SHED Cells Labeled with MIRB can be Tracked in Repairing Periodontal Bone Defects in Rats

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Research

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Abstract

Background: Chronic periodontitis could lead to alveolar bone resorption and even tooth loss. Stem cells from exfoliated deciduous teeth (SHED) are the proper seed cells for bone regeneration because of their potential in osteogenic differentiation. However tracking the survival, migration and differentiation of the transplanted stem cells is necessary to improve the transplantation success.

Methods: Superparamagnetic iron oxide particles (SPIO) Molday ION Rhodamine-B^{TM} (MIRB) were used for labeling and monitoring SHED cells in vivo by magnetic resonance imaging (MRI). Proper labeling concentration of MIRB was determined by cell viability, proliferation, osteogenic differentiation and MRI analysis in vitro after SHED cells were labeled with MIRB at different concentration of 12.5, 25, 50, 100μgFe/mL. MIRB labeled SHED were transplanted to the periodontal bone defect model in rats and tracked by MRI in vivo. The regeneration of periodontal bone were calculated with HE and immunohistochemical analysis. The survival of transplanted SHED cells in vivo was verified with Prussian blue staining.

Results: After testing 25μg Fe/mL MIRB was used in vivo cells tracking. After transplanted to the periodontal bone defect model in rats, the MIRB labeled SHED could be tracked in vivo through the artifact of the low intensity signal caused by Fe^{3+} at 6 and 9 weeks post-surgery. HE and immunohistochemical analysis showed that both SHED labeled and unlabeled with MIRB could promote regeneration of periodontal bone defect. Prussian blue staining further verified the survival of transplanted SHED cells in vivo.

Conclusions: Overall, SHED cells could promote the regeneration of periodontal bone in rats and the survival of SHED cells could be tracked by labeling with MIRB in vivo. However the distribution of the positive cells at the edge of the regenerated new bone remind us the SHED cell could promote the regeneration of new bone by factors section.

Background

Periodontisis is a chronic inflammation which occurs in deep periodontal tissues and could lead to attachment tissue loss, alveolar bone resorption and even tooth loss[1, 2]. Regeneration of alveolar bone is the main objective for periodontal therapy. Tissue engineering has provided new technologies to regenerate periodontal tissue defects by transplantation of appropriate cells and scaffolds[3, 4].

Mesenchymal stem cells (MSCs) are one kind of adult stem cells which have the ability to differentiate into multiple specialized cell types [5]. Stem cells isolated from exfoliated deciduous teeth (SHEDs) are one kind of MSCs. SHEDs could differentiate into adipocytes, osteoblasts, chondrocytes, neural cells and odontoblasts under appropriate induction conditions[6–9].When transplanted into calvarial critical-size defect in immunocompromised mice, SHEDs are capable of producing substantial bone in vivo, suggesting the potential for bone regeneration[10]. Previous studies showed that SHEDs were able to transplant and regenerate bone for repairing mandibular defects in dog models [11]. However, there are

few reports on transplantation of SHEDs for repairing periodontal bone defect. In this study we have transplanted SHEDs into periodontal defect area and examine whether SHED-mediated bone regeneration can be utilized for periodontal defect treatment.

Scaffold is an important influence factor for the success of stem cell transplantation. Fibrin gel, a network formed by fibrinogen when it is activated by thrombin in the presence of Factor XIII and Ca^{2+} ion at the same time[12]. It provides a suitable three-dimensional structure for cell migration, adhesion and differentiation [13, 14]. In previous reports fibrin gel has been reported to stimulate proliferation and osteogenic differentiation of mesenchymal stem cells (MSC) so it has been used as the scaffold for bone regeneration engineering in previous reports [15–17].

However, after transplanted to the defect region, the survival of stem cells is a prerequisite for the success of stem cell therapy [18]. Therefore, the tracking of transplanted stem cells *in vivo* is very important for the development of stem cell therapies. With excellent resolution and localization, magnetic resonance imaging (MRI) has become the main solution for tracking stem cells in live animals and clinical research [19]. MIRB (Molday ION Rhodamine B; BioPAL,Worcester, MA, USA), a new magnetic nanoparticles have been widely used in transplanted cells labeling and tracking [20–22]. Many different types of cells have been labeled with MIRB successfully. The proliferation, phenotype, and differentiation of cells have been investigated after labeling [23–25]. However, little knowledge is known about the biological influences of MIRB on SHED cells. In our study, we characterized the biological properties of MIRB on SHED cells. At the same time, the proliferation and odontogenesis/osteogenesis differentiation of SHED cells were qualitatively and quantitatively analyzed. Furthermore, we transplanted MIRB labeled SHED cells into a periodontal bone defect model in rats, and evaluated the potential of imaging and monitoring of the transplanted SHED cells by MRI *in vivo*.

Methods

1. Cells culture and osteogenic differentiation

Deciduous teeth were obtained from healthy children of 6–8 years old through a procedure authorized by the Ethics Committee of Liaocheng People's Hospital (Liaocheng, China).The SHEDs were isolated from the dental pulp by outgrowth method as described in previous report[26]. For osteogenic induction, SHED cells were cultured in osteogenic induction medium for two weeks and the medium was replaced twice weekly. Osteogenic differentiation medium composition: 90% DMEM (Gibco), 10% fetal bovine serum (Gibco), 100 U/ml streptomycin (Gibco) and 100 U/ml penicillin (Gibco), 10 mM β-glycerol phosphate, 100 nmol/l dexamesone, 50 μM l-ascorbic acid 2-phosphate and 50 nM vitamin D3 (Sigma-Aldrich). Two weeks later after osteogenic induction, mineral deposit formation was identified by Alizarin Red S staining. The cells were first fixed with 4% paraformaldehyde (m/v; Sigma-Aldrich), and then stained with 40 mM Alizarin Red S (pH 4.2; Sigma-Aldrich) at room temperature for 15 min. Finally, images were captured with an inverted fluorescence microscope (Nikon Ti, Japan).

2. Western blot analysis

Total protein was extracted from osteo-induced cells with RIPA buffer and quantified using a BCA protein assay kit (Beyotime, Beijing, China). Total protein about 20μg extracted from osteogenic differentiation and control group were separated with 10% sodium dodecyl sulfate-polyacryl amide gel electrophoresis (SDS-PAGE) and then transferred onto a polyvinylidene fluoride (PVDF)membrane (0.45μm, Milipore, Germy) using the Bio-Rad protein assay system (Bio-Rad, USA). Runx2 (1: 1000, Abcam, Cambridge, MA), Alp (1 : 1000, Abcam, Cambridge, MA), and β-actin (1 : 2000, Santa CruzBiotechnology, Santa Cruz, CA) were used as primary antibodies. Horseradish peroxidase- (HRP-) linked goat anti-rabbit or anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA) were used as the secondary antibodies. All bands were analyzed using Image Lab Software (version 1.6 NIH) to determine the relative level by comparing with the internal control β-actin.

3.Fluorescence microscope observation

Cells were trypsized and seeded in 6-well plates at the density of 2×10^4 cells/cm^2. Molday ION Rhodamine B (MIRB, BioPAL Co., Worcester, MA, USA) stock solution (2mgFe/mL) was added to the normal culture medium (DMEM/F12 + 10% FBS) for preparing labeling medium with the final concentrations of 0, 12.5, 25, 50 and 100 μgFe/mL respectively. SHED cells were incubated in labeling mediun for 24h under standard culture conditions (37℃, 5% humidified CO_2). The cells were washed 3 times with PBS and then fixed with 4% paraformaldehyde for 15 min. After wash 3 times with PBS, the cells were blocked with 5% BSA dissolved in Triton X-100 for 1 hour. Then FITC-phalloidin conjugate solution (Enzo, New York, NY, USA) was used to stain Filamentous actin at room temperature for 1 hour. After washed 3 times with PBS, cells were observed with fluorescence microscope**.**

4. Prussion blue staining and labeling efficiency

After labeled with various concentration of MIRB, SHED cells were fixed with 4% paraformaldehyde. Wash 3 times with PBS. SHED cells were incubated with Perl's Prussian blue reagent (2% potassium ferrocyanide was dissolved in 6% hydrochloric acid) (Leagene Biotechnology, Beijing,China) for 30min. The distribution of intracellular Fe^{3+} was observed with light microscopy. In general 10 random fields of view were selected under a 10x microscope to count Prussian blue positive staining cells, and the labeling efficiency was calculated as following: Labeling efficiency = (number of Prussian blue positive cells/ number of total cells) × 100%.

5. Detection of intracellular iron content

The average intracellular iron content was measured with an iron assay kit (Biovision,Inc., CA, USA). The detailed detection method is as follows: First, SHED cells were seeded in a 6-well plate at the density of 2×10^4 cells/cm^2 and incubate with MIRB labeling medium for 24h. Then labeled SHED cells were lysed with 65μL Iron Assay Buffer. Centrifuge the lysed product at 16000g for 10 minutes. Add 50 μL supernatant to the well of 96-well plate and add an appropriate volume of Iron Assay Buffer to make the final volume 100 μl. Add 5μL iron reducer to each well for transforming Fe^{3+} into Fe^{2+}. Add 100 μL Iron

Probe into each hole, and incubate for 1 h at room temperature. Absorbance was read with a spectrometer at 593nm. Meanwhile iron content standard curve was drawn according to the instructions in the kit. Calculate the total iron content in each hole according to the standard curve. Count the number of cells in each well with a hemocytometer. Iron content of a single cell = total iron content per well/number of cells per well.

6. Trypan blue staining and viable cells percentage calculation

Prepare 4% trypan blue stock reagent by adding 4g trypan blue (Merck KGaA, Darmstadt, Germany) to 100mL double-distilled water and filter with filter paper. Before use dilute to 0.4% with PBS. Five groups of cells in the control and labeled group were trypsinized to prepare single cell suspension and diluted appropriately. Mix 1 part of cell suspension and 1 part of 0.4% trypan blue and allow the mixture incubate at room temperature for about 3 minutes. Then count the live and dead cells separately (the microscope shows that the dead cells are stained light blue, and the viable cells are colorless after staining). Viable cells percent (%) = number of viable cells/(number of viable cells + number of dead cells) × 100%.

7. The effect of MIRB labeling on the proliferation of SHED cells

Seed SHED cells into the 96-well plates at the density of 1000 cells/well. The SHED cells are divided into 5 groups: 0, 12.5, 25, 50, 100μg Fe/mL respectively. There are 8 multiple holes in each group, each hole is 100μL. After SHED cells adhered to the wall, 4 groups of cells were labeled with different concentrations of MIRB. After 24h incubation, change to normal culture medium and continue culture for 1, 3, 5, and 7 days. Then add 10μL CCK-8 solution (Yeasen, Shanghai, China) to each well at each time point, incubate at 37°C for 4 hours, and detect the absorbance at 450nm with a microplate reader.

8. The effect of MIRB labeling on the osteogenic differentiation of SHED cells

SHED cells were trypsinized and seeded in a 6-well plate at the density of 2×10^4cells/cm^2. When the cells reach 80% confluence, change the culture medium to MIRB labeling solutions with concentrations of 0, 12.5, 25 and 50μg Fe/mL respectively. After incubate for 24 hours, MIRB labeling medium was replaced with the osteogenic induction fluid, and the induction was terminated after 14 days. Fix the cells with 4% paraformaldehyde for 30 minutes and wash three times with PBS. Add 2mL Alizarin Red S solution (pH 4.2; Sigma-Aldrich) to each well and stain for 15 minutes. Wash 3 times with PBS, take pictures and record the staining of each well. For semi-quantitative analysis of Alizarin Red S, add 600 μL cetylpyridinium chloride solution (100g/L; Sigma-Aldrich) to each well and incubate at room temperature for 15 min. Detect the absorbance of the supernatant at the wavelength of 562nm.

9. Magnetic Resonance Imaging of MIRB labeling SHED cells in vitro

SHED labeled with different concentrations of MIRB (0, 12.5, 25, 50μg/mL) were collected and counted with a hemacytometer. One million cells per group were transferred to 1.5ml centrifuge tubes (Eppendorf, Westbury, NY, USA) respectively. After centrifuged at 150×g for 5 min, SHED cells were resuspended in

15μL thrombin solution. Then the 15 μL thrombin solution containing 1×10^6 cells and 15 μL fibrinogen solution were injected into the bottom of the 1.5ml centrifuge tube at the same time. The mixture condensed into a jelly in 1-2 minutes. Then the tubes were imaged with a 1.5 T system (Achieva, Philips Healthcare, the Netherlands) with a 8-channel Wrist Coil(repetition time [TR] =20ms, International time [TE] = 8.1ms, flip angle = 25, field of view [FOV] =80 × 80×30mm ^{3} , slice thickness = 4mm, Mat: 208 × 208).

10. Animals and group design

Forty eight SD rats about 6-8 weeks old (200-250g, male) were used in our study. For investigating the effect of transplanted SHEDs on periodontal bone regeneration, all rats used in this research were numbered and divided into four groups randomly. Each rat has one defect at the right mandibles. The four group rats were: (1) SHED(MIRB labeled), (2) SHED, (3) Fibrin and (4) PBS. Six rats in each group were anesthetized at 0, 3, 6 and 9 weeks after the operation, and the defect healing was captured by MRI. At 2 and 4 weeks after operation, 3 rats in each group were perfused to obtain samples of periodontal bone defects for Histological and Immunohistochemical analysis.

11. Preparation periodontal bone defects in rats

Periodental bone defects were constructed as the following procedures. Rats were anesthetized with 10% chloral hydrate (at the dose of 4ml/kg) by intraperitoneal injection. An extra-oral incision about 2cm long was made which was parallel to the inferior edge of rat mandibles. Separate the subcutaneous tissues and masseter muscles from the surface of mandible. A periodontal wound defect (length×height×depth: 5mm×4mm×1mm) was constructed with a dental drill at a low speed accompanying with lasting irrigation of physiological saline. The defect was located at 1mm behind the front of the mandible and 1mm below the crest of the alveolar bone. Muscle and skin were sutured separately and the wound was clean with Iodophor after the surgery. In order to avoid being rejected of the transplanted cells by the immune system, the rats were injected with cyclosporine A (10 mg/kg; Sigma) intraperitoneally one day before transplantation and every day after transplantation until the rats were sacrificed.

12. Preparation of fibrin glue containing SHED cells for transplantation

Fibrin glue was prepared using a Fibrin sealant kit (SHANGHAI RASS, Shanghai, China) according to the instructions of manufacturer, by ejecting equal volumes (15 μl) of a fibrinogen solution (40 mg/ml) and thrombin solutions (450 IU/ml) through a two-channel syringe. For cell transplantation, one million SHED fresh cells (MIRB labeled of unlabeled) were resuspended with 15μL thrombin solution. After preparation of periodontal bone defects in rats, the thrombin solution containing SHED cells (labeled or unlabeled) were injected into the defect region along with equal volume fibrinogen solution at the same time. In Fibrin group only fibrin glue without SHED cells and in PBS group only equal volume PBS was injected to the defect region.

13. MRI of MIRB-Labeled SHED *in vivo*

MRI of anesthetized rats was performed at 0, 3, 6 and 9 weeks after transplantation. All rats undergoing MRI testing were performed on a 1.5 T system (Achieva, Philips Healthcare, the Netherlands) using an 8-channel wristband coil. Rats were positioned head forward in a prone position. The axial and coronal three-dimensional fast field echoT1-weighted imaging“black bone” sequence was scanned. Sequence specifications are listed in Table 1.

Table 1. The dedicated parameters for the 3D T1 FFE sequence.

Axial 3D T1 FFE
Acquisition time 4:44min (02:48 min and 01:56min respectively)
FOV 80 ×80×30mm^3
Matrix 208× 208
Voxel size 0.385 × 0.385 × 0.75mm^3
Acq voxel 0.385 × 0.385 × 1.50mm^3
REC voxel 0.16 × 0.16 × 0.75mm^3
Reconstuction Matrix 512
Number of signal averages 2
TR 20 ms
TE 8.1ms
Flip angle(deg) 25
WFS (pix)/bandwidth (Hz) 1.004/216.3

14. MRI Image Analysis and Quantification

For image analyzing, the obtained DICOM datasets were reconstructed in the axial and coronal plane. The reconstruction slice number was 40 and the thickness was 0.5 mm. The qualitative and quantitative evaluations were performed separately by two radiologists certified by the Radiology Committee without interfering with each other. Imaging parameters comprised the defect area in defined regions of interest (ROIs) and signal intensity value.

15. Samples collection and preparation for histological and immunohistochemical analysis

Rats were sacrificed at 2 and 4 weeks after operations. First rats were anesthetized with chloral hydrate (at the dose of 4ml/kg) and then perfused with 200ml normal saline and finally fixed with 200ml 4% paraformaldehyde. The right mandible of each rat was excised and fixed with 4% PFA for 24h. Then these mandibles were decalcified in 10% ethylenediaminetetraacetate (EDTA) at room temperature for 4 weeks.

The decalcification solution was replaced twice a week. After decalcification is completed, the bone tissue was dehydrated with gradient ethanol and immersed in paraffin wax for 2hours. The tissue was embed in paraffin and cut into sections of 3-4μm thick. These paraffin sections were placed in a 62℃ oven for 20min for HE, or 2hours for immunohistochemical staining.

16. Histological analysis

In order to evaluate the regeneration of periodontal tissue in each group, every fifth slices were selected and stained with hematoxylin and eosin kits(Beyotime, China). Then the specimens were observed under Nikon Ti microscope (Nikon,Japan) and the area of newly formed bone was measured by Image Pro-plus 6.0 software (Silver Spring, USA). The regeneration of defect area was calculated with the flowing equation: Percentage of bone regeneration = area of newly formed bone /area of total defect×100%.

17. Immunohistochemical staining

The osteogenesis of the mandibular defect was estimated by immunohistochemical analysis of Runx2 and Ocn. The paraffin sections were dewaxed with xylene treatment and rehydrated in gradient ethanol. Immunohistochemical staining was performed with Biotin-Streptavidin HRP Detection Systems (ZSGB-BIO, Beijing, China) according to the instruction of the kit. Briefly, to inhibit endogenous peroxidase, the sections were incubated with 3% hydrogen peroxide for 10 min. To block nonspecific staining, the sections were blocked in 1% goat serum for at least 30 min. Then these sections were incubated with primary antibodies of Runx2 (1:1000) and Ocn (1:500) (Abcam, Cambridge, MA, USA) for 2 hours at room temperature. Negative controls were incubated with PBS instead of the primary antibodies. The sections were then incubated with biotinylated goat anti-mouse or goat anti-rabbit immunoglobulin G (IgG) as a secondary antibody at 37°C for 15 min. The Immunoreactions were detected with diaminobenzidine (DAB) substrate kit (Maxim biotechnology Co., Ltd., Fuzhou, China). After being rinsed with running water for 5 min, the sections were counterstained with hematoxylin for 30 sec. Images were taken with a Nikon microscope (Nikon, Japan). Integrated optical densities (IOD) of Ocn and numbers of Runx2 positive cells in four groups were measured or counted using the Image-Pro Plus 6.0 software (Media Cybernetics, Silver Spring, MD, USA).

18. Prussian blue staining of iron ions in vivo

Prussian blue staining was performed according to the instruction of the staining kit (Leagene Biotechnology, Beijing,China). Briefly, mix the A1 solution (2% potassium ferrocyanide) and A2 solution (6% hydrochloric acid) in the kit in a 1:1 volume to form the Perl's staining solution. After the paraffin sections are deparaffinized and rehydrated, add Perl's staining solution and incubate at room temperature for 30 minutes. Rinse three times with PBS, and finally counter-stain with solution B (nuclear fast red) in the kit for 5 minutes. Rinse three times with PBS, mount the slide after dehydration, and observe the staining under a microscope.

19. Statistical analysis

Data were expressed as the mean ± standard error of the mean and all the data were analyzed using SPSS software (SPSS Inc., IL, USA). Statistical significance was tested by Independent-Samples t-test. P < 0.05 was considered statistically significant.

Result

1. Isolation of stem cells from human exfoliated teeth and osteogenic induction analysis

SHEDs were isolated from 6-8 years old children as described in previous report[26](Figure 1A). In order to evaluate the osteogenic capability of SHEDs, cells were cultured in osteogenic differentiation medium for two weeks. Alizarin red S staining results showed that SHEDs were able to form obvious calcified nodules after induction (Fig.1 B). Western blot showed that expression of Runx2 and Alp was increased to10 times and 4.5 times respectively after osteogenic induction treatment than non-treated control (Fig.1 C, D).

2. Morphological observation and labeling efficiency statistics of MIRB labeled cells

After 24 hours incubation with different concentrations of MIRB, the cytoskeleton and morphology of cells was stained with FITC-phalloidin which showed green fluorescence. On the other hand, the accumulation of MIRB in the cell was indicated by red fluorescence which showed by MIRB particles itself. With the increase of MIRB concentration, there is no change in cell morphology as showed by green fluorescence (Figure 2 A). The red fluorescence of MIRB became brighter and brighter with the increase of labeling concentration, indicating that MIRB gathered more and more in cells (Figure 2 A). Merged images of nuclei (blue), cytoskeleton (green) and MIRB (red) showed that MIRB labeled positive cells was all nearly 100% under various labeling concentrations. A similar result was observed in calculating the percent of positive cells that were stained with Prussian blue staining (Figure 2 B).

3. Intracellular iron content

In order to find the relationship between the intracellular iron content and the MIRB concentration, iron assay kit was used according to the instructions of manufacturer. The detection of iron content showed that with the increase of labeling concentration, the iron content gradually increased, and the highest iron content was found under 100μg/ml labeling (Figure 2 C).

4. Cell viability of MIRB-labeled SHED

To determine the effect of MIRB labeling on the growth of SHED cells, Trypan blue staining was performed to identify the living and dead cells. At the concentration of 12.5-100 μg/mL, trypan blue staining showed that 100μg/ml MIRB affected the survival of cells and the ratio of living cells decreased. Other concentrations had little effect on cell survival rate (Figure 2D).Therefore MIRB concentration below 100μg/mL was safe for SHED labeled.

5. Cell proliferation of MIRB-labeled SHED

The cell growth curve was determined under the MIRB concentration of 12.5-100μg/ml. The results showed that the cell growth was not affected by the MIRB concentration of 12.5-50μg/ml, but the cell growth was significantly inhibited after 100μg/ml MIRB labeling (Figure 3 A).The result suggested that high concentration of MIRB will affect the growth of cells after transplantation. Therefore, 100 μg /ml MIRB is not suitable for cell labeling and tracking in vivo. The concentration of 100ug/ml will not be used to label cells in the following experiments.

6. Osteogenic differentiation of MIRB-labeled SHED

In order to determine the effect of MIRB labeling on the osteogenic differentiation of SHED cells, Alizarin red S staining and quantitative analysis were performed after 14 days osteogenic induction of labeled SHED cells. The results showed that there is no significant difference in the osteogenic differentiation ability of the labeled and unlabeled SHED cells ($P>0.05$) (Figure 3 B, C). The MIRB labeling concentration of 12.5-50 μg/ml has no harmful effect on cell growth and differentiation, so it can be used as a safe concentration for cell tracking *in vivo*.

7. MRI analysis of SHED labeled with MIRB in vitro

The above experiments prove that MIRB is safe for cell growth and osteogenic differentiation within the concentration range of 12.5-50μg/ml. In order to save experimental costs, we need to find the lowest MIRB marker concentration that can guarantee the effect of MRI imaging. Therefore, we observed 1×10^6 cells at three concentrations of 12.5, 25 and 50 μg/ml. The labeled cells were resuspended with 15ul thrombin, and then prepared into a jelly mixture with 15ul fibrinogen for MRI Shoot. The area containing MIRB-labeled cells appeared as a low signal intensity area on the MRI image. The low signal area of cells labeled with MIRB could be observed and the signal intensity increased with the increasing of MIRB concentrations (Figure 4). Considering the signal intensity after labeling and the effect of labeling on cell growth and osteogenic differentiation, we decided to use 25 μ g / ml MIRB to label cells in vivo.

8. Construction of periodontal bone defect and preparation of stem cell transplantation complex

The experiment were divided into four groups: SHED(MIRB labeled), SHED, Fibrin, PBS. A defect 5 mm × 4 mm × 1 mm (length × width × depth) was constructed on the mandible bone (Figure 5 A.B).Fibrinogen solution 15μL and thrombin solution 15μL containing 1×10^6 SHED cells were injected into the defect region at the same time (Figure 5 C). The liquid will turn into a gel after 1-2 minutes. The muscle layer and skin layer were sutured respectively.

9. Tracing the survival state of transplanted cells

After transplantation of fibrin glue complex containing MIRB-labeled and unlabeled SHED cells, MRI images were taken at 0,3,6,9 weeks respectively after surgery. Compared with Fibrin and PBS groups at the same time point, the percent of bone healing in the SHED(MIRB) and SHED (unlabeled) group was higher at 3 and 6 weeks post-surgery (Figure 6). It is suggested that SHED transplantation to periodontal

bone defect is helpful to repair the defect. Since Fe^{3+} can reduce the signal, the artifact of the low intensity signal can be observed at 6 and 9 weeks post-surgery as red arrows indicated in Figure 6 A.

10. Evaluate the regeneration of the defect with HE and immunohistochemistry

In addition to MRI, HE and immunohistochemistry are also used to analyze the healing of bone tissue *in vivo*. Statistics of the percentage of new bone in the defect area 4 weeks after surgery showed that there was no significant difference in the percentage of new bone between the SHED (MIRB) group and the SHED group. The percentage of new bone in SHED (MIRB) and SHED groups was significantly higher than that in Fibrin and PBS groups ($P<0.05$) (Figure 7 A.B). Runx2 immunohistochemistry was performed 2 weeks after the operation, and the number of Runx2 positive cells was statistically analyzed. The statistical results showed that the number of Runx2 positive cells in SHED (MIRB) and SHED groups was significantly higher than that in Fibrin and PBS groups ($P<0.01$) (Figure 7 A.D). The optical density of OCN immunohistochemical staining at 4 weeks after operation was statistically analyzed. The results showed that the expression of OCN in SHED (MIRB) and SHED groups was significantly higher than that in Fibrin and PBS groups ($P<0.01$) (Figure 7 A.C).The results indicated that transplantation of labeled or unlabeled SHED cells can promote the healing of bone tissue.

11. Demonstrate the presence of MIRB-labeled cells by Prussian blue staining

In order to verify the survival of SHED labeled with MIRB, Prussian blue staining was performed on the tissue 3 weeks after surgery. The results showed that MIRB-labeled cells existed in the tissue of the SHED (MIRB) group, but were not detected in the SHED, Fibrin, and PBS groups. Most of the SPIO-positive cells exit in the edge of the bone tissue while very few SPIO-positive cells were detected in the interior of the bone tissue (Figure 8). The results showed that SHED cells can repair bone defects by regulating the local microenvironment or activating endogenous progenitor cells by secreting biological factors.

Discussion

Destruction of alveolar bone is a common hallmark of human periodontitis which is the major cause of tooth loss in human[27]. With the development of tissue engineering in recent years, stem cell based therapy has become a hot spot in the study of periodontal bone regeneration [4]. However, before the application of stem cells to clinical treatments, the survival, migration, and differentiation of transplanted stem cells are important subject that we need to explore[28]. In this study, we used SPIO to monitor the in vivo behavior of implanted SHED cells and investigated their fate for success of cell transplantation therapies.

Superparamagnetic iron oxide (SPIO) is an ideal tracing marker for MSCs and the SPIO-labeled MSCs could be observed by magnetic resonance imaging (MRI). Molday ION Rhodamine-B™ (MIRB), a new SPIO tracer marker which is labeled with a fluorescent dye rhodamine B, can be visualized by both MRI and fluorescence imaging [29, 30]. However, a high intracellular free iron concentration was toxic to the labeled cells. In the current study, various concentrations of 12.5, 25, 50 and 100 μg Fe/ml were used to

incubate with SHED cells to determine the proper labeling concentration. Our results showed that the viability and proliferation of the cells were both affected by the concentration of 100 μg Fe/ml which is consistent with previous reports[31].

MSCs are important cell resources in periodontal tissue engineering. Among them, SHEDs are a candidate cell source for periodontal regeneration and have been proven to be an ideal seeding cells for periodontal tissue engineering [32, 33]. In our previous study we have isolated and characterized SHEDs cells and proved the self-renewal and multi-lineage differentiation ability. In this study, we have transplanted SHED cells to repair periodontal bone defect. HE and Immunohistochemistry staining results showed that both SHED labeled or unlabeled with MIRB could promote the regeneration of new bone issue.

In recent years, MRI has provided a non-invasive method to monitor the survival and migration of transplanted cells labeled with SPIO [34]. MRI signal intensity is directly related to the amount of intracellular SPIO in the surviving cells [35]. In the present study, we have proved that different MIRB concentration of 12.5, 25, 50,100 μg Fe/ml labeled SHED precipitations in tubes were visualized with high-resolution MRI in vitro. Additionally in vivo, the transplanted SHED cells were successfully tracked by MRI, and Prussian blue staining further indicated that the SHED could adhered to edge of the periodontal bone defect. In our study, the combination of MRI and histological observation can accurately determine the survival and location of the implanted SPIO-labeled SHED.

HE and Immunohistochemistry staining results showed that labeled and unlabeled SHED provided a possible therapeutic benefit in periodontal bone regeneration. However most of the SPIO-positive cells exit in the edge of the bone tissue while very few SPIO-positive cells were detected in the interior of the bone tissue. The therapeutic effects of transplanted cells may depend on factors secretion of various biological factors for modulating local microenvironment or activating of endogenous progenitor cells.

Conclusions

In the present study, the transplanted SHED cells promoted periodontal bone regeneration. SPIO-labeled SHED cells injected into the periodontal bone were successfully and non-invasively tracked by MRI. In further, implanted SHED cells adhered to the edge of the regenerated bone tissue and suggested that stem cells may promote bone regeneration by improving microenvironment in periodontal bone regeneration defects. Our studies provides theoretical basis for clinical application of SHED cells in periodontal bone defect regeneration.

Abbreviations

SHED
Stem cells from exfoliated deciduous teeth
SPIO
superparamagnetic iron oxide particles

MIRB
Molday ION Rhodamine-BTM
MSCs
Mesenchymal stem cells; MRI:magnetic resonance imaging

Declarations

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Contributions

Nan Zhang and Fabin Han were responsible for the study design and manuscript writing. Nan Zhang, Li Xu, Hao Song and Chunqing Bu were responsible for performing the experiments, analysis and interpretation of the data. Chuanchen Zhang, Jie Kang and Xiaofei Yang also provided help for perfoemance of the experiments. All authors read and approved the final manuscript

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Ethics Declarations

Ethics approval and consent to participate

All experimental procedures in this study were approved and performed in accordance with the guidelines of the Ethics Committee of Liaocheng People's Hospital.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

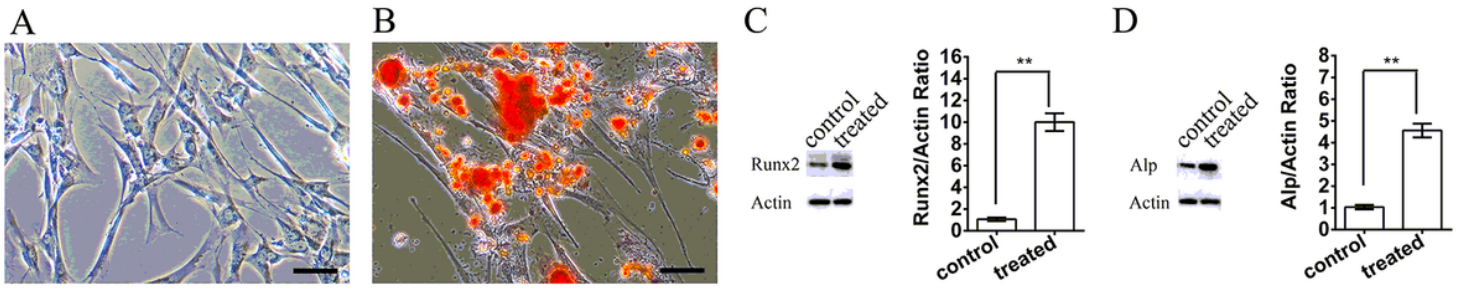

Figure 1

SHEDs culture and osteogenic differentiation A. The morphology of SHEDs isolated from dental pulp tissue of Children. B. Alizarin Red S staining of SHEDs after osteogenic induction. C.D. The variety of Runx2 and Alp expression before and after osteogenic induction.

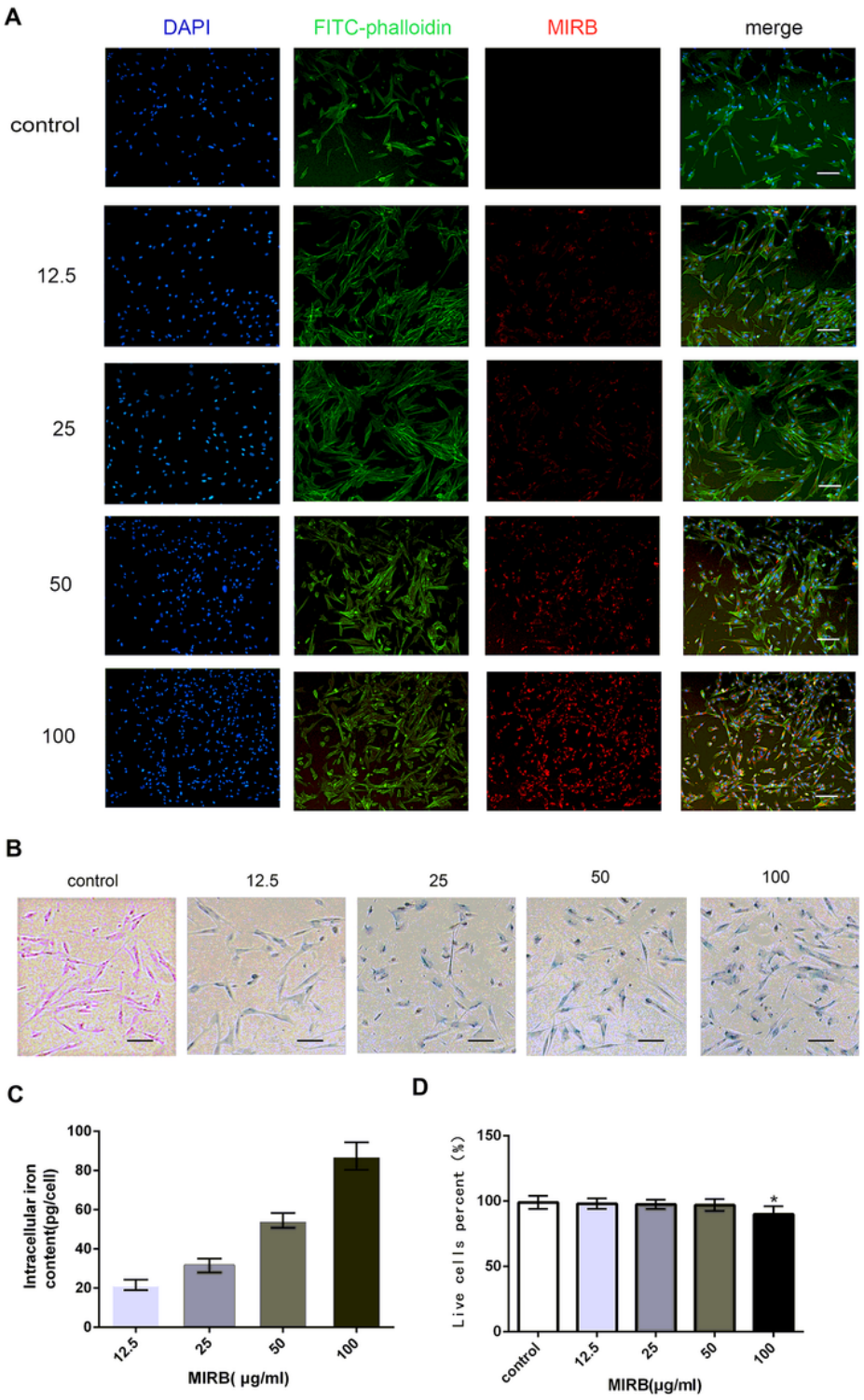

Figure 2

Detection of morphology, iron content and living cell ratio of SHED cells treated with various concentrations of MIRB. A. Morphology of SHED cells exposed to various concentrations of MIRB. The nuclei of SHED are stained with DAPI (blue); the cytoskeleton is stained with FITC-phalloidin (green); the MIRB particles show red. B. Fe3+ particles were detected with Prussian blue staining. C. Iron content

analysis of SHED cells. D. Living cells percent was analyzed with Trypan blue staining. The scale bar indicates 100 μm.

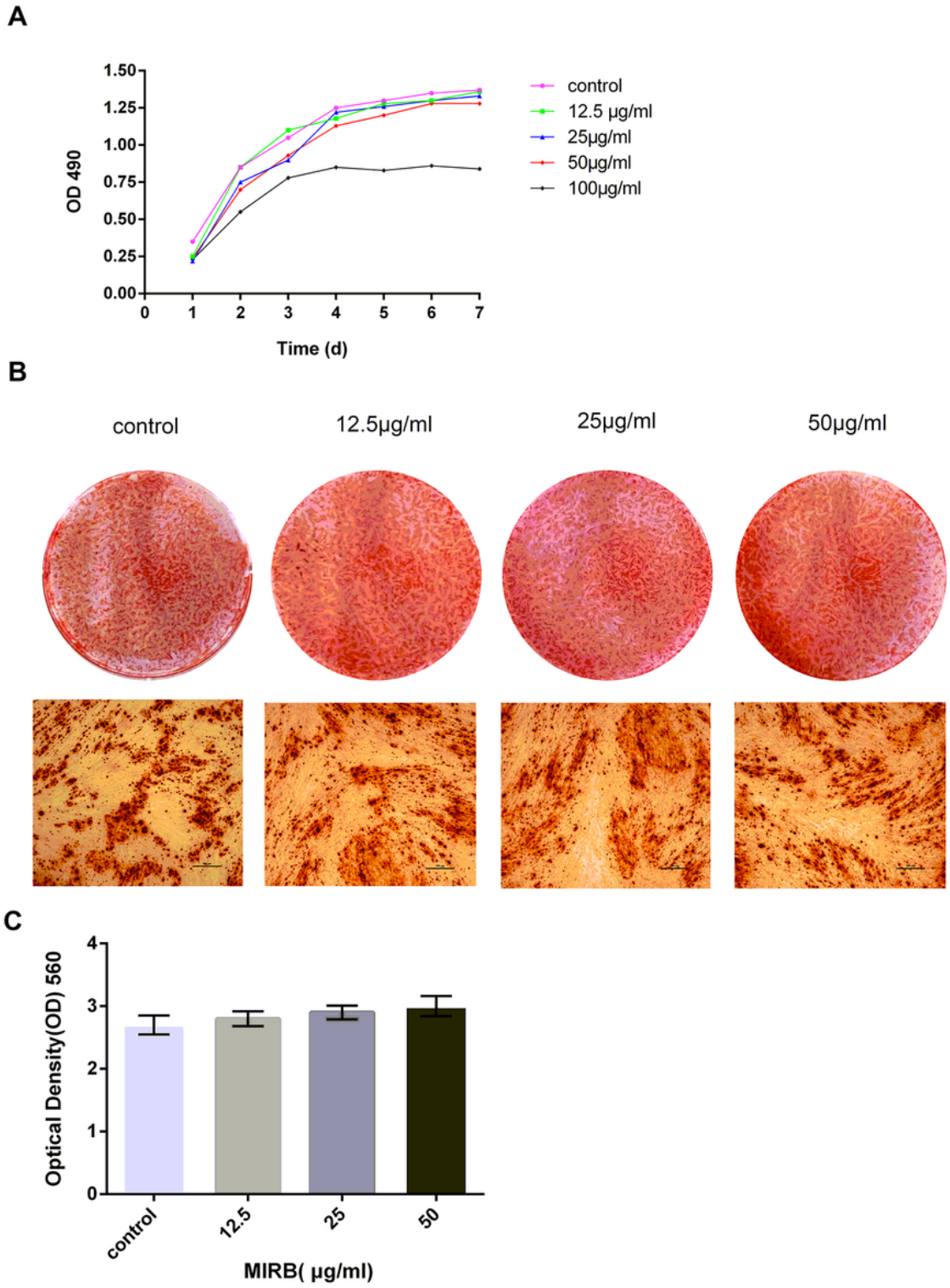

Figure 3

Growth curve and osteogenic differentiation of SHED cells labeled with various concentrations of MIRB. A. Growth curve of SHED cells labeled with various concentrations of MIRB. B.C. Alizarin red S staining

and quantitative analysis of SHED cells labeled with various concentrations of MIRB after osteogenic differentiation induction. The scale bar indicates 100 μm.

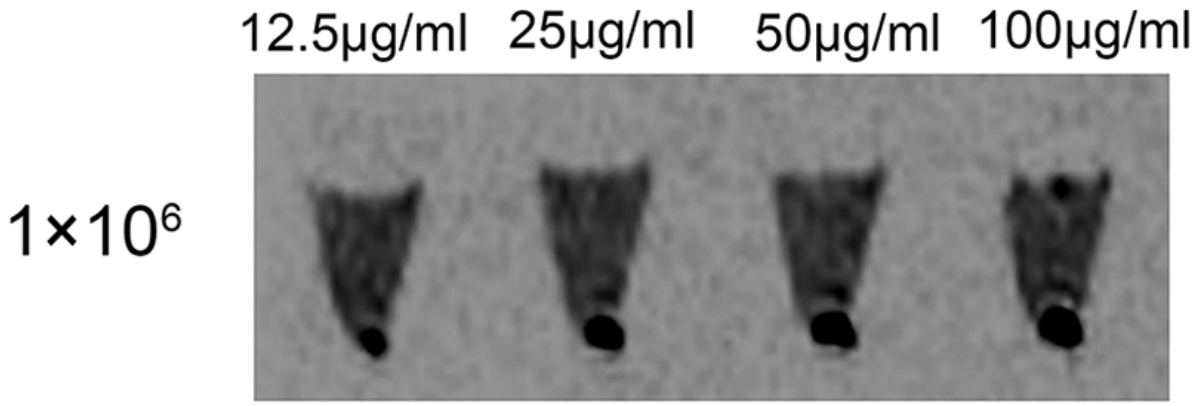

Figure 4

MRI of 1×106 SHED cells labeled with various concentration of MIRB in vitro.

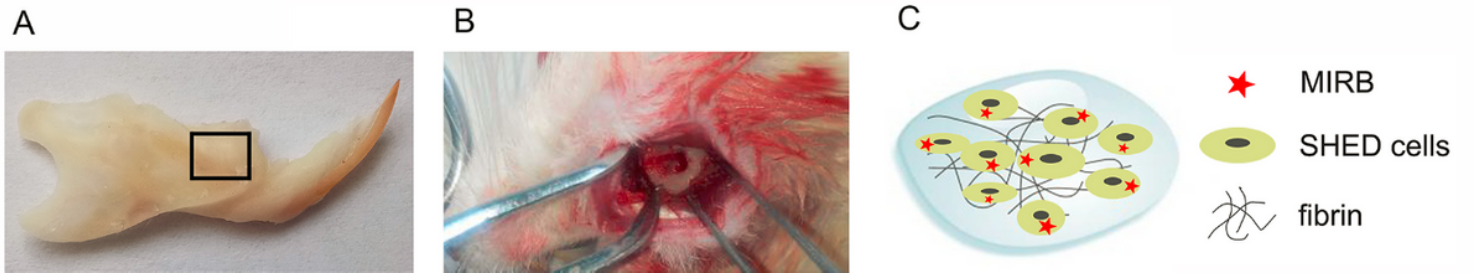

Figure 5

The construction of periodontal bone defect in rats A. The location and shape of the defect on the mandible of rats. B. The created periodontal fenestration defect. C. Illustration of transplantation complex SHED cells labeled with MIRB in experimental group.

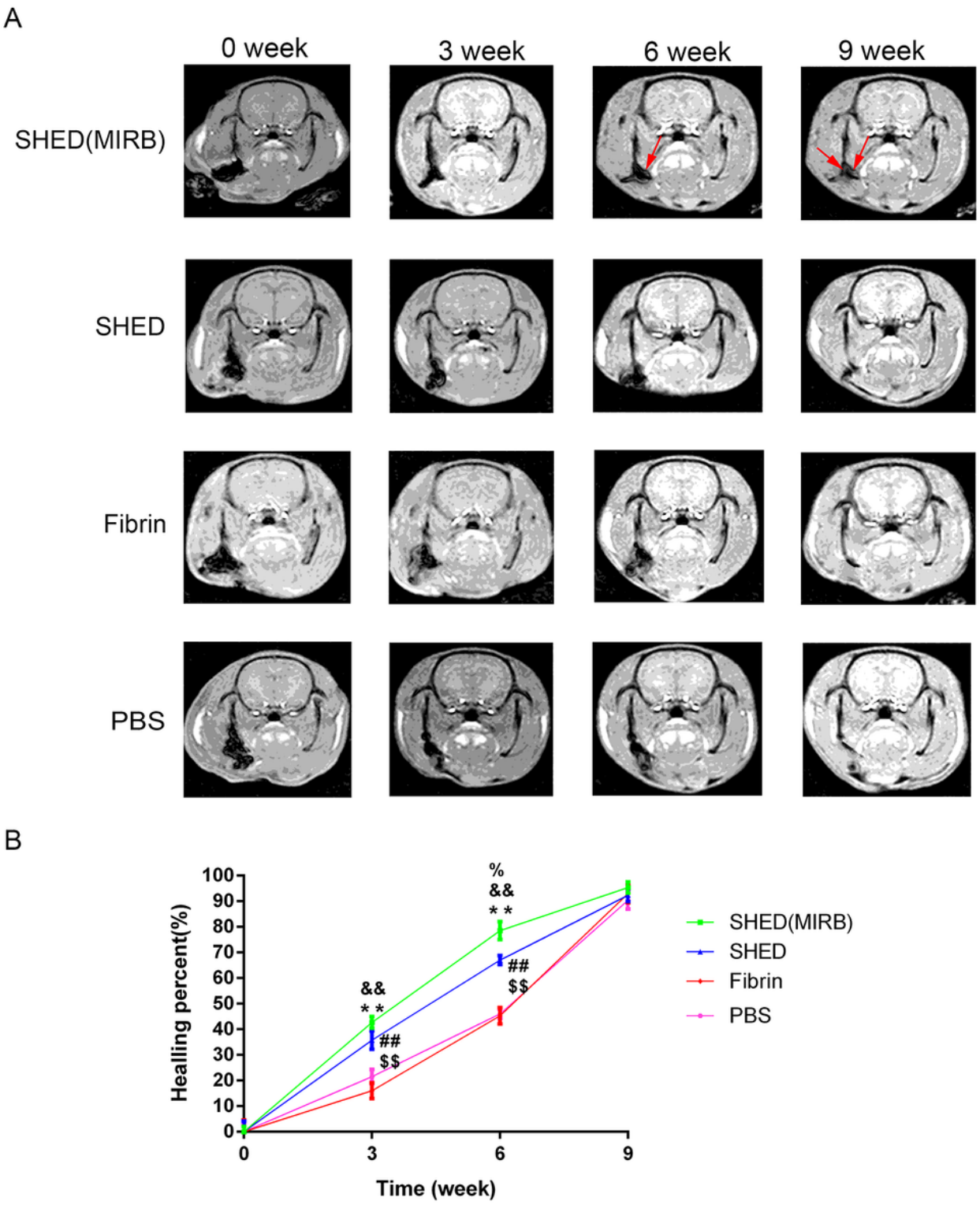

Figure 6

MRI examination of bone tissue recovery in vivo A. In vivo MRI images of rats at 0,3,6,9 weeks after transplantation. MRI image of SHED(MIRB) at 6 and 9 weeks post-surgery has a reduced signal intensity (red arrows) region. This region indicates the presence of SPIO-labeled SHED at the periodontal bone defect. B. The average healing percent of bone tissue at each time point was calculated according to the results of MRI. Healing percent= (total defect area-present defect area)/total defect area. %,&,*

:SHED(MIRB)group compare with SHED,Fibrin,PBS group respectively. #,$:SHED group compare with Fibrin,PBS group respectively. %,&,*,#,$ P<0.05. %%,&&,**,##,$$ P<0.01

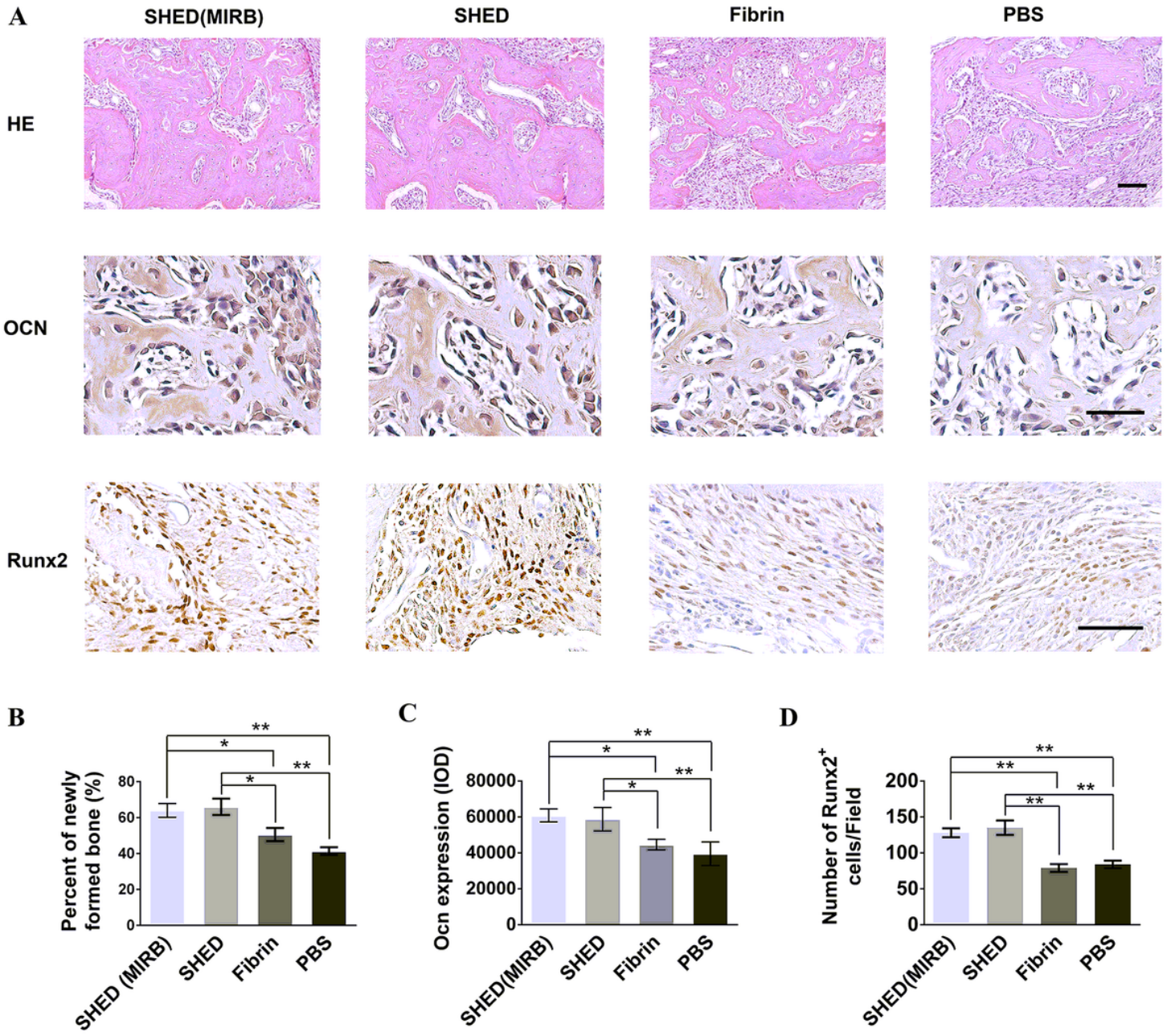

Figure 7

The effects of SHED (MIRB labeled and unlabeled) cells on bone regeneration. A. HE staining(at 4 weeks post-surgery) and Immunohistochemical staining of OCN (at 4 weeks)and Runx2(at 2 weeks). HE staining and quantitatively analysis of the four groups of new bone regions. C. Quantitative analyses of OCN expression in four groups. D Comparison of number of Runx2+ cells in four groups. B-D. ** P<0.01,*P<0.05. The scale bar indicates 100 μm.

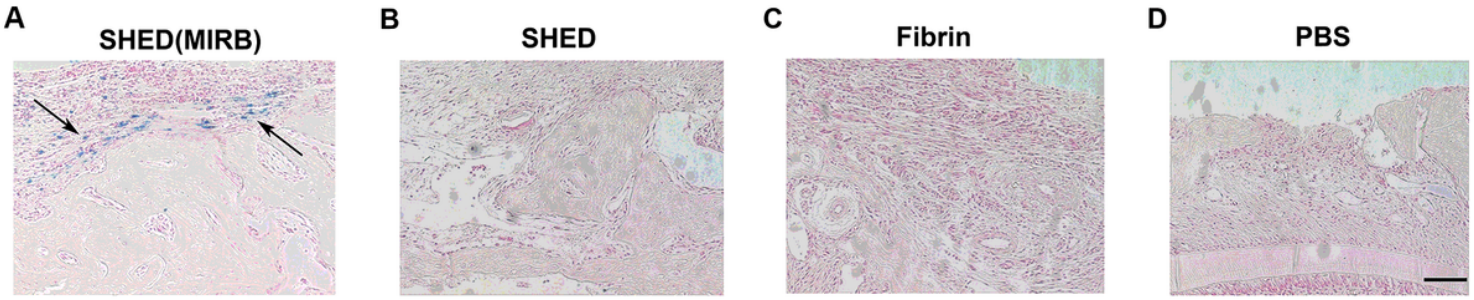

Figure 8

Prussian blue staining of periodontal bone defect specimens after transplanted with SHED (MIRB labeled), SHED, Fibrin and PBS respectively 3 weeks post surgery. Arrows indicated the positive cells of Prussian blue staining. The scale bar indicates 100 μm.