



Elevated osteogenic potential of stem cells from inflammatory dental pulp tissues by Wnt4 overexpression for treating bone defect in rats

Tianyu Zhong^{1,2,3}, Yunan Gao^{1,2}, Hu Qiao^{1,2}, Hong Zhou^{1,2}, Yong Liu^{1,3}

¹Key Laboratory of Shaanxi Province for Craniofacial Precision Medicine Research, Xi'an Jiaotong University, Xi'an, China; ²Department of Orthodontics, The Affiliated Stomatological Hospital of Xi'an Jiaotong University, Xi'an, China; ³Institute of Neurobiology, Xi'an Jiaotong University Health Science Center, Xi'an, China

Contributions: (I) Conception and design: T Zhong, Y Liu, H Zhou; (II) Administrative support: Y Liu, H Zhou; (III) Provision of study materials or patients: T Zhong, Y Gao, H Qiao; (IV) Collection and assembly of data: T Zhong; (V) Data analysis and interpretation: T Zhong; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Yong Liu. Institute of Neurobiology, Xi'an Jiaotong University Health Science Center, #76 Yanta West Road, Xi'an, China. Email: liuy5599@mail.xjtu.edu.cn; Hong Zhou. Department of Orthodontics, The Affiliated Stomatological Hospital of Xi'an Jiaotong University, Xi'an, China. Email: zhouhong@mail.xjtu.edu.cn.

Background: The osteogenic capacity of inflammatory dental pulp stem cells (DPSCs-IPs) is reported lower than that of normal dental pulp stem cells (DPSCs-NPs). Down-regulation of Wnt4 may be the key factor affecting the osteogenic ability of DPSCs-IPs. In order to prove that the restoration of Wnt4 expression could improve the osteogenic potential of DPSCs-IPs, Wnt4 overexpressed inflammatory dental pulp stem cells (Wnt4-DPSCs-IPs) were performed to reconstruct bone defects in rats.

Methods: Human DPSCs-IPs were cultured and transfected with Wnt4 overexpression lentiviral vector. Stem cell characterization was performed by flow cytometry and induction of multidirectional differentiation. Wnt4-DPSCs-IPs were loaded onto poly (hydroxybutyrate-co-valerate) (PHBV). The compounds were grafted into artificially-created defect in alveolar bone. The effectiveness of Wnt4-DPSCs-IPs/PHBV in bone regeneration was assessed by micro-CT and immunohistochemical staining of osteocalcin, a representative osteogenic marker.

Results: Collecting data showed that Wnt4 overexpression didn't change stem cell characteristics of DPSCs-IPs. Wnt4-DPSCs-IPs retain osteogenic, adipogenic and chondrogenic differentiation abilities. Wnt4-DPSCs-IPs/PHBV were more effective than DPSCs-IPs/PHBV in repair of rat bone defects by 3 months' post-surgical reconstruction.

Conclusions: Restoration of Wnt4 expression could improve the osteogenic potential of DPSCs-IPs. Wnt4 restored DPSCs-IPs may be a feasible resource of seed cells for bone regeneration in future clinical application.

Keywords: Inflammatory dental pulp stem cells (DPSCs-IPs); Wnt4; bone regeneration

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Introduction

Recently evidence indicated that inflammatory dental pulp tissues retained tissue regeneration potential by containing a certain proportion of mesenchymal stem cells, inflammatory

dental pulp stem cells (DPSCs-IPs) (1,2). However, the osteogenic potential was inhibited to some extent compared with normal dental pulp stem cells (DPSCs-NPs) (3,4). DPSCs-NPs have been proved to be a good source of seed

cells in tissue engineering (5-10). While the main challenge in clinical application is the availability, since patients must sacrifice dental pulp tissues in normal tooth for culturing DPSCs-NPs (11-13). However, there are numerous patients who have already had their dental pulp infected and the extracted pulp turned medical waste. If we can find a way to improve the osteogenic capacity of DPSCs-IPs, we can turn waste into treasure in the clinic.

Previous studies showed that the down-regulation of Wnt4 accounted for the impaired osteogenic potential of DPSCs under inflammatory conditions (14-16). Wnt signaling plays a key role in the osteogenic differentiation of mesenchymal stem cells (17). Studies have found that Wnt4 (a non-classical Wnt member) was crucial in promoting the osteogenic differentiation of MSCs isolated from craniofacial tissues (18). In our previous study, Wnt4 regulated C-Jun pathway was down regulated in inflammatory conditions (19). In addition, we also found that Wnt4 rescue promoted the osteogenic differentiation of DPSCs-IPs as well as the related gene expression. Our present study aims to figure out whether the restore of Wnt4 in DPSCs-IPs engrafted in poly (hydroxybutyrate-co-valerate) (PHBV) has the developed ability of reconstructing bone defect in rats. We present the following article in accordance with the ARRIVE reporting checklist (available at <http://dx.doi.org/10.21037/apm-20-460>).

Methods

Cell culture

Dental pulp (normal and inflamed) were obtained from Stomatological Hospital, Xi'an Jiaotong University, and was approved by the Ethics Committee of Stomatological Hospital, College of Medicine, and Xi'an Jiaotong University. Extracted pulps were digested by 3 mg/mL dispase II (Roche, Germany) and 3 mg/mL type IV collagenase (STEMCELL Technologies, Canada). After 1 h digestion, cells were suspended in 10 cm dish with Dulbecco's-modified Eagle's media (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (all from Gibco, USA). The full-length sequence of Wnt4 was amplified by PCR and connected to the lentiviral vector (Genechem, China). DPSCs-IPs at passage 3 were collected to transduce recombinant lentiviral Wnt4. Stably over-expressing Wnt4 cell line was

screened out.

Flow cytometry for characterization of Wnt4-DPSCs-IPs

Passage 1 of Wnt-DPSCs-IPs was collected to detect surface markers by flow cytometry. Primary antibodies used were: mouse monoclonal anti-human STRO-1, CD105 and CD45 and rabbit monoclonal anti-human CD34 and CD90 (all from Abcam, Cambridge, MA, USA).

Multidirectional differentiation of Wnt4-DPSCs-IPs

Osteogenic, adipogenic and chondrogenic differentiation induction were also performed to characterize Wnt4-DPSCs-IPs. For osteogenic differentiation, cells were cultured with induction media (HUXDP-90021, Cyagen, China) for 14 days for Alizarin Red S (ARS) staining. For adipogenic differentiation, cells were cultured with induction media (HUXDP-90031, Cyagen, China) for 14 days for oil-red O staining. For chondrogenic differentiation, cells were cultured with induction media (HUXDP-90041, Cyagen, China) for 14 days for toluidine blue staining. For ARS staining, calcified nodules were evaluated, for oil-red O staining, red lipids were calculated, and for Toluidine Blue staining, blue-purple chondrocytes were counted.

Preparation and evaluation of complex Wnt4-DPSCs-IPs/PHBV

One cm² PHBV (thickness: 0.2 μm) was placed in one well in a 24-well plate, Wnt4-DPSCs-IPs were engrafted on PHBV. Culture medium was generally changed every 3 days. Fourteen days later, the complex was scraped for scanning electron microscope analysis.

Cell proliferation and ALP assay

Zero point three cm² PHBV (thickness: 0.2 μm) was placed in one well in a 96-well plate, Wnt4-DPSCs-IPs were seeded at a density of 5×10³ cells per well. Cells were cultured at 37 °C for 1, 3, and 7 days. Cell proliferation was detected by MTT analysis. Cells cultured with osteogenic induction media for 14 days were used to detect ALP activity by alkaline phosphatase (ALP) detection kit (G5610, Solarbio, China) according to the product instructions.

Generation of a bone defects in rats

Twenty 4-week-old male Sprague Dawley rats (weighing 200 g) were used in the experiment. They were all treated in accordance with the guidelines of Xi'an Jiaotong University College of Medicine for Experimental Animals. Bone defect model was standardized built with a size of 2×1×1 mm at retromolar area in maxillary.

In vivo transplantation and evaluations

There are four treatment groups: (I) PHBV (n=5): treatment with only PHBV; (II) DPSCs-IPs/PHBV (n=5): DPSCs-IPs engrafted on PHBV; (III) DPSCs-NPs/PHBV (n=5): DPSCs-NPs engrafted on PHBV; (IV) Wnt4-DPSCs-IPs/PHBV (n=5): Wnt4 overexpressed DPSCs-IPs engrafted on PHBV. Micro-CT was used to examine bone regeneration 3 months post-transplantation. Maxillary were fixed in 4% paraformaldehyde. Tissues were scanned on a micro-CT system. The parameters are set as follows: 70 kV, 114 μA, 12 μm resolution. CTAn software was used to analysis.

Immunohistochemistry

Deparaffinize and rehydrate slides. Wash slides for 3 times each. Incubate with 0.1% Trypsin (T8150, Solarbio, China) at 37 °C for 10 min. Rinse slides with PBS 3 times for 2 minutes each. Incubate slides with serum blocking solution (SL038, Solarbio, China) for 30 min. Incubate with Osteocalcin primary antibody (1:200, ab13420, Abcam, USA) at 4 °C overnight. Wash and incubate with biotinylated second antibody (1:2,000, ab205719, Abcam, USA). Wash and incubate with streptavidin-enzyme conjugate.

Statistical analysis

One-way ANOVA was used to analyze the significance of differences among groups. A P value of less than 0.05 was considered significant. SPSS 16.0 software (SPSS Inc., Chicago, IL, USA) was used for analysis.

Results

Characterization of Wnt4-DPSCs-IPs

Five surface markers were chosen to characterize Wnt4-DPSCs-IPs. STRO-1, CD90 and CD105 were selected as positive markers to characterize mesenchymal stem cells,

hematopoietic CD34 and CD45 were chosen as negative markers. As shown in *Figure 1*, positive reaction was displayed by 89.93% of Wnt4-DPSCs-IPs for STRO-1, 85.89% for CD90 and 77.36% for CD105. While negative reaction was found for CD34 and CD45. The results suggested that Wnt4 overexpression did not change stem cell characteristics of DPSCs-IPs.

Multi-differentiation potential of Wnt4-DPSCs-IPs

We further tested the multi-differentiation potential of Wnt4-DPSCs-IPs. As we expected, Wnt4-DPSCs-IPs could be positively stained by Alizarin red, oil-red O and toluidine blue (*Figure 2*). Wnt4-DPSCs-IPs still retain the potential of multidirectional differentiation.

Characterization of Wnt4-DPSCs-IPs/PHBV

We first measured cell proliferation of Wnt4-DPSCs-IPs on PHBV. MTT showed that no significant difference was detected on OD value between Wnt4-DPSCs-IPs and Wnt4-DPSCs-IPs/PHBV at 1, 3 and 7 days (*Figure 3A*). PHBV did not affect cell proliferation of Wnt4-DPSCs-IPs. Next, ALP activity was tested with Wnt4-DPSCs-IPs and Wnt4-DPSCs-IPs/PHBV. No significant difference was shown (*Figure 3B*). Last, we performed scanning electron microscope with Wnt4-DPSCs-IPs/PHBV. In *Figure 3C*, we may find the rough surface of the PHBV. A large number of porous structures were detected as well as the cell attachment.

Wnt4-DPSCs-IPs in bone regeneration in rats

Micro-CT and histopathology were performed. *Figure 4A* showed that the PHBV group demonstrated certain bone repair; while DPSCs-IPs/PHBV has less bone repair capacity than DPSCs-NPs/PHBV, but better capacity than PHBV; Wnt4-DPSCs-IPs/PHBV showed the improved bone repair potential which was close to DPSCs-NPs/PHBV. Bone mineral density (BMD) and bone volume over total volume (BV/TV) were analyzed to show the bone repair potential of each group. Micro-CT analysis showed that BMD and BV/TV were decreased in DPSCs-IPs/PHBV compared with DPSCs-NPs/PHBV and were restored in Wnt4-DPSCs-IPs/PHBV compared with DPSCs-IPs/PHBV (*Figure 4B*). The immunohistochemistry results showed that osteocalcin expressed at a higher level in DPSCs-NPs/PHBV as well as in Wnt4-DPSCs-IPs/PHBV,

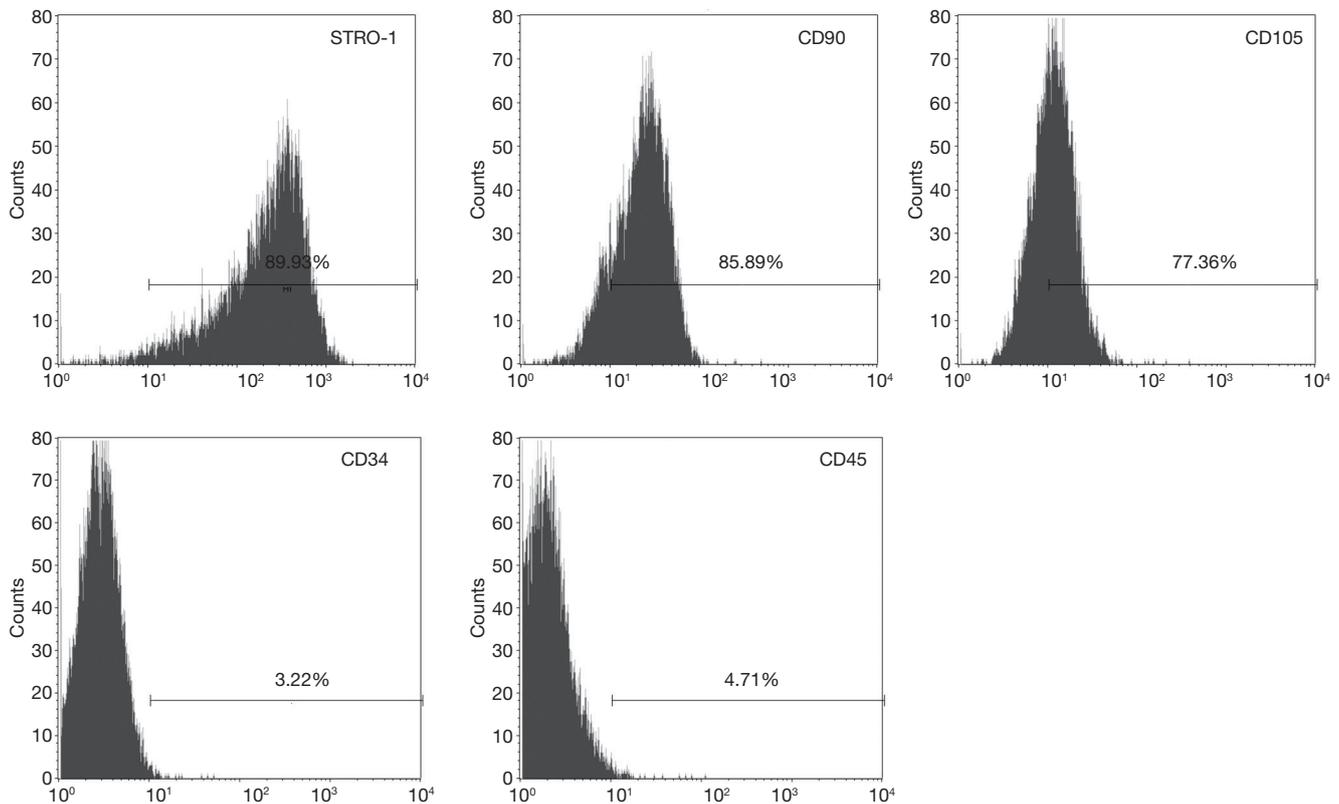


Figure 1 Characterization of Wnt4-DPSCs-IPs. Flow cytometry for characterization of Wnt4-DPSCs-IPs. Expression of surface markers STRO-1, CD90, CD105, CD34 and CD45 were detected. DPSCs-IPs, inflammatory dental pulp stem cells.

while a lower level in PHBV and DPSCs-IPs/PHBV, suggested that new bone formation were more active in both DPSCs-NPs/PHBV and Wnt4-DPSCs-IPs/PHBV (Figure 4C).

Discussion

In the present study, a possible strategy to improve the impaired osteogenic potential of DPSCs-IPs was tested, the feasibility and effectiveness were verified *in vivo*. Wnt4 restoration promoted the osteogenic differentiation of DPSCs-IPs. Wnt4-DPSCs-IPs/PHBV showed developed ability of reconstructing bone defect in rats.

Inflammatory dental pulp tissue-derived stem cells have broadened the source of mesenchymal stem cells. They have great potential in the application since no damage to normal tissues was needed any more (20,21). However, the only problem lies in the decrease of osteogenic capacity. Therefore, finding key factors to improve osteogenic ability of DPSCs-IPs can fundamentally solve the current

problems. Wnt4 has been shown to inhibit bone resorption and its expression was also down regulated in the DPSCs-IPs. The expression of Wnt4 is closely related to several stem cell osteogenic markers. We believe that Wnt4 is a key factor in increasing osteogenic potential of DPSCs-IPs.

Next, we investigated whether restoring Wnt4 expression would affect the stem cell characteristics of DPSCs-IPs. We found that Wnt4 overexpression would not change the characteristics of DPSCs-IPs as stem cells. In addition, Wnt4-DPSCs-IPs still retained multidirectional differentiation potential, especially osteogenic differentiation, which has laid a good foundation for its future clinical application.

The effect of PHBV on the proliferation and osteogenic differentiation of Wnt4-DPSCs-IPs also needs to be elucidated before *in vivo* studies (22,23). Our results showed that after loading Wnt4-DPSCs-IPs on PHBV, the proliferation and osteogenic differentiation ability of the complexes were not statistically different from Wnt4-DPSCs-IPs, which suggested that we may use the complexes *in vivo* to repair bone defect.

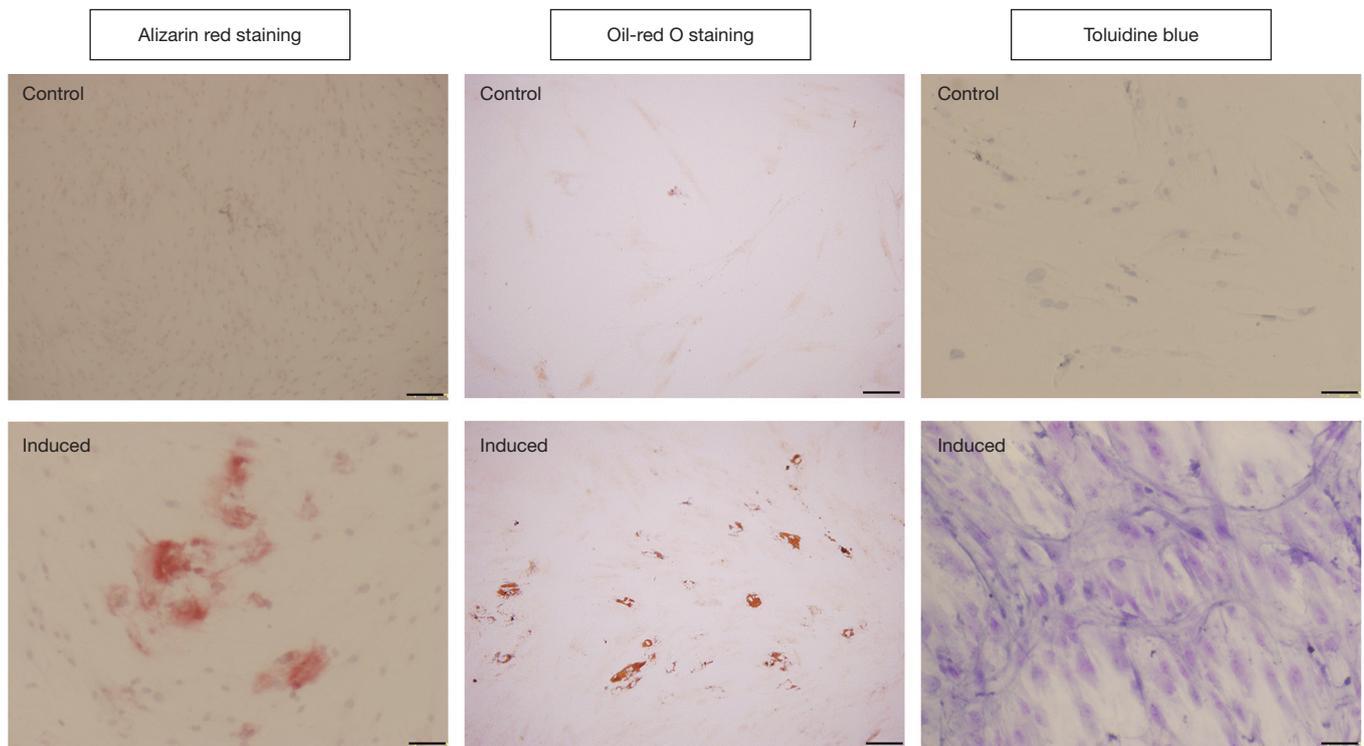


Figure 2 Multi-differentiation potential of Wnt4-DPSCs-IPs. Two weeks after induction of multidirectional differentiation, Alizarin red, oil-red O and toluidine blue were stained with Wnt4-DPSCs-IPs. Scale bars =100 μ m. DPSCs-IPs, inflammatory dental pulp stem cells.

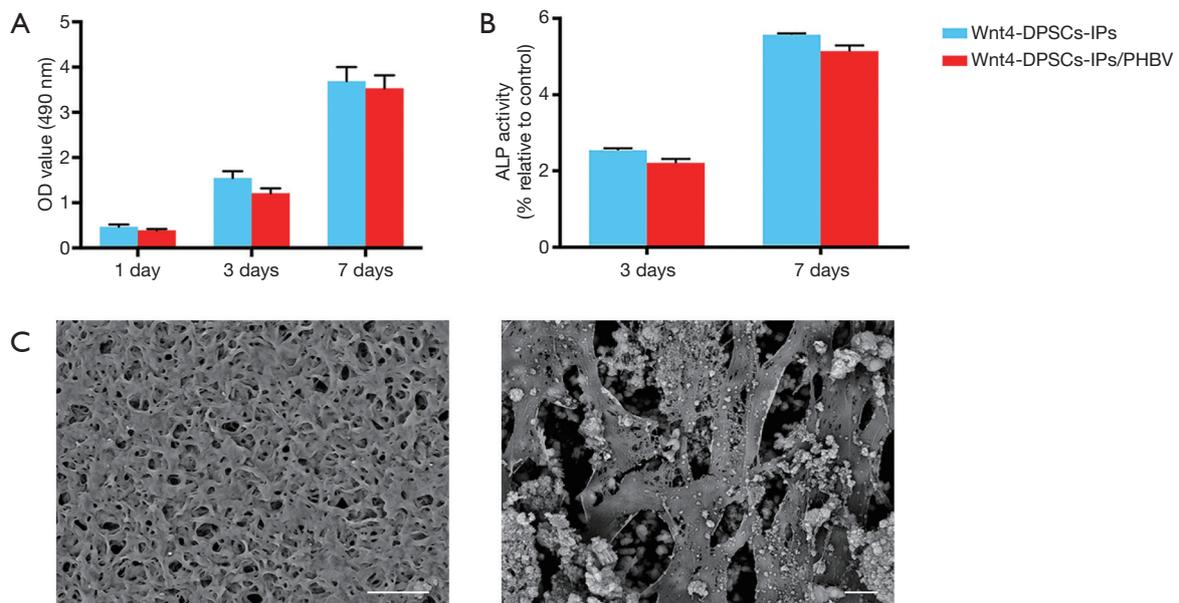


Figure 3 Characterization of Wnt4-DPSCs-IPs/PHBV. (A) Wnt4-DPSCs-IPs proliferation on PHBV; (B) osteogenic potential of Wnt4-DPSCs-IPs on PHBV; (C) scanning electron microscopy of Wnt4-DPSCs-IPs/PHBV. Scale bars =1 μ m. DPSCs-IPs, inflammatory dental pulp stem cells; PHBV, poly (hydroxybutyrate-co-valerate).

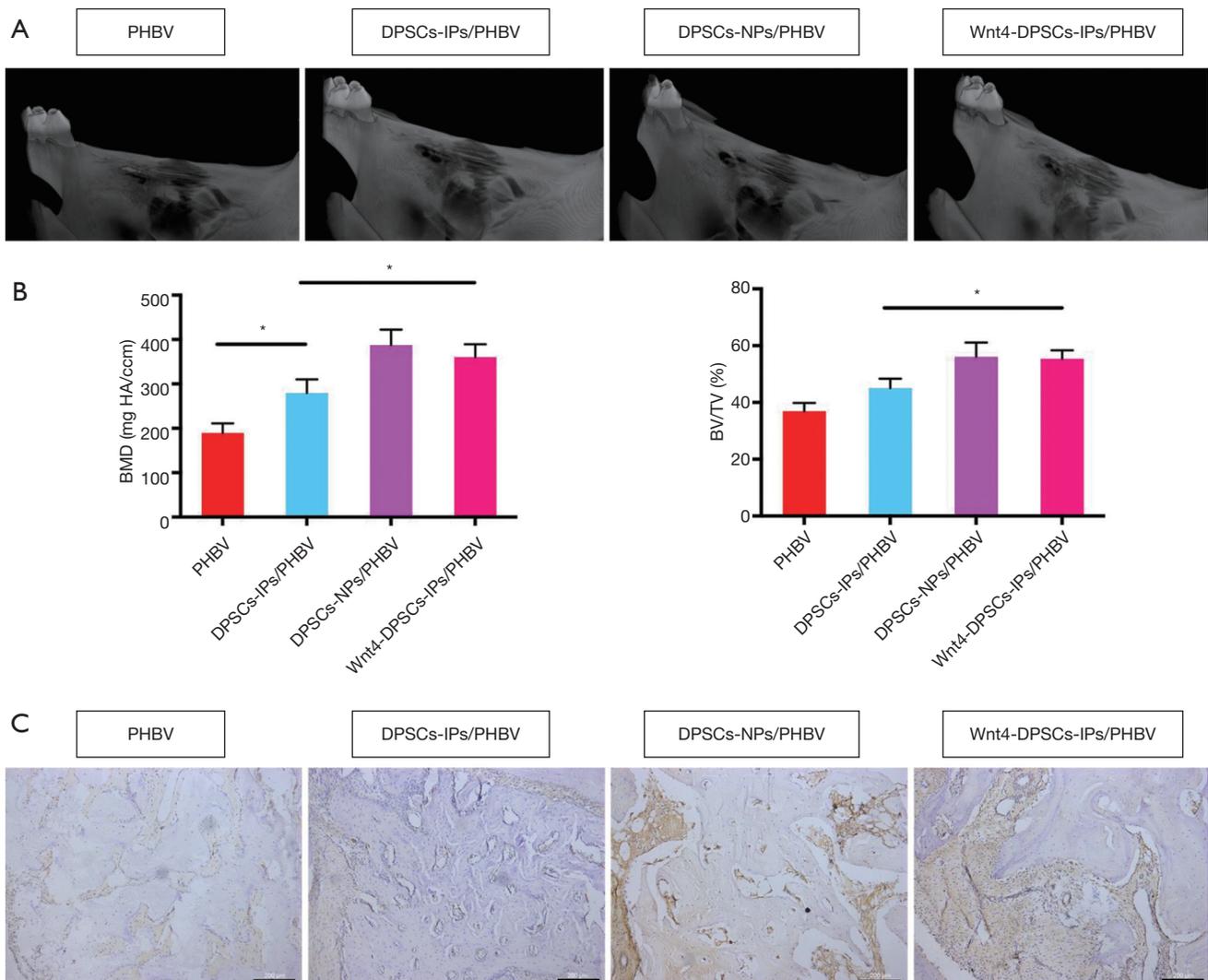


Figure 4 Wnt4-DPSCs-IPs in bone regeneration in rats. (A) Micro-CT images of bone defects 12 weeks after transplantation. Bone defects are clearly marked; (B) BMD and BV/TV were calculated; (C) osteocalcin expression in new bone formation area. Scale bars =200 μ m. *, $P < 0.05$. DPSCs-IPs, inflammatory dental pulp stem cells; DPSCs-NPs, normal dental pulp stem cells; PHBV, poly (hydroxybutyrate-co-valerate); BMD, bone mineral density; BV/TV, bone volume over total volume.

The results of *in vivo* experiments showed that the PHBV group had no bad influence on bone repair, proving that PHBV is biologically safety *in vivo* and may be used in tissue regeneration in the clinic. While DPSCs-IPs/PHBV, DPSCs-NPs/PHBV and Wnt4-DPSCs-IPs/PHBV have much better effect. DPSCs-IPs/PHBV has a slightly decreased bone repair effect than DPSCs-NPs/PHBV, since the osteogenic capacity was inhibited in inflammatory environment. Wnt4 restoration improved the osteogenic potential of DPSCs-IPs *in vivo*.

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Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at <http://dx.doi.org/10.21037/apm-20-460>

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/apm-20-460>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All animals are treated in accordance with the guidelines of Xi'an Jiaotong University College of Medicine for Experimental Animals. All procedures performed were in accordance with the ethical standards of Xi'an Jiaotong University College of Medicine for Experimental Animals. The present study does not contain human participants.

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