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IGF-Loaded Silicon and Zinc Doped Brushite Cement: Physico-Mechanical Characterization and *In Vivo* Osteogenesis Evaluation

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Abstract

Dopants play critical roles in controlling the physical, mechanical, degradation kinetics, and *in vivo* properties of calcium phosphates. The aim of the present study was to evaluate the effects of Silicon (Si) and Zinc (Zn) dopants on physico-mechanical, and *in vivo* osteogenesis properties of brushite cements (BrCs) alone and in combination with insulin like growth factor 1 (IGF-1). Although addition of 0.5 wt.% Si did not alter the setting time, β -TCP content, and compressive strength of BrCs significantly, 0.25 wt. % Zn incorporation was accompanied by a significant decrease in mechanical strength from 4.78 ± 0.21 MPa for pure BrC to 3.78 ± 0.59 MPa and 3.28 ± 0.22 MPa for Zn-BrC and Si/Zn-BrC, respectively. The *in vivo* bone regeneration properties of doped BrCs alone and in combination with IGF-1 were assessed and compared using chronological radiography, histology, scanning electron microscopy and fluorochrome labeling after 2 and 4 months post implantation in rabbit femoral defect model. Based on different *in vivo* characterization, better osteogenesis and vasculogenesis was observed for Si-BrC and Si/Zn-BrC, whereas moderate bone regeneration was found in Zn-BrC as compared to pure BrCs. Excellent bone regeneration was observed when doped BrCs were combined with IGF-1. Our findings signify that addition of Si and/or Zn alters the physico-mechanical properties of BrCs and promotes the early stage *in vivo* osseointegration and bone remodeling properties. Moreover, addition of IGF-1 further improved the performance of BrCs in terms of bone regeneration in animal model.

Keywords

Brushite cement; Si and Zn dopants; IGF-1; setting time; compressive strength; *in vivo* osteogenesis

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1. Introduction

Calcium phosphate (CaP) based biomaterials are being widely used due to their distinguished biocompatibility and similar composition to that of bone. Among various CaPs, the cement form has appeared as a prospective bone graft substitute, specifically in management of small-scale bone defects and stabilizing load bearing prosthetic implants due to excellent mouldability, low setting temperature, and easy deformability at the application site [1,2,3,4,5]. Therefore, the cements are easier to handle than prefabricated CaP granules or scaffolds. Moreover, calcium phosphate cements (CPCs) are resorbable, osteoconductive, and noncytotoxic. They also generate chemical bonds to the host bone and restore contour [4,6,7].

Apatite (ApC) and brushite (BrC) are the two major forms of calcium phosphate cements. Even though AP cements show higher mechanical strength, *in vivo* resorption rates are very slow and hinder the bone regeneration process [8,9]. On the other hand, BrCs are resorbed much faster *in vivo* due to the presence of metastable CaP phases [10,11,12]. Nonetheless, the major limitations of BrC are local decrease in pH and its quick setting properties [5,13,14,15]. Several modifiers like sodium hydrogen phosphate, compounds of sulfates and citrates, as well as various metal ions have been added to modulate the setting time of BrCs [1,16,17,18].

BrCs have shown new bone formation *in vivo* through attachment and proliferation of bone-forming cells. However, the recent trend in developing a new generation of resorbable biomaterials demands osteoinductivity [19]. Osteoinduction can be accomplished by the addition of growth factors such as bone morphogenetic protein (BMP), insulin like growth factor (IGF), vascular endothelial growth factor (VEGF), and transforming growth factor β (TGF- β) [20,21]. Metallic ion substitutions in CaP cements have recently garnered a great deal of attention in biomedical research because of their significant role in overall bone turnover. Among various metal ions, strontium (Sr), zinc (Zn), magnesium (Mg) and silicon (Si) are predominantly studied [16,17,18, 22, 23]. Zn has stimulatory effect on proliferation of osteoblast cells *in vitro* and inhibits bone resorption through retarding osteoclastogenesis *in vivo* [24,25]. Moreover, the zinc ion plays a critical role in various proteins and metallo-enzymes such as alkaline phosphatase (ALP) activity essential in bone regeneration. Si is another important trace element that is involved significantly in bone regeneration/ calcification and induces biological activity by enhancing the solubility of the material and increasing the electronegativity of surface [26]. In addition, it promotes cellular activities such as proliferation, differentiation and mineralization of osteoblasts and mesenchymal stem cells [27,28,29]. In addition to single doped CaPs, binary doped systems have been also studied. Co-substitution of strontium (Sr) and zinc (Zn) ions in BrC reduces the setting time, enhances the compressive strength and induces osteoblast maturation [16,17].

Furthermore, bone derived growth factors such as BMP, IGF, VEGF, and (TGF- β) trigger many biological activities leading to enhanced bone formation [20,21]. IGF-1 is a well-known cytokine, enhancing bone and cartilage growth, and plays vital role during embryonic patterning and early skeletal formation [30]. Delivery of growth factors to local

fracture sites is an effective approach for early bone healing. However, their effective integration for getting optimal outcome is very challenging task.

The purpose of the present work is to develop Si and/or Zn doped brushite cements and to investigate the effects of dopants on the physico-chemical properties of BrCs including phase composition, setting time, and compressive strength. The capability of BrCs as IGF-1 carrier along with their effectiveness on *in vivo* new bone formation in rabbit bone defect model was studied over 4 months. We hypothesize that metal ion release from doped BrCs will trigger osseointegration at early stages of bone healing process. In addition, IGF-1 release from BrCs along with presence of dopants will enhance the *in vivo* bone regeneration. To validate our hypothesis, in the present work we report the effects of dopants on phase composition, setting time, and microstructural and mechanical properties of BrCs. We have also investigated the effects of Si and/or Zn along with IGF-1 on *in vivo* bone regeneration in rabbit distal femur models using chronological radiographs, fluorochrome labeling, scanning electron microscopy and histology. To the best of our knowledge, the effects of Si and/or Zn on physico-chemical, mechanical and *in vivo* bone regeneration properties of BrCs, alone or in presence of IGF-1 is not investigated.

2. Materials and methods

2.1. Cement Preparation and Characterization

The brushite cement powders were synthesized as described in the supporting data in detail. 0.5 wt. % Si, 0.25 wt. % Zn, and 0.5 wt. % Si/0.25 wt. % Zn doped brushite cements were made by mixing relative amount of SiO₂ and ZnO with precursors of β -TCP. The cement paste was prepared by mixing 2 wt. % polyethylene glycol (PEG) solution with the powder and then molded into 6mm diameter and 12 mm height cylindrical molds. From now on pure, 0.5 wt. % Si, 0.25 wt. % Zn, and 0.5 wt. % Si/0.25 wt. % Zn doped brushite cements will be denoted as BrC, Si-BrC, Zn-BrC, and Si/Zn-BrC, respectively.

Phase analysis, microstructure and compressive strength of pure and doped BrCs were evaluated using an X-ray diffractometer (XRD), field emission scanning electron microscope (FESEM), and a screw-driven universal testing machine as described in supporting data.

2.2. Setting Time

According to ASTM C266, a Gillmore needle was used to measure initial and final setting time of the BrCs. The cement paste was poured into the mold as described above. Initial and final setting times were recorded as the time that needles with 113.4 g weight (and 2.12 mm diameter) and 453.6 g weight (and 1.06 mm diameter) could not leave an impression on the surface of the cement paste, respectively.

2.3. In Vivo Study

Animal studies were performed following the standards of the Institutions Animal Ethical Committee of the West Bengal University of Animal and Fishery Sciences, India. The study was conducted on thirty six mature New Zealand white rabbits with an average weight

ranging from 1.5–2 kg. All the animals underwent bilateral surgery with control BrC in right limb and Si-BrC, Zn-BrC, and Si/Zn-BrC cement in left limb. In each group, 3 rabbits were euthanatized at 2 and 4 months to assess and compare the progressive healing potentialities. The effect of IGF-1 on bone healing process was assessed using IGF-1 loaded BrCs. 30 µg recombinant human IGF-1 (G-Biosciences, USA) was dissolved in 0.1% sterile bovine serum albumin in PBS and was then mixed with pure and doped BrCs in sterile condition. Similarly to previous part, IGF-1 loaded pure BrC was used as control in right limb and IGF-1 loaded doped BrCs were applied as treatment in left limb.

The animals were kept off feed for 6 hours prior to surgery. The lateral aspect of the distal femur bone of both hind limbs was prepared aseptically and the area below the operation site up to paw was protected with sterile protective bandage. The animals were anaesthetized with the combination of injection xylazine hydrochloride at 6 mg/kg body weight (Xylaxin®, Indian Immunologicals, India) and ketamine hydrochloride 30 mg/kg body weight (Ketalar®, Parke-Davis, India) intramuscularly. The operative site was prepared aseptically and painted with povidone iodine 5% solution.

A motorized dental drill was used to create (19 mm²) bone defect in lateral aspect of the both distal femur of all animals. The cements were implanted in the defects followed by suturing to close the wound. Treated animals were administered intramuscularly with cefotaxime sodium (Mapra India, India) at 20 mg/kg body weight for 5 days at 12 hour intervals, and meloxicam at 0.2 mL (Intas Pharmaceuticals, India) once daily. All animals were sacrificed after 2 and 4 months of experiment.

Bone healing in the operated limb defect was monitored using chronological radiographs taken immediately after implantation and once a month up to 4 months. The radiographs were examined at the two time points of 2 and 4 months to assess implant status, implant-bone interface and new bone formation. Fluorochrome (oxytetracycline dehydrate; Pfizer India, India), 25 mg/kg body weight, was intramuscularly injected 3 weeks before sacrifice at 2 and 4 months. Undecalcified sections prepared from implanted segments were ground to 20 µm thickness. To determine the amount and source of the newly formed bone, the sections were observed using an Orthoplan microscope (Excitation filter, BP- 400 range, Leitz, USA). After sacrificing the rabbits, bone samples were collected at two time points to study the interface between bone and cement. Samples were fixed in glutaraldehyde solution followed by gradual ethanol series drying. Dried samples were gold coated before imaging using FESEM (FEI 200F, FEI, OR). For histological analysis, the bone specimens were fixed in 10% formalin until full demineralization. The bone tissues were then decalcified using Goodling and Stewart's fluid, followed by fixation in 4% paraformaldehyde. Paraffin wax was used to embed the bone samples and 4 µm sections were cut from the mount and stained with haematoxyline and eosin. Additionally, a scoring system was developed from the histological slides to assess the osteogenesis and vasculogenesis activity of specimens. Each activity was scored accordingly on a scale from 0–4: (0) absent, (1) mild, (2) moderate, (3) marked, and (4) severe.

2.4. Statistical analysis

Compressive strength measurement was applied on at least 8 samples. Histological images of all groups were taken with $n=3$ per experiment and experimental group. All data were presented as means $\pm$ standard deviations using one-way ANOVA.

3. Results

3.1. Setting Time

Effects of Si and/or Zn on initial and final setting times of BrCs are presented in Table 1. Si addition decreased the initial setting time and increased the final setting time slightly. However, presence of Zn as single or binary dopant in Zn-BrC and Si/Zn-BrC increased both initial and final setting significantly.

3.2. Phase Analysis and Microstructure

Figure 1 shows the XRD spectra of pure and doped BrCs after 1 day of incubation in PBS. β -TCP (JCPDS no. 09-0169) and brushite (DCPD, JCPDS no. 09-0077) were the only phases present in all samples. Presence of Si and/or Zn in cements did not introduce any new phase. β -TCP content in cements with different dopants is presented in Table 1. Although addition of 0.5 wt. % Si did not affect the β -TCP amount, 0.25 wt. % Zn increased the β -TCP content to 66–67% in both Zn- and Si/Zn-BrCs.

FESEM micrographs of cements after 1 day of incubation in PBS are presented in Figure 2. BrC sample showed the TCP granules embedded in more compact structure of DCPD. Compared to BrC, individual needle/plate like DCPD crystals were noticed on TCP granular structure in all doped samples.

3.3. Compressive Strength

To analyze the mechanical properties of bone cements according to ASTM standard (ISO 5833), BrCs should set at least for 24 h, preferably under physiological condition before mechanical testing. Figure 3 represents the compressive strength of BrCs. Compressive strengths of BrC and Si-BrC were 4.78 ± 0.21 MPa and 4.32 ± 0.63 MPa, respectively. Although Si addition did not change the compressive strength of BrC significantly, Zn incorporation resulted in notable decrease in compressive strength.

3.4. In Vivo Study

3.4.1 Radiological Examination—To investigate the bone-material interaction, *in vivo* experiment in a standardized animal model is necessary. Among various characterization tools, radiology is one of the vital noninvasive methods to investigate the host bone-material interfaces after implantation [31]. Figure 4 shows the radiographs of cement implanted rabbit distal femur as a function of implantation time and cement composition. The bone defect implanted with different BrCs showed presence of radiodense material extending up to the medullary cavity without any spillage on the day of surgery. At 2 months, the shape of the BrC changed from cylindrical to oval. The periphery of the cement showed initiation of radiolucency. Si-BrC had irregularity of the margin of the implant without any change in radiodensity, whereas Zn-BrC revealed irregularity in the periphery of the cement pack with

moderately increased radiolucency. After 4 months, pure BrC showed bridging of the gap with the presence of a faint cortical line and reduction of cement size. The radiodensity of Si-BrC decreased and the periphery of the implant was very irregular. In addition, most of the cement material was still present. However, the adjoining medullary cavity of the bone was well established in Zn-BrC sample. In Si/Zn-BrC, there was presence of nil to traces of the cement material in the bony defect.

Figure 5 shows the radiographs of IGF-1 loaded BrCs in rabbit femoral defect. Up to 2 months post implantation, no significant change was found except establishing continuity in medullary cavity in BrC-IGF-1. Appreciable continuity of marrow cavity at the adjacent defect area was observed at 4 months with formation of newly formed bony tissue. The Si-BrC-IGF-1 radiograph showed gradual diminution of radiodensity of material which was initiated from 1 month (presented in supporting data) and became homogenous at 4 months. The process of resorption of the implant started at 1 month and completely remodeled with continuous homogenous cortex at the defect area after 4 months. The Zn-BrC-IGF-1 radiograph did not reveal any remarkable change of implant in terms of radiodensity and quantum up to 2 months. However, the process of remodeling of medullary cavity continued and ended at 4 months. Binary doped Si/Zn-BrC-IGF-1 showed appreciable diminution of radiodensity of material at 2 months and completely re-established medullary cavity at the defect site at 4 months.

3.4.2 Fluorochrome Labeling Study—Fluorochrome labeling studies provide a way to study bone mineralization [32]. When incorporated, the labeled old and new bones emit dark-sea green and bright golden-yellow fluorescence under UV light, respectively. Figure 6 shows the fluorochrome labeling results after 2 and 4 months post implantation. The new bone was formed moderately from both ends in pure BrC (Figure 6a). As shown in Figure 6b, the deep golden yellow color in center of Si-BrC indicated newly formed osseous tissue on the endosteal side and sea green appearance at the outer border represented old bone on the periosteal side. Figure 6c shows that the presence of golden yellow color on both the endosteal and periosteal sides of Zn-BrC, although the intensity was less than Si-BrC. In Si/Zn-BrC samples as shown in Figure 6d, extensive new bone formation was noticed on the endosteal surface, originating from the host bone towards the implant. As shown in Figure 6 (e-h), new bone formation was enhanced in all samples as the area with golden yellow fluorescence became larger after 4 months of implantation. Wider regions of newly formed bone was found in Si/Zn-BrC compared to pure BrC and Zn-BrC. Si-BrC also showed intense new bone formation compared to previous time point.

Microphotographs observed under fluorescent light imparted a two-tone golden yellow fluorescence in a narrow linear zone in the defect site in BrC-IGF-1 samples after 2 months and the remaining areas looked dark sea green indicating presence of host bone (Figure 7a). Compared to Si-BrC-IGF-1 (Figure 7b) and Zn-BrC-IGF-1 (Figure 7c), the intensity of newly formed bone was more in Si/Zn-BrC-IGF-1 samples (Figure 7d) after 2 months. At 4 months, reasonably higher amount of new bone formation was observed in all BrCs as compared to 2 months. It was also observed that intensity of new bone formation was much higher in Si/Zn-BrC-IGF-1 samples than that of Si-BrC-IGF-1 and Zn-BrC-IGF-1 samples

(Figure 7 e-h). Comparing the IGF-1 loaded doped samples with unloaded doped BrCs, better new bone formation was found in loaded ones.

3.4.4 Histological Observations—Histological experiments were performed on host bone-cement cross sections to understand the mechanism of bone remodeling through evaluation of cellular and vascular activities. Figures 8a-d and Table 2 show the histological evaluation at the bone-implant interface after 2 months. As shown in Figure 8a, BrC showed well formed osseous tissue with osteoblast and osteoclast accumulation within the matrix of the osseous tissue. Fibrovascular proliferation around the haversian system developed moderately. Infiltration with mononuclear cells and blood cells along with exudation with serum/plasma was noticed. As shown in Figure 8b, Si-BrC depicted well formed osseous tissue embedded around haversian systems and canalicular structures. Some portion of medulla was infiltrated with mononuclear cells, blood cells and osteocytes along with accumulation of multinucleated osteocytes. Fibrin and osteoblast deposition in osseous lamina were moderate. Fibrovascular reaction particularly in medullary plate was high. Zn-BrC samples exhibited well-formed vascular osseous plates invaded by numerous osteoclasts and osteocytes. Fibrocytic proliferations were abundant in the bony matrix. Cellular infiltration by mononuclear cells, osteoblasts, osteocytes, red blood cells (RBCs) and mucin were prominent, as presented in Figure 8c. Figure 8d shows the histological images of Si/Zn-BrC cement with well developed bony structure, less vascular medullary sinuses and occasional fibroblastic proliferation around bony matrix. Clusters of osteoblasts initiated the development of permanent bony plates by replacing immature osseous stroma and angiogenesis was scanty. Histological evaluations at 4 months for the pure and doped BrCs are shown in Figures 8 e-h and Table 2. In comparison to results of 2 months, a visibly higher angio-proliferation component of bony tissue was noticed for all the BrCs. Numerous haversian systems, canalicular structures, infiltration with fibroblasts, osteoblasts, and mononuclear cells were noticed in all three doped BrCs compared to pure BrC. Figures 9 a-d and Table 3 show the histological evaluation at the bone-implant interface after 2 months with IGF-1 loaded BrCs. At 2 months, IGF-1 loaded doped samples showed fibrovascular proliferation of osteon, well formed haversian canals with lacunae, infiltration with osteoblasts, RBCs, few osteoclastic cells and marked angioinvasion in comparison to pure BrC. At 4 months, the IGF-1 loaded doped section depicted highly vascular osseous tissues with clumps of robust osteoblastic network invading the whole stroma. Fibrosis around the cortical region is mild and the total stroma are encapsulated by varying types of endothelial stroma i.e. blood vessels. The medullary region is quite normal and characterized by infiltration with mononuclear cells, osteoclasts, and mucinous network as compared to BrC-IGF-1 (Figures 9 e-h and Table 3).

From the histological score sheet as presented in Tables 2 and 3, which is based on osteoclastic activity, vascularity and osteoblastic activity, more bone regeneration was observed in Si/Zn-BrC followed by Si-BrC, Zn-BrC, and BrC at both time points. Similar activities of bone regeneration were also observed in IGF-1 loaded BrCs.

4. Discussion

BrCs are biocompatible, osteoconductive, bioresorbable, and more soluble than ApCs at physiological pH [8,9]. Its resorption is closely followed by new bone formation *in vivo* [12]; however, short setting time, low mechanical strength, and *in vivo* rapid resorption are the major limitations of BrCs for clinical applications.

In general, addition of trace elements to CaPs results in controllable degradation kinetics and mechanical strength. Furthermore, it can alter the total new bone formation by affecting osteogenic and/or angiogenic properties of CaPs [24,33, 34,35,36]. The purpose of this study is to investigate the effects of Si and/or Zn dopants on physical, mechanical, and *in vivo* osteogenic and angiogenic properties of BrCs alone and in combination with IGF-1.

Based on our previous results of the effects of PEG concentration and incubation time on physical, mechanical, and biological properties of BrCs, 2 wt. % PEG solution was added to powder to form the cement paste, followed by incubation in PBS for 24 hours at 37 °C [37]. XRD results indicate formation of β -TCP and DCPD as the only two phases present in structure. No other phases containing Si and/or Zn dopants were formed, which is due to the low amount of added dopant. However, Zn addition increases the β -TCP percentage in cement. Brushite cement forms through three steps: 1- dissolution of cement precursors, 2- formation of a supersaturated gel, and 3- nucleation and growth of DCPD. In monocalcium phosphate monohydrate (MCPM)/ β -TCP system, cement formation begins with MCPM dissolution, followed by β -TCP dissolution, and finally DCPD precipitation [2]. Doped BrCs can be synthesized through two different approaches: mixing the cement powder with the salt containing the dopant [38], or doping the cement precursors with desired dopants [16]. In current work, Si and Zn were added to β -TCP precursors to form the doped TCP. Si^{4+} substitutes for P^{5+} in β -TCP lattice and creates defects by either formation of O^{2-} vacancies or the presence of excess Ca^{2+} , and thus increases the β -TCP solubility [26,39]. However, Zn^{2+} stabilizes the β -TCP structure through substitution for Ca^{2+} . The structure stabilization retards the β -TCP dissolution [40,41]. Due to the increased β -TCP dissolution rate by Si substitution, a slight increase in final setting time may be related to its inhibitory effect on DCPD precipitation. However, our results show that Zn^{2+} presence as single or binary dopants increases both initial and final setting time significantly, which may be due to its retarding effect on TCP dissolution and/or DCPD precipitation.

In the present work, cements were set for 1 hour at room temperature under 100% humidity, followed by 24 hours immersion in PBS, prior to compressive strength evaluation. Ionic substitution, presence of polymer, polymer concentration, and cement components chemistry alter the mechanical properties of cements. Pure and doped BrCs were synthesized through mixing the pure and doped TCP powder with the other components. In other words, the type of dopant was the only variable to be considered as the effective parameter on compressive strength of cements. Dopants alter the TCP phase purity and stability, and as a result affect the final strength of cements. It has been already reported that increase in TCP content in BrCs is accompanied with decrease in compressive strength [37]. In addition, a better compaction of structure is seen in pure BrC. Thus, formation of higher

amounts of TCP in Zn-BrC and Si/Zn-BrC results in drop of compressive strength in Zn doped BrCs.

To investigate the interaction between host bone and cements, *in vivo* experiment was performed in rabbit distal femur model. Radiology was used as a noninvasive method to study the interface between host bone and cement after implantation at different time points. In addition, fluorochrome labeling was applied to investigate bone mineralization [42]. When incorporated, the fluorochrome binds directly to undergoing calcification areas at the bone/osteoid interface. Tetracycline is an effective marker of skeletal features, bone mineralization, and its remodeling processes which gets absorbed to the areas where mineralized tissue is depositing [43,44,45]. In this study, the shape of the bony defect has changed from cylindrical to oval with moderately increased radiolucency after 2 months, indicating onset of resorption of implant material in single and binary doped BrCs. In addition, fluorochrome labeling results show the moderate new bone formation in pure BrC, relatively higher in Si-BrC and Zn-BrC cement samples, and extensive in Si/Zn-BrC. At 4 months "taking up" of the implant by the host tissue occurs in more efficient way in Si- and Zn-BrCs indicating better process of resorption and remodelling of the bony defect. Si/Zn-BrC samples show the presence of nil to traces of the cement material in bony defect, representing almost complete resorption of the material by the host tissue and healing of bony defect. Moreover, doped samples provide better results as compared to pure samples due to stimulatory effects of zinc and silicon [46]. Similarly, fluorochrome labeling results showed the greater area of new bone formation in all doped samples, specially in Si/Zn-BrC samples indicating rapid bone regeneration in binary doped BrC. Zn ions have stimulatory effect on proliferation and differentiation of osteoblast cells and inhibitory effect on osteoclast activity [24,25,47, 48,49]. Furthermore, Zn ion is involved in many metallo-enzymes and proteins. Similarly, Si can stimulate proliferation and differentiation of osteoblast cells and osteogenic differentiation of mesenchymal stem cells [27, 28, 29]. The induced bone regeneration by Si at early stage is possibly due to the synthesis and/or stabilization of collagen [27,50]. In addition, it can effectively encourage angiogenesis by upregulating nitric oxide synthase (NOS) leading to increased VEGF production [36]. In some cases, leakage of the brushite cement has been observed into the adjacent tissues, but it is ultimately resorbed without causing any significant complications [51]. Similar observations were also noted in microstructural evaluation using SEM. In Si-BrC and Si/Zn-BrC, new osseous tissue formation starts with no interfacial gap between bone and implant which may be due to early release of metal ions to initiate new bone formation. In Zn-BrC, there is no close association between bone and implant; however, collagen fibrils are present on the cement surface indicating initiation of new bone formation. Extensive new bone apposition through SEM studies is also noted with injectable calcium phosphate bone substitute in a critical-sized bone defects at the distal end of rabbit femurs [52]. The interfacial gap was almost absent in IGF-1 loaded BrCs due to its effective role as a positive catalyst for soft tissue formation. In all the cases, IGF-1 helped the formation of collagen network and gave a matrix for bone cell colonization which was completed after 4 months as can be seen from microstructure.

To understand the mechanisms of bone remodeling, histological study from the retrieved bone samples was carried out. Histological evaluation at the bone-implant interface confirms osteoblastic activity at 2 and 4 months in Si/Zn-BrC cement followed by Si-BrC, Zn-BrC and pure BrC. Si actively takes part in initial bone formation [26]. Moreover, addition of Si to biomaterials enhances their bioactivity [53] and osteogenic properties [54,55]. Zn, another essential trace element plays a vital role in new bone formation [56] and maturation utilizing metalloenzymes like alkaline phosphatase (ALP). ALP creates an alkaline environment that supports the precipitation and consequent mineralization of inorganic phosphates onto the extracellular matrix (ECM). The release of zinc from the implant material is thought to stimulate the osteoblastic bone building process and to stop the osteoclastic resorption process [47,48]. Various studies on zinc-doped CaPs have also demonstrated increased *in vitro* osteoblastic response and *in vivo* new bone formation [24,57]. Higher osteoblastic activity and lamellar bone formation is prominent in Si/Zn-BrC which is due to combining effects of both dopants on bone formation process and mineral aggregation. In general, woven bone is substituted by lamellar bone as growth and remodeling continues which corroborates the present findings in binary doped samples [58]. The superior osteoblastic activity in IGF-1 loaded BrCs is presumably due to sustained local release of growth factor [59]. IGF promotes cell proliferation and chemotactic migration, and facilitates bone metabolism [60]. In this study, statistical analysis was performed on the different cellular events based on the histological images observed. The score sheets provide an indication of bone regeneration based on different cellular events. Though not an exact quantitative analysis, they provide a quantifiable basis to explain the bone regeneration activity.

5. Conclusions

This study examines the effects of Si and Zn dopants on physical, mechanical, and *in vivo* osteogenesis and vasculogenesis properties of brushite cement alone and in combination with IGF-1. Addition of Si did not alter the setting time, β -TCP content, and compressive strength of BrCs significantly. However, Zn incorporation was accompanied by significant decrease in mechanical strength from 4.78 ± 0.21 MPa for pure BrC to 3.78 ± 0.59 MPa and 3.28 ± 0.22 MPa for Zn-BrC and Si/Zn-BrC, respectively. Zn addition increased the final setting time and β -TCP content in final product. All BrCs showed *in vivo* new bone formation during 4 months. Based on the microstructural, histological, radiological and fluorochrome labeling results, Si and/or Zn doped BrCs showed accelerated early bone formation at the rabbit model defect site and Si/Zn BrC and Si-BrC proved to be more effective than the other two compositions. Incorporation of IGF-1 in pure and doped BrCs further enhanced bone regeneration. Our findings suggest that incorporation of Si and Zn in BrCs can alter the physico-mechanical characteristics, and can effectively enhance early stage *in vivo* osseointegration and bone remodeling properties. Additional benefits in terms of enhanced bone regeneration can be achieved by incorporation of IGF-1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Insight, innovation, integration

This study was designed to study the effects of silicon and zinc on physical, mechanical, and in vivo osteogenic properties of brushite cements. In addition, the effect of insulin like growth factor-1 (IGF-1) presence along with dopants was investigated on bone formation in rabbit distal femur model. Addition of zinc increases the initial and final setting time; whereas silicon does not affect it significantly. Addition of dopants, specifically silicon in single or binary system enhances the total new bone formation, notably in rabbit distal femur model. Further bone formation at early stages of cement implantation was found in IGF-1 loaded doped cements. The results suggest that doped brushite cements, alone and in combination with IGF-1 are promising materials for bone tissue engineering applications.

Figure 1.
XRD spectra of BrCs after incubation in PBS at 37 °C for 1 day.

Figure 2.
FESEM micrographs of a) BrC, b) Si-BrC, c) Zn-BrC, and d) Si/Zn-BrC. Black and white arrows show the DCPD and TCP, respectively.

Figure 3. Compressive strength of BrCs after incubation in PBS at 37 °C for 1 day (* $P < 0.2$ and ** $P < 0.05$).

Figure 4.
X-ray images depicting implanted BrCs in the distal femur over the course of 120 days (arrow shows the implantation site and subsequent bone formation).

Figure 5.
X-ray images depicting IGF-1 loaded BrCs implanted in the distal femur over the course of 120 days (arrow shows the implantation site and subsequent bone formation).

Figure 6. Fluorochrome labeling images of BrCs after 2 and 4 months post implantation (1-Golden yellow fluorescence: new bone, and 2- Sea green fluorescence: old bone).

Figure 7.
Fluorochrome labeling images of IGF-1 loaded BrCs after 2 and 4 months post implantation
(1- Golden yellow fluorescence: new bone, and 2- Sea green fluorescence: old bone).

Figure 8.

Histological images depicted bone formation after 2 and 4 months post implantation. a) BrC 2 months, b) Si-BrC 2 months, c) Zn-BrC 2 months, d) Si/Zn-BrC 2 months, e) BrC 4 months, f) Si-BrC 4 months, g) Zn-BrC 4 months, h) Si/Zn-BrC 4 months.

Figure 9. Histological images depicted bone formation after 2 and 4 months post implantation in IGF-1 loaded samples. a) BrC 2 months, b) Si-BrC 2 months, c) Zn-BrC 2 months, d) Si/Zn-BrC 2 months, e) BrC 4 months, f) Si-BrC 4 months, g) Zn-BrC 4 months, h) Si/Zn-BrC 4 months.

Table 1

β -TCP wt. %, initial and final setting times of BrCs.

Composition	Initial setting time (min)	Final setting time (min)	β -TCP wt. % (± 1.5)
BrC	5 $\pm$ 1	11 $\pm$ 1	61.7
Si- BrC	4 $\pm$ 1	12 $\pm$ 1	61.1
Zn- BrC	7 $\pm$ 1	19 $\pm$ 1	67.1
Si/Zn- BrC	6.5 $\pm$ 1	17.5 $\pm$ 1	66.3

Table 2

Mean with standard deviation of the score sheet* for different cellular events at 2 and 4 months after observing the histological images for BrCs and doped BrCs.

Cellular events	BrCs		Si-BrCs		Zn-BrCs		Si+Zn-BrCs	
	2month	4 month	2month	4 month	2month	4 month	2month	4 month
Fibro-vascular proliferation	1.0 ^a ± 0.32	1.0 ^a ± 0.32	1.0 ^a ± 0.32	1.8 ^b ± 0.20	1.0 ^a ± 0.32	1.2 ^{ab} ± 0.20	1.0 ^a ± 0.32	1.0 ^a ± 0.00
Infiltration with mononuclear cells	1.0 ^a ± 0.32	1.0 ^a ± 0.00	2.0 ^b ± 0.32	1.2 ^a ± 0.20	1.0 ^a ± 0.32	1.8 ^b ± 0.20	1.0 ^a ± 0.32	0.80 ^a ± 0.20
Osteoclastic activity	2.0 ^b ± 0.32	1.2 ^a ± 0.20	2.0 ^b ± 0.32	2.0 ^b ± 0.32	2.0 ^b ± 0.32	1.0 ^a ± 0.00	3.0 ^c ± 0.32	1.8 ^b ± 0.20
Mucin deposit	1.0 ^a ± 0.32	1.2 ^a ± 0.20	1.0 ^a ± 0.32	1.0 ^a ± 0.00	2.0 ^b ± 0.32	1.0 ^a ± 0.00	1.0 ^a ± 0.32	1.0 ^a ± 0.00
Vascularity	1.0 ^a ± 0.32	2.8 ^c ± 0.20	1.0 ^a ± 0.00	1.0 ^a ± 0.00	1.0 ^a ± 0.32	1.8 ^b ± 0.20	2.8 ^c ± 0.20	2.8 ^c ± 0.20
Osteoblastic activity	1.0 ^a ± 0.00	1.0 ^a ± 0.00	1.8 ^b ± 0.20	1.8 ^b ± 0.20	1.8 ^b ± 0.20	2.0 ^b ± 0.00	1.8 ^b ± 0.20	1.8 ^b ± 0.20

The score value with different superscripts (a, b, c) within a row differs significantly p<0.05.

* The score sheet value is considered as (0) Absence, (1) mild, (2) moderate, (3) marked, (4) Severe.

Table 3

Mean with standard deviation of the score sheet* of different cellular events at 2 and 4 months after observing the histological images for BrCs-IGF-1 and doped BrCs-IGF-1.

Cellular events	BrCs+IGF-1		Si-BrCs+IGF-1		Zn-BrCs+IGF-1		Si+Zn-BrCs+IGF-1	
	2month	4 month	2month	4 month	2month	4 month	2month	4 month
Fibro-vascular proliferation	1.0 ^a ± 0.32	1.0 ^a ± 0.32	2.0 ^b ± 0.32	1.8 ^b ± 0.20	1.0 ^a ± 0.32	1.8 ^b ± 0.20	2.0 ^b ± 0.32	1.0 ^a ± 0.00
Infiltration with mononuclear cells	1.0 ^a ± 0.32	1.8 ^b ± 0.20	2.0 ^b ± 0.32	2.0 ^b ± 0.32	2.0 ^b ± 0.32	2.0 ^b ± 0.00	1.0 ^a ± 0.32	2.0 ^b ± 0.32
Osteoclastic activity	1.0 ^a ± 0.00	1.2 ^a ± 0.20	1.0 ^a ± 0.00	2.0 ^b ± 0.32	1.8 ^b ± 0.20	1.0 ^a ± 0.00	1.8 ^b ± 0.20	1.8 ^b ± 0.20
Mucin deposit	0.0 ^a ± 0.00	1.2 ^{ab} ± 0.20	1.0 ^b ± 0.32	2.0 ^b ± 0.00	1.0 ^b ± 0.32	1.0 ^a ± 0.32	1.0 ^a ± 0.32	1.0 ^a ± 0.32
Vascularity	1.0 ^a ± 0.00	1.0 ^a ± 0.00	1.8 ^b ± 0.20	1.8 ^b ± 0.20	1.8 ^b ± 0.20	1.8 ^b ± 0.20	2.8 ^c ± 0.20	2.8 ^c ± 0.20
Osteoblastic activity	1.0 ^a ± 0.00	2.0 ^b ± 0.00	1.0 ^a ± 0.32	1.0 ^b ± 0.00	1.0 ^a ± 0.32	1.8 ^b ± 0.20	1.0 ^a ± 0.32	1.0 ^a ± 0.00

The score value with different superscripts (a, b, c) within a row differs significantly p< 0.05.

* The score sheet value is considered as (0) Absence, (1) mild, (2) moderate, (3) marked, (4) Severe.