Original Article

Effects of Blood Contamination on Apatite Formation, pH and Ion Release of Three Calcium Silicate-based Materials

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Abstract

The aim of this study was to assess the effects of blood contamination on the apatite formation, pH and ion release of three calcium silicate-based materials. ProRoot MTA (WMTA), Biodentine and TotalFill BC RRM putty (TRRM) were exposed to blood (blood-contaminated condition) or normal saline (non-blood-contaminated condition) for 24 hours. Samples were divided into 6 groups: WMTA without blood, WMTA with blood, Biodentine without blood, Biodentine with blood, TRRM without blood and TRRM with blood. Three samples of each group were analysed for apatite formation using X-ray diffractometer (XRD) and an energy-dispersive X-ray spectoscope (EDX) integrated into a scanning electron microscope (SEM) after immersion in phosphate-buffered saline (PBS) for 1, 7, 14 and 28 days. Five samples of each group were used to measure pH, calcium and silicon ion release using a pH meter and inductively coupled plasma-optical emission spectrometer (ICP-OES) after immersion in deionized water for 1, 7, 14 and 28 days. Apatite formation was found in the blood-contaminated groups later than the non-blood-contaminated groups. In both conditions, WMTA demonstrated apatite formation earlier than other materials. The pH and calcium ion release of the materials were not significantly affected by blood contamination ($P>0.05$). Silicon ion release was reduced in all blood-contaminated groups ($P<0.05$). Blood contamination delays apatite formation and decreases silicon ion release of calcium silicate-based materials.

Keyword: Apatite formation, Blood contamination, Calcium silicate-based materials, Ion release

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Introduction

A characteristic of bioactivity is the ability to form apatite when in contact with a serum-like solution.[1] The development of a carbonated apatite surface after immersion in biological fluids is one of the key elements for tissue adaptation.[2] Apatite formation provides clinical advantages by creating an appropriate environment for cell differentiation and supporting hard tissue formation.[3,4]

Mineral trioxide aggregate (MTA) is a calcium silicate-based material that is widely used in endodontic procedures.[5] An advantage of MTA is its bioactivity, which results in the formation of a bond between mineralized tissue and the material.[6] The *In vitro* bioactivity of MTA has been reported after immersion in phosphate-containing fluids.[7,8] Other advantages of MTA are the release of inorganic ions and generating an alkaline pH.[9] Calcium ion release induces osteoblast proliferation, differentiation and extracellular matrix mineralization.[10] while silicon ions are essential for metabolic processes associated with bone calcification and inducing apatite precipitation.[11]

Several new calcium silicate–based materials have been developed, aiming to improve MTA drawbacks of a long setting time and tooth discoloration. Biodentine (Septodont, Saint-Maur-des-Fosses, France) has a reduced setting time by adding calcium chloride as a setting accelerator and maintains tooth color stability with an alternative radiopacifier.[12,13] Another material is TotalFill BC RRM putty (TRRM) (BUSA, BRASSELER, Savannah, GA, USA), which is produced as a premixed putty product to avoid mixing inconsistency. These materials are recommended for the same clinical applications as MTA because they exhibit similar properties.[14,15]

In clinical procedures such as endodontic surgery, perforation repair, vital pulp therapy and regenerative endodontics, MTA may come into contact with blood during placement. The contamination of MTA by blood has been investigated in a number of laboratory studies in terms of the effects on its properties.[16-18] Blood contamination had a detrimental effect on the surface microhardness of MTA and caused a change in the surface microstructure[16] and compressive strength.[17] In addition, exposure to blood during setting has a negative effect on marginal adaptation of MTA.[18]

In endodontic applications, it could be difficult to completely avoid material surface contamination with blood. Different calcium silicate-based materials may show varying degrees of bioactivity, because of differences in their compositions and ion releasing properties.[19,20] Thus, the aim of this study was to assess the effects of blood contamination on the apatite formation, pH and ion release of WMTA, Biodentine and TRRM.

Materials and Methods

Specimen Preparation

The study protocol was approved by the Human Research Ethics Committee of the Faculty of Dentistry, Chulalongkorn University (HREC-DCU 2017-051). Whole fresh blood was collected from a healthy volunteer after informed consent.

Three calcium silicate-based materials, ProRoot MTA (WMTA) (Dentsply Sirona Endodontics, Tulsa, OK, USA), Biodentine and TRRM were used. The compositions of the test materials were given in Table 1.

Table 1 *Composition of test materials.*

Material	Composition
ProRoot MTA (WMTA)	Powder: tricalcium silicate, dicalcium silicate, tricalcium aluminate, bismuth oxide, calcium sulphate dihydrate or gypsum Liquid: water
Biodentine	Powder: tricalcium silicate, dicalcium silicate, calcium carbonate, zirconium oxide, calcium oxide, iron oxide Liquid: water, calcium chloride, a hydrosoluble polymer
TotalFill BC RRM (TRRM)	Calcium silicate, zirconium oxide, tantalum pentoxide, calcium phosphate monobasic, filler agents

The materials were prepared in accordance with the manufacturer's instructions. WMTA was mixed with distilled water using a 3:1 powder-to-water ratio. Biodentine was mixed by adding 5 drops of liquid into the powder capsule and mixed for 30 seconds using an amalgamator at 4,000 rotations/minute. TRRM was a premixed putty material.

Plaster of Paris ring moulds[21] (5 mm in diameter, 2 mm in height) were prepared and stored at 37°C in a water bath for 24 hours. The moulds were placed on a glass slide and then dropped with 10 µL of whole fresh blood (blood-contaminated specimens) or 10 µL of normal saline (non-blood-contaminated specimens). The moulds were then filled with their respectively prepared materials as follows.

Group 1: WMTA exposed to normal saline (WMTA/non-blood)

Group 2: WMTA exposed to blood (WMTA/blood)

Group 3: Biodentine exposed to normal saline (Biodentine/non-blood)

Group 4: Biodentine exposed to blood (Biodentine/blood)

Group 5: TotalFill BC RRM exposed to normal saline (TRRM/non-blood)

Group 6: TotalFill BC RRM exposed to blood (TRRM/blood)

Each specimen was placed in an incubator for 24 hours (37 ± 1°C, >95 % relative humidity) to set. After setting, the disc-shaped specimens were sterilised with ultraviolet light for 30 minutes per side.

Apatite formation

Three specimens per group were individually immersed in 5 ml of PBS [22] and stored at 37 ± 1°C for 1, 7, 14 and 28 days. The PBS was changed weekly. At each time point, the specimens were dried in a vacuum desiccator for 1 day. Apatite formation was analyzed using a X-ray diffractometer (XRD) and an energy-dispersive X-ray spectoscopy (EDX) that was integrated into a scanning electron microscope (SEM).

XRD. Specimen phase composition was evaluated using XRD (D8 Discover, Bruker, Karlsruhe, Germany). The scan range was 10-65°2θ with a scan speed of 2°2θ per minute. Phase identification was accomplished using search match software using the ICDD database (International Centre for Diffraction Data, Newtown Square, PA).

SEM and EDX. The specimens were mounted on aluminum stubs and coated with platinum. The specimen surface was observed using SEM (JSM-6610LV, JEOL, Tokyo, Japan) at 10,000x. Elemental analysis was performed using EDX (X-MaxN 50, Oxford instruments, Oxfordshire, United Kingdom) and the data were used to calculate the surface calcium-to-phosphorus atomic (Ca/P) ratio (stoichiometric hydroxyapatite Ca/P ratio is 1.67).[23]

pH and Ion Release

Five specimens per group were individually immersed in 5 ml of deionized water. The solutions were collected after 1, 7, 14 and 28 days for evaluation. The deionized water was replaced at the end of each time point. The pH measurements were performed on the collected solutions with a pH meter (710A, Thermo Scientific Orion, USA). Calcium and silicon ion release were measured by ICP-OES (OPTIMA 7300DV, PerkinElmer, USA).

Statistical Analysis

The pH and ion release were presented as means and standard deviation. Two-way analysis of variance with the Student *t*-test was used to determine the significant differences between groups using IBM SPSS software version 22.0 (IBM Corp., Armonk, NY, USA). The results were considered statistically significant if the *p*-value was less than 0.05.

Results

Apatite formation

After immersion in PBS for 1, 7, 14 and 28 days, the crystal structure of hydroxyapatite on the materials was determined by XRD. Surface morphology and elemental composition of the materials were examined by SEM and EDX. Representative data of each group were shown in Figure 1, 2 and 3.

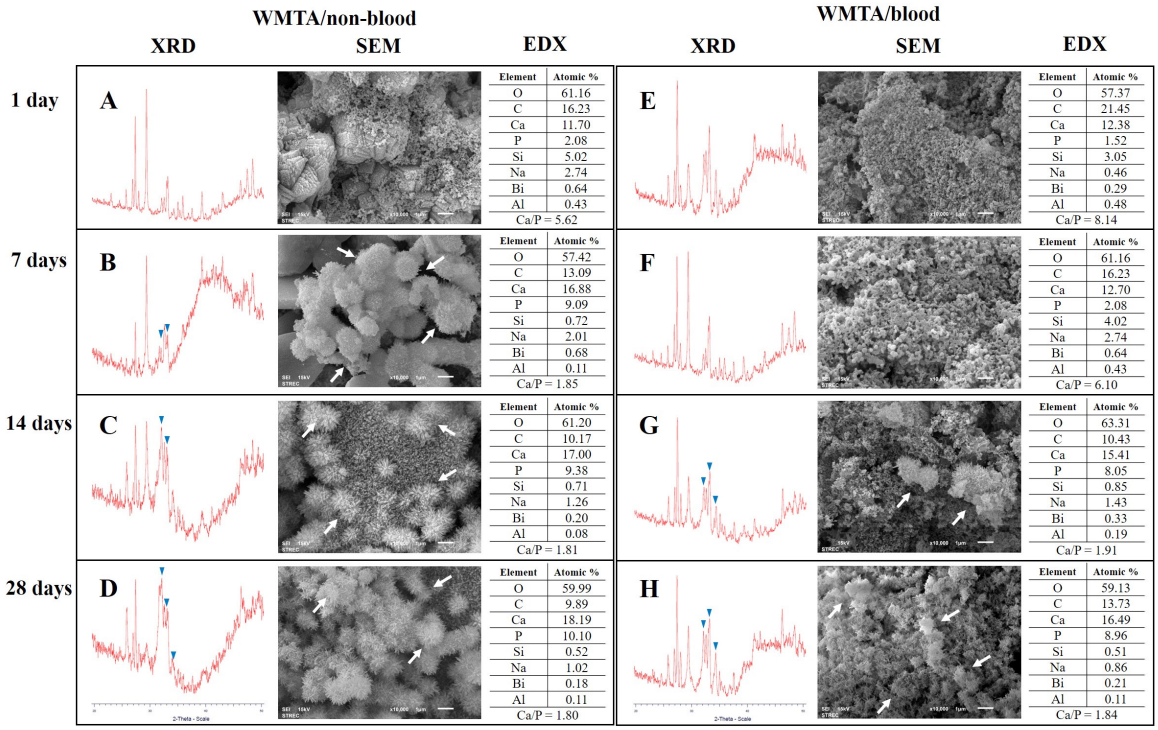

Figure 1 *Representative XRD, SEM and EDX data of WMTA/non-blood after immersion in PBS for 1, 7, 14 and 28 days (A, B, C and D respectively) and WMTA/blood after immersion in PBS for 1, 7, 14 and 28 days (E, F, G and H respectively). ▼ = hydroxyapatite. SEM images of the surface of test materials at x10,000 magnification, white arrow = spherical precipitates.*

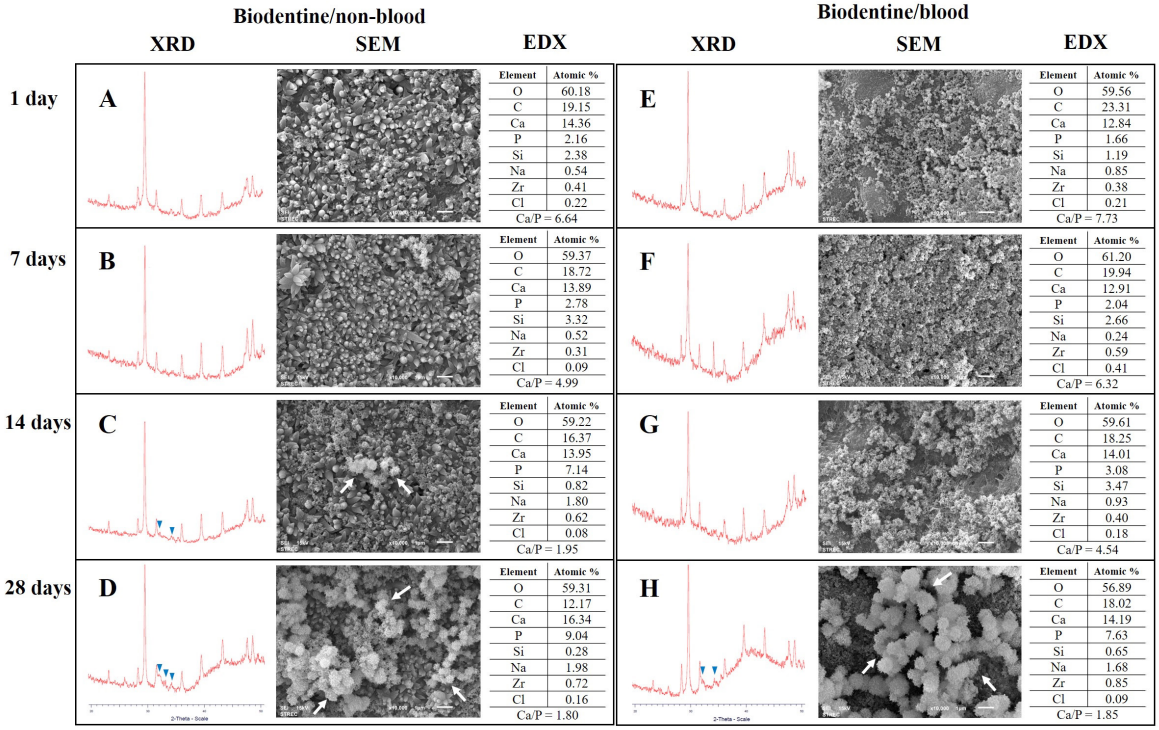

Figure 2 *Representative XRD, SEM and EDX data of Biodentine/non-blood after immersion in PBS for 1, 7, 14 and 28 days (A, B, C and D respectively) and Biodentine/blood after immersion in PBS for 1, 7, 14 and 28 days (E, F, G and H respectively). ▼ = hydroxyapatite. SEM images of the surface of test materials at x10,000 magnification, white arrow = spherical precipitates.*

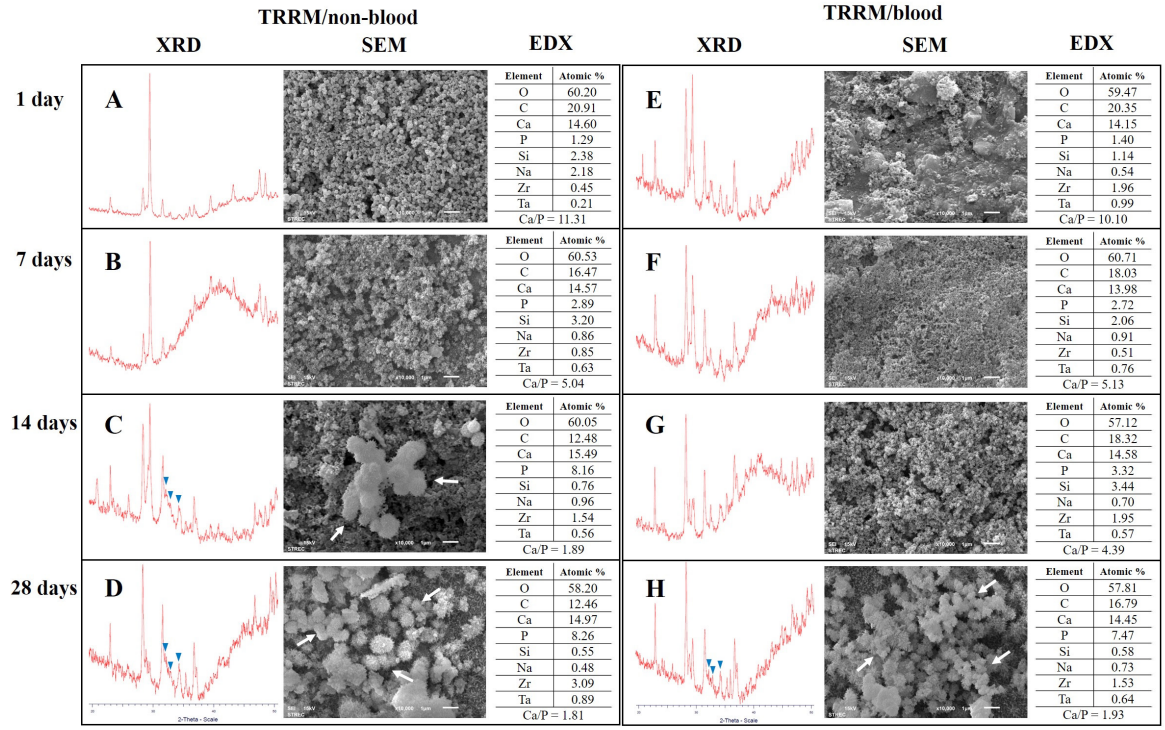

Figure 3 *Representative XRD, SEM and EDX data of TRRM/non-blood after immersion in PBS for 1, 7, 14 and 28 days (A, B, C and D respectively) and TRRM/blood after immersion in PBS for 1, 7, 14 and 28 days (E, F, G and H respectively).* ▼ *= hydroxyapatite. SEM images of the surface of test materials at x10,000 magnification, white arrow = spherical precipitates.*

The WMTA/non-blood group

XRD detected hydroxyapatite crystals ($Ca_{10}(PO_4)_6(OH)_2$, ICDD: 00-003-0747) at days 7, 14 and 28, but not at day 1 (Fig. 1A – 1D). SEM demonstrated the amorphous and irregular surface at day 1 (Fig 1A), then at days 7, 14 and 28, spherical precipitates formed over the surface (Fig. 1B – 1D). EDX indicated the presence of elements of oxygen (O), carbon (C), calcium (Ca), phosphorus (P), silicon (Si), sodium (Na), bismuth (Bi) and aluminum (Al). Ca/P ratio was 5.62 at day 1, then, decreased to 1.85, 1.81 and 1.80 at days 7, 14 and 28, respectively.

The WMTA/blood group

XRD found hydroxyapatite crystals at days 14 and 28, but not at days 1 and 7 (Fig. 1E – 1H). SEM displayed spherical precipitates on the surface at days 14 and 28. EDX showed the presence of O, C, Ca, P, Si, Na and Bi with Ca/P ratio of 8.14, 6.10, 1.91 and 1.84 at days 1, 7, 14 and 28, respectively (Fig. 1E – 1H).

The Biodentine/non-blood group

XRD detected hydroxyapatite crystals at days 14 and 28, but not at days 1 and 7 (Fig. 2A – 2D). SEM showed spherical precipitates over the surface at days 14 and 28. EDX revealed the presence of O, C, Ca, Si, P, Na, zirconium (Zr) and chlorine (Cl) with Ca/P ratio of 6.64, 4.49, 1.95 and 1.80 at days 1, 7, 14 and 28, respectively (Fig. 2A – 2D).

The Biodentine/blood group

XRD detected hydroxyapatite crystals at day 28, but not at days 1, 7 and 14 (Fig. 2E – 2H). At days 28, spherical precipitates were found over the surface under SEM. EDX revealed the presence of O, C, Ca, Si, P, Na, Zr and Cl with Ca/P ratio of 7.73, 6.32, 4.54 and 1.85 at days 1, 7, 14 and 28, respectively (Fig. 2E – 2H).

The TRRM/non-blood group

XRD detected hydroxyapatite crystals at days 14 and 28, but not at days 1 and 7 (Fig. 3A –3D). SEM showed spherical precipitates over the surface at days 14 and 28 (Fig. 3C –3D). EDX revealed the presence of O, C, Ca, P, Si, Na, Zr and tantalum (Ta) with Ca/P ratio of 11.31, 5.04, 1.89 and 1.81 at days 1, 7, 14 and 28, respectively (Fig. 3A – 3D).

The TRRM/blood group

XRD detected hydroxyapatite crystals at day 28, but not at days 1, 7 and 14 (Fig. 3E – 3H). At day 28, spherical precipitates were found over the surface under

SEM. EDX revealed the presence of O, C, Ca, P, Si, Na, Zr and Ta with Ca/P ratio of 10.10, 5.13, 4.39 and 1.93 at days 1, 7, 14 and 28, respectively (Fig. 3E – 3H).

pH and Ion Release

The pH of specimens immersed in deionized water for 1, 7, 14 and 28 days were shown in Table 2.

Each material exhibited an alkaline pH (11.03-12.14) at each time point. No significant differences was found between conditions of the same material at any time point. Biodentine and TRRM displayed a significantly higher pH compared with WMTA at each time point ($P<0.05$).

The calcium ion releases of each material immersed in the deionized water for 1, 7, 14 and 28 days were determined (Table 3).

There was no significant difference in calcium ion release between conditions of the same material at each time point. However, the Biodentine/non-blood and Biodentine/blood groups showed a significantly higher calcium ion release compared with the WMTA/non-blood and WMTA/blood groups at each time point ($P<0.05$).

The silicon ion releases of each material immersed in the deionized water for 1,7,14 and 28 days were displayed in Table 4.

Table 2 *pH of test materials immersed in deionized water.*

Period (day)	WMTA /non-blood	WMTA /blood	Biodentine /non-blood	Biodenine /blood	TRRM /non-blood	TRRM /blood
1	11.24 ± 0.2^{a}	11.36 ± 0.11^{a}	11.47 ± 0.06^{b}	11.43 ± 0.04^{b}	11.46 ± 0.07^{b}	11.51 ± 0.04^{b}
7	11.49 ± 0.22^{a}	11.67 ± 0.12^{a}	12.05 ± 0.08^{b}	12.07 ± 0.11^{b}	12.12 ± 0.03^{b}	12.14 ± 0.03^{b}
14	11.27 ± 0.25^{a}	11.32 ± 0.21^{a}	11.73 ± 0.2^{b}	11.83 ± 0.01^{b}	11.69 ± 0.14^{b}	11.7 ± 0.15^{b}
28	11.03 ± 0.34^{a}	11.3 ± 0.21^{a}	11.63 ± 0.1^{b}	11.59 ± 0.07^{b}	11.55 ± 0.1^{b}	11.41 ± 0.15^{b}

Mean ± SD, n = 5.

Mean values followed by different superscripted letters in the same row were significantly different (P<0.05).

Table 3 *Calcium ion release (mg/L) of test materials immersed in deionized water.*

Period (day)	WMTA /non-blood	WMTA /blood	Biodentine /non-blood	Biodenine /blood	TRRM /non-blood	TRRM /blood
1	32.24 ± 18.01^{a}	52.76 ± 39.27^{a}	184.92 ± 41.22^{b}	190.08 ± 23.53^{b}	62.32 ± 32.38^{a}	74.25 ± 22.39^{a}
7	66.55 ± 56.72^{a}	84.71 ± 12.93^{a}	215.3 ± 31.33^{b}	197.58 ± 57.24^{b}	195.36 ± 13.15^{b}	193.1 ± 30.91^{b}
14	60.69 ± 39.13^{a}	72.39 ± 57.72^{a}	159.73 ± 54.09^{b}	160.73 ± 52.76^{b}	124.97 ± 33.87^{ab}	121.74 ± 32.59^{ab}
28	38.82 ± 22.69^{a}	39.96 ± 8.12^{a}	114.83 ± 23.29^{b}	113.3 ± 32.15^{b}	83.22 ± 18.1^{c}	65.82 ± 18.55^{c}

Mean ± SD, n = 5.

Mean values followed by different superscripted letters in the same row were significantly different (P<0.05).

Table 4 *Silicon ion release (mg/L) of test materials immersed in deionized water.*

Period (day)	WMTA /non-blood	WMTA /blood	Biodentine /non-blood	Biodenine /blood	TRRM /non-blood	TRRM /blood
1	1.21 ± 0.12^{a}	0.87 ± 0.13^{b}	0.8 ± 0.1^{b}	0.41 ± 0.14^{c}	0.78 ± 0.07^{b}	0.48 ± 0.09^{c}
7	1.64 ± 0.18^{a}	1.11 ± 0.2^{b}	0.94 ± 0.13^{b}	0.64 ± 0.13^{c}	0.96 ± 0.12^{b}	0.57 ± 0.14^{c}
14	1.4 ± 0.2^{a}	0.76 ± 0.18^{b}	0.83 ± 0.13^{b}	0.44 ± 0.08^{c}	0.76 ± 0.11^{b}	0.49 ± 0.04^{c}
28	1.49 ± 0.25^{a}	0.85 ± 0.15^{b}	0.79 ± 0.14^{b}	0.4 ± 0.05^{c}	0.7 ± 0.12^{b}	0.41 ± 0.08^{c}

Mean ± SD, n = 5.

Mean values followed by different superscripted letters in the same row were significantly different (P<0.05).

Within the same material, the non-blood-contaminated groups had significantly higher silicon ion releases compared with the blood-contaminated groups at each time point ($P<0.05$). In each group, the pH, calcium, and silicon ion release increased to a maximum level at day 7, then, gradually decreased.

Discussion

The present study tested three calcium silicate-based materials to compare the effects of blood contamination on apatite formation, pH and ion release. In the majority of endodontic applications, material comes into contact with blood or tissue fluid during placement. Many previous studies investigated MTA's properties when it was mixed with blood.[16,17] However, mixing MTA with blood represents a situation that may be far from the clinical reality in which the material comes concomitantly into contact with fluids. Therefore, this study designed the method of contamination by placing the materials into the moulds that were filled with human whole fresh blood immediately after mixing to most closely simulate the clinical conditions.

MTA is one of the calcium silicate-based materials considered to be bioactive. Previous studies reported that, when it came into contact with phosphate-containing fluids, MTA produced hydroxyapatite that represented bioactivity.[7,8] For *in vitro* evaluation of apatite formation, various methods have been used to identify the deposits on the material surface.[19,24,25] XRD identifies the phases related to the materials,[26] while SEM with EDX provides images of a material's surface microstructure and quantifies the elements on its the surface layers.[5] Spherical precipitates observed under SEM were recognised as apatite crystals (apatite spherulites) as mentioned in previous studies.[26-28] Apatite precipitates have been previously observed on MTA, Biodentine and EndoSequence Root Repair Material surfaces immersed in phosphate-buffered solutions.[22,29,30] In the present study, hydroxyapatite crystals were detected under XRD in all test materials at different immersion times. The results showed that detection of hydroxyapaptite crystal by XRD concurred with SEM and EDX analyses. SEM micrographs displayed spherical precipitates over the material surface and EDX revealed Ca/P ratio similar to stoichiometric hydroxyapatite[23] at all-time points of which XRD detected the hydroxyapatite crystals.

In this study, when apatite was detected on the material via XRD, EDX concurrently demonstrated the Ca/P ratio that is similar to stoichiometric hydroxyapatite,[23] suggesting the presence of calcium phosphate deposites.[27] Meanwhile, Si was decreased from the surface, suggesting its accumulation mainly in the subsurface calcium silicate hydrate phase.[27] Calcium silicate-based materials have been made based on a composition of calcium and silicate. MTA is one of the most popular calcium silicate-based materials used in endodontics which mainly composed of tricalcium silicate, dicalcium silicates, tricalcium aluminate and bismuth oxide. Meanwhile, Biodentine is a powder and liquid system that consists of tricalcium silicate, dicalcium silicate, calcium oxide, calcium carbonate and zirconium oxide as radiopacifier. The liquid contains calcium chloride which decreases the setting time and a hydrosoluble polymer as a water reducing agent. TRRM is produced as a premixed product in putty consistency which is composed of calcium silicates, zirconium oxide, tantalum oxide, calcium phosphate monobasic and filler agents. In this study, although all materials had bioactivity potential, WMTA demonstrated apatite precipitation earlier than other materials in both conditions. Notably, it is well-known that ionic dissolution is an initial step in the apatite precipitation.[31] Different calcium silicate-based materials have shown varying degrees of bioactivity, because compositional differences may influence the ion dissolution.

An apatite layer on a calcium silicate-based material forms as a result of the chemical reactions between the material and solution. During the hydration reaction, the silanol groups (Si-OH) on calcium silicate hydrate are deprotonated in an alkaline pH.[32] Negative charges (SiO-) interact with calcium ions, resulting in an increase in cations that can act as nucleation sites for apatite formation from amorphous calcium phosphate.[1] In the present study, the blood-contaminated groups exhibited apatite precipitation later than the non-blood-contaminated groups. Furthermore, prior to detecting

apatite formation, blood-contaminated groups displayed a lower Si atomic percentage than non-blood-contaminated groups. Thus, blood might impede Si on material's surface during the hydration reaction leading to delayed apatite formation. The presence of silicon is crucial for a material to exhibit bioactivity.[32] Dissolving the material in an alkaline environment releases silicon ion that form the silica gel surface layer in calcium silicate hydrate gel and creates silanol groups.[32] Our results indicated that for each material, silicon ion release from the non-blood-contaminated group was significantly higher than the blood-contaminated counterpart. These results suggest that blood may reduce silicon ion release, and inhibit the development of the silica gel layer on the material's surface. The role of silicon ions in hard tissue formation was suggested in early bone calcification and inhibition of osteoclastogenesis.[11,33] Our study showed that the release of silicon ions from WMTA was greater than that from Biodentine and TRRMno matter whether there was blood contamination or not. Increased silicon ion release facilitates osteoblast differentiation and inhibits osteoclastogenesis, thus promoting the healing of periapical tissues.[10, 33] However, whether the reduced silicon ion release in blood-contaminated calcium silicate-based materials has an impact on hard tissue formation requires further studies.

The results of this study showed that blood contamination delays apatite formation and decreases silicon ion release of calcium silicate-based materials, but not their pH or calcium ion release. Calcium ion and hydroxide exchange occur following calcium silicate hydration. MTA has an alkaline pH, resulting from calcium ion release and calcium hydroxide formation.[34] After the cement hydration reaction, it continues to release calcium and silicon ions.[32] Our study demonstrated that all test materials released calcium and silicon ions and maintained an alkaline pH that reached its maximum value at day 7 and decreased over time. WMTA's calcium ion release pattern is in accordance with a previous study.[35] Our results demonstrated that Biodentine and TRRM had a higher pH and calcium ion release compared with WMTA at all experimental time points. This finding is similar to a previous report where Biodentine showed a higher calcium ion release than that of WMTA.[22] The higher calcium ion release from Biodentine may be attributable to the calcium chloride added as an accelerator.[36] It has been demonstrated, by both *in vitro* and *in vivo* studies, that pulpal wound healing by the deposition of mineralized apatite depends on pH and calcium ion release.[37,38]

Lack of solubility has been mentioned as a preferred characteristics of root-end filling and root repair materials.[39] Though solubility of TRRM has not been previously reported, some studies have reported low or no solubility of WMTA.[40,41] While, Biodentine demonstrated higher solubility compare with WMTA.[42,43] High solubility could contribute to higher calcium ion release of Biodentine found in our study. Nonetheless, there was no report of solubility of these calcium silicate-based materials when contaminated with blood. Even though we did not find any significant difference in pH and calcium ion release between blood contaminated and non-contaminated conditions of the same material in this study, the solubility of these materials under blood contaminated condition remains to be identified.

The importance of hydroxyapatite in supporting osteoblastic differentiation which leads to bone bonding is well-known. The presence of an extensive apatite layer on biomaterials promotes the adsorption of proteins and the formation of a protein layer that favours osteoblast adhesion.[44,45] Moreover, their deposition over time improved sealing at the interface between dentin and materials.[8] Therefore, the delay of an apatite formation may affect the osteoblast behaviour and the sealing ability between dentin and materials. Further studies are required to examine the effects of blood contamination on cell-material as well as dentine-material interactions.

Conclusions

Blood contamination delays apatite formation and decreases silicon ion release of calcium silicate-based materials, but not their pH or calcium ion release. WMTA demonstrates earlier apatite formation and greater silicon ion release compared with Biodentine and TRRM when exposed to blood. Based on these findings, we hypothesize that blood contamination of calcium silicate-based

materials interrupts ion exchange during hydration and reduces the formation of the silanol groups that act as sites for nucleation of apatite formation. Blood exposure reduces a material's silicon ion release. Therefore, blood may inhibit the development of a silica gel layer on a material's surface. Thus, hemorrhage control is needed before placing the materials in the clinical situations.

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