Microstructure and color stability of calcium silicate-based dental materials exposed to blood or platelet-rich fibrin

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Research Article

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Abstract

Objectives To investigate the effects of blood and platelet-rich fibrin (PRF) on the hydration, microstructure, and color stability of three hydraulic calcium silicate cements (HCSCs), OrthoMTA, RetroMTA, and TotalFill-BC-RRM.

Materials and methods The HCSCs were prepared and placed into polyethene molds and transferred to Eppendorf tubes containing PRF, blood, or PBS then incubated for 1 week or 1 month. The microstructure and hydration of the cements were studied by scanning electron microscopy (SEM), Energy-dispersive X-ray spectroscopy (EDS) and X-ray diffraction (XRD). The chromatic alteration of materials was also measured using a spectrophotometer. The data for color stability were analyzed using 2-way analysis of variance.

Results There was no significant difference between the color stability of cements exposed to PBS ($p > 0.05$). The chromatic alteration of cements exposed to blood was significantly greater than those exposed to PRF and PBS ($p < 0.001$). In the presence of blood and PRF, the color change of OrthoMTA was significantly greater than that of RetroMTA and TotalFill ($p < 0.05$), with no significant difference between RetroMTA and TotalFill ($p > 0.05$). XRD analysis of all cements, revealed a calcium hydroxide peak after 1-week and 1-month exposure to the media; however, OrthoMTA and TotalFill exposed to blood and PRF for 1 month were associated with a reduction of the calcium hydroxide peak. SEM images revealed cements exposed to PBS had a different surface microstructure compared to those exposed to blood and PRF. Furthermore, the surface microstructure of HCSCs was influenced by the type of cement radiopacifier (bismuth oxide or zirconium oxide). EDS analysis of the elemental composition in all groups displayed peaks of Ca, O, C, Si, P and Al.

Conclusions Color stability, hydration behavior and microstructure of HCSCs were affected by exposure to PRF and blood and the type of cement radiopacifier.

Clinical relevance As some important physicochemical properties of HCSCs could be influenced by the environmental conditions and the type of radiopacifier, alternatives to blood clot and HCSCs containing substitutes for bismuth oxide might be more suitable in RETs.

Introduction

Management of immature teeth with necrotic pulps is one of the most challenging endodontic treatment modalities [1]. Chemo-mechanical root canal disinfection and subsequent filling of the root canal using conventional techniques is difficult and has led to the introduction of regenerative endodontic treatments (RET) [2]. The main goal of RET is to resolve periradicular disease, whilst ensuring continuing thickening of the dentine making up the root canal walls, increasing the length of the root, and allowing the root apex to continue developing. RET is based on recruiting stem cells into the bacteria-free root canal to populate a resorbable scaffold inside the root canal system [3]. This is then followed by protecting the

scaffold through the placement of a coronal barrier, normally a hydraulic calcium silicate cement (HCSC), followed by a conventional coronal restoration [4].

A blood clot, created by provoking bleeding from the periapical tissues into the root canal system, has been used as a biological scaffold [5]. Over time, the advantages of platelet derivatives such as platelet-rich plasma (PRP) and platelet-rich fibrin (PRF), as an important source of growth factors that have been used to enhance the regeneration of various tissue defects, including the dentine-pulp complex, have been reported in RET [6]. PRF, as an easy-to-use autologous scaffold [7, 8] with a strong three-dimensional fibrin matrix, contains a high concentration of growth factors that are gradually released [9] that encourages the migration, proliferation and differentiation of stem cells [7, 10]. PRF has several advantages compared to blood and PRP [11], and clinical studies [11, 12] have used PRF as a scaffold in RET and achieved successful results.

Sealing and protecting the scaffold with an appropriate dental material is crucial to create a suitable environment for regeneration. Mineral trioxide aggregate (MTA), an HCSC, has been used as a coronal barrier in most of the studies in the field of RET [13]. The radiopacifier bismuth oxide found in the formulation of some HCSCs has been associated with tooth discoloration [14, 15]. Therefore, other HCSCs have been developed with lower potential for tooth discoloration by replacing the bismuth oxide with alternative radiopacifiers such as zirconium oxide [16].

Hydraulic calcium silicate-based cements need moisture to set, which they acquire physiologically from tissue fluids and blood. Calcium hydroxide, as a hydration by-product of HCSCs, combines with phosphate in the environment and forms hydroxyapatite as the key element in inducing hard tissue formation [17]. Studies have used various media such as phosphate-buffered saline (PBS) to simulate tissue fluids or induce the bioactivity of HCSCs [18].

As HCSCs are in close contact with scaffolds, the interaction between them might affect the physical properties of the cement [19, 20]. Indeed, the adverse effects of blood contamination on the physical and biological properties of HCSCs, such as bioactivity, hydration, setting time as well as tooth discoloration have been investigated [18–20, 21]. Several studies have reported the effect of PRF, as a beneficial natural scaffold, on color stability [22] and bioactivity potential [20] of some HCSCs. However, there is insufficient information on the effect of platelet-rich fibrin on the physical properties and colour stability of HCSCs containing various radiopacifiers. This study was designed to evaluate the effects of blood and PRF compared with those of PBS on the microstructure and colour stability of HCSCs containing bismuth oxide (OrthoMTA) to those that do not contain bismuth oxide (RetroMTA and TotalFill BC RRM). The null hypothesis was that would be no difference regarding the microstructure and color stability of OrthoMTA, RetroMTA, and TotalFill exposed to PBS, PRF, or blood.

Materials And Method

The materials investigated were OrthoMTA ® (BioMTA, Seoul, Korea), RetroMTA ® (BioMTA, Seoul, Korea), TotalFill BC RRM ® putty (FKG Dentaire, La Chaux-de-Fonds, Switzerland).

OrthoMTA and RetroMTA cements were prepared according to the manufacturer's instructions. TotalFill was available as a ready-to-use putty and did not require prior preparation. The materials were placed into polyethene cylindrical molds (3 mm diameter and 3 mm height) with minimal pressure. The polyethene cylinders containing the material were then transferred to 0.2 mL Eppendorf tubes that were filled with either blood, PRF, or PBS so that the lower surfaces of the test materials were just in contact with blood, PRF, or PBS. The Eppendorf tubes were then incubated at 37 °C and fully saturated humidity for 1 week or 1 month.

The whole fresh human blood used in this study was collected from a healthy consented volunteer. This study was approved by the Ethics Committee of Tehran University of Medical Sciences (Ethics code: IR.TUMS.DENTISTRY.REC.1398.027) and University of Jordan. To prepare PRF, fresh human blood was poured into a test tube without the addition of anticoagulants and immediately centrifuged (PRF Centrifuge DUO Quattro, Nice, France) at 1300 rpm for 8 min. To remove the exudates, the PRF clot was separated from the platelet-rich plasma and red blood cell layers and compressed in a compression box.

The surface microstructure and elemental composition of specimens were qualitatively assessed using scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopic (EDS), respectively. Also, the hydration process and phase composition of specimens were evaluated using X-ray diffraction analysis (XRD). Assessment of color stability was conducted by spectrophotometry.

SEM and EDS analysis

Specimens exposed to blood, PRF, or PBS for 1 week (n = 2 for each group) and 1 month (n = 2 for each group) were mounted on aluminum stubs, carbon-coated, and analyzed using a scanning electron microscope (Mira3 XMH SEM; TESCAN, Brno, Czech Republic) fitted with an energy-dispersive X-ray detector (SEM-EDS; TESCAN). The microstructure of surfaces exposed to blood, PRF, or PBS were examined with several magnifications (×5000 to ×25000) and element analysis was performed by EDS.

XRD analysis

For phase composition analysis of unhydrated cements (n = 2 for each group), 1-week (n = 2 for each group) and 1-month specimens (n = 2 for each group) exposed to blood, PRF, or PBS, the cements were removed from the polyethene cylindrical moulds, dried under a vacuum, and then crushed into a very fine powder. The powders were tested using a Philips X'Pert Pro diffractometer (PANalytical, Netherlands) which monochromatized Cu Kα radiation conditions (λ = 1.54 Å, operated on 35 mA and 40 kV current). The diffraction angles (2θ) were scanned from 15º to 65º. Phase identification was accomplished using Xpert HighScore software for XRD analysis. In addition to hydrated specimens, the phase composition of unhydrated OrthoMTA and RetroMTA powder and TotalFill putty were determined.

Assessment of color stability

The color of the lower surface of HCSCs exposed to blood, PRF, or PBS (n = 12 for each cement exposed to each medium), was measured by spectrophotometry (VITA Easyshade V; Zahnfabrik, Bad Säckingen, Germany). The color assessment was performed by the same investigator under steady laboratory conditions and the device was calibrated before use for each specimen. Color measurements were performed prior to exposure of HCSCs to blood, PRF or PBS as the baseline color and 1 month after exposure.

The chromatic alteration (ΔE) between the initial and the second measurements was calculated using the measurement of the L^*, a^* and b^* values and the following formula:

$$\Delta E = [(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2]^{1/2}$$

In this color measurement, L^* indicates the value of lightness-darkness, a^* indicates greenness-redness, and b^* indicates blueness-yellowness.

Statistical analysis

Data were evaluated using SPSS software (PASW Statistics 18; SPSS Inc, Chicago, IL). Two-way ANOVA and Tukey post hoc tests were used to evaluate the effects of cements and media variables on chromatic alteration (ΔE). The level of significance was set at $p < 0.05$.

Results

XRD analysis

The results of XRD analysis of three HCSCs are shown in Fig. 1.

OrthoMTA

XRD analysis of the unhydrated powder demonstrated the phases present, namely tetra-calcium aluminoferrite, tri-calcium aluminate, bismuth oxide, dicalcium silicate, and tricalcium silicate. One-week specimens exposed to PBS, PRF, and blood and 1-month specimens exposed to PBS, displayed di-calcium aluminate and calcium hydroxide, in addition to components presented in the unhydrated powder. However, calcium hydroxide peaks were greatly reduced in the specimens exposed to PRF and blood for 1 month.

RetroMTA

XRD analysis of the unhydrated powder revealed peaks of calcium carbonate, calcium zirconium complex, zirconium oxide, dicalcium silicate, and tricalcium silicate. In addition to the ingredients of the unhydrated powder, calcium hydroxide was observed in the specimens exposed to PBS, PRF, and blood

for 1week and 1 month. Also, the specimens exposed to PBS for 1 month, had carbonate apatite depositions.

TotalFill BC RRM

Analysis of the unhydrated putty revealed peaks of monobasic calcium phosphate, tantalum pentoxide, zirconium oxide, dicalcium silicate, and tricalcium silicate. In addition to the phases present in the unhydrated putty, calcium hydroxide was identified in all hydrated specimens after 1 week and 1 month, except the specimens exposed to PRF and blood for 1 month when calcium hydroxide deposits were substantially reduced.

SEM analysis

OrthoMTA

SEM images revealed accumulations of coral-like particles after 1 week of exposure to PBS. One-month specimens exposed to PBS revealed hexagonal and globular particles within clusters of crystalline structures at higher magnifications. OrthoMTA specimens exposed to PRF or blood for 1-week and 1-month had no prominent crystalline structures (Fig. 2).

RetroMTA

After 1 week of exposure to PBS, accumulations of plate-like crystals among coral-like shaped aggregates on the surface of RetroMTA were formed. Spherical aggregates composed of minute particles along the periphery within a multi-globular matrix were observed on the surface of 1-month specimens of RetroMTA. Fused globular aggregates were observed on the surface of the RetroMTA specimens exposed to PRF for 1 week. Smaller more compacted fused globular particles were demonstrated on 1-month RetroMTA samples exposed to PRF. After 1 week of exposure to blood, islands of unstructured surfaces mixed with small crystalline particles were observed. One-month RetroMTA specimens exposed to blood had fused globular aggregates (Fig. 3).

TotalFill BC RRM

Bundles of string-like aggregates and aggregates of coral-like particles were present after 1-week and 1-month exposure to PBS, respectively. The material had an unstructured surface after 1-week exposure to PRF. However, aggregates of single and multiple globular structures on the surface of specimens exposed to PRF for 1 month were found. Specimens exposed to blood revealed small globular aggregates after 1 week; however, larger and more fused globular particles were seen on the surface of 1-month samples (Fig. 4)

EDS analysis

Analysis of the elemental composition of precipitates formed on the cement surfaces in all groups displayed high peaks of Ca, O, C (Fig. 5 a-c). In addition, a high peak of Si was observed on the surface of

OrthoMTA specimens. Precipitates on the surface of OrthoMTA exhibited moderate peaks of Bi and Al and low peaks of P.

EDS analysis of RetroMTA specimens revealed moderate peaks of Si and low peaks of Zr, P, and Al. Whereas, precipitates on TotalFill BC RRM displayed moderate peaks of Si and Zr, and low peaks of Al, P, and Ta.

Color stability assessment

The mean values for the chromatic alteration in each subgroup are illustrated in Fig. 6. Analysis of the chromatic alteration in the various groups after 1-month exposure to PBS, PRF and blood revealed a significant difference between the types of cement in the presence of different media ($p = 0.016$).

Chromatic alteration (ΔE) of samples after one month by type of media

In the present study, there was no significant difference between the chromatic alteration (ΔE) of the three types of cement exposed to PBS ($p > 0.05$). There was a significant difference between the chromatic alteration produced by the different cements exposed to blood and PRF. In the presence of blood, the chromatic alteration of OrthoMTA specimens was significantly greater than RetroMTA and TotalFill specimens ($p < 0.001$) as well as in the presence of PRF ($p < 0.005$ and $p < 0.003$, respectively for RetroMTA and TotalFill. No significant difference was found between RetroMTA and TotalFill ($p > 0.05$).

Chromatic alteration (ΔE) of samples after one month by cement

The chromatic alteration of all cements exposed to blood was significantly greater than those exposed to PRF and PBS ($p < 0.001$). No significant difference was found between cements exposed to PBS or PRF ($p > 0.05$)

Photos of a specimen from each group are shown in Fig. 7.

Discussion

The current study exposed cements for two periods of time, 7 days and one month, in order to assess the progress of the hydration process and microstructure of the materials exposed to various media.

To simulate the intracanal coronal barrier used in RET, HCSCs were placed in polythene cylindrical molds and transferred to Eppendorf tubes so that their lower surface was in contact with fresh human whole blood or PRF, which is used as a natural scaffold in regenerative treatments.

Hydraulic calcium silicate-based cements need moisture to set, which they acquire from physiological tissue fluids and blood. The term bioactivity usually refers to the release of OH^- and Ca^{2+} ions, which interact with the mineral components of dentine to form a mineral bond [23]. Calcium hydroxide, as a hydration by-product of HCSCs, combines with phosphate in the environment and forms hydroxyapatite

as the key element in inducing hard tissue formation [17]. PBS is a simulated tissue fluid containing phosphate that mimics clinical conditions in laboratory studies and was considered an ageing medium in the control group of the present study [20, 24].

The present results revealed that in contrast to PBS and PRF, blood contamination significantly increased chromatic alteration associated with all materials. This finding is in accordance with the results of studies that reported increased discoloration following blood contamination of HCSCs [20, 21, 25–27].

Fe^{3+}, a dark brown by-product of natural oxidation and reduction of erythrocyte ferrous (Fe^{2+}) may cause chromatic alteration of materials [26, 27]. Also, it has been demonstrated that diffusion of blood components into the porosities of partially hydrated cements may exacerbate chromatic alteration [25]. The partial absence of erythrocytes in platelet derivatives such as PRF might be the cause for less color change compared to whole human blood [22].

The chromatic alteration of OrthoMTA specimens exposed to blood and PRF was significantly more pronounced than TotalFill and RetroMTA. This finding may be linked to the content of tetracalcium aluminofrrite and bismuth oxide in OrthoMTA, which is not present in other test cements. Presence of metal components such as iron and bismuth oxide is one of the important factors affecting the color of endodontic cements and subsequently tooth crown discoloration. The oxidation of residual iron components, in the set material, relating to the calcium aluminoferrite phase of the powder, has been considered as a possible mechanism for tooth discoloration by tooth-colored MTAs [26]. Moreover, relating to the mechanism of discoloration caused by bismuth oxide, the theory of bismuth oxidation has been proposed. This reaction results in the creation of unstable oxygen, the reaction of oxygen with carbon dioxide, and then the production of bismuth carbonate as a discoloration agent [28, 29]. In this regard, some studies suggest that MTA chromatic alteration may be related to bismuth oxide, which has been added to both white and grey MTA as a radiopacifier [30]. While several studies have shown less tooth discoloration in the cement containing zirconium oxide or tantalum oxide than bismuth oxide containing cements [16, 21, 28]. It should be noted that the final discoloration of the tooth can be due to the discoloration of the cement itself, the interaction of bismuth oxide in some types of cement with dentin, or the penetration of blood and its components into dentinal tubule. Therefore, discoloration caused by blood may mask the effects of cements with or without bismuth oxide in tooth discoloration and prevent an accurate study of the color stability of HCSCs.

In the current study, XRD analysis of the three cements revealed high peaks of calcium hydroxide, after 1-week exposure to PBS, PRF and blood. However, the analysis of OrthoMTA and TotalFill exposed to blood and PRF after 1 month confirmed that the intensity of calcium hydroxide peaks reduced qualitatively. Like the present study, it has been reported that no peaks of calcium hydroxide formed on ERRM cements, which is similar to TotalFill exposed to blood, after 28 days, but contrary to cements in contact with water and HBSS [18]. Regarding the reduction of calcium hydroxide in the specimens of OrthoMTA and TotalFill exposed to PRF and blood for 1-month, it seems that the hydration process of calcium silicate cements arrested, and dissolution of calcium hydroxide commenced. Nekoofar *et al.* [19] found no peaks of

calcium hydroxide in specimens mixed entirely with blood, which is considered to be due to the inhibition of calcium hydroxide formation and/or its dissolution. In addition, the formation of amorphous calcium silicate hydrate (CSH) following to the hydration of HCSC should be considered, because only crystalline compounds are traceable in the XRD patterns [19]. Also, the lack of this amorphous content as well as absence of ettringite, as hydration reaction indicators, could be due to the short time of incubation and incomplete hydration [19, 31]. On the other hand, the effects of blood and PRF on the amounts of calcium hydroxide at different time intervals, may be related to differences in the chemical composition of HCSCs. In assessing the hydration behavior of bismuth contained HCSCs, bismuth remains an unreacted powder in the hydrated composition of these cements, affecting the MTA hydration mechanism. This element enters the structure of hydrated calcium silicate and forms calcium silicate hydrate-bismuth (CSH-Bi), which can affect the formation and dissolution rate of calcium hydroxide and, consequently, the bioactivity of the hydrated material [32], thus preventing complete hydration [19, 33].

All cements tested in the study had a different surface microstructure after 1 week and 1 month of exposure to PBS, which may be due to differences in chemical composition. This finding might be related to the bioactivity of HCSCs and the deposition of apatite crystals in the presence of fluid containing phosphate [20, 24]; as seen in the present study where RetroMTA cement was exposed to PBS for one month. Indeed, over time, the size of the precipitated crystals and surface microstructure as well as the chemical composition of the PBS- exposed specimens changed.

Also, all cements exposed to PBS had different surface microstructures compared to cements exposed to blood and PRF, indicating that the cements were affected by blood and PRF. The crystalline microstructure was seen in all cements exposed to PBS while it was not observed in the cements exposed to blood and PRF. This result is in accordance with Nekoofar *et al*. [19] who revealed the unfavorable effects of blood contamination on the microstructure and hydration behavior of MTA. Furthermore, the surface microstructure of specimens exposed to PRF and blood for 1 month varied compared to the 1-week samples. This indicates changes in the hydration process, over time, which resulted in different surface microstructures [24, 31].

EDS analysis of the material revealed that the surface precipitations in all groups, mainly contained high peaks of carbon (C), oxygen (O) and calcium (Ca), which reflect the nature of HCSCs, as reported in similar studies [18, 24, 34]. It has also been reported that high peaks of calcium, oxygen, and silicon occur during EDS analysis of pure Portland cement exposed to PBS and PRF [20], which might be due to the release of hydrated calcium silicate and calcium hydroxide following hydration reaction of the HCSCs [35]. In the present study, the peaks of bismuth (Bi) were observed specifically in OrthoMTA specimens, while the peaks of zirconium (Zr) in RetroMTA, TotalFill, and the peaks of tantalum (Ta) was detected only in TotalFill specimens, which can be justified by the radiopacifier content of these cement [18, 24, 34].

Aluminum (Al) was seen in all specimens, which can play a role in the formation of the ettringite crystalline microstructure [19]. Aluminum contributes to the formation of tetra-calcium aluminoferrite and

tricalcium aluminate in the OrthoMTA cement [28], this may be considered a reason for greater amounts of Al in the EDX analysis. However, Camilleri stated that the presence of aluminum in HCSCs is generally rare [31].

In the present study, phosphorus was observed in the EDS analysis of all specimens, which could be due to the presence of phosphates in PBS, blood, and PRF. Previous studies have also shown the presence of phosphorus in blood and PRF [18, 20, 27]. Furthermore, TotalFill BC RRM contains phosphorus as monobasic calcium phosphate [18, 24, 34]. High peaks of oxygen can be attributed to the presence of oxygen (O) in the chemical composition of all calcium silicate materials [34]. The presence of carbon (C) may also be related to carbon dioxide and carbon in blood and its derivatives, and subsequently the presence of calcium carbonate deposits, which are formed by the reaction of calcium and carbonate ions in the environment [18]. In addition, carbon is related to use of the carbon grid before SEM-EDS analysis.

Conclusions

According to the XRD analysis of OrthoMTA and TotalFill, there was a reduction in the peak of calcium hydroxide, after 1-month exposure to blood and PRF. The carbonate apatite crystals were observed only on the surface of 1-month PBS exposed RetroMTA specimens.

The surface microstructure of three studied cements exposed to PBS was different from the cements exposed to blood and PRF. The microstructure of HCSCs was affected by exposure to PRF and blood.

The chromatic alteration of all cements exposed to blood was significantly greater than those exposed to PRF and PBS. RetroMTA and TotalFill specimens had less color change when exposed to blood and PRF compared to OrthoMTA.

Declarations

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Ethical approval The study was performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. This study was approved by Ethics Committee of Tehran University of Medical Sciences (Ethics code: IR.TUMS.DENTISTRY.REC.1398.027) and University of Jordan.

Consent for participate Written informed consent was obtained from all individual participants included in the study.

Author contributions

All authors contributed to the study conception and design. Material preparation and data collection were performed by Noushin Shokouhinejad, Shima Saber Tahan, and Fatemeh Mohandes. All authors contributed to the interpretation of the data. Noushin Shokouhinejad, Mohammad H. Nekoofar, Shima Saber Tahan, and Fatemeh Mohandes wrote the main manuscript text. Noushin Shokouhinejad and Fatemeh Mohandes prepared figures. Ibrahim Abu Tahun and Paul M.H. Dummer critically reviewed the manuscript. All authors read and approved the final manuscript.

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Figures

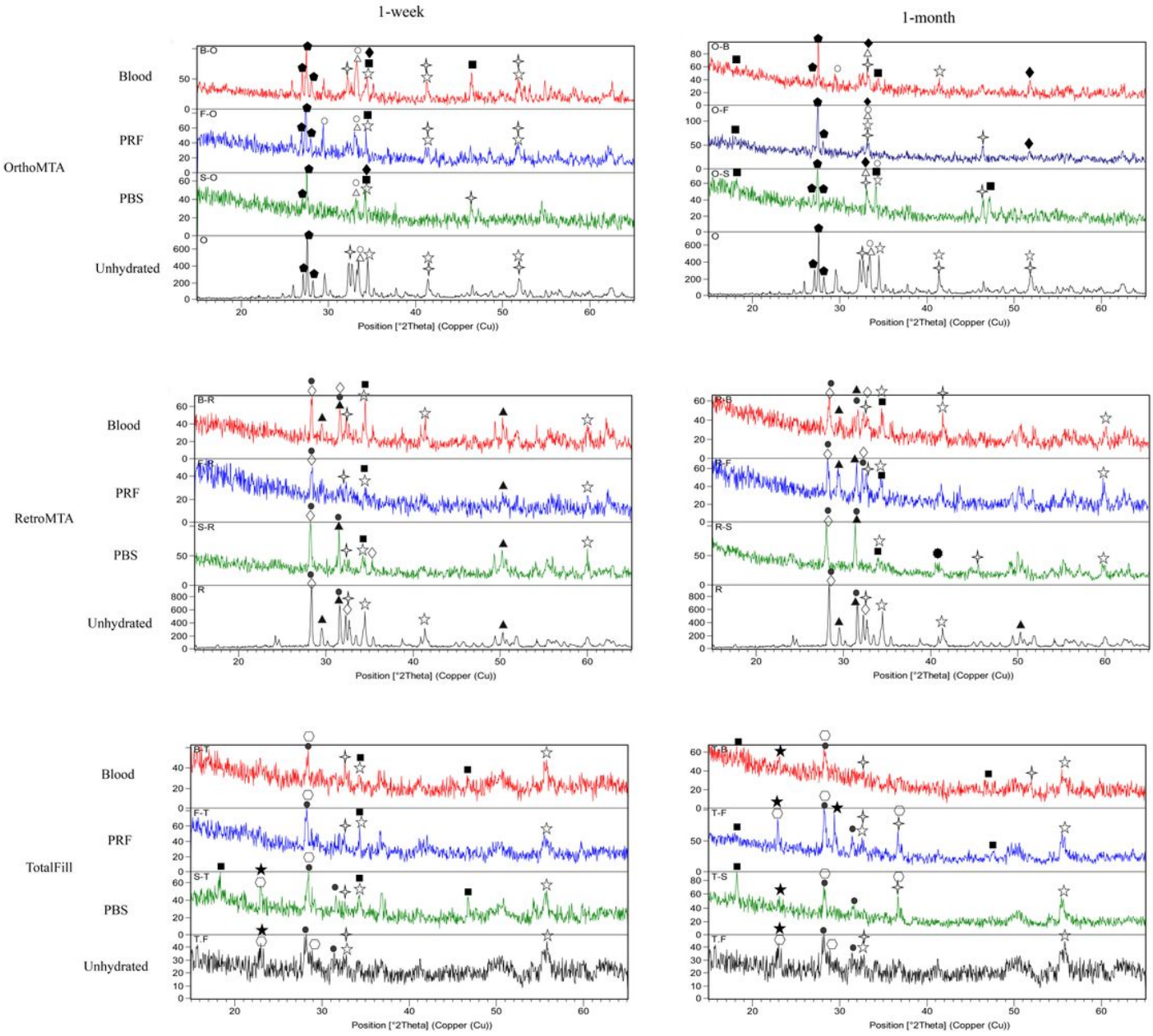

Figure 1

XRD analysis of unhydrated OrthoMTA, RetroMTA, and TotalFill (black), exposed to PBS (green), PRF (blue), and blood (red) for 1 week and 1 month.

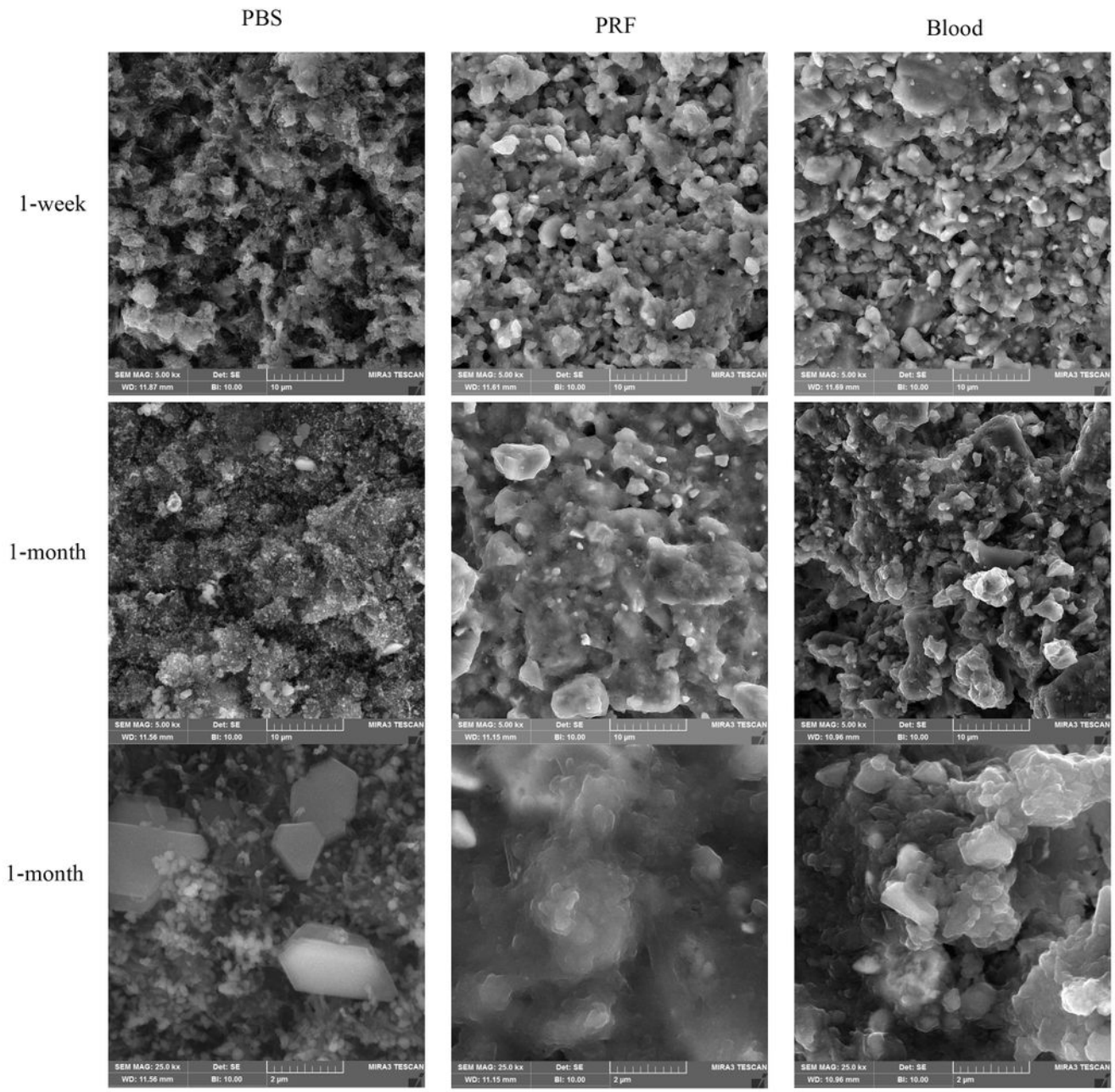

Figure 2

SEM images of OrthoMTA specimens exposed to PBS, PRF, and blood after 1 week and 1month

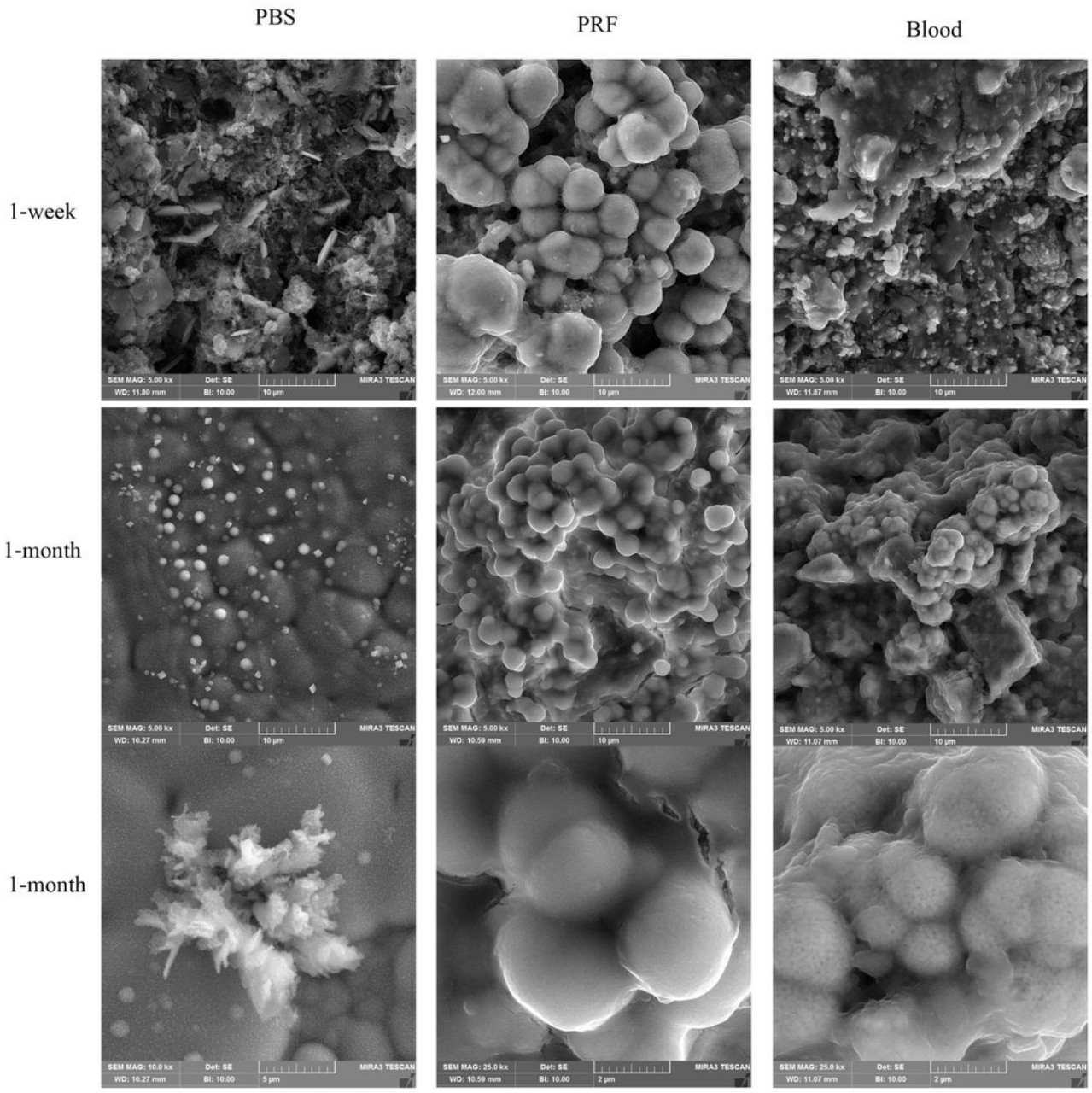

Figure 3

SEM images of RetroMTA specimens exposed to PBS, PRF, and blood after 1 week and 1 month

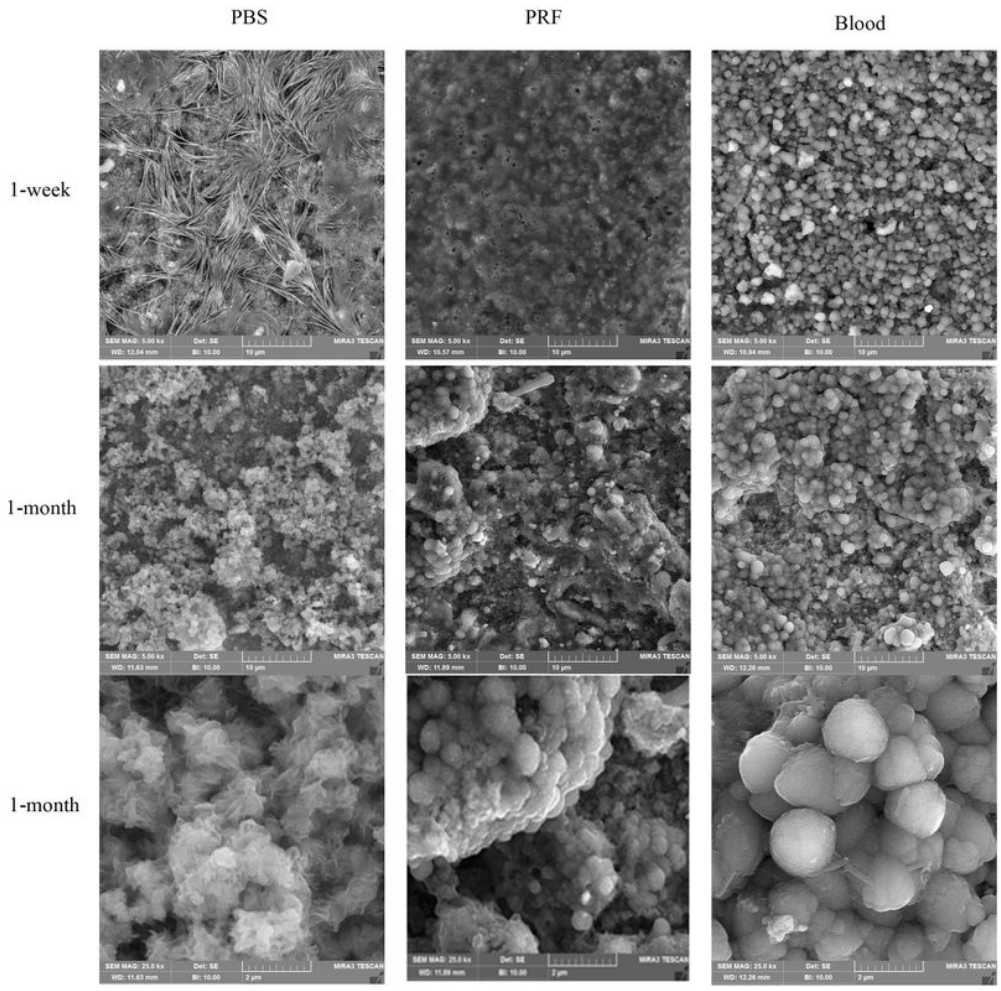

Figure 4

SEM images of TotalFill BC RRM specimens exposed to PBS, PRF, and blood, after 1 week and 1 month

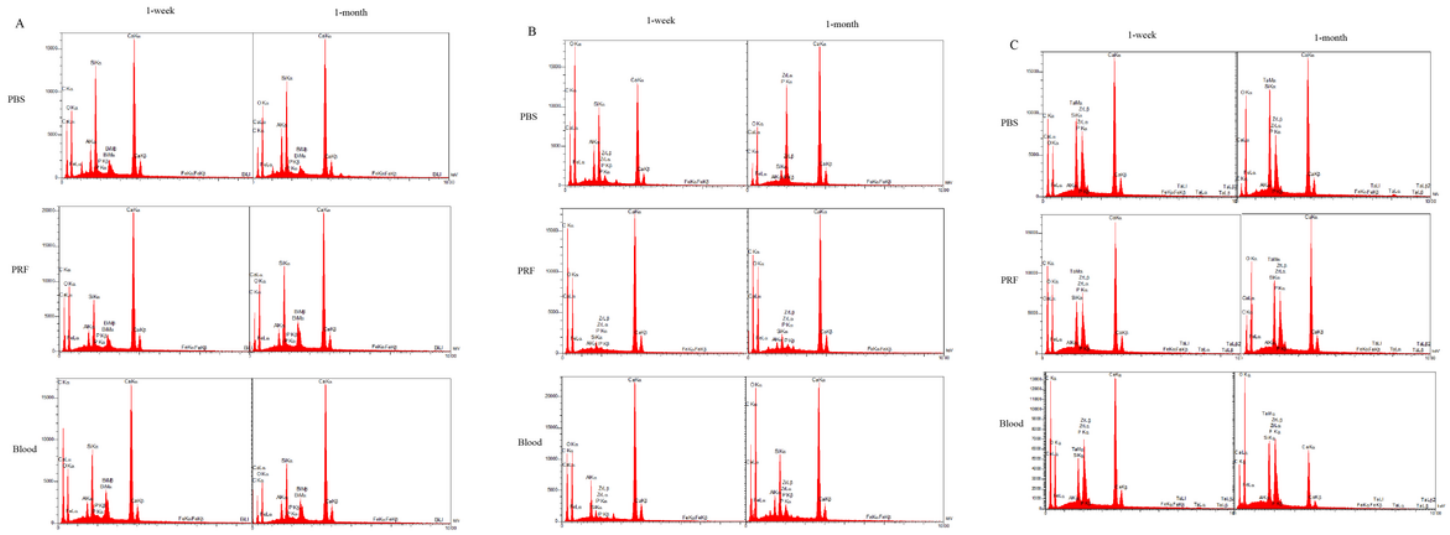

Figure 5

EDS analysis of OrthoMTA (a), RetroMTA (b) and TotalFill (c) exposed to PBS, PRF or blood for 1 week and 1 month.

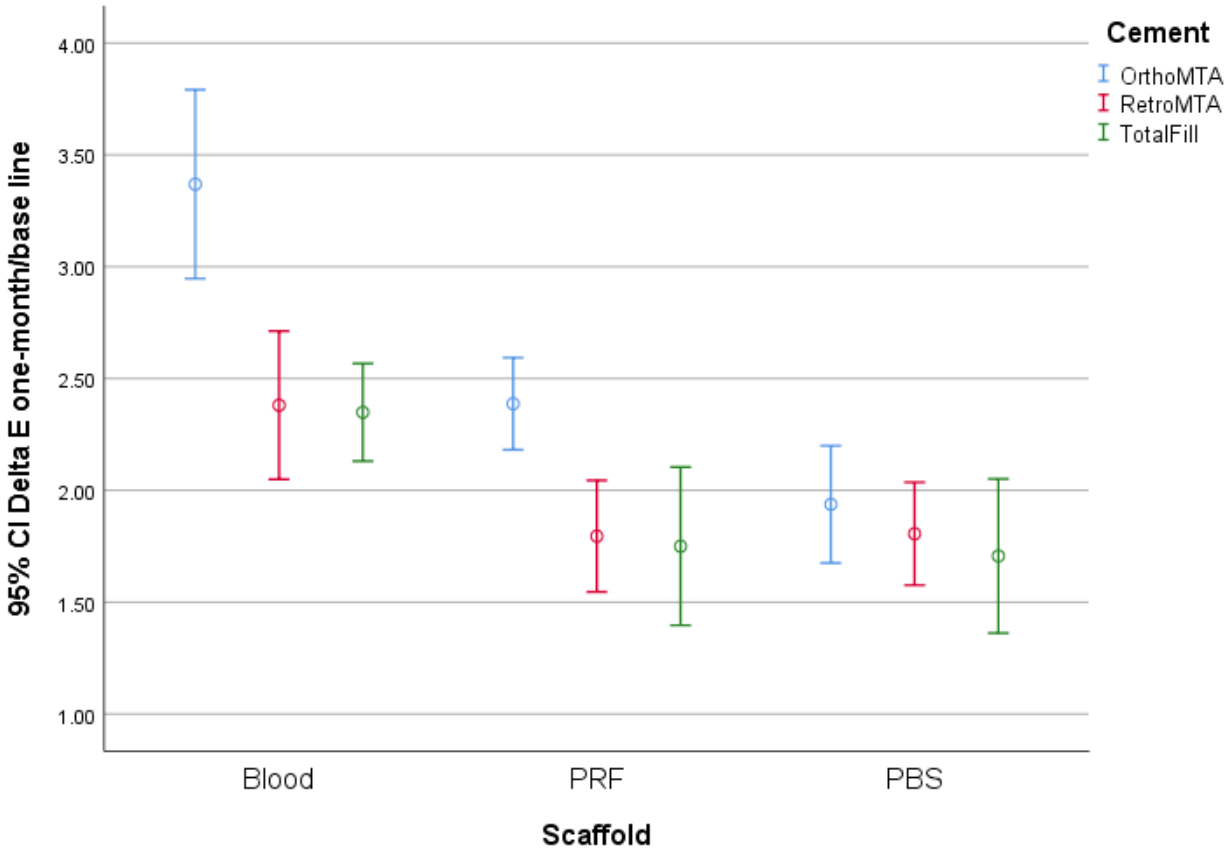

Figure 6

ΔE values (mean and standard deviation) of the experimental groups (*n* = 12).

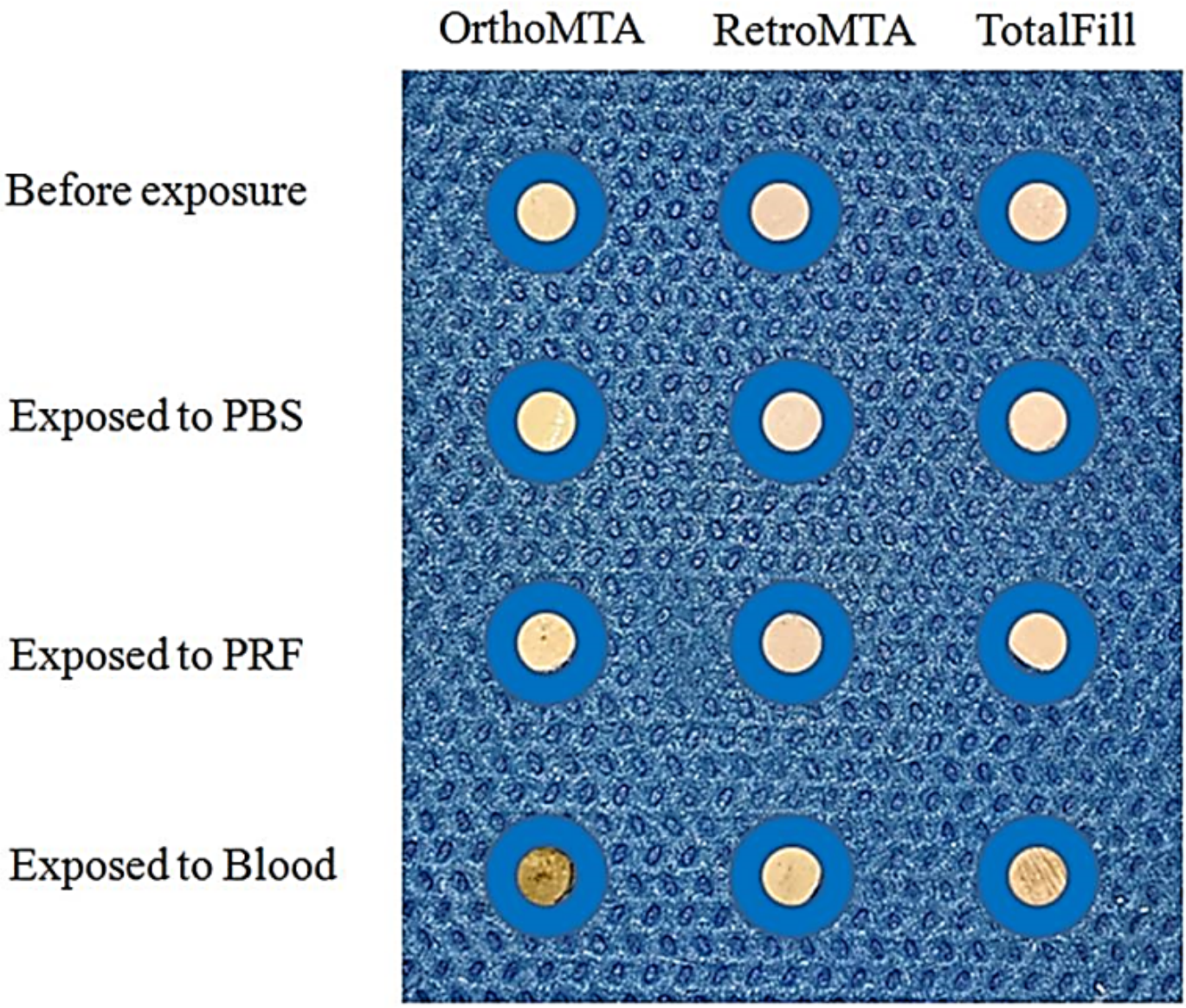

Figure 7

Photograph of samples in each group.