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Novel injectable calcium phosphate scaffold with human periodontal ligament stem cell encapsulation in microbeads for bone regeneration

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Objectives: 1) Develop a novel construct of human periodontal ligament stem cells (hPDLSCs) encapsulated in degradable alginate microbeads (DAMB) with human platelet lysate (hPL) and injectable calcium phosphate cement (ICPC); 2) Investigate the proliferation and osteogenic differentiation of hPDLSCs in ICPC with hPL as a xeno-free supplement and animal serum replacement for bone tissue engineering applications.

Methods: hPDLSCs were encapsulated in alginate-fibrin microbeads (DAMB + fibrin), alginate-hPL degradable microbeads (DAMB + hPL), or alginate-fibrin-hPL microbeads (DAMB + fibrin + hPL). The proliferation and osteogenic differentiation of hPDLSCs were investigated in culturing with the ICPC scaffold.

Results: Flexural strength of ICPC was 8.4 ± 0.91 MPa, and elastic modulus was 1.56 ± 0.1 GPa, exceeding those of cancellous bone. hPDLSCs had higher viability in DAMB + fibrin + hPL group than in DAMB + fibrin. ALP was 69.97 ± 16.96 mU/mg for ICPC + DAMB + fibrin + hPL group, higher than 30.68 ± 2.86 mU/mg of ICPC + DAMB + fibrin ($p < 0.05$) and 4.12 ± 1.65 mU/mg of control ($p < 0.01$). At 7 days, osteogenic gene expressions (*ALP*, *RUNX2*, *COL1*, and *OPN*) in ICPC + DAMB + fibrin + hPL and ICPC + DAMB + fibrin were 4–11 folds that of control. At 21 days, the hPDLSC-synthesized bone mineral amounts in ICPC + DAMB + fibrin + hPL and ICPC + DAMB + fibrin were 13.2 folds and 11.1 folds that of control group, respectively.

Conclusion: The novel injectable CPC scaffold encapsulating hPDLSCs and hPL is promising to protect and deliver hPDLSCs. The hPL-based medium significantly enhanced the osteogenic differentiation of hPDLSCs in ICPC + DAMB + fibrin + hPL construct, suggesting a promising xeno-free approach for bone tissue regeneration applications.

KEYWORDS

periodontal ligament stem cells, calcium phosphate scaffold, alginate microbeads, osteogenic differentiation, human platelet lysate

Introduction

Bone tissue engineering involves the use of biomaterials, cells and bioactive factors and represents a promising approach for bone regeneration. Injectable calcium phosphate cement (ICPC) is used for bone repair due to their similarity to bone minerals (Szpalski et al., 2012a; Szpalski et al., 2012b; Wang P. et al., 2014). The first ICPC was developed in 1986 and consisted of a mixture of tetracalcium phosphate [TTCP, $\text{Ca}_4(\text{PO}_4)_2\text{O}$] and dicalcium phosphate anhydrous (DCPA, CaHPO_4). ICPC was approved by the Food and Drug Administration (FDA) for the repair of craniofacial defects due to its excellent biocompatibility, osteoconductivity, resorbability, and injectability (Friedman et al., 1998).

Various ICPC formulations were reported with optimizing the porosity, surface architectures, resorption rates, and mechanical strength (Barralet et al., 2002; Bohner et al., 2005; Bodde et al., 2009; Ginebra et al., 2010). Furthermore, cell therapy was employed for bone defect treatment with ICPC to increase the bone regeneration efficiency (Humbert et al., 2019). Due to its self-renewal and multiple differentiation potential, mesenchymal stem cells (MSCs) have been used for cell-based therapy. The seeding of ICPC with stem cells included human bone marrow MSCs (hBMSCs) (He et al., 2013), human umbilical cord MSCs (hUCMSCs) (Zhao et al., 2010), human embryonic stem cells (hESCs) (Liu et al., 2014), and human-induced pluripotent stem cells (hiPSCs) (Liu et al., 2013a).

In stem cell therapy, MSCs need to proliferate for an optimal therapeutic dose and differentiate into multiple cell lineages for effective tissue repair and clinical treatment. Therefore, the culture medium is essential to supply nutrients for cell metabolism, proliferation, and differentiation *in vitro*. Currently, cell culture medium supplements are based on animal serum, mostly fetal bovine serum (FBS), which consists of a large number of low and high molecular weight biomolecules with different physiological activities (Gstraunthaler, 2003; van der Valk et al., 2018). However, it has reported the FBS could evoke the xenogeneic immunological reactions and raise the risk of transmitting bovine infections (Brunner et al., 2010). As ICPC have been widely investigated in a number of clinical trials for bone regeneration, ICPC scaffolds seeding with MSCs represent a new strategy to promote pre-vascularization and enhance bone tissue engineering efficacy (Lin

et al., 2019). Therefore, it is urgently needed to switch the culture medium to a FBS-free, xeno-free alternative, thus enable the MSCs seeding ICPC product not cause any potential infections, allergies or malignancies and ultimately facilitate the clinical application.

Human platelet lysate (hPL) is a valuable, non-xenogenic alternative to FBS in cell culture (Bernardo et al., 2007; Rauch et al., 2011). The advantages of hPL includes that it is human-derived, serum-free, and cost-effective. An increasing number of studies demonstrated that hPL showed a growth-promoting effect on a multitude of cell lines (Fernandez-Rebollo et al., 2017). Among the various types of MSCs, periodontal ligament stem cells (hPDLSCs) play a key role in dental and periodontal tissue regeneration. Seo et al. (2004) identified hPDLSCs in the periodontal ligament (PDL) and demonstrated that hPDLSCs implanted into nude mice generated cementum/PDL-like structures that resembled the native PDL. Compared with other MSCs, hPDLSCs have a marked ability to generate multiple tissue types, including alveolar bone, cementum, and Sharpey's fibers, making them an ideal source for cell-based dental therapy. However, there has no report that investigated the effect of hPL as a cell growth supplement on the proliferation and osteogenic differentiation of hPDLSCs in culture with ICPC.

In stem cell-based bone tissue engineering, the number of functioning cells in the bone injury site appears to be of critical importance. However, it was reported that few transplanted cells survived *via* local administration of cell suspensions. One study demonstrated that only 50% of initial donor BMSCs remained alive 48 h after implantation, and only 5% survived after 8 weeks (Giannoni et al., 2010). Another study showed that less than 1% of human multipotent stromal cells were detectable after 30 days post-implantation (Becquart et al., 2012). Thus the preferred approach was delivering a large number of cells to the target site *via* a carrier scaffold. Alginate hydrogel is highly hydrated with excellent biocompatibility and has shown great potential as a carrier for cell delivery (Izeia et al., 2020; Xu et al., 2021). Previous studies have reported that alginate microbeads are an ideal vehicle to localize the cells at the target defect site for cell-based therapy (Li et al., 2012; Pal et al., 2014). In addition, it has been reported that the incorporation of hPL could improve the viability, adhesion, and proliferation of a various types of cell in the alginate hydrogel (Sandri et al., 2015; Saporito et al., 2019).

However, there has been no report that investigated the alginate hydrogel incorporation of hPL as a cell delivery system with ICPC for bone tissue regeneration.

The excellent biocompatibility of ICPC renders it suitable for cell and growth factor delivery. hPL has been used as a cell nutrient supplement for cell expansion, as well as a source of growth factors for tissue engineering applications. To date, a literature search revealed no report on the development of injectable hPDLSC-hPL-CPC-microbead constructs for bone tissue engineering. There has been no report on hPL-containing degradable microbeads as a vehicle to deliver hPDLSCs. There has been no report of investigating hPL as a cell growth supplement for hPDLSCs in culture with CPC, using the FBS-free and xeno-free cell culture medium alternative to avoid the risk of transmitting bovine infections and the initiation of xenogeneic immunological reactions.

Therefore, the objectives of the present study were to: 1) Develop a novel construct of hPDLSCs encapsulated in hydrogel microbeads with hPL and ICPC; 2) Investigate the proliferation and osteogenic differentiation of hPDLSCs in microbeads in ICPC with hPL as the xeno-free supplement for bone regeneration. The following hypotheses were tested: 1) Encapsulating hPDLSCs in alginate microbeads would protect the hPDLSCs during the setting reaction of ICPC paste; 2) The incorporation of hPL could improve the viability of hPDLSCs in the alginate microbeads; 3) The hPDLSCs released from the degradable alginate microbeads in ICPC would be able to proliferate and undergo osteogenic differentiation when using a xeno-free growth medium with hPL.

Materials and methods

Fabrication of injectable calcium phosphate cement scaffold

The ICPC powder consisted of a mixture of TTCP and DCPA at a TTCP:DCPA molar ratio of 1:1 because this ratio was shown to produce apatite minerals (Xu and Simon, 2005). The TTCP powder was synthesized from a solid-state reaction between CaHPO_4 and CaCO_3 (Baker Chemical Company, NJ, United States) and then ground to obtain a median particle size of 17 μm . The DCPA powder (Baker Chemical) was ground to obtain a median particle size of 1 μm . Chitosan was used as cement liquid as previous studies have indicated that the addition of chitosan to CPC enhanced the strength and durability of ICPC (Weir and Xu, 2008). Two cement liquids at 0 and 7.5% chitosan were used. The ICPC liquid consisted of chitosan malate mixed with sterile distilled water at a chitosan/(chitosan + water) mass fraction of 7.5%. The ICPC powder was mixed with the chitosan liquid at a mass ratio of 2:1. Each paste was placed into $3\text{ mm}^3 \times 4\text{ mm}^3 \times 25\text{ mm}^3$ stainless steel molds to make flexural specimens. Each specimen was covered with a glass slide on

each side, clamped, and incubated in a humidifier with 100% relative humidity at 37°C for 24 h.

Load-bearing properties

The hardened specimens were demolded and immersed in distilled water for 24 h. A three-point flexural test was used to fracture the specimens in a universal Testing Machine (MTS, Eden Prairie, MN). Three-point bending strength $S = 3F_{\text{max}}L / (2bh^2)$, where F_{max} is the maximum load on the load-displacement (F-d) curve, L is the span, b is the specimen width and h is the thickness. Elastic modulus $E = (F/d) (L^3 / [4bh^3])$, where load F divided by displacement d is the slope (Xu et al., 2002).

hPDLSC isolation from extracted human teeth

hPDLSCs were isolated from the PDL tissues on extracted human premolars collected from healthy patients. The procedures were approved by the Institutional Review Board of the University of Maryland Baltimore (HP-00052180). All patients or their guardians were informed with written consent. The PDL tissue was scraped off the middle third of the root surfaces (Chen et al., 2020). The scraped tissues were digested in a solution of 3 mg/ml collagenase I (Worthington Biochem, Freehold, NJ, United States) and 4 mg/ml dispase (Roche, Mannheim, Germany) for 1 h at 37°C in a humidified atmosphere with 5% CO_2 . Then the PDL samples were placed into culture dishes with growth medium. The medium consisted of Dulbecco's modified Eagle's medium (DMEM, GIBCO BRL, Grand Island, NY, United States) supplemented with 20% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, United States) and 1% penicillin/streptomycin (P.S, GIBCO BRL). The samples were incubated at 37°C with 5% CO_2 . Single cells were observed 3 days later, and cell colonies were formed at 7–10 days. The individual cell colonies were digested to a single cell suspension using filter paper (Whatman, TISCH Scientific, North Bend, Ohio, United States) with 0.25% Trypsin-EDTA (GIBCO BRL), and transferred to 24-well culture plates, and the cells were passaged when they reach to 80% confluency. It was shown in our previous study that the hPDLSCs were positive for STRO-1, CD146⁺, and OCT4 and negative for CD34 and CD45 (Chen et al., 2020). Passage 3 cells were used in subsequent experiments.

hPDLSC encapsulation in alginate microbeads

Alginate was made degradable by oxidation at 7.5%, following a method described in a previous study

(Bouhadir et al., 2001). Three types of alginate-based microbeads were fabricated to encapsulate the hPDLSCs: 1) Alginate-fibrin microbeads (DAMB + fibrin); 2) Alginate-hPL microbeads (DAMB + hPL); 3) Alginate-fibrin-hPL microbeads (DAMB + fibrin + hPL). A 1.2% (mass fraction) sodium alginate solution was prepared by dissolving oxidized alginate in saline (155 mmol/L NaCl) containing 0.1% fibrinogen for preparing type-1 microbeads. For type 2 microbeads, oxidized alginate was dissolved by 50% dilution of hPL with saline. For type 3 microbeads, fibrinogen was added at a concentration of 0.1% to the alginate-hPL solution. hPDLSCs were encapsulated at a density of 100,000 cells/mL of alginate solution. The alginate-cell solution was loaded into a syringe that was connected to a bead-generating device (Var J). Nitrogen gas was fed to the gas inlet and a pressure of 8 psi was established to form a coaxial airflow to break up the alginate droplets (Zhou and Xu, 2011). For type 1 and type 3 microbeads, the droplets fell into a well of 6-well plate containing 8 ml of 200 mmol/L calcium chloride solution plus 1 NIH units/mL of thrombin (Millipore Sigma) and cross-linked for 20 min to form DAMB + fibrin and DAMB + fibrin + hPL. For DAMB + hPL, the droplets fell into a well of 200 mmol/L calcium chloride solution and crosslinked for 20 min to form microbeads. A microscope (Eclipse TE-2000S, Nikon, Melville, NY) was used to measure the size of the microbeads.

Viability of hPDLSCs inside alginate microbeads

The ICPC paste was extruded through a sterile syringe into a 12 well-plate with 0.05 ml of paste per well. The cement was set in an incubator for 30 min. The hPDLSC-encapsulating microbeads were suspended in the culture medium and added into the 24 well-plate containing CPC for co-culture. The viability and proliferation of hPDLSCs were tested in four groups:

1. ICPC + DAMB + fibrin + DMEM group (Alginate-fibrin microbeads encapsulating 1×10^5 hPDLSCs with 0.05 ml ICPC scaffold in DMEM);
2. ICPC + DAMB + fibrin + FBS group (Alginate-fibrin microbeads encapsulating 1×10^5 hPDLSCs with 0.05 ml ICPC scaffold in growth medium containing 10% FBS);
3. ICPC + DAMB + hPL group (Alginate-hPL microbeads encapsulating with 1×10^5 hPDLSCs with 0.05 ml ICPC scaffold, cultured in DMEM for the first three days and then in growth medium containing 2.5% hPL);
4. ICPC + DAMB + fibrin + hPL group (Alginate-fibrin-hPL microbeads encapsulating with 1×10^5 hPDLSCs with 0.05 ml ICPC scaffold, cultured in DMEM for the first three days and then in growth medium containing 2.5% hPL).

After culturing for 1, 7, and 14 days, cells were stained with a live/dead kit (Invitrogen) and observed *via* epifluorescence microscopy (Eclipse TE-2000S, Nikon). Two images were taken at random locations for each sample, with four samples yielding 8 images at each time point for each group. Live and dead cells were counted *via* Image-Pro Plus software. The percentages of live cells (P_{Live}) were calculated. $P_{\text{Live}} = \text{number of live cells} / (\text{number of live cells} + \text{number of dead cells})$.

The proliferation rate of hPDLSCs was investigated *via* Cell Counting Kit-8 (CCK-8, Dojindo, Tokyo, Japan), following the manufacturer's protocol. At 1, 3, 7, and 14 days, the culture medium was carefully removed and placed with 500 μ l DMEM containing 50 μ l CCK-8 dye. After 2 h incubation at 37°C, a 200 μ l aliquot from each well was placed in a 96-well plate and the absorbance at an optical density of 450 nm (OD450 nm) was measured with a microplate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA, United States).

To evaluate the number of hPDLSCs released from the degrading alginate microbeads, the samples were imaged *via* microscopy (Eclipse TE-2000S, Nikon). Two images were taken at random locations for each sample, with four samples yielding 8 images at each time point for each group. The number of the hPDLSCs with spindle-shape morphology was counted *via* Image-Pro Plus software. The density of the released hPDLSCs was calculated: $D_{\text{Release}} = N_{\text{Release}}/A$, where A is the area of the view field for D_{Release} .

Alkaline phosphatase activity assay

The hPDLSC-microbeads and ICPC scaffold were cultured in 24 well-plate with an osteogenic differentiation medium containing 10% FBS or 2.5% hPL. The basic osteogenic medium was DMEM growth medium with 100 nM dexamethasone (Millipore Sigma), 10 mM β -glycerophosphate (Millipore Sigma), 0.05 mM ascorbic acid (Millipore Sigma), 10 nM 1 α , 25-dihydroxy vitamin D3 (Millipore Sigma) and 1% P.S. Heparin was added to the hPL-based osteogenic medium at a final concentration of 2 U/mL. Negative control cultures were maintained in a medium containing 10% FBS. The medium was changed three times weekly. Four groups were tested to evaluate the osteogenic differentiation of hPDLSCs:

- (1) DAMB + fibrin + FBS + control group (hPDLSCs cultured with DMEM +10% FBS +1% P.S.);
- (2) ICPC + DAMB + fibrin + FBS + osteo group (1×10^5 hPDLSCs encapsulated in DAMB + fibrin with 0.05 ml ICPC scaffold and cultured in DMEM +10% FBS +1% P.S + 100 nM dexamethasone +10 mM β -glycerophosphate +

0.05 mM ascorbic acid +10 nM 1 α , 25-dihydroxy vitamin D3);

- (3) ICPC + DAMB + hPL + osteo group (1×10^5 hPDLSCs encapsulated in DAMB + hPL with 0.05 ml ICPC scaffold and cultured in DMEM +2.5% hPL +1% P.S + 2 U/mL heparin +100 nM dexamethasone +10 mM β -glycerophosphate + 0.05 mM ascorbic acid +10 nM 1 α , 25-dihydroxy vitamin D3);
- (4) ICPC + DAMB + fibrin + hPL + osteo group (1×10^5 hPDLSCs encapsulated in DAMB + fibrin + hPL with 0.05 ml ICPC scaffold and cultured in DMEM +2.5% hPL +1% P.S + 2U/mL heparin +100 nM dexamethasone +10 mM β -glycerophosphate + 0.05 mM ascorbic acid +10 nM 1 α , 25-dihydroxy vitamin D3).

At 1, 7, and 14 days, cells were lysed in a 0.2% Triton X-100 (Millipore Sigma) solution. The ALP activity of the cell lysate was measured by using an ALP Assay kit (QuantiChrom, BioAssay Systems, Hayward, CA, United States) with p-Nitrophenylphosphate (pNPP) as a substrate. The ALP activity was determined by measuring the absorbance at an optical density of 405 nm using a microplate reader (SpectraMax M5). The protein of cell lysate was quantified using Protein Assay Kit (Pierce BCA, Thermo Scientific, Rockford, IL, United States) following the manufacturer's protocol. The ALP activity was normalized to the protein amount and reported as mU/mg protein (Zhao et al., 2019).

Quantitative real-time PCR

The ICPC scaffold and hPDLSC-microbeads were cultured in 12 well-plates. Three groups were tested for the osteogenic gene expressions and Alizarin Red S (ARS, Millipore Sigma) staining:

1. ICPC + DAMB + fibrin + FBS + control group (2×10^5 hPDLSCs encapsulated in DAMB + fibrin and cultured with 0.1 ml ICPC scaffold in FBS-based growth medium)
2. ICPC + DAMB + fibrin + FBS + osteo group (2×10^5 hPDLSCs encapsulated in DAMB + fibrin and cultured with 0.1 ml ICPC scaffold in FBS-based osteogenic medium);
3. ICPC + DAMB + fibrin + hPL + osteo group (2×10^5 hPDLSCs encapsulated in DAMB + fibrin + hPL microbeads and cultured with 0.1 ml ICPC scaffold in hPL-based osteogenic medium).

The total cellular RNA of the cells was extracted using TRIzol reagent (Invitrogen) and reverse-transcribed into cDNA using a High-capacity cDNA Reverse Transcription kit (Applied Biosystems) in a thermal cycler (GenAmp PCR

2720, Applied Biosystems). RT² SYBR[®] Green ROX qPCR Mastermix (Qiagen, Germantown, MD, United States) was used to quantify the transcript levels of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), alkaline phosphatase (*ALP*), runt-related transcription factor-2 (*RUNX2*), Collagen type 1 (*COL1*), and Osteogenicpontin (*OPN*). The human-specific primers were synthesized commercially (Millipore Sigma), and the sequences of the primers are listed in Table 1. The qPCR data collection and analyses were performed using a QuantStudio™ 3 Real-Time PCR System (Thermo Scientific). Relative expressions were calculated using the $2^{-\Delta\Delta CT}$ method and normalized by the C_t value of the housekeeping gene *GAPDH*. The C_t value of hPDLSCs in the control group at 1 day served as the calibrator.

Bone mineral synthesis by hPDLSCs

At 1, 14, and 21 days, the hPDLSCs were fixed with 4% paraformaldehyde for 15 min and stained with ARS. After staining for 30 min, the ARS solution was removed and rinsed with distilled water to remove any loose ARS. The samples were imaged *via* microscopy (Eclipse TE-2000S, Nikon). For quantification, the stained mineralization was de-stained in 10% cetylpyridinium chloride (Millipore Sigma) for 30 min and the concentration was measured at an optical density of 652 nm using the microplate reader (SpectraMax M5). The ARS concentration of the control group at 1 day served as the calibrator.

Statistical analysis

One-way and two-way analyses of variance (ANOVA) were performed to detect the significant differences, followed by Tukey's test as a post hoc comparison. Statistical analyses were performed by SPSS 19.0 software (SPSS, Chicago, IL, United States) at an alpha of 0.05.

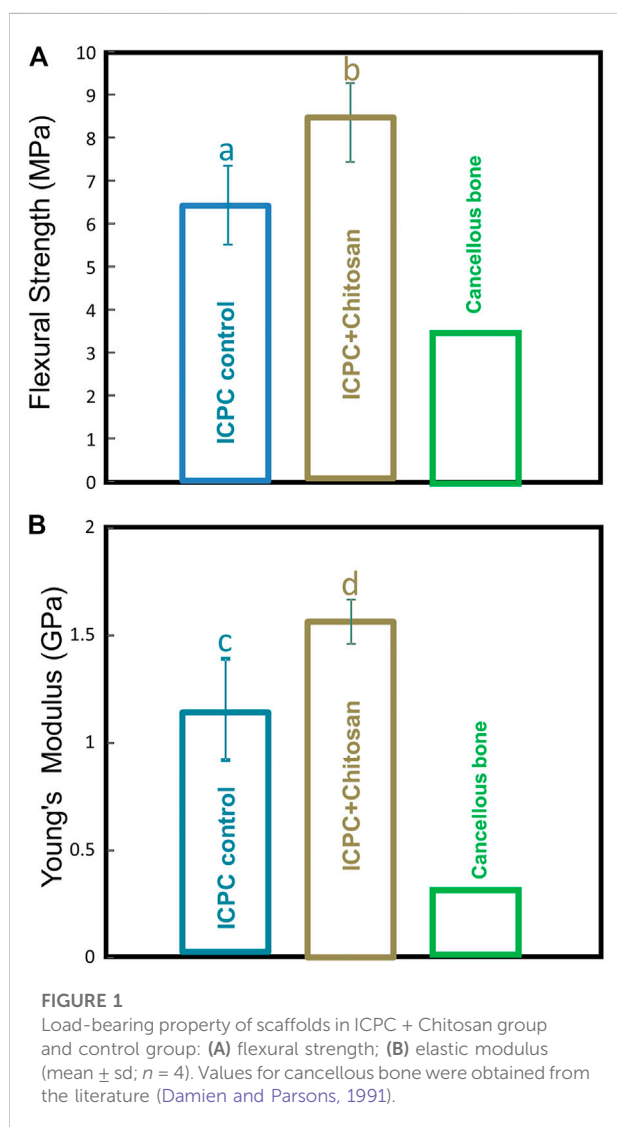
Results

Load-bearing properties

The flexural strength and elastic modulus of the ICPC scaffolds are plotted in Figure 1. Flexural strength and elastic modulus of ICPC scaffolds was 8.4 ± 0.91 MPa and 1.56 ± 0.1 GPa, significantly higher than 6.4 ± 0.43 MPa and 1.12 ± 0.23 GPa of the control group, respectively ($p < 0.05$). This demonstrates that the incorporation of chitosan improved the mechanical properties of the ICPC scaffold. Both of these values are higher than those of cancellous bone reported in the literature (Damien and Parsons, 1991).

TABLE 1 List of primer sequences used in Real-time PCR.

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
GAPDH	GCACCGTCAAGGCTGAGAAC	ATGGTGGTGAAGACGCCAGT
ALP	TCAGAAGCTAACACCAACG	TTGTACGCTTGGAGAGGGC
RUNX2	TCTGGCCTTCCACTCTCAGT	GACTGGCGGGGTGTAAGTAA
COL1	CTGACCTTCCTGCGCCTGATGTCC	GTCTGGGGCACCAACGTCCAAGGG
OPN	TCACCTGTGCCATACCAGTTAA	TGAGATGGGTCAGGGTTTACG



Encapsulated hPDLSC proliferation and viability in microbeads

The diameter of 100 random-selected alginate microbeads was measured by magnification for the three groups. These

values (mean \pm sd; $n = 100$) which were $312 \pm 58 \mu\text{m}$, $367 \pm 56 \mu\text{m}$ and $297 \pm 646 \mu\text{m}$ for DAMB + fibrin, DAMB + hPL, and DAMB + fibrin + hPL respectively.

Representative live/dead staining images from 1 to 14 days are shown in Figures 2(A–F). At 1 day, there were numerous live cells (stained green) and a few dead cells (stained red) in all groups (Figures 2A–E). At 7 days, the hPDLSCs were released from the degradable microbeads in the ICPC + DAMB + fibrin + hPL, ICPC + DAMB + hPL, and ICPC + DAMB + fibrin + FBS. The cells exhibited a polygonal morphology and a relatively high viability (Figures 2F–H). At 14 days, the degradation of microbeads accelerated and most of the cells were released (Figures 2J–L). The PLive increased with time due to proliferation in the ICPC + DAMB + fibrin + hPL, ICPC + DAMB + hPL, and ICPC + DAMB + fibrin + FBS. At 1 day, the PLive of hPDLSCs in ICPC + DAMB + fibrin + hPL and ICPC + DAMB + hPL was slightly higher than that in the ICPC + DAMB + fibrin + FBS group, indicating that incorporation of hPL into the microbeads could better protect the hPDLSCs from the pH change and ion activities during the ICPC setting (Figure 2M). In contrast, very few cells were observed to attach and spread in the ICPC + DAMB + fibrin + DMEM, the PLive of the hPDLSCs was lower, due to the lack of nutrition supplements.

The cell growth rate was evaluated *via* the CCK-8 assay (Figure 2N). At 1 day and 3 days, the proliferation of hPDLSCs was low in the groups of ICPC + DAMB + fibrin, ICPC + DAMB + hPL, and ICPC + DAMB + fibrin + hPL. At 7 days, as more cells were released from the degradable microbeads, the cell growth was significantly enhanced. The proliferation rate in the ICPC + DAMB + fibrin and ICPC + DAMB + fibrin + hPL groups was higher than that of the ICPC + DAMB + hPL group ($p < 0.05$), indicating that the incorporation of fibrin accelerated the hPDLSCs migrated out from the microbeads. The absorbance of the hPDLSCs in the ICPC + DAMB + fibrin + DMEM group was lower over time, indicating that the DMEM did not support the proliferation of the hPDLSCs encapsulated in the microbeads.

The D_{Release} was evaluated for ICPC + DAMB + fibrin, ICPC + DAMB + hPL, and ICPC + DAMB + fibrin + hPL. At 7 days, hPDLSCs were released from the degradable microbeads (Figures

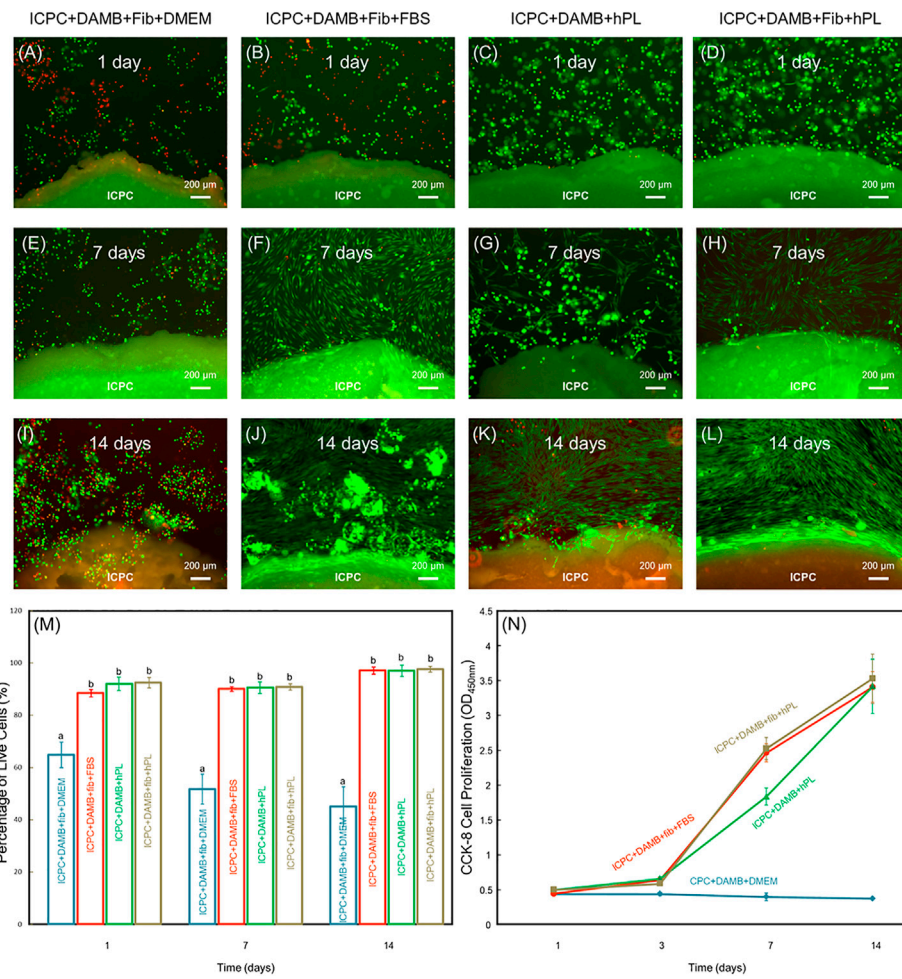


FIGURE 2 Live/dead results of human periodontal ligament stem cells (hPDLSCs) in ICPC + DAMB + fibrin + DMEM, ICPC + DAMB + fibrin + FBS, ICPC + DAMB + hPL, and ICPC + DAMB + fibrin + hPL constructs (A–L). Percentage of live cells at 1, 7, and 14 days (M). Each value is (mean ± sd; n = 5). CCK-8 cell viability (absorbance at 450 nm) increased with culture time (N). Each value is (mean ± sd; n = 6). Bars with dissimilar letters in the plot indicate values that are significantly different from each other (p < 0.05).

3A–F). The $D_{Release}$ of ICPC + DAMB + fibrin + hPL was significantly higher than that of ICPC + DAMB + hPL (Figure 3G). This demonstrated that adding fibrin increased the degradation rate of the microbeads. As the microbeads degraded, cells were released, and the $D_{Release}$ of each group increased. There was no difference in $D_{Release}$ between each group at 14 days (Figure 3G).

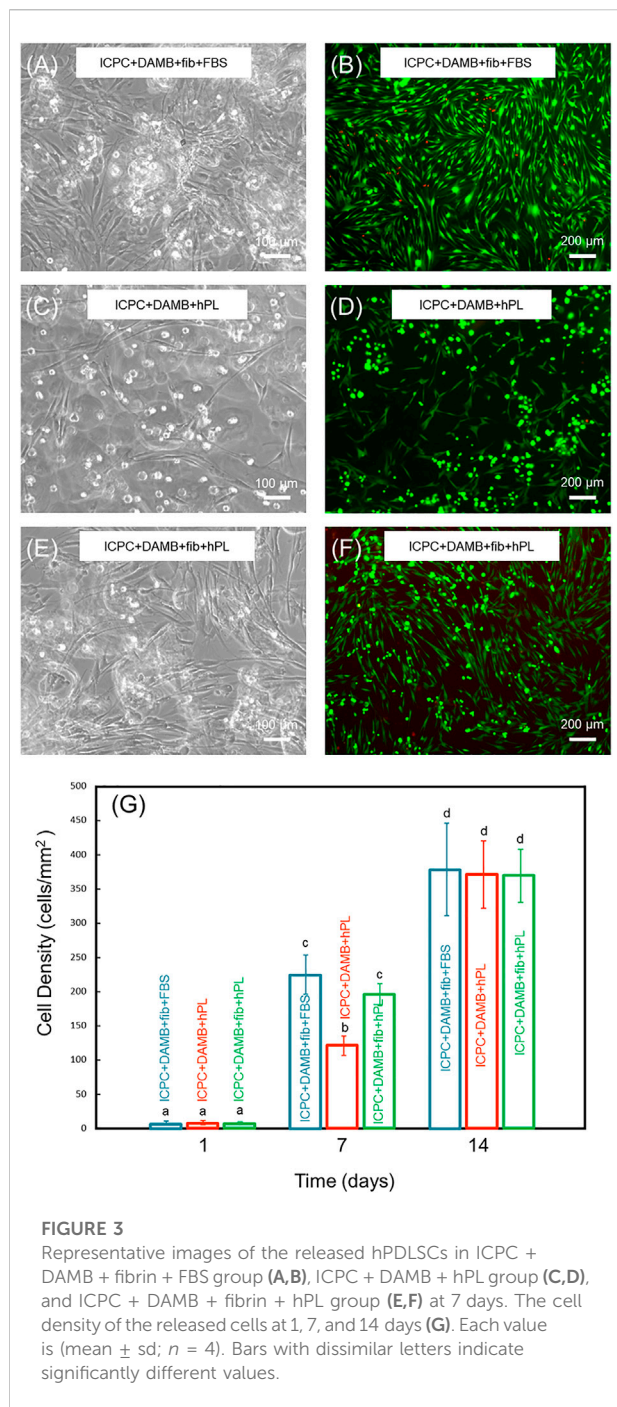
Alkaline phosphatase activity of hPDLSCs

The ALP activity of hPDLSCs encapsulated in various types of alginate-based microbeads increased with time in osteogenic medium supplemented with 10% FBS or 2.5% hPL, indicating successful differentiation towards osteoblasts (Figure 4). At

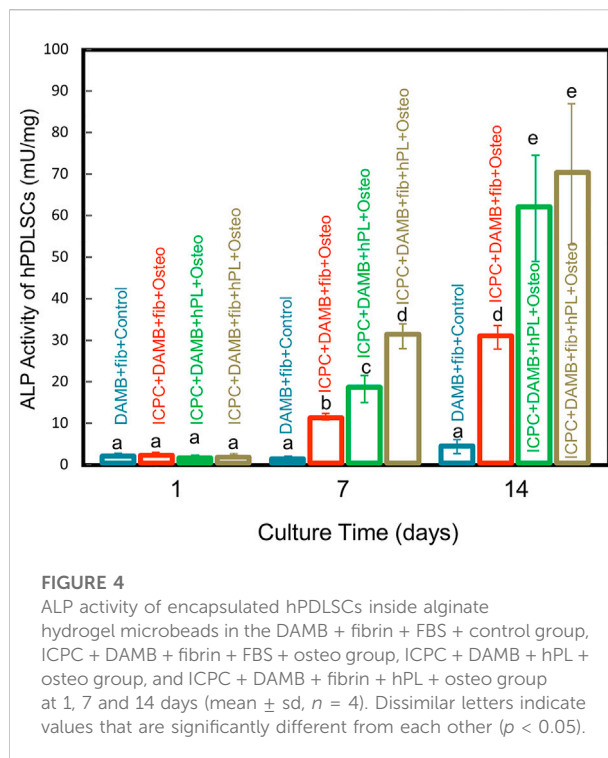
14 days, the ALP activity was 69.97 ± 16.96 mU/mg for the ICPC + DAMB + fibrin + hPL group, significantly higher than 30.68 ± 2.86 mU/mg of the ICPC + DAMB + fibrin group (p < 0.05) and 4.12 ± 1.65 mU/mg of the control group (p < 0.01). These results indicate that the hPL-based osteogenic medium significantly enhanced the osteogenic differentiation of the hPDLSCs in ICPC scaffold.

Osteogenic gene expression

Up-regulations of ALP, RUNX2, COL1, and OPN were observed in hPDLSCs in the ICPC + DAMB + fibrin + FBS + osteo group and ICPC + DAMB + fibrin + hPL + osteo group. At 7 days, the values of ALP, RUNX2, COL1, and OPN in the ICPC +



DAMB + fibrin + hPL + osteo group and ICPC + DAMB + fibrin + FBS + osteo group were 6–11 folds and 4–7 folds those of the control group, respectively (Figure 5). Furthermore, significantly higher expressions of ALP and RUNX2 were observed in ICPC + DAMB + fibrin + hPL + osteo than in ICPC + DAMB + fibrin + FBS + osteo. This indicates that the hPL promoted higher osteogenic differentiation of hPDLSCs than the commonly used 10% FBS.



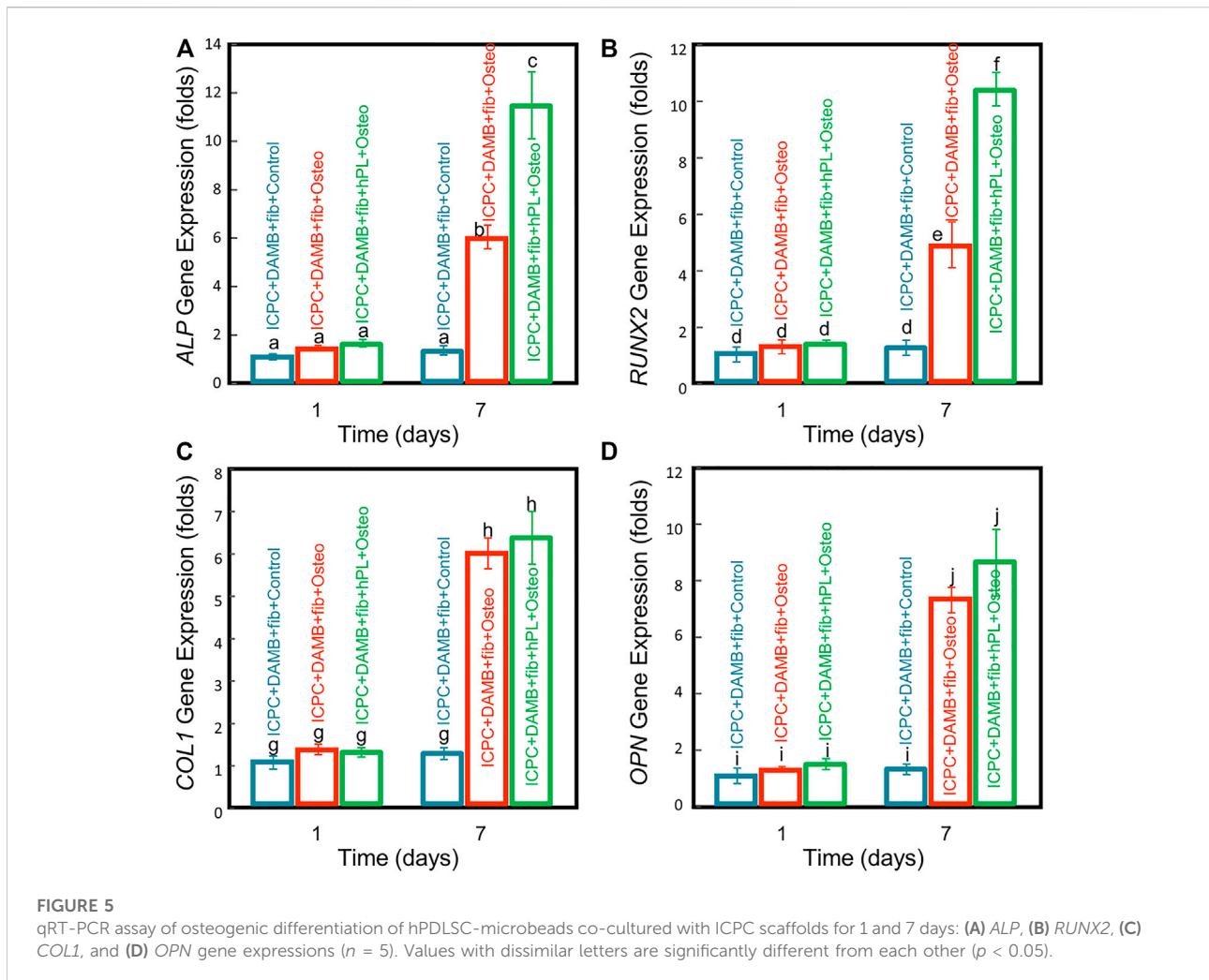
Alizarin Red staining of minerals synthesized by hPDLSCs

At 21 days of osteogenic differentiation, the hPDLSCs from the microbeads exhibited a high viability and proliferation. Figures 6A,D,G show that the hPDLSCs released from the microbeads significantly proliferated and adhered to ICPC scaffold. This indicates that the ICPC scaffold was cytocompatible.

Representative ARS staining images of bone mineral secretion by hPDLSCs in the 12-well plate with ICPC are shown in Figures 6B,E,H. The red staining of mineralized nodules formed by the hPDLSCs was denser in the ICPC + DAMB + fibrin + hPL + osteo group than that of the ICPC + DAMB + fibrin + FBS + osteo group Figures 6C,F,I. The synthesized bone mineral in ICPC + DAMB + fibrin + FBS + osteo and ICPC + DAMB + fibrin + hPL + osteo increased with culture time from 1 to 21 days. The synthesized bone mineral amount in the ICPC + DAMB + fibrin + hPL + osteo was 6.9-fold and 13.2-fold that of the control group, at 14 days and 21 days, respectively (Figure 7). These results demonstrate that the hPL-based osteogenic medium significantly enhanced the osteogenic differentiation of the hPDLSCs.

Discussion

This study represents the first report to investigate degradable alginate hydrogel incorporated with hPL inside an



injectable calcium phosphate scaffold as a cell delivery system for bone regeneration. The hPDLSCs showed excellent viability while being encapsulated in the DAMB + fibrin + hPL microbeads in ICPC scaffold. When the setting of the ICPC was complete, the hPDLSCs were gradually released from the degradable hydrogel microbeads and grew rapidly in the scaffold. Furthermore, the osteogenic differentiation of hPDLSCs was significantly enhanced by the osteogenic medium supplemented with hPL. This study is schematically shown in Figure 8. These findings demonstrate a promising and novel xeno-free approach to delivering hPDLSCs in ICPC scaffold for bone regeneration.

The ICPC paste can be filled into a bone defect with an intimate adaptation to complex defect cavities and set *in situ* to form bioresorbable hydroxyapatite (Weir et al., 2006; Xu et al., 2006). However, ionic activities and pH variations during the setting of ICPC paste could exert a cytotoxic effect on the cells (Matsuya et al., 2000; Simon et al., 2004; Przekora, 2019). TTCP and DCPA dissolved in the chitosan solution as Ca^{2+} , PO_4^{3-} , and

OH^- ions (Weir et al., 2006), which then re-precipitated to form hydroxyapatite: $2\text{Ca}_4(\text{PO}_4)_2\text{O} + 2\text{CaHPO}_4 \rightarrow \text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ (Weir et al., 2006). It was reported that the pH during the setting of ICPC could be increased to approximately 10 (Simon et al., 2004). Therefore, there was a need to protect the cells from the ICPC setting reaction.

Alginate is among the most common natural polymers for the encapsulation and delivery of cells because of its many outstanding properties such as biocompatibility, gel-forming ability, non-toxicity, and ease of process (Gasperini et al., 2014; Izeia et al., 2020; Xu et al., 2021). However, the absence of alginate degrading enzyme (alginase) in the human body limits the degradability of the alginate. As the setting reaction of ICPC paste is largely complete after 1 day, it would be desirable for the alginate microbeads to quickly degrade, thus releasing the cells from the microbeads for enhanced cell viability and proliferation. A previous study reported that alginate could be chemically modified using oxidizing agents such as sodium periodate to produce oxidized alginate that was hydrolytically degradable

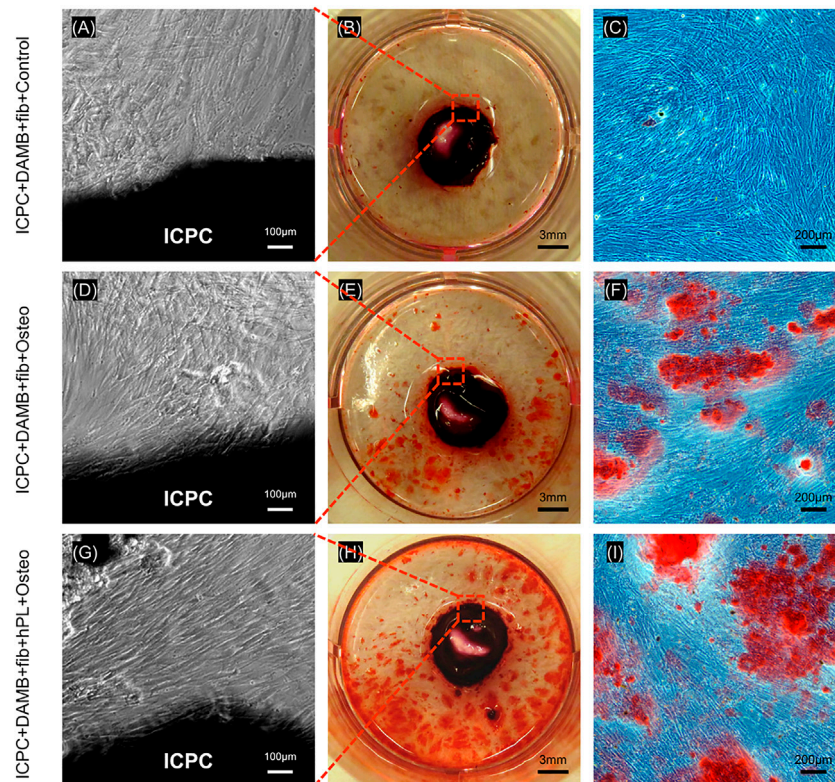


FIGURE 6

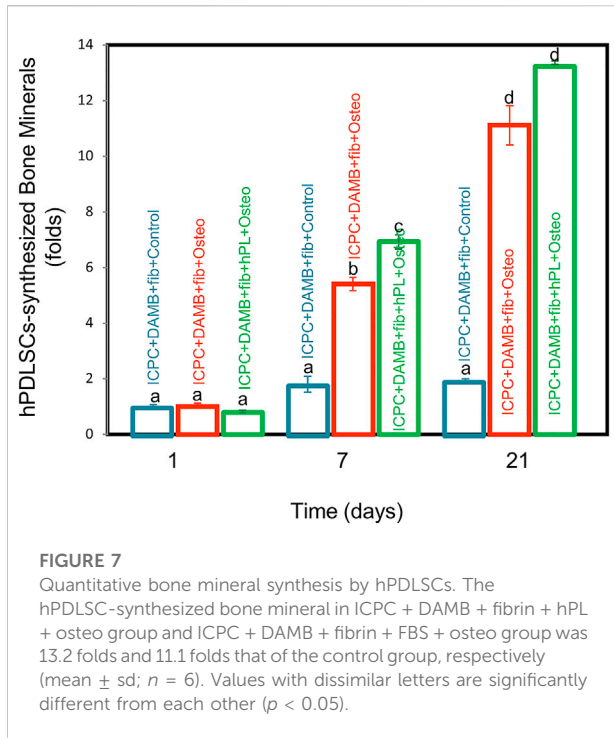
hPDLSCs showed high proliferation in ICPC for 21 days (A,D,G). Representative ARS staining images of mineral synthesis by hPDLSCs with ICPC (B,E,H). Representative images of mineralized nodules formed by the hPDLSCs. More mineralized nodules were present in the ICPC + DAMB + fibrin + hPL + osteo group than in ICPC + DAMB + fibrin + FBS + osteo group and control group (C,F,I).

(Zhou and Xu, 2011). In addition, it has been reported that adding fibrin could improve the attachment and proliferation of the cells in the alginate hydrogel, as well as to accelerate the release of cells from the alginate-based microbeads (Liu et al., 2013b). Fibrinogen was converted to fibrin *via* the mediation of thrombin and self-assembles into fibrin mesh, providing cell binding sites for cell attachment, migration, and proliferation (Li et al., 2015). As the cells migrated out of the microbeads, the porosity of the hydrogel increased, thus accelerating the degradation of the hydrogel. A previous study showed that the cell-encapsulating-alginate-fibrin-microbeads had diameters of about 100–500 µm, and the microbeads started to degrade at day 4 and released the encapsulated hiPSCs (Wang et al., 2016). Another study encapsulated hUCMSCs into the oxidized alginate-fibrin microbeads of about 300 µm in sizes (Chen et al., 2012). The microbeads started to release the cells at 4 days and completely degraded at 21 days (Chen et al., 2012). Therefore, microbeads with sizes of several hundred microns were suitable for injection, and were quickly degraded to release the cells.

FBS is the most widely-used supplement for the cultivation and expansion of eukaryotic cells. FBS contains essential

components for cell growth and maintenance such as hormones, vitamins, transport proteins, trace elements, spreading, and growth factors (Brunner et al., 2010), which is effective on most types of human and animal cells. However, FBS-expanded MSCs could evoke immune responses against xenogenic serum antigens in the human body (Spees et al., 2004; Tonti and Mannello, 2008). In addition, FBS is a potential source of microbial contaminants such as fungi, bacteria, viruses, or prions. Indeed, a 20%–50% contamination rate of the virus was reported for FBS (Even et al., 2006). Furthermore, the ingredients of FBS are not precisely defined, and lot-to-lot variability and unintended interactions with test substances can lead to unexpected or undesired outcomes in the clinical application (Jochems et al., 2002). Therefore, it is critically important and highly desirable to develop a FBS-free medium as a cell nutrition supplement for cell-based therapies.

The use of hPL for MSC expansion was first reported by Doucet et al., in 2005 (Doucet et al., 2005). Since then, hPL has been proven as a viable alternative to FBS, enabling efficient propagation of MSC under animal serum-free conditions for clinical application. There are several advantages for hPL for cell growth supplement: 1) hPL can be easily obtained and produced

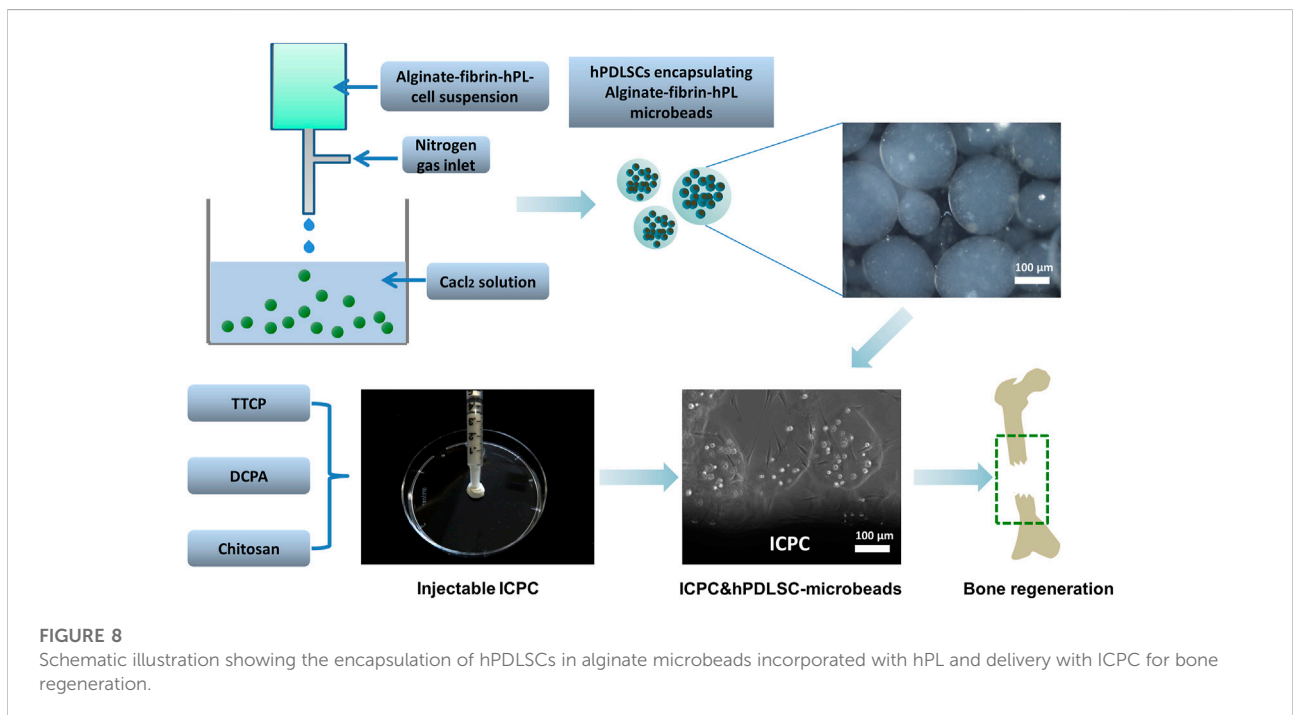


using freeze-thaw procedures (Dessels et al., 2016); 2) Because hPL is derived from humans, neither bovine viruses nor immune reactions against bovine proteins are a concern (Saury et al., 2018); 3) hPL can be used in autologous settings to minimize the

risk of immunological reactions (Sánchez et al., 2003); 4) hPL is more efficient in terms of costs and proliferation rate than FBS for certain types of MSCs (Burnouf et al., 2016).

In the present study, 2.5% hPL promoted the proliferation of hPDLSCs when compared with the 10% FBS. In a previous study, the use of 10% FBS showed a slightly higher proliferation of hPDLSCs as compared to 5% hPL. However, the difference in colony number was not statistically significant (Abuarqoub et al., 2019). In another study, hPDLSCs were cultured using a medium supplemented with 10% FBS, 5% PL + 5% FBS, or 10% PL. It was revealed that the media containing 5% PL + 5% FBS resulted in more significant stimulation of cell growth, when compared with those containing either 10% FBS or 10% PL. Furthermore, a tendency toward enhanced proliferation was exhibited in media containing 10% PL as compared with media containing 10% FBS (Wu et al., 2017). The differences of hPL concentration or hPDLSCs could be associated with the difference in the preparation method, the variability in the donors, and the storage conditions. Hence, the quality of hPL used by different laboratories reported in the literature may be somewhat different from each other (Christgau et al., 2006; Ogino et al., 2006; Schallmoser et al., 2007).

The implantation of ICPC scaffold with MSCs has achieved ectopic and orthotopic bone formation and critical-sized defect healing (Wang P. et al., 2014; Xu et al., 2017). The seeded MSCs could directly deposit bone matrix minerals in the scaffold due to their osteogenic differentiation potential. The therapeutic benefit of the transplanted MSCs was associated with a paracrine mechanism that stimulated the recruitment of host cells. These host cells



included osteoblast progenitors, endothelial cells, and osteoclasts, which took over the responsibility of subsequent bone formation and remodeling (Wang et al., 2011; Wang J. et al., 2014). Interestingly, it has been revealed that MSCs could enhance bone repair by modulating the foreign body reaction to ICPC, attracting circulating monocytes, and inducing their differentiation into osteoclasts, thus favoring bone formation (Gamblin et al., 2014).

In the present study, the hPDLSCs in ICPC were gradually released from the degradable hydrogel microbeads and underwent differentiation into osteogenic lineage by the hPL-based medium, without exposure to the animal serum. Moreover, higher ALP activity, osteogenic expression, and bone mineralization were achieved in ICPC + DAMB + fibrin + hPL construct than in ICPC + DAMB + fibrin + FBS construct. Therefore, the novel hPDLSC-hPL-microbeads-ICPC construct is a highly promising xeno-free approach for bone regeneration. Future *in vivo* studies are needed to evaluate the bone regenerative capacity of the hPDLSC-hPL-microbeads-ICPC construct for the treatment of bone defects in animal models.

Conclusion

This study demonstrated for the first time the hPDLSC-encapsulation in degradable alginate hydrogel microbeads with hPL inside an injectable calcium phosphate scaffold for bone regeneration. The ICPC scaffold was biocompatible, mechanically load-bearing, while supporting hPDLSC attachment, proliferation, and osteogenic differentiation with the hPL as xeno-free cell supplement. The microbeads incorporating with hPL protected the hPDLSCs from the setting reaction of ICPC. The encapsulated hPDLSCs in ICPC with hPL-based osteogenic medium underwent successful differentiation into the osteoblast lineage, with highly elevated *ALP*, *RUNX2*, *COL1*, and *OPN* as well as bone mineral synthesis. Therefore, the novel hPDLSC-hPL-microbeads-ICPC construct is highly promising for bone regeneration without the risk of infection from unknown pathogens by using animal-origin serums.

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Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

Author contributions

Study conception and design: GQ, MH, JL, and HX
Acquisition of data: GQ, TM, and DK
Analysis and interpretation of data: GQ, TM, MW, AS, and TO
Critical revision: GQ, PW, LZ, HX, and YX.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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