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Role Of Iron on Physical and Mechanical Properties of Brushite Cements, And Interaction with Human Dental Pulp Stem Cells

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# Abstract

Improving the physical, mechanical and biological properties of brushite cements (BrC) is of a great interest for using them in bone and dental tissue engineering applications. The objective of this study was to incorporate iron (Fe) at different concentrations (0.25, 0.50, and 1.00 wt%) to BrC and study the role of Fe on phase composition, setting time, compressive strength, and interaction with human dental pulp stem cells (hDPSCs). Results showed that increase in Fe concentration increases the β-tricalcium phosphate (β-TCP)/dicalcium phosphate dihydrate (DCPD) ratio and prolongs the initial and final setting time due to effective role of Fe on stabilizing the β-TCP crystal structure and retarding its dissolution kinetic, in a dose dependent manner where the highest setting time was recorded for 1.00 wt% Fe–BrC sample. Addition of low concentrations of Fe (0.25 and 0.50 wt%) did not have adverse effect on compressive strength and strength was in the range of 5.7–7.05 (±~1.4) MPa; however, presence of 1.00 wt% Fe decreases the strength of BrC from 7.05 ± 1.57 MPa to 3.12 ± 1.06 MPa. Interaction between the BrCs and hDPSCs was evaluated by cell proliferation assay, scanning electron microscopy, and live/dead staining. Low concentrations of 0.25, and 0.50 wt% of Fe did not have any adverse effect on cell attachment and proliferation; while significant decrease in cellular activity was evident in BrC samples doped with 1.00 wt %. Together, these data show that low concentrations of Fe (equal or less than 0.50 wt %) can be safely added to BrC without any adverse effect on physical, mechanical and biological properties in presence of hDPSCs.

# Keywords

Brushite cement, Iron, Setting reaction, Human dental pulp stem cells, Cellular proliferation

# 1. Introduction

Brushite cements (BrCs) are calcium phosphate ceramics (CPCs) which are being widely used in bone and dental tissue engineering due to their osteoinductivity, injectability and molding properties [[1], [2], [3], [4]]. Compared to other CPCs, such as hydroxyapatite (HA) and tricalcium phosphate (TCP), BrC is significantly more resorbable in the physiological environment and its osteoinductivity is superior to that of TCP [5,6].

Though being widely used, properties of brushite cements such as mechanical properties, injectability, setting time, dissolution rate, and biological responses need to be improved for their clinical applications [1,7,8]. In addition, using the cements as carriers for drugs and stem cells is still under investigation [[9], [10], [11]]. One way to alter the properties of BrCs is to modify their chemical composition by adding trace elements as dopants. This not only affects the phase composition and mechanical properties, but also the setting reactions and biological responses can be significantly altered [[12], [13], [14], [15], [16]]. Magnesium (Mg) doping in BrC increases setting time of the cement in a dose dependent manner, and enhances the compressive strength, regardless of its concentration [13]. 0.5 wt% of silicon (Si) enhances osteoclast activity of BrC, while higher concentrations do not have significant effect [17]. Co-doping of BrC with Si and zinc (Zn) increases setting time of BrC, decreases the compressive strength, and upregulates alkaline phosphatase activity and overall bone formation and vascularization, *in vivo* [12,18].

Recently, iron (Fe) was added to BrC to evaluate the possible antimicrobial activities [14]. Fe is an essential trace element and is involved in various biological processes in the body. However, its concentration plays a critical role in its efficiency. Fe overload results in deposition of Fe in vital organs such as liver and kidney. It weakens the bone and has severe effect on postmenopausal women, mainly by degrading the type I collagen [19]. On the other hand, Fe deficiency and resultant anemia causes the disruption in bone turnover through its adverse effect on vitamin D metabolism and collagen synthesis [20,21]. Severe iron deficiency, alone or in combination with vitamin B12 can destroy the periodontal tissue, significantly [22]. Fe deficiency also alters the bone formation in zebrafish [23] and increases the adsorption of manganese (Mn) and toxic metals such as cadmium (Cd) and lead that leads to neudevelopment deficits in children [24]. Fe incorporation to scaffolds enhances the osteogenic and angiogenic properties of bone substitutes. Fe upregulates the osteoblastic differentiation of mesenchymal stem cells, vascular endothelial growth factor (VEGF) secretion, and alkaline phosphatase expression in various bone constructs [[25], [26], [27], [28]]. Fe in the form of iron oxide has received significant attention due to its antimicrobial properties against several types of bacteria and inhibitory effect on bone tumor development [[29], [30], [31], [32], [33], [34]]. Apart from the nanoparticles of iron oxide, Fe addition to brushite cements inhibits the growth of *Staphylococcus aureus* and *Pseudomonas aeruginosa* [14]. Fe is an essential element in oral health as well. Fe with concentration of 118 and 93 ppm in enamel and dentine [35], is believed to play significant role in preventing dental caries. Iron deficiency results in higher risk of dental caries in young mouse model, suggesting the relationship between Fe and childhood dental health [36,37]. Due to beneficiary effects of Fe on bone and dental tissue, in this work we have incorporated Fe3+ to substitute Ca2+ in TCP to further investigate beneficiary effect of new composition for possible clinical application.

In this study, we hypothesize that the presence of Fe at different concentrations in brushite cement will alter its phase composition, compressive strength, setting time, dissolution behavior, and interaction with human dental pulp cells (hDPCs). To validate our hypothesis, Fe dopant was incorporated into TCP as a precursor of the final brushite product. Phase composition, compressive strength, and setting time of cements were evaluated by X-ray diffractometer, universal testing machine, and Gillmore needle, respectively. The interaction with human dental pulp cells was examined by attachment and proliferation assays.

# 2. Materials and methods

## 2.1. Tricalcium phosphate preparation

Pure and doped β-TCP were synthesized using a solid-state synthesis method, as explained before [38,39]. Preparation began with one mole of calcium carbonate (CaCO3, Sigma-Aldrich) and two moles of calcium phosphate dibasic (CaHPO4, Sigma-Aldrich) mixed and ball-milled for 2 h with a milling media:powder ratio of 4:1. The powder was then calcinated in a muffle furnace at 1050 °C for 24 h and cooled naturally to room temperature. The 0.25, 0.50, and 1.00 wt% Fe-doped TCP (Fe-TCP) was prepared with relative amounts of iron (III) oxide (Fe2O3, Sigma-Aldrich). A molar ratio of Ca/P and (Ca + Fe)/P remained at 1.5 for pure and different concentrations of iron.

After calcination, each composition was divided in half and further processed. Half of each composition remained as calcined, i.e. coarse, while the remaining was mixed with ethanol at an ethanol:powder:milling media ratio of 1.5:1:5 for 6 h. After milling, the mixture dried for 2–3 days at 65 °C to obtain the fine TCP powder.

## 2.2. Cement preparation

Pure and Fe-doped brushite cements (Fe–BrCs) were prepared as explained in detail in our previous work [17,40]. The pure and Fe-doped β-TCP powders were mixed with calcium phosphate monobasic monohydrate (MCPM, Sigma-Aldrich), in addition to few other precursors to control the setting reaction [2]. The cement paste was prepared by combining cement powder and 2 wt% polyethylene (PEG) solution at powder:liquid ratio of 3.33:1. The paste was mixed and poured into 6 mm diameter and 12 mm height cylindrical molds. Molded samples were covered and incubated at 37 °C for 1 h under 100% humidity. Samples were removed and immersed in a phosphate buffer solution (PBS) at 37 °C for 24 h to complete the setting reaction. From now on, 0.25 wt%, 0.50 wt%, and 1.00 wt% Fe doped brushite cements will be denoted as 0.25-Fe, 0.50-Fe, and 1.00-Fe, respectively.

## 2.3. Cement characterization

Phase composition was analyzed using X-ray diffractometer (XRD) using a 0.05° step size and count time of 1 s per step. Compressive strength of the samples was measured by universal testing machine (MTS Criterion Model 43) at 10 kN load and 0.01 mm/s speed. Setting time of the BrC samples was measured using the Gillmore needle. According to ASTM C266, two needles of 113.4 g and 453.6 g with diameter of 2.12 mm and 1.06 mm were used to measure the initial and final setting time respectively. Initial and final setting times were determined as the time the needles could not leave impressions on the surface of the freshly molded samples.

## 2.4. Dissolution study

Three samples of each composition were fully immersed in 4 mL of phosphate buffer solution with a pH of 7.4 ± 0.2, and placed in an incubator shaker at 37 °C and 75 rpm. The pH of the phosphate buffer was measured at 1.5 h, 3 h, 6 h, 24 h, 2 days, 5 days, 8 days, 14 days, and 16 days using a pH-meter (Seven Easy pH, Mettler Toledo). The solution was collected and replaced with equal amount for each iteration. Samples were removed from the phosphate buffer and dried at room temperature after 16 days of incubation. After drying, the phase composition of cements was analyzed using XRD as explained above.

## 2.5. Cytocompatibility assays

Human dental pulp stem cells (hDPSCs) characterized earlier in Dr. Tayebi's Lab were used for cytocompatibility studies on scaffolds (at passage # 5). Sterilization of scaffolds were done using 70% (v/v) ethanol for 30 min, washing with phosphate buffer saline (PBS) for three times, and then drying overnight under a laminar flow hood. After detachment of hDPSCs and determination of the number of cells in suspension using the C-Chip DHC-NO1 hemocytometer (INCYTO, Korea), cells were seeded on the scaffold at a concentration of 1 × 105 cells/samples in 50 μL of culture medium (DMEM with 4.5 gl −1 glucose, l-glutamine, and sodium pyruvate (Corning) supplemented with 10% fetal bovine serum (FBS, Gibco Invitrogen) and 1% Antibiotic-antimycotic (HyClone)). After one-hour incubation for allowing cell adhesion, samples were immersed in media and incubated at 37 °C and 5% CO2 for predetermined times (1, 3, and 7 days). After 24 h, samples were transferred to a new plate. The cells attached on the bottom of the plate which were in contact with the samples during 24 h were imaged using optical microscope (EVOS FL, Life Technologies). For assessment of cell proliferation in the cell/scaffold constructs, PrestoBlue™ assay (Invitrogen, Life Technologies) was used. Briefly, after washing cells with PBS, and adding PrestoBlue solution (1:9 in DMEM without phenol-red), samples were incubated at 37 °C (95% humidity and 5% CO2) for 2 h. During this time, cells repair the PrestoBlue® which result in its color change. With transferring 100 μL of the solution into a 96‐well plate, the fluorescence intensity was evaluated with the microplate reader (SYNERGY-HTX, Biotek, USA) at 540 nm (excitation)/590 nm (emission).

Cell viability of DPSCs seeded on scaffolds were assessed using a Live/Dead® viability/cytotoxicity Kit (Invitrogen). After 7 days of cell seeding, samples were rinsed with PBS and incubated in a mixture of 5 μM calcein-AM and 4 μM ethidium homodimer-1 (EthD-1) in PBS for 15 min (room temperature). After washing again with PBS, live (green) and dead (red) cells were imaged with fluorescence microscopy (Evos Fluorescent, life technologies).

Finally, Cell adhesion on the scaffolds were investigated qualitatively by scanning electron microscopy (SEM). For this purpose, after rinsing cell-seeded samples with PBS, fixation in Karnovsky's fixative (glutaraldehyde + paraformaldehyde) for 24  h at 4 °C, and washing again with PBS, a series of increasing concentrations of ethanol (30, 50, 70, 90, and 100%v/v) were used for dehydration. The samples were dried completely, sputter-coated with gold, and observed under microscope (SEM, JEOL-JSM6510, Japan).

## 2.6. Statistical analysis

Compressive strength of samples was measured for at least 10 samples. Setting time was recorded for 3 samples per composition. Data for setting time and compressive strength are presented as mean ± standard deviation and statistical analysis was performed by student's t-test. P values < 0.05 and < 0.01 were considered significant and very significant, respectively.

Statistical analysis of the difference in cell proliferation after certain time points (1, 3, and 7 days), was performed using GraphPad Prism® software (ANOVA, *t*-test), considering *P* < 0.05 for statistically significant difference.

# 3. Results

## 3.1. Phase analysis

Fig. 1a shows the XRD spectra of the BrCs after 24 h of incubation in PBS. The only phases present in all samples were β-TCP and dicalcium phosphate dehydrate (DCPD). Additional phases were not noticed within the spectra. A decrease in the relative DCPD peaks’ intensity is noticed with increasing amounts of Fe dopant concentration. The β-TCP/DCPD ratio for samples, calculated by using the values of the most intense TCP (2θ = 31.1°) and DCPD (2θ = 21°) peaks was 1.84, 2.03, 2.56, and 3.69 for pure, 0.25-Fe, 0.50-Fe, and 1.00-Fe, respectively.

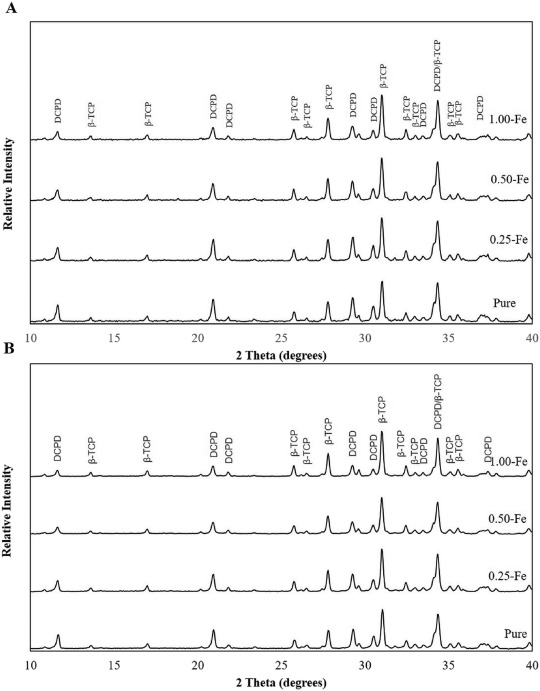


Fig. 1. XRD spectra of pure and Fe-doped BrCs after, a) 24 h incubation in PBS to allow the setting of the cement. The spectra show presence of β-TCP and DCPD as the only two phases, where the ratio of β-TCP/DCPD increases with increase in Fe concentration. b) after 16 days dissolution in PBS. The β-TCP/DCPD ratio has further increased as compared to before dissolution samples.

## 3.2. Setting time

The initial and final setting times of each composition are presented in Fig. 2. Addition of 0.25 wt% Fe did not change the initial and final setting time of BrC, significantly. However, the initial and final setting time of BrC significantly increased from 3.51 ± 0.40 min and 7.58 ± 0.22 to 5.74 ± 0.07 and 13.05 ± 0.05 with adding 0.5 wt% of Fe. The highest setting times of 6.88 ± 0.04 min and 24.16 ± 0.08 min were recorded for 1.00 Fe–BrC.

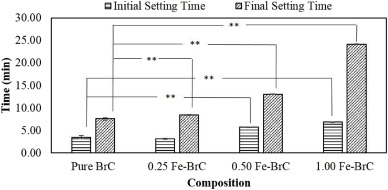


Fig. 2. Initial and final setting time of pure and Fe-doped BrC samples. Low concentration of 0.25 wt% Fe does not affect the setting time, where 0.5 wt% addition increases the initial and final setting times. The 1.00 Fe–BrC has the highest setting time (\**P* < 0.05 and \*\**P* < 0.01).

## 3.3. Compressive strength

Compressive strength of samples is presented in Fig. 3. Addition of 0.25 and 0.5 wt% of Fe does not have significant effect on the compressive strength of samples. The compressive strength of pure, 0.25-Fe, and 0.50-Fe, are 7.05 ± 1.57 MPa, 5.66 ± 1.26 MPa, 6.41 ± 1.75 MPa, and, respectively. However, addition of 1.00 wt% Fe significantly decreased the compressive strength to 3.12 ± 1.06 MPa.

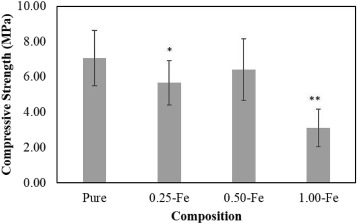


Fig. 3. Compressive strength of each composition after 24 h in PBS. Addition of 0.25 and 0.5 wt% of Fe does not affect the compressive strength, while 1.00 wt% of Fe dopant decreases the compressive strength, significantly (\**P* < 0.05 and \*\**P* < 0.01).

## 3.4. Dissolution study

The change in pH over the course of 16 days in PBS is presented in Fig. 4. In general, the pH of the PBS solution was lower for all Fe-doped samples. The 1.5 h and 14 d markers had the most drastic change in pH. After pH measurement at 16 day, samples were removed and dried at room temperature, and phase composition was analyzed by XRD (Fig. 1B). Similar to as-fabricated BrCs, TCP and DCPD were the only two phases in the samples. The TCP/DCPD ratio after dissolution was 2.08, 2.49, 3.21, 4.45 for pure, 0.25-Fe, 0.50-Fe, and 1.00-Fe, respectively. As noticed in Fig. 1S, compressive testing on samples was not possible due to the irregular change in cross section of samples.

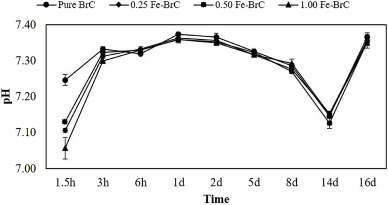


Fig. 4. Change in pH during 16 days of immersion in PBS.

## 3.5. Cytocompatibility assays

Fig. 5 shows the results of PrestoBlue assay on scaffolds. Addition of 0.25 and 0.5 wt% of Fe does not affect cell proliferation in comparison to pure samples. However, 1.00 wt% of Fe dopant decreases significantly the cell proliferation in different time points. Images of the cells attached on the bottom of the plate which were in contact with the samples during 24 h, Live/Dead staining on the surface of samples after 7 days, and SEM confirmed less cell proliferation on the group of 1.00 wt% of Fe dopant (Fig. 6a, b, and Fig. 7).

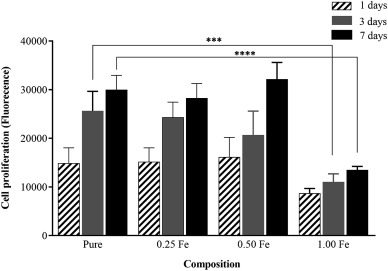


Fig. 5. Cell proliferation on scaffolds in different time points.

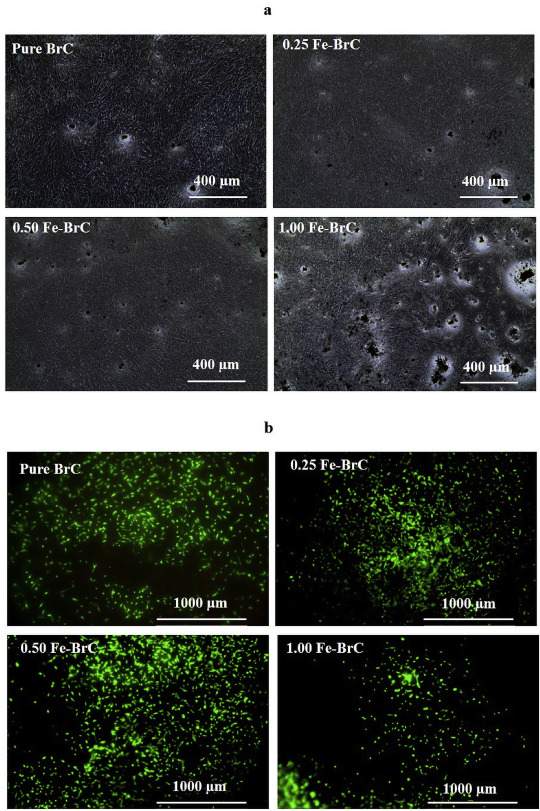


Fig. 6. Morphology of the cells attached on the bottom of the plate which were in contact with the samples during 24 h (a). Images of live (green) and dead (red) cells seeded on scaffolds after 7 days (b). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

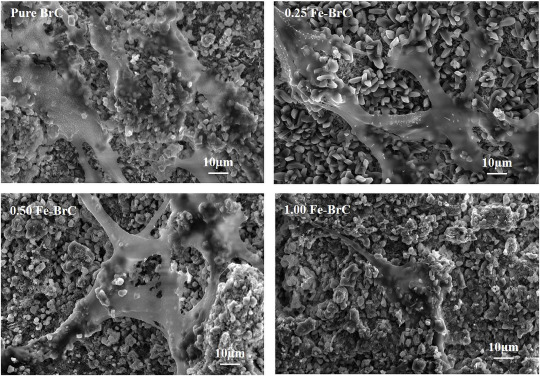


Fig. 7. The SEM micrographs showing cell attachment of DPSCs after 24 h seeding on different samples.

# 4. Discussion

Fe is a natural trace element in the body with the highest concentration in the blood. However, it is also present in other tissues like dentine and enamel in lower concentrations. Despite the low amount, Fe is believed to play a critical role in oral health through its antimicrobial effects and prevention of dental caries [14,36,37]. In this work, we examined the role of Fe presence and its concertation on physical, mechanical and biological properties of BrC for possible dental tissue engineering applications.

Samples were prepared based on our previous studies, where 2 wt% PEG solution was added to the cement powder to create a paste, followed by 24 h incubation in PBS at 37 °C [18,40,41]. XRD results indicate only β-TCP and DCPD phases are present within the samples. No new phases were present due to the addition of Fe, likely due to the low content of dopant. The XRD patterns indicate an increase in the β-TCP/DCPD ratio with the dopant, which will affect the physical and mechanical properties of the BrC. Increase in the β-TCP/DCPD ratio with increase in Fe concentration is directly related to the Fe substitution in TCP crystal structure. Fe3+ as a trivalent ion with the radius of 0.64 Å substitutes the Ca2+ site (size of 0.99 Å), and results in shrinkage, and eventually stabilizing the β-TCP structure which is more prominent in samples with 0.5 and 1.0 wt % Fe [38]. Formation and setting of BrC includes the dissolution of TCP and MCPM, followed by precipitation of DCPD [5,7]. As Fe incorporation stabilizes the crystal structure of β-TCP, the decrease in dissolution of β-TCP and thus increase in TCP/DCPD ratio as a result of higher concentration of Fe is expected, which is in line with our previous data that Co substitution has similar effect on dissolution behavior of TCP [40,41]. Similarly, presence of Fe alters the setting time of the BrC that is controlled by dissolution-precipitation processes. Setting time of BrCs is an important property which determines if the material is suitable to use in clinical applications. The ideal setting time should not be too long as the patient may be under anesthesia, nor should it set too soon as it will be difficult to work with [1,5,6,17]. Increase in setting time with addition of Fe is directly related to decrease in TCP dissolution as the first step of setting reaction. As Fe was doped in β-TCP and not present in MCPM, it can be concluded that increasing the setting time with Fe addition is due to Fe retarding effect on TCP dissolution and/or DCPD precipitation.

The compressive strength of brushite decreased most significantly within the 1.00 Fe–BrC from 7.05 ± 1.57 MPa to 3.12 ± 1.06 MPa. Change in mechanical properties can be affected by a number of variables such as dopants, chemical composition, minor phases, polymer presence and its concentration, and TCP/DCPD ratio. Dopants alter the phase purity and stability of brushite cements, thus leading to a change in strength of the material. Minor phases could not be detected with XRD spectra and additives to create the cement powder were kept at consistent ratios between the samples, as well. Thus, the only difference between samples is the amount of dopant and the resultant change in other properties, mainly the TCP/DCPD ratio and the setting time. Increase in TCP/DCPD ratio results in decrease in compressive strength [2] and prolonged setting reaction, inhibits the crystal growth and thus, enhances the strength [2,42]. In current research, addition of Fe increases both TCP/DCPD ratio and the setting time, in a dose dependent manner that shows there are two counteracting parameters that will affect the strength. In general, TCP/DCPD ratio has stronger effect and we notice a general decrease in compressive strength with addition of Fe. However, in 0.50 Fe–BrC sample where the initial and final setting time increases significantly (as compared to pure and 0.25 Fe–BrC samples), the setting time shows a stronger effect as TCP/DCPD ratio, and compressive strength increases. We have previously reported the decrease in compressive strength with increase in TCP/DCPD by addition of other dopants such as silicon (Si), zinc (Zn), and cobalt (Co) [18,41], and the counteracting role of the TCP/DCPD ratio and setting time in BrCs with different concentrations of Si [17].

Dissolution was performed in PBS at pH 7.4 to understand the role of Fe and its concentration on change in pH of buffer solution, and morphology and compressive strength of samples. The pH of solution was in the range of 7.2–7.4 throughout the experiment for all samples, except for the two timepoints of 1.5 h, and 14 days. At 1.5 h, the pH of Fe–BrC was less than that of pure BrC, with higher drop with increasing in Fe content. This shows that an acidic reaction was happening or accelerated in these samples. Comparing the XRD data after 16 days of dissolution, the TCP/DCPD was further increasing with higher Fe concentration as compared to primary samples, proving that the presence of Fe has retarding effect on TCP dissolution and/or DCPD precipitation which is in line with our initial data. As the DCPD precipitation is accompanied by pH increase in dissolution-precipitation process [5,9], it may be concluded that Fe prevents DCPD dissolution and as a result, the pH does not increase significantly, as compared to pure BrC. Moreover, the dissolution was not uniform throughout the samples and changed the cross section (Fig. 1S). As a result, the uniform cross section of samples was not satisfied to measure the compressive strength after the dissolution.

To evaluate the efficacy of the prepared samples for bone and dental tissue engineering applications, the *in vitro* interaction of hDPSCs with BrCs was evaluated by cell attachment, viability, and proliferation. hDPSCs were first discovered in 2005 [43]. As compared to bone marrow stem cells that are one of the most studied stem cells, hDPSCs are derived from dental pulp [44]. These cells have better proliferative potential and odontogenic capability as compared to MSCs [45,46]. Recently, hDPSCs have received significant attention for their potential use in different tissue engineering applications, including nervous system, retina, dental pulp, bone, and cartilage [44]. Thus, evaluating their interaction with bone and dental substitutes is very important. Incorporating magnetic and non-magnetic iron oxide nanoparticles into dicalcium phosphate anhydrous cements does not have cytotoxic effect on human dental pulp cells in comparison to pure sample, however high concentration of Fe could result in lower cell attachment and proliferation. In addition, regardless of magnetic properties of the cements, presence of nanoparticles enhances the osteogenic differentiation of hDPSCs at days 7 and 14, significantly, which is attributed to the surface nanotopography of scaffolds and cell internalization in presence of nanoparticles, through WNTβ-catenin signaling pathway [47,48]. These data is in line with our findings where we noticed significantly decrease in proliferation of hDPSCs as early as day 3 of culture, where lower concentrations did not have significant effect as compared to the pure sample. Dose dependency of cellular interaction with Fe doped calcium phosphates is also reported elsewhere. Basu et al. have shown that Fe incorporation in biphasic calcium phosphate up to 2 mol % enhances the MC3T3 cell viability and increasing the content to 20 mol % decreases the cellular activity significantly [49]. Fe is an essential element for survival of human pluripotent stem cells as well [50]. We have previously shown that Fe presence in tricalcium phosphate enhances proliferation and differentiation of osteoblast cells in a dose dependent manner, and upregulates type-I collagen expression *in vivo* [38,39].

# 5. Conclusion

In this work, different concentrations of Fe were incorporated to BrC and physical, mechanical, and biological properties of BrC were evaluated in presence of Fe. Stabilizing the crystal structure of TCP by increase in Fe amount, increases the TCP/DCPD ratio and prolongs the setting reaction, in a dose dependent manner. The highest TCP/DCPD ratio of 3.69 and final setting time of ~24 min are found for BrCs that contain 1.00 wt% Fe. Compressive strength decreased with Fe addition, except for 0.50 Fe–BrC where the strength was not significantly different as compared to that of the pure BrC. HDPSCs were well attached to the pure, 0.25 and 0.50 BrCs. Together, our current results suggest that 0.25 and 0.5 wt% Fe addition to BrC does not have adverse effect on setting time, compressive strength and proliferation of hDPSCs and along with stimulatory effect of Fe on proliferation and differentiation of osteoblasts, these compositions can be considered as suitable bone and dental substitutes. In addition, more studies on the effect of different concentration of Fe on differentiation of stem cells should be considered in future studies.

# Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: None.

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