffusion. In addition, the DMSO impact on cells is concentration-dependent; lower concentrations of DMSO had a lower damaging effect on cell membranes 46, therefore, the diluted forms of DMSO were noncytotoxic. Notably, ethanol had a cytotoxic effect at similar concentrations (50% v/v). When diluted in water, interestingly, (50% v/v) dilution in DMSO had no cytotoxic effect, which means that DMSO could show a protective effect against the cytotoxicity of ethanol. This might be related to the ability of DMSO to form hydrogen bonds to ethanol and dentin. In addition, this study series evaluated the effect of the combination of solvent pretreatments and bioactive materials on trans-dentinal cytotoxicity based on the idea that solvents have the ability to improve the material interaction with the dentin. In this Thesis, dentin pretreatments showed effects on the trans dentinal cytotoxicity, and the DMSO or its aqueous solutions (50% v/v) had mild to no effect on the cell viability of the tested bioactive materials. However, pure DMSO significantly reduced the cell viability of TheraCal; this is related to the ability of DMSO to penetrate deeper into the methacrylate-based monomers present in TheraCal into

dentinal tubules, which are known for their cytotoxicity (Gallorini *et al.* 2014). The ethanol and its water-diluted form (50% v/v) showed a similar cytotoxic effect on MTA and S-PRG. Controversially, milder effects of ethanol diluted in water were seen on TheraCal, which is cytotoxic in origin. However, pure ethanol significantly compromised the cell viability of TheraCal even more.

6.3 Push-out bond strength testing (study III)

It is very important for the success of an endodontic procedure to have a good bond between the material and the dentin (Ferracane. 2005; Reyes-Carmona *et al.* 2010). Consequently, mechanical testing of the interface can help in proper material selection and prediction of the outcomes (Mastoras *et al.* 2012; Assmann *et al.* 2012). Recently, Microtensile bond strength is the standard method to evaluate the bond strength of various dental materials (Armstrong *et al.* 2010). However, it is inappropriate to be used for intracanal filling materials, as this is related to the high variations in test results and premature bonding failures (Soares *et al.* 2008). A more suitable test for the evaluation of the bonding in the root canals is the push-out test that was modified from the shear bond strength (Goracci *et al.* 2004). It is considered the most reliable test for the evaluation of root canal sealers, root repair materials, and posts (Collares *et al.* 2015). Therefore, the push-out bond strength test methodology was utilized in this thesis to assess the effect of solvent-containing pretreatments (DMSO, ethanol, and their aqueous dilutions) on the bond strength of different bioactive materials at different root levels. Solvent pretreatments were combined with bioactive materials to benefit from the ability of solvents to improve material-dentin interactions. Studies have shown that even low concentrations of DMSO can improve the bond strength of resin-based materials to coronal dentin (Stape *et al.* 2018a; 2018b; Salim Al-Ani *et al.* 2018). Calcium silicate-based materials are well known to interact with dentin to promote the incorporation of calcium inside dentinal tubules (Han *et al.* 2011), which helps in dentin remineralization, intrafibrillar apatite deposition (Tay *et al.* 2007), and formation of intratubular tag-like structure (Reyes-Carmona *et al.* 2009; Han *et al.* 2011). In addition, the nucleation of ap apatite at the interface helps in reducing voids, which therefore increase the bonding ability (Gandolfi *et al.* 2013). Although TheraCal is a resin modified calcium silicate cement, it showed the highest overall bond strength compared to the other tested materials, this might be attributed to its composition since it contains methacrylate based hydrophilic monomers, which can improve the penetration into dentinal tubules, therefore enhancing the bond strengths (Gasperi *et al.* 2020). MTA showed higher bond strength when compared to the S-PRG, which is mainly related to the relatively fast expansion (Iacono *et al.* 2010) and biomineralization ability (Reyes-Carmona *et al.* 2010) through calcium release of

MTA. A previous study showed similar results (Yassen *et al.* 2016). The relatively low bond strength of the S-PRG might be related to the short incubation period (7 days). The storage period in this study was chosen to comply with the most commonly used storage period to evaluate the bond strength of silicate-based materials (El-Ma'Aita *et al.* 2013; Radulica *et al.* 2024). Previous studies suggested the better bonding outcomes of the S-PRG at a longer incubation period due to the ability of the S-PRG to release several ions, including strontium, sodium, fluoride, and silicon, that can incorporate deeper into dentin only after1-3 months of application (Han *et al.* 2011; Yassen *et al.* 2016). In addition, the bonding of the bioactive materials tested varied according to the position in the root. This variation is mainly related to the nature and anatomy of dentinal tubules; the density of the tubules is higher apically compared to the coronal part of the root (Ferrari *et al.* 2000; Mannocci *et al.* 2004), also the orientation and the degree of tubular sclerosis are different between the coronal and apical thirds of the root (Paqué *et al.* 2006). In this study, TheraCal and S-PRG had a significant reduction of the bond strength between the coronal and the apical thirds. At the same time, MTA was not affected by the root canal morphology. Another reason for the difference between the coronal and the apical thirds of the TheraCal is the fact that TheraCal is a light-curable resin-based material, Therefore, the increased distance between the light source and the bonding surface of the apical third might lead to a lower conversion of the methacrylate monomers which adversely affect the intraradicular bonding. Previous reports have shown similarity with lower bonding efficiency at the apical third compared to the coronal third in TheraCal (Bennett *et al.* 2004). However, a previous study evaluated the S-PRG and showed no significant difference in the push-out bond strength between the thirds (Yassen *et al.* 2016). In this study, the SEM imaging didn't show any tag formation in the bonded interface of the apical third, which is related to the relatively large S-PRG filler sizes that exceed the cross-sectional dimension of the dentinal tubules in the apical third. Also, the paste-like consistency of the S-PRG might have caused difficulty in tag formation, which resulted in the lower bond of the S-PRG apically. MTA didn't show any differences among the root thirds, which goes along with previous findings (El-Ma'Aita *et al.* 2013; Yassen *et al.* 2016). MTA-based materials setting mechanism causes the formation of a mineral infiltration zone with a tag-like structure at the interface with the dentin (Reyes-Carmona *et al.* 2009; Ulusoy *et al.* 2016). The SEM imaging clearly identified the formation of the tag-like structure at the bonded dentin interface, which confirms the ability of MTA to penetrate dentinal tubules. The pretreatments used had different effects on the bioactive materials. Diluted DMSO improved the bond strength on the apical third for TheraCal and wasn't different from coronal or middle thirds, this might be related to the ability of DMSO to increase the monomer conversion at the resin dentin interface (Stape *et al.* 2021) and reduce the termination

rates of free radical polymerization of methacrylates (Gupta *et al.* 1970) which improve the polymer formation of TheraCal (Ferrari *et al.* 2000). It is worth mentioning that the bond strength improvement was only seen in the apical third while all the pretreatments was applied similarly in all root's thirds. In contrast, the undiluted DMSO had a negative effect on the bond strength of TheraCal on the apical third, this might be related to the low vapor pressure of TheraCal that keeps it pooled in the apical third and therefore alters the composition of TheraCal and compromises the formation of polymer chains. However, none of the pretreatments had an effect on the MTA except for undiluted ethanol, which lowered the bond strength of the apical third in MTA. This is because of the lowered intrinsic wetness of the root canal since MTA is highly miscible in water which makes it replace the residual water molecules in dentin and that effect the setting reaction of MTA specially at the apical third due to the higher dentinal density and higher overall water content at the coronal and middle thirds which reduced the dehydration level. Moreover, DMSO didn't have any effect on the bond strength of MTA since DMSO doesn't cause dentin dehydration. None of the pretreatments affected the S-PRG bonding to dentin; further studies to evaluate the long-term bonding ability of bioactive materials are needed.

7 Conclusions

Based on the series of studies described in this Ph.D project, the following conclusions were drawn:

1. The ions released from the S-PRG fillers have the potential to partly inhibit the endogenous enzymatic activity, more specifically effective on MMPs in demineralized dentin matrices.

2. Pure ethanol or its aqueous dilutions (50% v/v) should preferably not be applied close to the pulp, due to its cytotoxic effect. However, relatively high DMSO dilutions (50% v/v) had minimal to no effects on cell viability regardless of the bioactive material. Therefore, solvent selection must be carefully performed to avoid unnecessary increases in cytotoxicity levels.

3. Intraradicular bond strengths of the different bioactive materials varied according to the different compositions and the tested root segment. The use of diluted DMSO can improve the bond strength of the resin-based silicate cement, however, the use of pretreatments should be applied with caution.

4. Although many bioactive materials are classified as MTA cements, their interactions with dentin vary significantly depending on their specific chemical compositions. The choice of a bioactive material would depend on the specific requirements of the dental procedure and the desired effects on tissue response and bioactivity.

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Original Publications

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The inhibitory effects of various ions released from S-PRG fillers on dentin protease activity

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This study investigates the effect of ions released from S-PRG fillers on host-derived enzymatic degradation of dentin collagen matrices. Dentin beams (n=80) were demineralized and distributed to eight groups following baseline dry mass and total MMP activity assessments. Each group treated with boron, fluoride, sodium, silicone, strontium, aluminium, or S-PRG eluate solutions for 5 min. Untreated beams served as control. After pre-treatment, MMP activity was reassessed, beams were incubated in complete medium for 1 week, dry mass was reassessed. Incubation media were analyzed for MMP and cathepsin-K-mediated degradation fragments. Data were analyzed with ANOVA and Tukey's test. All pretreatment groups showed significant reduction in total MMP activity (p<0.05) that was sustainable after incubation in all groups except for boron and silicone groups (p<0.05). Cathepsin-K activity did not differ between control or treatment groups. The results indicated that ions released from S-PRG fillers have the potential to partly inhibit MMP-mediated endogenous enzymatic activity.

Keywords: Enzymes, Collagen degradation, Cysteine cathepsins, Matrix metalloproteinases, S-PRG filler

INTRODUCTION

The goal of contemporary caries management is to utilize noninvasive remineralization technologies or minimal invasive selective caries removal techniques to preserve the structural integrity of the dental hard tissues. When dentin is demineralized *via* bacteria-derived acids during caries process, endogenous proteolytic enzymes; matrix metalloproteinases (MMPs) and cysteine cathepsins (CCs) are uncovered and activated[1,2] resulting in progressive degradation of extracellular matrix components, compromising the structural integrity and future remineralization potential of dentin. Therefore, significant research efforts have been focused on enzyme inhibition[3,4] or biomimetic remineralization strategies[5-8] to slow down the degradation and preserve demineralized matrix for future attempts to remineralize the cavitated lesions. The use of ion-releasing dental materials (*e.g.* liners, bioactive adhesives, and restoration materials) have been advocated for effectively increasing the mineral content of the sealed carious dentin[9], and for slowing the collagen degradation by dentin proteases in the hybrid layer[8,10].

Surface pre-reacted glass ionomer (S-PRG) filler was introduced as a biofunctional addition with ion-release and ion-recharge abilities and was incorporated into resin composites[11,12], root canal sealers[13], or fissure sealant[14]. S-PRG filler features a glass-ionomer phase around the glass core, which slowly releases multiple ions, such as aluminum ions (Al_3^+), boric acid ions (BO_3^{3-}), sodium ions (Na^+), silicate ions (SiO_2^{2-}), strontium ions (Sr^{2+}) and fluoride ions (F^-)[15-17]. The released ions exert various effects such as acid-buffering capacity, antimicrobial effect[18], inhibition of bacterial adhesion, remineralization[19], enhanced bone formation[20,21], as well as tertiary dentin formation[22]. However, the effect of S-PRG or/and its releasing ions on the degradation of dentin collagen matrices by host-derived enzyme activity has not been studied yet.

Therefore, the aim of this study was to investigate the effect of S-PRG fillers and ions released from S-PRG fillers on host derived enzyme-mediated degradation of dentin collagen matrices. The null hypotheses was; pretreatment with various ions released from SPR-G fillers would not inactivate dentin matrix-bound MMPs, or cathepsin K and induces no changes in demineralized matrices.

MATERIALS AND METHODS

Preparation of specimen

Eighty extracted non-carious human molars were stored at 4°C in 0.9% NaCl containing 0.02% sodium azide (NaN_3) to prevent microbial growth and were used within one month after extraction. The extracted teeth were collected from anonymous donors and, therefore, were exempt from ethical notification according to the Finnish law (Tissue act, section 20). The enamel and superficial dentin were removed by horizontal sectioning 1 mm below the deepest central fissure and dentin beams (1×2×6 mm) were prepared from mid-coronal dentin, using a low-speed saw (Isomet, Buehler, Lake Bluff, IL, USA) under water-cooling. Dentin beams were demineralized with 10% H_3PO_4 for 24 h at 20°C under

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constant stirring. After demineralization, specimens were rinsed with distilled water for 24 h at 4°C.

Preparation of treatment solutions

Standard solutions containing 100 ppm of boron, fluoride, sodium, silicon, strontium, and 10 ppm solution of aluminum were prepared in distilled water to simulate the various ions released from S-PRG fillers. To prepare the S-PRG filler eluate, distilled water was mixed with S-PRG fillers (1 μm) in ratios of 1:1 (1L:1,000 g)[15,16]. The mixture was stirred for 24 h at 23°C and centrifuged to precipitate the S-PRG fillers, and the supernatant S-PRG filler eluate was collected. The supernatant was filtered (pore size 0.45 μm) to remove any residual insoluble material and the resulting filtrate was used as S-PRG filler eluate. The concentrations of various ions in S-PRG-filler eluate were determined using an emission spectrophotometer (ICPS-8000, Shimazu, Kyoto, Japan) and an F- electrode (Model 9609BNWP, Orion Research, Espoo, Finland) with ion selective electrode meter (Model 720A, Orion Research). Ion concentrations in the S-PRG filler eluate were: Al^{3+} (19.4 ppm), BO_3^{3-} (1,480.2 ppm), Na^+ (549.2 ppm), SiO_2^{3-} (14.5 ppm), Sr^{2+} (151.5 ppm), and F^- (135.5 ppm).

Relative activity of MMPs

After demineralization and rinsing, demineralized dentin beams were used as the MMP source to assess the dentin matrix-bound MMP activity following various treatment solution[23]. The total MMP activity was evaluated using a chromogenic thiopeptide MMP substrate (Sensolyte Generic MMP assay, Anaspec, San Jose, CA, USA). To measure the baseline MMP activities, demineralized dentin beams were incubated in 150 μL of the substrate and 150 μL assay buffer for 5 min[24] at 37°C, and 200 μL of incubation solution were read every 15 min using a microplate reader (Synergy HT, BioTek Instruments, Winooski, VT, USA) at 412 nm wavelength until peak value obtained individually. After baseline measurements, beams were divided into 8 groups (n=10/group) so that the baseline activity was not significantly different among different treatment groups. After rinsing to remove the substrate solution, each group was treated for 5 min with 200 μL of irrespective ion or S-PRG filler eluate. The group treated only with distilled water served as control. After pretreatment, samples were rinsed, blot-dried and the enzymatic activity was re-measured as described above. Changes in the total MMP activity of dentin beams were calculated as a percentage of the baseline measurement of the samples individually for each sample to assess the relative inhibition or the activation of MMPs after pretreatment compared to the control group according to the following equation Relative MMP activity (%)=(MMP activity after treatment-initial MMP activity)/control group (100%)

Loss of dry dentin mass

Loss of demineralized dry dentin mass is an indirect method to measure the degradation of collagen matrix by endogenous dentin proteases. Eighty demineralized dentin beams were prepared as described above and then were dried in the desiccator for 72 h. The initial dry mass of samples was measured to the nearest 0.01 mg on an analytical microbalance (XP6 Microbalance, Mettler Toledo, Hightstown, NJ, USA), and ten beams were assigned to each of 8 groups (n=10/group) so that the mean dry mass of each group was statistically similar. After the rehydration in distilled water for 2 h at 4°C, dentin beams were treated with corresponding treatment solution for 5 min. The group treated with distilled water served as control. Following the treatment dentin beams were incubated in individually labeled tubes containing 1 mL of the simulated body fluid containing 5 mM HEPES, 2.5 mM $CaCl_2 \cdot H_2O$, 0.05 mM $ZnCl_2$, 0.68 mM KH_2PO_4, 30 mM KCl and 0.3 mM NaN_3 (pH 7.4) for 1 week[25]. Following the incubation, dentin beams were rinsed free of buffer salts in distilled water under constant stirring at 4°C for 8 h and then were placed in a vacuum desiccator containing dry silica beads for 72 h. The dry mass of each dentin beam was measured with an analytical balance (XP6 Microbalance, Mettler Toledo). The percent reduction in dry mass was calculated with regard to the baseline dry mass values for each sample individually under the same protocol.

Measurement of released fragments of type I collagen C-terminal telopeptide

After incubation, aliquots of dentin beams were used to determine specific collagen degradation fragments for ICTP and CTX–fragments. Cathepsin K is the only known source of CTX fragments from C-terminal peptides of collagen[26,27] which are the end-product of cathepsin K-mediated degradation of collagen in dentin matrix[28,29]. Therefore, the amount of liberated telopeptide fragments due to cathepsin-K activity was analyzed using enzyme-linked immunoassay specified for CTX detection (Serum CrossLaps ELISA Immunodiagnostic System, Farmington, UK). Ten to fifty microliter of the incubation medium were used for CTX ELISA assay. The amount of CTX release was calculated with a standard curve using standards with known concentrations provided in the kits. The assays were performed in duplicates using 10 samples/group.

To determine MMP-mediated degradation on demineralized dentin, the amount of solubilized type I collagen C-terminal cross-linked telopeptides (ICTP) was measured using the ICTP RIA kit, a radioimmunoassay kit (ICTP RIA, Orion Diagnostica, Espoo, Finland). The amount of ICTP in the incubation medium was calculated with 5-point fitting curve with known concentrations standard from 1 to 50 ng/mL with the limit 0.6 ng/mL by a gamma counter (WIZARD 1470 Wallac, PerkinElmer Finland, Turku, Finland). Each measurement was performed in duplicate (n=10/group).

Statistical analyses

The relative MMP activity, loss of dry mass (%), release of ICTP (ng telopeptide/mg dry dentin), and CTX (pg/mg dry dentin) were analyzed separately using Kolmogorov-Smirnov test for normality, and the modified Levine test

for homoscedasticity. Since the normality and equality of variance assumptions of the data were valid, they were subjected to one-way analysis of variance (ANOVA). *Post hoc* comparisons were performed with Tukey test using SPSS (SPSS Statistics for Mac, Version 27, IBM, NY, USA). Statistical significance was pre-set at α=0.05.

RESULTS

The relative MMP activity

The change in total MMP activity (±SD) after different treatments is shown in Fig. 1. All treatment groups showed significant reduction in total MMP activity compared to untreated control group (p<0.05). During incubation, MMP activity of control group increased 99.8±13%, whereas the increase in S-PRG filler eluate-treated group was limited to 23.2±4.6%. Fluoride-treated group showed the lowest value and reduced its activity to −3.5±2.3% after treatment.

The loss of dry mass

The mean loss of dry mass (±SD) of demineralized dentin beams after different pretreatments is shown in Fig. 2. Cumulative loss of dry mass of dentin beams was measured and expressed as a percentage of the initial mass after 1-week incubation period. Mass loss of fluoride-treated group was significantly lower compared to the strontium and the control group (p<0.05). The lowest mass loss was observed at the fluoride group with only 4±1.5% loss of mass confirming a partly inhibited enzymatic activity with this group. There was no statistical significant difference between the other tested groups and the control (p>0.05); however, strontium showed the highest mass loss among the tested groups.

Inactivation of endogenous telopeptide of dentin

The release of ICTP and CTX fragments were used as indirect measurement of enzyme specific degradation of collagen matrix and were calculated based on assays' instructions. The amount of released CTX peptide (±SD) during 1-week incubation is shown as pg/mg dentin in Fig. 3. There was no significant difference between the groups (p>0.05). However, S-PRG group with 122.6±41.3 pg CTX/mg dentin showed the lowest CTX release, whereas the maximum release of CTX fragments were measured in the strontium group with 186.9±50.1 pg CTX/mg dentin, followed by the control with 165.5±17.5 pg CTX/mg dentin. All other groups showed almost similar amounts of CTX release.

Figure 4 shows the mean ICTP release (±SD) to the incubation medium from the demineralized dentin beams. During incubation, the highest ICTP release

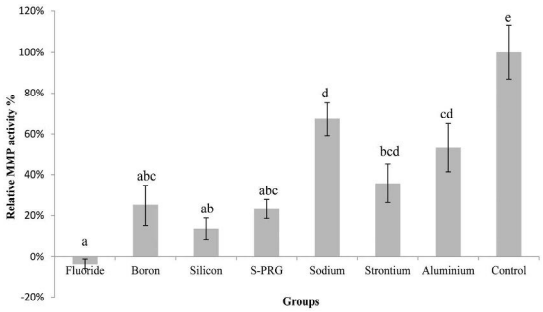

Fig. 1 Relative MMP activity percentage after 5 min treatment.

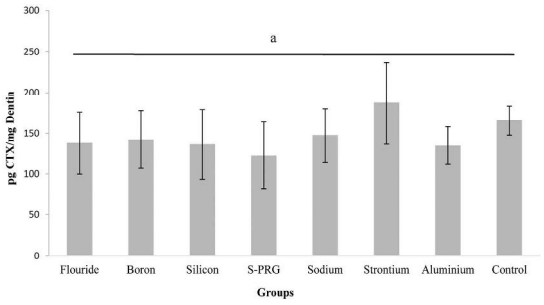

Fig. 3 The mean of CTX fragments released to the incubation medium from the demineralized beams during 1-week incubation in pg/mg dentin.

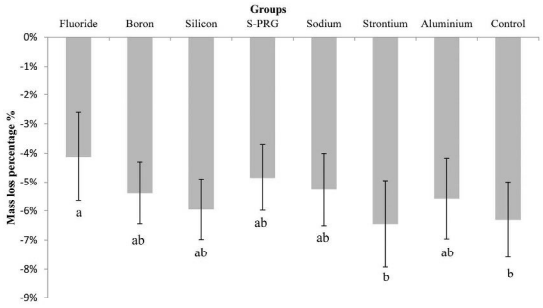

Fig. 2 Dry mass loss percentage after 1-week incubation period.

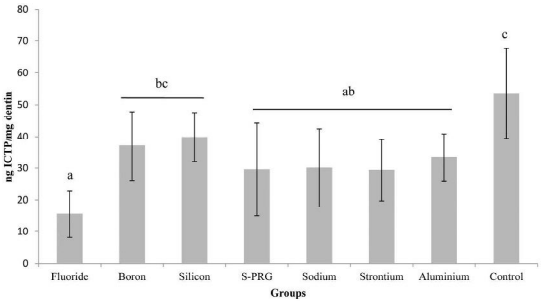

Fig. 4 The release of ICTP telopeptide fragments after 1-week incubation.

occurred in the control group with 53.5±14.1 ng ICTP/mg dentin. The fluoride pretreated group showed the lowest ICTP release among all other groups with 15.4±7.3 ng ICTP/mg dentin and was significantly different compared to untreated group, boron- and silicon-treated groups ($p<0.05$). Strontium-, sodium-, and S-PRG filler eluate-treated groups showed similar trend on ICTP release and were significantly lower compared to untreated control ($p<0.05$). Among treatment groups, silicon with 39.8±7.7 ng ICTP/mg dentin showed the highest ICTP release following the control ($p>0.05$).

DISCUSSION

With the increased understanding of the role of host-derived enzymatic degradation in structural integrity of demineralized dentin matrices, there has been an increased focus on utilizing biofunctional materials and technologies such as ion-releasing dental materials to prevent degradation of dentin collagen matrices[30]. In the present study, we investigated, for the first time, multiple ion releasing S-PRG filler and its content ions effect on the host-derived enzymatic degradation of dentin. Our results warrant partial rejection of the null hypothesis; while a significant reduction was observed in MMP mediated activity following pretreatment, the effect on cathepsin-mediated activity was not significant.

To evaluate direct inactivation of MMPs in demineralized dentin, a generic MMP activity assay was used[31]. The results of MMP activity assay revealed that S-PRG filler or/and all released ions can effectively inactivate matrix bound-MMPs in dentin following a pretreatment. Among all tested ions, fluoride showed the most efficient inactivation during incubation in line with the previous research findings which reported inactivation of MMP by fluoride in saliva[32], and in dentin[33,34]. MMPs depend on calcium to maintain their three dimensional configuration for enzymatic activity. The fluoride ions could complex with dentin-bound ionized calcium as well as the calcium ions at the incubation medium to form CaF_2 resulting in the complete or partial inhibition of the enzymatic activity in a concentration-dependent manner[32]. Similarly, strontium was previously shown to reduce the MMP-1 and MMP-2 production and had a positive effect on MMP-TIMP balance[35]. Tissue inhibitors of MMPs (TIMPs) are endogenous protein regulators for local control of MMP activity. Although several MMPs are present in demineralized dentin, MMP-2 and MMP-9 are the most abundant MMPs in dentine[36]. A recent report also showed strict association of TIMP-1 and MMP-2 in demineralized dentin matrices[37]. Therefore, a partial initial inhibition obtained with strontium might be due to its effect on MMP-2-TIMP balance[36]. Boron was previously shown to induce MMP-9 whereas showed limited effect on MMP-2[38], which could partly explain partial inhibition obtained in this study. Since MMPs contain structural zinc groups, previous work suggested that boron could act as a zinc chelator which results in the inhibition of the activity[39]. Therefore, almost 77% inhibition of total MMP activity with S-PRG fillers compared to control could be explained by the synergetic effect of the ions in the solution.

To evaluate if the inhibitory effect is sustainable over time, the MMP and cathepsin K activities of treated groups were evaluated indirectly by measuring the release of MMP-mediated ICTP and cathepsin –mediated CTX telopeptide fragments, respectively[28,29,40].

In line with the initial MMP inhibition, all pretreatment solutions significantly decreased the degradation of the collagen matrix by MMPs after 1-week of incubation as was confirmed with the reduction in ICTP release compared to control. Among treatment groups, fluoride-treated group showed the lowest ICTP release, which could be explained with inactivation of zinc- and calcium-dependent MMP by fluoride. The possible mechanism could be the interaction between Zn^{2+} and Ca^{2+} in the catalytic side of MMPs and highly electronegative fluoride result in reversible enzyme inactivation at lower and irreversible at higher concentrations[31]. Another study evaluating the effect of fluoride on dentinal MMPs and CCs, also showed reversible MMP inhibition during the incubation period in terms of both total MMP activity assay and ICTP telopeptide release[33]. Although the mechanism between MMPs and S-PRG fillers or other ions are not well known, we speculated that they could have similar interaction result in the inhibition of the catalyst action on MMPs.

On contrast to the reduction in MMP mediated degradation, after one week of incubation no significant difference was observed in CTX release between the control and pretreatment groups. S-PRG filler eluate pretreatment showed the lowest release among all the tested groups. Previously only very high concentrations of fluoride was shown to inactivate CC activity in dentin, whereas lower concentrations did not have much inhibitory effect[32]. Since the fluoride ion concentration tested in this study is much lower, it is not surprising to see no significant effect on CTX release. Furthermore, CTX fragments are much smaller than ICTP fragments, and therefore may easily diffuse through the collagen matrix[41]. It should also be noted that, much lower release rates of CTX compared to ICTP could be related to the incubation medium pH being 7.4 rather than optimum pH of 5.5 for CC activity[42,43]. Nevertheless, pretreatment with various ions had no significant inhibition effect on cathepsin activity after one-week incubation period. The loss of dry mass after one week incubation was similar for all groups compared to control except for the fluoride which showed the lowest dry mass reduction among all groups and significantly lower mass loss than the control and the strontium groups ($p>0.05$). Other pretreated groups had a slight reduction in dry mass loss ($p>0.05$) after 1-week incubation. Considering to the amount of total degradation by MMPs and Cathepsin K, the changes on dentin mass could be relatively negligible in a short term, but significant for extended incubation according to previous findings[24,33,44]. On the other hand, ion treated dentin matrices might further interact with the ions in

incubation medium, resulting in mineral precipitation on demineralized dentin surface. This could further mask the possible dry mass loss of the specimens during short-term incubation. Therefore, future work should focus on longer incubation periods to evaluate if the initial inhibition of MMPs will be reversible over time.

CONCLUSION

Within the limitation of the study, we can conclude that the ions released from the S-PRG fillers have the potential to partly inhibit the endogenous enzymatic activity, more specifically effective on MMPs in demineralized dentin matrices.

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Effect of solvent pretreatments on trans-dentinal cytotoxicity of bioactive materials.

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Effect of solvent dentinal pretreatment on the transdentinal cytotoxicity of bioactive materials

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Data availability statement: Data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethical approval statement: The extracted teeth, obtained from anonymous donors, were exempt from ethical notification according to Finnish law (Tissue Act, section 20).

Abstract

This study aimed to evaluate the effects of pretreatment with different concentrations of dimethyl sulfoxide (DMSO) and/or ethanol (EtOH) on the transdentinal cell viability of bioactive materials in simulated deep clinical cavities. Dentin discs were prepared, balanced, and autoclaved. Three-dimensional cultures of odontoblast-like cells adhered to the dentin discs inside perfusion split chambers designed for dentin-barrier cytotoxicity tests. Dentin pretreatments included ethanol, DMSO, 50% DMSO dissolved in water (DMSO/H_2O) or ethanol (DMSO/EtOH), and an aqueous 50% ethanolic solution (EtOH/H_2O). After applying 1.5 µL of pretreatments, a 1 mm increment of light-curable methacrylate-based silicate (TheraCal), a mineral trioxide aggregate (MTA), or an experimental pre-reacted glass-ionomer filler (S-PRG) was applied following the manufacturers' instructions. Cell viability was assessed via MTT and analyzed spectrometrically. Trans-dentinal cytotoxicity was evaluated via the Kruskal–Wallis test (α=0.05), assessment of cell damage followed ISO 7405. TheraCal was considered severely cytotoxic, MTA moderately cytotoxic, and S-PRG noncytotoxic. Assessment of cell damage caused by solvents was ranked as EtOH=EtOH/H_2O>DMSO>DMSO/EtOH=DMSO/H_2O. The cell viability of bioactive materials decreased depending on the solvent type, particularly with solutions containing ethanol *($p<0.05$)*. While ethanol was harmful, 50% DMSO had a minor effect on cytotoxicity. Solvents must be used with caution in deep cavities.

Keywords: Cell viability; Ethanol; MTA; DMSO; S-PRG; TheraCal.

Introduction

Selective caries removal techniques are becoming more popular clinical options to delay or even prevent unnecessary root canal treatments. Given the increasing emphasis on preserving tooth vitality [1], the development of new bioactive and biocompatible biomaterials has gained significant attention. While external factors such as caries can harm pulp cells [2], biomaterials may also influence pulp cell viability [3]. Successful vital-pulp therapy hinges on material biocompatibility, the absence of bacterial contamination, and the patient's general health [4]. Ensuring the compatibility of these materials with pulp cells is crucial for creating a conducive environment for effective tissue repair [5].

Current methods for assessing the cell viability of biomaterials in vitro typically involve collecting eluates from "fully set" or "freshly mixed" biomaterials [5]. This approach, however, overlooks the protective impact of dentin on pulp cells. While substantial progress has been made in understanding the effects of biomaterials on pulp cell compatibility, translating results from conventional cell culture cytotoxicity tests to real clinical applications can be problematic. The protective dentin-barrier effect plays an important role in determining the compatibility of bioactive materials. Therefore, trans-dentinal cytotoxicity setups help bridge the gap between in vitro and in vivo studies by simulating more realistic clinical simulations [6]. Under such testing conditions, bioactive materials initially interact with dentin before releasing ions that diffuse toward pulp cells, mimicking in vivo applications more realistically.

Since the introduction of the first hydraulic silicate cement in 1993 [7], various material modifications have aimed to enhance handling and setting properties, reduce discoloration, lower solubility, and improve sealing. Effective interactions with mineralized dental tissues and achieving a good seal are crucial for the long-term success of biomaterials [4]. Dimethyl sulfoxide [$(CH_3)_2SO$] (DMSO) is a polar aprotic colorless solvent that enhances dentin wettability [8] and facilitates the penetration of high molecular-weight molecules into dentin [9]. The mechanisms by which DMSO improves the interactions between restorative materials and dentin [8,10–13] may also contribute to better sealing between bioactive materials and dentin. Since the main reason for failure after vital pulp therapy

is reinfection of pulp tissue by bacteria [4,14], lowering the risk of leakage could reduce long-term reinfection risk after material placement. Additionally, more intimate contact between bioactive materials and dentin could favor complete reparative dentin bridge formation within shorter times. Exploring such potential benefits of DMSO pretreatment on biomaterial–dentin interactions would be promising as long as cytocompatibility remains at acceptable levels. While low DMSO concentrations have been found to have little or no effect on pulp-derived cells [15,16], the effects of relatively high DMSO concentrations remain unknown. Consequently, this study aims to evaluate the effects of dentin pretreatments containing different solvents (DMSO, ethanol, and their aqueous dilutions) along with bioactive materials on transdentinal cell viability in simulated deep clinical cavities. The null hypotheses tested were that the composition of (i) bioactive materials or (ii) dentin pretreatments would have no effect on trans-dentinal cytotoxicity.

Materials and methods

Preparation of dentin discs

Two hundred and seventy-six extracted sound third molars collected from anonymous donors and exempt from ethical notification according to local regulations (Tissue Act, section 20) were collected. All experiments were performed in accordance with relevant guidelines and regulations. Tooth extraction was not related to this study. The teeth were stored in 0.9% NaCl supplemented with 0.02% sodium azide and stored at 4 °C. The dentin discs were sectioned immediately above the pulp horns of each tooth (500 µm thick via a precision diamond saw (Isomet 1000 Precision Saw, Buehler, Lake Bluff, IL, USA)) under water cooling. Discs with perforations, indicating pulpal exposures, were discarded and replaced. The occlusal surface of the dentin discs was polished with 320-grit SiC abrasive paper (CarbiMet, Buehler Ltd; USA) to achieve a final thickness of 300 µm (±15 µm). To remove the smear layer, 50% citric acid was applied for 30 s, and the samples were rinsed for 30 s.

Dentin permeability measurement

Dentin permeability was assessed to ensure a homogenous distribution of the dentin discs into

the tested groups. The application of 50% citric acid to the dentin discs for 30 s was performed to eliminate the smear layer and smear plugs, thereby reopening the dentinal tubules. The permeability of the dentin disks was evaluated via a flow-measurement infiltration apparatus. (SLI-1000 Liquid Flow Meter, Sensirion, Switzerland) within a modified split-chamber unit connected to a deionized water container, simulating a hydrostatic pressure of 20 cm [17]. After maximum permeability measurement, the dentin discs were rinsed with distilled water for 15 s and autoclaved in 0.9% sodium chloride at 121 °C for 25 min. Dentin discs (n = 12 discs/group) were then allocated into 23 balanced groups considering statistical similarities in terms of dentin permeability.

Experimental design

The experimental design was composed of two study factors defined as follows: (i) "dentin pretreatment" at six levels (no pretreatment; ethanol; dimethyl sulfoxide; 50% aqueous ethanol; 50% aqueous dimethyl sulfoxide; 50% ethanolic dimethyl sulfoxide), (ii) "bioactive material" at four levels (no material; light-curable silicate-based cement (TheraCal); mineral trioxide aggregate (MTA); and an experimental prereacted glass-ionomer filler (S-PRG). A total of 23 experimental groups were obtained (n=12 discs/group). **Figure 1** shows a summary of the experimental design. The compositions of the test materials and application procedures are shown in **Table 1.** A light-curable methacrylate-based silicate (TheraCal LC, Bisco, Chicago, Illinois, USA; TheraCal), a mineral trioxide aggregate (MTA Orbis, Superior Dental materials GmbH, Hamburg, Germany), and an experimental prereacted glass-ionomer filler (S-PRG, Shofu Inc., Higashiyama-ku, Kyoto, Japan; S-PRG) were used following the manufacturer's instructions as indirect pulp-protecting materials. A hydrophobic polyvinylsiloxane material (Imprint 4 super quick ultralight; 3 M ESPE, Neuss, Germany) was employed as a negative control, and an experimental glass-ionomer cement was used as a positive control according to ISO 7405 standards [18]. Negative controls had no effect on cell viability, whereas positive controls reduced cell viability by approximately 50% after 24 h of exposure [19].

Pulp-derived three-dimensional cell culture

Clonal large T-antigen bovine pulp cells (SV40) derived from calf dental papilla [18,20] were stored in liquid nitrogen until use. The cells were maintained in growth medium (αMEM; Sigma–Aldrich, New Road, Gillingham, UK) supplemented with 20% fetal bovine serum, 150 IU/ml penicillin, 150 mg/ml streptomycin, 0.125 mg/ml amphotericin B and 0.1 mg/ml geneticin (Sigma–Aldrich). The cell cultures were incubated in 5% CO_2 and 100% humidity at 37 °C until use. Polyamide nylon meshes with a pore size of 150 µm and a diameter of 8 mm were prepared. The nylon meshes were cleaned with 0.1 M acetic acid for 30 minutes, washed three times with sterile water, and coated with 0.03 mg/ml fibronectin (bovine plasma fibronectin, 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. A Millicel insert was carefully placed at the bottom of the well plate to support the initial growth of the cells on the meshes. Four meshes were then inserted into each cell culture insert (Greiner bio-one, Nurtigen, Germany) in sufficient nutritional medium. The suspension of pulp-derived cells was adjusted to 80,000 cells/20 µL for each mesh, and the cultures were incubated for 48 hours to allow proper cell growth over the polyamide nylon mesh (37 °C, 5% CO_2, and 100% humidity). After incubation, each polyamide nylon mesh was separately placed in a 24-well tissue plate. In each well, 1 ml of MEM α and 10% FBS medium were added to feed the cells. The medium was changed three times a week for 14 days in the incubator to produce three-dimensional cells on the mesh. Extra membranes were prepared for each test to ensure the presence of enough cells on 8 membranes for each testing cycle. The number of cells on the meshes was calculated with a TC20 automated cell counter (Luminex xMAP, Bio-Rad, Hercules, CA, USA) by mixing 15 µl of the cell suspension and 15 µl of 0.4% trypan blue and then pipetting 10 µl of the mixture into the counting slide chamber.

Dentin pretreatment solutions

Five dentin pretreatment solutions containing DMSO (dimethyl sulfoxide, Sigma–Aldrich, St. Louis, MO, USA; DMSO) and/or ethanol (ethanol 99.8%, Sigma–Aldrich; EtOH) were prepared and stored at room temperature until use. The solvent concentrations (v/v) used were as follows: pure ethanol

(EtOH), pure dimethyl sulfoxide (DMSO), 50% aqueous ethanol (EtOH/H_2O), 50% aqueous dimethyl sulfoxide (DMSO/H2O), and 50% ethanolic dimethyl sulfoxide (DMSO/EtOH) [8].

Dentin barrier cytotoxicity test

Trans-dentinal cytotoxicity evaluation was carried out according to ISO 7405 [18,19]. In this method, the tested materials/pretreatments are applied to dentin discs and allowed to pass through dentinal tubules to evaluate the cell response inside split-chamber compartments **(Figure 1).** After a 14-day incubation period, polyamide meshes containing three-dimensional cell cultures were placed in the lower compartment of commercially available cell culture perfusion chambers (Minucells and Minutissue, Bad Abbach, Germany) in direct contact with the pulpal side of the dentin disc and held in place by a stainless-steel holder. The pulpal compartment was perfused with assay medium supplemented with 5.96 g/L hydroxyethyl piperazine ethanesulfonic acid (HEPES) at a rate of 0.3 ml/h for 24 h via a precision pump (Minucells and Minutissue, Bad Abbach, Germany). The perfusion was then briefly switched off, and treatment solutions (1.5 µl) were applied for 10 s over the dentin slice inside the chamber, followed by the tested biomaterials according to the experimental groups. Test materials (MTA, TheraCal, and SPR-G) were applied following the manufacturer's recommendations in a 1 mm-thick layer **(Table 1).** The MTA was then covered with a wet–cotton pellet (sterile NaCl 0.9%). TheraCal was light-cured for 20 s via an LED light-curing unit (Elipar; 3 M ESPE) at 1200 mW/cm^2. After the split chambers were closed, the cells were perfused at a rate of 0.2 ml/h for 24 h. The cell meshes were gently sectioned into 4 mm^2 circular shapes with metallic inserts retrieved from the stainless-steel holder. Therefore, only the part of the mesh in contact with the dentin disc where the tested solvent pretreatment and/or the bioactive material was retrieved, and stored for 2 h in a cell incubator at 5% CO_2 and 100% humidity at 37 °C. Each experiment was repeated six times, and two replicates were performed (n = 12). Positive and negative controls were also included in each test.

Trans-dentinal cell viability (MTT assay)

Cell viability was determined by the MTT assay. Meshes covered with cells were removed from

the metallic inserts, placed into 48-well plates containing 500 µl of prewarmed MTT solution (0.5 mg/ml growth medium) and incubated for 2 h at 37 °C. The blue formazan precipitate was extracted from the mitochondria using 250 µl of dimethyl sulfoxide (DMSO; Sigma–Aldrich) on a shaker at room temperature for 30 min. After that, 200 µl of this mixture was transferred to a 96-well plate, and the absorption at 570 nm was determined spectrophotometrically (Synergy HT; BioTek Instruments). The percentage of cell viability was calculated on the basis of the optical density of the negative control samples.

Statistical analysis

The assessment of cell damage was further categorized as noncytotoxic, moderately cytotoxic, or severely cytotoxic according to ISO 7405 [18]. Since the trans-dentinal datasets were not normally distributed, the data were analyzed via the Kruskal–Wallis test (α=0.05). Calculations were performed with IBM SPSS Statistics for Windows, version 26 (IBM Corp., Armonk, NY, USA).

Results

Assessment of cell damage

Significant differences were observed between the negative and positive control groups *($p<0.05$).* bioactive materials, pretreatment solutions, or their combined use significantly affected cell damage. Assessments of cell damage according to ISO 7405 [18] are reported in **Table 2**.

Trans-dentinal cell viability

The trans-dentinal viability of pulp-derived cells after 24 h is shown in **Figure 2.** The results of the Kruskal–Wallis test revealed that bioactive materials, pretreatment solutions, and their combined use significantly affected cell viability *($p<0.001$).* The greatest reduction among pretreatment solutions was observed for EtOH and EtOH/H_2O *($p<0.05$).* No significant differences were observed between the DMSO/H_2O, DMSO/EtOH, or DMSO groups *($p>0.05$).* S-PRG was the least cytotoxic bioactive material, followed by MTA, without significant differences *($p>0.05$).* TheraCal presented the greatest reduction in cell viability among the tested materials *($p<0.05$).* In general, EtOH and EtOH/H_2O produced

the lowest overall cell viabilities, as opposed to DMSO/H_2O and DMSO/EtOH, which had higher values, irrespective of the bioactive material used *($p<0.05$).* EtOH and DMSO significantly decreased the viability of the cells treated with TheraCal *($p<0.05$),* whereas DMSO/H_2O, DMSO/EtOH, and EtOH/H_2O had no significant effect *($p>0.05$).* EtOH and EtOH/H_2O significantly reduced the viability of the cells produced by MTA *($p<0.05$),* whereas DMSO/H_2O, DMSO/EtOH, and DMSO had no significant effect *($p>0.05$).* DMSO/H2O and DMSO/EtOH had no significant effects on the viability of the cells treated with S-PRG *($p>0.05$),* unlike EtOH, EtOH/H_2O, and DMSO, which significantly decreased cell viability *($p<0.05$).*

Discussion

Since the tested bioactive materials affected the trans-dentinal viability of pulp-derived cells, the first null hypotheses were rejected. In accordance with the ISO 7405 standards, S-PRG was noncytotoxic, whereas TheraCal and MTA were deemed severely and moderately cytotoxic, respectively. Cell activities and responses can be modulated by certain properties of biomaterials, including pH, ion release, surface topography, and stiffness. For example, pH can affect enzymatic activity and cellular metabolism, whereas ion release is crucial for providing signals necessary for cell differentiation and mineralization [21]. MTA and TheraCal are well known for their calcium release. Compared with TheraCal, MTA generally resulted in lower initial calcium release [22–24]. S-PRG is a surface-prereacted glass that does not contain leachable calcium ions [25]. The composition of the materials was a decisive factor, with TheraCal distinguished by its hydrophilic monomer/matrix, which exhibited greater calcium release over 28 days than MTA [26].

Silicate-based materials are well known for their alkalinity due to the release of calcium, hydroxyl, and other ions [27]. It has been suggested that the positive effect of MTA cements on the proliferation of human dental pulp cells is potentially enhanced by the continuous and constant release of calcium ions. Calcium ions react with carbonates in pulp tissue to form calcium carbonate; this process influences pulp cell proliferation and contributes to the progression of mineralization [28–31]. Despite

their similar pH profiles [26], resin-containing biomaterials such as TheraCal are generally more cytotoxic [5]. This might be due to the leaching of uncured hydrophilic monomers, causing cell death [32]. While uncured monomers are cytotoxic to pulp cells, their cured versions present milder effects [33]. The greater cytotoxicity of TheraCal corroborates the findings of previous eluate-based cell culture studies. Importantly, the effects experienced by cells differ depending on the type of ion, compound, and concentration. Relatively high concentrations of calcium ions could lead to extensive cell death or a reduction in initial proliferation [31,34,35]. The complex structure of the resin–dentin interface likely influences TheraCal's cytotoxicity because of different gradients of monomer diffusion and conversion rates in the presence of moisture [11,36]. Uncured methacrylate monomers can increase reactive oxygen species production and oxidative DNA damage [37], impacting cell metabolism and protein expression [38]. Compared with eluate-based cell culture studies, the trans-dentinal cytotoxicity setup used in this study allowed a more realistic assessment of the uncured-monomer effect on pulp cells [5]. Notably, less efficient light curing conditions might further reduce cell viability, suggesting potential increased cytotoxicity for TheraCal in clinical scenarios [39].

Although MTA was classified as moderately cytotoxic, no significant differences in cell viability between MTA and TheraCal were observed at 24 h. The prolonged hydration reaction of MTA results in the release of byproducts such as calcium hydroxide [40], altering cell processes. Even though the higher pH might be buffered by pulp fluid, the leaching of uncured monomers from TheraCal may accumulate over time, exhausting the detoxifying glutathione metabolism and other defense mechanisms of the cells [41]. Therefore, TheraCal's cytotoxicity, which can be attributed to both the high pH and the uncured monomers, surpasses MTA's moderately cytotoxic nature. Pulp cell viability also depends on exposure time [38]. When cytotoxic eluates are exposed directly to cells, peak reductions in cell viability occur at 7 days [38]. The likely slower diffusion of cytotoxic components did not result in lower cell viability for TheraCal at 24 h, as observed in eluate-based cell cultures [38,42]. In any case, TheraCal's cytotoxicity should not be underestimated, as clinical applications naturally characterize longer exposure times. In

contrast, S-PRG demonstrated noncytotoxic behavior, indicating promising applications for various dental applications [43] because of its ion release and exchange abilities [44,45].

DMSO concentrations were chosen on the basis of a series of studies [8,11,13] showing that high concentrations significantly improve interactions between resin-based materials and dentin and enhance wetting. Low concentrations of DMSO (0.008%) were previously shown to have no effect on pulp-cell cytotoxicity [15]. The safety of higher DMSO concentrations, however, remains uncertain. Since dentin pretreatment significantly affected transdentinal cell viability, the second null hypothesis was rejected. This study highlighted the moderate cytotoxicity of DMSO, while 50% v/v dilutions with water or ethanol resulted in noncytotoxic binary solutions. In contrast, pure ethanol and its dilution in water (50% v/v) were severely cytotoxic according to ISO 7405 [18]. The solvent type is a determining factor for pulp cell viability and should be considered carefully depending on the clinical application. This study provides evidence that aqueous or ethanolic dilutions of DMSO (up to 50% v/v) resulted in no measurable signs of trans-dentinal cytotoxicity against pulp-derived cells. Solvent cytotoxicity can be related to their interaction with membranes and the consequent effects of cell permeability and diffusion. Undiluted DMSO was moderately cytotoxic. The reduction in the cytotoxicity response after DMSO dilution may be explained by the impact of DMSO on cell membranes at concentration-dependent doses [46]. A lower concentration of DMSO resulted in less cell membrane damage [46] and consequently lower cytotoxicity. Notably, DMSO presented significantly lower trans-dentinal cytotoxicity than ethanol at comparable concentrations (50% v/v). Interestingly, DMSO had a protective effect against the high cytotoxicity of ethanol, potentially through its ability to form hydrogen bonds with both dentin and ethanol. This finding warrants further investigation into its underlying mechanisms. The rationale for combining bioactive materials and solvent pretreatments was to benefit from the ability of solvents to potentially improve material interactions with dentin. Although trans-dentinal cytotoxicity findings indicate that DMSO itself does not necessarily harm pulp tissue, the effects of combining DMSO and ethanol with bioactive materials are unknown.

Dentin pretreatment affects the transdentinal cytotoxicity of bioactive materials. Severely cytotoxic solutions (EtOH/H_2O and EtOH) significantly reduced the viability of MTA and S-PRG cells. However, EtOH/H_2O had no additional effect on the viability of TheraCal cells, while EtOH produced significantly lower values. Since TheraCal itself was severely cytotoxic, only pure ethanol (EtOH) was able to further compromise cell viability. Pretreatment with pure or diluted ethanol increased the transdentinal cytotoxicity of bioactive materials, which may be useful for clinical applications. In contrast, moderately cytotoxic DMSO or its noncytotoxic aqueous or ethanolic dilutions (50% v/v) had milder or no effects on the viability of the tested bioactive materials. DMSO is well established as a penetration enhancer that may have positive or negative effects on cell viability depending on the carried substances. Owing to the cytotoxicity of methacrylate-based monomers [37], the use of DMSO in deep cavities along with the use of resin-based materials has raised important concerns about its safety. The effect of DMSO pretreatment on the viability of TheraCal-treated cells was concentration dependent. While pure DMSO significantly reduced the viability of TheraCal cells, aqueous (DMSO/H_2O) or ethanolic (DMSO/EtOH) dilutions had no significant effects. Considering the considerably low thickness of the dentin barrier employed here (300 μm), it is possible to assume that diluted DMSO pretreatment (up to 50% v/v) would not necessarily increase the risk to pulp cells when resin-based materials are used in vivo. Nonetheless, future studies should aim to combine DMSO pretreatments with materials containing higher resin contents to exclude any possible concentration-dependent effects of methacrylate monomers on trans-dentinal cytotoxicity. In contrast, DMSO pretreatment did not affect the viability of MTA cells, which was certainly due to the absence of methacrylate monomers. Only pure DMSO decreased S-PRG cell viability, whereas diluted DMSO pretreatment had no effect. Higher DMSO concentrations may facilitate the diffusion of filler ions, such as Al^{3+}, increasing overall cytotoxicity. Notably, DMSO induces osteoblastic differentiation with similar effects on odontoblast-like cells [15]. Therefore, low doses that diffuse through dentin could increase the rate of secondary dentin formation rather than damage the dentin–pulp complex, making DMSO not only a safer option but also a more beneficial dentin

pretreatment than ethanol.

Conclusion

The choice between bioactive materials depends on the specific requirements of the dental procedure and the desired effects on the tissue response and bioactivity. Solvent selection must be carefully performed to avoid unnecessary increases in transdentinal cytotoxicity levels. While pure ethanol or its aqueous dilutions (50% v/v) should preferably not be applied in close proximity to the pulp, relatively high DMSO dilutions (50% v/v) had minimal to no effects on cell viability regardless of the bioactive material.

Figures and tables legends

Table 1. Composition of test materials and application procedures.

Table 2. Assessment of cell damage* (SV40 pulp-derived cells) after different solvent treatments were used in combination with bioactive materials.

Figure 1. Flowchart of the experimental design and scheme for the perfusion system used for the dentin barrier cytotoxicity test according to ISO 7405.

Figure 2. Box-plot diagram representing the distribution of trans-dentinal viability for pulp-derived cells produced by different bioactive materials used in combination with different solvent pretreatments (n = 12). The line inside each box plot represents the group median. Lines above the box plots represent no significant differences between groups (p>0.05). Statistical comparisons were performed via the Kruskal–Wallis test (α=0.05).

Author contributions statements

IS and AT testing methodology. IS and RS validation of the test data. TS and MM worked on Investigation and data curation, IS wrote the original manuscript. AT and MM worked on the review & Editing of the manuscript. RS, TS, and AT provided supervision of the work. All authors have read and agreed to the published version of the manuscript.

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Table 1. Composition of bioactive materials and application procedures

	Composition	Application procedures
Resin-modified calcium silicate-based cement (TheraCal LC, Bisco)	Portland cement type III (20–60%), poly(ethylene glycol) dimethacrylate (10–50%), bis-GMA (5–20%), and barium zirconate (1–10%)	Apply a 1 mm-thick layer over moist dentin and light cure for 20 s.
Mineral Trioxide Aggregate (MTA, Orbis)	Tricalcium silicate, dicalcium silicate, calcium carbonate filler, zirconium oxide, iron oxide	Mix 1 full spoon with 2 drops of the liquid. Apply a 1 mm thick layer over moist dentin. Cover the MTA layer with a sterile wet-cotton pellet.
Surface prereacted glass (S-PRG, Shofu Inc)	Powder: Fluoro-boro-aluminosilicate glass core, prereacted glass-ionomer phase, SiO_2. Ion release: Al^{3+}, BO_3^{3-}, Na^+, SiO_2^{3-}, Sr^{2+} and F^-. liquid: polyacrylic acid	Mix 2.8 g of powder with 1 g of liquid and apply a 1 mm-thick layer over moist dentin.

Table 1. Composition of test materials and application procedures.

Table 2. Assessment of cell damage* (SV40 pulp-derived cells) after different solvent treatments used in combination with bioactive materials.

Material	*Treatment*	*Cell damage*
-	DMSO/H_2O	Non cytotoxic
	DMSO	Moderately cytotoxic
	DMSO/EtOH	Non cytotoxic
	EtOH/H_2O	Severely cytotoxic
	EtOH	Severely cytotoxic
Theracal LC	No treatment	Severely cytotoxic
	DMSO/H_2O	Severely cytotoxic
	DMSO	Severely cytotoxic
	DMSO/EtOH	Severely cytotoxic
	EtOH/H_2O	Severely cytotoxic
	EtOH	Severely cytotoxic
MTA	No treatment	Moderately cytotoxic
	DMSO/H_2O	Moderately cytotoxic
	DMSO	Moderately cytotoxic
	DMSO/EtOH	Moderately cytotoxic
	EtOH/H_2O	Severely cytotoxic
	EtOH	Severely cytotoxic
S-PRG	No treatment	Non cytotoxic
	DMSO/H_2O	Non cytotoxic
	DMSO	Moderately cytotoxic
	DMSO/EtOH	Non cytotoxic
	EtOH/H_2O	Severely cytotoxic
	EtOH	Severely cytotoxic

*According to ISO 7405. *Abbreviations:* DMSO = dimethyl sulfoxide; EtOH = ethanol; MTA = mineral trioxide aggregate; S-PRG = prereacted glass ionomer fillers. DMSO/H_2O, DMSO/EtOH and EtOH/H_2O followed 50% (v/v) dilutions.

Table 2. Assessment of cell damage* (SV40 pulp-derived cells) after different solvent treatments used in combination with bioactive materials.

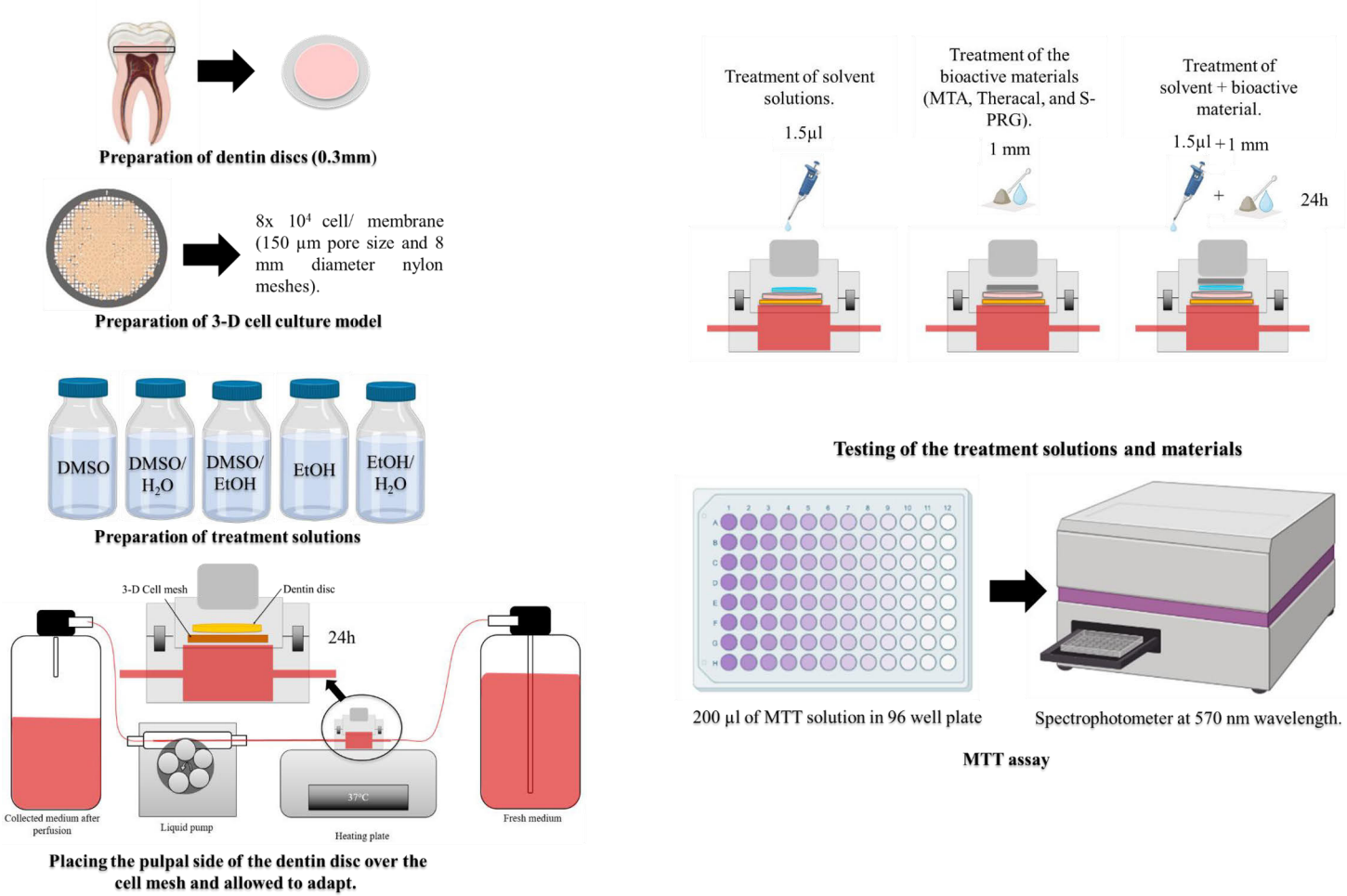

Figure 1. Flowchart of the experimental design and scheme for the perfusion system used for the dentin barrier cytotoxicity test according to ISO 7405.

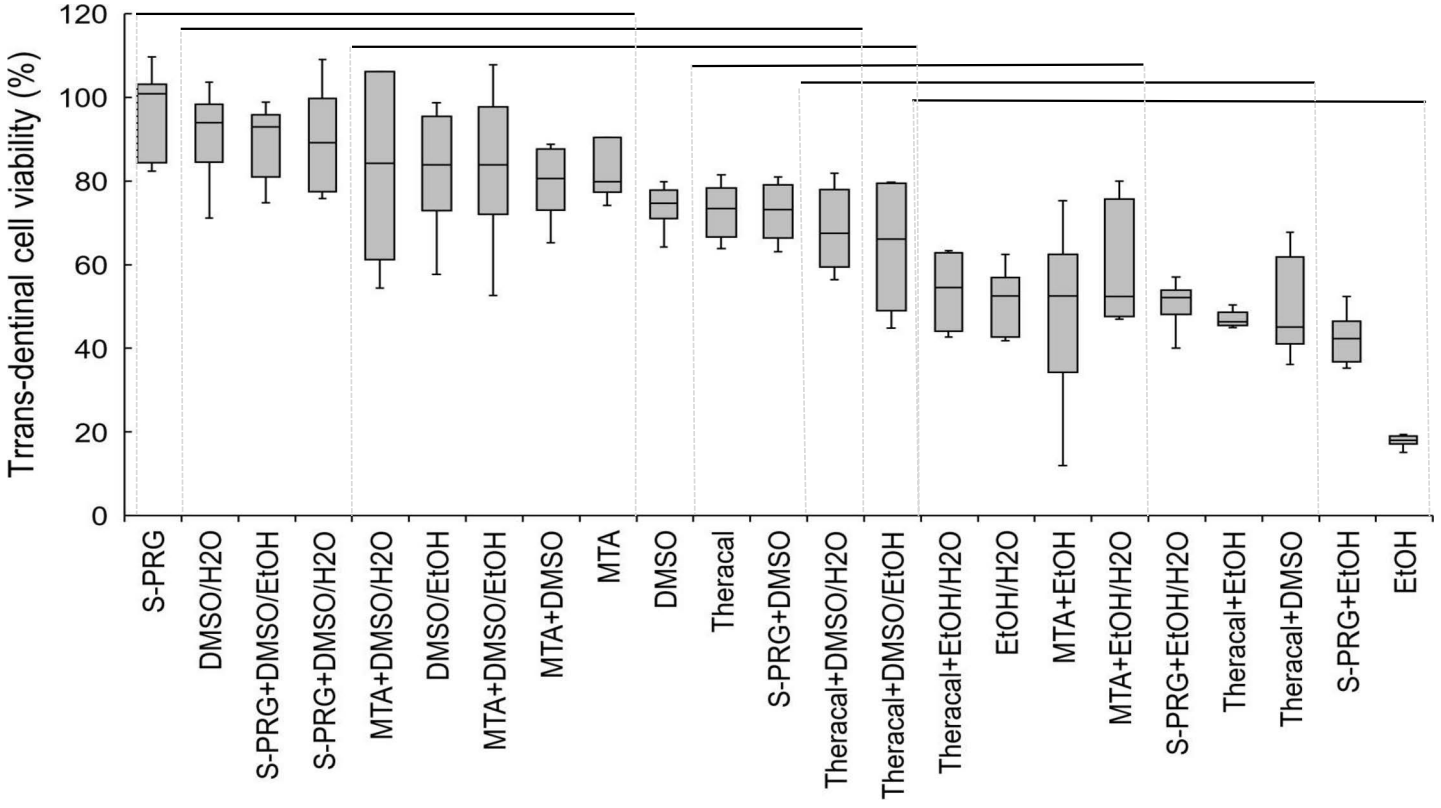

Figure 2. Box-plot diagram representing the distribution of trans-dentinal viability for pulp-derived cells produced by different bioactive materials used in combination with different solvent pretreatments (n=12). The line inside each box plot represents the group median. Lines above box plots represent no significant differences between groups (p>0.05). Statistical comparisons were performed via the Kruskal–Wallis test (α=0.05).

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Effect of solvent pretreatments on intraradicular bond strength of bioactive cements

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ABSTRACT

Bioactive materials are gaining interest in dentistry due to their ability to interact with dental tissues promoting healing and regeneration; albeit their ability to bond to root dentin remains unclear. This study investigated the effect of solvent-containing pretreatments on intraradicular bond strengths of bioactive cements. Sound premolars were decoronated, root canals were instrumented and irrigated using sodium hypochlorite and water. Roots were randomly divided into eighteen groups (n = 7/group) based on dentin pretreatments (no treatment, dimethyl sulfoxide (DMSO), ethanol (EtOH), EtOH/H_2O, DMSO/H_2O and DMSO/EtOH) and bioactive cements; a light-curable calcium silicate (Theracal LC, Bisco, Chicago, Illinois, USA), a mineral trioxide aggregate (Orbis MTA, P.L. Superior Dental Materials GmbH, Hamburg, Germany), and an experimental surface pre-reacted glass ionomer root canal material (S-PRG, Shofu Inc, Higashiyama-ku, Kyoto, Japan). Root canals were filled with bioactive cements and stored for 7 days at 37 °C. Filled roots were sectioned perpendicular to their longitudinal axis to obtain 1-mm thick slices. Intraradicular bond strengths were measured by the push-out bond strength test (0.5 mm/min). Analysis of failure mode was performed using a stereomicroscope. Characterization of bonded interfaces was performed via SEM. Bond strength data was analyzed by Kruskal Wallis test and ANOVA on ranks ($\alpha = 0.05$). Bond strengths varied according to bioactive cements, Theracal > MTA > S-PRG, and root thirds ($p < 0.05$). Aqueous (DMSO/H_2O) or ethanolic (DMSO/EtOH) dilutions of DMSO increased Theracal bond strengths to apical root segments ($p < 0.05$). While MTA bond strengths were not affected by pretreatments, undiluted ethanol reduced bond strengths of S-PRG to apical root segments ($p < 0.05$). Intraradicular bonding of bioactive cements may vary according to root canal depth and material composition. Solvent-based pretreatments containing DMSO might improve intraradicular bonding of resin-modified bioactive cements in a concentration-dependent manner.

1. Introduction

In endodontics, an ideal filling material must bond to dentin and seal the communication pathway of the root from its surrounding tissue [1,2]. In addition, the filling material should meet certain criteria including biocompatibility, leakage prevention, optimal adaptation to the tooth structure, and high bond strength against dislodging movement, especially in cases of perforation [3]. Moreover, the material should have the ability to chemically interact with the living tissues of the body which is known as the bioactivity, this ability results from the release of ion that led to the formation of a mineralized layer on he surface of the material [4]. Calcium-silicate materials offer excellent sealing properties. In addition, they can stimulate the formation of apatite-like precipitates when they react with phosphate-containing liquids. This reaction releases ions that promote the growth of dentin bridges [5]. Multiple calcium silicate-based materials, particularly mineral trioxide aggregate (MTA), have been

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developed with those goals in mind due to their biocompatibility [6], sealing ability [7], regenerative capabilities [8], and antibacterial activity [9]. However, MTA's handling difficulties [10,11], and prolonged setting time [12] pose practical challenges for clinical applications [13].

Resin-modified calcium silicate-based cements (RMCS) were introduced to address these drawbacks by combining calcium-silicate materials properties with enhanced handling through a resin-based matrix [14]. Apart from its easy handling and precise placement characteristics, RMCS has demonstrated effective sealing capability, reduced interfacial microleakage, lower solubility, and increased release of calcium ions compared to MTA and Dycal [15,16]. It also enables immediate placement of the final restorative material, eliminating the need for a waiting period required by other calcium-silicate cements. A new emerging bioactive material, surface pre-reacted glass ionomer fillers (S-PRG), are known for their ion-releasing properties [17]. S-PRG fillers are employed in various dental applications, including composite resins [18], fissure sealants [19], and tooth coating materials [20], due to their antibacterial activity [18], acid buffering capacity [18,21], and multiple ion releasing ability [21–23]. Although several studies evaluated the physical and mechanical properties of the S-PRG fillers, the bonding ability of the S-PRG cement to root dentin remains unclear.

Previous studies have shown that the use of aqueous DMSO solutions can improve both immediate and long-term bond strengths of resin-based materials to dentin [24–26]. DMSO preserves bond strengths [27,28] by improving resin penetration and monomer diffusion into the collagen matrix [29]. Ethanol is another solvent that was suggested to control dentin moisture and enhance bond strength [30]. Several previous studies evaluated the effect of solvent pretreatments containing DMSO, ethanol or their combinations on dentin bonding [27,31,32]. However, the effect of these pretreatments on bonding of bioactive materials to root dentin remains inconclusive. Getting a good adhesion of the materials in the root canal is difficult due to its complex structure. The dentin in the middle third of the root has a higher density of dentinal tubules with a greater diameter than that in the apical third [33], the bond strength measurement of bioactive cements is important to enable the quantification of the dentin/material interaction. Therefore, the aim of this study was to assess the effect of solvent-containing pretreatments (DMSO, ethanol, and their aqueous dilutions) on the push-out bond strength of different bioactive cements. The tested null hypotheses were that; (i) the different compositions of the bioactive cements or (ii) the type of root canal pretreatments (DMSO, ethanol, or their aqueous dilutions) before bioactive cement application does not significantly affect the intraradicular push-out bond strength or interfacial integrity.

2. Materials and methods

2.1. Teeth selection and root canal preparation

One hundred twenty-six sound premolars with completely formed apex, collected from anonymous donors, and exempt from ethical notification, were obtained following local regulations (Tissue Act, section 20) were collected. Teeth were stored in 0.9 % NaCl supplemented with 0.02 % sodium azide at 4 °C until use. Teeth with calcified canals, cracks or fractures, developmental defects, multiple canals, and root caries were excluded. All teeth were horizontally decoronated at the level of 0.5 mm radicular to the cemento-enamel (CE) junction using a 0.3-mm-thick, diamond-coated slow-speed band saw (Buehler Ltd., Lake Bluff, IL, USA) generating 16 mm (±1 mm) long roots. Apical patency was verified using an ISO size-10 K-file (Dentsply Maillefer, Ballaigues, Switzerland). The working length of each root was determined by visualizing the tip of a size 10 K file extending beyond the apical foramen and subtracting 1 mm from that length of the file. Canals were instrumented using rotary nickel-titanium instruments (Protaper SX, S1, S2, F1, F2, F3, F4; Dentsply Maillefer) using a low-speed rotary endodontic handpiece (X-smart; Dentsply Maillefer). The master apical instrument was the Protaper F4. Carbide fiber post preparation burs (3M ESPE RelyX, St. Paul, USA) up to size #3 (1.9 mm in diameter) were used at low speed to standardize the canal diameter. Irrigation was performed using 27-G Endo-Eze irrigator tips (Ultradent Products Inc, South Jordan, UT) alternating 2 mL 2.5 % sodium hypochlorite and Glyde File Prep (Dentsply Maillefer, Ballaigues, Switzerland) between the use of each instrument and at the end of instrumentation with 2 mL 17 % EDTA (Inter-Med Inc/Vista Dental Products, Racine, WI) for 1 min, followed by 2 mL normal saline. The root canals were dried with 2 paper points size F4 (Dentsply, York, PA, USA). Roots were then randomly divided into eighteen groups (n = 7/group) according to the bioactive cement used and the pretreatment.

2.2. Preparation of pretreatment solutions

A total of five dentin pretreatment solutions were prepared and stored at room temperature until use, containing dimethyl sulfoxide (DMSO, Dimethyl sulfoxide, Sigma-Aldrich, St. Louis, MO, USA) and/or ethanol (Ethanol 99.8 %, Sigma-Aldrich). The concentrations (v/v) of the solvents were as follows: pure ethanol (EtOH), pure dimethyl sulfoxide (DMSO), 50 % aqueous ethanol (EtOH/H2O), 50 % aqueous dimethyl sulfoxide (DMSO/H2O), and 50 % ethanolic dimethyl sulfoxide (DMSO/EtOH) [34].

2.3. Experimental design and root filling

The experimental design was composed of two study factors: (i) dentin pretreatment at six levels (no pretreatment; ethanol; dimethyl sulfoxide; 50 % aqueous ethanol; 50 % aqueous dimethyl sulfoxide; 50 % ethanolic dimethyl sulfoxide) and (ii) bioactive cements at three levels (light-curable resin-modified calcium silicate; mineral trioxide aggregate, and an experimental surface pre-reacted glass ionomer). A total of 18 experimental groups were obtained (n = 7 roots/group). The composition of test materials and application procedures are shown in Table 1, while a flowchart outlining the experimental design is presented in Fig. 1. A light-curable calcium silicate (Theracal LC, Bisco, Chicago, Illinois, USA), a mineral trioxide aggregate (Orbis MTA, P.L. Superior Dental Materials GmbH, Hamburg, Germany), and an experimental surface pre-reacted glass ionomer root canal material (S-PRG,

Table 1
Composition of calcium silicate biomaterials and application procedures.

	Composition	Application procedure
Resin-modified calcium silicate-based cement. Theracal L.C, Bisco Inc, Schamburg, IL, USA	Light cured resin-modified calcium silicate. Tricalcium silicate particles in a hydrophilic monomer.	The material comes in a premixed syringe, apply a 2 mm thick layer inside the canal and light cure for 20 s using a light curing unit (Elipar; 3M ESPE) at 1200 mW/cm^2
Mineral Trioxide Aggregate Orbis MTA, P.L. Superior Dental Materials GmbH, Hamburg, Germany	Di- and Tricalcium silicate and Tricalciumaluminate Portland Cement Clinker, and bismuth oxide	Mix 1 full spoon present in the kit with 2 drops of the liquid present in the kit. Increments of the material were placed inside the canals using amalgam carrier and a plugger, to ensure the whole canal's filling. A wet cotton plate was placed over the MTA.
Surface pre-reacted glass (S-PRG) Shofu Inc, Higashiyama-ku, Kyoto, Japan	Fluoro-boro- aluminosilicate core glass, pre-reacted glass-ionomer phase, SiO_2. Ion release: Al^{3+}, BO_3^{3-}, Na^+, SiO_2^{3-}, Sr^{2+} and F^-.	Mix 2.8 g powder with 1 g liquid, increments of the material were placed inside the canal using an amalgam carrier and a plugger to ensure the filling of the whole canal.

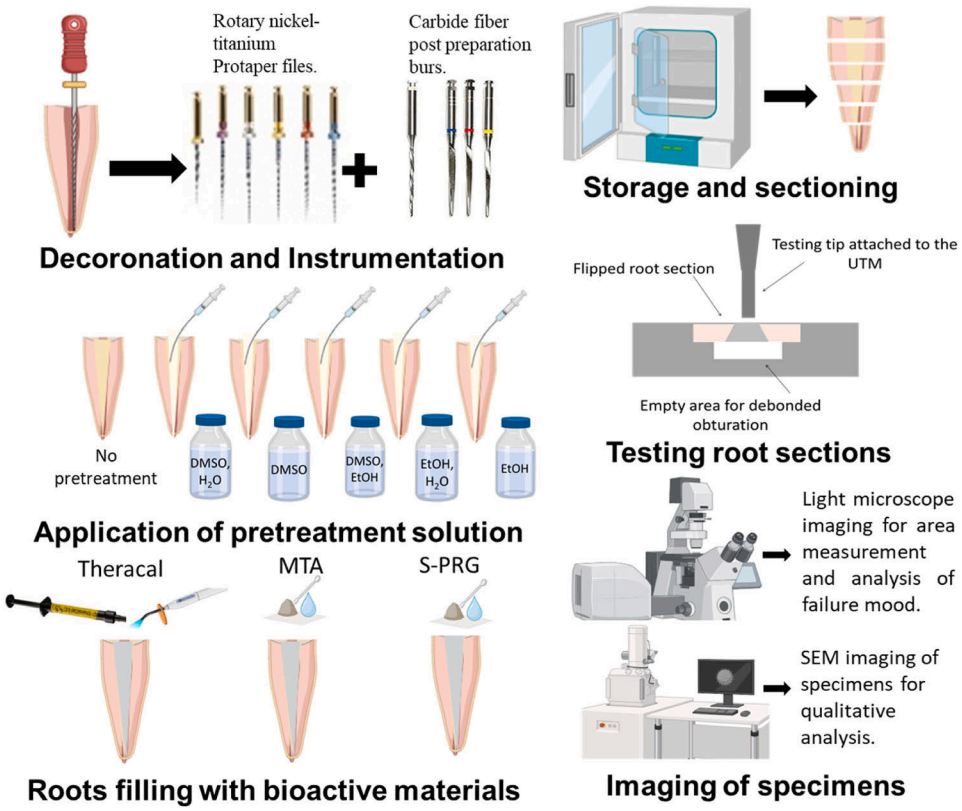

Fig. 1. Flowchart of the experimental design and scheme of testing used for push-out evaluation.

Shofu Inc, Higashiyama-ku, Kyoto, Japan) were used following manufacturer's instructions.

Root fillings were performed by a single operator. One ml of the tested pretreatment solution was applied inside the canal using a tuberculin syringe with a 30-G blunt-tip needle, carried to the working length, and gently injected into the root canal while slowly withdrawing the syringe. Flooding of the pretreatment solution was visually verified and left in the canal for 60 s. The excess pretreatment solutions were removed with paper points, followed by the placement of the filling bioactive material. MTA and S-PRG were mixed according to the manufacturers' instructions and manually plugged into the root canals in their respective groups using hand pluggers. Untreated roots served as control. Roots were kept at 100 % relative humidity and stored at 37 °C for 7 days to allow complete setting of MTA. Following the storage period, each root was sectioned horizontally perpendicular to the longitudinal axis using a low-speed diamond-coated saw (Isomet 2000, Buehler Ltd, Lake Bluff, IL, USA) under water cooling at three different levels (namely: coronal, middle, and apical) to obtain 6 slices of 1 ± 0.1 mm in thickness. The thickness of each root cylinder was measured to the nearest 0.01 mm using a digital caliper.

2.4. Push-out bond strength testing

The push-out technique was performed by applying a load using a universal testing machine (AGS-10, Shimadzu Corp., Kyoto, Japan), each specimen was subjected to a compressive loading apico-coronally at a crosshead speed of 1 mm/min until failure and the maximum load was recorded in newton (N). After taking images of the specimens using a stereomicroscope (Leica M165C, Leica Microsystems GmbH., Wetzlar, Germany) with 20x magnification. The smaller and larger root canal diameters were measured using digital image-analysis software (ImageJ; National Institute of Health, Bethesda, Maryland, USA). The adhesion surface area was calculated in the same way as measuring the area of a trapezium following the equation: adhesion surface area (mm^2) = ((D1 + D2)/2) πh where D1 and D2 are the largest and smallest canal diameter, respectively, π is the constant 3.14, and h is the thickness of the root slice [35,36]. To obtain the push-out bond strength in mega Pascal (MPa), the load (N) at failure was divided by the surface area (mm^2) of the root canal at the filling-dentin interface.

2.5. Analysis of failure modes

The failure modes were analyzed under a stereomicroscope at 40x magnification. Failure modes were categorized as follows: adhesive (failure at the cement-dentin or the sealer-core material interface), cohesive (failure within bioactive cement or dentin), or mixed (failure in both the cement and dentin) [37].

2.6. Characterization of bonded interfaces (SEM)

Qualitative analysis of the interface between dentin and bioactive cement was performed using SEM imaging. Representative discs from each group were embedded in epoxy resin and wet-polished with 600, 1200, 2000, and 4000-grit SiC paper, followed by 0.05 μm aluminum

oxide polishing paste (Buehler Ltd, USA). Beams were ultrasonically cleaned in distilled water for 10 min after each polishing step. Bonded interfaces were treated with 50 % H_3PO_4 for 5 s and 3 % NaOCl for 10 min, dried in silica overnight, mounted on stubs, and analyzed on backscattering mode at 15 kV (Phenom ProX, Phenom-World, USA [31, 34]. A series of sequential micrographs of the bonded interfaces (2000 × magnification) were obtained from each resin-dentin beam by an experienced blinded operator. Sequential micrographs (2000 magnification) were obtained from all groups to analyze the interface.

3. Statistical analysis

Push-out bond strength values for all tested groups were analyzed using IBM SPSS Statistics for Windows, version 26 (IBM Corp., Armonk, NY, USA). Both root segments and whole root data were evaluated and failed in normality and homoscedasticity testing ($p < 0.05$). Kruskal Wallis test and ANOVA on ranks for pair-wise comparisons were performed with statistical significance set at $\alpha = 0.05$.

4. Results

4.1. Push-out bond strength

The results of the push-out bond strengths are shown in Fig. 2. Material-wise, the control group where Theracal was used without the application of any pretreatment showed significantly higher bond strengths (29.1 MPa) compared to the control MTA (17.88 MPa) or the control S-PRG (7.22 MPa) bond strength value ($p < 0.05$). None of the pretreatments significantly affected the intraradicular bond strengths of the bioactive cements ($p > 0.05$).

4.2. Push-out bond strength at different root thirds

Push-out bond strengths of the tested materials at different root thirds (coronal, middle, and apical) are shown in Table 2. Intraradicular bond strengths of bioactive cements varied according to root third and dentin pretreatments ($p < 0.05$). For Theracal, intraradicular bond strengths were higher at the coronal third compared to the apical third in all groups except for the DMSO/H2O and DMSO/EtOH treated groups ($p < 0.05$). DMSO pretreatment produced lower intraradicular bond strengths of Theracal at the apical third compared to the apical third of the control group ($p < 0.05$). Dentin pretreatments had no significant effects on bond strengths of Theracal to coronal or middle root thirds ($p < 0.05$).

For MTA, no significant differences were seen between root thirds (coronal, middle, or apical) within dentin pretreatments, except for EtOH ($p < 0.05$). EtOH caused significant bond strength reductions at the apical third ($p < 0.05$). Dentin pretreatments produced comparable intraradicular bond strengths within root thirds ($p > 0.05$).

For S-PRG, intraradicular bond strengths were generally lower than Theracal and MTA ($p < 0.05$). Intraradicular bond strengths at apical thirds were significantly lower than coronal thirds, regardless of dentin pretreatments ($p < 0.05$). Dentin pretreatments had no effects within root thirds except for the DMSO-treated group which caused a reduction in the bond strength of the middle and apical third compared to control ($p < 0.05$).

4.3. Analysis of failure modes

Failure modes for all tested materials are shown in Fig. 3. Generally, in all tested groups, the least seen failure mode was the adhesive failure. Mixed failures were the most common failure mode for Theracal at around 50 % for all pretreatments except for the ethanol-treated group (EtOH), where cohesive failure was found in 95 % of specimens, with failure occurring within the Theracal. A different trend was seen in the MTA groups where the most commonly seen failure mode was mixed failure with more than 60 % for groups where ethanol was used at its different dilutions, when DMSO was used as a pretreatment, both adhesive and cohesive failure within the material were recorded at a similar percentage of around 48 %. The S-PRG groups consistently failed within the material, as indicated by the percentage of cohesive failures (more than 40 % with all pretreatments). The control group exhibited a

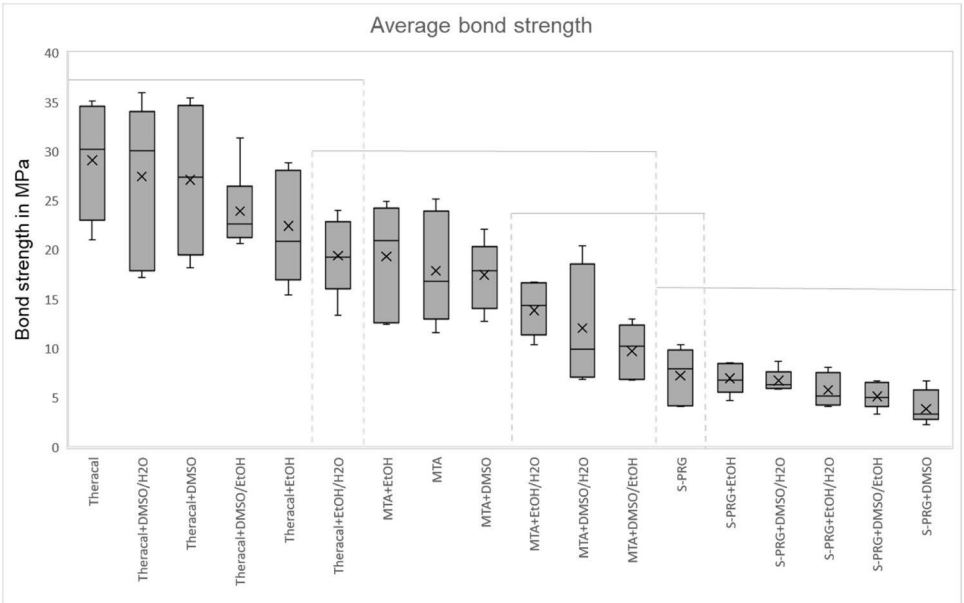

Fig. 2. Push-out bond strength value in mega Pascal for the different tested silicate-based materials.

Table 2
Means and standard deviations (±) of push-out bond strengths (MPa) at different root thirds.

	Theracal			MTA			S-PRG		
	Coronal	Middle	Apical	Coronal	Middle	Apical	Coronal	Middle	Apical
Control	31.46 ^{Aa} ±10.49	25.45 ^{Aab} ±9.22	20.52 ^{Ab} ±8.97	20.12 ^{Aa} ±11.9	21.88 ^{Aa} ±10.21	12.08 ^{Aa} ±4.81	8.10 ^{Aa} ±3.25	8.59 ^{Aa} ±3.22	4.18 ^{ABb} ±1.98
DMSO/H_2O	27.81 ^{Aa} ±10.32	28.38 ^{Aa} ±11.16	22.27 ^{ABa} ±6.7	12.16 ^{Aa} ±5.82	13.57 ^{Aa} ±7.13	11.61 ^{Aa} ±3.11	7.44 ^{Aa} ±2.01	6.66 ^{ABab} ±3.02	4.96 ^{Ab} ±2.24
DMSO	28.71 ^{Aa} ±8.52	25.54 ^{Aa} ±8.69	14.82 ^{Bb} ±5.12	17.78 ^{Aa} ±4.20	17.62 ^{Aa} ±5.31	17.39 ^{Aa} ±5.57	5.36 ^{Aa} ±3.55	4.33 ^{Bab} ±1.51	2.99 ^{Bb} ±1.07
DMSO/EtOH	25.75 ^{Aa} ±8.91	25.45 ^{Aa} ±12.81	22.14 ^{Aa} ±7.31	11.70 ^{Aa} ±6.81	15.78 ^{Aa} ±7.01	13.80 ^{Aa} ±6.45	7.11 ^{Aa} ±2.84	6.11 ^{Aa} ±1.05	3.82 ^{Ab} ±1.02
EtOH/H_2O	27.92 ^{Aa} ±15.06	26.80 ^{Aa} ±9.58	18.02 ^{ABb} ±6.02	13.73 ^{Aa} ±5.72	15.38 ^{Aa} ±5.20	11.61 ^{Aa} ±5.37	7.71^{Aa} ±3.34	6.28 ^{ABa} ±3.21	3.79 ^{Ab} ±0.92
EtOH	30.06 ^{Aa} ±9.29	26.08 ^{Aa} ±4.81	22.39 ^{Ab} ±4.41	18.55 ^{Aa} ±11.34	19.99 ^{Aa} ±9.30	12.39 ^{Ab} ±8.81	8.40 ^{Aa} ±4.31	8.91^{Aab} ±1.23	4.56 ^{Ab} ±1.81

Different capital letters represent significant differences between different pretreatments within the root third (column) for each silicate-based material according to ANOVA on ranks ($p < 0.05$). Different lowercase letters represent significant differences between root thirds within dentin pretreatments (row) for each silicate-based material according to ANOVA on ranks ($p < 0.05$).

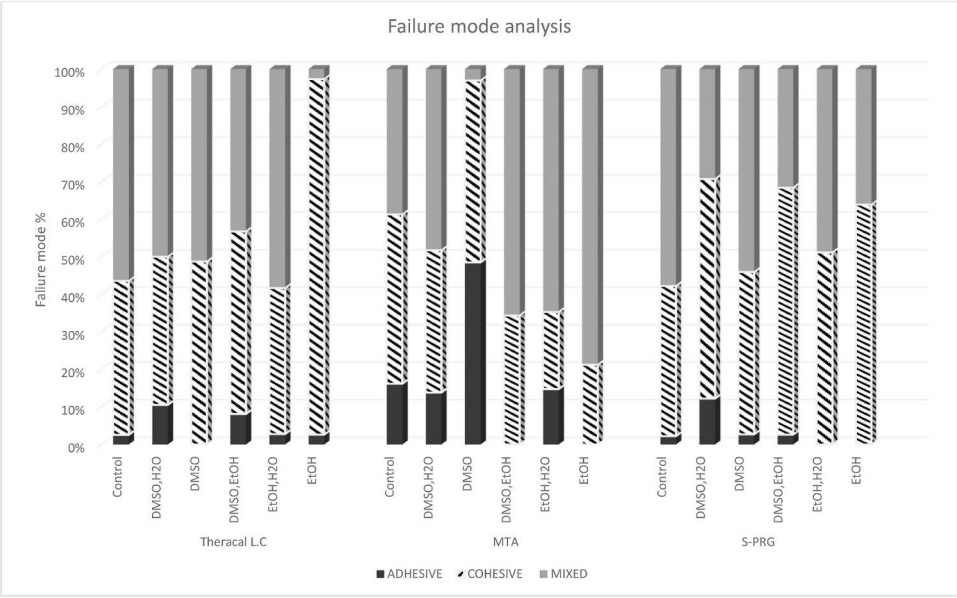

Fig. 3. Push-out fracture mode percentages in different groups.

combination of cohesive and mixed failures, with mixed failure occurring in more than 57 % of the specimens.

4.4. Characterization of bonded interfaces (SEM)

Representative scanning electron microscopic images of all tested materials and pretreatments are shown in Fig. 4. A clear penetration of the Theracal into the dentinal tubules was seen in the control group, DMSO diluted in water (DMSO/H2O), and DMSO treated group (DMSO). Smaller tags were seen at the bonded interface between the Theracal and dentin for ethanol-treated groups. MTA showed the deepest penetration in the group with ethanol and the control group where no pretreatment was applied. Small tags or accumulations of the material on the dentin-material margin were seen in all other treated groups. In the experimental S-PRG groups, little to no penetration was observed.

5. Discussion

Since the composition of bioactive cements affected intraradicular bond strengths, the first null hypothesis was rejected. The rationale for employing the push-out test was to verify whether variations of dentin morphology inside root canals (cervical, middle, or apical thirds) could affect the bonding performance of silicate-based materials. The push-out setup is a commonly used method to test intraradicular bond strengths [38]. It is the most reliable mechanical test to rank the dislodgement resistance of various endodontic materials applied to root dentin such as root canal sealers, root repair materials, and intraradicular posts [39]. There were significant differences in intraradicular bond strengths between the tested bioactive cements when root thirds were polled together: Theracal > MTA > S-PRG ($p < 0.05$). Theracal's higher bond strengths can be attributed to the presence of methacrylate-based hydrophilic monomers, which can improve their penetration into dentinal tubules, enhancing bond strengths [40]. In addition, previous

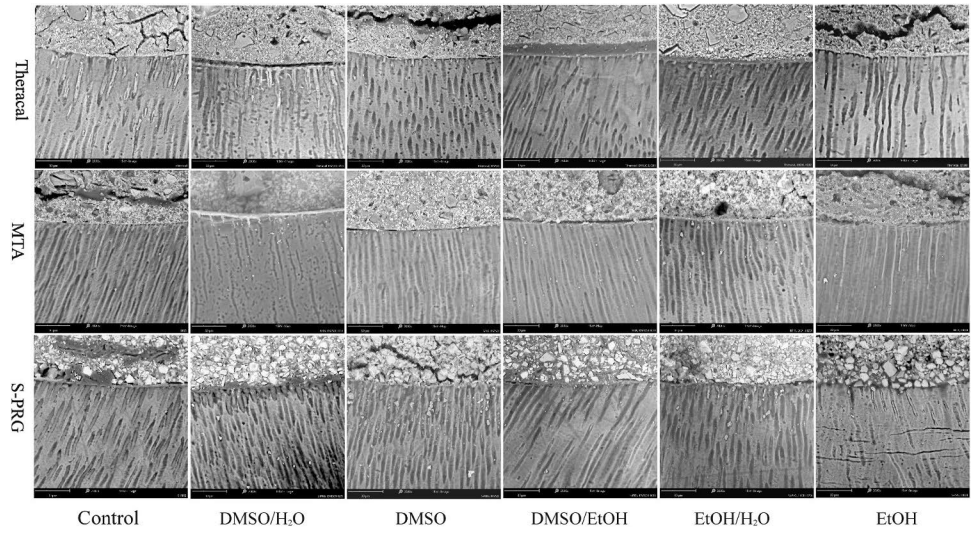

Fig. 4. Representative SEM images of the different tested materials and pretreatments.

reports [36] corroborate the obtained higher bond strengths of MTA compared to S-PRG. This may be related to the biomineralization abilities [41] and the relatively fast expansion of MTA [42]. The low bond strengths of S-PRG cement could be related to the short storage period (7 days) in this study. This period was chosen to comply with the most commonly used storage time to evaluate the bond strengths of silicate-based cements [35,43]. Additionally, ion release from S-PRG including fluoride, strontium, and silicon was suggested to incorporate deeper into root dentin only after 1–3 months of application [44]. Relatively longer storage times may contribute to better bonding outcomes for S-PRG [36,44].

Not only did bond strengths vary among bioactive cements, but the depth inside root canals also affected bonding efficiency. While Theracal and S-PRG presented significant reductions in bond strengths at the apical third, MTA was not affected by changes in dentin morphology inside root canals ($p < 0.05$). Theracal is a light-curable resin-based cement. The increased distance between the light source and the apical region leads to a lower conversion of methacrylate monomers, thereby compromising intraradicular bonding. This is in agreement with previous reports [45], which corroborate the lower bond strengths in the apical third compared to the coronal third for Theracal. In addition, the significant differences in dentinal tubule density between the coronal third and apical third [46,47], the orientation of the tubules [46], and the degree of tubular sclerosis [48] between apical and coronal thirds of the roots may be attributed to the significant change of the bond strength in the apical third. Surface pre-reacted glass ionomer fillers (S-PRG) were introduced as bioactive materials that retain their basic properties by forming a stable glass ionomer phase on the treated surface through the acid-base reaction between the fluoro-boro-alumino-silicate glass and polyacrylic acid [21]. Although the S-PRG formulation used in this study did not incorporate light-curable components, bond strengths were still lower at the apical region. This is not in agreement with a previous report showing no differences between root segments [36]. In the present study, S-PRG-tag formation in the apical third was not easily identified at bonded interfaces given the relatively large S-PRG filler sizes. The filler size easily exceeds the cross-sectional area of dentin tubules at the apical region. Additionally, the paste-like consistency of the tested experimental S-PRG cement may have prevented optimum tag formation. Since dentinal tubule density and size decrease at the apical third, S-PRG retention was likely compromised resulting in lower bond strengths. Differently, MTA showed no changes in intraradicular bond strengths among root segments. This is in agreement with previous studies [35,36]. MTA-based materials bond by the deposition of hydroxyapatite which causes the formation of a mineral infiltration zone with a tag-like structure at the interface with the dentin [49,50]. The tested MTA was a resin-free version, not relying on light curing for setting, but rather depending only on the slow hydration reaction of the hydrophilic MTA particles [51]. MTA-tag formation at the apical third was easily identified at bonded MTA-dentin interfaces, showing MTA's capacity to penetrate dentinal tubules.

The reason for combining bioactive cements and solvent pretreatments was to benefit from the solvents' ability to improve material-dentin interactions. Solvents, such as DMSO, can increase dentin wettability [34], which is a primary requirement for good adhesion. Recent studies have shown that even low concentrations of DMSO can improve immediate and long-term bond strengths of resin-based materials to coronal dentin [24–26]. DMSO concentrations were chosen based on previous studies [24,29,52] showing that the use of relatively higher DMSO concentrations (50 % v/v) substantially improves interactions between resin-based materials and dentin. Since the effect of solvents used as dentin pretreatments on intraradicular bond strengths of bioactive cements depended not only on cement composition but also on the solvent type and dilution, the second null hypothesis was rejected. The most promising effects were obtained for Theracal combined with diluted DMSO pretreatments. DMSO/H2O and DMSO/EtOH were able to increase the bond strengths of Theracal on the apical third, producing comparable bond strengths to coronal or middle thirds. DMSO can increase monomer conversion at resin-dentin interfaces [52] and lower the termination rates in free radical polymerization of methacrylates [53], likely improving polymer formation of Theracal on such challenging root segments [46]. Interestingly, bond strength improvements for Theracal were only observed for the apical third even though pretreatments were similarly applied in all root segments. Future studies must evaluate the sealing ability of pretreated dentin bonded with bioactive cements to investigate potential improvements that were not reflected as higher bond strengths. It is important to note that undiluted DMSO reduced bond strengths at the apical third for Theracal. Undiluted DMSO has a low vapor pressure. Unlike ethanol, it does not evaporate easily. Even after removal with paper points, undiluted DMSO may remain pooled and the apical third acts as a strong solvent. As a result, the components of Theracal may be diluted,

which can comprise the formation of polymer chains and bond strengths.

A different trend was observed for MTA, pretreatments had no effects on bond strengths, except for undiluted ethanol (EtOH). Lower bond strengths at the apical third were obtained after pretreating dentin with undiluted ethanol. Ethanol is highly miscible in water and replaces residual water molecules inside dentin tubules. This drastically reduces the intrinsic wetness of root canals [54]. Since MTA relies on proper hydration for adequate setting [55,56], ethanol dehydration resulted in lower bond strengths at the apical third. Higher dentinal tubule density at the coronal and middle thirds certainly contributed to lower dehydration levels due to the overall higher water content inside tubules. Higher moisture levels certainly prevented bond strength reductions. Differently, diluted ethanol had no negative effects on the bond strengths of MTA at the apical third due to the presence of water in the pretreatment solution. Since DMSO does not have the ability to dehydrate dentin, but rather acts by displacing water molecules, no negative effects were observed for MTA despite root segments.

Intraradicular bond strengths of S-PRG were not affected by any of the dentin pretreatments. None of the pretreatments were able to revert reductions in bond strength at the apical third. The bond strength of S-PRG at the apical third for treated and untreated were comparable. Further studies of the push-out bond strength at longer incubation times are needed to evaluate the long-term bonding ability of bioactive cements to root dentin.

6. Conclusion

Intraradicular bond strengths of the different bioactive cements varied depending on their composition and the root canal depth. Solvent-based pretreatments containing DMSO might improve intraradicular bonding of resin-modified bioactive cements in a concentration-dependent manner. However, different pretreatment methods should be applied with extreme caution, especially when combined with hydraulic bioactive cements.

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CRediT authorship contribution statement

Ikram Aqel Salim: Writing – original draft, Resources, Methodology, Investigation. **Anas Aaqel Salim:** Writing – original draft, Methodology, Investigation. **Thiago Henrique Scarabello Stape:** Writing – review & editing, Validation, Methodology, Formal analysis. **Mustafa Murat Mutluay:** Supervision, Formal analysis, Data curation. **Arzu Tezvergil-Mutluay:** Writing – review & editing, Supervision, Resources, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Ikram Aqel Salim I.A, Capitanio M, Seseogullari-Dirihan R, Mutluay M.M & Tezvergil-Mutluay A.
The direct inhibitory effect of various bioactive materials on dentin matrix and dentin protease activity.
Manuscript

IV

-Bioactive materials impact on dentin matrix collagen integrity

-Understanding the Effects of Bioactive Materials on Collagen Integrity and Enzymatic Activity

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Abstract

Silicate-based bioactive materials are used in various endodontic applications. These materials are alkaline and might adversely affect dentin collagen matrix integrity over time. This study investigates the prolonged contact effects of these materials on dentin collagen matrix integrity. Dentin beams (0.3 × 3 × 7 mm) were partially demineralized and distributed into five groups following baseline dry mass and total MMP (matrix metalloproteinase) activity assessments. Beams were placed in direct contact with fresh materials, including: a pre-mixed mineral trioxide aggregate (Bio-C), an experimental root canal material (S-PRG), a traditional mineral trioxide aggregate (Orbis MTA), and a resin-modified calcium silicate (TheraCal L.C), for 15 minutes and then stored as assemblies with material blocks in artificial saliva for 24 hours, 1 week, 1 month, 2 months, and 6 months. Untreated beams served as controls. At each time point, incubation media were analyzed for MMP and cathepsin-K-mediated degradation fragments. The mass loss, pH, total extractable protein, and hydroxyproline were also assessed, along with the modulus of elasticity. The calcium leaching profile was measured for each material up to 28 days. Data were analyzed using Kruskal-Wallis one-way analysis of variance on ranks and ANOVA. The results indicated that both the material and aging time significantly affected the integrity of the dentin beams. Bio-C and Orbis appeared to cause a faster degradation of the collagen matrix. Prolonged contact with the calcium-silicate-based bioactive materials might compromise the integrity of the collagen matrix; caution is advised when applying these materials to thin dentinal walls.

Keywords: dentin matrix, collagen degradation, cysteine cathepsins, matrix metalloproteinases, bioactive materials.

1. Introduction

Calcium silicate-based materials are widely used in endodontics due to their biocompatibility [1,2], bioactivity, and regenerative potential [3,4], and antimicrobial properties [5–7]. Mineral trioxide aggregate (MTA) is the first bioactive calcium silicate-based cement (CSC) originally formulated as a root-end filling material [8]. Its hydraulic setting properties, good sealing ability, and regenerative capabilities extend its use for broader clinical applications such as pulp capping, pulpotomy, endodontic repairs, or root fillings. However, shortcomings in handling properties [9,10], extended setting time [11,12], and discoloration [2,13,14] led to development of new materials that could overcome some of these shortcomings. Since then, several calcium silicate-based materials such as MTA Angelus (Londrina, PR, Brazil), Endosequence BC Sealer (Brasseler, Savannah, GA), Biodentine (Septodont, Saint Maur des Fosses, France), and TheraCal L.C (Bisco Inc., Schaumburg, IL, USA) have been introduced to the market. Among those, TheraCal is a resin-modified calcium silicate-based cement (RMCS) that combines the desirable properties of the silicate cement and the handling properties of the resins [15,16]. These calcium silicate-based cements are well known to stimulate the formation of apatite-like crystals and secondary dentin due to the calcium release and high alkalinity (pH 10-11) [15,17]. On the other hand, some other bioactive materials are based on ion release and recharge abilities, such as surface pre-reacted glass ionomer (S-PRG) fillers. S-PRG consists of glass-ionomer phase around the glass core that is proven to release several ions, including aluminum ions (Al_3^+), boric acid ions (BO_3^{3-}), sodium ions (Na^+), silicate ions (SiO_2^{3-}), strontium ions (Sr^{2+}), and fluoride ions (F^-) [18,19]. Some of the ions released from the S-PRG fillers can partially inhibit the endogenous enzymatic activity, specifically targeting MMPs in demineralized dentin [20]. Furthermore, some studies suggested that the ions released from S-PRG exhibit antibacterial effects, albeit with limited impact on bacterial adhesion and biofilm formation [21–23].

These bioactive materials are used in applications that require the materials to be in contact with dentin tissue as a permanent material. Previous studies reported a severe reduction in dentin

fracture resistance after prolonged applications [1,24]. Additionally, some earlier reports showed the activation of host-derived dentin enzymes and initiation of collagenolytic and gelatinolytic activities in dentin following the use of acidic or alkaline root sealers or obturation materials. Matrix metalloproteinases (MMPs) and cysteine cathepsins (CCs) are key enzymes responsible for proteolytic activities in demineralized dentin matrices [25]. Therefore, the present study investigated the effect of prolonged contact with bioactive cements on dentin matrix integrity, mechanical properties, and dentin protease activity over time. The null-tested hypothesis was that bioactive silicate materials would neither inactivate dentin matrix-bound MMPs, or cathepsin K, nor induce changes in demineralized dentin matrices over time.

2. Materials and Methods

2.1 Dentin specimen preparation

Fifty extracted caries-free human molars were stored at 4 °C in 0.9 % NaCl containing 0.02 % sodium azide (NaN_3) to prevent microbial growth and utilized within one month after extraction. The extracted teeth, obtained from anonymous donors, were exempt from ethical notification according to Finnish law (Tissue Act, section 20). Enamel and superficial dentin were removed by horizontal sectioning 1 mm below the deepest central fissure, and 50 dentin beams (0.3 × 3 × 7 mm) were prepared from mid-coronal dentin using a low-speed saw (Isomet, Buehler, Lake Bluff, IL, USA) under water cooling **(Figure 1, A&B)**. Dentin beams were demineralized with 0.5 M ethylenediaminetetraacetic acid (EDTA) and rinsed with distilled water, both for 2 hours at 4 °C under constant stirring. Ten beams were assigned to each of the five groups (n=10/group) in which the mean dry mass of each group was statistically similar.

2.2 Preparation of bioactive materials blocks

Four bioactive materials were utilized in this study **(Table 1)**, including a pre-mixed mineral trioxide aggregate (Bio-C Repair, Angelus Indústria de Produtos Odontológicos S/A, Londrina, Brazil; Bio-C), an experimental surface pre-reacted glass-ionomer filler (S-PRG, Shofu Inc, Higashiyama-ku,

Kyoto, Japan; S-PRG), a traditional mineral trioxide aggregate (Orbis MTA, Superior Dental Materials GmbH, Hamburg, Germany; Orbis), and a light-curable methacrylate-based silicate (TheraCal LC, Bisco, Chicago, Illinois, USA; TheraCal). Silicone molds were used to create ten blocks (1 × 3 × 7 mm) for each material **(Figure 1C)**. Orbis and S-PRG were mixed following the manufacturer's instructions. Bio-C and TheraCal were suspended in the mold from the premixed syringe. Bio-C, Orbis, and S-PRG were maintained in a humidified chamber for 2 hours until set, while TheraCal was placed without moisture and light-cured for 20 seconds using a light-curing unit (Elipar; 3M ESPE) at 1200 mW/cm^2. For immediate application, dentin beams were initially positioned in the mold, and the materials were applied on the occlusal surface of the dentin beams for 15 min immediately after preparation. Subsequently, they were washed in a sequence of spray, ultrasound, and distilled water under constant stirring for 1 hour and 45 minutes.

2.3 Aging of dentin-material assemblies

The occlusal surface of each dentin beam was positioned in contact with the corresponding material block to simulate a clinical scenario in which coronal dentin was treated with the same materials **(Figure 1D)**. Each beam and material were fastened together using a parafilm tie and then aged within screw cap propylene tubes containing 0.5 ml of calcium and zinc-containing media (CM) at 37 °C for 24 hours, 1 week, 1 month, 2 months, and 6 months in a shaking-water bath (60 cycles/minute). The CM contained 5 mM HEPES, 2.5 mM $CaCl_2 \cdot H_2O$, 0.02 mM $ZnCl_2$, and 0.3 mM NaN_3 (pH 7.2). The control groups consisted of demineralized dentin beams without contact with materials, aged as described previously.

2.4 Loss of dry mass

The dry mass loss of the beams after each incubation time point was considered an indirect indicator of demineralized dentin matrix degradation [26]. Following the generic MMP activity tests, the incubated beams were rinsed in distilled water (at 4 °C for 8 hours) and then dried in a desiccator containing dry silica beads for 72 hours. The dry mass of the samples was measured to the nearest 0.01

mg on an analytical microbalance (XP6 Microbalance, Mettler Toledo, Hightstown, NJ, USA), and the percentage of dry mass changes was calculated based on the respective baseline dry mass of each beam. Subsequently, the beams were rehydrated in distilled water at 4 °C for 1 hour, transferred to the corresponding fresh CM, and incubated until the next time point.

2.5 Generic MMP assay

The demineralized dentin beams served as the MMP source for the generic colorimetric MMP assay (Sensolyte Generic MMP assay; Anaspec, San Jose, CA, USA) to assess the catalytic activity of dentin matrix-bound MMPs. The intrinsic total MMP activity of each specimen was determined by incubating the beams in 200 µL of chromogenic thiopeptide substrate and assay buffer in a 96-well plate for 60 minutes at 25 °C. After 60 minutes, 150 µL of the medium was removed from the wells, and the baseline MMP activity of each specimen was measured at 412 nm (Synergy HT; BioTek Instruments, Winooski, VT, USA) and repeated every 15 minutes until peak values were individually obtained. The same protocol was performed after contact with fresh materials for 15 minutes and after each incubation period.

2.6 pH assessment

The pH measurements of each CM were conducted immediately after collecting the specimens for dry mass. The pH values were determined using a previously calibrated electrode pH meter (PHM210; Radiometer Analytical SAS, France) with an accuracy of ± 0.01. Following each measurement, the electrode was rinsed with distilled water to prevent subsequent contamination. The mean pH of the groups was calculated for each incubation period. The CM was stored frozen (-80 °C) until the end of the experiment, when the media were analyzed for ICTP, CTX, hydroxyproline, and micro-BCA.

2.7 Solubilized telopeptides of collagen

2.7.1 ICTP assay

Enzyme-linked immunosorbent assays (ELISAs) were used to measure the enzyme-specific degradation product of collagen molecule C-terminal telopeptide fragments in the incubation media. The specific marker for MMP-dependent degradation of type I collagen is the crosslinked carboxy-terminal telopeptide (ICTP); these telopeptide markers have been used to identify the enzymes responsible for type I collagen degradation [27,28]. At the end of each incubation period, 100 µL of incubation media was collected from each vial and analyzed using human serum ICTP assay kits (ICTP RIA, Orion Diagnostica, Espoo, Finland). The amount of ICTP in the CM was calculated using a 5-point fitting curve with known concentrations standard from 1 to 50 ng/mL, with a limit of 0.6 ng/mL, determined by a gamma counter (1470 WIZARD; PerkinElmer, Wallac Oy, Finland). Each measurement was performed in duplicate (n=10/group).

2.7.2 CTX assay

It is well known that cathepsin K is the only known source of CTX fragments from the C-terminal peptides of collagen. Additionally, the incubation medium of dentin beams can be utilized as an indirect indicator for CC-dependent degradation of the dentin matrix [29,30]. The CM of each beam was analyzed using human serum CTX assay kits (Serum CrossLaps ELISA, Immunodiagnostic System, Farmington, UK) with a plate reader (Synergy HT, BioTek Instruments, Winooski, VT, USA). Ten to fifty µL of the incubation medium was used for the CTX ELISA assay. The amount of CTX release was calculated using a standard curve with known concentrations provided in the kits. The assays were performed in duplicates.

2.8 Collagen solubilization quantitation (HYP)

Collagen solubilization was assessed by hydroxyproline (HYP) quantification. Solubilized collagen peptide fragments were assessed following a previously described HYP quantification protocol [31]. Briefly, aliquots of HYP standards (2–20 µg) prepared from stock solutions, and 25 µl

of re-suspended solutions were mixed with 25 µl of 4 N NaOH (2 N final concentration) in a total volume of 50 µl in 2 mL Nalgene O-ring tubes. The samples were hydrolyzed by autoclaving at 120 °C for 20 minutes. Then, 450 µl of chloramine-T was added to the hydrolyzed tubes and mixed gently to allow oxidation for 20 minutes at room temperature. Subsequently, 500 µL of Ehrlich's aldehyde reagent was added to each specimen for chromophore formation by incubating the specimens at 65 °C for 20 minutes. Absorbance values were obtained in a spectrophotometer (Model UV-A180, Shimadzu, Tokyo, Japan) at 550 nm and plotted against the standard HYP curves to determine the HYP release (ng/mg of dry dentin).

2.9 Quantitation of total extractable protein (Micro BCA)

Total protein detection and quantitation of the extracts were determined using a Micro BCA Protein Assay Kit (Thermo Scientific, IL, USA). In brief, 150 µl of each standard and sample CM were pipetted into a microplate well, and another 150 µl of working reagent was mixed on a plate shaker for 30 seconds. The plate was covered and incubated at 37 °C for 2 hours. After cooling at room temperature, the absorbance was measured at 562 nm on a plate reader (Synergy HT, BioTek Instruments, Winooski, VT, USA), and a standard curve was used to determine the protein concentration of each sample.

2.10 Modulus of elasticity (E)

Each demineralized dentin beam was placed on a miniature three-point flexure device with a 5 mm span length between the supports. Using a 5 N load cell (SMT1-5N, Interface, Scottsdale, AZ, USA) mounted on a universal testing machine (Autograph AGS-X, Shimadzu, Japan), the beams were loaded to a 3 % strain at a displacement rate of 0.5 mm min^{-1} while immersed in distilled water in a way that the surface of each beam that was in contact with the material was the surface that received the tensile force. After maximum displacement, the load was immediately returned to 0% stress within 15 seconds without further holding to prevent creep of the demineralized collagen matrix. Load-displacement curves were converted to stress-strain curves and the apparent modulus of elasticity (E)

in MPa was calculated using the following formula: $E = mL^3/4bh^3$ where m is the slope of the linear portion of the load-displacement curve (N/mm), L is the span length (5 mm), b is the width of the specimen and h is the beam thickness [32]. Specimens were tested in duplicates for each beam initially, after fresh application, and after each incubation time point. The averages were used as the final E values.

2.11 Assessment of calcium leaching

Assessment of calcium ion leaching was performed using an atomic absorption spectrophotometer (AA6800; Shimadzu, Tokyo, Japan) equipped with a calcium-specific hollow cathode lamp. Ion chromatography on leachates recovered from newly prepared cylindrical specimens of 10 mm diameter and 2 mm thickness incubated in 5 ml distilled water for 3, 7, 14, and 28 days. A blank solution was also tested, and the amount of calcium released from the tested materials was reduced.

3. Statistical analysis

Kruskal-Wallis one-way analysis of variance on ranks was used to verify whether differences existed between groups at different time points. In case of significant differences, pairwise multiple comparisons were conducted using Dunn's method. These analyses were carried out after validating the normality and equality of variance with Shapiro-Wilk and Brown-Forsythe's tests, respectively. All statistical analyses were performed with Sigma plot (version 14.0; Systat Software, Inc.), and significance was pre-set at $\alpha=0.05$.

4. Results

4.1 Loss of dry mass

The changes in dry mass over time are presented in **Figure 2.** Both the incubation time and different materials showed a significant effect on mass loss *($p<0.05$).* The control group showed a steady increase in mass loss, showing 2.9 % after 24 h, which reached up to 4.8 % after 6 months of incubation. On the other hand, the Bio-C group exhibited a 3 % mass loss after 24 h, which rapidly

increased to 35 % after one month and reached 63 % after 6 months of incubation. Similarly, Orbis showed a 1.8 % mass loss after 24 h, which increased to 6.6 % after one month and 28.9% after 6 months. Both S-PRG and TheraCal showed steadily low dry mass loss, 1.7 % for S-PRG after 24 h, which increased up to 2.23 % while TheraCal ranged between 1.4 % in 24 h to 1.6 % after 6 months.

4.2 Generic MMP assay

Except for the control group, which maintained consistent MMP activity throughout the entire experiment (p=0.98), the other groups exhibited different behaviors **(Figure 3).** Overall, all the materials consistently inhibit MMP activity compared to the respective time points in the control group, except for Bio-C, which showed 120.5 % activation at 6 months. The minimal MMP activity for the Bio-C and TheraCal groups occurred at one month of incubation and was statistically different from their highest readings and the control *($p<0.05$).* The minimal MMP activity for S-PRG and Orbis occurred at 24 hours and 2 months, respectively, and it was also statistically different from their initial readings *($p<0.05$).* Furthermore, when compared to the control activity level, all of the experimental groups showed the highest MMP inhibition at one month (Bio-C 84 %; Orbis 88.6 %; TheraCal 90.1 %; S-PRG 85.7 %), and only S-PRG did not show a statistical difference from the control group at one month *($p>0.05$).*

4.3 pH assessment

Results are shown in **Table 2**. The treatments increased the average pH from 2 % to 68 % compared to the control group (pH 7.32). Bio-C had the highest pH average after all storage periods (pH 12.31). It was statistically similar to Orbis (pH 11.63) *($p>0.05$),* but higher than TheraCal (pH 10.93), S-PRG (pH 7.47), and control *($p<0.05$).*

4.4 Solubilized telopeptides of collagen

The release of ICTP and CTX fragments was used as an indirect measurement of enzyme-specific degradation of collagen matrix and was calculated based on assays' instructions.

4.4.1 ICTP assay

The mean ICTP release (±SD) from the demineralized dentin beams to the incubation medium is shown in **Figure 4.** The highest overall cumulative ICTP release occurred in the TheraCal (7471.8 pg/mg dry dentin) and control (6920.9 pg/mg dry dentin) groups, while the smallest release was seen in the Orbis (1389.5 pg ICTP/mg dry dentin) and the S-PRG groups (1478.2 pg ICTP/mg dry dentin). In the control group, the highest release of ICTP occurred at 2 months of incubation and was significantly higher than the 6 months *($p<0.05$).* Differently, Bio-C had the highest ICTP fragments release at 24 hours and significantly decreased to less than 95 pg ICTP/mg dentin dry weight at the one-month reading *($p<0.05$).* A similar trend was observed in the S-PRG, Orbis, and TheraCal groups, where the highest ICTP release occurred at 24 hours and reached the minimum at 6 months *($p<0.05$).* Moreover, there was a significant difference between TheraCal (2538.2 pg ICTP/mg dry dentin) and Orbis (427.5 pg ICTP/mg dry dentin) at 24 hours *($p<0.007$).* At 1 week, TheraCal (1843.3 pg ICTP/mg dry dentin) also released significantly more ICTP than S-PRG (382.1 pg ICTP/mg dry dentin) and Orbis (307.1 pg ICTP/mg dry dentin) *($p<0.05$).* At one month, both Control (2611.1 pg ICTP/mg dry dentin) and TheraCal (1282.1 pg ICTP/mg dry dentin) groups showed a significantly higher release compared to the Bio-C group (93.5 pg ICTP/mg dry dentin) *($p<0.05$).* The highest ICTP release at 6 months was seen in the Bio-C group (552.8 pg ICTP/mg dry dentin), which was significantly different from the Control and TheraCal groups, both with zero ICTP release *($p<0.05$).*

4.4.2 CTX assay

The release of CTX peptide (±SD) is shown in **Figure 5**. The highest cumulative CTX release was in the control group (122.4 pg CTX/mg dry dentin), followed by TheraCal (86.2 pg CTX/mg dry dentin). While in the control group, the CTX release progressively increased over the first month of incubation, the other groups showed a different behavior, with CTX decreasing after 24 hours of incubation. The amount of the released CTX gradually increased, reaching the highest at one-month incubation for the control group (57.1 pg CTX/mg dry dentin), then significantly decreased at 2 months

(p=0.02). After the 24-hour readings, Bio-C, Orbis, and S-PRG groups showed a decrease in CTX release until they reached the minimum at 1 month, 2 months, and 6 months of incubation, respectively.

4.5 Collagen solubilization quantitation (HYP)

The mean (±SD) HYP levels measured in the CM are presented in **Figure 6**. In general, Bio-C exhibited the highest cumulative HYP release (23.3 µg HYP/mg dry dentin), while the least HYP release was observed in the control group (2.5 µg HYP/mg dry dentin). Except for TheraCal, all the experimental groups followed a similar trend in which the HYP gradually increased to reach the highest level (between 1.3 and 7.6 µg HYP/mg dry dentin) at one month of incubation, and then it decreased again. There was a significant difference between the initial and 1-month HYP readings for control, Bio-C, and Orbis groups *($p<0.05$).* The TheraCal group had the highest HYP release at 6 months; however, it was not statistically different from the other groups *($p>0.05$).*

4.6 Quantitation of total extractable total protein (Micro-BCA)

The effect of the materials on the total extractable proteins is shown in **Figure 7**. Following extraction, there was a notable difference in the cumulative release between groups, wherein Bio-C showed an extractable protein level of 38.5 protein µg/mg dentin dry weight, a value 483 % higher than the control group (6.6 protein µg/mg dry dentin). S-PRG showed similar behavior to the control group at all time points *($p>0.05$)* with a cumulative release of 5.0 protein µg/mg dry dentin. However, all groups exhibited variations in protein release between the incubation periods. Except for TheraCal, all groups released more proteins in the first week or one month of incubation. At one week and one month, Bio-C showed higher protein release than the control and S-PRG groups *($p<0.05$).* TheraCal demonstrated a different behavior with a progressive decrease in release until the second month.

4.7 Modulus of elasticity (E)

The elastic modulus (E) of the tested dentin beams remained consistent from the initial to one-week incubation values *($p>0.05$)* **(Figure 8)**. At one month, Bio-C exhibited a dramatic decrease in E, showing significant differences from the control and TheraCal groups *($p<0.05$)*. After two months,

the Bio-C group lost all elasticity, with all dentin beams either broken or degraded inside the tubes. Orbis showed a similar behavior to Bio-C and lost the majority of its elasticity at the end of the experiment. Both Bio-C and Orbis showed significant differences from their initial and 6-month E values (p=0.002), while control, S-PRG, and TheraCal groups remained the same *(p>0.05).*

4.8 Calcium leaching

The results for calcium ion leaching in water are shown in **Table 3**. The leaching of calcium was significantly affected by the material type and the tested time point *(p<0.05).* TheraCal had significantly higher overall release of calcium compared to both Orbis and Bio-C *(p<0.05).* A significant interaction was found between material type and periods *(p<0.05).* Calcium leaching exhibited a strong time-dependent increase *(p<0.001),* with values rising progressively from 14.77 ± 1.24 mg (Day 3) to 38.49 ± 1.28 mg (Day 28). All pairwise time comparisons were statistically significant *(p<0.001).* On day 14, TheraCal released significantly more calcium ions than Bio-C and Orbis, and on day 28, both TheraCal and Bio-C leached significantly more calcium than Orbis (p<0.05). There was no detected release of calcium ions from S-PRG.

5. Discussion

The increased focus on bioactive materials in dentistry began with the introduction of the mineral trioxide aggregate (MTA), mainly due to its favorable characteristics, including the good sealing ability [33], biocompatibility, and low cytotoxicity [34]. With the development of new bioactive materials, several studies have evaluated their chemical and mechanical properties [35–39]. Recognizing the materials' composition can influence tissue response, our study investigated the physical and chemical properties of demineralized dentin after prolonged exposure to bioactive silicate-based cements with different formulations. Our findings revealed that the tested materials only partially inhibited the solubilization of collagen telopeptides and reduced MMP activity compared to the control group. Therefore, the null hypothesis that prolonged contact with bioactive silicate-based

materials would neither inactivate dentin matrix-bound MMPs or cathepsin K, nor induce changes in demineralized dentin matrices over time, was rejected.

Tricalcium silicate and dicalcium silicate, contained in MTA, react with water to form calcium silicate hydrate and calcium hydroxide ($Ca(OH)_2$) [40]. $Ca(OH)_2$ is known for its biological properties, including antimicrobial activity and tissue-dissolving ability [41]. The formation of $Ca(OH)_2$ in MTA is correlated to the release of calcium ions and alkalinization of surrounding fluids [15]. In contrast, materials such as TheraCal do not form $Ca(OH)_2$ during setting; however, they still release calcium ions, which contribute to surface calcium phosphate deposition [42]. This behavior is related to the resin matrix in TheraCal, which alters its setting mechanism [42]. At optimal concentrations, calcium ions provided by $Ca(OH)_2$ can also promote remineralization of dental hard tissues by facilitating the formation of apatite-like crystals [15,43,44], emphasizing the importance of controlled ion release in biomaterials. The evaluation of calcium leaching from bioactive materials has been previously reported, with MTA typically presenting lower initial calcium release compared to TheraCal [15]. This is consistent with the findings of the present study, which demonstrated a higher cumulative calcium leaching from TheraCal. S-PRG, on the other hand, is a surface pre-reacted glass that does not include leachable calcium ions in its composition [18].

Calcium ions released from calcium silicates have the ability to reduce enzyme-mediated collagen degradation [45,46]. Since MMPs require two Zn^{2+} ions for enzymatic activity and one Ca^{2+} ion for structural stability [47,48], the release of Ca^{2+} ions during the hydration process of calcium silicate-based materials may disrupt MMP activity by altering the ionic environment required for optimal Zn^{2+} and Ca^{2+} binding within the enzyme's active site. MMPs and cysteine cathepsins work synergistically during processes such as caries formation [49] and may be activated during dental procedures [50] or even inactivated by specific crosslinkers [51]. This enzymatic activity is critical, as unregulated degradation could compromise the long-term structural integrity of demineralized dentin. A direct MMP activity assay revealed a significant reduction in MMP activity in $Ca(OH)_2$ release

groups, with inhibition rates reaching (84 % - 90.1 %) after three weeks *($p<0.05$).* This inhibition is attributed to the release of calcium ions. Since S-PRG does not release Ca^{2+}, it maintains a consistent MMP inhibition rate, but lower than the other materials. This suggests an alternative mechanism, possibly related to its bioactive glass composition or ion exchange capacity. Furthermore, the EDTA used for demineralizing the beams have the ability to inhibit MMPs by chelating the catalytic zinc ions of the MMPs and removing calcium ions from the collagen matrix [48,52]. However, the high water solubility reverses their inhibitory effect [26,53].

Opposite to the MMPs, which can only fragment the substrate in their molecular mobility zone [54], Cathepsin K can cleave collagen at multiple sites. Furthermore, 98 % of cathepsin protease activity is related to cathepsin K, since it cleaves helical collagen [29], while other cathepsins cleave only the non-helical telopeptides [55]. MMP-mediated and CC-mediated activities can be analyzed separately using assays for their specific degradation products, ICTP and CTX, respectively [28]. Previous studies reported that demineralized dentin releases CTX telopeptides, although at a lower rate compared to the release of ICTP [26,56]. In this study, the results of CTX and ICTP followed a similar trend, with TheraCal showing the highest initial release for both CTX and ICTP. The rates of ICTP and CTX release by matrix-bound endogenous proteinase are affected by storage time and specimen surface area [57]. Cleaved collagen fragments near the surface of the specimen might be released to the incubation medium while those far from the surface remain entrapped in to the collagen matrix. In addition, the sources of MMPs and cathepsins in peripheral dentin are limited due to the lack of cellular or odontoblastic activities [54].

This might be related to the lower solubility of TheraCal and reduced ion release compared to several other types of MTA, like ProRoot MTA, Angelus MTA, and Biodentine. Moreover, the water sorption and porosity of TheraCal are similar to ProRoot MTA and Biodentine but lower than Angelus MTA [15,58]. Hence, TheraCal might act as a scaffold for the formation of the reparative dentin, dentinal

fluids are absorbed, causing the release of calcium and forming an apatite layer, which plays an important role in remineralization and dentin repair [59].

The further dissociation of the $Ca(OH)_2$ in calcium silicate-based cements into calcium ions (Ca^{2+}) and hydroxyl ions (OH^-) [15,60] raises the pH of the surrounding tissues [61]. The ability to maintain a high pH in the dentin is related to $Ca(OH)_2$ diffusion capacity through dentin tubules [61]. In this study, pH measurements revealed that both traditional and pre-mixed MTAs exhibited higher pH (~10) compared to TheraCal, whereas S-PRG produced a neutral pH (~7), similar to the control group. This was expected, as $Ca(OH)_2$ is not a byproduct of S-PRG's setting reaction. The pH can influence enzymatic activity and cellular metabolism, whereas ion release plays a crucial role in cell differentiation and mineralization [62]. However, the alkaline nature of the $Ca(OH)_2$ reaction may also contribute to chemical degradation of dentin by disrupting the bond between hydroxyapatite crystals and the collagenous network [63], by neutralizing, dissolving, or denaturing some of the acidic components that act as bonding agents [64]. To investigate potential pH-related degradation, we first quantified extractable total protein after incubation. A micro-BCA assay revealed higher cumulative protein levels in alkaline solutions, particularly in the Bio-C group. To determine whether these proteins included collagen fragments, we performed a hydroxyproline (HYP) assay, since 90 % of the dry mass of demineralized dentin collagen comprises type I collagen, containing 9.6 % mass hydroxyproline [65]. (Butler, 2000). Again, higher HYP levels were observed in media with elevated pH, confirming that a substantial portion of proteins detected by micro-BCA were type I collagen fragments, likely due to chemical degradation under alkaline conditions. The cumulative HYP released in the Bio-C group was 9.32 times higher than in the control. In contrast, S-PRG did not significantly increase collagen solubilization compared to the control group *($p>0.05$)*. Moreover, the loss of dry mass, used as an indirect measurement of collagen solubilization by dentinal proteolytic enzymes over time [53,66] was higher for alkaline groups, increasing at 1 month and peaking at 6 months, in parallel with elastic modulus results. A previous study showed that incubation of dentin beams in calcium and

zinc-containing media resulted in a relatively rapid and significant decrease in stiffness and an increased amount of mass loss [67].

Given that the flexural strength of dentin depends in part on the link between the hydroxyapatite and the collagen matrix, the reduced organic support due to chemical degradation can negatively impact the mechanical properties of dentin [63,64]. Several studies have reported decreased dentin fracture resistance and flexural strength following exposure to $Ca(OH)_2$ [24,63,64,68]. Although some authors observed minimal effects on the elastic modulus under short-term exposure, attributing this to the limited penetration of $Ca(OH)_2$ affecting only the surface and not the bulk of dentin [67], others have reported a gradual loss of elasticity after three months of exposure, possibly as a result of protein denaturation within dentin matrix [68]. Our findings indicate that exposure to bioactive materials releasing $Ca(OH)_2$ significantly affects the elastic modulus of demineralized dentin over time. A progressive loss of elasticity was observed, with the Bio-C and Orbis groups exhibiting complete loss of elasticity at 2 and 6 months, respectively. Variations among studies may be due to methodological differences. In the present study, the use of the flexural test was due to its ability to establish a state of pure tension at the convex surface of the beam [69], enabling accurate assessment of structural integrity. However, the progressive disintegration of the dentin beams at later incubation stages prevented measurement, lowering the average elastic modulus values. Moreover, the organic matter of the dentin, such as the collagen molecules, is usually protected from degradation by intrafibrillar and extrafibrillar apatite crystallites in the mineralized dentin [70,71]. Demineralized dentin used in this study had reduced buffering capacity due to the loss of hydroxyapatite, and the use of thin 0.3 mm demineralized beams allowed more effective diffusion of $Ca(OH)_2$ through the matrix. It is possible that $Ca(OH)_2$ (molecular weight, 56.1 Da) is capable of penetrating collagen fibrils and altering their three-dimensional conformation [72], which may explain the significant reduction in elastic modulus observed in this study.

Although many bioactive materials are classified as MTA cements, their interactions with dentin vary significantly depending on their specific chemical compositions. The choice of a bioactive material would depend on the specific requirements of the dental procedure and the desired effects on tissue response and bioactivity. Our findings demonstrate that both material composition and exposure duration are critical factors influencing dentin matrix integrity. The observed interaction between material and time shows the dynamic behavior of these effects, suggesting that the dentin response is influenced by the exposure period and material used. However, it remains unclear whether these differences will be clinically significant or result in better long-term performance for any of the materials.

6. Conclusion

Although bioactive materials are well known for their properties, the alkaline degradation caused by bioactive materials should be kept in mind. Material type had significant effects on the collagen matrices over time, which could compromise the mechanical integrity of the restoration. Bioactive materials are still valid materials for clinical use in dentistry. However, caution is advised when they are used to obturate root canals with thin dentinal walls and in filling the full length of the canal to prevent collagen degradation that might lead to root fracture. Material type and time significantly influence Ca leaching, with TheraCal showing the highest potential for sustained calcium release.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability statement

Data will be made available on request.

Figures and tables legends

Table 1. Composition of test materials and application procedures.

Table 2. pH at room temperature values of bioactive endodontic materials at different time points. *Note:* values represent the mean and ± standard deviation. Different letters represent statistical differences between groups ± $p<0.001$. Different capital letters represent differences between the PH values of the different materials at same time point. Different small letters represent the difference for each material at different time points.

Table 3. Leaching of calcium ions at different time points measured in µg/ml. Different capital letters represent differences between materials at the same time point, different small letters represent differences for each material at the different time points.

Figure 1. Protocol configuration. (A) preparation of a tooth slice; (B) preparation of a dentin beam; (C) preparation of a set CSM block; (D) incubation of dentin-material assemblies in artificial saliva.

Figure 2. Dry mass loss values by endodontic materials after storage in CM at 37 °C after 24 hours, 1 week, 1 month, 2 months, and 6 months. The chart columns represent the mean loss values, and the error bars represent the standard deviation. Different letters represent statistical differences between groups ($p<0.005$). Different capital letters represent differences for each material at different time points. Different small letters represent the differences between the values of the different materials at the same time point.

Figure 3. The mean values and the standard deviation of the MMP activity after storage in CM at 37 °C at different tested time points. Different letters represent statistical differences between groups ($p<0.005$). Different capital letters represent differences for each material at different time points. Different small letters represent the differences between the values of the different materials at the same time point.

Figure 4. The mean values and the standard deviation of the release of ICTP telopeptide fragments after different incubation time points. Different letters represent statistical differences between groups ($p<0.005$). Different capital letters represent differences for each material at different time points. Different small letters represent the differences between the values of the different materials at the same time point.

Figure 5. CTX type I collagen fragments were released after storage in CM at 37 °C after 24 hours, 1 week, 1 month, 2 months, and 6 months. The chart columns represent the mean values, and the error bars represent the standard deviation. Different letters represent statistical differences between groups ($p<0.005$). Different capital letters represent differences for each material at different time points. Different small letters represent the differences between the values of the different materials at the same time point.

Figure 6. The mean values and the standard deviation of the Hydroxyproline released after storage in CM at 37 °C after 24 hours, 1 week, 1 month, 2 months, and 6 months. Different letters represent statistical differences between groups ($p<0.005$). Different capital letters represent differences for each material at different time points. Different small letters represent the differences between the values of the different materials at the same time point.

Figure 7. The mean values and the standard deviation of the MBCA after storage in CM at 37 °C after 24 hours, 1 week, 1 month, 2 months, and 6 months. Different letters represent statistical differences between groups ($p<0.005$). Different capital letters represent differences for each material at different time points. Different small letters represent the differences between the values of the different materials at the same time point.

Figure 8. Elastic modulus values by endodontic materials after storage in CM at 37 °C after 24 hours, 1 week, 1 month, 2 months, and 6 months.

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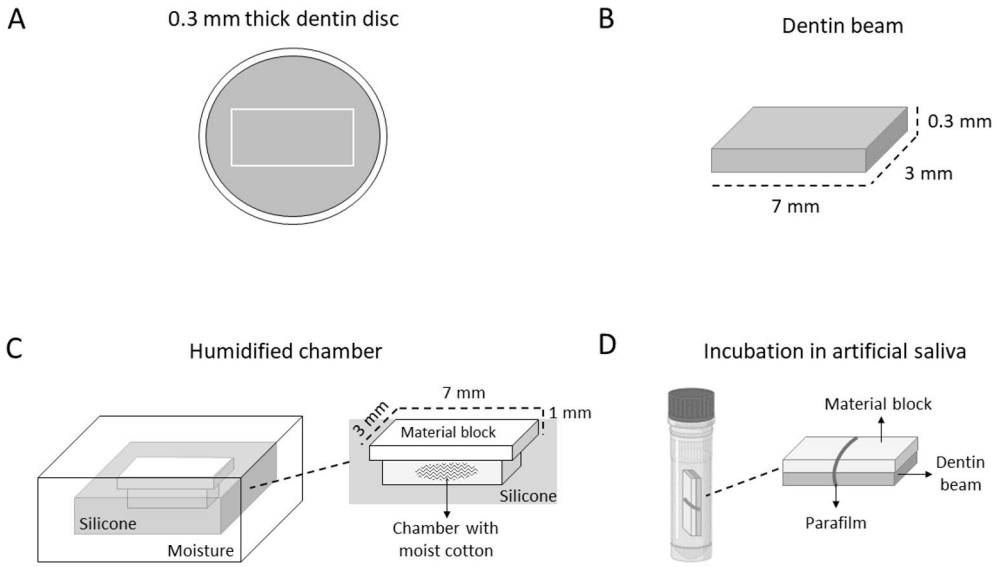

Figure 1. Protocol configuration. (A) preparation of a tooth slice; (B) preparation of a dentin beam; (C) preparation of a set CSM block; (D) incubation of dentin-material assemblies in artificial saliva.

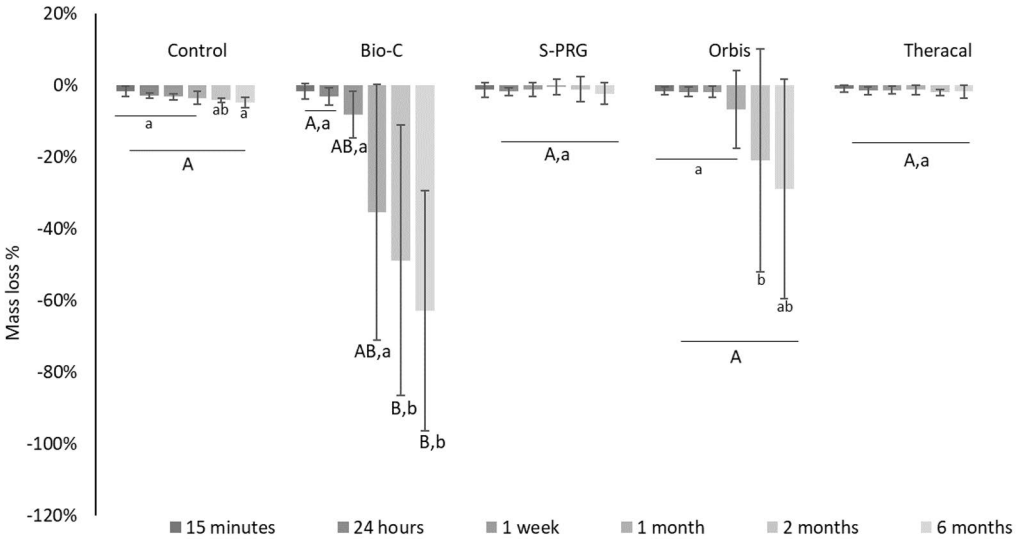

Figure 2. Dry mass loss values by endodontic materials after storage in CM at 37 °C after 24 hours, 1 week, 1 weeks, 2 months, and 6 months. The chart columns represent the mean loss values and the error bars represent standard deviation. Different letters represent statistical differences between groups ($p<0.005$). Different capital letters represent difference for each material at different time points. Different small letters represent the differences between the values of the different material at same time point.

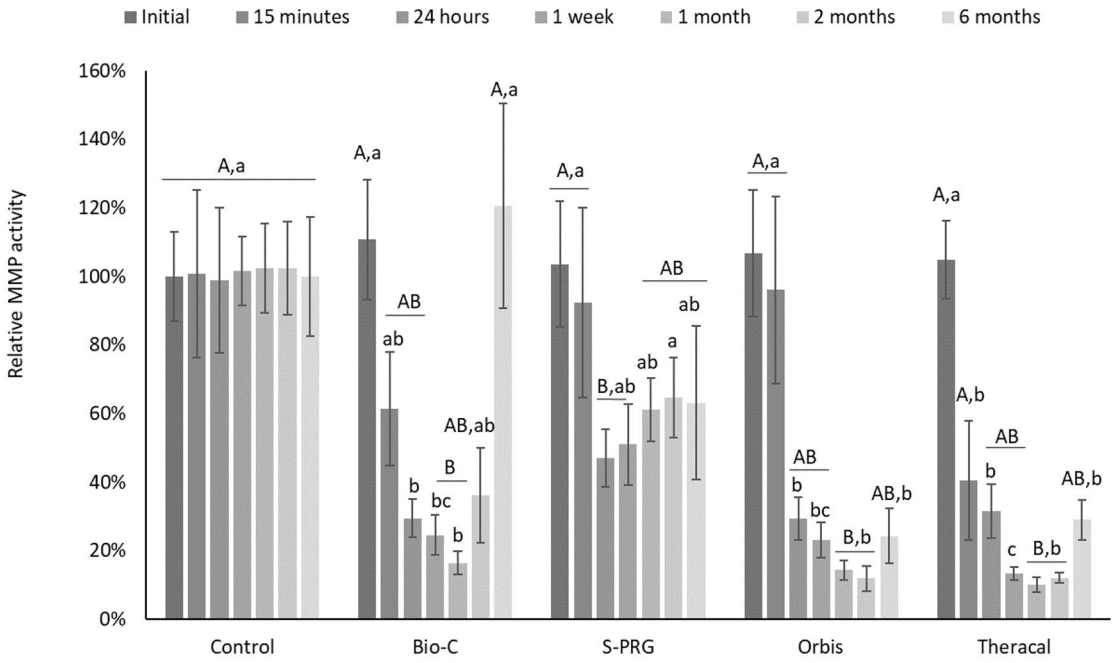

Figure 3. The mean values and the standard deviation of the MMP activity after storage in CM at 37 °C at different tested time points. Different letters represent statistical differences between groups ($p<0.005$). Different capital letters represent difference for each material at different time points. Different small letters represent the differences between the values of the different material at same time point.

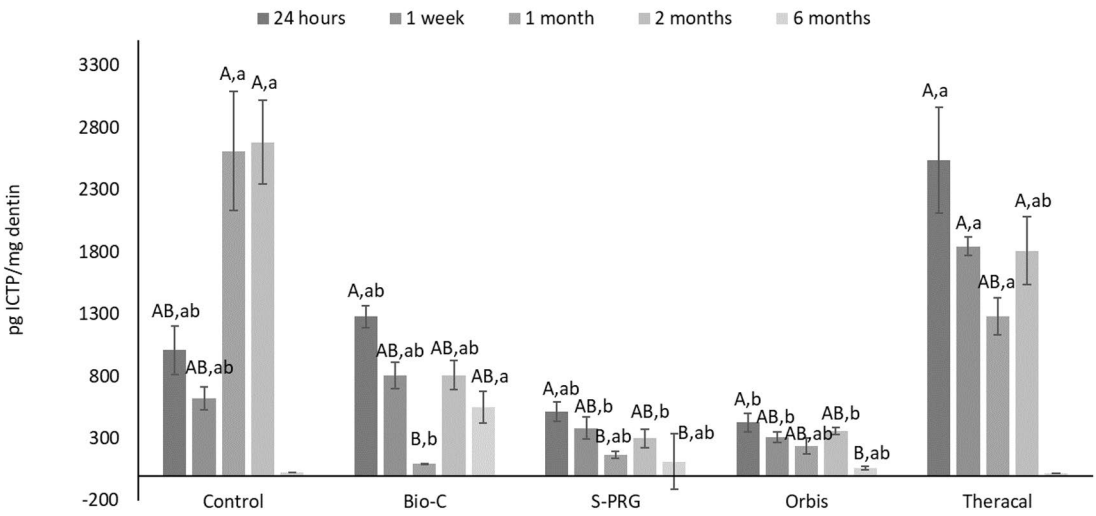

Figure 4. The mean values and the standard deviation of the release of ICTP telopeptide fragments after different incubation time points. Different letters represent statistical differences between groups ($p<0.005$). Different capital letters represent difference for each material at different time points. Different small letters represent the differences between the values of the different material at same time point.

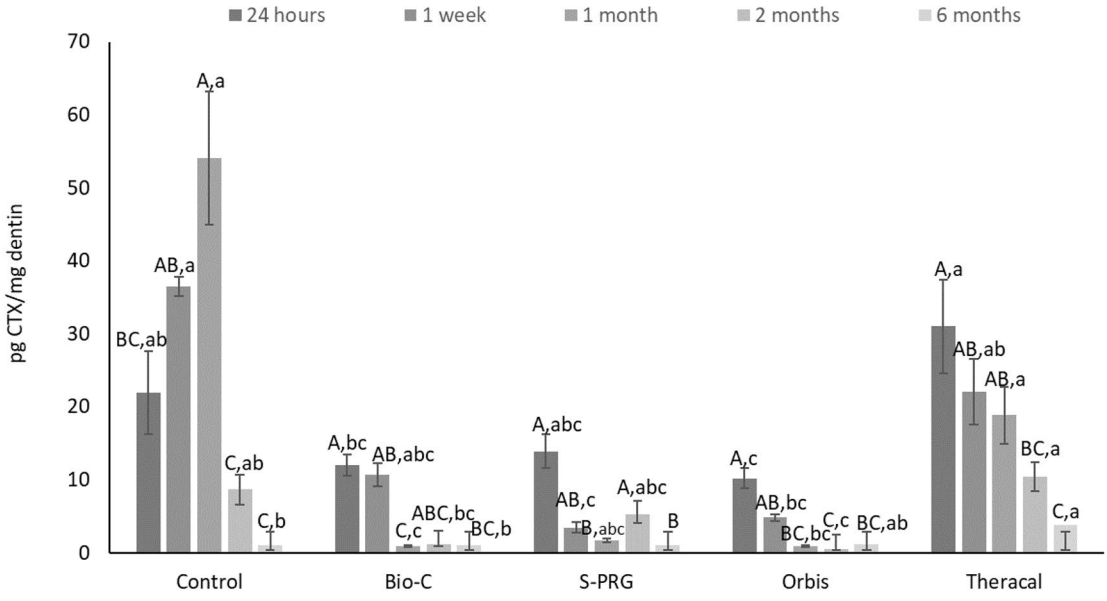

Figure 5. CTX type I collagen fragments released after storage in CM at 37 °C after 24 hours, 1 week, 1 month, 2 months, and 6 months. The chart columns represent the mean values and the error bars represent standard deviation. Different letters represent statistical differences between groups ($p<0.005$). Different capital letters represent difference for each material at different time points. Different small letters represent the differences between the values of the different material at same time point.

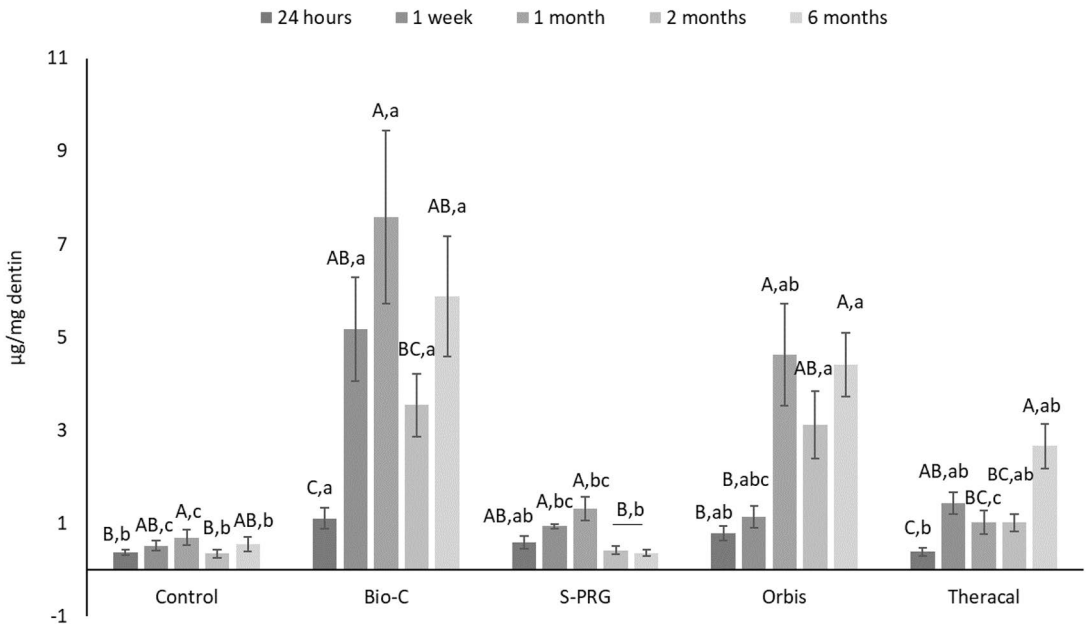

Figure 6. The mean values and the standard deviation of the Hydroxyproline released after storage in CM at 37°C after 24 hours, 1 week, 1 month, 2 months and 6 months. Different letters represent statistical differences between groups ($p<0.005$). Different capital letters represent difference for each material at different time points. Different small letters represent the differences between the values of the different material at same time point.

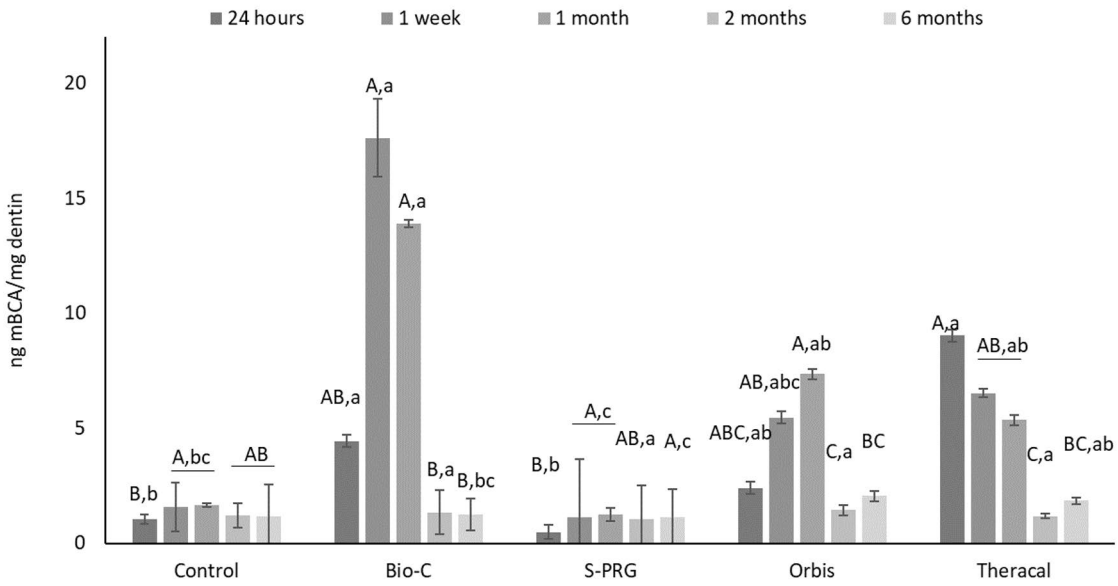

Figure 7. The mean values and the standard deviation of the MBCA after storage in CM at 37°C after 24 hours, 1 week, 1 month, 2 months and 6 months. Different letters represent statistical differences between groups ($p<0.005$). Different capital letters represent difference for each material at different time points. Different small letters represent the differences between the values of the different material at same time point.

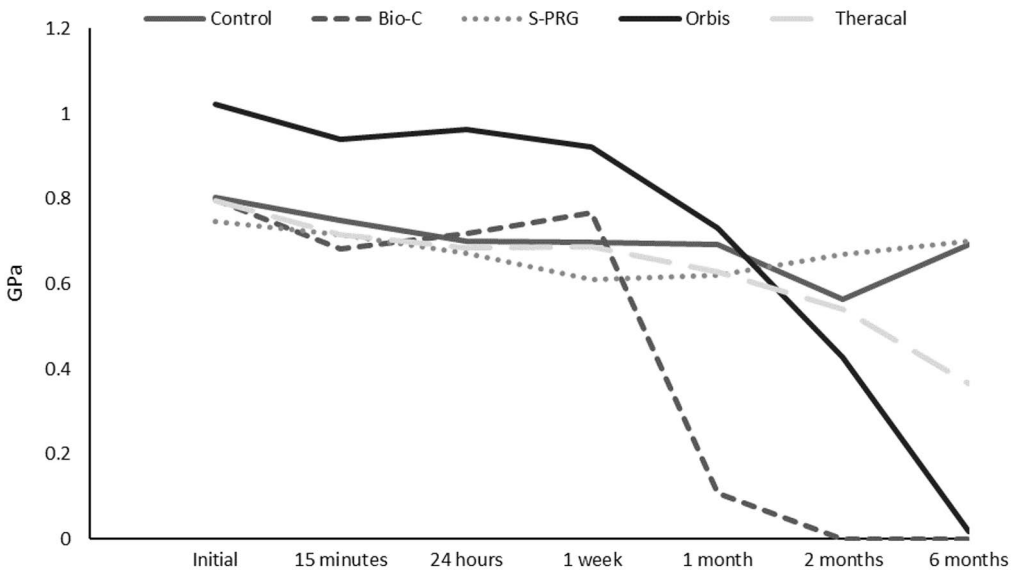

Figure 8. Elastic modulus values by endodontic materials after storage in CM at 37 °C after 15 minutes, 24 hours, 1 week, 1 month, 2 months, and 6 months.

Table 1. Composition of calcium silicate biomaterials and manufacturer

Material	*Composition*	*Manufacturer*
Mineral trioxide aggregate. (MTA, Bio-C)	calcium silicates, calcium aluminate, calcium oxide, zirconium oxide, iron oxide, silicon dioxide, and a dispersing agent	Angelus Indústria de Produtos Odontológicos S/A, Londrina, Brazil
Surface pre-reacted glass (S-PRG, Shofu Inc)	Powder: Zinc oxide-based inorganic compound filler, S-PRG filler, additives. Liquid: Poly carboxylic acid derived solution, water	Shofu Inc, Higashiyama-ku, Kyoto, Japan
Mineral Trioxide Aggregate (MTA, Orbis)	Tricalcium silicate, dicalcium silicate, calcium carbonate filler, zirconium oxide, iron oxide	MTA; Orbis oy, Espoo, Finland.
Resin-modified calcium silicate-based cement (TheraCal LC, Bisco)	Portland cement type III (20–60%), poly (ethylene glycol) dimethacrylate (10–50%), bis-GMA (5–20%), and barium zirconate (1–10%)	Theracal L.C Bisco Inc, Schamburg, IL, USA

Table 1. Composition of tested materials and manufacturer.

Table 2. PH value of the different tested endodontic materials at the different time points.

	24 hours	1 week	1 month	2 months	6 months
Control	7.33 ^{ABd} ±0.04	7.28 ^{Bd} ±0.03	7.39 ^{Ac} ±0.07	7.26 ^{Bc} ±0.04	7.36 ^{Ac} ±0.02
Bio-C	12.50 ^{Aa} ±0.02	12.19 ^{Ba} ±0.13	12.28 ^{Ba} ±0.13	12.26 ^{Ba} ±0.10	12.32 ^{ABa} ±0.11
S-PRG	7.78 ^{Acd} ±0.05	7.52 ^{ABcd} ±0.20	7.46 ^{ABCc} ±0.15	7.29 ^{Cc} ±0.09	7.32 ^{BCc} ±0.05
Orbis	12.28 ^{Aab} ±0.07	11.22 ^{Bab} ±0.45	11.75 ^{ABab} ±0.19	11.48 ^{Bab} ±0.13	11.42 ^{Bab} ±0.16
Theracal	11.80 ^{Abc} ±0.04	9.93 ^{CDbc} ±0.12	11.09 ^{ABbc} ±0.04	10.87 ^{CDbc} ±0.04	10.95 ^{BCbc} ±0.04

Table 2. pH at room temperature values of bioactive endodontic materials at different time points. *Note:* values represent the mean and ± standard deviation). Different letters represent statistical differences between groups ±$p<0.001$). Different capital letters represent differences between the PH values of the different material at same time point. Different small letters represent the difference for each material at different time points.

Table 3. Leaching of calcium ion at different time points measured in µg/ml. Different capital letters represent differences between materials at the same time point, different small letters represent differences for each material at the different time points.

	Leaching of calcium Ion			
	3 days	7 days	14 days	28 days
Bio-C	17.51^{Ab} ±3.26	22.99^{Ab} ±1.90	34.27^{ABa} ±4.85	37.06^{Ba} ±5.87
S-PRG	0	0	0	0
Orbis	13.87^{Ab} ±3.91	24.08^{Aa} ±4.58	30.83^{Ba} ±4.02	31.31^{Ba} ±4.10
TheraCal	12.93^{Ad} ±4.49	29.93^{Ac} ±4.32	39.09^{Ab} ±5.99	47.10^{Aa} ±4.11

Table 3. Leaching of calcium ions at different time points measured in µg/ml. Different capital letters represent differences between materials at the same time point, different small letters represent differences for each material at the different time points.

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