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Development of injectable bone substitutes with improved biocompatibility and faster tissue healing for bone tissue defects

Abstract

Tissue engineering is a promising strategy for the reconstruction of bone tissue defects. Calcium phosphate cement (CPC) can be molded or injected to form a scaffold in situ, which imminently conforms to complex bone tissue defects. Here, we have prepared a bone cement with the citric acid-treated human amniotic membrane (HAM), monocalcium phosphate monohydrate (MCPM), and β-tricalcium phosphate (β-TCP). Different physicochemical properties such as setting time, injectability, phase composition, compressive strength, disintegration resistance, biodegradability, bioactivity, *in vitro* biocompatibility of the bone cement were determined. The bone cement was transplanted in a rabbit long bone defect model to evaluate the new bone formation. The CPC and CPC+HAM both showed the same level of osteo conductivity within long bone defects. At 4 and 12 weeks post-implantation, new bone formation was comparable between the CPC and CPC+HAM based bone substitutes. We conclude that CPC+HAM have excellent potential for use as cement in bone defect reconstruction.

Keywords: bone cement, human amniotic membrane, monocalcium phosphate monohydrate, β-TCP, hydroxyapatite, injectability, bioactivity, biocompatibility, long bone defect

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Abbreviations: CPC, calcium phosphate cement; HAM, human amniotic membrane; MCPM, monocalcium phosphate monohydrate; TCP, tricalcium phosphate; ECM, extracellular matrix, TECP, tetra calcium phosphate; MSCs, mesenchymal stem cells; HA, Hydroxyapatite; DCPA, dicalcium phosphate anhydrous; CA, citric acid

Introduction

Bone disease and its complications are still a significant clinical problem.[1,2] Large bone defects or injuries are most commonly caused by old age, traffic accidents, bone tissue excision, and other factors, all of which harm one's health and quality of life.[3] As tissue engineering technology has advanced, bone tissue engineering has become a well-known strategy to heal or replace biological tissues.[4,5] Scaffolds are important in bone tissue engineering because they imitate the structure and function of the natural bone extracellular matrix (ECM), which can create a three-dimensional environment.[6,7] Biomaterials, which are the building blocks of scaffolds, are critical in bone tissue engineering. Polymers, metals, and carbon-based ceramics are among the most common biomaterials utilized in the bone tissue engineering field. However, these materials have some disadvantages, such as weak mechanical characteristics, low biocompatibility, and poor tissue adhesion.[8] Calcium phosphate biomaterials, on the other hand, have begun to emerge as suitable biomaterials for overcoming these problems.

In approximately 60% of all native human bones, calcium phosphate is the main inorganic substance.[9] Hydroxyapatite (HA), tricalcium phosphate (TCP), tetra calcium phosphate (TECP), dicalcium phosphate anhydrous (DCPA), monocalcium phosphate monohydrate (MCPM), and other calcium phosphate biomaterials are commonly utilized for orthopedic reconstruction due to their good biocompatibility, osseo integration and osteoconduction.[10–12] Calcium phosphates also help mesenchymal stem cells (MSCs) to differentiate into osteogenic cells.

Calcium phosphate cement (CPC) is defined as a paste made up of one or more calcium phosphate powders that, when mixed with a liquid phase, form a paste that can self-set and harden in the bone defect location to form a scaffold.[13] Scientists previously created a conventional CPC made up of a powdery blend of TECP [$Ca_4(PO_4)_2O$] and DCPA [$CaHPO_4$]. Another CPC with increased hardness and tensile strength was produced utilizing TCP and MCPM.[14]

The human amniotic membrane (HAM) is derived from the interior layer of fetal membranes.[15] It has anti-inflammatory, immune-modulatory, hypo-immunogenic, multi-potent, and non-tumorigenic characteristics.[16] The ECM of the HAM and its components, such as physiologically active mediators and growth factors, indicate that the HAM is a good candidate for tissue engineering. Moreover, HAM is a biomaterial that is easy to obtain, process, and transport.[17,18] It was previously discovered that HAM possesses the osteo inductive capacity for bone repair.[19] Furthermore, multiple studies were performed by using acellular HAMs as scaffolds revealed that stem cells loaded on HAMs differentiate into osteogenic and chondrogenic tissues.[20]

Several forms of CPCs have recently been developed by combining various ingredients.[21–25] HAM has already been demonstrated as a potential osteo inductive biomaterial for bone regeneration.[18,19] Incorporation of citric acid (CA) treated HAM with the CPC material can be newly and helpful technique for healing damaged bone. The concentration of citric acid in the liquid component influences both the mechanical properties and biocompatibility of the cement.[26] Additionally, the proper amount of the CA might also promote osteogenic differentiation.[27]

In the present study, a unique CPC material was prepared by mixing CA-treated HAM solution with a TCP+MCPM mixture. Different physical properties of newly developed CPC material (setting time, injectability; microstructure, phase composition, compressive strength, biodegradability, bioactivity, in-vitro biocompatibility) were

determined and then transplanted in a rabbit long bone defect model to evaluate the new bone formation.

Materials and methods

Preparation of CA treated HAM solution

CA treated HAM was prepared according to the method followed by Murphy et al.[28] Briefly, hospital staffs collected placentas from seronegative (HIV, Syphilis, Hepatitis B and C viruses) donors willing to donate placenta for research purpose and put down in a sterile collection bag. Soon after the collection, placental tissues were washed several times with saline water to eliminate blood clots from the membrane. Then, HAMs were carefully detached from chorionic membranes by using sterile scissors and were sliced into 5 cm × 5 cm pieces. Then, HAMs were rinsed numerous times with saline water. After that, HAMs were treated with penicillin and streptomycin solution for at least 15 minutes. Then, the membranes were frozen at ultra-low temperature (-80ºC) for at least 24 hours. Frozen HAMs had underwent into "freeze drying" at -55°C for 24 hours. After being freeze dried, membrane pieces were pulverized by using mortar and pestle. Following pulverization, 220 mg of HAM and 22 mg of pepsin (Cat# P7000, Sigma, Darmstadt, Germany) were added into a 15 ml tube. The tube was then gamma irradiated at 25 kGy. Following gamma radiation, all subsequent steps were performed in sterile conditions. Ten milliliters of sterilized 10% of CA was added to the separate tube. The materials within the tube were then mixed, allowing digestion for 48 hours at 37°C. The digest was centrifuged at 4,500 rpm for 10 minutes. The supernatant was removed and placed in another 15 ml tube. Finally, CA treated HAM solution was stored in aliquots at −80°C until further use.

Synthesis of calcium phosphate cement

In this study, CPC was prepared by following the method as described by Fukuda et al.[22] with some modification. Briefly, CPC was prepared by mixing solid powders of MCPM and TCP with different concentration of CA solution. To make a homogenous paste, the CPC composite powder was combined with different concentrations (5%, 10%, 15%, and 20%, w/v) of CA solutions with a solid-liquid ratio of 0.6 mL/g. The paste was then placed into a mold and set in a 100% relative humidity box at 37°C for 24 h, at which time the hardened CPC composite was obtained. However, to prepare the CPC+HAM material, CA treated HAM solution was used rather than CA solution with following same procedure.

Phase and microstructure characterization

The as-prepared CPC samples were evaluated using X-ray diffraction analysis (X'Pert Pro, PANalytical, Netherlands). The parameter was CuK α radiation with a wavelength of 1.78896 Å and over a range of 20 from 10° to 70° angle, step size 0.02/s with 40 kV voltages and 30 mA current.

Setting time

The setting time of the cement paste was evaluated following the method as described earlier.[22] The setting time was determined by measuring how long it took the paste to harden to the point where a needle (300 g, W=1 mm) could not penetrate deeper than 1 mm into the sample. Each specimen was measured five times and the average value was calculated.

Injectability

The injectability of composite cement was evaluated according to the literature.[24,25] Briefly; approximately 4g of the homogeneous cement pastes were prepared and put into a 5 ml syringe with an opening nozzle diameter of 2 mm. After 3 minutes since the beginning of mixing the composite powders and liquid, the cement was extruded by hand until it was too hard to push the syringe. The percentage of cement that could be extruded from the syringe was used to evaluate the injectability coefficient, using the following equation:

$$Injectability\ (\%) = \frac{M_1 - M_2}{M_1 - M_0} \times 100\%$$

Where M_0 is the mass of the empty syringe, M_1 is the total mass of syringe and cement, and M_2 is the remaining mass of syringe and cement after extrusion.

Compressive strength test

A universal testing machine (MTS-858, MTS System Inc., USA) was used to measure the compressive strength of the freshly prepared samples at a loading rate of 1 mm/minutes. Each measurement was carried out five times and the average value was calculated.

Disintegration resistance

The degree of cohesiveness or disintegration resistance of CPC specimens in liquid was investigated by injecting 1 g freshly made cement into phosphate-buffered saline (PBS, pH = 7.2 7.4) at 37 °C.[24] The integrity of the cement was examined with naked eyes after soaking in PBS for 24 hours, and the amount of non-decayed cement was carefully collected, dried, and weighed (W_1). As a control, 1g freshly made cement was dried and weighed (W2). Each measurement was carried out five times. The following equation was used to calculate the disintegration resistance (D):

$$D\% = \frac{W_1}{W_2} \times 100\%$$

Biodegradation in simulated body fluid

Simulated body fluid (SBF), which has ion concentrations and a pH value similar to those of human blood plasma, was prepared following the procedure reported by Oyane et al.[29] Briefly, paste specimens (10 mm diameter and 3 mm thickness) were soaked in an SBF solution for 7 and 14 days at 37°C with a weight-to-volume ratio of 0.2 g/mL and the solution was refreshed after 24 hours. The samples were removed after incubation for the given periods, rinsed with deionized water, and dried at room temperature until a consistent weight was achieved for *in vitro* bioactivity evaluation. Surface morphologies of the specimens were examined by using SEM (JSM 6490LA, Jeol, Japan). Specimen surfaces were coated with platinum to make them conductive before being placed inside the SEM chamber. For the measurement of *in vitro* degradation, the 7-day-set paste specimens were immersed in SBF solution at 37°C for 28 days with a weight-to-volume ratio of 0.2 g/mL and the solution was replenished every day. After incubation for the given periods, the samples were removed, rinsed with deionized water, dried at 60°C for 24 hours, and weighed. The following equation was used to calculate in vitro degradation:

$$Degradation\ Rate\ (\%) = \frac{W_0 - W_t}{W_0} \times 100\%$$

Where, D is the degradation rate and W_0 and W_t are the dry weight of the initial specimen and the degraded specimen, respectively. All values presented are the average of five tests performed for each sample.

In vitro cytotoxicity and biocompatibility assay for CPC material

Cytotoxicity test of the CPC materials on brine shrimp (*Artemia salina*) was performed following the process depicted by Khan et

al.[30] In brief, brine shrimps eggs were hatched in a conical shaped vessel (1L), filled with sterile artificial seawater (pH was adjusted at 8.5 using 0.1N NaOH) under constant aeration for 48 hours. After hatching, active nauplii free from eggshells were collected from the brighter portion of the hatching chamber and used for the assay. The CPC powder was dissolved in artificial seawater at 0.25, 0.50, 0.75, and 1.0 mg/mL concentration and was taken in Petri plates where the active nauplii were inoculated. After overnight incubation, the nauplii were counted.

In this study, heparinized human blood was used for *in vitro* blood biocompatibility assay. CPCs powders were diluted with different ratios of blood. The blood sample of the same donor was also diluted at the same ratios with deionized water and PBS for positive and negative control respectively. Samples were incubated for 2 hours at RT. Finally, one drop of samples was spread on glass slides and investigated under a light microscope (Leica microsystem, Germany).

In vivo grafting of the CPC+HAM into rabbit condyle bone critical-sized defect model and post-implantation analysis

A surgically produced critical-sized defect in the condyle section of the long bone in rabbits (Oryctolagus cuniculus) was used as the experimental model in this work. The institutional animal ethics committee of the Bangladesh Atomic Energy Commission granted ethical permission before the study. The surgery protocol was carried out following animal welfare regulations.

The rabbits were kept in cages with pelleted food, hay, and water at room temperature in a humidity-controlled room during the experiment. All of the animals were put through a 12-hour day/night cycle. Importantly, the animals were given a 15-day acclimatization period before surgery. A total of 12 adult male rabbits (1.5–2.0 kg) were used in the investigation, and they were divided into two groups: (1) CPC and (2) CPC+ HAM. The rabbits were initially anesthetized intravenously with 2 percent pentobarbital sodium (30 mg/kg) under aseptic conditions. The hair around the leg was clipped. After that, a 20 mm longitudinal skin incision was created and subcutaneous tissues were carefully separated along the upper edge of the rabbit long bone. An adequate defect size of 5 mm diameter was created using an orthopedic hand drill machine with a drill bit size of 5 mm. The scaffolds were drenched in blood that seeped out of the incision during surgery to make them more compliant and resilient. After that, the scaffold constructs were inserted into the defect. Then, using horizontal mattress sutures, the skin incision was sutured with nylon. The surgical site was cleaned and dressed with nitrofurazone ointment after being washed with povidone-iodine (5%). Animals were kept in solitary pens for 7 days after surgery to limit activity during the early phases of recovery, and then moved to group pens for the balance of the trial. Following surgery, analgesia and antibiotics were given. Ceftriaxone 250 mg injection (TRIZON VET, ACME Laboratories Ltd, Bangladesh) was given twice daily for seven days. Intramuscular (I/M) Ketoprofen injections (K-Pain Vet, ACI Limited, Bangladesh) (0.5 mL) was given once a daily. On day 8, the sutures were removed. The experimental animals were sacrificed after 4 and 12 weeks, the skin was removed, and long bone specimens from the CPC-treated area were surgically taken for histological tests.

Histological examination

All extracted specimens were preserved in 10% formalin solution and decalcification with 15% ethylene-di-amine tetra acetic acid (pH 7.4) (EDTA, BDH Laboratory Supplies, UK). After that, the samples were processed using an automated tissue processor (ASP300, Leica, Germany), and then embedded in paraffin wax blocks. Then, using a rotary microtome (Leica RM2235, Leica Biosystems, Germany) tissue slices were cut and put on L-polylysine coated glass slides (Sigma-Aldrich, USA). Following dewaxing, slices were stained histologically with hematoxylin and eosin (H&E) to investigate repair tissue morphology, composition, arrangement, cell infiltration, and ECM production and CPC degradation. Microscopically, sections were inspected using light microscopy, and digital images were generated (Leica microsystem, Germany).

Results

Physical characterization

Setting time, injectability, and compressive strength at different percentages of CA

The setting time of CPC materials were measured with varying concentrations of CA at 100% humidity at 37°C. The setting times for all CPC composite pastes rose as the concentration of CA increased; for example, when the concentration of CA changed from 5% to 20% at solid/liquid (S/L) ratio of 0.6 mL/g, the time increased from 7 to 32 minutes (Figure 1A). The injectability of CPC materials were improved from 65.08 to 94.39% (Figure 1B). When the CA concentration was adjusted from 5% to 20%, the mechanical strength of the cement was reduced from 6.42 to 3.17 MPa (Figure 1C). Based on the above analysis, 10% concentration of CA solution was selected for preparation of CPC+HAM material. Finally, CPC composites prepared by 10% concentration of CA solution (CPC) and CPC+HAM were used for further analysis.

X-ray diffraction analysis

After 24 hours of curing at 37°C and 100% relative humidity, X-ray diffraction patterns of cement paste indicated that the hydration product of cement was weakly crystallized HA (Figure 1D), which was remarkably comparable to the inorganic character of human bone.

Physical characterization of CPC and CPC+HAM

On the other hand, the injectability of the CPC and CPC+HAM was 90.12% and 88.67% respectively, and there was no phase separation when the cement samples were extruded from syringe. The initial and final setting time of the CPC and CPC+HAM was 5 minutes and 14 minutes respectively. With the inclusion of HAM, the initial and final setup times were reduced to 4 and 11 minutes, respectively. The CPC and CPC+HAM have compressive strengths of 5.51 MPa and 4.73 MPa, respectively (Table 1).

Table 1 Major parameters of CPC and CPC+HAM

Parameter	Time	CPC	CPC+HAM
Setting Time	Initial time (minutes)	5±0.5	4±0.2
(Mean ± SD)	Final time (minutes)	14±2	11±1.5
Injectability (%)		90.12±2.0	88.67±1.8
Compressive Strength (MPa)		5.51±0.75	4.73±0.68

Furthermore, it was found that the cement could nearly maintain its original form and there was no visible deterioration. After two weeks, the disintegration resistance of CPC and CPC+HAM cement was 63.94% and 65.57%, respectively; whereas, after eight weeks, it was 58.25% and 61.2%, indicating high disintegration resistance in PBS (Figure 2A). There was also a considerable weight loss in the sample; however, after a period of time had passed, there was no significant weight loss between the two types of cement.

In vitro degradation

The *in vitro* degradation rate of the CPC and CPC+HAM composite pastes were measured at different time intervals. The degradation rate of the CPC+HAM cement sample (18.21%) was slightly greater than that of the CPC paste sample (16.67%) after 4 weeks, indicating that the presence of solubilized amniotic membrane in CPC+HAM composite enhanced the degradation rate (Figure 2B).

In vitro bioactivity

In vitro bioactivity was evaluated by analyzing SEM micrographs (Figure 2C). Both the CPC and CPC+HAM composites had nano-sized clumps of bonelike apatite on both sides. The quantity and grain size of apatite particles on the CPC and CPC+HAM composite surfaces increased with increased immersion time, resulting in denser apatite layers. After immersion for 7 and 14 days, the apatite aggregates on the CPC+HAM composite surface were higher in number and denser than those on the CPC surface. Furthermore, numerous crystals formed agglomerates and then congregated to create a layer on the surface of the CPC+HAM composite, and the density of the apatite structures increased with amniotic membrane concentration.

In vitro cytotoxicity and blood biocompatibility assay of the calcium phosphate cement

In the present work, *in vitro* cytotoxicity assessment of cement material was tested by the brine shrimp lethality bioassay method. The death of the nauplii due to toxicity was nil at 0.25- 0.75 mg/mL concentration suggesting that CPC and CPC+HAM had no cytotoxic effect. The small number of death of nauplii was found at higher concentrations of the tested materials (1.0 mg/mL) (Figure 3A).

In vitro blood biocompatibility assay revealed that the shape of red blood cells incubated with cement materials nano-powder was unaltered (Figure 3B), which indicates the compatibility of the cements for *in vivo* applications.

Bone regeneration and resorption of cement *in vivo*

For the *in-vivo* study, a group of rabbits were treated with the CPC+HAM composite and CPC and kept in observation. Healing of all surgical sites was observed without any adverse reactions or postoperative complications such as abnormal bleeding or infection. Signs of inflammation such as swelling appeared to be minimal, and the grafted materials were confirmed to be intact within the defects. After 4 weeks surgical area of each rabbit was healed completely without any scar mark and was covered by new hairs (Figure 4E).

From the *in vivo* investigation of implanted samples, CPC+HAM composite and CPC implants showed no foreign body reaction, no apparent inflammatory response, rejection, or necrosis in the nearby host tissue, and they were effectively integrated with the surrounding tissue (Figure 5A). With the prolonged time to 12 weeks, the borders between CPC+HAM composite specimens and normal surrounding tissue were unclear due to specimen deterioration and subsequent ingrowth of new bone. All composite specimens were covered with a tissue layer that was indistinguishable from surrounding tissue (Figure 5B).

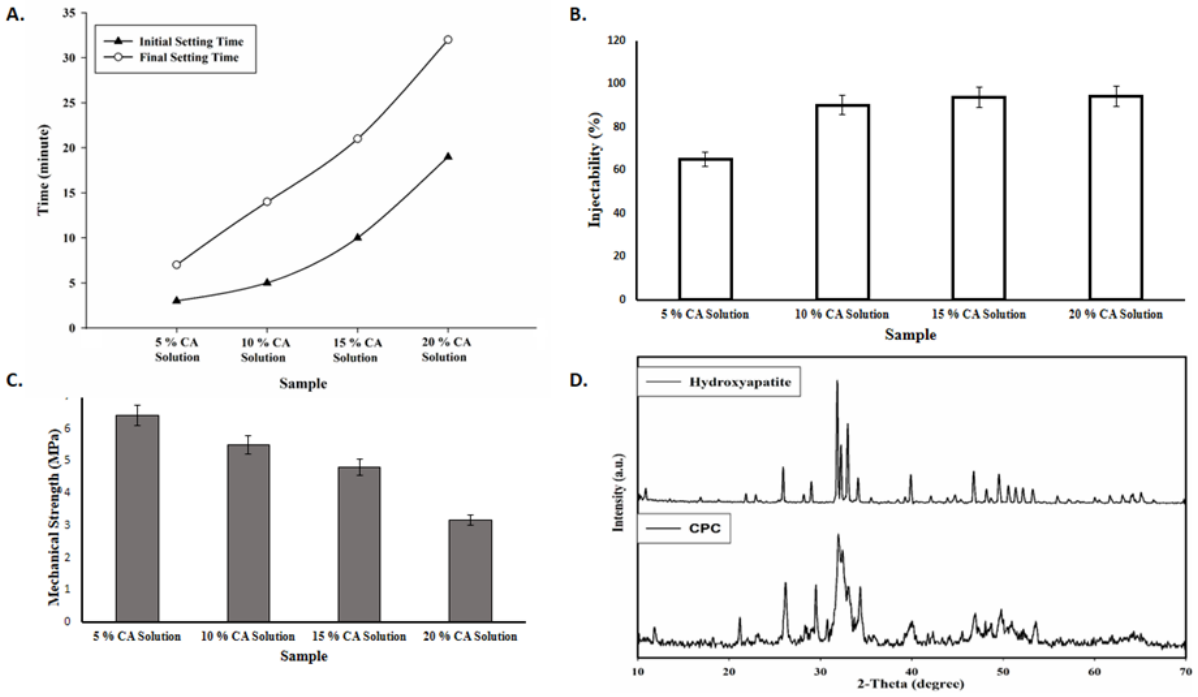

Figure 1 Characterization of Calcium Phosphate Cement: (A) Setting Time; (B) Injectability; (C) Mechanical strength at different concentrations of CA; (D) X-ray diffraction patterns of the Hydroxyapatite and CPC material.

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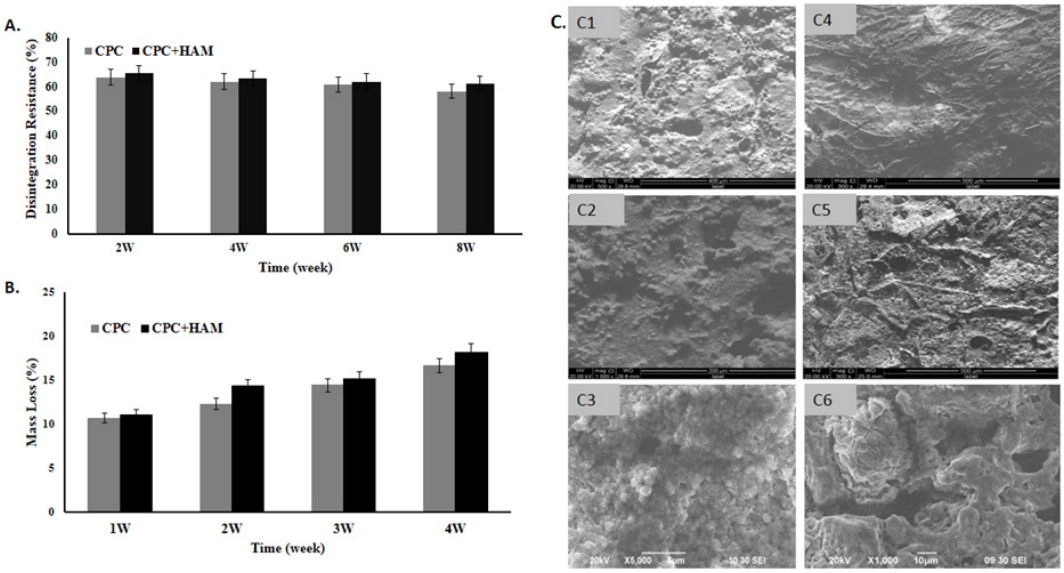

Figure 2 Characterization of calcium phosphate cement: (A) Disintegration Resistance; (B) Biodegradability; (C) SEM micrographs of cross-sections of CPC and CPC+HAM composite specimens that were hardened for 24 h (C1 and C2) and specimens immersed in SBF for different times (C2-C6). (C1) CPC; (C2) CPC after 7 days; (C3) CPC after 14 days; (C4) CPC+HAM; (C5) CPC+HAM after 7 days; (C6) CPC+HAM after 14 days.

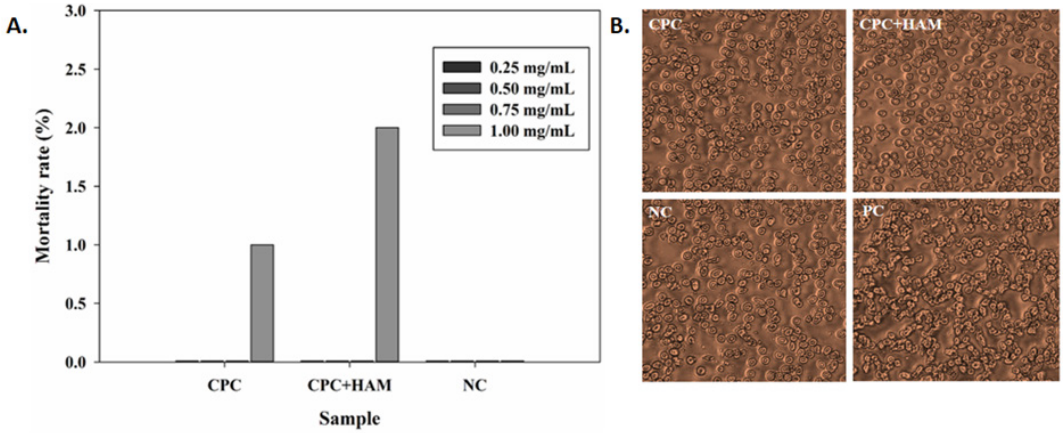

Figure 3 *In vitro* cytotoxicity study (A) Different concentrations of the CPCs powder were added with brine shrimp to quantify the degree of toxicity. (B) Blood biocompatibility analysis: Magnification 20X.

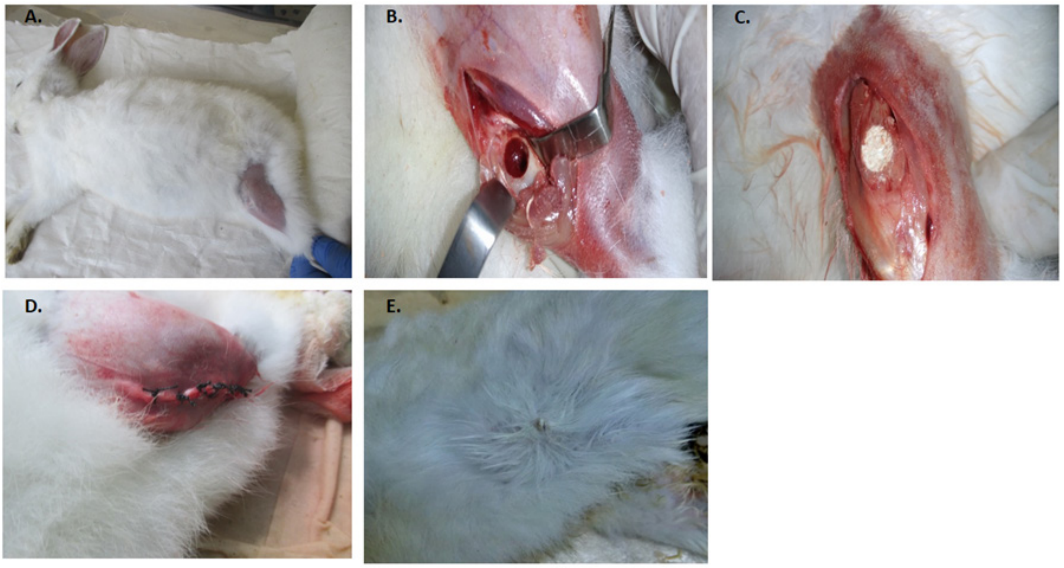

Figure 4 Images of the surgical and implantation procedures: *In vivo* grafting of scaffold in the surgically created rabbit long bone defect model (A) Removal of hair showed the site of bone to be drilled; (B) Drilled defect chamber in the long bone of rabbits; (C) Post implanted defected Bone cement composite filled hole; (D) Sutured incision; (E) Post grafting recovery observation on day 28.

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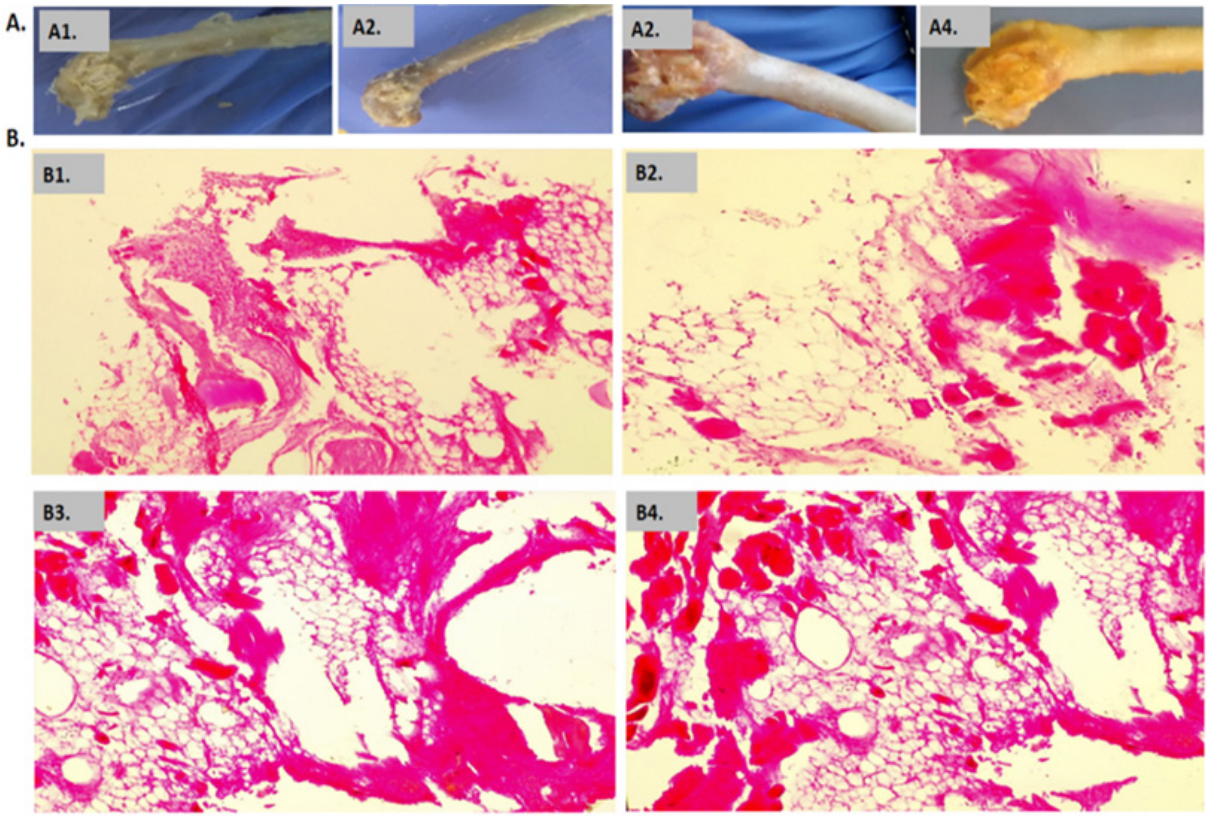

Figure 5 (A) Images of the post-surgical bone tissues treated with calcium phosphate cement composites; (A1) CPC for 4 weeks; (A2) CPC for 12 weeks; (A3) CPC+HAM for 4 weeks; (A4) CPC+HAM for 12 weeks; (B) Histological analyses of the scaffold implanted sites collected after 4 and 12 weeks from surgery. (B1) CPC for 4 weeks; (B2) CPC+HAM for 4 weeks; (B3) CPC for 4 weeks; (B4) CPC+HAM for 12 weeks.

Discussion

CPC composites have been employed in the treatment of bone deformities due to their outstanding characteristics.[31,32] However, one of the disadvantages of the CPC is its poor bioactivity, which has limited its applicability.[33] Previously, pectin, strontium, and bioactive glass, etc. have been added to CPC to improve its characteristics.[34–36] The current work incorporates HAM powder with CPC to alter its physicochemical characteristics, bioactivity, and osteo conductivity. The setting time of CPC is known to be dependent on the concentration of CA.[37]

The initial setting time of the prepared cement was varied from 3 to 19 minutes as the increasing percentage of CA (5 to 20%). It has been observed that dental applications require times closer to 3 minutes, whereas orthopedic applications demand periods closer to 8 minutes for clinical usage of CPC.[38] The injectability was approximately 65 percent at a low concentration of CA; however, as the concentration of CA increased, the injectability rate increased to 90 to 94 percent, but the mechanical strength decreased from 6.42 to 3.17. This data implies that raising the amount of CA alters the crystal shape of MCPM while maintaining its involvement in TCP linkages. Based on the above analysis, 10% CA solution containing CPC was selected for preparation of CPC+HAM composites. The initial and final setting time were 5 &14 minutes for CPC and 4 & 11 minutes for CPC+HAM composites; whereas the commercially available cement (chronOS™ Inject) has an initial setting time of 6 minutes and a final setting time of 12 minutes.[39]

On the other hand, if the cement is used for bone restoration, the mechanical characteristics of the hardened cement are a required index. Human trabecular bone compressive strength has been estimated to be between 0.1 to 16 MPa.[40] The compressive strength of CPC and CPC+HAM cement was 5.51±0.75 and 4.73±0.68 MPa, respectively, which is similar to that of chronOS™ (3.0±0.6 MPa).[39] The results show that the CPC and CPC+HAM cement groups have values that are comparable to commercial available cement and human trabecular bone.

For bioactive substitution materials, it is important to generate a bone-like apatite layer on the surface that can form chemical bonds to bone tissue at the early stage of the implantation.[41] In the current investigation, the quantity and grain size of the apatite aggregates on CPC and CPC+HAM composite surfaces increased with prolonged immersion duration. After immersion for 7 and 14 days, the number of apatite aggregates on the surface of the CPC+HAM composite was higher than the CPC composite surface. These findings suggest that the addition of HAM could result in a bioactive composite with controllable bioactivity. Furthermore, the biomaterial should degrade over time, allowing freshly produced bone tissue to progressively replace it.[42] One of the most significant aspects of bone healing applications is proper degradation in a physiological environment.[43] Bone cement, which is made up of TECP and DCPA, has a slow decomposition rate.[44] The breakdown rate of the CPC+HAM composite was quicker than that of CPC in this investigation, which might be due to the increased bioactivity of HAM.

Biocompatibility is a factor related to the response of cells that interact with the biomaterial. It has been reported that the surface of biomaterials could have an influence on the behavior and morphology of cells grown on their surface.[45] Furthermore, the surface properties

of biomaterials *in vitro* might impact cellular responses to them. Biomaterials also have an active interaction with cells, causing them to grow and proliferate.[46] The cell attachment stage is the first point of contact between the cells and the biomaterial, and the quality of this stage can have a direct impact on cell development, morphology, proliferation, and differentiation.[47] To confirm the applicability of the CPC materials nauplii lethality assay and RBC compatibility were also experimented. The CPC materials were RBC biocompatible and non-toxic to brine shrimp larvae, which indicates the compatibility of the calcium phosphate cement for healing of tissue defects. These results indicated that CPC+HAM composite have the potentialities to be used as a safe biomaterial for bone tissue engineering applications.

The macroscopic examination results from the *in vivo* investigation revealed that neither the CPC+HAM composite nor the CPC implants displayed any apparent inflammatory response, rejection, or necrosis within the nearby host tissue and that they integrated well with the surrounding tissue. According to the histological analysis, the CPC+HAM composite specimens were engulfed by the surrounding bone tissue, indicating that the new bone was in direct contact with the implant 4 weeks after implantation. After 12 weeks of implantation, new bone tissue development increased considerably in conjunction with the resorption of the CPC+HAM composite implant.

According to macroscopic examination results, the CPC+HAM composite implant developed a tight and direct connection with the surrounding host bone without the intervention of soft tissue. These findings indicated that the CPC+HAM composites exhibited improved osteogenesis and osteo integration at the defect location, in addition to quicker biodegradability. It is well recognized that resorption of the bone substitute material is required in the replacement of bone tissue because bone ingrowth into the defect region necessitates the release of space.[5,48]

Considering the differences in bone metabolism between healthy and impaired bone, the biological performance of the CPC+HAM composite should be evaluated for compromised situations such as osteoporosis. Long-term investigations of CPC+HAM composites for bone regeneration are also necessary. As an attractive implanted biomaterial option for bone regeneration, the CPC+HAM composites discussed herein conferred high biocompatibility and osteo conductive characteristics and promoted bone ingrowth into the implant. The produced material was also demonstrated to be resorbable, and it was replaced by new bone in a creeping substitution manner. According to our findings, CPC+HAM might be a noble and viable bioactive material for bone regeneration in future clinical situations.

Conclusion

The combination of polymer and CPC, whether dissolved in the liquid phase or as a second solid phase, has proven to be an interesting strategy for developing bone substitute materials with improved properties. While CPCs have excellent biocompatibility and osteo conductivity, they do have certain inherent limitations that can be overcome by using a polymer in their composition. The variety of characteristics that may be changed by adding a polymer is extensive, including rheological or mechanical behavior, resorption rate, and cell and tissue response. As a result, the incorporation of these various types of polymers might be a helpful tool for controlling the distribution of various physiologically active compounds. According to our findings, the incorporation of CA and HAM in CPC increased its biocompatibility and osteo conductivity of BC.

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Ethics approval

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Conflicts of interest

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