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Original Research

Exosomes Released by Periodontal Ligament Stem Cells in Low-Inflammatory Microenvironment Promote Bone Tissue Regeneration

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Abstract

Background: This study investigates the effects of periodontal ligament stem cells preconditioned with low-inflammatory stimuli and their derived exosomes on jaw bone regeneration. **Methods:** Periodontal ligament stem cells (PDLSCs) were treated with varying concentrations of inflammatory induction medium to establish low-inflammatory PDLSCs, from which exosomes were subsequently extracted. The osteogenic effects of the exosomes derived from i-PDLSCs (i-PDLSCs-exo) on osteoblasts were evaluated *in vitro*. **Results:** The findings demonstrated that PDLSCs subjected to low-inflammatory conditions (i-PDLSCs) exhibited a significantly increased expression of osteogenesis-related genes, such as alkaline phosphatase (ALP) and Osteocalcin (OCN), and showed enhanced mineralized nodule formation. Additionally, there was an increase in the secretion of i-PDLSCs-exo, and at an optimal concentration of 20 μg/mL, they were taken up by hFOB1.19 cells, enhancing mineralized nodule formation and osteogenic gene expression. *In vivo*, a chondroitin sulfate hydrogel loaded with i-PDLSCs-exo was implanted into periodontal bone defects in Sprague–Dawley rats. This treatment group demonstrated superior bone regeneration compared to both the chondroitin sulfate hydrogel alone and the blank control groups. **Conclusion:** Collectively, these findings suggest that short-term pretreatment with low concentrations of inflammatory factors enhances the osteogenic potential of PDLSCs and their exosomes, promoting the regeneration of jaw bone defects in rats. This approach offers a promising therapeutic strategy for repairing bone tissue defects.

Keywords: periodontal ligament stem cells; exosomes; inflammation; osteoblasts; bone regeneration

1. Introduction

Periodontitis is a chronic and progressive bacterial infection that targets the periodontal tissues. The sustained local inflammation associated with this condition disrupts the balance of bone homeostasis by promoting osteoclast (OC)–mediated bone resorption while simultaneously inhibiting osteoblast (OB)–mediated bone formation. This disruption results in the loss of periodontal attachment, the degradation of alveolar bone, and ultimately culminates in tooth loss [1,2]. The Global Burden of Disease Study (2016) identifies severe periodontitis as the eleventh most prevalent disease worldwide [3]. Achieving complete and predictable regeneration of periodontal bone defects remains a considerable clinical challenge [4,5].

Recent advances in stem cell biology and regenerative medicine have highlighted the potential of periodontal ligament stem cells (PDLSCs) for treating periodontal diseases [6,7,8,9,10]. The direct application of PDLSCs faces several practical and ethical concerns, including difficulties in cell harvesting, extended *in vitro* expansion, immune rejection, and ethical considerations. Consequently, there is a growing interest in stem cell–derived acellular therapies as alternative strategies. Increasing evidence suggests that exosomes derived from PDLSCs are essential for intercellular communication, immune regulation, and tissue homeostasis through the transfer of bioactive molecules. These characteristics enable PDLSC-derived exosomes to provide therapeutic benefits in various pathophysiological conditions, underscoring their potential as innovative regenerative agents [9,10,11]. For example, dental pulp stem cell-derived exosomes carry the BGN protein, which supports bone formation via the BMP pathway

[10]. Exosomes from DPSCs also directly reduce inflammation and reshape the immune microenvironment, thereby aiding PDLSCs in their osteogenic differentiation [12]. It is now widely recognized that the regenerative capacity of exosomes largely depends on the miRNAs they transport. For instance, miR-6879-5p from M2 macrophage exosomes, miR-21 from regulatory T-cell exosomes enhance the osteogenic potential of PDLSCs [13,14,15]. Additionally, studies have shown that miR-423-5p encapsulated in osteoblast-derived mineralizing exosomes stimulates vascular formation by modulating CXCL10, thus offering a novel strategy for managing bone defects [16]. Overall, as key paracrine effectors of stem cells, exosomes transfer proteins, miRNAs, and other bioactive molecules to modulate recipient cell functions and facilitate periodontal regeneration.

Periodontal tissue is highly sensitive to inflammatory stimuli, and its clinical phenotype changes significantly with the inflammatory environment, which makes tissue homeostasis difficult to maintain [17,18]. Traditionally, inflammatory microenvironments have been considered detrimental to stem cell–mediated tissue regeneration. However, emerging evidence suggests that acute or low-grade inflammation may actually enhance the immunomodulatory and reparative capacities of stem cells and their secreted products. This enhancement can significantly contribute to the facilitation of early tissue healing processes. Notably, the pretreatment of human muscle stem cells (hMuSCs) with tumor necrosis factor-α (TNF-α) + interferon-γ (IFN-γ) (10 ng/mL) for 48 h markedly alleviated symptoms of inflammatory bowel disease (IBD) in rat models [19]. Moderate inflammatory stimulation can activate the intrinsic defense and repair mechanisms in PDLSCs, resulting in the secretion of functionally enhanced exosomes.

Previous studies have shown that TNF-α stimulation significantly increases exosome secretion from gingival mesenchymal stem cells. This process drives macrophages toward an anti-inflammatory, reparative phenotype, thereby shaping an immune microenvironment conducive to bone regeneration. Additionally, exosomes derived from lipopolysaccharide-preconditioned PDLSCs have been found to facilitate reparative macrophage polarization and osteogenic differentiation through the activation of the NF-κB signaling pathway. During the osteogenic induction of PDLSCs, the exosomes secreted can also regulate osteoclastogenesis via circRNA–miRNA regulatory axes, underscoring the multifaceted roles of exosomes in maintaining bone metabolic balance [20,21,22].

In summary, we propose an innovative approach involving the short-term, low-concentration pretreatment of periodontal ligament stem cells (PDLSCs) with TNF-α and IFN-γ. Our objective is to examine how varying concentrations of these inflammatory factors influence the proliferation and osteogenic differentiation of PDLSCs *in vitro*. Additionally, we aim to explore the effect of i-PDLSC-exosomes on the osteogenic capacity of hFOB1.19 cells.

2. Materials and Methods

2.1 Animals

Experiments used Sprague–Dawley (SD) rats (10 weeks old, 250–270 g) supplied by Nanjing Huimiaoxin Biotechnology Co., Ltd. All experimental procedures were reviewed and approved by the Ethics Committee of Liaocheng People's Hospital (Approval Nos. 2025310).

2.2 Reagents

All reagents utilized in this study are commercially available and were purchased from the specified companies. The reagents include Dulbecco's modified Eagles medium (DMEM; Gibco, Grand Island, NY, USA), α-minimum essential medium (α-MEM; Procell, Wuhan, Hubei, China), fetal bovine serum (FBS; E600001-0500, BBI, China), exosome-depleted FBS (iCell-0650, iCell, Shanghai, China), human mesenchymal stem cell adipogenic and chondrogenic differentiation media (HUXXC-90031, Cyagen, Santa Clara, CA, USA), trypsin (Thermo Fisher Scientific, Shanghai, China), Alizarin Red (C0138), Oil Red O, BCIP/NBT alkaline phosphatase staining kit (C3206), Alcian blue, paraformaldehyde (Beyotime, Shanghai, China), neutral protease, type I collagenase, pKH67, lipopolysaccharide (LPS; Solarbio, Beijing, China), dexamethasone (D4902), β-glycerophosphate, ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA), Hematoxylin and Eosin staining solution, Masson's trichrome staining kit, sodium pentobarbital (Servicebio, Wuhan, Hubei, China), as well as antibodies raised against CD90, CD45, CD29, and CD73 from BioLegend, San Diego, CA, USA; and chondroitin sulfate (TCI, Shanghai, China) were also used.

2.3 Cell Culture and Identification

Premolars extracted for orthodontic purposes were sourced from donors aged 12 to 18 years at Liaocheng People's Hospital. Informed consent was obtained from both the patients and their legal guardians prior to the collection. The extracted teeth were intact and free of caries. Post-extraction, the isolation of PDLSCs was carried out using two primary methods: the tissue explant method and the enzymatic digestion method (n = 6) and were mycoplasma-free. All procedures were approved by the Ethics Committee of Liaocheng People's Hospital (Approval No. 2025301). The hFOB1.19 cell line was derived from ATCC CRL-11372 and cultured in DMEM. The hFOB1.19 cell line was maintained at 37 °C in air, 95%; carbon dioxide, 5% and were mycoplasma-free. All cell lines were authenticated shortly before use by the STR technique, carried out by Cobioer Biosciences Co., Ltd. (Nanjing, China).

2.3.1 Tissue Explant Method and Enzymatic Digestion Method

Periodontal ligament tissues were obtained from the mid-root region, and subsequently digested with a solution containing 3 mg/mL type I collagenase and 4 mg/mL dispase. Alternatively, the tissues were seeded into T25 culture flasks that were filled with α-MEM, which was supplemented with 20% FBS and 1% penicillin–streptomycin.

2.3.2 Flow Cytometry

PDLSCs at passages 3–4 were resuspended and incubated with antibodies (PE-CD45, PE-CD29, PE-CD73, APC-CD90). The expression of surface markers was then analyzed using a flow cytometer (LSR Fortessa, Becton Dickinson, NJ, USA).

2.4 Cell Proliferation Assay

After a 24-hour culture period, Cell Counting Kit-8 (CCK-8) reagent was added to the PDLSCs. Assessment of absorbance at 450 nm was then performed at 24, 48, and 72 hours post-treatment.

2.5 Trilineage Differentiation of PDLSCs

PDLSCs were then cultured in osteogenic induction medium (α-MEM supplemented with 10% FBS, 20 nM dexamethasone, 10 mM β-glycerophosphate, and 50 μg/mL ascorbic acid. After 7 days, alkaline phosphatase (ALP) staining was performed. Following 21 days, Alizarin Red S (ARS) staining was performed.

For adipogenic differentiation, PDLSCs were first cultured until they reached 100% confluence. Following this, the cells were subjected to a 30-day adipogenic induction. After induction, they were fixed in paraformaldehyde and processed for Oil Red O staining.

For chondrogenic differentiation, PDLSCs were harvested through centrifugation into 15 mL conical tubes and subsequently resuspended in 0.5 mL of chondrogenic differentiation medium (Oricell). The cells were centrifuged, a process that was repeated twice to create distinct cell pellets. These pellets were cultured at 37 °C in a humidified atmosphere with 5% CO_2 for 28 days. After this incubation period, samples were fixed with formaldehyde, embedded in paraffin, sectioned, and stained using an Alcian blue staining kit (Beyotime, Shanghai, China) to assess chondrogenic differentiation.

2.6 Preparation and Collection of PDLSC-Conditioned Medium Containing Exosomes

PDLSCs were treated with an inflammatory induction medium composed of 1 ng/mL TNF-α and IFN-γ for a duration of 24 h. Following this induction period, the medium was substituted with α-MEM that had been supplemented with exosome-depleted FBS. The cells were then incubated for an additional 24 h before the supernatant was collected for subsequent analysis.

2.7 Isolation and Characterization of Exosomes

Supernatants from healthy PDLSCs (h-PDLSCs) and i-PDLSCs were collected as described in section 1.6. Exosomes were isolated using differential ultracentrifugation (Optima XE, Beckman, CA, USA). Then, flow cytometry, transmission electron microscopy (TEM), and nanoparticle tracking analysis (NTA) were performed.

2.8 Uptake of Exosomes by hFOB1.19 Cells

hFOB1.19 cells were seeded on coverslips in a 24-well plate and cultured for 24 h. Following this, pKH67-labeled exosomes were added to serum-free α-MEM and incubated with the cells at 37 °C for 6–8 h. The cells were fixed using 4% paraformaldehyde for 30 min and subsequently permeabilized with 0.2% Triton X-100 for 5 min. Following permeabilization, the cells were incubated with phalloidin for 45 minutes and DAPI for 1–2 minutes to stain the cytoskeleton and nuclei, respectively. After performing thorough PBS washes, exosome internalization in hFOB1.19 cells was evaluated using confocal laser scanning microscopy.

2.9 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Analysis

PDLSCs were treated with an inflammatory induction medium that included TNF-α and IFN-γ at concentrations of 1, 5, 10, or 15 ng/mL for a period of 24 h. Following this, i-PDLSCs were subjected to osteogenic induction for 5 days. Total RNA extracted from the i-PDLSCs was then analyzed using quantitative PCR (qPCR). Meanwhile, the expression levels of inflammation-related genes in the 1 ng/mL and 10 ng/mL groups were analyzed by qRT-PCR (ABI-7500, USA).

Primer sequences used in this study are listed in Table 1.

2.10 Establishment of the JawBone Defect Animal Model

A total of 48 adult male SD rats were utilized in this study. All rats were housed under specific pathogen-free conditions (22 °C, 12 h light/12 h dark cycle, 50%–55% humidity) with free access to food and water.

2.10.1 Preparation of Hydrogel Scaffolds and Exosome Loading

The hydrogel scaffold was prepared at the School of Chemical Engineering, Liaocheng University. Chondroitin sulfate-based hydrogel (CS-DMAA) was synthesized using an enzymatic initiation method involving a glucose-triggered enzyme complex [23]. Subsequently, exosomes derived from PDLSCs were incorporated into the hydrogel at a final concentration of 80 μg/mL, resulting in the formation of exosome-loaded CS-DMAA hydrogels.

Table 1. Related Gene Primer Sequences.

Gene	Upstream of the gene	Downstream of a gene
ALP	GCCTCTTGTAAGAAGTGTCTCG	TCTTTTCAGAGATGCCCTCGAT
COL1A1	ATCAACCGGAGGAATTTCCGT	CACCAGGACGACCAGGTTTTC
OCN	GGCGCTACCTGTATCAATGG	GTGGTCAGCCAACTCGTCA
OPN	CTCCATTGACTCGAACGACTC	CAGGTCTGCGAAACTTCTTAGAT
RUNX2	CCGCCTCAGTGATTTAGGGC	GGGTCTGTAATCTGACTCTGTCC
TNF-α	CGTGGAGCTGGCCGAGGAG	AGGAAGGAGAAGAGGCTGAGGAAC
IL-6	GGTGTTGCCTGCTGCCTTCC	GTTCTGAAGAGGTGAGTGGCTGTC
β-actin	CTTTGCGGATGTCCACGTCA	TGAGGCACTCTTCCAGCCTT

ALP, alkaline phosphatase; *COL1A1*, Collagen type I alpha 1 chain; *OCN*, Osteocalcin; *OPN*, Osteopontin; *RUNX2*, Runt-related transcription factor 2; *IL-6*, Interleukin 6.

2.10.2 Creation of Mandibular Jaw Bone Defects in SD Rats.

Forty-eight 10-week-old SD rats were acclimated for one week before undergoing surgery. Sodium pentobarbital (50 mg/kg, 2% w/v) was administered intraperitoneally to induce general anesthesia. The body weight of rats typically ranges from 250 grams to 270 grams. For routine procedures, an intraperitoneal injection of pentobarbital sodium at a dosage of 50 mg/kg corresponds to 13 mg. In contrast, when used for euthanasia, the intraperitoneal injection is administered at a higher dosage of 180 mg/kg, corresponding to 45–50 mg, and cervical dislocation was performed on the basis of deep anesthesia. A standardized jaw bone defect (4 × 4 × 1 mm^3; length × width × depth) was established in the mandibular angle region. Rats underwent randomization and were allocated to the following groups: (1) blank control group; (2) CS-DMAA group; (3) CS-DMAA + h-PDLSCs-exo group; (4) CS-DMAA + i-PDLSCs-exo group. We sacrificed the rats at 2, 4, and 8 weeks post-operation, with 4 rats in each group at each time point, using the random number table method for allocation. The left mandibles were collected for micro-computed tomography (micro-CT) scanning (VNC-102, Pingsheng Medical Technology) and subsequent histological analyses. The inclusion criteria for animal results were uneventful recovery from anesthesia, no abnormal behavior, and normal eating and drinking.

We calculated the effect size (Cohen's d) for each time point between treatment groups and the control group using the primary endpoint, BV/TV. The results showed that the vast majority of comparisons had effect sizes far greater than 0.8 (the smallest being 0.79), indicating large effects. According to Cohen's criteria, a sample size of 4 per group is sufficient to provide adequate statistical power (>80%) for large effect sizes. Furthermore, this sample size selection also adheres to the 3R principle. Blinding was not used.

2.11 Histological Analysis of Bone Tissue

The rat mandibles were fixed in paraformaldehyde, decalcified using EDTA, dehydrated, and then embedded. Sections with a thickness of 4 μm were cut and stained with hematoxylin and eosin (H&E) as well as Masson's stain for histological evaluation. To evaluate the presence of osteoblasts in the bone defect area, an OCN antibody (1:100) was employed for immunohistochemical staining.

2.12 Statistical Analysis

All data are presented as the mean ± standard deviation (SD). Statistical analyses were performed using Student's *t*-test for comparisons between two groups and one-way analysis of variance (ANOVA) for comparisons involving more than two groups. Each experiment was independently conducted a minimum of three times, with representative results displayed. A *p*-value of less than 0.05 was considered statistically significant.

3. Results

3.1 Characterization and Isolation of PDLSCs

Primary PDLSCs were isolated using both the tissue explant method and enzymatic digestion. The primary cells exhibited a spindle-shaped morphology with abundant cytoplasm (Fig. 1A). Compared to isotype controls, the cells showed positive expression of CD29 (44%), CD73 (95%), and CD90 (79%), while the hematopoietic marker CD45 was minimally expressed (1.5%) (Fig. 1B), suggesting a mesenchymal stem cell phenotype consistent with PDLSCs. The multipotent differentiation potential of PDLSCs was further evaluated. Following osteogenic induction, both ALP and ARS staining revealed significant mineralized nodule formation. Numerous bead-like lipid droplets were observed through Oil Red O staining. Alcian blue staining of paraffin-embedded cartilage pellets revealed structures resembling typical cartilage (Fig. 1C). These findings collectively affirm that the isolated cells exhibit the distinct trilineage differentiation potential typical of PDLSCs.

3.2 Low-Inflammatory Conditions Promote the Osteogenic Potential of PDLSCs

To assess the effects of various inflammatory conditions on the osteogenic capacity of PDLSCs, cells were stimulated with a mixed inflammatory induction medium

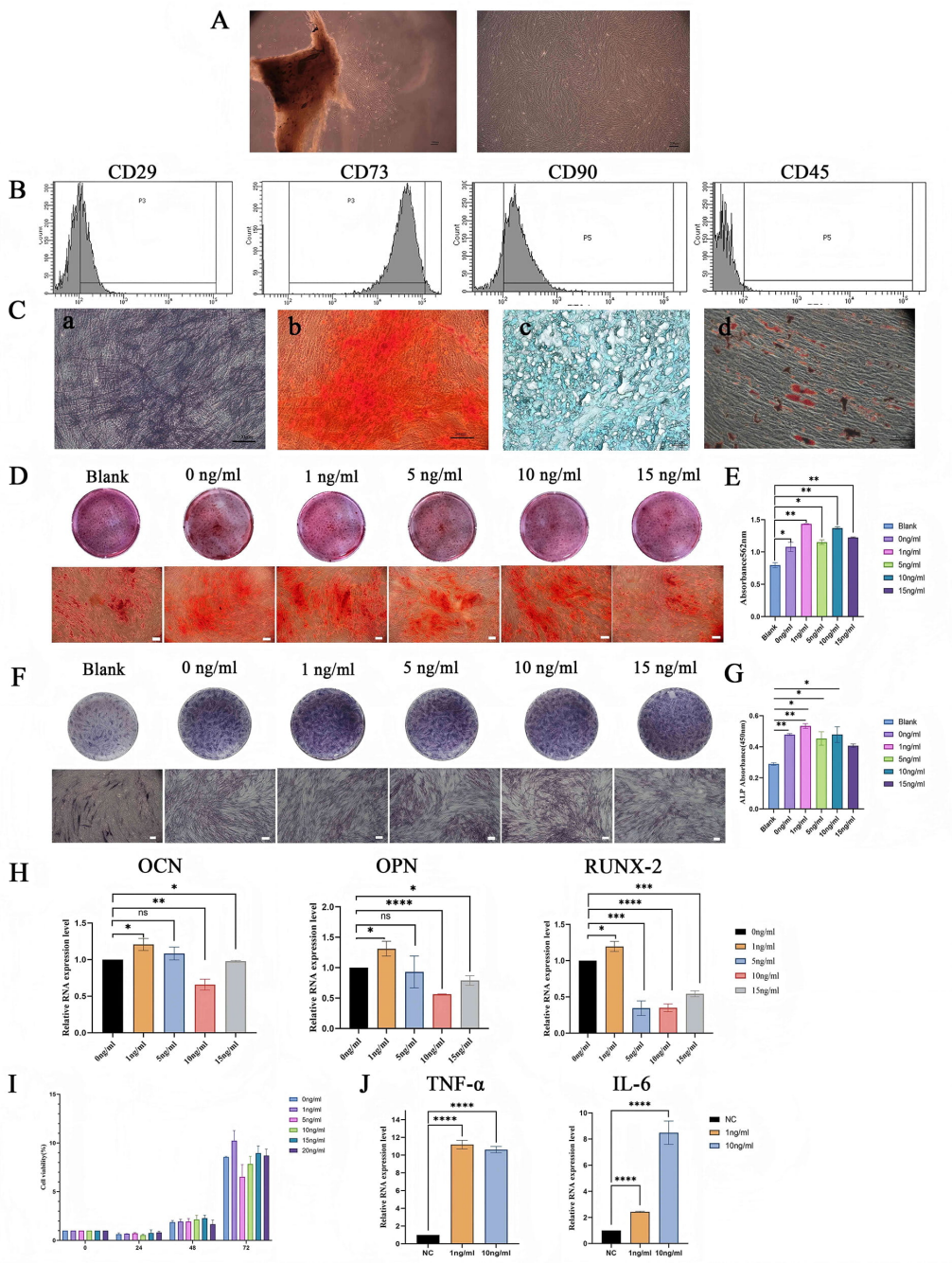

Fig. 1. Characterization and isolation of periodontal ligament stem cells (PDLSCs) and the impact of inflammatory factors on their osteogenic differentiation. (A) Primary PDLSCs were isolated using tissue explant outgrowth and enzymatic digestion methods (Scale bar: 200 μm). (B) Flow cytometric analysis of surface markers confirming PDLSC identity. (C) Tri-lineage differentiation of PDLSCs indicating stemness. (a) ALP staining for osteogenic differentiation (Scale bar: 100 μm); (b) Alizarin Red S (ARS) staining for mineralized nodule formation (Scale bar: 200 μm); (c) Representative Alcian Blue staining images of chondrogenic differentiation (Scale bar: 25 μm); (d) Adipogenic Differentiation of PDLSCs (Scale bar: 50 μm). (D) ARS staining images of PDLSCs after 14 days of osteogenic induction under varying concentrations of inflammatory factors (Scale bar: 100 μm). (E) Quantitative analysis of ARS staining. (F) Representative ALP staining images of PDLSCs after 7 days of osteogenic induction under different concentrations of inflammatory induction (Scale bar: 100 μm). (G) Quantitative analysis of ALP staining. (H) Real-Time polymerase Chain Reaction (RT-PCR) analysis showed that inflammatory stimulation at varying concentrations differentially affected the expression of osteogenic genes in PDLSCs. (I) Cell Counting Kit-8 (CCK-8) assay demonstrating the proliferation curves of PDLSCs under different concentrations of inflammatory induction. (J) Expression of inflammation-related genes in PDLSCs after inflammatory induction (n = 3). Data are shown as mean ± standard deviation (SD). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, ns, $p \geq 0.05$.

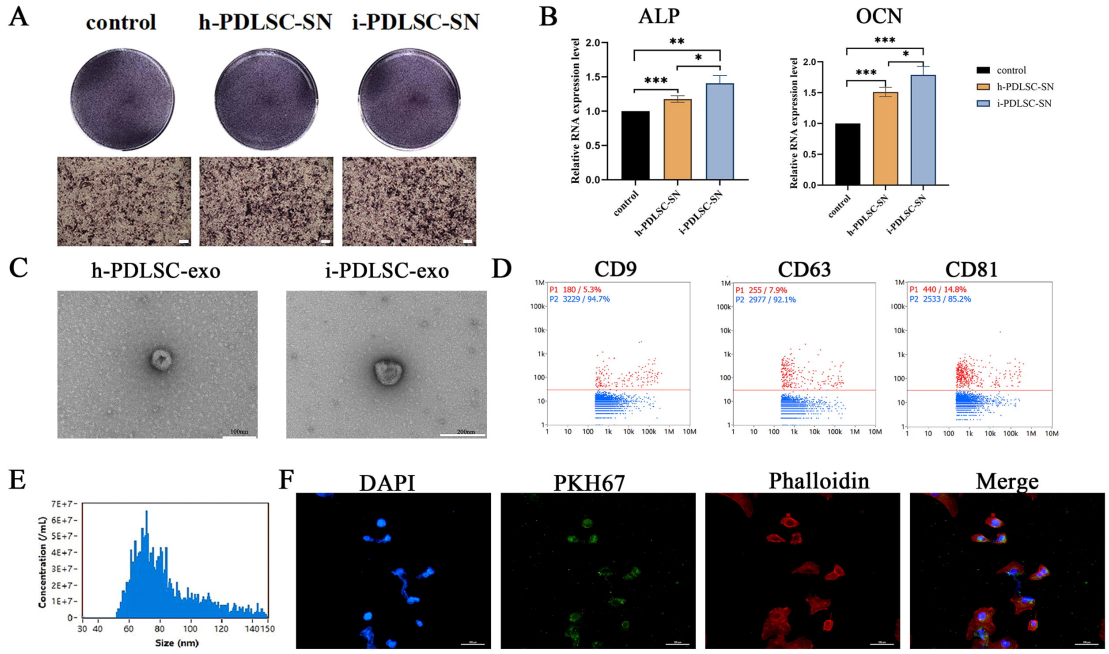

Fig. 2. Isolation, characterization, and uptake of exosomes derived from h-PDLSC (h-PDLSC-exo) and exosomes derived from i-PDLSC (i-PDLSC-exo). (A) The effects of conditioned medium from healthy PDLSCs (h-PDLSC-SN) and low-inflammatory PDLSCs (i-PDLSC-SN) on hFOB1.19 osteogenic activity (scale bar: 200 μm). (B) Treatment with h-PDLSC-SN or i-PDLSC-SN promoted osteogenesis in hFOB1.19 cells, according to qRT-PCR. (C) Representative transmission electron microscopy (TEM) images of h-PDLSC-exo and i-PDLSC-exo (Scale bar: 100 nm, 200 nm). (D) Flow cytometry analysis of exosome surface markers. (E) Nanoparticle tracking analysis (NTA) showing size distribution and mean particle diameter of exosomes. (F) Uptake of pKH67-labeled exosomes by hFOB1.19 cells (Scale bar: 100 μm). Data are presented as mean ± SD, n = 3, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

containing TNF-α and IFN-γ at different concentrations. Compared to healthy PDLSCs (h-PDLSCs), PDLSCs subjected to low-inflammatory conditions (1 ng/mL TNF-α and IFN-γ; i-PDLSCs) exhibited a significant increase in mineralized nodule formation, further supported by semi-quantitative analysis (Fig. 1D–G). Consistently, following induction with 1 ng/mL inflammatory factors, i-PDLSCs showed significant upregulation of osteogenic markers, including Runt-related transcription factor 2 (RUNX2), OCN, and Osteopontin (OPN) (Fig. 1H). The CCK-8 assay revealed that i-PDLSCs exhibited a higher proliferation rate than h-PDLSCs (Fig. 1I). Additionally, i-PDLSCs showed upregulation of inflammatory genes, including TNF-α and interleukin-6 (IL-6), compared to h-PDLSCs (Fig. 1J).

Collectively, these results indicate that inflammatory factor–induced PDLSCs *in vitro* can partially mimic the *in vivo* inflammatory microenvironment, and that low-inflammatory conditions promote PDLSC proliferation and osteogenic differentiation.

3.3 Conditioned Medium From i-PDLSCs Contain Abundant Exosomes and Promote Osteogenesis of hFOB1.19 In Vitro

Before exosome isolation, the conditioned medium was collected from healthy PDLSCs (conditioned medium from healthy PDLSCs [h-PDLSC-SN]) and low-inflammatory PDLSCs (i-PDLSC-SN) and co-cultured with hFOB1.19 cells. ALP staining showed that the staining effect was more pronounced in hFOB1.19 cells treated with i-PDLSC-SN (Fig. 2A). qRT-PCR analysis showed that osteogenesis-related genes were significantly upregulated in hFOB1.19 cells treated with i-PDLSC-SN (Fig. 2B). Exosomes were isolated from h-PDLSC- and i-PDLSC-conditioned media using ultracentrifugation and characterized by TEM, flow cytometry, and NTA. TEM images revealed the typical cup-shaped morphology of both h-PDLSCs-exo and i-PDLSCs-exo (Fig. 2C). The classical exosomal markers CD9, CD63, and CD81 were positively expressed (Fig. 2D). i-PDLSCs-exo had an average diameter of approximately 83.3 nm (Fig. 2E). The yield of exosomes per unit volume of conditioned medium was higher for i-PDLSCs-exo (4.978 mg/mL) compared to h-PDLSCs-exo (4.136 mg/mL). These findings demonstrate that both h-PDLSCs and i-PDLSCs are rich in exosomes, and that low-inflammatory induction (1 ng/mL TNF-α/IFN-γ) can enhance exosome secretion from PDLSCs.

3.4 i-PDLSCs-Derived Exosomes Effectively Enhance Osteogenesis of hFOB1.19

To further explore the osteogenic potential of i-PDLSC-exo *in vitro*, exosomes were labeled with pKH67

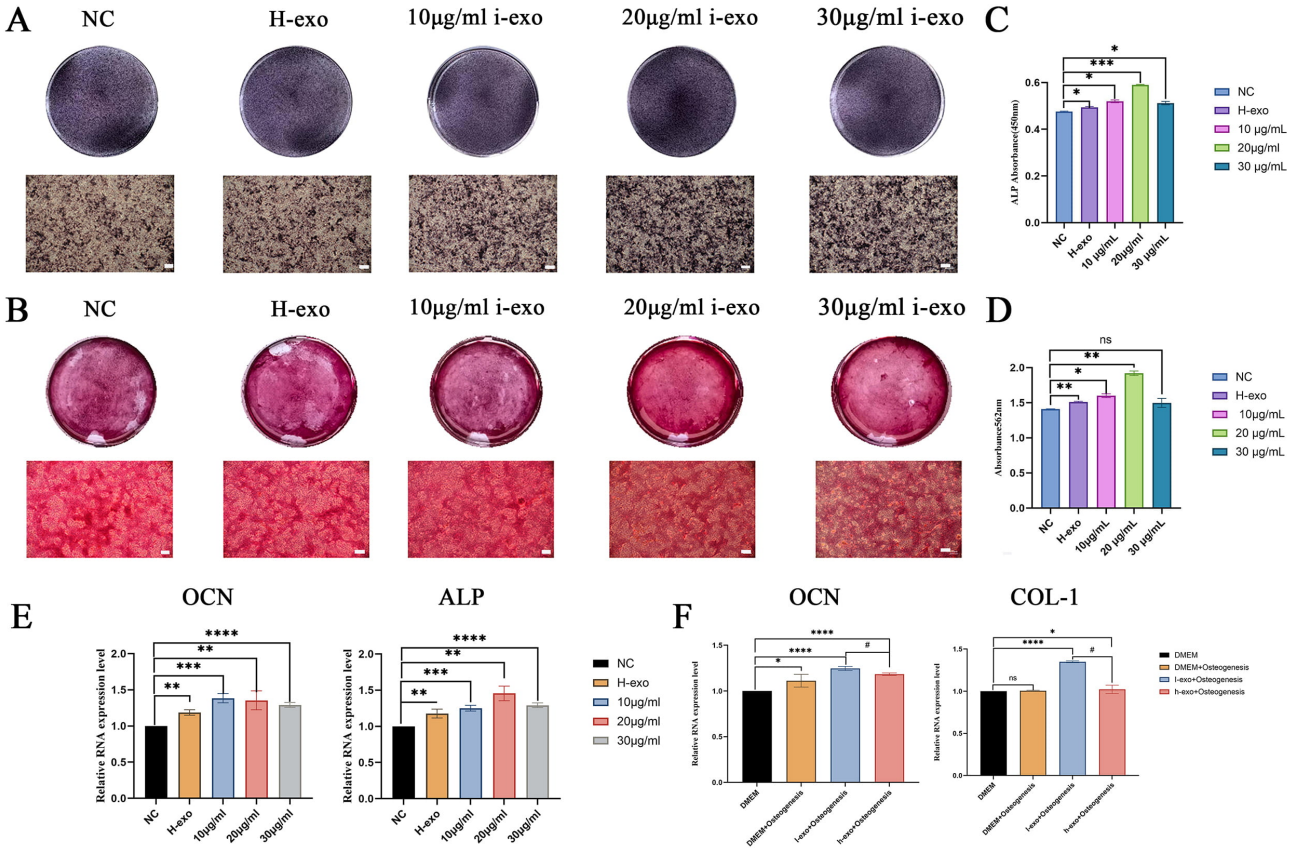

Fig. 3. h-PDLSC- and i-PDLSC-derived exosomes regulate osteogenic differentiation of hFOB1.19 *in vitro*. (A) Representative ALP staining images and quantification of hFOB1.19 of osteogenic induction with different concentrations of i-PDLSC-exo (Scale bar: 100 μm). (B) Representative ARS staining images and quantification of hFOB1.19 of osteogenic induction with different concentrations of i-PDLSC-exo. (Scale bar: 100 μm). (C) Quantitative analysis of ALP staining. (D) Quantitative analysis of ARS staining. (E) RT-PCR analysis indicated that 20 μg/mL i-PDLSC-exosomes markedly enhance osteogenic activity in hFOB1.19 cells. (F) The expression of osteogenesis-related genes in hFOB1.19 treated with 20 μg/mL h-PDLSC-exo or i-PDLSC-exo (n = 3). Data are shown as the mean ± SD, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, ns, $p \geq 0.05$.

and added to hFOB1.19 cultures to assess cellular uptake. Immunofluorescence analysis revealed that hFOB1.19 cells efficiently internalized exosomes within 6 hours (Fig. 2F). Next, hFOB1.19 cells were treated with i-PDLSC-exo at varying concentrations (10, 20, and 30 μg/mL) to assess osteogenic differentiation.

ALP and ARS staining demonstrated that the treatment with 20 μg/mL i-PDLSC-exo resulted in the most significant formation of mineralized nodules (Fig. 3A–D), which was further supported by semi-quantitative analysis. Further analysis using qRT-PCR confirmed that this concentration of i-PDLSC-exo significantly upregulated the expression of genes associated with osteogenesis (Fig. 3E). Following this, hFOB1.19 cells were co-cultured with either h-PDLSCs-exo or i-PDLSCs-exo at a concentration of 20 μg/mL for a period of 7 days to induce osteogenic differentiation. The results from both qRT-PCR and ALP/ARS analyses indicated that i-PDLSCs-exo significantly enhanced mineralization and elevated the expression of osteogenic markers, in comparison to both h-PDLSCs-exo and control groups (Fig. 3F). These results suggest that i-PDLSCs exhibit a greater intrinsic osteogenic potential compared to h-PDLSCs, and that their secreted exosomes effectively facilitate the osteogenic differentiation of hFOB1.19 cells, highlighting a possible mechanism for accelerated bone regeneration.

3.5 i-PDLSC-Derived Exosomes Promote Bone Defect Regeneration In Vivo

In vitro experiments demonstrated that low-inflammatory PDLSCs exhibited enhanced osteogenic potential, and their secreted exosomes were abundant and capable of promoting osteogenesis in human osteoblasts. To validate these findings *in vivo*, the i-PDLSC-exosomes were loaded into a hydrogel and administered to rat bone defects to evaluate their effects on bone regeneration.

3.5.1 Hydrogel Synthesis and Characterization

Hydrogel-based delivery systems effectively preserve the structure and biological activity of cells or bioactive

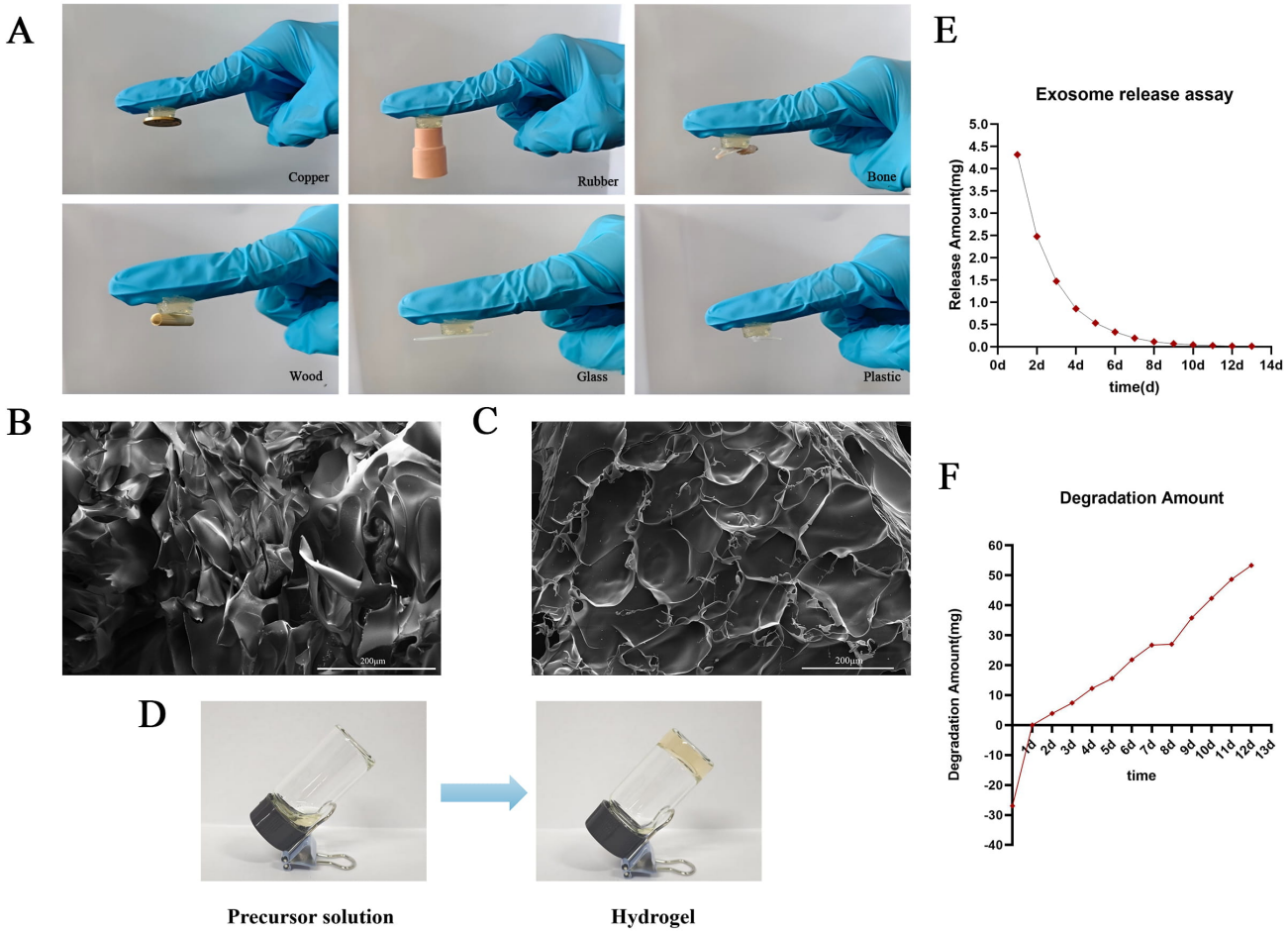

Fig. 4. Encapsulation and characterization of exosomes in chondroitin sulfate-based hydrogel (CS-DMAA). (A) Adhesion properties of CS-DMAA hydrogel. (B) The CS-DMAA hydrogel exhibited a porous structure (Scale bar: 200 μm). (C) Representative Scanning Electron Microscopy (SEM) image of CS-DMAA hydrogel encapsulating exosomes (Scale bar: 200 μm). (D) Gelation of CS-DMAA hydrogel. (E) Release profile of exosomes from CS-DMAA hydrogel. (F) Degradation curve of CS-DMAA hydrogel.

molecules. They enhance delivery efficiency and enhance therapeutic efficacy, ensuring greater tissue specificity for injury repair [24,25,26]. Here, a CS-DMAA hydrogel was selected, which was synthesized by polymerizing acryloylated chondroitin sulfate (Acryloylated-CS) and N, N-dimethylacrylamide (DMAA) catalyzed by glucose oxidase and ferrous glycinate. It demonstrated good adhesiveness, enabling it to conform securely to the defect site (Fig. 4A). This hydrogel functioned as a carrier for PDLSC-derived exosomes, allowing for stable and sustained delivery to the bone defect site. The CS-DMAA hydrogel was characterized for its structure, adhesion, degradation rate, and exosome release profile. Scanning Electron Microscopy (SEM) revealed that the hydrogel exhibited a porous structure (Fig. 4B). After embedding exosomes, SEM revealed that the exosomes embedded in the hydrogel were uniformly distributed (Fig. 4C). The comparison before and after gelation is distinct, indicating excellent gelation performance (Fig. 4D). The degradation rate was appropriate for sustained exosome release, and its excellent biocompatibility contributed to maintaining a favorable microenvironment within the bone defect. A combined analysis of the hydrogel's degradation and exosome release curves (Fig. 4E,F) indicated that exosomes could be effectively released over a 14-day period, thereby prolonging their bioactivity and ensuring their sustained presence at the defect site. However, the variation in the exosome release rate at different stages highlighted the need for further optimization to achieve a consistent and controlled release.

3.5.2 Exosome-Loaded Hydrogel Sustains Bone Regeneration in Rat Mandibular Defects

Alveolar bone defects often arise from chronic progressive bone loss related to periodontitis. These defects are frequently associated with a diminished osteogenic potential of endogenous stem cells and osteoblasts under inflammatory conditions. To explore the therapeutic potential of i-PDLSC-derived exosomes *in vivo*, researchers established a unilateral mandibular bone defect model (4 × 4 × 1 mm^3) in rats. A CS-DMAA hydrogel loaded with 80

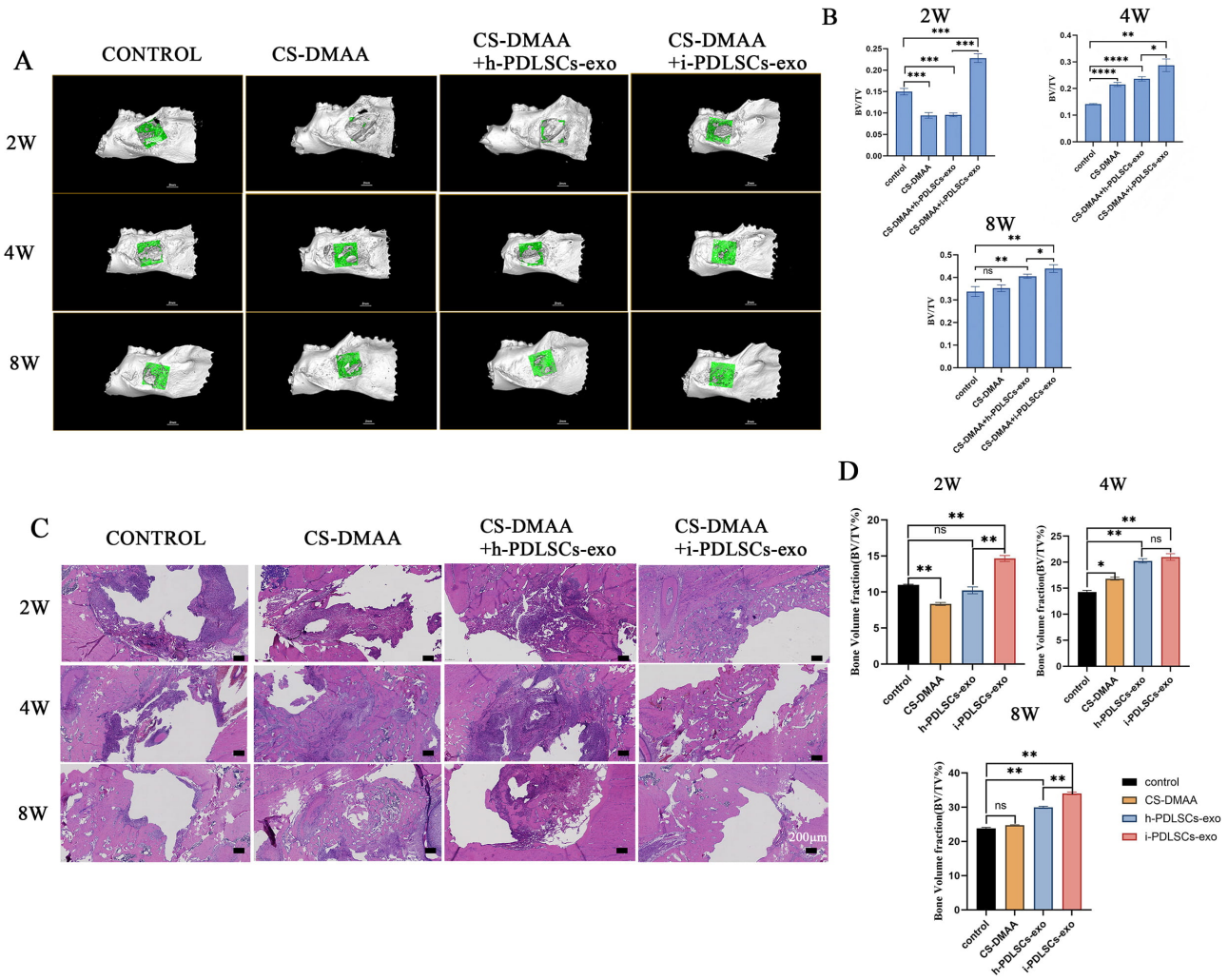

Fig. 5. h-PDLSCs-exo and i-PDLSCs-exo combined with CS-DMAA hydrogel promote jaw bone regeneration *in vivo*. (A) Imaging analysis of new bone in jaw bone defects. (Scale bar: 2 mm). (B) Analysis of bone volume fraction (BV/TV) in jaw bone regeneration. (C) Representative hematoxylin and eosin (H&E) staining image of the defect area. (Scale bar: 200 μm). (D) Percentage of bone volume to tissue volume in H&E staining. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, ns, $p \geq 0.05$, n = 4.

μg/mL of h-PDLSCs-exo or i-PDLSCs-exo was implanted into the defect site. The hydrogel alone served as a negative control, while untreated defects acted as a blank control.

Micro-CT evaluations revealed that at 4–8 weeks, the new bone formation in both the h-PDLSCs-exo and i-PDLSCs-exo groups was significantly higher than that in the hydrogel-only and blank control groups (Fig. 5A,B). Histological analysis further confirmed increased jaw bone volume and density in the exosome-treated groups (Fig. 5C,D; Fig. 6A,B), with the i-PDLSCs-exo group demonstrating more pronounced osteogenic effects than the h-PDLSCs-exo group. These findings indicate that i-PDLSC-exosome-loaded hydrogel can sustain and enhance bone regeneration *in vivo*, highlighting its potential as a therapeutic strategy for jaw bone defects.

Subsequently, we conducted an immunohistochemical analysis of the osteogenic factor OCN in the bone defect regions. Immunohistochemical staining showed a significantly greater number of OCN-positive cells in both the h-PDLSCs-exo and i-PDLSCs-exo groups compared to the hydrogel-only and blank control groups (Fig. 6C,D). Notably, the i-PDLSCs-exo group demonstrated the highest number of OCN-positive cells, indicating that i-PDLSCs-exo can effectively enhance osteogenesis in localized bone defect areas.

4. Discussion

Achieving alveolar bone regeneration, whether in inflammatory or non-inflammatory conditions, continues to pose a significant challenge in clinical dentistry. PDLSCs are regarded as the “gold standard” for periodontal regeneration because of their ability to differentiate into osteoblasts, cementoblasts, and periodontal ligament fibroblasts. The interactions of PDLSCs with other bone-regenerative cells, including immune cells, osteoclasts, and

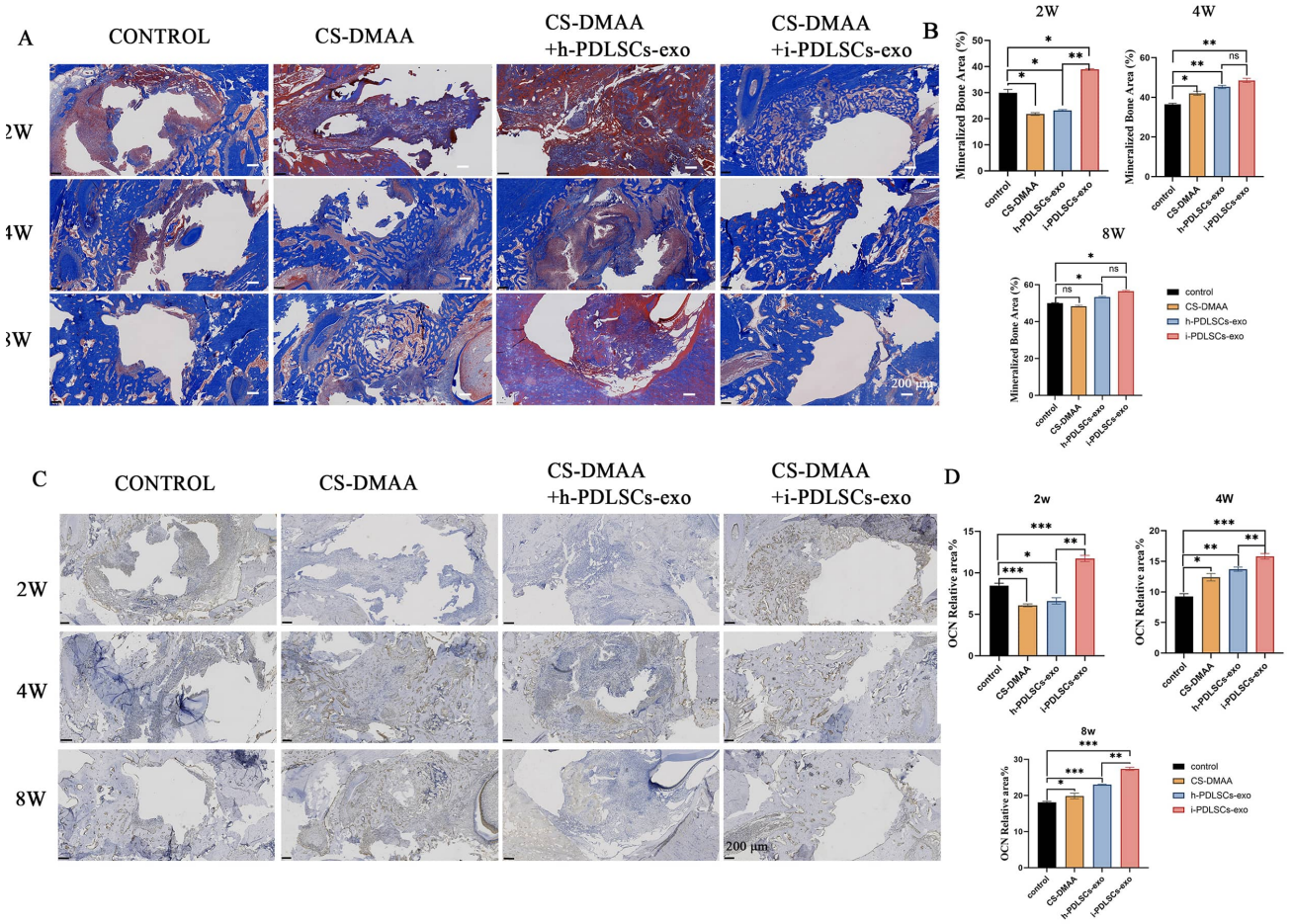

Fig. 6. h-PDLSCs-exo and i-PDLSCs-exo combined with CS-DMAA hydrogel promote jaw bone regeneration *in vivo*. (A) Histological examination of newly formed bone in the defect area as determined via the Masson staining assay (Scale bar: 200 μm). (B) Percentage of mature bone tissue volume to tissue volume in Masson staining. (C) An immunohistologic assay was performed to detect the expression of osteogenic marker OCN in the bone defect area (Scale bar: 200 μm). (D) Immunohistochemical quantitative analysis. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, ns, $p \geq 0.05$, n = 4.

osteoblasts, along with their association with the extracellular matrix (ECM), are essential for the effective repair of periodontal tissues [22]. Early studies aimed to apply PDLSCs directly to bone defects, utilizing either scaffolds or cell sheets. This approach aimed to exploit their differentiation potential for bone regeneration [22,27,28]. However, clinical translation of mesenchymal stem cells (MSCs) is limited by *in vitro* expansion, donor-dependent heterogeneity, and lack of standardized protocols [20].

Previous studies indicate that preconditioning MSCs with hypoxia, inflammatory cytokines, or genetic modifications can significantly enhance their immunoregulatory and regenerative capabilities. Notably, MSCs subjected to hypoxic preconditioning not only produce larger quantities of exosomes but also exhibit changes in their functional characteristics [29]. Preconditioning MSCs with specific inflammatory cytokines, particularly TNF-α and IFN-γ, activates intracellular signaling pathways (e.g., PI3K-AKT). This process modulates cellular metabolism, regulates cytokine secretion, and influences mitochondrial function, thereby enhancing it enhances the immunomodulatory, anti-inflammatory, and tissue repair functions of MSCs [30,31,32]. Additionally, low-inflammatory preconditioning enhances bone formation by effectively modulating the Wnt/β-catenin and NF-κB pathways. This approach sustains a balanced level of Wnt activation, which encourages bone formation and mitigates the effects of inflammatory suppression, all while preventing excessive activation that could inhibit bone formation [19,33,34,35]. Simultaneously, it inhibits abnormal NF-κB activation and prevents its nuclear translocation through essential regulators such as GSK-3β, thereby mitigating its adverse effects on bone formation. Together, these actions transform inflammation from a barrier to bone formation into a facilitator of regeneration [36,37]. Exosomes secreted by MSCs serve as key paracrine mediators of tissue regeneration [6,38,39]. In recent years, significant progress has been made in the treatment of periodontitis using PDLSCs and their secre-

tory products. In terms of osteogenesis promotion, PDLSC-derived exosomes can carry miRNAs such as miR-126 and miR-155-5p to target and inhibit axis inhibition protein 2 (AXIN2), thereby significantly enhancing the osteogenic differentiation capacity of PDLSCs. Additionally, PDLSC-derived exosomes drive osteogenic differentiation by delivering non-coding RNAs to activate the PI3K/AKT signaling pathway [40]. Based on these findings, we hypothesized that i-PDLSCs-exo could act as a therapeutic agent for treating bone defects.

In this study, we successfully induced h-PDLSCs into a low-inflammatory state (i-PDLSCs) and isolated their exosomes for application in treating jaw bone defects. Our findings indicated that i-PDLSCs displayed improved proliferation rates and increased osteogenic capacity. Furthermore, i-PDLSCs-exo markedly enhanced the osteogenic differentiation of hFOB1.19 cells *in vitro*, thereby supporting bone repair in rat models. These results imply that i-PDLSCs-exo could serve as a promising cell-free alternative in periodontal stem cell therapy.

In this study, we tested inflammatory cytokine concentrations at 1, 5, 10, and 15 ng/mL. We found that concentrations exceeding 20 ng/mL progressively impaired the proliferation and osteogenesis of PDLSCs. RT-PCR analysis of osteogenic genes and ALP/ARS staining indicated that 1 ng/mL of TNF-α and IFN-γ significantly enhanced the osteogenic potential of PDLSCs. Notably, i-PDLSCs generated a greater yield of exosomes compared to h-PDLSCs. When these exosomes were applied to hFOB1.19 cells at an equivalent concentration, i-PDLSCs-exo significantly enhanced osteogenic activity in a dose-dependent manner, with 20 μg/mL demonstrating the most pronounced effect. In contrast, higher concentrations, such as 30 μg/mL, resulted in a slight reduction in osteogenic promotion. These findings suggest that i-PDLSCs-exo not only yield a higher quantity of exosomes but also exhibit considerable osteogenic potential.

For *in vivo* studies, we selected CS-DMAA hydrogel as a carrier for PDLSCs-exo due to its rapid gelation, excellent adhesion, and porous structure [21], which facilitated implantation into bone defects and exosome delivery. We observed that the degradation rate of the CS-DMAA hydrogel was slower than the rate of exosome release. Consequently, remnants of the hydrogel remained for 4 to 6 weeks following implantation, with the duration varying based on the volume of the implant. Subsequent research should focus on optimizing the sustained release of exosomes while minimizing any potential space-occupying effects.

In *in vivo* experiments, the CS-DMAA + h-PDLSCs-exo group produced substantial new bone formation, further supporting the osteogenic potential of i-PDLSCs-exo. This study demonstrates that exosomes derived from low-inflammatory PDLSCs possess therapeutic effects on alveolar bone defects. A brief, low-level inflammatory induction was shown to enhance the secretion of i-PDLSCs-exo, while exosome therapy addresses the immunogenic and ethical challenges associated with cell-based treatments. Therefore, preconditioned PDLSC-derived exosomes may offer a promising approach for the treatment of periodontal bone defects.

5. Limitations

Our current study primarily focuses on validating the expression of i-PDLSCs-exo in the context of bone defect repair. However, it does not examine potential direct targets or investigate the upstream and downstream regulatory mechanisms involved. Moreover, during our *in vitro* experiments, we encountered a challenge related to hydrogel space occupation. Specifically, we found that the hydrogel's degradation rate is slower than the exosome release rate. This prolonged presence of the hydrogel may inhibit new bone growth, likely due to the excessive amount of hydrogel utilized during the modeling process. Addressing this issue will be essential for enhancing future studies.

6. Conclusion

In summary, low-concentration inflammatory induction promoted PDLSC proliferation, exosome production, and expression of osteogenesis-related genes, such as ALP and OCN. i-PDLSCs-exo strongly increased the osteogenic potential of hFOB1.19 cells *in vitro*. *In vivo*, compared with the chondroitin sulfate hydrogel group, the blank control group, and the h-PDLSCs-exo group, the CS-DMAA hydrogel loaded with either i-PDLSCs-exo facilitated pronounced bone formation in rat jaw bone defects. These findings suggest that preconditioned PDLSC-derived exosomes represent an effective therapeutic approach for jaw bone regeneration.

Abbreviations

PDLSCs, periodontal ligament stem cells; h-PDLSCs, healthy PDLSCs; i-PDLSCs, PDLSCs subjected to low-inflammatory conditions; OB, osteoblast; OC, osteoclast; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; IL-6, interleukin-6; i-PDLSCs-exo, exosomes derived from i-PDLSCs; h-PDLSCs-exo, exosomes derived from h-PDLSCs; SD, Sprague–Dawley; ALP, alkaline phosphatase; ARS, Alizarin Red S; TEM, transmission electron microscopy; NTA, nanoparticle tracking analysis; SEM, Scanning Electron Microscopy; Acryloylated-CS, acryloylated chondroitin sulfate; DMAA, N, N-dimethylacrylamide; CS-DMAA, Chondroitin sulfate-based hydrogel; Micro-CT, micro-computed tomography; H&E, hematoxylin and eosin; h-PDLSC-SN, conditioned medium from healthy PDLSCs; i-PDLSC-SN, low-inflammatory PDLSCs; ECM, extracellular matrix; MSCs, mesenchymal stem cells.

Availability of Data and Materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions

KL: Conceptualization; Funding Acquisition; Supervision; Writing—Review & Editing; MZT: writing—original draft; Formal Analysis; Methodology; Validation; Writing—review and editing. JRG: Formal Analysis; Project Administration; CSW, QZ, NZ: Project Administration; Resources; Analyzed the data; WZJ, HLG, JQZ, XW and YJH: provided help on validation; BF: Review & Editing; Validation and Supervision. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The study was conducted in accordance with the Declaration of Helsinki. All procedures involving human tissues and experimental animals were reviewed and approved by the Ethics Committee of Liaocheng People's Hospital (Approval Nos. 2025301 and Approval Nos. 2025310), and all of the patients and their legal guardians provided signed informed consent. The experiments were conducted in strict accordance with the 3R principles – Replacement (using non-animal models whenever possible), Reduction (minimizing the number of animals through optimal experimental design), and Refinement (alleviating animal suffering by improved housing, anesthesia, analgesia, and humane endpoints). The study was carried out in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and reported in accordance with the ARRIVE guidelines.

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Conflicts of Interest

The authors declare no conflicts of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/FBL50928.

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