Proliferation and differentiation of mesenchymal stem cells on scaffolds containing chitosan, calcium polyphosphate and pigeonite for bone tissue engineering

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Abstract

Objectives: Treatment of critical-sized bone defects with cells and biomaterials offers an efficient alternative to traditional bone grafts. Chitosan (CS) is a natural biopolymer that acts as a scaffold in bone tissue engineering (BTE). Polyphosphate (PolyP), recently identified as an inorganic polymer, acts as a potential bone morphogenetic material, whereas pigeonite (Pg) is a novel iron-containing ceramic. In this study, we prepared and characterized scaffolds containing CS, calcium polyphosphate (CaPP) and Pg particles for bone formation in vitro and in vivo.

Materials and methods: Chitosan/CaPP scaffolds and CS/CaPP scaffolds containing varied concentrations of Pg particles (0.25%, 0.5%, 0.75% and 1%) were prepared and characterized by SEM, XRD, EDAX, FT-IR, degradation, protein adsorption, mechanical strength and biomineralization studies. The cytocompatibility of these scaffolds with mouse mesenchymal stem cells (mMSCs, C3H10T1/2) was determined by MTT assay and fluorescence staining. Cell proliferation on scaffolds was assessed using MUSE™ (Merck-Millipore, Germany) cell analyser. The effect of scaffolds on osteoblast differentiation at the cellular level was evaluated by Alizarin red (AR) and alkaline phosphatase (ALP) staining. At the molecular level, the expression of osteoblast differentiation marker genes such as Runt-related transcription factor-2 (*Runx2*), ALP, type I collagen-1 (*Col-I*) and osteocalcin (OC) was determined by real-time reverse transcriptase (RT-PCR) analysis. Bone regeneration was assessed by X-ray radiographs, SEM and EDAX analyses, and histological staining such as haematoxylin and eosin staining and Masson's trichrome staining (MTS) in a rat critical-sized tibial defect model system.

Results: The inclusion of iron-containing Pg particles at 0.25% concentration in CS/CaPP scaffolds showed enhanced bioactivity by protein adsorption and biomineralization, compared with that shown by CS/CaPP scaffolds alone. Increased proliferation of mMSCs was observed with CS/CaPP/Pg scaffolds compared with control and CS/CaPP scaffolds. Increase in cell proliferation was accompanied by G0/G1 to G2/M phase transition with increased levels of cyclin(s) A, B and C. Pg particles in CS/CaPP scaffolds enhanced osteoblast differentiation at the cellular and molecular levels, as evidenced by increased calcium deposits, ALP activity and expression of osteoblast

marker genes. In vivo implantation of scaffolds in rat critical-sized tibial defects displayed accelerated bone formation after 8 weeks.

Conclusion: The current findings indicate that CS/CaPP scaffolds containing iron-containing Pg particles serve as an appropriate template to support proliferation and differentiation of MSCs to osteoblasts in vitro and bone formation in vivo and thus support their candidature for BTE applications.

1 | INTRODUCTION

Defects in the skeletal system hamper normal bone functions. Recent statistical surveys have substantially evidenced the increased prevalence of such critical-sized bone defects around the world.¹ Owing to the innumerable constraints associated with conventional grafting techniques and the increasing rates of non-self-healing bone defects, bone tissue engineering (BTE) indisputably continues to gain clinical significance. Development of temporary matrices known as scaffolds is one of the key processes of the tissue engineering approach for reconstruction of the defective bone. Therefore, strenuous efforts are being made by researchers to design an ideal guiding matrix to restore bone defects.

Chitosan (CS) is a natural, semi-crystalline, cationic polysaccharide with biocompatibility and structure similar to that of glycosaminoglycans. It also exhibits antibacterial properties and is biodegradable.¹⁻⁴ Polyphosphate (PolyP) was recently identified as a naturally occurring, morphogenetically active, inorganic polymer composed of linearly arranged phosphate units linked together by high-energy phosphoanhydride bonds. Chemically or enzymatically prepared PolyP has emerged as potential bone morphogenetic material for the treatment of human bone disorders like osteoporosis.⁵ Several studies have identified the presence of PolyP-hydrolysing enzymes in osteoblast-like cells. The action of exopolyphosphatases and endopolyphosphatases of the body facilitates breakdown of PolyP units to orthophosphates, which are similar to inorganic phosphates in inducing mineralization in bone cells, whereby PolyP can replace β -glycerophosphate (β -GP), a conventionally used ingredient of the osteogenic activation cocktail.⁶ PolyP (Ca^{2+} complex) in a stoichiometric ratio has been shown to promote hydroxyapatite (HAp) mineralization to a considerably higher extent than β -GP and could also inhibit osteoclasts.^{7,8} Iron plays an important role in maintaining bone physiology by serving as a cofactor for collagen synthesis and by improving bone mineral density (BMD).^{9,10} Iron oxide (FeO)-reinforced polycaprolactone (PCL) nanofibers, FeO/Collagen/HAp and PCL/magnetic nanoparticles (MNP/FeO NPs) have been explored in recent years as iron-based biocomposites for bone tissue regeneration.¹¹⁻¹⁴ Nanophase ceramics have gained increasing attention because of their superior bioactivity as well as structural and compositional similarity to bone extracellular matrix. The biological role of ceramics like nBGCs, nHAp, diopside (Dp), mesoporous wollastonite (m-Ws) in BTE has been reported.¹⁵⁻¹⁸ Pigeonite (Pg), containing repeating units of $(\text{Ca,Mg,Fe})(\text{Mg,Fe})\text{Si}_2\text{O}_6$, is a novel iron-containing nanophase ceramic, and its role in bone regeneration

has not yet been determined. Hence, in this study, we developed CS-based scaffolds containing calcium polyphosphate (CaPP) and Pg particles and tested their potential towards promoting bone formation in vitro and in vivo.

2 | MATERIALS AND METHODS

2.1 | Preparation and characterization of CS/CaPP and CS/CaPP/Pg scaffolds

Pg particles were prepared by the co-precipitation method,¹⁹ as shown in Table S1, and the particle size was reduced by the wet-ball milling process (Figure S1). CaPP was prepared as described previously.²⁰ Briefly, 5% CS solution in acetic acid was mixed with 5% CaPP solution in a ratio of 4:1 v/v. Pg particles at concentrations 0.25%–1% were dispersed into the existing solution and stirred for 1 hour to ensure homogeneous dispersion. The solution was transferred to 24-well culture plates, frozen at -20°C overnight and lyophilized. The scaffolds were cross-linked in 2% alginate dialdehyde (ADA), as reported earlier.²¹ The pH was neutralized using 0.1 mol/L NaOH and the scaffolds were lyophilized. The prepared CS/CaPP and CS/CaPP/Pg scaffolds were subjected to SEM, XRD and EDAX studies, as described previously.¹⁵⁻¹⁸

2.2 | Preparation of conditioned media and assessment of ionic dissolution by ICP-OES

Freeze-dried CS/CaPP and CS/CaPP/Pg scaffolds (0.5 g) were incubated for 24 hours in the culture media at 37°C . The dissolved products from the media were collected, syringe-filtered using a $0.2\ \mu\text{m}$ syringe filter and used for further experiments. The supernatant was then analysed using an inductively coupled plasma optical emission spectrometer (ICP-OES; PerkinElmer, Optima 5300 DV, Shelton, USA) to measure the levels of calcium (Ca), silicon (Si), phosphorus (P), magnesium (Mg) and iron (Fe) released from the scaffolds.

2.3 | Biocompatibility assessment

Cytotoxicity of scaffolds was determined by the MTT assay.¹⁷ Fluorescein diacetate (FDA) and Hoechst 33 342 were used as the cytoskeletal and nuclear stains, respectively. The dyes were added to cover the cell monolayers and the cells were incubated for 30 minutes at 37°C in the dark.^{22,23} The excess stain was removed, and the cells

were washed with PBS and observed under a fluorescent microscope under the 40× objective lens.

2.4 | Cell viability, cell count and cell cycle phase analyses

Mouse mesenchymal stem cells (mMSCs; C3H10T1/2) were seeded in 12-well plates and serum starved for 6 hours in Dulbecco Modified Eagle Medium (DMEM) containing 0.1% FBS to obtain a synchronous population. These cells were incubated in medium (25 mg/mL) conditioned with scaffolds (CS/CaPP, CS/CaPP/0.25%Pg) as described previously.²⁴ Cells without treatment served as control. After incubation, cells were harvested and subjected to cell count and viability assays by using MUSE™ Cell Analyzer (Merck-Millipore, Germany) according to the manufacturer's protocol. The trypsinized cells were subjected to cell cycle phase analysis, as described previously.²⁵

2.5 | Alizarin red staining and ALP staining

Mouse mesenchymal stem cells were cultured in the absence or presence of conditioned media in DMEM with 10% FBS for 7 days. The medium was changed once in 2 days. The cultured cells were then subjected to Alizarin red (AR) and ALP staining, as described previously.^{18,21}

2.6 | Real-time RT-PCR and western blot analyses

Total RNA was isolated from mMSCs, using TRIzol. cDNA synthesis and real-time PCR were performed as described previously.²⁶ The primers used in the study are shown in Table 1. The relative mRNA expression was calculated using the $\Delta\Delta C_t$ method.²⁷ Whole cell lysates were prepared from mMSCs, and the proteins were subjected to western blot analysis as described previously.²⁷ The proteins of interest were detected using an enhanced chemiluminescence detection kit (WESTAR SUPERNOVA, Cyanagen, Bologna, Italia) and quantified using Image Lab version 4.1 (BioRad, Hercules, CA, USA) software. α -Tubulin was used for normalization.

2.7 | Animal studies

2.7.1 | Animals and surgical procedure

Forty male albino Wistar rats weighing ~200 g were procured from the National Institute of Nutrition (NIN), Hyderabad. All animal experimental procedures were approved by the Animal Ethical Committee, Kovai Medical College and Hospital, Coimbatore, Tamil Nadu, India, and the experiments were performed according to the Institutional Guidelines and Regulations for the Care and Use of Laboratory Animals. Prior to surgery, the rats were anaesthetized with 5% isoflurane and subjected to perforation of the right tibia, using a dental drill with 3 mm diameter, under constant saline irrigation (0.9% NaCl), as described previously.^{18,22,28} The defect was left

TABLE 1 Primer sequences used for real-time reverse transcriptase PCR analysis

Gene	5'-3' sequence
Cyclin A2	Forward-CCTGCAAAGTCTGCAAAGTTGA Reverse-AAAGGCAGCTCCAGCAATAA
Cyclin B1	Forward-GAGATGTACCCTCCAGAA Reverse-CCATGTCGTAGTCCAGCA
Cyclin C	Forward-TGCCTACATGTAGCCTGTGT Reverse-GCTGTAGCTAGAGTTCTGAC
Cyclin D1	Forward-TGAACCTACCTGGACCGCT Reverse-GCCTCTGGCATTCTTGAG
Cyclin E1	Forward-GGGAGACCTTTTACTTGGC Reverse-GGCAGTCAACATCCAGGAC
Runx2	Forward-CGCTCACAACAACACACAG Reverse-TCACTGTGCTGAAGAGGCTG
ALP	Forward-TGTGCCAGAGAAAGAGAGAGA Reverse-GTTTCAGGGCATTCTTCAAGGT
Col-1	Forward-TAACCCCTCCCCAGCCACAAA Reverse-TTCCTCTTGCCGTGCGTCA
OC	Forward-ATGGCTTGAAGACCGCTAC Reverse-AGGGCAGAGAGAGAGGACAG
RPL13AB	Forward-CCTGTTTCCGTAGCTCATG Reverse-AAGTACCAGGCAGTGACAG

ALP, alkaline phosphatase; Col-1, collagen-1; OC, osteocalcin.

completely unfilled (Group 1), filled with CS/CaPP (Group 2) or filled with CS/CaPP/Pg (Group 3). Each group contained 8 animals. After 8 weeks, the animals were euthanized by anaesthesia overdose, and the tibiae were immediately removed, radiographed and fixed in neutral 10% buffered formalin for 48 hours at room temperature for histological analyses.

2.7.2 | Histological processing

The implanted bone/scaffold implants were collected from animals and subjected to haematoxylin and eosin (H&E) staining and Masson's trichrome staining (MTS) individually, as described previously.^{18,22,28}

2.7.3 | SEM and EDAX analyses

The calcified and unstained sections of the bone were analysed by SEM-EDAX for identifying the extent of mineralization at the implant-tissue interface. The sections were sputter-coated with a thin layer of gold and observed in HR-SEM Quanta 200 FEG Instrument (FEI, Eindhoven, the Netherlands).¹⁸

2.8 | Statistical analysis

All experiments were performed in triplicates, and the results were expressed as the mean $\pm$ standard deviation (SD). Statistical significance

was calculated by ANOVA and Student's *t* test. A *P* value lower than .05 was considered significant.

3 | RESULTS

3.1 | Physical characterization of scaffolds and their cytocompatibility assessment

The synthesized Pg particles depicted a mean size of about 400 nm, as assessed by SEM (Figure S1A) and Dynamic Light Scattering (DLS)

(Figure S1B). The EDS analysis of Pg particles identified the presence of Fe, Ca, P, Si and Mg (Figure S1C). Scaffolds containing CS/CaPP with varied concentrations of Pg particles (0.25%, 0.5%, 0.75% and 1%) were prepared and characterized by SEM, XRD and FT-IR analyses. CS/CaPP, CS/CaPP/0.25%Pg, CS/CaPP/0.5%Pg, CS/CaPP/0.75%Pg and CS/CaPP/1%Pg scaffolds exhibited pore size >60 μm (Figure 1Aa-e). The fine dispersion of CaPP and Pg particles in CS matrix could be observed from the SEM images at higher magnification (Figure 1Ba-c). The spectral patterns analysed by XRD suggested a semi-crystalline nature of the fabricated scaffolds (Figure 1C). In the FT-IR spectra of

FIGURE 1 SEM, XRD and FT-IR analyses of scaffolds. A, (a)-(e) represent the SEM images of surface morphology of Chitosan (CS)/CaPP, CS/CaPP/0.25%Pg, CS/CaPP/0.50%Pg, CS/CaPP/0.75%Pg and CS/CaPP/1%Pg scaffolds. The pore size was found to be >60 μm . B, Dispersion of CaPP and Pg particles in CS matrix. (a), (b) and (c) represent the SEM images of CS, CS/CaPP and CS/CaPP/0.25%Pg scaffolds, respectively, at higher magnification (4000 $\times$). C, XRD spectra of Pg particles were found to be in accordance to the standard JCPDS. No. 83-0100. All the scaffolds exhibited semi-crystalline nature. The characteristic peaks of CS, CaPP and alginate dialdehyde (ADA) were observed in the scaffolds. D, FT-IR spectra of CS/CaPP, CS/CaPP/0.25%Pg, CS/CaPP/0.50%Pg, CS/CaPP/0.75%Pg and CS/CaPP/1%Pg scaffolds. The characteristic fingerprint regions of the functional groups of individual compounds and their respective wavenumbers are represented. The presence of imine group (C = N) due to Schiff's base formation was identified

the composite scaffolds (Figure 1D), the disappearance of 2 distinct peaks corresponding to the N-H group of CS and the appearance of bands between the regions 1665–1674 cm^{-1} corresponded to the

C = N stretching that resulted in the formation of Schiff's base and thus confirmed the crosslinking of the composite mediated by the addition of a 2% ADA solution.²⁹

FIGURE 2 Degradation, protein adsorption, mechanical strength and biomineralization of scaffolds. (A) Effect of Pg particles on percent degradation of CS/CaPP scaffolds at 7 days in $1\times$ PBS containing lysozyme (10 000 U/L). Addition of Pg particles at 0.25%, 0.5% and 0.75% in Chitosan (CS)/CaPP scaffolds decreased their degradation. (B) Effect of Pg particles on the protein adsorption ability of CS/CaPP scaffolds at 1 h, 3 h and 24 h. Equally weighed scaffolds were incubated in DMEM containing 1% serum. The amounts of unadsorbed proteins were calculated by Bradford assay followed by determination of adsorbed proteins by an indirect method. Addition of Pg particles promoted the protein adsorption ability in CS/CaPP scaffolds. (C) Effect of Pg particles on the mechanical strength of CS/CaPP scaffolds. Rectangular disks of about 30 mm length and 25 mm width were subjected to compressive strength testing at crosshead speed of 0.1 mm/min. The load was applied until the scaffolds were compressed to 30% of its original thickness. Addition of Pg particles (0.25%) improved the compressive strength and compressive modulus of CS/CaPP scaffolds. (D) SEM and (E) EDS analyses of (a) CS/CaPP, (b) CS/CaPP/0.25%Pg, (c) CS/CaPP/0.50%Pg, (d) CS/CaPP/0.75%Pg and (e) CS/CaPP/1%Pg scaffolds after 7-d incubation in SBF. EDS analysis identified the presence of Ca and P on the deposits.

*Significant increase compared with control ($P < .05$)

It is a pre-requisite for any implantable scaffold material to exhibit appropriate swelling and controlled degradation behaviour to enable infiltration of cells, favour nutrient infusion, ensure circulation of surrounding body fluids and maintain the structural integrity until the completion of healing process at the site of injury. Addition of Pg particles at 0.25%, 0.5% and 0.75% displayed a significant decrease in the degradation behaviour of CS/CaPP scaffolds, whereas no significant difference was observed at 1% (Figure 2A). Addition of Pg particles resulted in increased adsorption of proteins by the scaffolds at all wt% at 1- and 3-hour incubation (Figure 2B). CS/CaPP/0.25%Pg scaffolds showed increased compressive strength and modulus (Figure 2C). Biomineralization is the ability of the substrates (scaffolds) to initiate hydroxyapatite crystal nucleation and growth on the interfacial surface from the surrounding body fluid, which in turn would render an osteoconductive surface for subsequent calcification. The SEM images of the scaffold surfaces identified mineralized deposits. The distribution of these deposits was more prominent and dense with the addition of Pg at 0.25% and 0.5% in the CS/CaPP scaffolds (Figure 2D). The presence of Ca and P/HAp was also confirmed by EDS analysis (Figure 2E).

Before the in vitro testing of any implantable biomaterial, it is crucial to evaluate the biocompatibility of the biomaterial in terms of the non-toxic concentration. As CS/CaPP scaffolds containing 0.25% and 0.5%Pg particles showed enhanced bioactivity (protein adsorption and biomineralization), the cytotoxicity of these scaffolds was assessed by the MTT assay. CS/CaPP/0.25%Pg scaffolds at the concentrations

from 1 µg/mL to 25 mg/mL showed increased cell viability in mMSCs after 24 hours (Figure 3). Fluorescent staining of cells with FDA (Figure S2A) and Hoechst 33342 (Figure S2B) showed no change in their morphology when they were grown in conditioned media obtained from the scaffolds. Light microscopic and FDA staining of mMSCs directly grown on scaffolds also showed no change in their morphology (Figure S2C,D). Despite the potential bioactive nature of ceramics, it is generally anticipated that the release of the constituent ions is expected to be well within the physiological levels to avoid discernible cytotoxic effects. Hence, the levels of the constituent ions, namely Ca, Mg, Si, Fe and P, released from the scaffolds were assessed at

TABLE 2 Assessment of dissolute ions by inductively coupled plasma optical emission spectrometer (ICP-OES)

	Release of dissolved ions in ppm			
	CS/CaPP		CS/CaPP/Pg	
	24 h	72 h	24 h	72 h
Ca	55.08	111.5	29.39	68.13
P	57.41	68.4	47.16	72.54
Mg	14.39	14.39	17.88	19.04
Fe	0.004	0.004	0.113	0.114
Si	0.010	0.010	0.526	5.35

CS/CaPP/Pg, chitosan/calcium polyphosphate/pigeonite.

FIGURE 3 In vitro cytotoxicity of mouse mesenchymal stem cells (mMSCs; C3H10T1/2). mMSCs at 4×10^4 cells/cm² were treated with 1 µg/mL to 25 mg/mL of conditioned media obtained from CS/CaPP, CS/CaPP/0.25%Pg and CS/CaPP/0.5%Pg scaffolds for 24 h followed by MTT assay. Cells treated with Triton X-100 and culture medium served as positive and negative controls, respectively. *A significant increase compared to control ($P < .05$)

24 hours and 72 hours by ICP-OES (Table 2). Scaffolds containing Pg particles showed lesser ionic dissolutions of Ca and P than CS/CaPP at 24 hours and 72 hours. This could be attributed to the previously observed reduction in the degradation ability of CS/CaPP/Pg (Figure 2A) because of a strong physical interaction due to the addition of ceramics. Release of Mg, Fe and Si from Pg particles was found to be within the physiological permissible levels. Notably, Ca, Si and P ions released from the scaffold have previously been reported to exert a stimulatory response to cell proliferation and differentiation.³⁰⁻³²

3.2 | CS/CaPP/0.25%Pg scaffolds enhanced cell proliferation

As CS/CaPP/0.25%Pg scaffolds showed increased cell viability (Figure 3), we next determined their effect on cell proliferation.

Figure 4A shows a representative plot of the percentage of live and dead cell populations excluded from debris in the control and treated groups. Both CS/CaPP and CS/CaPP/0.25%Pg scaffolds showed a significant increase in cell number, compared with control, whereas CS/CaPP/0.25%Pg scaffolds showed significantly increased cell number compared with control and CS/CaPP scaffolds after 24-hour and 48-hour treatments (Figure 4B). Cell cycle analysis depicted an increase in the percentage of cells at the G2/M phase by CS/CaPP/0.25%Pg scaffolds (Figure 4C,D). Because there was a change in the cell cycle phases by CS/CaPP and CS/CaPP/0.25%Pg scaffolds, we next determined the mRNA expression of cyclin genes by real-time RT-PCR analysis. The inclusion of 0.25%Pg particles in CS/CaPP scaffolds increased mRNA levels of cyclins A and C in mMSCs (Figure 5A); this result was consistent with their protein expression analysed by western blot (Figure 5B). The expression of cyclin B was increased by both the scaffolds in these cells.

FIGURE 4 Effect of scaffolds on the proliferation and cell cycle phase transition of mouse mesenchymal stem cells (mMSCs). mMSCs at 1×10^5 cells/cm² were treated with conditioned media (25 mg/mL) obtained from CS/CaPP and CS/CaPP/0.25%Pg scaffolds for 24, 48 and 72 h. The cells were then subjected to cell count and viability assay using a MUSE™ automated cell counter. Precise gating adjustments were pre-set to exclude the debris, nucleated cells and live cells from dead cells. A, Representative plot showing the live cell populations and dead cell populations on the left and right quadrants, respectively. B, Number of viable cells/mL in the treated groups. C, After 72 h treatment, cells were fixed for 3 h at -20°C using ice-cold ethanol followed by cell cycle phase analysis using MUSE™ automated cell counter. Initial gating adjustments were made according to manufacturer's instructions. Representative plot depicting the cell populations distributed in various phases of cell cycle. D, The percentages of cell population at each cycle phase are summarized in the table. *A significant increase compared with the control ($P < .05$)

FIGURE 5 Effect of scaffolds on the expression of cyclin genes in mouse mesenchymal stem cells (mMSCs). Cells were treated with CS/CaPP and CS/CaPP/Pg scaffolds for 24 h in medium containing 10% serum. Untreated cells grown in media containing 10% serum served as control. A, Total RNA was isolated and subjected to real-time RT PCR using the primers for cyclin genes. RPL13B was used as internal control. The relative mRNA expression of cyclins was determined after normalization with RPL13B gene. *A significant increase compared with the control ($P < .05$). #Significant increase compared with CS/CaPP scaffolds ($P < .05$). B, Whole cell lysates were prepared and subjected to western blot analysis using the antibodies as indicated. α -Tubulin was used as internal control. The relative expression of cyclins was determined after normalization with α -tubulin by using Image J software. Results of western blotting analysis of cyclin(s) A, B and C proteins in response to scaffold treatment at 24 h are shown. mMSCs were treated with CS/CaPP and CS/CaPP/Pg scaffolds for 24 h in medium containing 10% serum. Untreated cells grown in media containing 10% serum served as control. The whole cell lysates were prepared and western blotting was performed for the antibodies of cyclins A, B and C. α -Tubulin was used as the internal loading control. C, D, E Relative expression of cyclin(s) normalized to α -tubulin by using Image J software

3.3 | CS/CaPP/0.25%Pg scaffolds enhanced osteoblast differentiation at the cellular and molecular levels in vitro

The effects of CS/CaPP and CS/CaPP/0.25%Pg scaffolds on osteoblast differentiation were determined at the cellular and molecular levels by AR and ALP staining of mMSCs at 14 days (Figure 6C). Both scaffolds significantly increased calcium deposits (Figure 6A,B) and ALP activity (Figure 6C) in these cells, whereas CS/CaPP/0.25%Pg

scaffolds showed a further significant increase of osteoblast differentiation compared with control and CS/CaPP scaffolds. At the molecular level, both the scaffolds significantly increased mRNA expression of Runx2 (Figure 6E), ALP (Figure 6F), Col-I (Figure 6G) and OC (Figure 6H) in mMSCs, whereas CS/CaPP/0.25%Pg scaffolds showed a further significant increase in their mRNA expression except ALP, compared with control and CS/CaPP scaffolds.

3.4 | CS/CaPP and CS/CaPP/0.25%Pg scaffolds promoted bone formation in vivo

On the basis of the above in vitro studies, we intended to study the role played by Pg particles in CS/CaPP scaffolds for bone formation in vivo. Critical-sized bone defect of 3 mm diameter in rat tibia was used as the model system in this study. The defects were left unfilled in the control group and filled with CS/CaPP and CS/CaPP/0.25%Pg scaffolds in the treated groups. The radiographs of the rat tibiae, 8 weeks post-implantation are shown in Figure 7A. The representative images depict the presence of partially radiolucent space at the defect region in the control, whereas the implanted groups showed dense radio-opacity at the defect regions (Figure 7A). Both CS/CaPP and CS/CaPP/0.25%Pg scaffolds promoted bone formation, as evidenced by filling of the defective hole with calcified bone. The relative BMD was calculated and was found to be significantly higher in the groups implanted with these scaffolds than in the control group (Figure 7B). There was a non-significant increase of BMD in CS/CaPP/0.25%Pg scaffolds compared with that in CS/CaPP scaffolds. The extent of mineralization on the surfaces of calcified bone of the control, implanted groups along with the elemental composition (Ca, P) was assessed by SEM (Figure 7C) and EDAX (Figure 7D). The decalcified tibial tissue sections of animals analysed by H&E (Figure 7E) indicated sparse bone formation in the control group compared with that in the CS/CaPP and CS/CaPP/0.25%Pg groups. The observed new bony areas in all the 3 groups were represented as 'NB'. Well-distinctive structural characteristics of bone, namely bone marrow elements and bone marrow spaces along with mature bone/osteoid, were revealed in the histological sections corresponding to CS/CaPP and CS/CaPP/0.25%Pg groups but not in the control. MTS also showed that areas of new bone formation in the scaffold groups were larger than those in the control (Figure 7F). Further, a quantification of the blue regions representing collagen-stained areas in the sections by Image J software (National Institutes of Health, Bethesda, Maryland, USA) showed increased collagen in the defective areas filled with scaffolds compared with the control (Figure 7G).

4 | DISCUSSION

Biomaterials incorporated with growth factors (GFs) have been widely used as guiding matrices in BTE to promote bone formation. However, their shorter half-life, decreased bioavailability and high cost limit their application.³³ Efforts to establish alternative yet equivalently effective approaches to GF delivery for BTE led to the investigation of naturally

FIGURE 6 Effect of scaffolds on osteoblast differentiation at the cellular and molecular levels. Mouse mesenchymal stem cells (mMSCs) were treated with conditioned media obtained from Chitosan (CS)/CaPP and CS/CaPP/Pg scaffolds for 14 days. They were subjected to Alizarin red (AR) and alkaline phosphatase (ALP) staining. A and B, The representative light microscopic image and the quantified areas of the AR staining, respectively. C and D, The representative light microscopic image and the quantified areas of the ALP staining, respectively. *A significant increase compared with the control ($P < .05$). #A significant increase compared with CS/CaPP scaffolds ($P < .05$). E, F, G and H, The relative mRNA expression of Runx2, ALP, type I Col-1 and OC, respectively. *A significant increase compared with the control ($P < .05$). #A significant increase compared with CS/CaPP scaffolds ($P < .05$)

occurring bone morphogenetic substances like Bio-silica and PolyP.^{6,34-36} PolyP-mediated modulation of osteoblast-specific gene expression has been studied in MC3T3E1, SaOS2 and MSCs.^{20,34,37,38} To the best of our knowledge, there were no reports available to support the role of Pg and its composite scaffolds in bone formation. In this study, we developed CS-based scaffolds and tested the components, CaPP and Pg particles, for their ability to promote bone formation in vitro and in vivo. CS/CaPP/0.25%Pg scaffolds were found to have bioactivity by protein adsorption and biomineralization (Figure 2B,D). Protein adsorption of scaffolds plays a key role for cell attachment and proliferation.^{17,22,39} The formation of silanol (Si-OH) groups in silica-rich ceramics upon interaction with SBF contributes to its superior bioactivity. Additionally, the larger surface area attributed to nanophase materials would have

facilitated increased CaPP crystal nucleation and growth, thereby promoting biomineralization. However, when the concentration of Pg increased beyond 0.5%, there was decrease in the deposits, which could be due to loss of nanophase by the possible aggregation of particles. Compared to the CS/CaPP scaffolds, CS/CaPP/0.25%Pg scaffolds showed increased compressive strength and modulus (Figure 2C). This increased mechanical property facilitates reduced degradation of the scaffolds under physiological conditions. These scaffolds were found to be non-toxic to mMSCs (Figure 3), and the cells under fluorescent microscopy showed no visible alteration in their cytoskeletal morphology or distortion in the intact structure of the nucleus (Figure S2A,B).

Cell cycle phase transition from G0/G1 to G2/M phases mediated by the treatment with the CS/CaPP/0.25%Pg scaffolds in mMSCs

FIGURE 7 In vivo assessment of calcification. A, A representative radiograph of tibial defect of control, CS/CaPP and CS/CaPP/Pg groups at 8 wk post-implantation. B, The relative bone mineral density (%) was quantified by Image J software. C, SEM analysis of biomineralization. D, EDAX analysis of mapping of implant-tissue interfaces. E and F, Haematoxylin and eosin and Masson's trichrome-stained sections of decalcified tibia of the respective groups. NB denotes the distribution of new bony areas; all the images were captured under the 10 $\times$ objective lens. G, Quantification of collagen-stained regions in the control and implant groups by Image J software. *A significant increase compared with control ($P < .05$)

seems to occur *via* the stimulation of expression of cyclins A, B and C (Figure 4C,D). The release of Ca and P from CS/CaPP scaffolds could be responsible for activation of intracellular components for cell

proliferation; other essential ions, namely Si, Mg and Fe as dissolved products of Pg particles, could have played a role for further increased cell proliferation (Figure 4A,B). It has been reported that dissolution of

bioceramic particles resulted in release of ions such as Ca, Mg, Si and P, which were internalized by cells and led to activation of intracellular signalling cascades, resulting in enhancement of cellular processes like cell proliferation, differentiation, mineralization and vascularization.⁴⁰ The increased metabolic activity of cells could have positively influenced the proliferation of cells.³¹⁻³³ We previously showed that ceramics like nBGC, diopside and wollastonite have similar positive effects in promoting cell attachment and proliferation.¹⁵⁻¹⁸ Further, the biological effects of these ceramics were enhanced when composited with CS-based natural polymers.^{17,22,41-44} PolyP-based inorganic polymers showed increased cell proliferation, which was found to be due to the stimulation of bFGF and increased intracellular accumulation of calcium and ATP.^{7,8,45,46} Iron-containing bioceramics have been recognized as potential candidates and they supported the proliferation and differentiation of stem cells and bone cells.⁴⁷⁻⁴⁹ FeO-reinforced PCL nanofibers, FeO/Collagen/HAp and PCL/magnetic nanoparticles (MNPs/FeO NPs) have been explored as iron-based biocomposites for bone tissue regeneration.¹¹⁻¹⁴

Runx2 is a key transcription factor required for the differentiation of multipotent MSCs towards the osteoblast lineage. Expression of Runx2 in turn upregulated the expression of several bone-specific marker genes of early and late stages of osteoblast differentiation.^{50,51} Although both CS/CaPP and CS/CaPP/Pg scaffolds promoted osteoblast differentiation at the cellular and molecular levels (Figure 6), CS/CaPP/Pg scaffolds showed an enhanced effect compared with CS/CaPP scaffolds. This suggests the activation of additional signalling pathways that promote osteoblast differentiation by Pg particles. Several signalling pathways such as those involving TGF- β , BMP, FGF and Wnt have been reported to play a role in the promotion of osteogenesis.^{52,53} To further correlate our in vitro findings with those in in vivo conditions, we used a rat tibial bone defect model system. Exposure of biomaterials to the surrounding body fluids results in dissolution of bioactive constituents, thus facilitating recruitment of stem cells, which in turn fasten tissue repair. The bone morphogenetic activity of CaPP and the bioactive nature of the ions released from Pg particles collectively contributed the enhanced calcification of bone at the drill-hole site, as evidenced by radiography, SEM and EDAX (Figure 7). Bone healing by other biomaterials using this model system has been reported in several studies.^{18,22,27} H&E staining showed bone formation with CS/CaPP and CS/CaPP/Pg scaffolds (Figure 7E). MTS was also supportive of the larger areas of new bone formation in terms of more collagen deposition in the scaffold groups (Figure 7F,G). Notably, the increase in bone formation observed in the CS/CaPP/Pg group, compared with CS/CaPP group, was not significant. Although Pg particles showed a stimulatory effect on bone formation by increased mMSC proliferation at early stages (Figure 4A,B) and enhanced osteoblast differentiation in vitro (Figure 6), bone formation was not significantly altered in vivo. The possibility of saturation of the bone healing effect of Pg particles after a long treatment period (8 weeks) cannot be ruled out. The inclusion of Pg particles in CS/CaPP scaffolds may have reduced the bone healing time compared to that required by CS/CaPP scaffolds alone.

In conclusion, this study evidenced the plausible roles of a novel iron-containing ceramic, pigeonite, and bone morphogenetic

substance, CaPP, in CS-based composite scaffolds for bone formation. The scaffolds containing Pg particles showed enhanced effect on both proliferation and osteoblast differentiation of mMSCs in vitro. The study also demonstrated the in vivo bone-forming potential of the scaffolds in rat critical-sized tibial defect. Thus, the observations of our current study conclusively support the suitability of CS/CaPP/Pg composite scaffolds in the field of stem cells and BTE, and suggest that these scaffolds could further be established as suitable candidates to be employed as stem cell-based composites, as bone substitutes for orthopaedic repair.

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AUTHORS CONTRIBUTIONS

S.D. performed the experiments, collected data and prepared the manuscript. K.N. and L.K. assisted in the experiments and analysed the data. M.V. assisted in the technical parts. N.S. designed the study, analysed data and edited the manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interests related to this study.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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