Original Article

The effectiveness of allogeneic mesenchymal stem cells therapy for knee osteoarthritis in pigs

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Background: Intraarticular injection of the mesenchymal stem cells (MSCs) has shown to be successful for treating osteoarthritis (OA). Nevertheless, many studies have been focusing on autologous MSCs. The following study investigates the safety and effectiveness of intraarticular injection of allogenic MSCs in a pig OA model.

Methods: Superparamagnetic iron oxide (SPIO) nanoparticles were labelled with bone marrow-derived mesenchymal stem cells (BM-MSCs) to allow cells tracking using magnetic resonance imaging (MRI). A pig OA model was established by bilateral medial meniscectomy. Next, SPIO-BM-MSCs were injected into the right knee, while the left knee was left untreated. MRI and radiography were used to assess the degree of OA and to evaluate the effectiveness of allogenic MSCs. Hematoxylin and eosin (H&E), safranin-o fast green staining, toluidine blue, and immunohistochemical staining were used to evaluate the therapeutic effect of the injections.

Results: At concentration of ≤20 μg/mL, SPIO caused no toxicity to BM-MSCs. Four weeks after surgery, OA changes were observed on MRI scan. The SPIO labeled BM-MSCs were found moving towards the impaired part of the cartilage 8 to 24 h after injections. In addition, no significant differences between the right side (therapeutic side) and the left side (untreated side) were observed following histological and immunohistochemistry analysis.

Conclusions: The suitable concentration of SPIO for labelling BMSCs was 20 μg/mL, while the allogenic MSCs could move towards and accumulate around the impaired cartilage. No significant difference was found between treatment and control group.

Keywords: Intra-articular injection; allogenic; mesenchymal stem cell (MSC); osteoarthritis (OA)

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Introduction

Knee osteoarthritis (OA) is a common chronic disease and a major cause of disability in people aged 45 and above (1). Management of knee OA can be roughly divided into nonsurgical and surgical interventions. The choice of treatment is based on symptoms, OA stage, and patient-related factors (2). Referral for consideration of surgical intervention (osteotomy, arthroscopic interventions, and knee arthroplasty) is indicated after more conservative treatment options have been exhausted (3). Surgical treatments such as total knee arthroplasty, are more suitable for patients with late stage knee OA; however this procedure may lead to the joint disfunction (4). Nonsurgical treatments include lifestyle modifications and exercise (losing weight, switching from running or jumping exercises to swimming or cycling), pharmacologic therapies (complementary and alternative medicine, nonsteroidal anti-inflammatory drugs), and physiotherapy (kneading and hot compress) (2,5).

Cell therapy and tissue engineering approaches have become an alternative option for treating cartilage defects (4). Intra-articular injections may include administration of stem cells collected from different sources, platelet-rich plasma (PRP), hyaluronan preparations, and ozone (6,7). Mesenchymal stem cells (MSC) are superior to others due to: (I) the self-renewal ability; (II) being essential for normal turnover and maintenance of cartilage; (III) being capable to migrate to the damaged area of cartilage; and (IV) having the ability to induce chondrocyte proliferation and extracellular matrix (ECM) synthesis (4).

There are a plethora of reports on management of knee OA using MSC which have reported conflicting results mainly due to heterogeneous methodologies used (8). In several studies bone marrow MSCs (BM-MSCs) has been used as a source of MSCs. Thus, an effective *in vivo* cell tracer technology is necessary to distinguish the donor MSCs and to observe its migratory pathway to the receptors. At present, there are two methods that can be applied for tracing the cell, namely nuclear medicine and magnetic resonance imaging (MRI) (9). Nuclear medicine imaging technology is characterized by high sensitivity and specificity, but has the disadvantages of radioactive decay, short imaging time and low spatial resolution; while MRI can provide a resolution of up to 25–50 μm (9). Therefore, MRI has the high spatial and temporal resolution, and promising application prospect for real-time cell tracing. For tracing transplanted cells *in vivo* with MRI, labeling transplanted cells with contrast medium and high safety is an important issue (10). Currently, the contrast agents used for stem cell magnetic labeling are mainly based on magnetic iron oxide as the negative contrast agent, resulting in a strong T2 negative contrast effect (11). Contrast agents are divided into two types according to the size of the particle diameter: superparamagnetic iron oxide (SPIO) with diameter greater than 50 nm, and ultra-small superparamagnetic iron oxide (USPIO) with a diameter that is less than 50 nm (12). SPIO has been widely used for labeling transplanted cells due to its low threshold concentration and small cytotoxicity to MRI (13).

The aim of the present study was to label SPIO to allogenic BM-MSCs in order to examine the cell migration and therapeutic effect in pig OA model.

Methods

Study design

Six female bama miniature pigs, 150–180 days old, weighing 20–25 g, were obtained from Pudong New Area, Shanghai, China [SCXK(HU)2013-0013] [SYXK(SU)2014-0051]. All animal studies (including the mice euthanasia procedure) were done in compliance with the regulations and guidelines of Nanjing University institutional animal care and were conducted according to the AAALAC and the IACUC guidelines [SYXK(SU)2014-0051].

MRI was used to examine the knee joints in all pigs. One week later, bilaterally medial meniscectomy and guided exercise (which lasted 7 days) were used to induce knee OA, after which pigs were allowed to move freely. Seven weeks later, the labeled BM-MSCs were injected into the right knee, while the left side was left untreated. The MRIs were carried out once a week for the next 28 days, after which all animals were euthanized, and knee samples were collected and further examined. The study designs are shown in *Figure 1*.

Preparation of SPIO labeled BM-MSCs

Allogenic porcine BM-MSCs (CELLBIO, CBR-131561) were used for intra-articular injection. Certain concentration of SPIO nanoparticles were used to label the cells for 24 h, to ensure real-time cells tracking using the MRI.

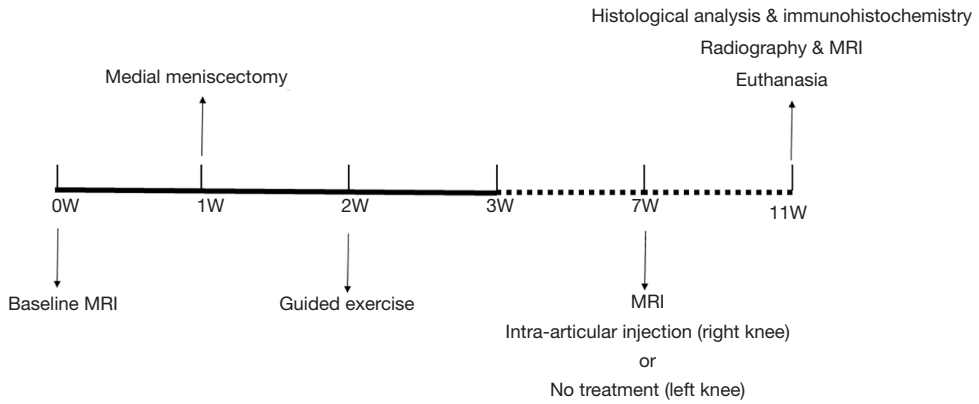

Figure 1 Timeline of the study. MRI, magnetic resonance imaging.

Cell viability

BM-MSCs were seeded in a 96-well plate at the density of 1×10^4 cells per well and cultured for 12 h for adherence. Cells were then exposed to gradually increased concentration (0, 2.5, 5, 10, 20 and 40 μg/mL) of SPIO for 24 h, after which they were examined using a Cell Counting Kit-8 (CCK-8) (KeyGen BioTech, Nanjing, China) according to the manufacturer's protocol.

Examining intracellular SPIO

For confirming the intracellular SPIO, the BM-MSCs were seeded on gelatin coated slides in 24-well plate at the density of 1×10^5 cells per well and cultured for 12 h for adherence. Next, the cells were exposed to gradually increased concentration (0 and 20 μg/mL) of SPIO for 72 h. The slides were then washed with PBS and stained with Prussian Blue staining (Solarbio, Beijing, China), according to the standard protocol. For *in vivo* experiment, the BM-MSCs were labeled with SPIO at the concentration of 20 μg/mL and cultured for 72 h.

Intra-articular injection of allogenic BM-MSCs

A total of 1×10^7 BM-MSCs (3 mL) were injected into the right medial compartment of the right knee joint once a week for a duration of 4 weeks. The joint was repeatedly flexed and extended to ensure cell dispersions throughout the joint after every injection.

MRI & radiography

Pre-detections were carried out before each surgery. Pigs were first anesthetized with 3.5 mg/kg Xylazine and the MRI (uMR770 3.0T, United Imaging, Shanghai, China) to evaluate the articular cartilage. Eight, 24 and 120 h post-injection, the knee joints were separately assessed using MRI. At the 11th week, the pigs were sacrificed and the isolated knee joints were appraised according to the Kellgren and Lawrence (K/L) system and Osteoarthritis Research Society International (OARSI) atlas (14).

Macroscopic examination, histological analysis & immunohistochemistry

All pigs were euthanized, and their knee joints were isolated and evaluated according to the International Cartilage Repair Society (ICRS) macroscopic score (15,16). Then, the samples were fixed in 4% paraformaldehyde for 24 h and decalcified in 10% methanoic acid for 1 month. Next, the tissues were embedded and sectioned into 5 μm thick slices. After that, the slices were stained with hematoxylin and eosin (H&E), safranin-o fast green staining and toluidine blue, and were evaluated according to the OARSI Osteoarthr Cartil Histopathology Assessment scoring system (17,18). Sections were covered with anti-collagen type I, II, and X antibody (Sigma Aldrich, USA) and incubated at 4 ℃ overnight. The sections were reacted with the secondary antibody (Sigma Aldrich, USA) for 30 min at room temperature. Immunohistochemical staining of type

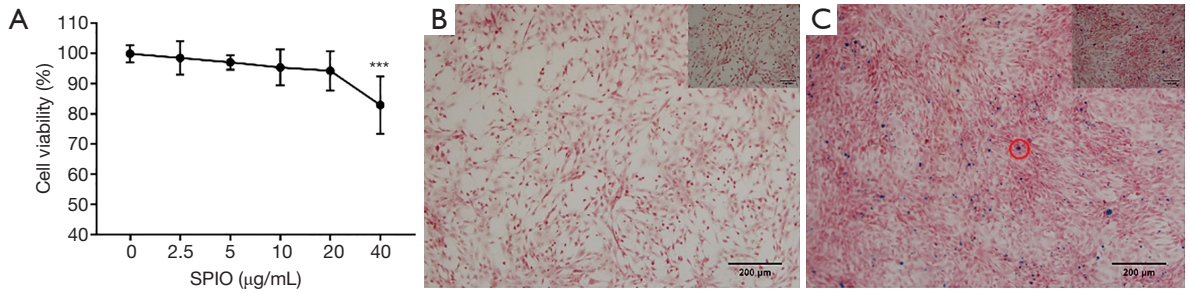

Figure 2 Biocompatibility and cellular uptake of SPIO. (A) Effects of SPIO on the cell viability after 24 h. ***, P<0.001; (B) Prussian Blue staining images of unlabeled BMSCs after 72 h; (C) Prussian Blue staining images of 20 μg/mL SPIO labeled BMSCs after 72 h (circled in red the loop). SPIO, superparamagnetic iron oxide; BMSC, bone marrow stem cell.

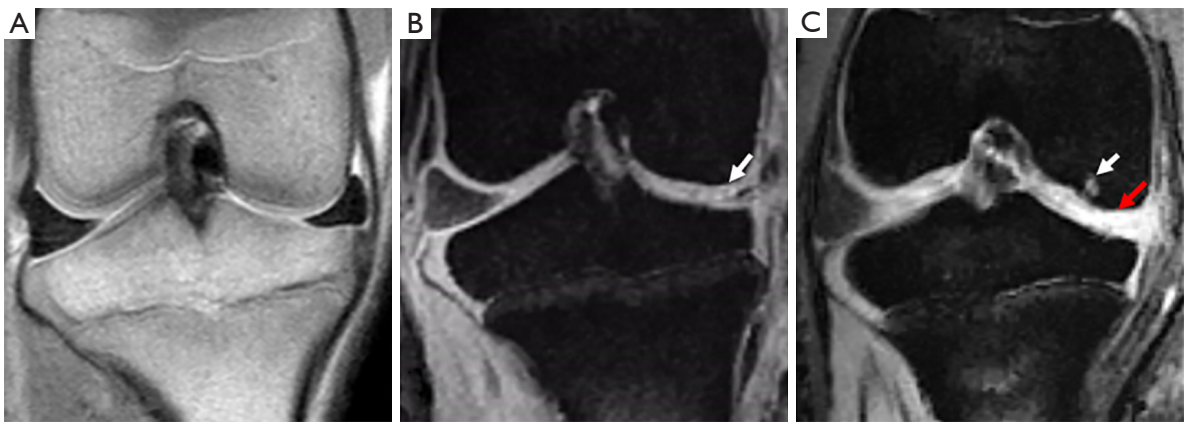

Figure 3 MRI of porcine knee at different stages. Red arrow marks typical OA changes of ruffled and thinner local cartilage; white arrows mark subchondral bone cystic degenerations. (A) Before surgery; (B) 1 week after surgery; (C) 1 week after the guided exercise. MRI, magnetic resonance imaging; OA, osteoarthritis.

I, II, and X were detected with the Vectastain ABC reagent (Vector Laboratories, USA).

Statistical analysis

Statistical analysis was performed using SPSS v22.0 (SPSS Inc., Chicago, IL, USA) and graphs were done with GraphPad Prism v7.0 (GraphPad Software Inc., La Jolla, CA, USA). Comparisons between the therapy side and the control side of the knee joint were preformed using unpaired *t*-test. A P value of <0.05 was considered statistically significant.

Results

The effect of SPIO on BM-MSCs viability

The effects of the SPIO on cell viability of bone marrow stem cells (BMSCs) are shown in *Figure 2*. Compared to concentration of 0–20 μg/mL, decreased cell proliferation was observed at concentration of 40 μg/mL.

To determine the presence of the particles within the cell, BMSCs were stained with Prussian Blue. Briefly, numerous blue granules were found within the cytoplasm after exposure to 20 μg/mL SPIO for 72 h (*Figure 2*), while no Prussian Blue-positive particles were observed in control group.

MRI & radiography

No cartilage damage was found before surgery (*Figure 3A*). Consequently, after preforming meniscectomy, the medial meniscus was observed (*Figure 3B*). In addition, the MRI demonstrated typical OA changes of ruffled and thinner local cartilage (red arrow in *Figure 3C*), as well as

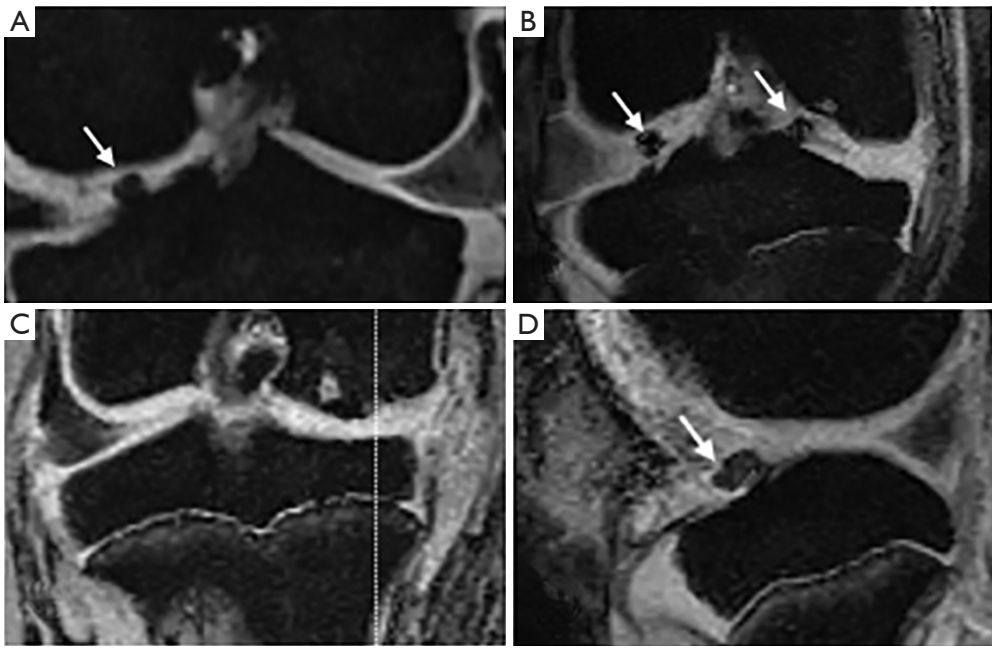

Figure 4 MRI of porcine knee after intra-articular injection. White arrows mark SPIO labeled BM-MSCs showed special signals on the MRI. (A) Eight h after injection; (B) 24 h after injection; (C) 120 h after injection; (D) The sagittal view of dotted line in (C). MRI, magnetic resonance imaging; SPIO, superparamagnetic iron oxide; BM-MSCs, bone marrow mesenchymal stem cells.

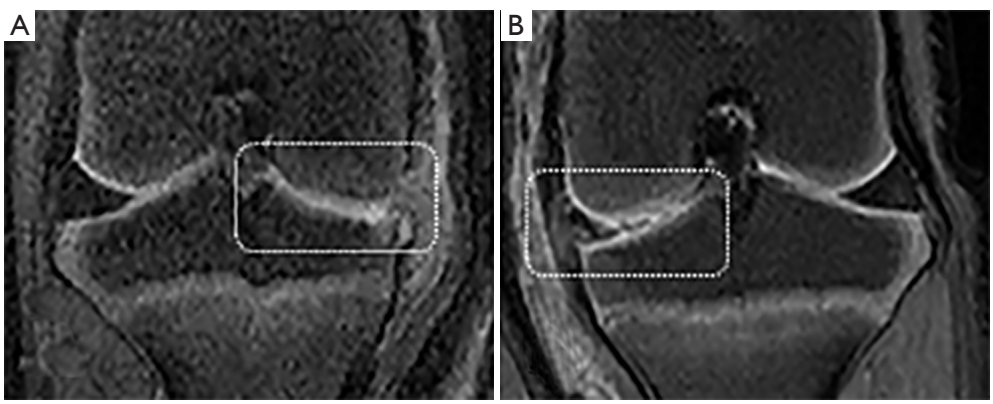

Figure 5 MRI of porcine knee after 4 weeks of intra-articular injections. White dotted boxes mark osteophytes and osteosclerosis around condyles and the tibial plateau. (A) Treated (right knee) side; (B) control (left knee) side. MRI, magnetic resonance imaging.

subchondral bone cystic degenerations (white arrow in *Figure 3C*).

Next, BM-MSCs were injected into the right medial compartment of the right knee joint once a week for a duration of 4 weeks. The movement of cells was examined using MRI. BM-MSCs were found moving towards the impaired part of the cartilage 8 to 24 h after injections. The SPIO labeled BM-MSCs showed special signals on the MRI (*Figure 4*).

As shown in *Figure 5*, 4 weeks post-injection, osteophytes and osteosclerosis were still visible around condyles and the tibial plateau. The results of K-L & Outer bridge grading of the knee are shown in *Figure 6*. In addition, no differences between the two groups were observed.

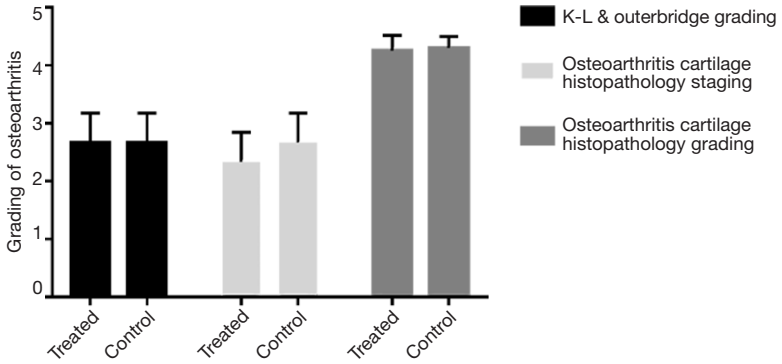

Figure 6 Imaging and histology grading of porcine knee after 4 weeks of intra-articular injections.

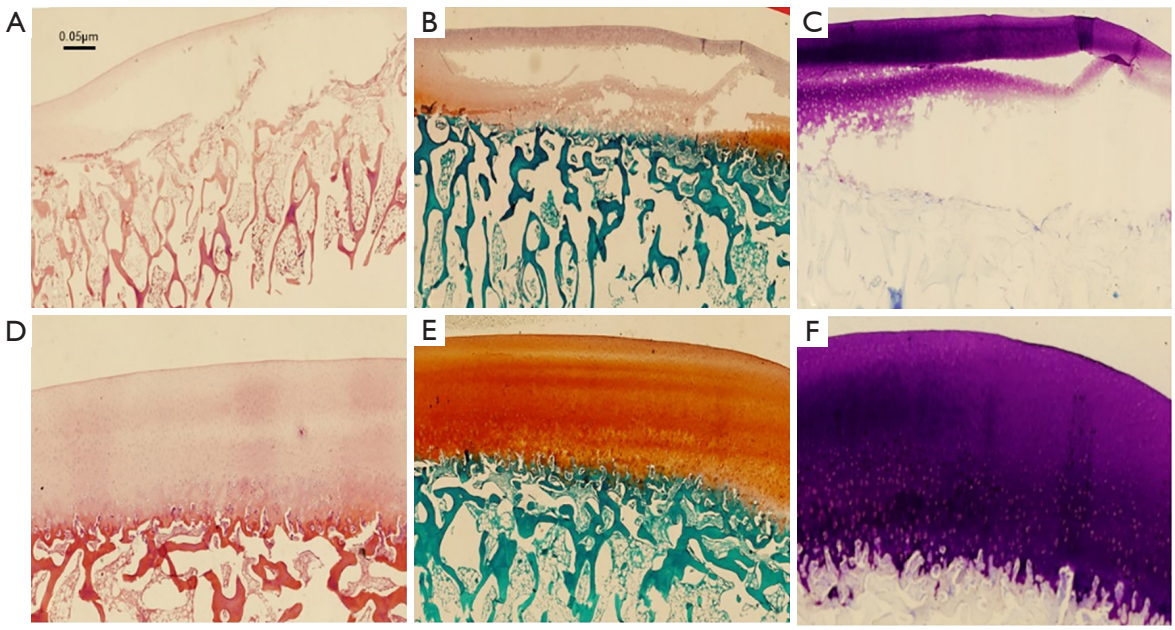

Figure 7 Slices of femoral condylar cartilages. (A,D) Hematoxylin and eosin (H&E) staining; (B,E) Safranin O-fast green staining; (C,F) Toluidine blue staining. (A,B,C) Treated (right knee) side; (D,E,F) control (left knee) side.

Macroscopic examination, histological analysis & *immunohistochemistry*

The gross specimens of knee joints were harvested after 4 weeks of injections. Dim rupture was found on the surface of both right and left side of the cartilage, however no significant difference between the right and left side was observed *(Figures 6*,*7)*.

Stained slices revealed the damage of the cartilage on both sides (*Figure 7*); again, no significant difference was found between the two groups. The immunohistochemical staining of femoral condylar cartilages showed the similar results (*Figure 8*).

Discussion

In the present study, we preformed meniscectomy to establish a pig OA model, and to evaluate the effectiveness of allogenic MSCs in treating OA. The obtained results revealed that 20 μg/mL of SPIO was a suitable level for labelling BM-MSCs. The SPIO labeled BM-MSCs were found moving towards the impaired part of the cartilage 8 to 24 h after the injections. Yet, there were no significant

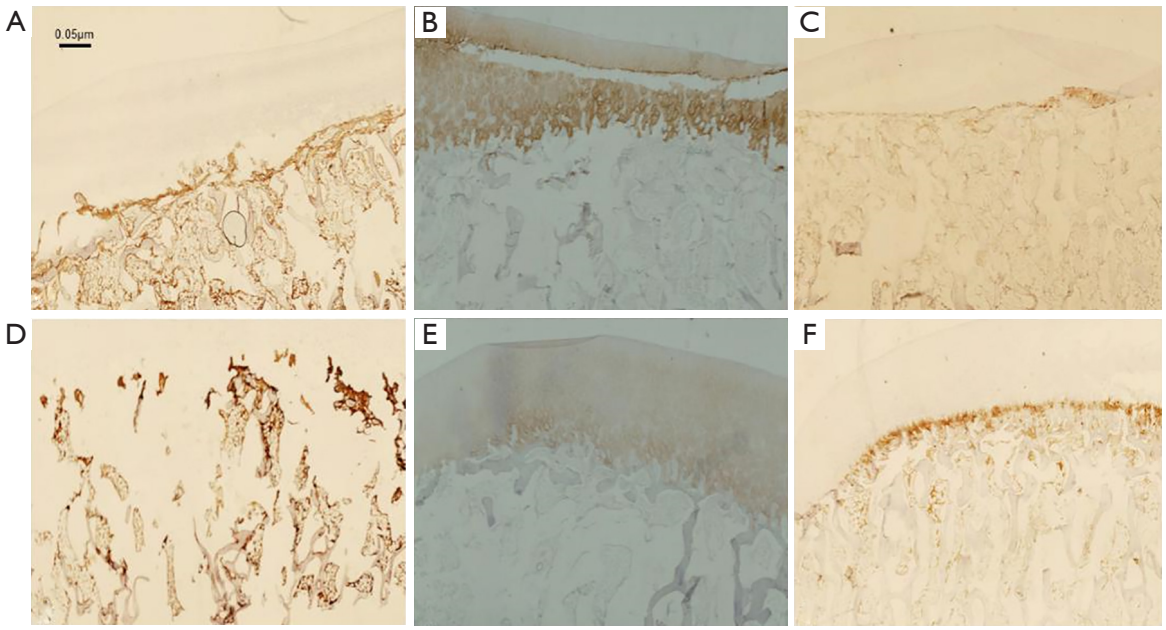

Figure 8 Immunohistochemical staining of femoral condylar cartilages. (A,D) Immunohistochemical staining of type I collagen; (B,E) immunohistochemical staining of type II collagen; (C,F) immunohistochemical staining of type X collagen. (A,B,C) Treated (right knee) side; (D,E,F) control (left knee) side.

differences between the treatment and control group.

Animal models are commonly used to study the pathogenesis, treatment and prevention of OA (19). There are many kinds of animal OA models, which are selected according to different study objectives (19). Certain models imply opening the articular capsule and damaging the intraarticular structures, such as cruciate ligament, meniscus, articular cartilage and so on (20), while others can be obtained via intra-articular injection of drugs that trigger OA (21). Although different models induce different mechanisms, ultimately cartilage degenerative changes and secondary bone changes occur (22). Small animals such as mice have a selection advantage when they need to be used in large quantities. Large animal models are suitable for the progression of OA, as their anatomical structure and mechanical structure are similar to the human structures (19-21). It has been demonstrated that pig models are suitable for the study of pathological process, histopathological characteristics, or changes in cartilage biochemical metabolism of OA (23-27). Partial or complete resection of the meniscus is a common method used for establishing an animal model of OA. After meniscal resection, the corresponding load of the patellofemoral joint is disordered, and the compressive stress is concentrated in an extremely small area, resulting in degenerate, rough fibrosis, or complete absence of the articular cartilage (28). Previous studies have shown that guiding the pig to exercise after meniscectomy could increase the load on the cartilage, thus accelerating the wear of the cartilage and inducing OA in a relatively short period of time (20).

Stem cell source, dosage, types of cell transplants (allogenic or autologous), and the feasibility of multiple injections are major issues related to BM-MSCs. The effects of MSC in treating cartilage degeneration and injury have been repeatedly reported in both, animal models and patients. Nevertheless, scholars and doctors have not yet reached a consensus. Jo and colleagues have reported that a high dose (1.0×10^8 AD MSCs) could improve the knee function in patients with OA (29). A multicenter randomized controlled clinical trial has also revealed that the similar dose of BM-MSCs together with hyaluronic acid lead to clinical and functional improvement (4). In the present study, the effectiveness of BM-MSCs was certainly worth considering. The intra-articular injection group did not show any significant differences in histological changes, which might be due to the subchondral bone damage, which has an import role in OA (30). Changes in subchondral bone can lead to the damage of cartilage. MRI showed collapse and cyst in subchondral bone, which suggested a late-stage of osteoarthritis. Some studies have indicated a

therapeutic effect of MSCs in early stage of OA (31). On the other hand, the allogenic BM-MSCs were used, unlike the common autologous ones. Allogenic BM-MSCs are easily manufactured in a large scale and are widely used in clinical applications. Udehiya and colleagues have illustrated a similar result of allogenic and autogenic BM-MSCs in repairing segmental bone defects in rabbits (32). Yang *et al.* have reported a protective role of allogenic BM-MSCs in the early stage of OA (31). Although we believed that allogenic BM-MSCs had promising application potential for treating OA, the obtained results were not consistent with our expectations. We did not find a significant difference between treatment and control group.

BM-MSCs, which are ideal for bone and cartilage tissue engineering, are more easily accessed, have more rapid expansion, and less immunological rejection (4). Some researchers believe that the MSCs can attach to the surface of cartilage and differentiate into chondrocytes to repair the defects, which is also known as stem cell homing (33). Stem cell homing includes four successive processes: cell recruitment, cell migration, cell proliferation and cell differentiation. Previous studies have shown that many tissues like heart, liver, brain, nerve, kidney, and epithelium go through the homing process of MSCs to promote the repair of injury (16), and have the potential for multi-directional differentiation, with highly self-renewing and proliferative ability (34). Still, the mechanism of stem cell homing remains unclear. In the present study, we found the directional migration of the MSCs *in vivo*. As *Figure 4* shows, BM-MSCs could be found in the articular cavity 8 h after injection. At 24 h after injection, it was possible to see the accumulation of BM-MSCs near the damaged side of the cartilage. Also, there was no obvious special signals till the 5th day after the injection.

The fact that BM-MSCs had no effect or were relatively inefficient could be explained by the following hypotheses: the intracellular concentration of SPIO could be diluted as a result of cell proliferation and differentiation (35); the mobility of joint might damage the BM-MSCs and then affect the biological activity of BM-MSCs. Furthermore, the SPIO released from dead cells might be phagocytosed by macrophages, which could also have an influence on the results of MRI. Arbab and other studies have found that iron is rapidly metabolized or degraded when iron-bearing cells die or macrophages phagocytize iron ions (36). At this time, the decrease of local MRI signal mainly occurs due to release of iron after cell death, suggesting that MRI low signal may be derived from iron containing cells, or from the iron released from dead cells, or from the hemoglobin in the bleeding process. Recently, there has been some debate about whether SPIO affects the chondrogenic differentiation of stem cells (37,38), which is why we selected the acceptable maximum dose (20 μg/mL) in order to guarantee a higher survival rate of cells, and to ensure a clearer MRI image. However, we only tested the ability of these cells to induce differentiation in the culture dish. Whether they have a tissue forming ability and whether they can be used as a tissue engineered seed cell for the repair of tissue defects needs to be further examined.

The present study has some limitations that need to be pointed out. First, the sample size was small, since the animal facility we used did not have enough room to keep too many pigs at the same time. Second, the pigs were not fastened after injection, which might have influenced the viability of BM-MScs. Third, we did not make the comparison of allogenic and autologous cells in one model, thus making the results less convincing. Moreover, we did not take the severity of OA into account, which might have impacted the treatment effects. Moreover, it was impossible to objectively evaluate the subjective feelings of the pigs using scoring scales.

In conclusion, 20 μg/mL of SPIO was the most suitable concentration for labelling BMSCs and the labelled allogenic BM-MSCs could be seen moving towards and accumulating around the lesion area of the cartilage. Yet, allogenic BM-MSCs was not significantly different between the treated group and control group. Thus, more studies are required to further investigate the therapeutic effect of allogeneic BM-MSCs *in vivo*.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: All animal studies (including the mice euthanasia procedure) were done in compliance

with the regulations and guidelines of Nanjing University institutional animal care and were conducted according to the AAALAC and the IACUC guidelines [SYXK(SU)2014-0051].

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