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Influence of blood contamination on the bond strength and biointeractivity of Biodentine used as root-end filling

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KEYWORDS

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Bond strength;
Root-end filling

Abstract *Aim:* To evaluate the influence of blood contamination on the bond strength and apatite forming ability of Biodentine used as root-end filling material.

Methodology: Eighty (n = 80) extracted single-rooted, sound human maxillary anterior teeth were prepared and obturated. Then, the roots were resected, retrograde cavities were prepared and Biodentine was inserted as the root-end filling material. Teeth were then randomly divided into 2 equal groups (n = 40) according to the setting environment of Biodentine i.e., group A where setting took place in human blood and group B where setting took place in deionized water (control group). Teeth were incubated at 37 °C for 45 min to ensure complete setting. Root discs with the filling material in their core were prepared. Push-out bond strength test was performed using a universal testing machine and failure mode was examined. Both groups were aged in HBSS for 30 days. Apatite nucleation was evaluated at one-day, 7-days, and 30-days interval using SEM for morphological analysis and EDX for elemental analysis. Calculation of the Ca/P ratios was performed in addition to XRD for crystal phase analysis.

Results: Blood contamination (group A) resulted in significant reduction of bond strength values. It also affected the amount of apatite deposition on the material surface and interfacial spaces with higher Ca/P ratios than that of the normal stoichiometric hydroxyapatite.

Conclusions: Blood contamination during setting of Biodentine had a detrimental effect on the bond strength and reduced the nucleation of apatite in comparison to non-contaminated group.

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1. Introduction

Endodontic surgery is performed when non-surgical treatment modalities or retreatments are proved to be unsuccessful or contra-indicated. Several materials have been proposed as root-end filling materials. According to Gartner and Dorn

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(Gartner and Dorn, 1992), an ideal root-end filling material should provide a bacterial tight seal and a fluid tight seal. It has to be non-carcinogenic, non-toxic, dimensionally stable and biocompatible. For clinical application, it should be simple to use as well as having an adequate degree of radio-opacity to be recognized on the radiograph (Galhotra et al., 2013).

Bioceramics, specially designed ceramics used for replacement of lost or diseased body parts, were proposed as a very promising option to be used in retro-grade cavities (Jitaru et al., 2016). The first generation of bioceramics used in endodontics was MTA (Parirokh and Torabinejad, 2010). It is a tri-calcium silicate-based cement which explains its sealing ability and biocompatibility. However, it suffered a number of drawbacks such as long setting time and difficult handling properties. New materials have developed to overcome these flaws. Biodentine (Septodont, Saint-Maur-des-Fossés, France), is a pure tricalcium silicate-based cement that was marketed as dentin substitute. Several investigations have previously reported its successful use for root-end filling (Caron et al., 2014).

During clinical application, exposure of root-end filling material to blood might affect its setting reaction. This might negatively affect the biological and physical properties of the material. Therefore, the aim of this study was to evaluate the influence of blood contamination on the bond strength and apatite forming ability of Biodentine used as root-end filling material. The null hypothesis tested is that blood contamination will not have an effect on the push out bond strength and apatite forming ability of Biodentine.

2. Materials and methods

2.1. Sample preparation

The current study was approved from the Research Ethics Committee of Ain Shams University (Cairo, Egypt), (approval number 01042017). Eighty (n = 80) single-rooted sound human maxillary teeth extracted for periodontal reasons were used. Teeth were cleaned mechanically using an ultrasonic scaler (Satelec, Cedex, France) to remove calculus or soft tissues. Teeth were immersed in 5.25% NaOCl solution for 30 min then stored in saline solution (El Nasr Pharmaceutical Chemicals Co., Cairo, Egypt) until used. Endodontic access cavities were prepared in all teeth. Working length was set 0.5 mm short to the apical foramen. Cleaning and shaping of teeth was performed using Protaper Universal rotary nickel titanium system (Dentsply Maillefer, Ballaigues, Switzerland) until finishing file F5. Irrigation was done with 5 ml of 2.5% sodium hypochlorite and final flush using 5 ml of EDTA 17% using a 27 gauge needle. Root canals were dried using paper points #50. Gutta percha cones (Meta Biomed, Chungcheongbuk-do, Republic of Korea) of #50/0.04 were used with Adseal resin sealer (Meta Biomed, Chungcheongbuk-do, Republic of Korea) in lateral compaction. Teeth were stored in 100% humidity at 37 °C for 7 days. Root-end resection was done by cutting 3 mm from the apical part of the root perpendicular to its long axis. Root-end cavities were prepared in all teeth under 8x magnification using a dental operating microscope (Zumax, Suzhou New District, China), using AS3D ultrasonic diamond coated retro-grade tip (Satelec, Cedex, France) attached to an ultrasonic unit (Satelec, Cedex, France) at a medium power setting. All cavities were standardized to 3 mm depth and 1 mm diameter. Dentin walls were left without conditioning. Biodentine (Septodont, Cedex, France; lot. B13547) was mixed and applied by first opening the capsule and placing it in its holder. A single liquid container was used for each capsule in which the mini bottle was opened and then 5 drops were carefully added to the powder in the capsule. The capsule was closed again and inserted in an amalgamator to be mixed for 30 s at a speed of 4000 oscillations per minute. The capsule was then reopened, and the plastic spatula supplied by the manufacturer was used to carry the paste out of the capsule and on to a mixing pad to be ready for insertion into the retrograde cavities. Biodentine was applied to fill the root-end cavities till the level of the resection and left untouched for 6 min according to the manufacturer's instructions.

2.2. Sample classification

Teeth were then randomly divided into 2 main groups (n = 40) according to the setting environment in which Biodentine root-end fills were allowed to set. Forty teeth were allowed to set inside tubes (Eppendorf, Hamburg, Germany) containing 1 ml of human blood with sodium citrate added as an anticoagulant (group A) while the other 40 teeth were allowed to set in tubes containing 1 ml of deionized water (group B) as a control. All tubes were stored in an incubator at 37 °C for 45 min to ensure final setting of the material. Then, all teeth were removed from the tubes and washed with saline (El Nasr Pharmaceutical Chemicals Co., Cairo, Egypt).

Human blood was obtained from two donors in the Clinical Pathology Department at El-Demerdash Hospital Ain Shams University (Cairo, Egypt). Blood samples were collected and tested negatively for blood diseases. It was included in the research after donor's approval.

A precision cutting machine was used to cut slices transversely from the mid-root perpendicular to the long axis of the tooth. Root discs 2 mm in thickness with the set material in the form of cylinders in its core were obtained.

Forty discs were used for bond strength testing (Akcay et al., 2016) and forty discs for apatite forming ability evaluation (Gandolfi et al., 2013) according to ISO 23317:2014 specification (International Organization for Standardization Dentistry, 2014).

2.3. Bond strength

Twenty blood-set root discs (group A) and 20 deionized water-set root discs (group B) were subjected to push-out bond strength testing. Push-out test was performed using a universal testing machine (Lloyd, Ametek, Kuala Lumpur, Malaysia). Each disc was placed with the apical part facing upwards and the root-end filling material was subjected to a load at a cross-head speed of 1 mm/min in an apical to coronal direction parallel to the long axis of the disc. A cylindrical attachment of 0.7 mm diameter was used to provide a clearance space of at least 0.2 mm from the borders of the dentinal wall ensuring contact only with the material to be tested. The test proceeded until dislodgment occurred. The maximum load applied to dislodge the root-end filling material was recorded in Newtons.

Push out bond strength was calculated using the following formula:

Maximum load to dislodge the material (Newtons) / bonding surface area (mm^2)

The bonding surface area was calculated for each sample as: $(\pi r_1 + \pi r_2) \times L$, where $L = \sqrt{(r_1 - r_2)^2 + h^2}$ in which π is the constant 3.14, r_1 and r_2 are the smaller and larger radii of the retro cavity respectively measured using digital microscope, and h is the height of each specimen (standardized at 2 mm).

Stereomicroscopic evaluation of root sections was then performed at x8 magnification to assess the failure mode. Two failure modes were recognized: Adhesive failure (between the filling and root dentin) and cohesive fracture (within the dentin or filling).

2.4. Apatite forming ability

Twenty blood-set root discs (group A) and 20 deionized water-set root discs (group B) were stored in tubes containing HBSS (Biochrom GmbH, Leonorenstr, Brelin, Germany) (Gandolfi et al., 2010). Analyses were performed at intervals of 1, 7, and 30 days changing the solution at each interval.

For morphological and elemental analysis, SEM connected with EDX unit (Quanta 250 FEG, FEI Company, Oregon, USA) was used. Discs were left to air dry for ten minutes. Discs were mounted on an aluminum stub using carbon sticky pads and inserted in the SEM/EDX unit. Air was expelled to allow analysis of the specimens in vacuum.

2.4.1. Morphological analysis by SEM

For morphological analysis, the surface was studied at low magnification (x200) to show the full surface of the filling as well as at high magnification (x1500) to show the interfacial layer between root dentin and Biodentine.

Three samples from each group (blood-set group, and deionized water-set group) were randomly selected. The percentage of the surface covered by the formed deposits was measured at day 1, 7 and 30 using Image J software (1.42a/Java 1.6.0-10 image analyzer software).

2.4.2. Elemental analysis by EDX

For elemental analysis, three areas were selected randomly from the Biodentine-dentin interface of each sample to measure the atomic percentage of calcium and phosphorus at each area as well as other elements like silica, magnesium, carbon and oxygen as seen in Fig. 1. Calcium to phosphorus atomic ratio was calculated from each area, average was calculated, and the value of each group was compared to the Ca/P ratio of normal stoichiometric hydroxyapatite (1.67) and bone-like carbonated apatite (Gandolfi et al., 2013). Calculated Ca/P ratios for each group were compared at different time intervals (1, 7, and 30 days) for intra-group comparison of the effect of time factor.

2.4.3. Phase analysis by XRD

After 30 days, root discs were gently fractured and the Biodentine filling was crushed into powder for phase analysis of the formed crystals by XRD. Diffraction lines produced by XRD were matched to those produced normally by stoichiometric hydroxyapatite and bone-like carbonated apatite. Phase identification was accomplished using a search-match software utilizing ICDD database (International Centre for Diffraction Data, Newtown Square, PA, USA).

2.5. Statistical analysis

Statistical analysis was performed using Minitab 17 statistical software. Significance was analyzed by one-way ANOVA and two-sample *t*-test analysis for bond strength and Ca/P ratio. Chi-square test was used for failure mode. The data were expressed by mean and standard deviation. Statistical significance was set at 5%.

3. Results

3.1. Bond strength

Blood-set roots (group A) showed lower push-out bond strength mean value (3.29 ± 2.24 MPa) compared to deionized water-set roots (group B) (8.58 ± 1.86 MPa). This difference was found to be statistically significant ($P < 0.001$).

Blood-set roots (group A) showed adhesive failure in 12/20 specimens while deionized water-set roots (group B) showed adhesive failure in 4/20 specimens. This difference was shown to be statistically significant ($P = 0.009823$).

3.2. Apatite forming ability

3.2.1. Morphological analysis by SEM

Low magnification images of both groups (blood-set and deionized water-set) revealed formation of surface deposits at 1-day interval. Surface deposits increased by time in both groups covering a larger area. However, blood-set samples (group A) revealed relatively smaller area of surface deposits at all observation intervals as shown in Figs. 2 and 3.

High magnification images allowed visualization of surface morphology and interfacial deposits at the Biodentine-dentin interface. Deionized water-set samples (group B) revealed deposition of globular precipitates on the Biodentine surface and interfacial spaces. Each globule was formed of numerous minute particles clustered together. These globules increased in number and coalesced forming larger globules by time. Blood-set samples (group A) showed smaller-sized interfacial deposits which were more irregular in shape and lacked the clusters of globules seen in the deionized water-set samples (group B) as shown in Fig. 4.

3.2.2. EDX analysis

Ca/P ratios (expressed as mean and standard deviation) calculated for both groups after 1, 7 and 30 days are showed in table 1. Blood-set samples (group A) showed significantly higher Ca/P ratios than that of the normal stoichiometric hydroxyapatite at all 3 intervals ($p < 0.001$). Deionized water-set samples (group B) showed lower Ca/P ratios that decreased by time approximating the ratio of normal hydroxyapatite; yet, slightly higher indicating the maturation of a carbonated-apatite phase (bone-like apatite) where carbonate replaces phosphate ions (Gandolfi et al., 2010).

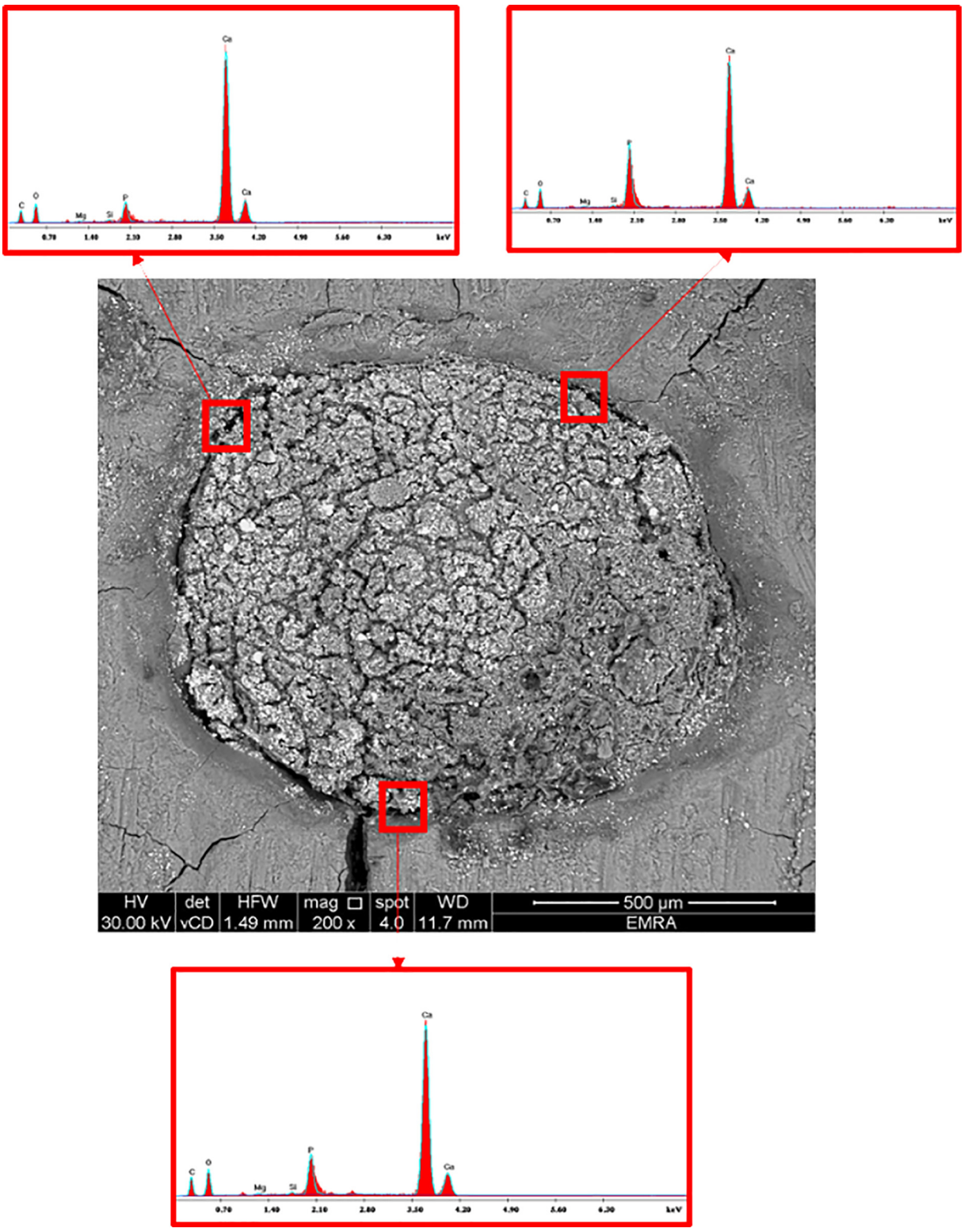

Fig. 1 Three randomly selected areas from the Biodentine-dentine interface for measuring the Ca/P atomic ratio within each area and calculating the average for the whole sample.

3.2.3. XRD analysis

Comparison between the diffraction lines produced by both groups after 30 days and those reported for normal hydroxyapatite of teeth and bone revealed that the nature of the formed crystals matches that of hydroxyapatite. This was demonstrated by broad and diffuse peaks produced at 2 theta = 25.9 degrees as well as 31.8–32.9 degrees which are characteristic peaks of apatite as shown in Fig. 5. Blood-set and deionized

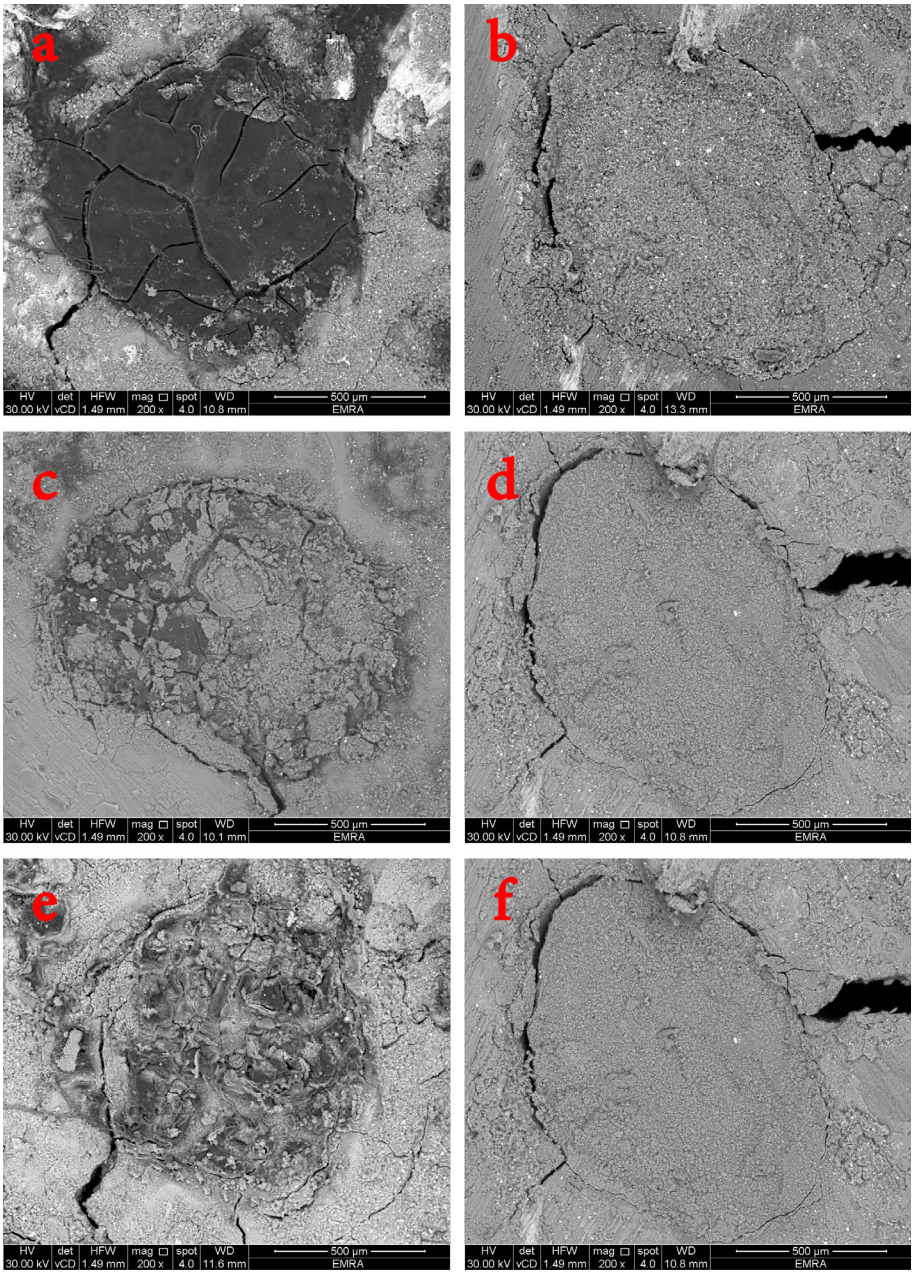

Fig. 2 Low magnification SEM images (a) blood-set at day 1, (b) deionized water-set at day 1, (c) blood-set at day 7, (d) deionized water-set at day 7, (e) blood-set at day 30, (f) deionized water-set at day 30.

water-set groups were able to form hydroxyapatite crystals; however, EDX results mentioned earlier revealed less amount of apatite formation for the blood-set group.

4. Discussion

Endodontic microsurgery allows exploration of the cause of a persistent pathosis affecting root canals as well as eliminating it when traditional retreatment methods fail or are contraindicated (Kim et al., n.d.). A very important step in this procedure is the preparation of a root-end cavity to be sealed with a root-end filling material.

Biodentine is one of the most commonly used tricalcium silicate based root-end filling materials in endodontics. Although setting of Biodentine is not affected by body fluids, ideally a root-end filling material should not be subjected to any kind of contamination throughout its placement and setting in order to avoid any possible deterioration in its biological or mechanical properties (Nekoofar et al., 2009, 2010). However, clinically, it could be considered utterly difficult, if not impossible, to achieve such an environment. Hence, the study of the effect of blood contamination on the properties of Biodentine root-end filling was considered of value.

In the present study, a hemorrhagic situation was simulated by inserting the tooth with the root-end filling material unset in

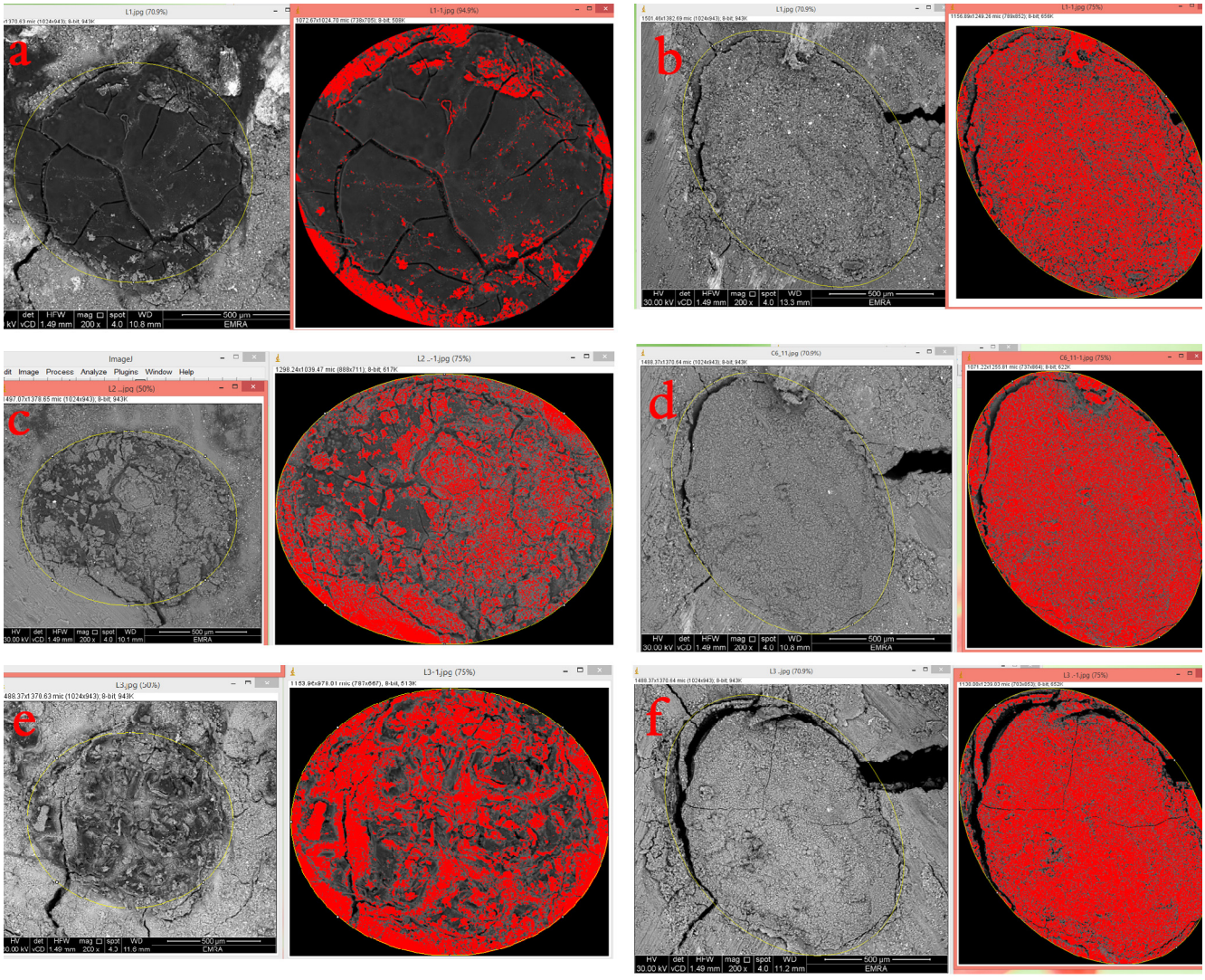

Fig. 3 Low magnification SEM images analyzed by Image J software showing the percentage of surface deposits on Biodentine (a) blood-set roots at day 1, (b) deionized water-set roots at day 1, (c) blood-set roots at day 7, (d) deionized water-set roots at day 7, (e) blood-set roots at day 30, (f) deionized water-set roots at day 30.

blood contained in Eppendorf tubes (Nekoofar et al., 2010). For non-contaminated samples, deionized water was used as a control medium so that no ions except those in HBSS would bond with the calcium ions in the material. It will also provide a source of hydration for the setting reaction to take place (do Carmo et al., 2017).

Formation of surface apatite is an important requirement for material bonding with adjacent tooth structure and vital bone tissue. Simulated body fluids (SBF) allow simulation of the in-vivo conditions were apatite forms by interaction of calcium from the material with phosphorus from the surrounding tissue fluid (Gandolfi et al., 2013). HBSS is a type of SBF solution prepared to mimic the ionic concentrations of the human blood plasma (Jalota et al., 2006). It simulates physiologic tissue fluids allowing the evaluation of in-vitro bioactivity of Biodentine as well as MTA (Huffman et al., 2009; Gandolfi et al., 2013; Camilleri, 2014).

Biodentine bonds with surrounding dentinal walls via micromechanical retention and apatite deposition at the interface. This interlocking improves the dislocation resistance of the root-end filling material within the canal (Saleh et al., 2003; Huffman et al., 2009). In the current study, the push-out method was used to assess the bond strength of the filling material to the dentinal walls because of its decreased premature failures and its ability to provide uniform stress distribution (Goracci et al., 2004; Cekic-Nagas et al., 2011). Although push-out method shows great variability in the design (plunger size and speed, slice thickness, timing of filling, and preparation method), it was used in the current study as pull-out and micro-tensile methods are not suitable for Biodentine (Akcay et al., 2016).

In the current study, blood contamination during setting had a significant negative effect on the push-out bond strength of Biodentine root-end filling to radicular apical dentin. These results were consistent with those of Akcay et al. (Akcay et al., 2016) where Biodentine was used as a root-end filling material. They attributed the reduction in bond strength to blood interference with complete hydration and therefore setting of the

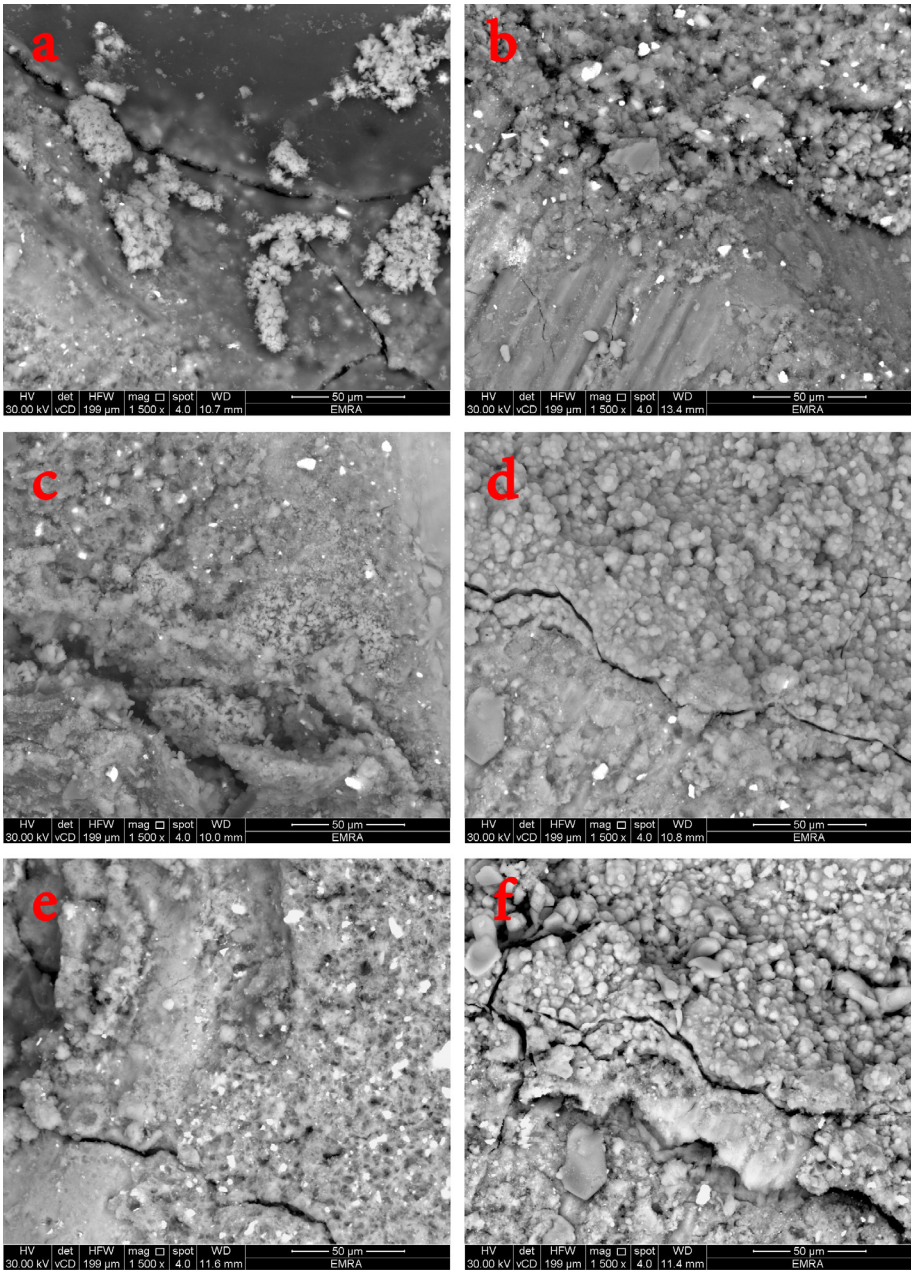

Fig. 4 High magnification SEM images (a) blood-set at day 1, (b) deionized water-set at day 1, (c) blood-set at day 7, (d) deionized water-set at day 7, (e) blood-set at day 30, (f) deionized water-set at day 30.

material, based on the surface differences observed in the blood-set group. This was confirmed with the statistically significantly higher adhesive failures showed by the blood contaminated samples. On the other hand, Aggrawal et al. (Aggarwal et al., 2013) showed no significant effect of blood contamination on bond strength values of Biodentine. They used Biodentine as a repair for furcal perforations and not as root-end filling. Results were attributed to the short setting time of Biodentine.

Bioactivity, as defined in the literature, is the ability of the material to interact with the surrounding tissue fluids forming apatite-like deposits and therefore bonding with the surrounding living tissues (Kokubo and Takadama, 2006; Chen et al., 2009). The ability of Biodentine to dissolve in SBF releasing its cationic components and allowing the deposition of hydroxyapatite on its surface and surroundings was reported (Gandolfi et al., 2013; Han and Okiji, 2013; Viegas, 2013). Therefore, it was of interest to evaluate the effect of blood contamination on the bioactive properties of Biodentine as well.

In the current study, SEM was used as a descriptive test for analysis of surface morphology. Low magnification images allowed visualization of the whole root-end filling material with the dentinal wall appearing all around it. This allowed the calculation of the percentage coverage of surface apatite in relation to the full surface area of the filling material using Image J software. It also allowed the comparison of the

Table 1 Mean and standard deviation values of the calculated Ca/P ratios for blood-set and deionized water-set samples after 1, 7 and 30 days.

	Blood-set (Group A)	Deionized water-set (Group B)	P value
Day 1	5.902 ± 2.01^{Ba}	3.56 ± 0.88^{Aa}	<0.005*
Day 7	4.99 ± 2.51^{Ba}	2.53 ± 0.57^{Ab}	<0.014*
Day 30	4.11 ± 1.45^{Ba}	1.79 ± 0.15^{Ac}	<0.001*
P value	>0.05 ns	<0.001*	

Different upper-case letters in the same row indicate statistically significance difference. Different lower-case letters in the same column indicate statistically significance difference.

* significant ($p < 0.05$), ns; non-significant ($p > 0.05$).

amount of surface apatite globules with and without blood contamination. It also allowed monitoring the increase in surface apatite coverage within each group over time. It was reported by Sarkar et al (Sarkar et al., 2005) and Reyes et al (Reyes-Carmona et al., 2009) that hydroxyapatite deposits were formed at the interface between the bioceramic material and dentin. SEM examination of deionized water-set samples revealed the deposition of globular precipitates formed of numerous minute particles clustered together. These results were consistent with the results reported by Sarkar et al (Sarkar et al., 2005) in their examination of surface morphology of MTA after interaction with SBF. It showed a surface coating of small but dense spherulites that became most prominent after 30 days. These results are also consistent with those of Gandolfi et al. (Gandolfi et al., 2013). On the other hand, blood-set samples in the current study showed lack of the characteristic morphology observed in the deionized water-set samples. These results are comparable to those reported by Nekoofar et al. (Nekoofar et al., 2010) showing lack of surface crystals in blood-contaminated MTA samples in comparison to non-contaminated ones.

EDX analysis is a semi-quantitative test that measures the atomic percentage of different elements on the material surface. Comparison of the calculated Ca/P ratio with that of normal stoichiometric hydroxyapatite allowed tracking the amount of hydroxyapatite formation with or without blood contamination (Gandolfi et al., 2013, 2014). Blood-set samples showed ratios further away from that of hydroxyapatite at all intervals which could be attributed to decreased hydration (Moinzadeh et al., 2016) as a result of adhesion of blood proteins to sites of crystal nucleation as explained by Nekoofar et al. (Nekoofar et al., 2011).

XRD test has the ability to detect hydroxyapatite crystalline phase produced. It has the advantage of analyzing the bulk of the material in addition to the surface. However, it only detects crystalline hydroxyapatite (Bozeman et al., 2006). In the current study, XRD was used to confirm that the crystals formed by the material in both conditions were hydroxyapatite in nature. A quantitative comparison was attempted to determine the difference in percentage of hydroxyapatite crystals formed in both groups after 30 days; however, it was rendered unsuccessful due to the small size of the specimen. XRD results confirmed the nature of the formed crystals to be similar to that of hydroxyapatite. It revealed broad and diffuse peaks at 2 theta = 25.9 degrees and 31.8–32.9 degrees which are characteristic peaks of apatite. Similar results were produced by Chen et al. (Chen et al., 2009) in their study. Our results were also consistent with those reported by Sarkar et al. (Sarkar et al., 2005) in their investigation of the nature of the crystals formed on the surface of bioactive bioceramics.

5. Conclusions

The null hypothesis tested in this study was rejected as blood contamination during setting of Biodentine had a detrimental effect on the bond strength and reduced the nucleation of apatite compared to the non-contaminated group. Therefore, blood contamination should be avoided during application of Biodentine as a root-end filling.

Ethics statement

The current study was approved from the Research Ethics Committee of Ain Shams University (Cairo, Egypt), (approvation number 01042017).

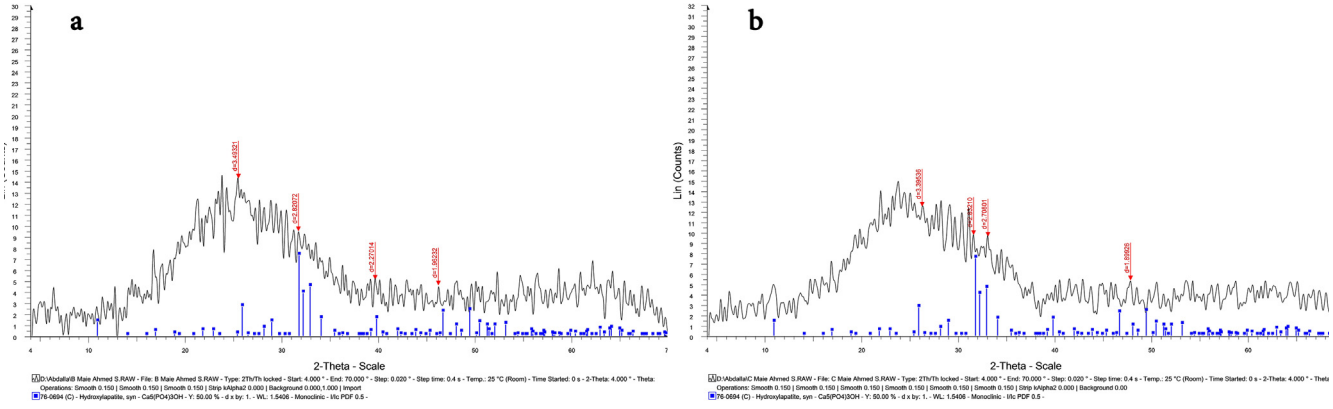

Fig. 5 XRD analysis of Biodentine powder denoting diffraction lines with broad and diffuse peaks produced at 2 theta = 25.9 degrees as well as 31.8–32.9 degrees which are characteristic peaks of apatite (a) blood-set group; (b) deionized water-set group.

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The authors deny any conflicts of interest related to this study.

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