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The osteogenic differentiation of dog bone marrow mesenchymal stem cells in a thermo-sensitive injectable chitosan/collagen/ β-glycerophosphate hydrogel: in vitro and in vivo

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Abstract Type I collagen was added to the composite chitosan solution in a ratio of 1:2 to build a physical cross-linked self-forming chitosan/collagen/β-GP hydrogel. Osteogenic properties of this novel injectable hydrogel were evaluated. Gelation time was about 8 min which offered enough time for handling a mixture containing cells and the subsequent injection. Scanning electronic microscopy (SEM) observations indicated good spreading of bone marrow mesenchymal stem cells (BMSCs) in this hydrogel scaffold. Mineral nodules were found in the dog-BMSCs inoculated hydrogel by SEM after 28 days. After subcutaneous injection into nude mouse dorsum for 4 weeks, partial bone formation was observed in the chitosan/collagen/β-GP hydrogel loaded with pre-osteodifferentiated dog-BMSCs, which indicated that chitosan/collagen/β-GP hydrogel composite could induce osteodifferentiation in BMSCs without exposure to a continual supply of external osteogenic factors. In conclusion, the novel chitosan/collagen/β-GP hydrogel composite should prove useful as a bone regeneration scaffold.

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1 Introduction

In recent years, fabrication of injectable sol–gel systems for biomedical applications has grown [1, 2]. Therapeutic drugs, growth factors or cells which are pre-incorporated in liquid sol–gel solution can be directly infused into the desired tissue, organ or body cavity in a minimally invasive manner and then formed into a gel scaffold in situ. To date, most studies of injectable material have focused on biopolymers.

Chitosan, an amino-polysaccharide derived from chitin, is a kind of pH-dependent cationic polymer characterized by its biocompatible [3], biodegradable [4], and antimicrobial properties [5]. In recent decades, chitosan has been widely used in tissue engineering and regenerative medicine [6, 7]. An acidic chitosan solution is commonly mixed with a basic β-glycerophosphate (β-GP) solution to prepare a thermally responsive pH-dependent sol–gel system [8]. Chitosan-GP hydrogel remains in solution at physiological pH, is in a liquid state below room temperature which is useful for encapsulating living cells and therapeutic agents, while it turns into solid gel implants in situ upon warming to body temperature. Chitosan shares a similar structure with glycosaminoglycans (GAGs) and its degradation time can be adjusted by changing its degree of deacetylation (DDA) to optimize its utility [4, 9]. Therefore, chitosan-based injectable hydrogels have also been widely used for cartilage repair, nucleus pulposus regeneration, drug release and other biomedical functions [10–14].

In bone tissue engineering, chitosan has shown excellent biological properties for use alone or combined with other bioceramics such as hydroxyapatite (HA) and tricalcium phosphate (TCP). Chitosan also showed excellent osteoconductivity and could accelerate osteoblasts growth in the scaffold [15]. Rat bone marrow mesenchymal stem cells

(BMSCs) were found to grow well in chitosan-GP gel [16] and calcium deposition could be found in rat muscle-derived stem cells with addition of osteoinductive agents [17]. However, it was argued that the calcium deposits observed in the pure chitosan-GP gel were probably a result of calcium release from cell necrosis [18], and direct interaction between chitosan particles and BMSCs was not the sole reason resulting in an enhancement of osteogenesis in vitro [19].

Type I collagen is a significant constituent of the natural bone extracellular matrix (ECM), provides a structural framework for connective tissue and plays a key role in the temporal events cascade leading to formation of new bone [20]. It has also been shown that integrin-mediated adhesion to type I collagen could enhance osteodifferentiation of human BMSCs [21]. Previously, type I collagen has been added into chitosan solution to improve its biological properties before β-GP initiated gelation [18]. In vitro assessments of gene and osteogenic markers expression showed that human BMSCs cultured in a collagen-chitosan composite gel differentiated better compared to those cultured in pure chitosan gel.

In this study, a thermo-sensitive injectable chitosan/collagen/β-GP hydrogel was fabricated and evaluated for its thermo-sensitivity, cell viability and morphology. Dog BMSCs were harvested, pre-osteodifferentiated and seeded into the chitosan/collagen/-GP composite hydrogel for in vivo histological evaluation. Osteogenesis in this novel hydrogel was identified and discussed.

2 Materials and methods

2.1 Materials and equipment

Chitosan with a deacetylation degree of approximately 91% was provided by Golden-Shell Biochemical Limited Company (China). Collagen type I was provided by MP Biomedical (France). Low glucose-Dulbecco's modified Eagle's medium (L-DMEM), fetal bovine serum (FBS) and phosphate buffered saline (PBS) were from Invitrogen (USA). Glycerol β-phosphate disodium salt hydrate (β-GP), L-glutamine, dexamethasone, L-ascorbic acid, acetic acid, calcein-AM and propidium iodide (live/dead) double cell stain kit were from Sigma–Aldrich (China). All other reagents were of analytical grade. Equipments used were as follows: pH meter (PHS-3C-LEIZI, China); SEM (Hitachi S-3400N, Japan); Freeze dryer (Chist, Germany).

2.2 Fabrication of the chitosan/collagen/β-GP hydrogel, pH value and gelation time

A 2.0 wt% totally dissolved chitosan solution was prepared by stirring powdered chitosan in 0.1 M aqueous acetic acid for 4 h at room temperature, followed by sterilization by autoclaving for 10 min at 121°C [22]. Sterilized collagen type I was dissolved in 0.02 M acetic acid to obtain a 3.5 mg/ml solution. The chitosan and collagen solution were mixed at a mass ratio of 2:1. Then the mixture was stirred for 10 min on ice, its pH measured and stored at 4°C for later use. A 2.0 wt% chitosan solution without collagen was prepared as a control. β-GP was dissolved in deionized water to obtain a 56 wt% solution and sterilized by passing through a 22 μm filter membrane prior to storage. Pre-cooled sterile β-GP solution was added drop wise to both the chitosan/collagen solution and the chitosan solution with stirring on ice so that the final β-GP concentration was adjusted to 8%. After determination of the pH, the obtained liquid solutions were sub-packaged into test tubes and transferred to a 37°C incubator. The gelation time was recorded and determined by evaluation of fluidity and the color change of the solution.

2.3 Cell isolation, passage and osteogenic induction

BMSCs harvested from the iliac of three beagle dogs were cultured. In detail, 4.5 ml of bone marrow was aspirated into a 10 ml syringe containing 5000 U of heparin, and then centrifuged to remove fat and heparin. The precipitated cells were cultured in a complete medium containing L-DMEM with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were incubated at 37°C in a humidified atmosphere containing 95% air and 5% CO_2. Once 80–90% cell confluency was reached, the cells were detached by treatment with 0.25% trypsin containing 0.01% EDTA, then sub-cultured and routinely passaged.

For cell osteogenic differentiation, the second passage of the cells were cultured in medium containing L-DMEM with 10% FBS, 100 nM dexamethasone, 5.0 mM β-GP, and 50 mg/ml L-ascorbic acid. The medium was replaced every 3 days. All animal procedures were done in accordance with the Animal Care Guidelines from PLA General Hospital.

2.4 Cell viability in hydrogel

The prepared liquid chitosan/collagen/β-GP and chitosan/β-GP were loaded with the third passage of dog-BMSCs to obtain 2×10^5 cells/ml. The mixed solution was then transferred to a 24-well plate in an incubator at 37°C with a 100% humidified chamber and 5% CO_2, with each well containing 0.5 ml solution. 1 ml complete medium was added to each well when gel formation was achieved after 20 min. The medium was changed every 2 days. Cell viability at day 1 and day 7 ($n = 3$) was assessed by calcein-AM and propidium iodide (live/dead viability kit). The cellular growth in the gel was observed by

fluorescence inverted microscope and analyzed by the image-pro-plus 6.0 system (IPP).

2.5 SEM investigation

Dog-BMSCs were seeded in both chitosan/collagen/GP and chitosan/GP gels for SEM observation. Microstructure of the gels and cell growth in the scaffold were observed after glutaraldehyde fixation at day 3. Cell growth under osteogenic conditions was observed by freeze-drying after dog-BMSCs were seeded into the two scaffolds cultured in osteogenic inductive medium for 28 days.

2.5.1 Glutaraldehyde fixed group

A total of 300 μl of chitosan/collagen/GP and chitosan/GP gel encapsulated 2×10^5 dog-BMSCs or cell free scaffolds were cultured in a 24-well plate with complete medium. On day 3, all gels were washed three times with PBS and then immersed into 2.5% glutaraldehyde for 2 h at room temperature. Then, the gels were washed again three times with PBS followed by a gradient-elution using ethanol and tertiary butyl alcohol. The final samples ($n = 3$) were vacuum dried, gold sputtered and observed by using SEM.

2.5.2 Freeze-dried group

The same volume of 300 μl of chitosan/collagen/GP and chitosan/GP gel encapsulated 2×10^5 dog-BMSCs were cultured separately in a 24-well plate with osteogenic inductive medium for 4 weeks. On day 28, all samples were transferred to −80°C for 2 h, and then lyophilized for 24 h. The dried gels ($n = 3$) were cut in half with a sharp blade to expose the internal microstructure and sputter coated with gold for SEM imaging.

2.6 In vivo procedures in nude mice

Ten nude mice were used for the in vivo experiment. Hydrogel implants of chitosan/GP, chitosan/collagen/GP, dog-BMSC-chitosan/GP and dog-BMSC-chitosan/collagen/GP were injected subcutaneously into four quadrants of a mouse dorsum. Passage two cells which had already been pre-cultured in osteogenic inductive medium for 7 days were used for the test. Cell density in the gel was 2×10^6 cell/ml.

A 1 cc syringe with a 26-gauge needle was used to inject 150 μl solutions per implant to mice that had been anesthetized with ethyl ether.

2.7 Histological analysis

All mice were sacrificed and the implants were individually dissected and removed from the subcutaneous dorsum at 4 weeks after implantation. The specimens were immediately fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin blocks. The embedded specimens were sectioned (5 μm thick) along the longitudinal axis of the implant. Half slides were stained by hematoxylin and eosin (H&E), and the other half were stained by von Kossa followed by toluidine blue.

2.8 Statistical analysis

All quantitative data were recorded and statistically analyzed by SPSS 13.0. A 2-way analysis of variance (ANOVA) at $P < 0.05$ level of significance was applied to indicate differences between treatment groups.

3 Results and discussion

3.1 Fabrication, gelation time and pH

Liquid chitosan/collagen/GP solution could be transformed to solid gel after incubation at 37°C as shown in Fig. 1a, b. The sol–gel process was accompanied by color transition from transparent to gray. The gelation time of the chitosan/collagen/GP gel was about 8 min while the chitosan-GP gel needed about 12 min for gelation (Fig. 1c). The gelation process was faster in the chitosan/collagen/GP group

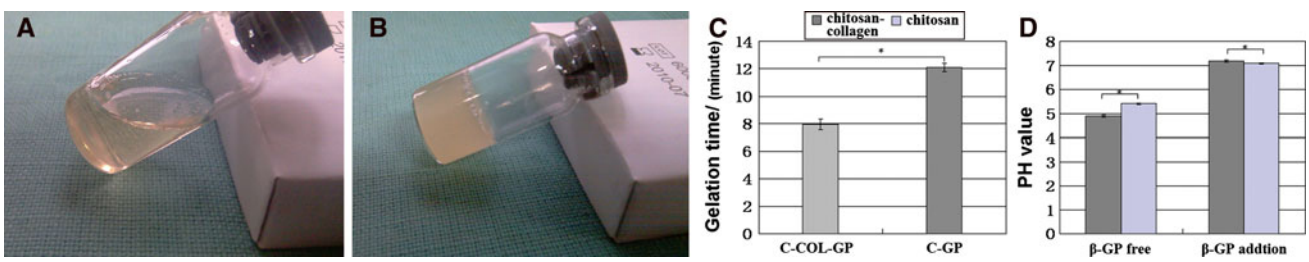

Fig. 1 **a** Chitosan/collagen/GP solution below room temperature. **b** Chitosan/collagen/GP transformed into hydrogel at 37°C. **c** Gelation time of chitosan/GP (C-GP) and chitosan/collagen/GP (C-COL-GP) hydrogel. **d** pH values of chitosan and chitosan-collagen solution before and after β-GP addition ($n = 5$; * statistically significant differences)

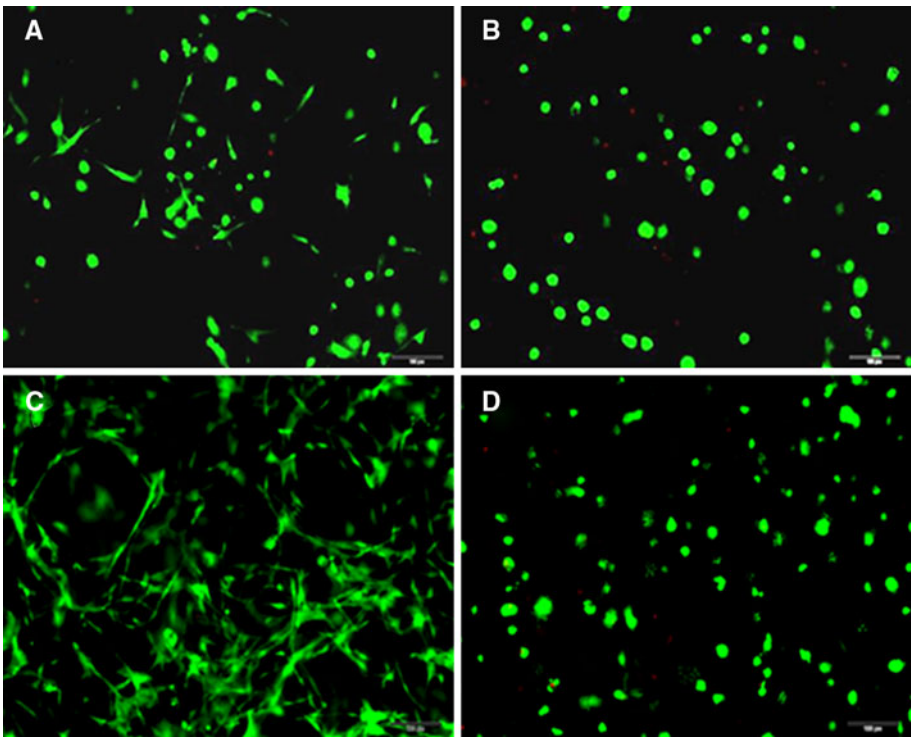

Fig. 2 Viability of dog-BMSCs cultured in chitosan/collagen/GP (**a**, **c**) and chitosan/GP (**b**, **d**) hydrogels by live/dead stain at day 1 (**a**, **b**) and day 7 (**c**, **d**). Live cells were *green* and dead cells were *red*. *Scale bar* = 100 μm (Color figure online)

which was consistent with the results of the Wang and Stegemann [18] but not with Song et al. [23]. pH values of both chitosan–collagen and chitosan only solution changed from acidic to physiological after the addition of β-GP (Fig. 1d), a process which should facilitate encapsulation of living cells or therapeutic proteins. About 8 min of gelation time was proven appropriate for handling the cell mixture and the subsequent injection before the solid gel formed.

3.2 Viability of BMSCs in the gel

Most dog-BMSCs grew well in chitosan/collagen/GP and chitosan/GP gels, and observation of dead cells were rare (Fig. 2a–d). Cells spread better in the collagen-containing gel (Fig. 2c) than in the pure chitosan gel (Fig. 2d) after a 7-day culture period. Cells in the chitosan/GP gel still maintained a round shape like originally on day 1, while cells in the collagen-containing gel showed typical spindle-shaped morphology and aligned in a vortex fashion. It has been reported that cell viability in a chitosan/GP scaffold could be affected by β-GP solution in a dose-dependent manner; and high concentration of β-GP could cause cytotoxicity [18, 24]. An assay of cell viability in this study showed 8% β-GP in the chitosan/collagen/GP gel had no negative effects on cell viability. The ratio of chitosan–collagen–GP hydrogel used in this study was appropriate to support the BMSCs growth and could offer proper space and support for cell proliferation.

3.3 Morphology of the gel

A typical heterogeneous microstructure of chitosan/GP gel was observed by SEM (Fig. 3b). The spherical chitosan microparticles formed a network in agglomerates and chains, which was also described in previous studies [25]. The chitosan/collagen/GP scaffold showed a more porous cobweb-like network constructed by collagen fibrils connected with chitosan microparticles (Fig. 3a). The diameter of the microparticles in the chitosan/collagen/GP scaffold (1.5–2 μm) was larger than that in the chitosan/GP scaffold (1–1.5 μm), which was consistent with the report of Crompton et al. [26] which stated that the size of the aggregates decreased with increasing chitosan concentration. The BMSCs were difficult to distinguish in the chitosan/GP gel (Fig. 3d), while they could be easily found inside the chitosan-collagen/GP hydrogel (Fig. 3c). Cells extended well and connected with each other in the chitosan/collagen/GP gel. Matrix release could be easily visualized at the surfaces of cells which indicated good cell function. These favorable biological properties were likely attributed to the physical cross-link method used in this study which allowed efficient combination of chitosan with collagen. Compared to covalent cross-linking [27] or lyophilization [28], physical cross-linking maintained the functional groups of chitosan and collagen during gelation.

It was also reported that collagen type I could be reconstituted by raising the temperature and pH [29]. In this study, collagen fibrillogenesis was initiated by adding

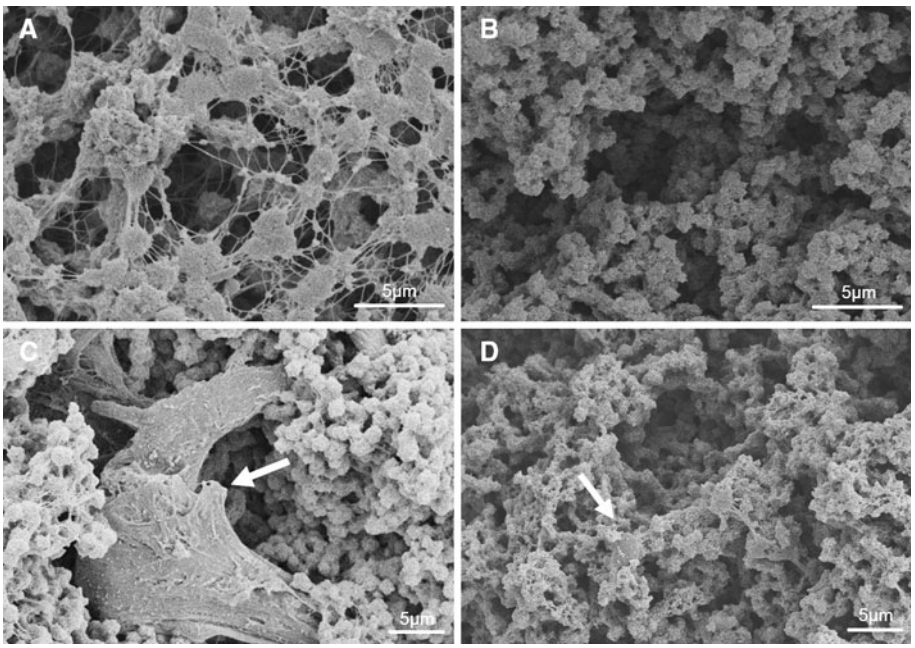

Fig. 3 SEM of chitosan/collagen/GP (**a**, **c**) and chitosan/GP (**b**, **d**) hydrogels treated by glutaraldehyde at day 3. **a** and **b** were cell free constructs; **c** and **d** were dog-BMSCs containing constructs. *White arrows* indicated the dog-BMSCs. Magnification: **a**, **b** ×5,000; **c**, **d** ×3,000. *Scale bar* = 5 μm

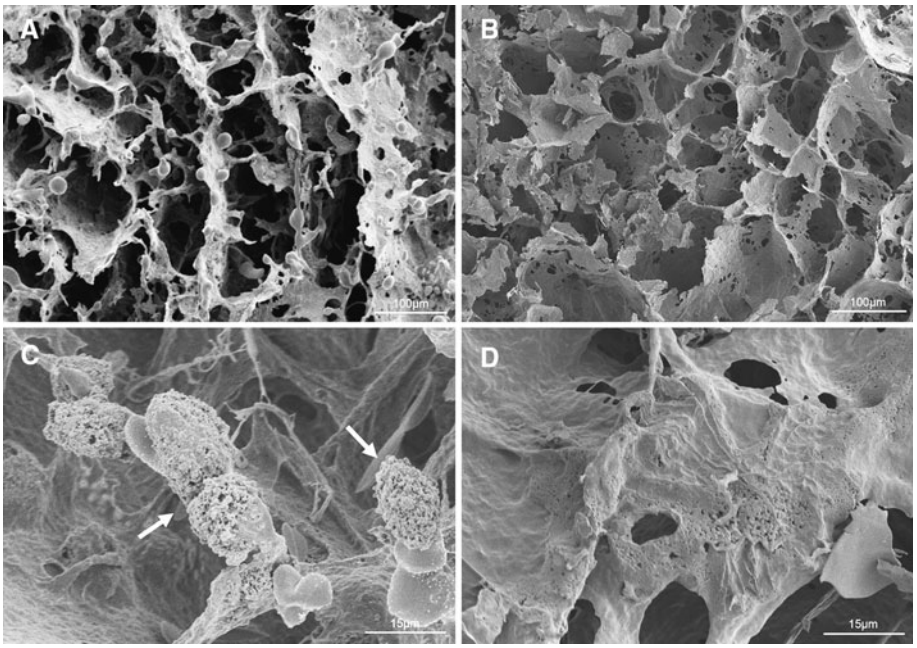

Fig. 4 SEM of dog-BMSCs seeded in chitosan/collagen/GP (**a**, **c**) and chitosan/GP (**b**, **d**) hydrogels cultured in osteogenic medium and treated by lyophilization at day 28. *White arrows* indicated mineral nodules. Magnification: **a**, **b** ×200; **c**, **d** ×1,500. *Scale bar*: **a**, **b** = 100 μm; **c**, **d** = 15 μm

basic β-GP to acidic collagen solution and raising the temperature simultaneously. A relatively higher porosity of chitosan/collagen/GP resulted which was also favorable for cell crawling and nutrition exchange.

After being cultured in osteogenic medium for 28 days, SEM images of freeze-dried gels showed differences in the structures of chitosan/GP gel (Fig. 4b) and chitosan/collagen/GP gel (Fig. 4a). The chitosan/GP scaffold exhibited a porous and honeycomb-liked network while the chitosan/collagen/GP scaffold exhibited a more regular and intensive sponge-like structure. In the latter composite construct, the previously described collagen fibrils embedded in the chitosan were gradually fused with the cells. In Fig. 4c, the higher magnification image showed a layer of cells with intercellular connections and many mineral nodules in the chitosan/collagen/GP scaffold, while mineral nodules were not found in the chitosan/GP scaffold (Fig. 4d). Deposition of bone mineral in dog-BMSCs encapsulated chitosan/collagen/GP suggested its good osteoconductivity, which was favorable as a scaffold for bone regeneration.

3.4 Implant gel in vivo and histology analysis

Gel injection was performed using a 26-gauge needle. The nude mice returned to normal activity after injection and were sacrificed by overdose of carbon dioxide at day 28. There was no infection occurrence around the implant area during the healing period. Gross observation (Fig. 5a) showed that the implants remained in situ subcutaneously after injection without displacement. Gels remained in the subcutaneous implant pockets after 4 weeks (Fig. 5b). A thin fibrous capsule containing blood vessels could be found around the retrieved specimen (Fig. 5c).

The entire specimen were fixed and sectioned. From H&E stain slides (Fig. 6a–d), a moderate chronic inflammatory reaction could be observed. Thin fibril capsules could be found around the implants and gels were infiltrated by host inflammation cells such as neutrophil periphery. These findings concurred with Vandevord et al. [3] who concluded that neutrophilic migration to chitosan appeared to be an inherent property because chitosan oligosaccharides were chemo-attractants for neutrophils. Significantly less inflammatory cells were found in cell-containing gel implants which were similar to the results of previous studies where the immune response was down regulated by BMSCs because of its immunosuppressive properties [16, 17].

Dog-BMSCs were maintained inside the gel in the cell-containing chitosan/GP group, which exhibited a more fibrous structure compared when there were no cells (Fig. 6b). There was no bone-like structure found in these chitosan/GP gels. However, ectopic bone-like mineral matrix could be found in the dog-BMSCs-chitosan/collagen/GP gel (Fig. 6d). Positive black dyeing by von Kossa stain confirmed the existence of the mineral matrix which was also found by H&E staining (Fig. 6h). In the other three groups, the von Kossa stain was negative (Fig. 6e–g). Purple dyeing by toluidine blue (Fig. 5h) suggested osteoid instead of bone formation inside the gels during in vivo

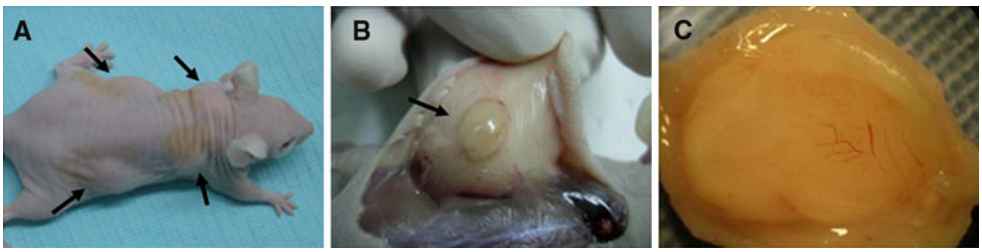

Fig. 5 Photographs of in situ formed chitosan-based hydrogels. **a** Subcutaneous injection of chitosan-based solution to a nude mouse. **b** Gel formed subcutaneously in the nude mouse and showed a spherical to ovoid shape after four weeks healing. **c** The gel was removed from the nude mouse on day 28. *Black arrows* indicated the implants

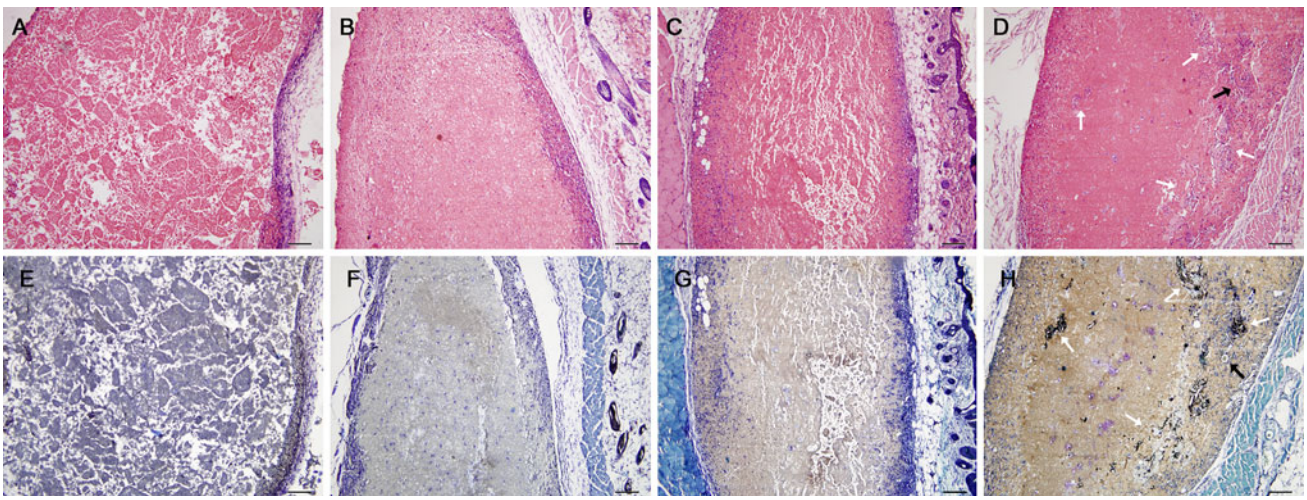

Fig. 6 Images of H&E stain and von Kossa counter stain with Toluidine blue of four different hydrogels after subcutaneous implantation in a nude mouse at 4 weeks. **a–d** H&E stain. **e–h** von Kossa and counter stain with Toluidine blue. **a**, **e** Chitosan/GP cell free scaffold. **b**, **f** Pre-osteodifferentiation of dog-BMSCs containing chitosan-GP scaffold. **c**, **g** Chitosan/collagen/GP cell free scaffold. **d**, **h** Pre-osteodifferentiation of dog-BMSCs containing chitosan/collagen/GP scaffold Vascularity could be observed in the bone forming area. *Black arrows* indicate blood vessels and *white arrows* indicate bone mineral matrix. Magnification: ×100. *Scale bars* 100 μm

culture. Dog-BMSCs containing constructs could be easily loaded with osteogenic factors in vitro to induce osteogenic differentiation by a method of immersion into the osteogenic medium. However, for bone regeneration in vivo, osteogenic media cannot be applied. Thus, it was favorable to fabricate an osteogenic cell-encapsulated construct and synthesize a bone matrix separately [30]. In this study, dog-BMSCs were cultured in osteogenic medium for a week before seeding in a chitosan–collagen–GP gel. In the subsequent in vivo culture, a considerable amount of bone marrow precursor cells differentiated into matured osteoprogenitor cells and synthesized bone minerals without application of any more osteogenic agent. This indicated that the pre-osteodifferentiated cells in this gel still maintained an osteogenic phenotype in vivo without any external usage of bone promotion factors such as BMP. In the future follow up study, more intensive investigations are necessary to further enhance the efficiency of bone formation in a chitosan/collagen/GP hydrogel.

4 Conclusions

A physical cross-linked thermo-sensitive chitosan/collagen/β-GP hydrogel was proven to be biocompatible in vitro and in vivo. The hydrogel also showed the ability for continual in vivo osteogenesis after being loaded with pre-osteodifferentiated dog-BMSCs in vitro. This novel construct could be used as a cell vehicle and injectable bone substitute for purposes of tissue regeneration.

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