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Original Article

Effect of Blood Contamination on Vickers Microhardness and Surface Morphology of Mineral Trioxide Aggregate

Jaehyun Seung[1], Seong-Jin Shin[2], Byounghwa Kim[1], Ji-Myung Bae[3], Jiyoung Ra[1,4]

[1]Department of Pediatric Dentistry, College of Dentistry, Wonkwang University, Iksan, Republic of Korea
[2]Institute of Tissue Engineering, Dankook University, Cheonan, Republic of Korea
[3]Department of Dental Biomaterials and the Institute of Biomaterial and Implant, College of Dentistry, Wonkwang University, Iksan, Republic of Korea
[4]Dental Research Institute, Wonkwang University, Iksan, Republic of Korea

Abstract

This study aimed to investigate the effects of blood contamination on the Vickers hardness and the surface morphology of premixed MTA and compare them with the effects on conventional MTA. The Vickers microhardness of Endocem MTA Premixed Regular (EP) and ProRoot MTA (PM) was assessed after immersion in fetal bovine serum (FBS) and saline. Stem cells from human exfoliated deciduous teeth (SHED) were seeded on MTA after immersion in FBS, saline, and deionized water (DW). Cell adhesion patterns and surface morphology were visualized via scanning electron microscopy (SEM). The surface microhardness of EP and PM in FBS was lower than in saline. However, short-term exposure of PM to FBS did not reduce the microhardness compared to saline. Angular crystals formed in water, while rounded crystals with more air voids appeared in FBS. Favorable SHED attachment occurred in all groups. Overall, the surface hardness of EP and PM decreased after FBS exposure, although PM was less influenced. We suggest minimizing the amount of bleeding when using MTA clinically; nevertheless, PM remains an option with more expected blood contamination than EP. In summary, exposure to FBS decreased mechanical performance but allowed cell adhesion for both MTAs, with PM being more resistant to these changes. [J Korean Acad Pediatr Dent 2024;51(2):165-175]

Keywords

Premixed mineral trioxide aggregate, Vickers microhardness, Fetal bovine serum, Stem cells from human exfoliated deciduous teeth

ORCID

Jaehyun Seung
https://orcid.org/0009-0005-7920-1538
Seong-Jin Shin
https://orcid.org/0000-0002-4140-5157
Byounghwa Kim
https://orcid.org/0000-0002-5606-4869
Ji-Myung Bae
https://orcid.org/0000-0002-8607-8604
Jiyoung Ra
https://orcid.org/0000-0002-2569-3259

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Introduction

Root canal treatment (RCT) has been considered the conventional treatment ap-

Corresponding author: Jiyoung Ra
Department of Pediatric Dentistry, College of Dentistry, Wonkwang University, 895 Muwang-ro, Iksan, Jeollabuk-do, 54538, Republic of Korea
Tel: +82-63-850-6633 / Fax: +82-63-858-2957 / E-mail: pedojy@wku.ac.kr

proach for managing dental pulp exposure attributed to deep dentin caries or trauma. However, RCT involves access opening and canal enlargement, which can lead to the destruction of tooth structure and loss of tooth vitality. On the other hand, vital pulp therapy (VPT) prevents excessive loss of tooth structure and maintains pulp vitality, thus preserving the tooth's defensive properties. As a more conservative option for primary and permanent teeth, VPT enables the preservation of the health, function, and esthetics of deciduous teeth and young permanent teeth with immature roots in children.

Mineral trioxide aggregate (MTA), introduced as a root repair material in 1993 by Torabinejad[1], exhibits excellent biocompatibility, sealing ability, and the capability to induce mineralization in teeth with pulp exposure[1,2]. Consequently, it has found widespread application in cases of VPT as well as a filling material for root canals, restoration of root perforations, and formation of root-end barriers in immature permanent teeth[3,4]. Recent clinical studies have demonstrated promising results using MTA in VPT of both permanent and primary teeth[5-8]. However, MTA is also associated with limitations, such as handling challenges, extended setting time, and the possibility of tooth discoloration[9].

Novel calcium silicate-based materials were formulated to address the constraints of conventional MTA. Premixed-type MTA, which comprises powdered calcium phosphate and a water-soluble liquid, is one such alternative. The presence of additives which serve as anti-washout agents or accelerators[10,11] in premixed MTA imparts several benefits, such as accelerated setting time, reduced influence of the mixing ratio, and improved cement delivery[10,12-14]. Furthermore, premixed MTA minimizes material wastage by facilitating accurate injection. These features can reduce chairside time, making it especially useful in pediatric treatment. Recently, several clinical studies have reported the success rate of premixed MTA to be similar to that of ProRoot MTA, which is considered conventional type[15-17].

The contamination of dental materials by blood must be avoided[18]. Complete isolation of teeth from contaminants is a fundamental tenet in clinical practice. However, achieving complete isolation from blood is difficult when placing materials within the tooth during clinical procedures, such as pulpotomy, apexification, and root perforation repairs.

Numerous investigations have explored the impact of blood contamination on MTA[19-24]. However, the majority of research has concentrated on conventional MTA, and studies on premixed MTA remain limited. Hence, this study aimed to investigate the effects of blood contamination on the Vickers hardness of premixed MTA and compare them with the effects on conventional MTA. In addition, the attachment patterns of stem cells from human exfoliated deciduous teeth (SHED) and alterations in the surface morphology of MTA under such conditions were evaluated.

Materials and Methods

1. Ethics approval

A deciduous tooth was extracted at Wonkwang University Dental Hospital due to the ectopic eruption of a successional tooth and was used for cell culture after receiving approval from the Institutional Review Board (WKIRB202304-03).

2. Materials and sample preparations

ProRoot® MTA (PM; Dentsply Tulsa Dental, Tulsa, OK, USA) and Endocem MTA® premixed regular (EP; Maruchi, Wonju, Korea) were used in this study. Fetal bovine serum (FBS; GIBCO, Grand Island, NY, USA) and 0.9% saline solution (JW Pharmaceutical, Seoul, Korea) were used as storage media (Table 1).

3. Vickers microhardness

MTA was mixed according to the manufacturer's instructions and injected into stainless-steel molds of 4.0-mm diameter and 6.0-mm height. The specimens in the molds were immersed in FBS, or saline-soaked floral foam. A subset of the samples immersed in the FBS-

Table 1. Materials used in the study

Trade Name	Category	Code	Composition	Manufacturer
ProRoot® MTA	Powder-liquid mix type MTA	PM	Tricalcium silicate, Dicalcium silicate, Bismuth oxide, Tricalcium aluminate, Calcium sulfate dehydrate, Tetra calcium aluminoferrite, Gypsum, Calcium oxide	Dentsply, Tulsa, OK, USA
Endocem MTA® Premixed Regular	Premixed type MTA	EP	Zirconium dioxide, Calcium silicate, Calcium aluminate, Calcium sulfate, Dimethyl sulfoxide, Lithium carbonate, Thickening agents	Maruchi, Wonju, Korea
Fetal bovine serum	Storage media	FBS	Bilirubin, Cholesterol, Creatinine, Urea, Glucose, Protein, Albumine, a-Globulin	GIBCO, Grand Island, NY, USA
Saline	Storage media	Saline	Sodium chloride 9 g	JW Pharmaceutical, Seoul, Korea
Deionized water	Storage media	DW	-	Cleanguy, Suwon, Korea

soaked floral foam for 5 minutes was subsequently immersed in saline-soaked floral foam. This subset was termed the "FBS-Saline" group. All experimental groups (n = 10) were stored in a cabinet with 100% humidity at 37°C for 4 days.

A digital microhardness tester (MXT70, Matsuzawa Seiki Co., Ltd., Tokyo, Japan) was used to determine the surface microhardness. The specimens were immobilized with a jig to prevent any movement during the measurement of the Vickers hardness. A force of 245.2 mN was applied from the following positions for 5 seconds using a pyramidal diamond indenter: 0.5, 2.0, and 4.0 mm from the surface in contact with the solution. The dimensions of the diamond impression were measured in the horizontal and vertical directions, and the Vickers microhardness (HV) values were derived using the following formula:

$$HV = \frac{2F \sin\frac{136^\circ}{2}}{d^2} = 1.854\frac{2F}{d^2}$$

where F represents the load and d represents the average length of the diagonal left by the indenter in millimeters (mm).

Fig.1 presents a schematic representation of the specimen fabrication process.

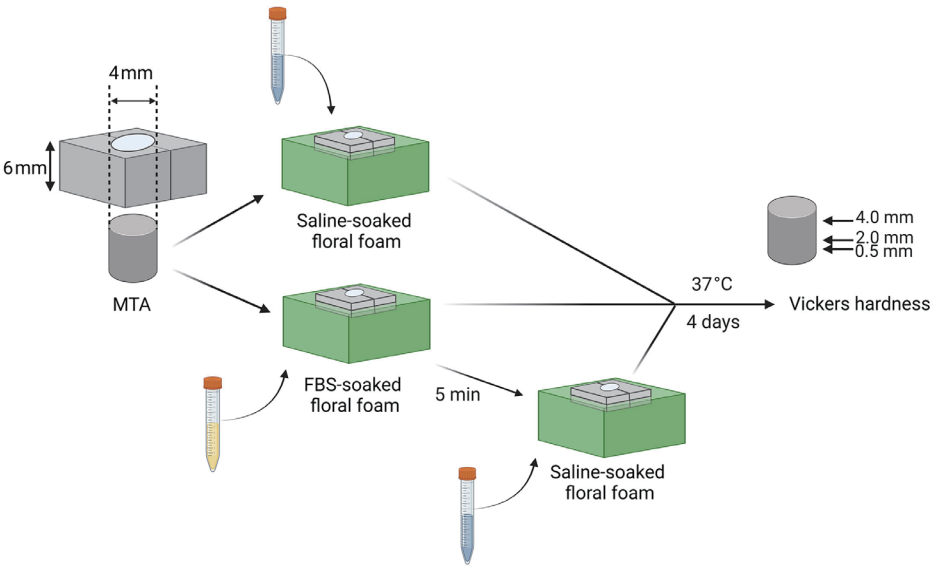

Fig. 1. Schematic illustration of Vickers microhardness test.
The specimens were immersed in fetal bovine serum (FBS)- or saline-soaked floral foam. A subset of the specimens immersed in FBS-soaked floral foam was immersed in saline-soaked floral foam after 5 minutes. All specimens were stored in 100% humidity at 37°C for 4 days. The Vickers hardness was measured at three points 0.5, 2, and 4 mm away from the contact surface.

4. Cell culture

The deciduous tooth specifically obtained for this study was preserved in MEM alpha (GIBCO) at 4°C before the extraction of SHED. Dental pulp tissue was ground into 1-mm fragments and placed in a tissue culture plate comprising MEM alpha supplemented with 10% FBS and 100 units of penicillin-streptomycin (GIBCO). The pulp tissue of the tooth was cultured in a 5% CO_2 incubator at 37°C.

5. Cell adhesion and surface morphology analysis

MTA was injected into Teflon molds with a diameter of 6.0 mm and a height of 2.0 mm after being mixed and stored in FBS, saline, or DW with a relative humidity of 95% at 37°C for 24 hours (n = 2). SHEDs were seeded onto discs in 48 well plates at a concentration of cells/well and stored for 1 day. The cells on the discs were incubated in 2.5% glutaraldehyde fixative in phosphate-buffered saline and then rinsed twice with the saline. The discs were then dehydrated in a series of graded ethanol solutions (60%, 70%, 80%, 90%, and 100%) for 10 minutes at each concentration and air-dried. The discs were coated with a 100 nm layer of platinum subsequently. The coated discs were examined by a scanning electron microscope (SEM, Hitachi S-4800, Hitachi, Tokyo, Japan) at an accelerating voltage of 20.0 kV and magnifications of ×500 and ×2,000. Fig. 2 presents a schematic representation of the cell adhesion and surface morphology analysis.

6. Statistical analysis

All statistical analyses were performed using SPSS Statistics software version 25.0 (IBM Co., Armonk, NY, USA). The effect of two factors, namely, the type of MTA and the contact solution, on the mechanical properties was determined using one-way and two-way analyses of variance (ANOVA) conducted at a 95% confidence level. Tukey's post hoc test was performed subsequently. Statistical analyses were conducted using an alpha significance level of 0.05.

Results

1. Surface microhardness

The surface hardness values of the FBS group were significantly lower than those of the saline group at all

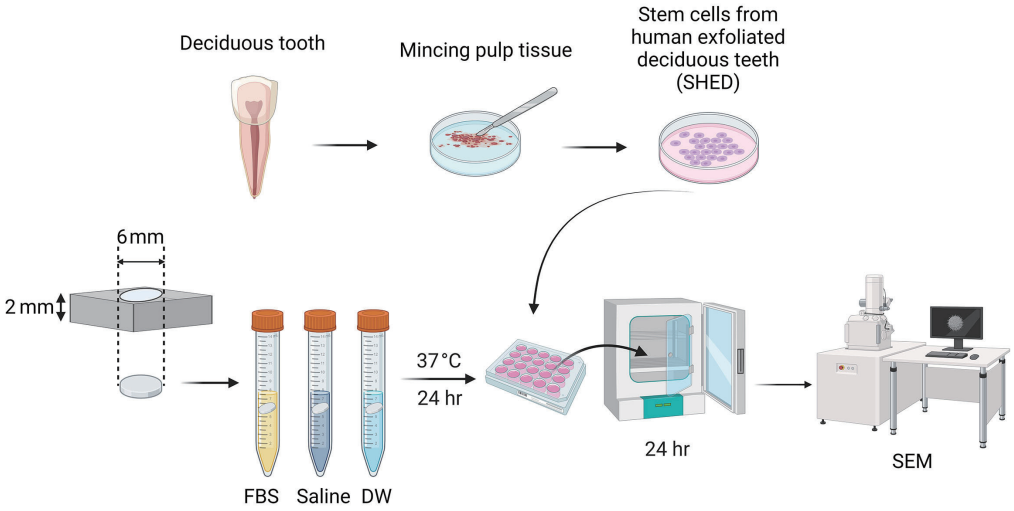

Fig. 2. Schematic illustration of cell attachment and surface morphology analysis.
The specimens were immersed in fetal bovine serum (FBS), saline, or deionized water (DW). Stem cells from human exfoliated deciduous teeth (SHEDs) were seeded onto the specimens, and all specimens were incubated for one day. The surface morphology and SHED attachment were observed using scanning electron microscopy (SEM).

points, regardless of the type of MTA (Table 2, Fig. 3). However, two-way ANOVA indicated no association between the type of MTA and the storage medium.

EP exhibited no hardening (labeled as "not measurable [NM]") at 0.5 mm from the contact surface with FBS. Conversely, the surface hardness of EP in the saline group was significantly higher than that in the FBS-saline group ($p = 0.025$). PM did not significantly differ between the saline and FBS-saline groups in terms of surface hardness.

In both PM and EP, no discernible difference was observed in the surface hardness measured at 2.0 mm from the contact surface between the saline and FBS-saline groups. Moreover, there were no significant differences between the FBS and FBS-saline groups of EP.

The microhardness of EP measured at 4.0 mm from the contact surface was significantly higher in the saline group than in the FBS-saline group ($p = 0.046$). However, no significant difference was observed between these groups of PM. The FBS and FBS-saline groups of EP did not differ significantly.

The surface hardness of PM was consistently higher than that of EP in all groups. With the exception of the values measured at 2.0 mm from the contact surface in the FBS and saline groups, significant differences were observed between the EP and PM values in most groups.

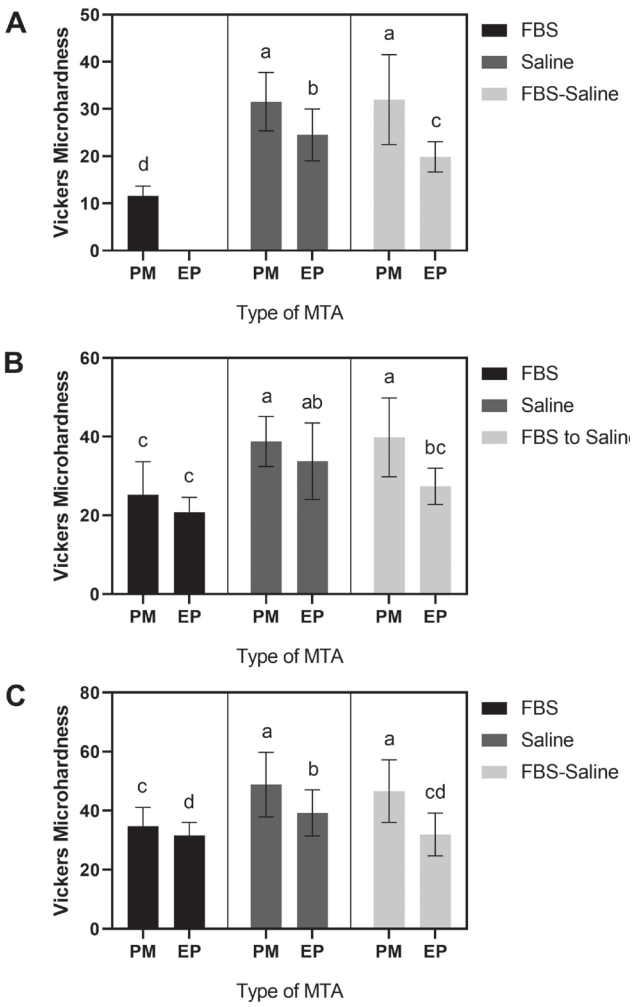

Fig. 3. Vickers microhardness at (A) 0.5, (B) 2, and (C) 4 mm from the bottom.
Different superscript letters indicate significant differences between groups at the same distance from the contact surface to the storage medium by 1-way ANOVA ($p < 0.05$).
PM: ProRoot MTA; EP: Endocem MTA Premixed Regular; FBS: Fetal bovine serum.

Table 2. The mean and standard deviation of Vickers microhardness of MTA in contact with storage media

		Length from the bottom (mm)		
Material	Storage media	0.5	2.0	4.0
PM	FBS	$11.62 \pm 2.07^{d,A}$	$25.29 \pm 8.35^{c,B}$	$34.80 \pm 6.27^{c,C}$
PM	Saline	$31.54 \pm 6.20^{a,A}$	$38.78 \pm 6.35^{a,B}$	$48.84 \pm 10.91^{a,C}$
PM	FBS→Saline	$31.97 \pm 9.53^{a,A}$	$39.83 \pm 9.99^{a,AB}$	$46.63 \pm 10.59^{a,B}$
EP	FBS	NM	$20.85 \pm 3.71^{c,A}$	$31.60 \pm 4.46^{d,B}$
EP	Saline	$24.51 \pm 5.48^{b,A}$	$33.75 \pm 9.70^{ab,B}$	$39.25 \pm 7.80^{b,B}$
EP	FBS→Saline	$19.86 \pm 3.22^{c,A}$	$27.36 \pm 4.61^{bc,B}$	$31.92 \pm 7.24^{cd,B}$

a - d: Different superscript letters indicate significant differences between the groups at the same distance from the contact surface to the storage medium by 1-way ANOVA ($p < 0.05$).
A - C: Different superscript letters indicate significant differences between the groups of the same material and storage media by 1-way ANOVA ($p < 0.05$).
PM: ProRoot MTA; EP: Endocem MTA Premixed Regular; FBS: Fetal bovine serum; NM: Not measurable.

2. Surface morphology by SEM

Fig. 4. presents the surface morphology of the MTA discs and the cell attachment of SHEDs. Distinct variations in the crystalline structures in the surface microstructure of MTA were observed among the different groups. The surfaces of PM and EP exhibited distinct crystalline forms upon exposure to DW or saline, which were characterized by the presence of angular, laminar, and acicular crystals within an uneven crystalline matrix (Fig. 4A - 4D, 4G - 4J). The globular crystals were distinct in all the groups exposed to FBS when compared with those of the control group (Fig. 4E, 4F, 4K, and 4L). In addition, the distinct acicular crystals in the DW or saline groups were not observed in the FBS group. A highly porous structure with a large number of air voids was observed.

The SHEDs on the discs exhibited a spindle-shaped morphology and adherence to the surface in all experimental groups. A consistent orientation in the cell

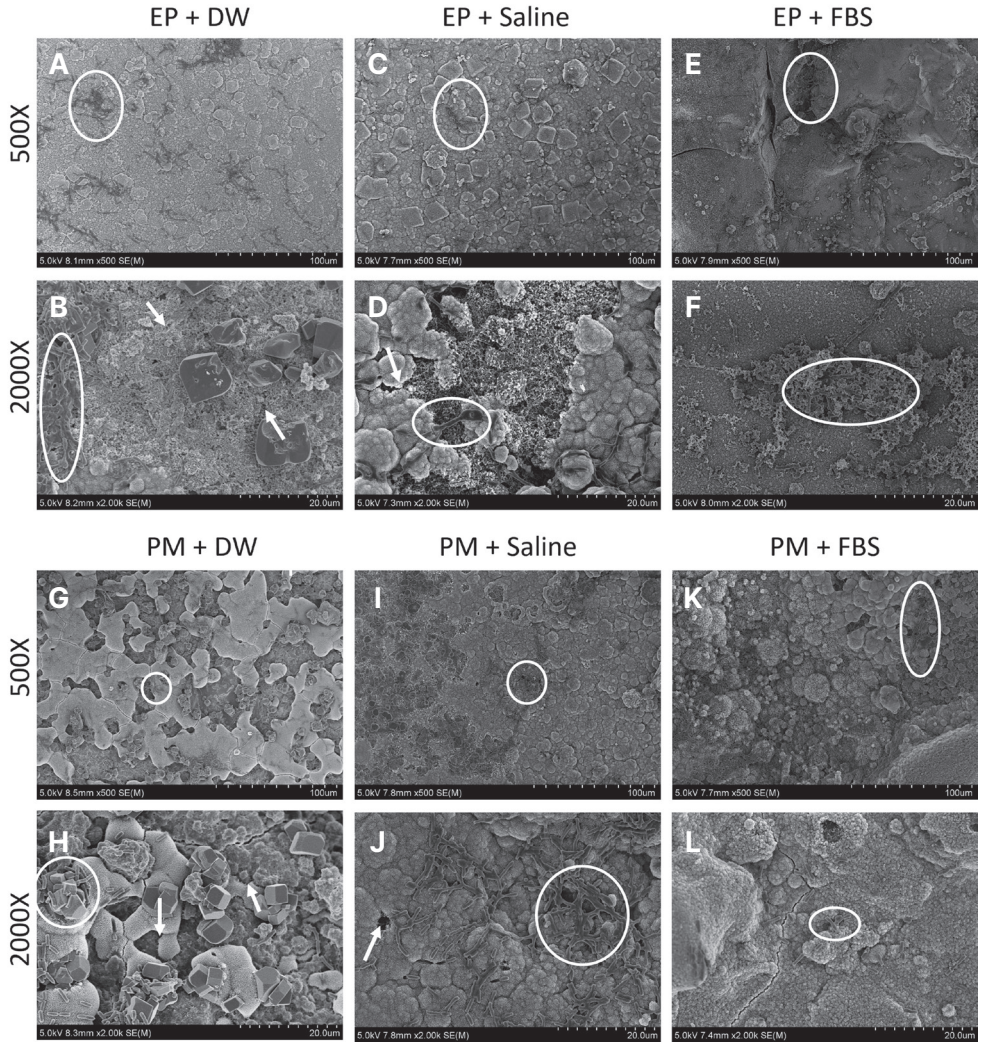

Fig. 4. SEM images of the surface of MTA. MTA discs were placed in contact with DW, saline, or FBS. SHEDs were seeded and incubated for 1 day. (A, B) EP in contact with DW. (C, D) EP in contact with saline. (E, F) EP in contact with FBS. (G, H) PM in contact with DW. (I, J) PM in contact with saline. (K, L) PM in contact with FBS. The arrows indicate acicular crystal formation. Circles indicate SHED attachments.
SEM: Scanning electron microscopy; PM: ProRoot MTA; EP: Endocem Premixed MTA Regular; FBS: Fetal bovine serum; DW: Deionized water; SHEDs: Stem cells from human exfoliated deciduous teeth.

attachment was not observed, as they extended and dispersed in various directions.

Discussion

The purpose of this study was to assess how blood contamination affected the surface hardness, surface morphology, and cell adhesion of premixed and conventional MTA. The Vickers microhardness of MTA was measured, and differences in the surface morphology and SHED attachment were evaluated following immersion in various solutions. The Vickers microhardness tended to decrease when EP and PM were in contact with FBS. The effect of exposure to FBS appeared to be higher on EP. In addition, a difference in the crystallization pattern of the surface was observed following exposure to FBS; however, the cell attachment pattern was even, with no significant difference being observed based on the contact solution.

MTA is utilized extensively for diverse applications, such as pulpotomy, restoration of root perforation, and root canal filling, owing to its exceptional biocompatibility, antibacterial characteristics, and sealing proficiency. However, MTA is often exposed to blood and tissue fluids during these procedures. Blood and tissue fluids comprise a diverse range of proteins, organic and inorganic compounds, and electrolytes[19] that affect the setting process of MTA. Several studies have investigated the effect of blood contamination on the characteristics of MTA[19-25]. Torabinejad et al.[22] proposed that achieving hemostasis and a dry environment were not mandatory during the application of MTA at the sites of root perforation. Nevertheless, it has been noted that contamination with human blood reduces the surface hardness of MTA[19]. In addition, variations in the crystalline configuration of MTA were observed following exposure to FBS[21].

The FBS solution employed in this study has a pH of 7.1 and contains various proteins and peptides, inorganic salts, carbohydrates, amino acids, and fatty acids and lipids. FBS was extensively used in the field of dentistry to mimic blood contamination owing to its biosafety, availability, and similarity to human serum in terms of its biochemical profile[25-28]. On the other hand, owing to its tendency to clot, whole blood could not be employed as an immersion solution[21]. DW and saline groups were designed to simulate situations involving contact with moisture without blood contamination, such as the clinical scenario of placing wet cotton to induce the curing of MTA[29-31].

When it comes to MTA materials used in this study, ProRoot MTA, the first commercial MTA product, is currently one of the most prominent products in many countries and has been studied extensively[32,33]. Among the premixed MTAs, we selected Endocem MTA Premixed Regular, which is widely used and distributed in the Republic of Korea and has been the subject of several research studies[16,34-36].

The Vickers microhardness test was utilized to evaluate the influence of the storage media on the setting characteristics of MTA at a specific point. The Vickers hardness scale, used to indicate the setting process[37], demonstrates how different setting conditions affect the overall strength of a material[38]. The Vickers microhardness was assessed after immersing the samples in the solution for 4 days in the present study. An increase in hardness has been reported at least 72 - 96 hours later[23]; however, no appreciable variation was reported between the microhardness values of MTA obtained after 4 and 180 days[19].

Compared with exposure to saline, a significant decrease in the Vickers microhardness value was observed following exposure to FBS. This finding suggests that long-term exposure to FBS hinders the setting process of MTA and that the inhibition is greater when the contact surface is closer. These findings are in accordance with those of Nekoofar et al.[19], where the microhardness of MTA decreased dramatically following exposure to human blood.

The presence of FBS has influenced the setting process of MTA, which is consistent with a previous study[21]. Short-term exposure groups, referred to as the FBS-saline group, were also included in the present study to simulate the clinical scenarios wherein MTA is exposed to blood before coagulation occurs. The presence of FBS

did not affect the setting of PM in the groups that were exposed to FBS for a short period. In contrast, the setting of EP was affected by brief exposure to FBS.

Several studies have demonstrated that the proteins present in blood disrupt the setting process of MTA and Portland cement; however, the exact mechanisms remain unclear. A reduction in compressive strength and an increase in cement porosity were reported when hemoglobin, which functions as an air entrainment admixture, was mixed with Portland cement[39]. Similarly, mixing Portland cement with red blood cell powder led to a substantial increase in setting time and a decrease in compressive strength[40]. These findings indicate that the porosity of calcium phosphate cement increases when combined with an air entrainment admixture[41].

In addition to the mechanical properties, the surface morphology differed during crystal formation. A descriptive approach was used, as no quantitative criteria were available to compare the groups in terms of surface morphology. In comparison to the other groups, the FBS group exhibited the formation of rounded crystals and the absence of acicular crystals (Fig. 4E, 4F, 4K, and 4L). The formation of angular and acicular crystals was observed in the DW and saline groups (Fig. 4A - 4D, 4G - 4J). These findings are consistent with previous studies on the influence of blood contamination on the microstructure of MTA[24]. Needle-shaped or acicular crystals, which are a characteristic feature of hydrated calcium sulfoaluminate, aid in the formation of inter-crystal bonds that influence the Vickers microhardness[42-44].

Several mechanisms have been attributed to the clinical success of MTA. Cellular adhesion plays a vital role in tissue repair under various physiological and pathological conditions[45]. The adhesion of dental pulp cells plays a crucial role during the initial phase of pulp capping. Cell adhesion is involved in cell development, differentiation, and proliferation, as well as the complex process of wound healing[46]. SHED is an appropriate source of cells for regenerative endodontics as it originates from dental tissues and possesses the ability to efficiently differentiate into odontoblast-like cells[47]. SHED exhibited viability under SEM after incubation on the MTA surface for 1 day. Cell proliferation and attachment to MTA were observed in all solutions, indicating effective adaptation to MTA, thereby affirming its excellent biocompatibility. Cells with extensions of cytoplasmic processes, such as lamellipodia and filopodial processes, were not observed. Thus, a longer incubation period is required to confirm cell differentiation after initial attachment.

This study had several limitations. FBS was used to simulate blood contamination during endodontic procedures in the present study. However, utilizing freshly obtained human blood products would have yielded greater clinical significance[20]. In addition, according to the manufacturer, flocculence may occur during thawing due to the denaturation of serum lipoproteins present in FBS. Moreover, only one type of premixed MTA was used in this study. Recently, premixed MTA with diverse compositions have been developed and studied[11,16]. Thus, studies comparing other types of premixed MTA are warranted. Furthermore, we employed SHED from a single donor; moreover, quantitative research and extended incubation times are required to assess the cellular properties. Hence, large-scale studies with multiple materials and SHED from different donors should be conducted in the future to address the limitations of the current study.

The purpose of this study was to determine the influence of blood contamination on the properties of PM and EP by evaluating the changes in the surface microhardness following exposure to FBS and by observing the surface morphology. Exposure to FBS reduced the Vickers microhardness of PM and EP. In addition, EP was more significantly affected by exposure to FBS.

Conclusion

Exposure to FBS has disrupted the setting of MTA, resulting in a lower Vickers microhardness and a modified surface without laminar or acicular crystals. The surface microhardness of EP was more significantly affected by exposure to FBS than PM. Thus, clinicians should attempt to control hemorrhage during the application of MTA in all clinical scenarios. PM may be a more favor-

able option than EP in cases where blood contamination is anticipated.

Conflicts of Interest

The authors have no potential conflicts of interest to disclose.

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