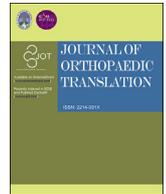




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Injectable adipose-derived stem cells-embedded alginate-gelatin microspheres prepared by electrospray for cartilage tissue regeneration



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ABSTRACT

Objective: To prepare adipose-derived stem cells (ADSCs)-embedded alginate-gelatin microspheres (Alg-Gel-ADSCs MSs) by electrospray and evaluate their feasibility for cartilage tissue engineering. To observe the efficacy of Alg-Gel-ADSCs MSs in repairing articular cartilage defects in SD rats.

Methods: ADSCs were isolated and characterized by performing induced differentiation and flow cytometry assays. Alginate-gelatin microspheres with different gelatin concentrations were manufactured by electrospraying, and the appropriate alginate-gelatin concentration and ratio were determined by evaluating microsphere formation. Alg-Gel-ADSCs MSs were compared with Alg-ADSCs MSs through the induction of chondrogenic differentiation and culture. Their feasibility for cartilage tissue engineering was analysed by performing Live/Dead staining, cell proliferation analysis, toluidine blue staining and a glycosaminoglycan (GAG) content analysis. Alg-Gel-ADSCs MSs were implanted in the cartilage defects of SD rats, and the cartilage repair effect was evaluated at different time points. The evaluation included gross observations and histological evaluations, fluorescence imaging tracking, immunohistochemical staining, microcomputed tomography (micro-CT) and a CatWalk evaluation.

Results: The isolated ADSCs showed multidirectional differentiation and were used for cartilage tissue engineering. Using 1.5 w:v% alginate and 0.5 w:v% gelatin (Type B), we successfully prepared nearly spherical microspheres. Compared with alginate microspheres, alginate gel increased the viability of ADSCs and promoted the proliferation and chondrogenesis of ADSCs. In our experiments on knee cartilage defects in SD rats in vivo, the Alg-Gel-ADSCs MSs showed superior cartilage repair in cell resides, histology evaluation, micro-CT imaging and gait analysis.

Conclusions: Microspheres composed of 1.5 w:v% alginate-0.5 w:v% gelatin increase the viability of ADSCs and supported their proliferation and deposition of cartilage matrix components. ADSCs embedded in 1.5 w:v% alginate-0.5 w:v% gelatin microspheres show superior repair efficacy and prospective applications in cartilage tissue repair.

The translational potential of this article: In this study, injectable adipose-derived stem cells-embedded alginate-gelatin microspheres (Alg-Gel-ADSCs MSs) were prepared by the electrospray. Compared with the traditional alginate microspheres, its support ability for ADSCs is better and shows a better repair effect. This study provides a promising strategy for cartilage tissue regeneration.

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1. Introduction

The role of articular cartilage in joints of the body is critical. However, the intrinsic healing ability of articular cartilage is limited due to its avascular nature. The management of cartilage damage is a challenge for orthopaedic surgeons [1]. In recent decades [2–5], tissue engineering (TE) technology has provided new methods to repair cartilage defects.

Mesenchymal stem cells (MSCs) are a promising new therapeutic strategy in the field of cartilage regeneration and show potential in the regeneration of injured and damaged tissues [6,7]. Adipose-derived stem cells (ADSCs) are a type of MSC that differentiate into chondrocytes and then treat cartilage defects [8]. The advantages of ADSCs in promoting cartilage regeneration have been shown, which will promote their clinical application [9]. The acquisition of adipose-derived cells is much less expensive than that of bone marrow-derived cells and chondrocytes and requires a less invasive operation [10]. Therefore, ADSCs have greater clinical value than other MSCs.

However, traditional two-dimensional (2D) cell growth tends to cause contact inhibition and even dedifferentiation. After the cells are transplanted into the damaged area, the lack of support from the carrier easily leads to the loss of stem cells. However, ADSC-embedded microspheres may prevent this issue. The microsphere is a microcarrier that has been used to embed cells [11]. In the field of cartilage TE, major advances have been achieved in the applications of microspheres [12,13]. Microspheres have unique advantages [11], such as providing a proper microenvironment for cell growth. In addition, due to the diameter of approximately 100–300 microns [14], microspheres reach the articular cartilage defect area after injection. Proper supportive matrices are important factors for microspheres. Alginate (Alg) is a polysaccharide with a structure similar to that of glycosaminoglycans (GAGs) in the natural cartilage extracellular matrix (ECM) [15]. However, the main disadvantages of Alg compared to cartilage are its poor support for cell proliferation and adhesion [16]. These drawbacks may be addressed by adding gelatine (Gel) [17].

In the present study, we first isolated ADSCs and determined whether they are able to differentiate after proliferation. ADSCs were induced to undergo chondrogenic differentiation to clarify their chondrogenic differentiation ability. Then, we designed and optimized Alg-Gel microspheres prepared using electrospray technology. The viability and proliferation of ADSCs cultured in Alg-Gel microspheres, Alg microspheres and traditional 2D well plates were compared. Furthermore, we generated ADSCs-embedded Alg-Gel microspheres for chondrogenic induction. Finally, ADSCs-embedded Alg-Gel microspheres and Alg-Gel microspheres were implanted directly into the knee cartilage defects of Sprague–Dawley (SD) rats. The cartilage regeneration ability was evaluated by performing a gait analysis, microcomputed tomography (micro-CT) assays, and histological evaluation and compared with that of the control group.

Thus, the purpose of this study was to initially explore the ability of ADSCs-embedded Alg-Gel microspheres to repair articular cartilage.

2. Materials and methods

2.1. ADSC isolation

ADSCs were prepared from adipose tissue using a previously reported method [18]. Briefly, the adipose tissue from the groin fat pad of SD rats (180 g–220 g, the cells used in each experiment were obtained from independent rat donors) was mechanically harvested and minced. The obtained adipose tissue was washed with phosphate-buffered saline (PBS) and then digested with 0.075% type I collagenase (Gibco, Thermo Fisher Scientific, MA) at 37 °C for 30 min. The cell suspension was filtered using a 70- μ m nylon mesh (Falcon, Corning, NY), 10% foetal bovine serum (FBS) was added to the medium (DMEM; Gibco, Thermo Fisher Scientific) for neutralization, and the mixture was centrifuged at 800 \times g for 5 min. Stromal cell pellets were resuspended in culture

medium to prepare the cell suspension. Cells were spread in a 75 square centimetre cell culture flask (Corning) and cultured with 5% carbon dioxide at 37 °C. After cultivation in DMEM containing 10% FBS for 24 h, the supernatant containing nonadherent cells was discarded, cells were rinsed with PBS three times, and then 12 ml of DMEM containing 10% FBS were added to continue the culture. Cells that were attached to the bottom of the culture flask were cultured with 5% CO₂ at 37 °C, and the medium was changed every 24 h. After the cells reached 80% confluence, they were digested with 0.25% trypsin/ethylenediaminetetraacetic acid and then reseeded in a new Petri dish at a density of 5 \times 10³ cells/cm². The cells were passaged to generation P3 before use.

The Institutional Animal Care and Use Committee of Chinese PLA General Hospital approved all experimental procedures involving animals in this study. The animals involved in the experiment were placed in a room with a controlled temperature and humidity, and a 12 h light and dark cycle, and they were provided standard food and water *ad libitum*.

2.2. Confirmation of ADSCs after amplification

ADSCs were analysed using flow cytometry. We analysed specific subpopulations using a combination of antibodies (BD, Biosciences) against CD31, CD34, CD45, CD90, and CD29. For staining, 1 \times 10⁶ cells in 50 μ l of PBS were mixed with 1 μ g of antibody and incubated in the dark at room temperature for 15 min. Then, 2 ml of PBS were added and the sample was centrifuged at 400 g for 5 min. After the supernatant was discarded, 500 μ l of PBS were added for analysis. All samples were analysed using a flow cytometer (BD, Biosciences).

2.3. Induction of ADSC differentiation

The differentiation potential of ADSCs was analysed in vitro using the differentiation induction method. For the preparation of adipogenic differentiation medium, 20 ml of FBS, 2 ml of penicillin–streptomycin, 2 ml of glutamine, 400 μ l of insulin, 200 μ l of dexamethasone, and 200 μ l of 3-isobutyl-1-methylxanthin were added to 175 ml of high-glucose DMEM (Sigma–Aldrich, MO). For the preparation of osteogenic differentiation medium, 2 ml of β -glycerophosphate, 20 μ l of dexamethasone and 400 μ l of ascorbate were added to 175 ml of DMEM. For chondrogenic differentiation, a micromass culture technique was used. Briefly, 4 \times 10⁵ cells were added to a 15 ml centrifuge tube for centrifugal precipitation, and complete chondrogenic differentiation medium (Cyagen) was added. After 36 h, a cluster of cells appeared at the bottom of the centrifuge tube. The bottom of the centrifuge tube was gently flicked to suspend the cartilage-like structures in the medium, and then, the medium was refreshed every three days. The three media described above were used to induce ADSC differentiation for 28 days at 37 °C with 5% CO₂. Osteoblasts, adipocytes, and chondrocytes were stained with Alizarin Red, Oil Red O, and Alcian Blue (Cyagen), respectively.

2.4. Preparation of microspheres

According to a published report [19], we selected sodium Alg (Sigma, USA) at a concentration of 1.5% w/v and calcium chloride at a concentration of 2% w/v to prepare intact and regularly shaped microspheres. We prepared an appropriate Gel concentration by dissolving Gel at concentrations of 0.5%, 1.5% and 2.5% (Type B, Sigma, USA) in the sodium Alg solution at a concentration of 1.5% w/v at 40 °C. The electrospray device (Nisco) consists of a high-voltage power supply (0–10 kV from the emitter to the collector) and a quantitative syringe propulsion pump. We used an electrospray device to spray the Alg solution and the abovementioned three concentrations of the Alg-Gel mixture into a calcium chloride solution. The voltage was set to 6.5 kV, the spray needle tip was placed 3 cm away from the calcium chloride liquid surface, and the solution advancing speed was 20 ml/h. We produced 1.5% w:v Alg microspheres (Alg MSs), 1.5% w:v Alg-0.5% Gel microspheres (Alg-Gel MSs), and 1.5% and 2.5% w:v Gel Type B microspheres using the

electrospray technique. The effect of the Gel concentration on microspheres was studied, and the morphology of the microspheres synthesized using the electrospray device was characterized using an optical microscope. Then, we identified the solute concentrations that formed intact microspheres. For ADSCs-containing microspheres, ADSCs (10^6 cells/ml) were first equally dissolved in Alg and Alg-Gel solutions with optimized Alg and Gel concentrations. Then, using the parameters described above, the electrospray device was used to spray the solutions into calcium chloride solution to form ADSC-embedded microspheres. Microspheres containing ADSCs were collected in calcium chloride solution shortly after formation (10 min).

2.5. Morphological characterization of microspheres

After electrospraying, the Alg-Gel microspheres, Alg microspheres and microspheres containing ADSCs were collected and imaged with a light microscope (Olympus, Japan). Thirty microspheres ($n = 30$) were randomly selected from each group with intact spheres, and their diameters were measured using image analysis software (ImageJ, USA).

2.6. Cell culture experiments

In this experiment, the proliferation of ADSCs was compared between the following groups over a 14-day period: (1) 5×10^6 ADSCs cultured in 2D flat culture flasks, (2) 5×10^6 cells embedded in 1.5% (w/v) Alg-0.5% (w/v) Gel microspheres with a density of 10^6 ADSCs/ml (Alg-Gel-ADSC MSs) were cultured in a 3D bioreactor, and (3) 5×10^6 cells embedded in 1.5% (w/v) Alg microspheres with a density of 10^6 ADSCs/ml (Alg-ADSC MSs) were cultured in a 3D bioreactor. The medium used for this experiment was DMEM supplemented with 100 U/ml penicillin/streptomycin and 10% FBS, and the medium was changed every two days. The Alg-ADSC MSs and Alg-Gel-ADSC MS groups were cultured in a rotary cell culture system (RCCS; Synthecon, Houston). The rotation speed of the RCCS was set to 15 rpm with continuous rotation in an incubator at 37 °C with 5% CO₂.

2.7. Cell proliferation and viability

The proliferation of ADSCs in each group in the cell culture experiment was measured by cell counting. Cell counts were performed on days 0, 7, and 14 of culture. Cells in the ADSCs group were counted using conventional cell counting methods. The cells were counted with a haemocytometer under a microscope at 10x magnification, and the total cell number was calculated [20]. The Alg-ADSC MSs and Alg-Gel-ADSC MSs groups were washed with a 0.9% sodium chloride solution 2 to 3 times, liquefied in 55 mmol/l sodium citrate solution for 15 min and then counted immediately. Three different fields of view were counted for each group, and 3 replicates were performed.

After the 1st, 7th and 14th days of culture, the viability of the ADSCs was determined by performing the Live/Dead Cell Viability Assay (Sigma, USA), where the Alg-ADSCs MSs and Alg-Gel-ADSCs MSs groups were compared. Briefly, for Live/Dead staining, the microspheres were washed with PBS and immersed in 1 ml of prepared Live/Dead staining solution in a 6-well plate. We incubated the sample with the Live/Dead staining solution for 15 min in the dark to ensure that the dye diffused throughout the cells. Then, we observed the samples under a fluorescence microscope. Finally, the groups underwent chondrogenic differentiation for 28 days. The cells embedded in microspheres and ADSCs were cultured in the aforementioned chondrogenic differentiation medium, and the medium was changed every 2 days.

2.8. Chondrogenic differentiation ability and GAG content of ADSCs-microspheres

The aforementioned four groups of microspheres (Alg-ADSCs MSs, Alg-Gel-ADSCs MSs, Alg- MSs, Alg-Gel MSs) were induced using the

chondrogenic differentiation medium described above for 21 days. The medium was changed every two days. After 21 days of induction, the two groups of microspheres were stained with Alcian Blue for 30 min and observed with an optical microscope. We used the same type and concentration of Alcian Blue staining solution for the two groups of microspheres and the same staining time to ensure the uniformity of the staining method between the two groups.

For quantitation, we used the DMMB Colorimetry kit (Genmed Scientific, Inc., Shanghai, China) to analyse the GAG content according to the manufacturer's instructions. Briefly, we digested the two groups (Alg-ADSC MSs and Alg-Gel-ADSC MSs) of samples in chondrogenic induction culture with sodium citrate solution at the aforementioned concentration overnight on days 0, 7, 14, and 21. Then, 1,9-dimethyl methylene blue (DMMB) was used to stain cells and prepare a standard curve based on the optical density value to calculate the GAG content per millilitre of the samples from the two groups.

2.9. Construction of a rat cartilage defect model and repair with microspheres

As mentioned above, the Institutional Animal Care and Use Committee of Chinese PLA General Hospital approved all experimental procedures. Excluding the losses to anaesthesia accidents, model failure, incision infection, and accidental death, thirty-six healthy rats (180 g–220 g) were used. After each loss of experimental animals, we immediately supplemented the corresponding groups according to the original method. After the rats were anaesthetized with 3% pentobarbital sodium, a sterile biopsy punch was used to create a cartilage defect with a diameter of 2 mm and a depth of 1 mm in the centre of the femoral trochlear groove of the left leg. We randomly divided the animals into three groups and implanted different materials to compare the repair effects: (1) the defect was filled with fibrin glue (control/defect group); (2) microspheres were prepared without any cells [1.5% (w/v) Alg-0.5% (w/v) Gel], injected into the defect and then maintained with fibrin glue (Alg-Gel MS group); and (3) the final group is shown in a schematic diagram in Fig. 1. Alg-Gel-ADSCs MSs were injected into the defect, and fibrin glue was then used to maintain the Alg-Gel-ADSCs MSs (Alg-Gel-ADSCs MSs group). The microspheres used in animal experiments were not pretreated for chondrogenic induction. Animals were euthanized by suffocation with carbon dioxide at 6 and 12 weeks after surgery and tissues were used in subsequent experiments. The details are shown in Table 1.

2.10. Fluorescence imaging and tracking of the cells in the ADSCs-Alg-Gel MSs in vivo

PKH26 is a lipophilic dye with strong fluorescence that stains membranes. This labelling method has been used to track cell migration in vivo [21]. We used PKH26 dye to label ADSCs and then encapsulated them in Alg-Gel MSs. Then, we implanted the microspheres into SD rats using the abovementioned surgical method. Three weeks after the operation, a Kodak In Vivo FX imaging system (Kodak, Tokyo, Japan) was used to immediately examine the knee joint to track the ADSCs labelled with PKH26 in the repair area. Moreover, we observed the slices of samples after staining with 4,6-diamidino-2-phenylindole (DAPI) and the merged signals using a fluorescence microscope.

2.11. Gross morphological evaluation and CatWalk gait analysis

The femoral trochlear cartilage from the rats in the three groups was observed after sacrifice to evaluate the surface smoothness, the filling conditions, and tissue integration at 6 and 12 weeks after surgery. We adopted the International Cartilage Repair Society (ICRS) and the modified Wakitani scoring system [22].

Gait analysis was performed with walking rats before surgery and at 0, 1, 6, and 12 weeks after surgery using the CatWalk method (CatWalk

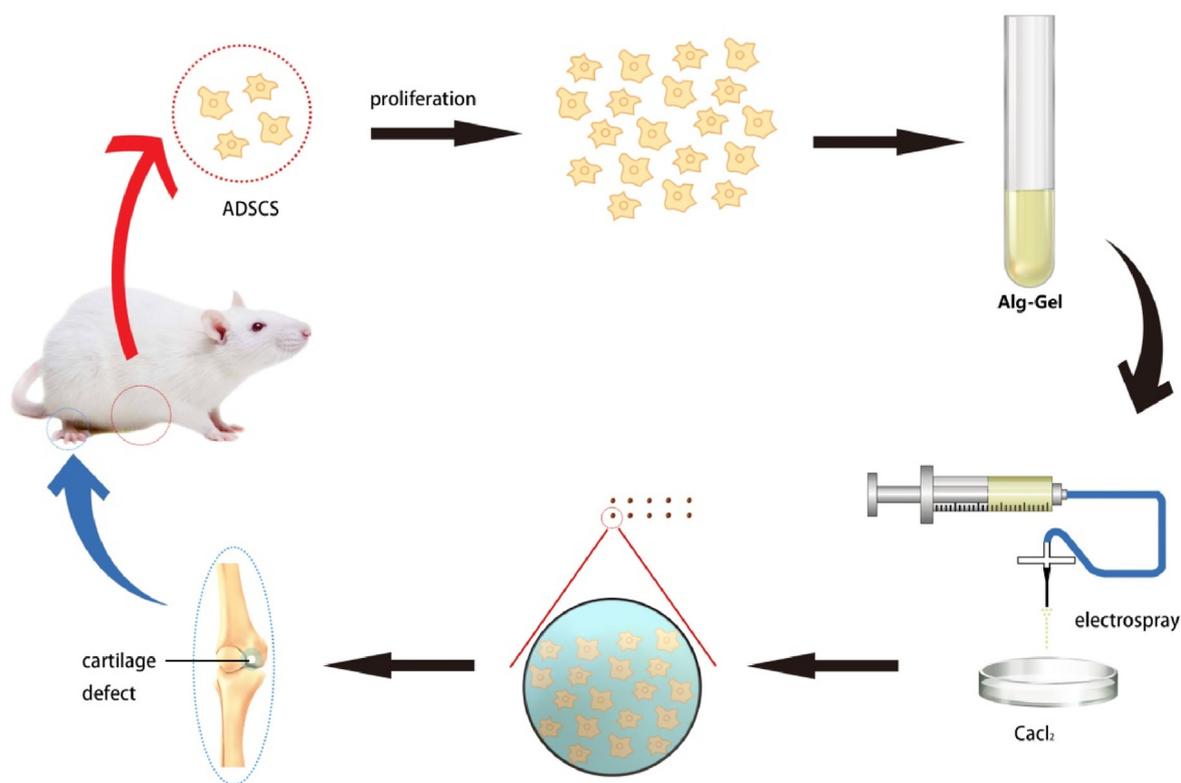


Fig. 1. Schematic diagram of the Alg-Gel-ADSCs MSs group repairing articular cartilage defects. The ADSCs from SD rats were separated, amplified, placed in Alg-Gel solution [1.5% (w/v) Alg-0.5% (w/v) Gel], and crosslinked with CaCl_2 through an electro-spray device to form cell-containing microspheres. The microspheres were injected into the cartilage defect of the SD rat.

Table 1

The number of experimental animals in each group.

Total = 36	control group	Alg-Gel MS group	Alg-Gel-ADSC MS group
6 weeks	6	6	6
12 weeks	6	6	6

XT; The Netherlands). The floor area contacted by the foot is recorded as the pressure exerted by the paw and paw area (cm^2), which are reflected by the mean intensity (arbitrary units, a. u.), and used to evaluate the joint function of the left leg in the experiment [23].

2.12. Histological and immunohistochemical evaluations

Samples ($n = 3$) were fixed for 3 days with 4% paraformaldehyde, decalcified in 10% (w/v) EDTA for 4 weeks, embedded in paraffin, and cut into 6- μm -thick sections. Based on a reported method [24], H&E staining, toluidine blue staining, Masson's trichrome staining and Sirius red staining were used for histological evaluations. For the analysis of collagen II in the repaired tissue, epitope unmasking was conducted by incubating sections with Proteinase K (2.0 mg/ml) for 1 h at 37 °C. The sections were then stained with primary antibodies (Abcam) against collagen II (1:100; Cat# ab34712) at 4 °C overnight. After 60 min of incubation with secondary antibodies, nuclei were counterstained for 5 min with Hoechst 33258 (Molecular Probes, Eugene, OR). Then, the samples were observed with a fluorescence microscope (Olympus). We also performed histological scoring on the repaired tissues in the defect using the Wakitani scoring system. The histological sections from the medial and lateral regions of each defect (25 images per group) were blindly scored by five independent evaluators.

2.13. Micro-CT assay

Subchondral bone generation was determined by performing a micro-CT (SkyScan, USA) analysis of the samples. The bone volume was calculated using DataViewer software (SkyScan) and the CTAn programme (SkyScan). A cylindrical region of interest (ROI) with a size of 2 mm in diameter and 2 mm thick was selected to analyse the bone volume.

2.14. Statistical analysis

All experiments were repeated at least three times in this study. All data are presented as the mean values \pm standard deviations. One-way analysis of variance was used (ANOVA) with significance levels of * $p < 0.05$ and ** $p < 0.01$.

3. Results

3.1. Characteristics and phenotypes of ADSCs

Phase microscopy revealed that ADSCs had a flattened fibroblast-like morphology. ADSCs grown in culture flasks showed autoregeneration (Fig. 2A). After ADSCs were cultured in adipogenic differentiation medium for 28 days, the presence of Oil Red O-positive intracellular lipid droplets in the cells confirmed adipogenic differentiation (Fig. 2B). After ADSCs were cultured in chondrogenic differentiation medium for 28 days, cartilage differentiation was confirmed by the detection of chondrogenic tissues rich in proteoglycan matrix stained with Alcian Blue (Fig. 2C). After ADSCs were cultured in osteogenic differentiation medium for 28 days, calcium deposits detected in cells stained with Alizarin Red S confirmed their osteogenic differentiation ability (Fig. 2D).

A phenotypic analysis using flow cytometry showed that the isolated ADSCs expressed the MSC markers CD90 and CD29, while the haematopoietic lineage marker CD45 and the endothelial cell marker CD31

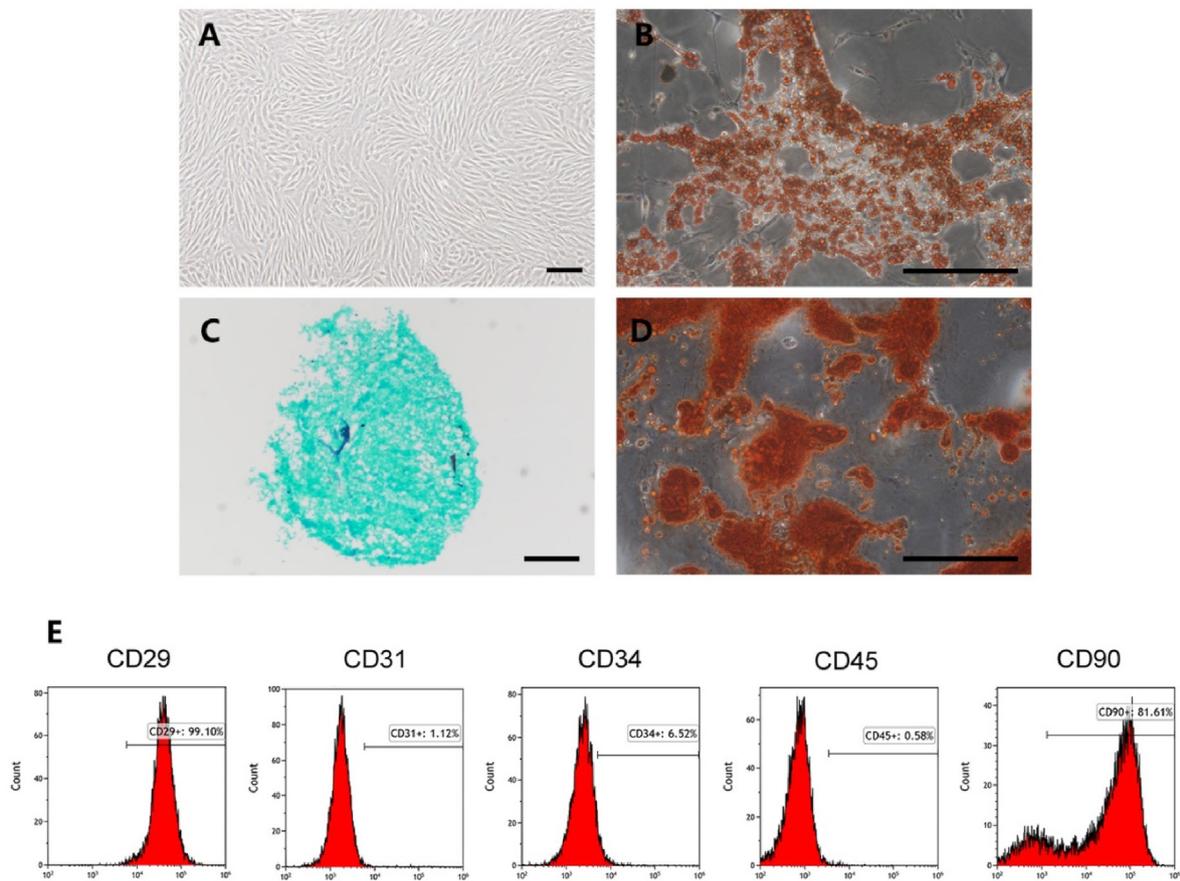


Fig. 2. Characterization of ADSCs. The morphology (A) of the cultured ADSCs was observed under a microscope. The staining results obtained after adipogenic differentiation (B), chondrogenic differentiation (C), and osteogenic differentiation (D) are shown. Scale bar = 100 μm (E) The immunophenotype of the ADSCs was analysed using flow cytometry to determine CD29, CD31, CD34, CD45, and CD90 expression.

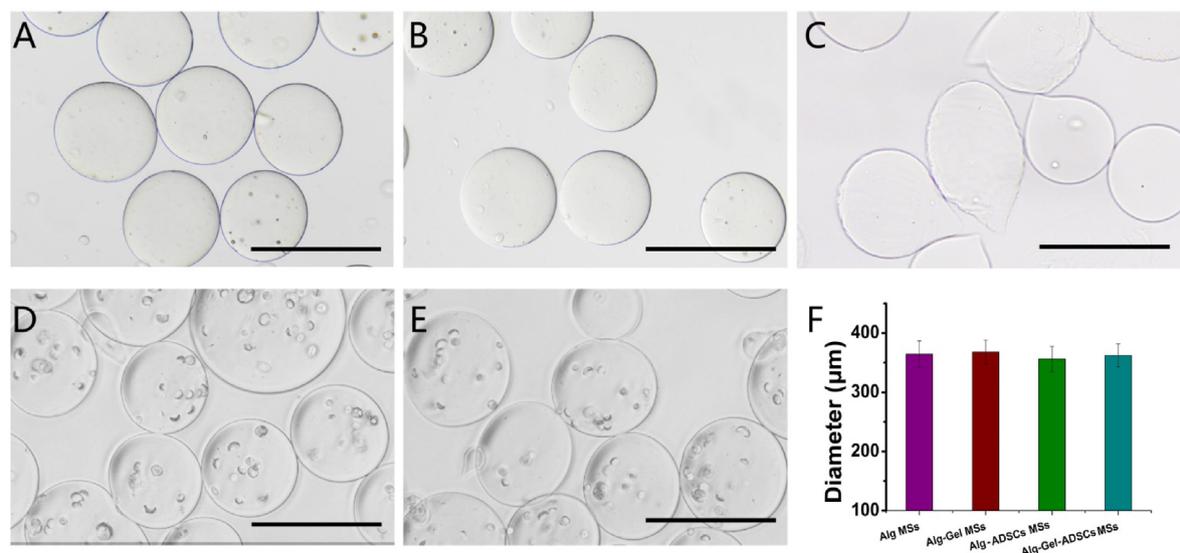


Fig. 3. Representative microscopy images of (A) 1.5% w:v Alg microspheres (B and C, respectively) 1.5% w:v Alg-0.5% microspheres and 1.5% w:v Gel Type B microspheres produced using the electrospray technique (D) Images of 1.5% w:v Alg-ADSC microspheres (Alg-ADSC MSs) and 1.5% w:v Alg-0.5% w:v Gel-ADSC microspheres (Alg-Gel-ADSC MSs). Scale bar = 500 μm (F) The diameters of the four types of microspheres.

were expressed at low levels. In addition, approximately 7% CD34⁺ cells were detected. The CD34 expression level is also consistent with that reported in other studies [25].

3.2. Morphological characterization of microspheres

We produced 1.5% w:v Alg, 1.5% w:v Alg-0.5%, and 1.5% and 2.5% w:v Gel Type B microspheres using electrospray. The 1.5% w:v Alg-2.5% w:v Gel group solidified at room temperature, resulting in clogging of the spray tube and a failure to prepare microspheres. The 1.5% w:v Alg microspheres (Alg MSs) and 1.5% w:v Alg-0.5% w:v Gel microspheres (Alg-Gel MSs) were similar in appearance and exhibited a complete shape (Fig. 3A–B). The 1.5% w:v Alg-1.5% w:v Gel group showed a loss of

circularity (Fig. 3C) and did not form microspheres. Through microscopic observations, we found that 1.5% w:v Alg-ADSC microspheres (Alg-ADSC MSs) and 1.5% w:v Alg-0.5% w:v Gel-ADSC microspheres (Alg-Gel-ADSC MSs) had similar morphologies (Fig. 3D–E). In the quantitative analysis, the diameters of the Alg MSs, Alg-Gel MSs, Alg-ADSCs MSs, and Alg-Gel-ADSCs MSs were similar, and the difference ($p > 0.05$) in diameter among the groups was not statistically significant (Fig. 3F).

3.3. Cell viability and proliferation

The results of Live/Dead staining (Fig. 4A) showed that the cell viability and number of cells cultured in the two groups of microspheres were highly similar in the initial culture. However, as the culture time

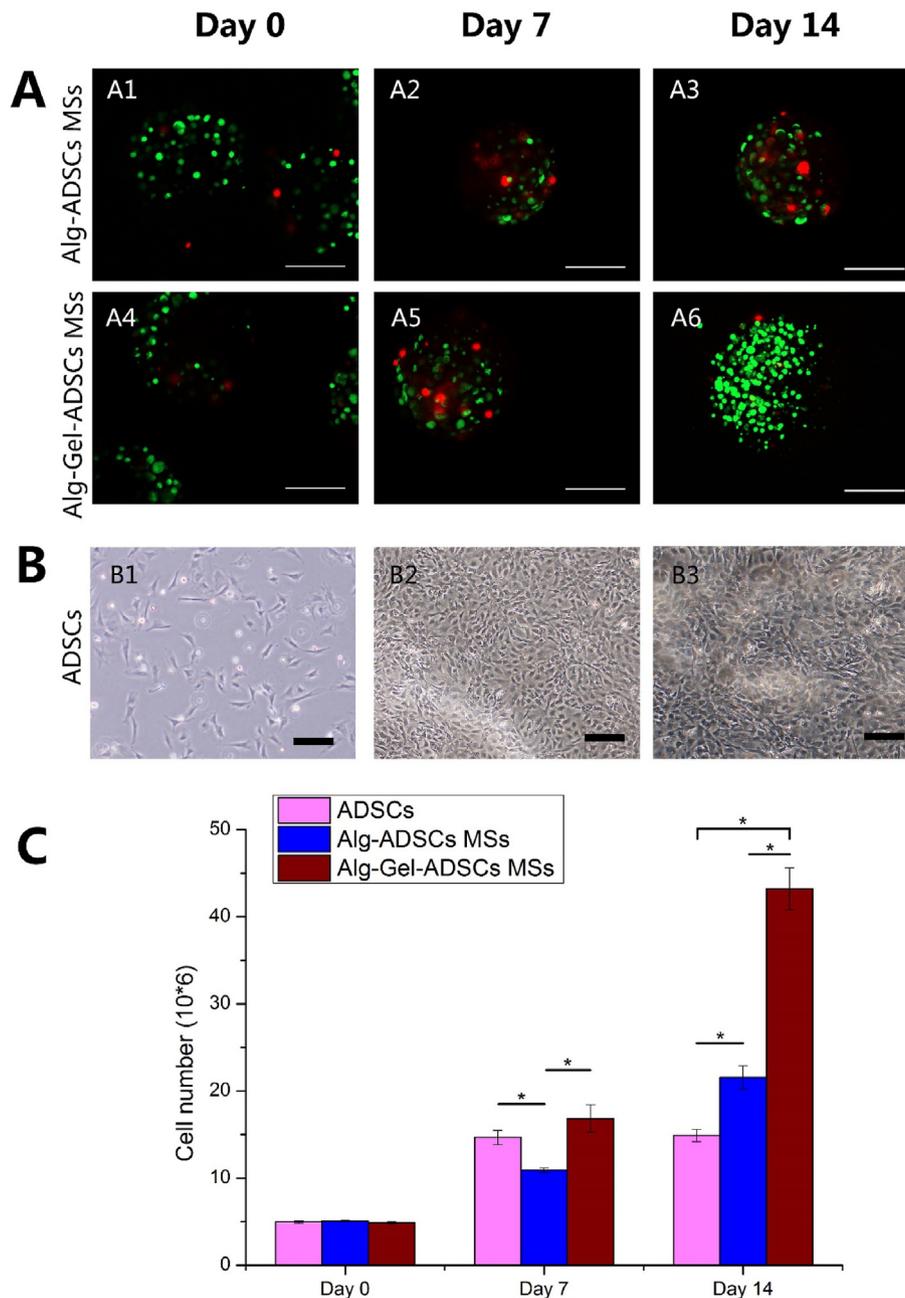


Fig. 4. Cell viability and proliferation. A Merged images of green fluorescence (live cells) and red fluorescence (dead cells) in Alg-ADSCs MSs (A1-3) and Alg-Gel-ADSCs MSs (A4-6) stained on days 0, 7 and 14 of culture. B Microscopy images of ADSCs cultured in a flask on days 0, 7 and 14 of culture (B1-3). Scale bar = 200 μ m. C Graph showing the statistical analysis of cell number.

increased, the number of cells in the two groups of microspheres increased significantly. On the seventh day, the number of cells in the Alg-Gel-ADSCs MSs group increased to a greater extent than that in the Alg-ADSCs MSs group. On the 14th day, both cell viability and cell number were substantially higher than those of the Alg-ADSCs MSs group. The microscopy analysis of cells grown using the traditional method of plate culture showed (Fig. 4B) that ADSCs had completely overgrown the bottom of the culture flask on the seventh day of culture, and the ADSCs had contacted each other. When the culture continued to the 14th day, an ECM on the cells was observed. Due to the phenomenon of contact inhibition, no visible increase in the number of cells was observed on the 14th day.

We also drew relevant conclusions from the graph showing the statistical analysis of the cell number (Fig. 4C). The number of cells in the three groups was approximately equal at first and then increased. On the seventh day, the number of cells grown using the traditional culture method and Alg-Gel-ADSCs MSs was similar, and the difference between the two groups was not significant, but both groups presented significantly higher values than the Alg-ADSCs MSs group ($p < 0.05$). On the 14th day of culture, the number of ADSCs in the microsphere groups was significantly higher than the number of cells in the traditional culture group ($p < 0.05$), and the number of ADSCs in the Alg-Gel-ADSCs MSs group was the largest. Compared with the number of cells cultured in Alg-ADSCs MSs and traditional methods, those in the other groups were not significantly different ($p < 0.05$).

For traditional culture, we used 75 cm² culture flasks, and we plated an initial number of 5×10^6 cells in five culture flasks. Based on the results of the cell counting analysis, approximately 3×10^6 ADSCs were counted in each 75 cm² culture flask on the seventh day. However, because of the large surface area of the microspheres, contact inhibition did not occur within 14 days of culture.

3.4. Chondrogenic differentiation ability

As shown in Fig. 5, all four groups of microspheres were stained positively with Alcian Blue. The microspheres containing ADSCs appeared more pigmented, possibly due to greater ECM deposition. Notably, the main explanation for this positivity is that Alg and Gel usually show positive Alcian Blue staining. Therefore, our GAG quantification experiments performed by dissolving the microspheres are more

accurate. In our analysis of the GAG content shown in Fig. 5C, both groups showed an increase in the GAG content as the induction time increased. Notably, the Alg-Gel-ADSCs MSs group had a higher GAG content than the other groups. The quantitative results indicate that Gel-containing microspheres are superior to Alg microspheres in cartilage formation and differentiation.

3.5. Detection of Alg-Gel-ADSCs MSs containing PKH26-labelled ADSCs in vivo

In the merged images of DAPI and PKH26 staining (Fig. 6A3), the prepared microspheres caused cells to remain in the cartilage defects for more than three weeks. However, the red fluorescence of PKH26 was not present in the control group (Fig. 6B1-3). Similarly, in the visualization of PKH26-labelled cells using the Kodak In Vivo Imaging System FX (Fig. 6A4, B4), we also observed that the ADSCs in the microspheres remained in the defects for up to three weeks.

3.6. Gross observation and histological evaluation

In the gross observation of cartilage repair samples of SD rats, we found that the cartilage defect persisted in the defect group at 12 weeks postoperatively, with clear edges and a rough surface. In the Alg-Gel MS group, the regenerated tissue was white and resembled hyaline cartilage, but obvious gaps were observed between the regenerated tissue and the normal tissue. The cartilage defect area of the Alg-Gel-ADSCs MSs group was almost completely filled with cartilage-like tissue. The surface of this cartilage-like tissue was relatively flat, and the boundary with the original cartilage was not obvious (Fig. 7A). At week 12, the Alg-Gel-ADSCs MSs group had significantly higher ICRS and modified Wakitani scores than the other two groups ($p < 0.05$) (Fig. 7F–G).

H&E and Masson's trichrome staining showed that the Alg-Gel-ADSCs MSs group and the Alg-Gel MSs group formed more cartilage-like tissues than the other groups and that the surface was flat. No obvious boundary was detected between the regenerated tissue and the surrounding normal cartilage in the Alg-Gel-ADSCs MSs group, and a number of cells were observed in the cartilage. In contrast, the Alg-Gel MSs group had fewer cells on the cartilage surface, while the control group had obvious defects and rough surfaces (Fig. 7B–C).

In terms of the immunohistochemical analysis shown in Fig. 7D, the

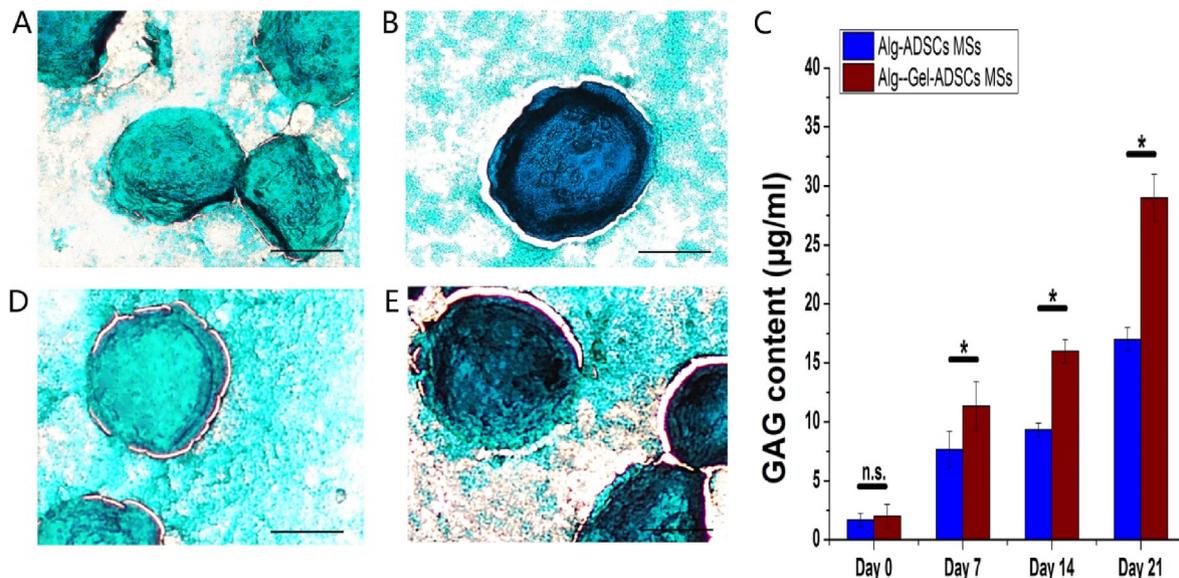


Fig. 5. Chondrogenic differentiation ability. Alcian Blue staining of Alg-ADSCs MSs (A), Alg-Gel-ADSCs MSs (B), Alg-MSs (D), and Alg-Gel MSs (E) on day 21 of chondrogenic induction. Scale bar = 200 µm (C) Graph showing the GAG content.

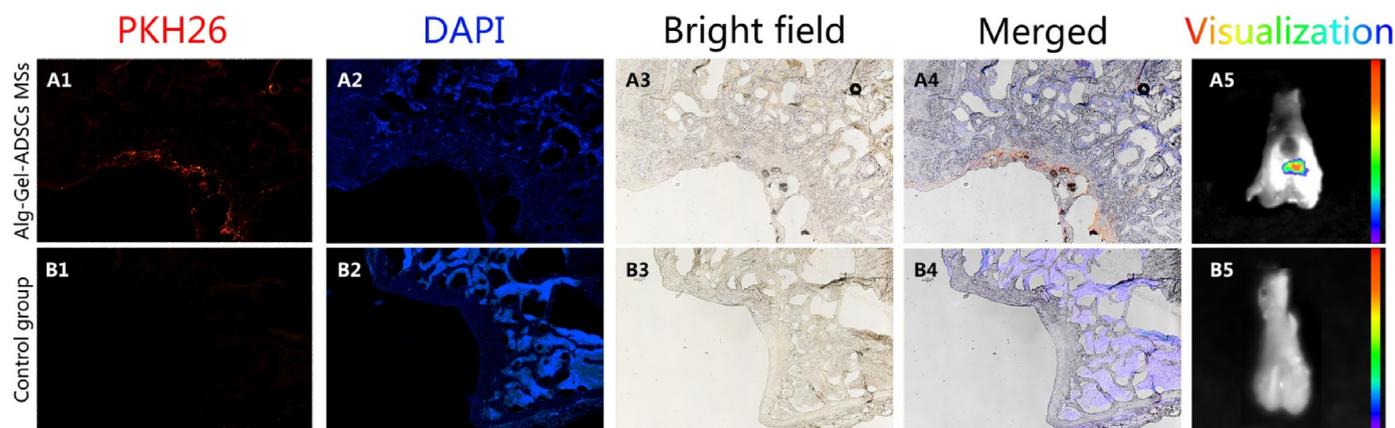


Fig. 6. ADSCs tracking. Image of the Alg-Gel-ADSCs MSs containing PKH26-labelled ADSCs (A1-4); images of the control group (B1-4).

repaired tissue cartilage surface was strongly positive for type II collagen in the Alg-Gel-ADSCs MSs group, similar to natural cartilage. The colour of type II collagen in the defect group and the Alg-Gel-ADSCs MSs group was lighter, and only extremely thin type II collagen was present on the cartilage surface (Fig. 7D).

Sirius red staining showed that repaired tissues from the defect group mainly expressed type I collagen and had strong refractive power, an irregular organization and defects. The repaired tissues from the Alg-Gel-ADSC MS group were rich in type II collagen, multicolour type II collagen fibres were observed in the repaired tissue, and the collagen fibres were arranged neatly, while the characteristics of the second group were between those of the other two groups (Fig. 7E).

3.7. Micro-CT and CatWalk evaluations

CatWalk was used to analyse the gait before and at 1, 6, and 12 weeks after surgery to evaluate the function of the left knee joint. The three-dimensional footprint intensity analysis showed that the recovery of the left back footprint of the Alg-Gel-ADSC MS group was better than that of the other two groups at 12 weeks after surgery (Fig. 8A–C). The quantitative analysis also confirmed these findings (Fig. 8G). As shown in Fig. 8G, cartilage defect surgery resulted in a prominent decrease in the footprint intensity of the left hind leg in each group one week after the operation. Over time, the intensity of this parameter in the Alg-Gel-ADSCs MS group showed a clear increasing trend. At 12 weeks post-operatively, the footprint intensity of the Alg-Gel-ADSCs group was significantly higher than that of the other two groups ($p < 0.05$), indicating that joint function had partially recovered.

Micro-CT images showed subchondral bone regeneration in all three groups after surgery (Fig. 8D–F). The bone volume fraction (BVF) of the Alg-Gel-ADSCs MS group was significantly higher than that of the other two groups. According to the quantitative analysis, the Alg-Gel MS group and the Alg-Gel-ADSCs MS group exhibited significantly higher ratios than the fibrin group ($p < 0.01$). Moreover, the Alg-Gel-ADSC MS group had a better bone volume fraction (BVF) value than the Alg-Gel MS group at 12 weeks postoperatively (Fig. 8H).

4. Discussion

The treatment of articular cartilage damage is very difficult, and this view is broadly accepted [26]. The construction of cartilage-like tissue to treat articular cartilage defects is a strategy with strong therapeutic potential in the field of cartilage regeneration.

As a cell population with good clinical application potential, ADSCs have been evaluated for their ability to be used in cartilage TE after isolation. We first analysed the phenotype of cells using flow cytometry

and found that ADSCs expressed some of the markers of MSCs. Furthermore, the differentiation of ADSCs was confirmed by performing induction experiments. Notably, ADSCs were induced to differentiate into chondrocytes for cartilage TE (Fig. 2C). The cells we separated in this experiment were expanded to obtain a larger number of purified ADSCs. This method is very simple, even if the purification effect is limited. Based on the results of flow cytometry (Fig. 2E), some adipose-derived stromal vascular fraction (SVF) components were still present in the obtained ADSCs [27]. Nevertheless, this population still differentiated successfully during the induced differentiation process. Therefore, studies are needed to determine whether SVF separated from adipose tissue can also be loaded into this material to repair articular cartilage defects in the future. If this method is feasible, namely, the cells are separated and the SVF-encapsulated microspheres are quickly prepared and injected into the cartilage defect area, this procedure, which can be completed in a single operation, will reduce the length of hospitalization and the economic burden on patients.

Researchers must mimic the required microenvironment to successfully expand stem cells. Alg and Gel have the advantages of good biocompatibility, low toxicity, low cost, and mild gel properties. These biomaterials have good application potential [28,29]. In addition, they can be crosslinked to form microspheres using the electrospray method.

In this study, we used an electrospray method to embed ADSCs. Compared with other methods, this type of physical preparation produces particles with a higher loading efficiency and stable particle size distribution [30]. Unlike some technologies, the electrospray method does not require the addition of surfactants that may damage cells; thus, it is a simple and good method for preparing cell-embedded microspheres [31–33]. Although Gel promotes cell adhesion and proliferation, complete microspheres were not prepared if excess Gel was added to Alg in the present study. This finding may be related to the solubility of Gel. Changing the temperature alters the solubility of Gel, but this change will also prevent the use of the electrospray method at room temperature, losing the important advantages of simplicity and speed. Therefore, we chose a 0.5% Gel concentration to complete the subsequent experiments. The concentration of Gel we selected had no effect on the diameter of the microspheres, and no significant difference in the diameter of the microspheres encapsulating cells and the microspheres without cells was observed ($p > 0.05$; Fig. 3).

Regarding cell proliferation, 3D cultured hydrogel microspheres indeed produced more cells after 14 days of culture (Fig. 4C). However, the 2D cultured cells grew to cover the culture plate after the seventh day of proliferation and produced a large amount of ECM after contact inhibition (Fig. 4B). A potential explanation for these results is that the microspheres have a relatively large surface area and do not cause early contact inhibition compared to 2D culture (Fig. 4A). For Alg

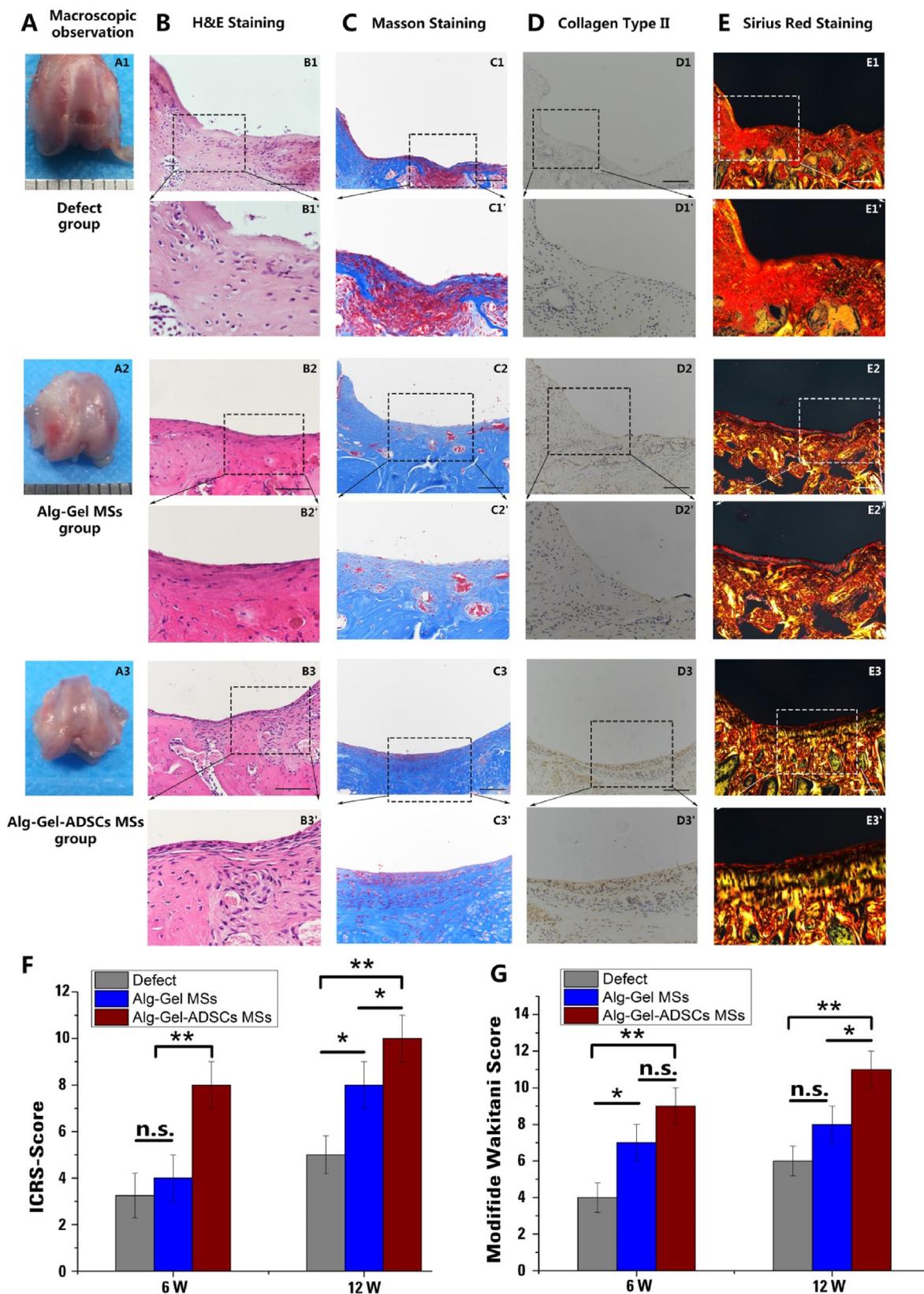


Fig.7. Macroscopic observation, histological evaluation and score at 12 weeks after surgery. Gross observation of the repaired cartilage defects (A1-A3). H&E staining (B1–B3, B1'–B3'), Masson's trichrome staining (C1–C3, C1'–C3'), immunohistochemical staining for type II collagen (D1–D3, D1'–D3') and Sirius red staining (E1–E3, E1'–E3') of repaired cartilage defects. The dashed rectangular area is displayed in the lower panels at higher magnification. Scale bars: 100 μ m in B1–B3, C1–C3, D1–D3, and E1–E3. ICRS scores (F) and modified Wakitani scores (G) recorded at 6 and 12 weeks after surgery are shown: * $p < 0.05$ and ** $p < 0.01$.

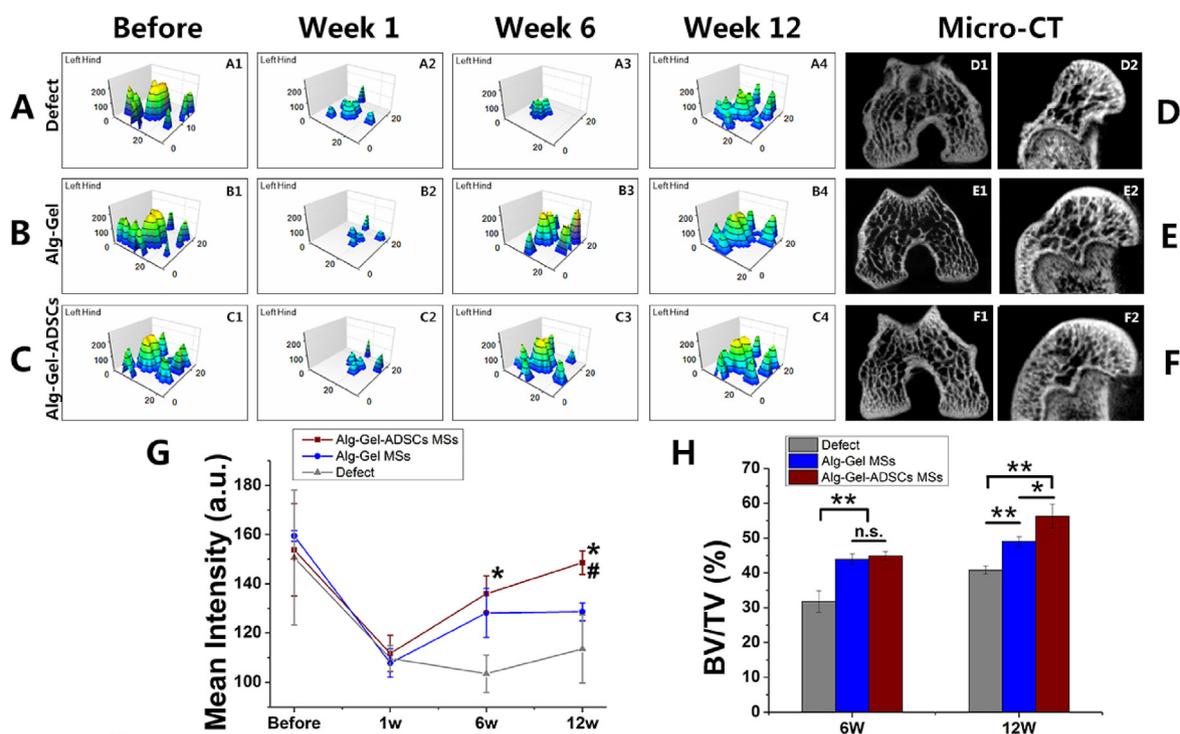


Fig. 8. Micro-CT imaging evaluation and CatWalk gait analysis. Three-dimensional images showing the footprint intensity of the left hind leg in the three groups preoperatively or at 1, 6 and 12 weeks postoperatively (A, B, C). The weight load was measured as the mean intensity preoperatively or at 1, 6 and 12 weeks postoperatively (G). The results are presented as the means \pm SD, # $p < 0.05$ compared to the Alg-Gel-ADSC MS group and * $p < 0.05$ compared to the defect group. Two-dimensional micro-CT images of the left knee of animals from the three groups at 12 weeks postoperatively (D, E, F) (H) bone volume fraction (BV/TV) at 6 and 12 weeks postoperatively. * $p < 0.05$, ** $p < 0.01$, n. s. not statistically significant.

microspheres, since the osmotic effect of the culture medium on the microspheres was not as direct as that of the 2D culture, a greater number of cells was detected in 2D culture on the seventh day (Fig. 4C). The addition of Gel compensated for the slower cell proliferation in the microspheres and increased cell viability (Fig. 4A). Moreover, previous reports have shown that 3D hydrogel culture of MSCs is superior to particle culture in preventing hypertrophy and terminal differentiation [34–36]. Furthermore, the method of adding Gel still has advantages in chondrogenesis (Fig. 5C).

The mechanism of most methods for the repair of cartilage damage involves MSC migration to the cartilage defect site and differentiation into chondrocytes [37,38]. Therefore, the residence of MSCs in the area to be repaired is a core factor contributing to the successful repair of defects. In our research, the microspheres we prepared remained in the cartilage defect for up to 3 weeks (Fig. 6). We conducted follow-up animal experiments to show its repair effect.

The process used to prepare the microspheres is simple and is an efficient method for cartilage TE. Hydrogel microspheres of suitable volume can be injected through a syringe and are expected to show their superior cartilage regeneration ability *in vivo*. Compared with the direct injection of cells, the effect of hydrogels on reducing shear stress has been confirmed [39]. Furthermore, we directly used ADSC-based Alg-Gel microspheres to repair cartilage defects in SD rats and evaluated the therapeutic efficacy. Hyaline-like cartilage repair by Alg-Gel-ADSCs MSs was evaluated histologically. According to the histological results, the repair effect of Alg-Gel-ADSCs MSs was better than that of the cell-free microsphere group and the defect group. Not only were the defects on the cartilage surface filled, but they also contained increased levels of type II collagen (Fig. 7D–E). In general, we explored the use of Alg-Gel-ADSCs MSs as the basis for cartilage regeneration by performing *in vivo* experiments. From the histological morphology and immunohistochemical evaluations, we conclude that Alg-Gel-ADSCs MSs perform better than Alg-Gel MSs in cartilage regeneration *in vivo*. Although Alg-Gel MSs

show some ability to repair cartilage defects (namely, they fill cartilage defects after a long period of repair), the integration between the regenerated tissue and the normal tissue is poor, and identifiable gaps were detected at the interface. This phenomenon results in a rough surface of the regenerated cartilage tissue. The results (Fig. 7) also showed regenerated cartilage-like tissues in the Alg-Gel-ADSCs MSs group. The collagen fibre structure was similar to that of natural cartilage and rich in type II collagen. Thus, Alg-Gel-ADSCs MSs effectively promote cartilage regeneration.

The melting point of gelatine is approximately 37 °C, which is very close to the physiological temperature, and thus gelatine may have dissolved during the culture period. We propose that this dissolution gave ADSCs more room for growth and resulted in greater infiltration of nutrients to increase the activity of ADSCs. Additionally, as reported by other scholars [17], gelatine components promote cell proliferation. The time of Alg degradation is suitable for cartilage repair, and it has been widely used in cartilage regeneration [40]. Alg helps to fill cartilage defects early and restore their barrier function, while it is gradually degraded as cells grow, forming ECM deposits.

Based on our study, ADSCs play an important role in newly regenerated cartilage. Based on the results of H&E staining and Masson's trichrome staining shown in Fig. 7, a large number of cells from the Alg-Gel-ADSCs MSs group were observed at the site of the regenerated cartilage. This finding was not observed in the other two groups without ADSCs. Furthermore, the immunohistochemical evaluation and Sirius red staining confirmed the presence of more collagen type II at the locations where cells were abundant on the surface of newly regenerated cartilage. This result may be due to the residence, differentiation and secretion of ECM by ADSCs in the cartilage defect.

The CatWalk gait analysis is also an important method to evaluate joint function. When SD rats touched the ground with their soles, the fluorescence intensity produced increased with the amount of force. The Alg-Gel-ADSCs MSs group had a significantly higher intensity than the

other two groups at week 12. Therefore, the repair mediated by the Alg-Gel-ADSCs MSs resulted in a firmer step of the left hind limb of the rat. Micro-CT analysis is often used to evaluate subchondral bone reconstruction. One important indicator of articular osteochondral repair is the bone volume fraction (BVF), which was calculated to evaluate the level of knee cartilage regeneration using micro-CT [41]. Notably, in the sixth week after surgery, no significant difference was observed between the Alg-Gel-ADSCs MSs group and the Alg-Gel MSs group in terms of micro-CT and CatWalk analyses. Whether the filling of articular cartilage defects in the early stage can also promote the recovery of articular subchondral bone and joint function requires further research.

The important factors of cartilage TE are as follows: excellent seed cells (including easy access to sources and chondrogenic differentiation potential), high-quality carrier materials, and a suitable microenvironment [42]. In this study, we selected ADSCs with abundant sources and potential for chondrogenic differentiation, as well as inexpensive and nontoxic hydrogels as carriers, and obtained a microenvironment suitable for proliferation and chondrogenic differentiation by adding Gel components. Moreover, this type of microsphere with a suitable volume can be injected into the cartilage defect site through a minimally invasive method, which has major advantages in treating cartilage damage.

Currently, relatively few studies have assessed tissue-engineered microspheres with alginate as a matrix to which gelatine has been added, and a series of studies on whether ADSCs effectively proliferate and differentiate in Alg-Gel microspheres prepared using the electrospray technique and whether they repair cartilage defects has not been conducted. Therefore, in this study, we isolated ADSCs from SD rats and used a simple and quick electrospray method to load the ADSCs into alginate-gelatine microspheres to construct injectable microspheres. The morphology, cell activity, proliferation and chondrogenic differentiation of different cell-loaded MSs were evaluated. Finally, Alg-Gel-ADSCs MSs and cell-free Alg-Gel microspheres were placed on the knee cartilage defects of SD rats. Through gross observation, a quantitative analysis of the gross score, cell tracing, histological evaluation, immunohistochemical evaluation, and microcomputed tomography (micro-CT) and gait analyses, the cartilage repair effect was evaluated.

5. Conclusions

A 1.5% Alg solution containing 0.5% Gel was prepared into microspheres using electrospray technology. Alg-Gel MSs support the attachment and proliferation of ADSCs and that ADSCs embedded in microspheres differentiate into chondrocytes; this treatment is superior to Alg MSs in terms of proliferation and differentiation. Cells transplanted in this carrier reside in cartilage defects. Alg-Gel-ADSCs MSs can effectively repair articular cartilage defects and represent a promising strategy for the treatment of cartilage damage.

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Declaration of competing interest

Author Sida Liao, Author Haoye Meng, Author Jun Zhao, Author Wancheng Lin, Author Xiuzhi Liu, Author Zhuang Tian, Author Lan Lan, Author Hanyu Yang, Author Yichi Xu, Author Xiao Gao, Author Shibi Lu and Author Peng Jiang declare that they have no conflicts of interest.

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