Int J Clin Exp Med 2016;9(6):10322-10332
www.ijcem.com /ISSN:1940-5901/IJCEM0023703

*Original Article*

# Synovium-derived mesenchymal stem cell sheet enhance autologous osteochondral transplantation in a rabbit model

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Received January 10, 2016; Accepted March 23, 2016; Epub June 15, 2016; Published June 30, 2016

**Abstract:** Objective: Autologous osteochondral transplantation (OCT) is an option to treat articular cartilage defects. However, poor graft integration at the cartilage defect/graft interface and secondary graft degeneration limit the use of autologous OCT. Cell sheet engineering offers hope in regenerative medicine. The purpose of this study was to evaluate the effect of synovium derived mesenchymal stem cell (SMSC) sheet on autologous OCT for (1) lateral integration and (2) graft degeneration. Method: Full-thickness osteochondral defects (3.5 mm in diameter, 3.0 mm in depth) were created at both femoral grooves of 12 rabbits. The right knees were used as the cell sheet group and the left knees were used as the control group. Osteochondral plugs were harvested when the defects were created and then were placed into their original locations with (cell sheet group) or without (control group) three-layered SMSC sheets. The animals were sacrificed at 4 and 8 weeks postoperatively (6 animals at each time point). Histological and immunohistochemical staining were used to evaluate the result. Results: Histological and immunohistochemical findings at the interface between the grafts and the original cartilage showed better integration in the cell sheet group. In addition, less graft and original cartilage degeneration were found 4 and 8 weeks after operation. The mean histological score was significantly higher in the cell sheet group. Conclusion: The animal model showed that the SMSC sheet enhanced the quality of autologous OCT by improving the integration of grafts to native cartilage and decreasing cartilage degeneration. Thus, the application of SMSC sheet can represent a therapeutic option for autologous OCT.

**Keywords:** Autologous osteochondral transplantation, synovium-derived mesenchymal stem cell sheets, integration, cartilage degeneration

## Introduction

Hyaline cartilage is easily injured, but due to its avascular and hypocellular nature, cartilage defects exhibit poor spontaneous healing instinct [1, 2]. If left untreated, even focal defects in weight-bearing areas will progress to secondary degeneration of the whole joint [3, 4]. Therefore, cartilage defects should be properly treated as soon as possible following injury. Numerous studies have been conducted on the repair of cartilage defects, and many repair strategies have been employed. However, cartilage regeneration remains a challenge [5]. Currently, the most common treatment options for cartilage injuries include articular debridement, microfracture, autologous chondrocyte implantation (ACL), and autologous osteochondral transplantation (OCT) [6-8]. However, no repair tissue that can withstand normal joint activity over prolonged periods was successfully regenerated in any of these therapies. For example: fibrocartilage is common for microfracture [9] and graft hypertrophy is one of the main obstacles for ACL [9, 10].

Autologous OCT is a standard procedure for the repair of small osteochondral defects [11, 12]. By transplanting with cylindrical osteochondral plugs obtained from low weight-bearing regions,

these defects can immediately obtain a smooth cartilage surface. Several clinical studies have demonstrated the efficiency of autologous OCT [11-14]; however, there are still some limitations. Actually, some studies have reported negative consequences of autologous OCT such as nonintegration at the defect margins and degeneration of the grafts [15-17]. In fact, it has been demonstrated that the integration of osteochondral grafts to the adjacent cartilage is one of the principle obstacles for OCT [16]. The low metabolic levels of cartilage, as well as its anti-adhesive extracellular matrix (ECM), may result in poor lateral integration [16, 17]. In addition, glycosaminoglycans were even found to directly inhibit cell adhesion [18]. Poor integration at the defect margins will eventually lead to degeneration of the grafts and adjacent cartilage. Several studies have been conducted to improve lateral integration with enzymatic treatment [17], platelet-rich plasma injection [19], and Mesenchymal stem cells (MSC) transplantation [20].

Recently, cell sheet engineering characterized by abundant ECM and cells has attracted a great deal of attention [21]. Layered cell sheets can form a 3D environment for tissue engineering without scaffolds. The cell sheet technique has been applied in the repair of heart, cornea, tooth root, and skin [22-25]. In addition, cell sheets derived from MSCs have been widely used in musculoskeletal regeneration such as bone, ligament, and cartilage [26, 27]. However, no studies have examined the combination of cell sheet technology with autologous OCT in cartilage defect restoration. This study hypothesized that synovium derived mesenchymal stem cell (SMSC) sheets could enhance lateral integration and decrease cartilage degeneration.

In this study, first transplanted autologous osteochondral cylinder wrapped with three-layered SMSC sheets was transplanted into osteochondral defects in an animal model. Then the lateral reconstruction and cartilage degeneration by histology and immunohistology at 4 and 8 weeks after operation were evaluated.

## Methods

### *Animals*

The study was carried out in strict accordance with the recommendations in the Guide for Care and Use of Laboratory Animals of the National Institutes of Health. All animal care and experiments were carried out in accordance with the guidelines and were approved by the Ethics Committee of Drum Tower Hospital, Medical School of Nanjing University, China. Total 16 skeletally mature New Zealand white rabbits (female, 12 weeks of age, 2.0-2.5 kg body weight) from the Jinling farm, Nanjing, China were enrolled in this study. Four rabbits were given overdose anesthetics to be sacrificed to harvest synovial tissue, and the other 12 were used to create cartilage defects in the trochlear grooves of both knees and obtain the osteochondral plugs under general anaesthesia. The knees were assigned to one of the two groups: (1) cell sheet group (right), and (2) control group (left). For the cell sheet group, OCT was performed after the graft was cylindrically wrapped with three-layered SMSC sheets; for the control group, OCT was performed without cell sheets. 4 and 8 weeks after surgery, the animals were given euthanasia and the repair quality was evaluated histologically and immunohistochemically. A modified O'Driscoll histological scoring system was used to assess the quality of transplantation at eight weeks.

### *Isolation of SMSCs*

The synovial tissue was obtained from the knee joints of four female rabbits under aseptic conditions and was placed into a transport medium containing Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) (Gibco, Life Technology, USA) with 1% (v/v) Penicillin-Streptomycin-Glutamine (PSG) solution (Gibco). After a brief storage at 4°C, the synovial tissue was transferred into a biosafety cabinet and rinsed with phosphate-buffered saline (PBS) for three times. After the adipose tissue was removed, the synovial tissue was minced into 1 × 1 $mm^2$ small pieces. The tissue fragments were incubated with 0.1% collagenase type I (Sigma, USA) overnight at 37°C in a humidified atmosphere (Thermo Scientific, USA). A 70-μm nylon filter was used to remove undigested tissue debris. The cell suspensions were centrifuged at 1500 rpm for 5 minutes and the isolated cells were resuspended in a complete medium containing DMEM/F12, 5% fetal bovine serum (Gibco), and 1% (v/v) PSG solution. After counting the number of cells using a manual cell counter under light microscopy, the cells were seeded in culture plate at a

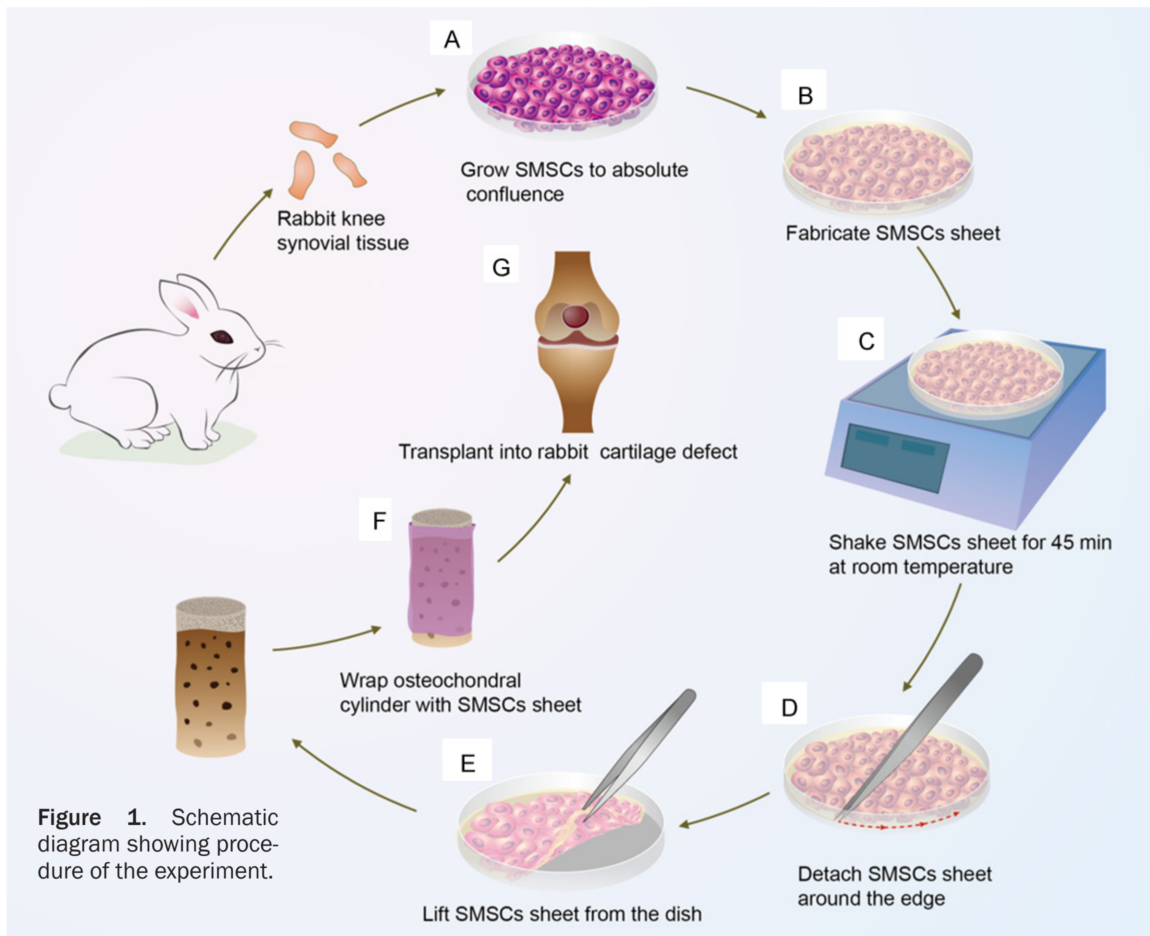


**Figure 1.** Schematic diagram showing procedure of the experiment.

low density (1000 cells per 60-mm dish). The culture medium was changed every 2-3 days to remove nonadherent cells and to purify the cells. The cell that could form a colony was selected and used as SMSC.

*Flow cytometry*

Passage 3 rabbit MSCs were harvested 7 days after plating. $1 \times 10^6$ cells were suspended in 100 ml PBS containing 0.5% bovine serum albumin (BSA), 2 mM EDTA and 20 ng/ml fluorescein isothiocyanate (FITC)-coupled antibodies against CD34, CD45, CD11b and CD90 (MACS, MiltenyiBiotec, Germany). FITC coupled nonspecific mouse IgG1 and mouse IgG2a antibodies (MACS) were used as isotype control. After incubation in the dark at 4°C for 10 minutes, the cells were washed with 2 ml PBS and centrifuged at 300 g for 10 minutes to discard the supernatant completely. The cells were resuspended in 500 ml PBS for analysis by fowcytometry (Becton Dickinson, USA). Data were analyzed using the flowjo software (Becton Dickinson).

*Preparation of SMSC sheet*

Rabbit SMSCs were amplified in a complete medium. The medium was changed every 2-3 days. After confluence was achieved, the cells were retrieved using trypsin–EDTA solution (0.25% trypsin, 1 mM EDTA; Hyclone, USA) and replated at $1 \times 10^6$ cells per 100 mm dish. The third passage cells were used to prepare cell sheets. $5 \times 10^5$ cells were plated in 60-mm dish and cultured in a complete medium supplemented with 5-ng/ml basic fibroblast growth factor (Pepro Tech, USA). When the cells achieved confluence at about 5 days' culture (**Figure 1A**), 20 µg/ml of vitamin C (Sigma) was added to the medium to induce the formation of a cell sheet [28]. Large amounts of adhesive proteins such as fibronectin, and binding proteins among cells will be secreted after 14 days of vitamin C induction [21, 28]. Thus, an SMSC

**Table 1.** Modified O'Driscoll Histological Grading Scale

| | Score |
|---|---|
| Nature of the predominant tissue | |
| Cellular morphology | |
| Hyaline articular cartilage | 4 |
| Incompletely differentiated mesenchyme | 2 |
| Fibrous tissue or bone | 0 |
| Safranin O staining of the matrix | |
| Normal or nearly normal | 3 |
| Moderate | 2 |
| Slight | 1 |
| None | 0 |
| Structure characteristics | |
| Surface regularity | |
| Smooth and intact | 3 |
| Superficial horizontal lamination | 2 |
| Fissures-25 to 100 percent of the thickness | 1 |
| Severe disruption, including fibrillation | 0 |
| Structure integrity | |
| Normal | 2 |
| Slight disruption, including cysts | 1 |
| Severe disintegration | 0 |
| Thickness | |
| 100 percent of normal adjacent cartilage | 2 |
| 50-100 percent of normal cartilage | 1 |
| 0-50 percent of normal cartilage | 0 |
| Bonding to the adjacent cartilage | |
| Bonded at both ends of graft | 2 |
| Bonded at one end, or partially at both ends | 1 |
| Not bonded | 0 |
| Freedom from cellular changes of degeneration | |
| Hypocellularity | |
| Normal cellularity | 3 |
| Slight hypocellularity | 2 |
| Moderate hypocellularity | 1 |
| Severe hypocellularity | 0 |
| Chondrocyte clustering | |
| No clusters | 2 |
| < 25 percent of the cells | 1 |
| 25-200 percent of the cells | 0 |
| Freedom from degenerative changes in adjacent cartilage | |
| Normal cellulariy, no clusters, normal staining | 3 |
| Normal cellularity, mild clusters, moderate staining | 2 |
| Mild or moderate hypocellularity, slight staining | 1 |
| Severe hypocellularity, poor or no staining | 0 |

sheet formed (**Figure 1B**). The 60-mm culture plate with a cell sheet was shocked on a horizontal rotator at 30 rpm at room temperature for 45 minutes (**Figure 1C**). A tweezer was used to detach the cell sheet from the culture plate by scratching around the edge of the culture plate (**Figure 1D**). Then the cell sheet can be easily lifted from the culture plate (**Figure 1E**).

Autologous OCT Surgery New Zealand white rabbits (12 female, 12-week old, 2.0-2.5 kg) were maintained in the animal center of Drum Tower Hospital for a week before operation. Osteochondral defects were created as previously described [29]. Briefly, the rabbits were anesthetized by intramuscular injections of 5-mg droperidol and 0.1-g ketamine and maintained with auricular vein injections of ketamine and diazepam during the operation. A medial parapatellar incision was performed at both knee joints after the rabbits were put in the supine position. The articular surfaces of the trochlear grooves were exposed by laterally dislocating the patellae. An osteoarticular transplantation system, which is 3.5 mm in diameter was used to create a full-thickness cartilage defect and to obtain an osteochondral plug in the trochlear groove of the knee. All of the plugs were placed into their original locations. In the control group, the osteochondral plugs were put directly back into the defects. In the cell sheet group, the osteochondral plugs were placed into culture plates and wrapped cylindrically with SMSC sheets using a tweezer (**Figure 1F**). The procedures were repeated three times. After wrapping, the osteochondral cylinders were immediately implanted into the defects (**Figure 1G**). As the diameter of the defects were larger than that of the osteochondral cylinders, the osteochondral cylinders wrapped with three-layered SMSC sheets could be properly pressed fit into the defect sites. The incisions were carefully closed, and the animals were allowed to have

free movements in their cages. The animals were sacrificed by over injection of ketamine at 4 and 8 weeks after surgery, and both knees were harvested. Six animals were sacrificed at each time point.

*Histological processing and scoring*

The samples were fixed with 10% formalin for 7 days at room temperature. Then, the samples were decalcified in 15% EDTA solution (Sunshine, Nanjing, China) for 14 days. After embedded in paraffin, the samples were cut into 5-μm sections serially. The sections were stained with hematoxylin and eosin (H&E), toluidine blue, and safranin O/fast green, according to the manufactures' recommendations, to examine graft integration and cartilage degeneration under light microscope (Olympus, Japan). A modified O'Driscoll histological scoring system was used to evaluate the quality of transplantation at 8 weeks postoperation (**Table 1**) [30]. The histological scoring system consisted of four categories: the nature of the predominant tissue, structural characteristics, graft degeneration, and adjacent cartilage degeneration. Three observers performed the histological scoring independently and blindly.

*Type II collagen immunohistochemical staining*

Eight weeks after operation, immunohistochemical staining of type II collagen was performed in the cartilage matrix. The slides were washed three times for 5 minutes in xylene. Serial ethanol was used to rehydrate the sections. Then, the sections were immersed into 0.4% pepsin (Sigma) solution at 37°C for 50 minutes to perform antigen retrieval. After blocked with 1% BSA at room temperature for 1 hour and washed, the tissue samples were incubated in monoclonal mouse type II collagen primary antibody (Calbiochem, Merck Millipore, 1:100 dilution) at 4°C overnight. After 1-hour incubation with a biotinylated secondary anti-mouse antibody (GE Healthcare; 1:500 dilution), the color was reacted using a 3', 3 -diaminobenzidine (DAB) solution (Sigma); 1% BSA solution was used as a control.

*Quantification of histological/immunohistochemical staining positive area*

The sections that were stained histologically or immunohistochemically were imaged for further quantification. The proteoglycan staining degree was evaluated by quantifying area fraction using the Image J software as previous reported [31]. Briefly, the image was opened by the software and the stained color was defined by selecting the stained area and clicking the Image/Adjust/Color threshold. The image was then transferred to an 8-bit binary image by clicking the Process/Binary/Make binary button. Then, the whole cartilage area was selected and the staining positive area fraction was calculated using the Analyze/Analyze particles tool.

*Statistical analysis*

The histological scores were summed. Unpaired *t*-tests were used to evaluate the differences in the cell sheet and the control groups. $P \leq 0.05$ was considered statistically significant difference. The statistical power of the study was 0.88. All analysis was performed using the SPSS software (version 20.0; IBM).

**Results**

*Animal health*

No animal failed the experiment. No complication related to surgical procedure was observed at any time point. No adverse effect related to the transplantation of SMSC sheets was detected.

*Cell culture and successful fabrication of the SMSC sheet*

SMSCs were successfully isolated from rabbit knee synovium tissue. The cells exhibited the spindle-like shape and proliferated in a swirl way (**Figure 2A**). Flow cytomeric analysis showed that the cells displayed MSCs' features (**Figure 2B**). After 2 weeks' induction with vitamin C, a SMSC sheet formed and was detached from the culture dish with a tweezer (**Figure 2C**). The osteochondral cylinder was cylindrically wrapped with three-layered SMSC sheets and transplanted into the osteochondral defect (**Figure 2D**).

*H&E and toluidine blue staining of the sections*

H&E and Toluidine blue staining 4 and 8 weeks after surgery revealed better lateral integration and less cartilage degeneration in the cell sheet group. H&E staining showed no clear

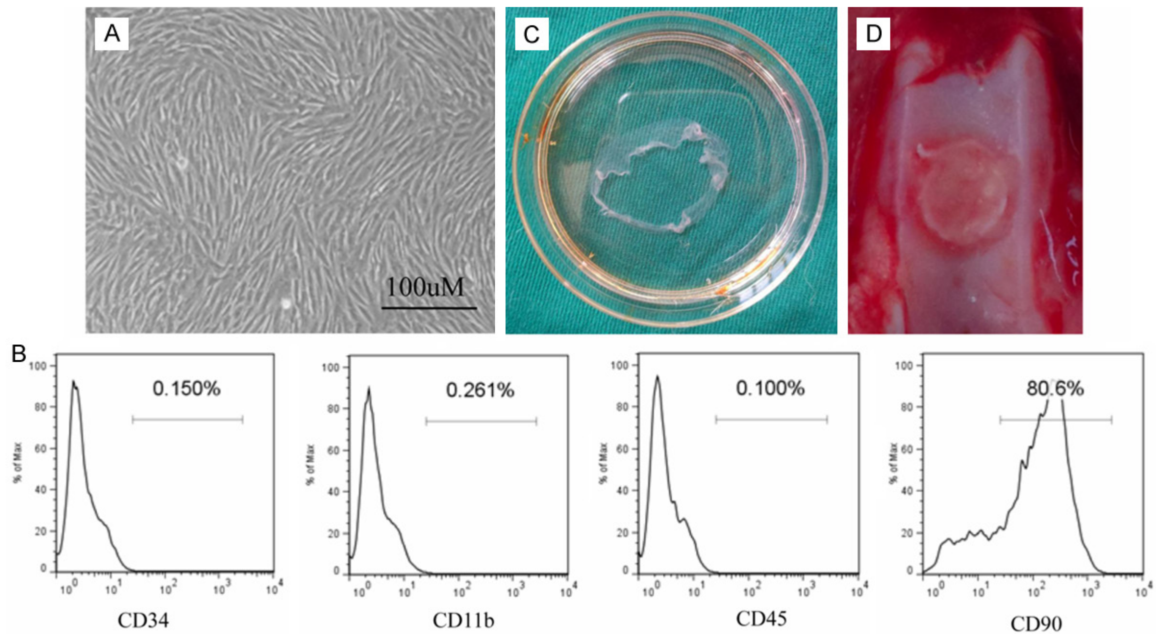


**Figure 2.** Preparation of a cell sheet with SMSCs. A: Spindle-like SMSCs reached confluence were ready for preparation of a cell sheet. B: Flow cytometric analysis of SMSCs at passage 3 showed that the majority of the cells were negative for CD34, CD11b, and CD45, but expressed CD90. C: A newly formed cell sheet was detached from a culture dish. D: An osteochondral cylinder was just transplanted into the osteochondral defect immediately after wrapped with three-layered SMSC sheets. Scale bar: 100 mm in A.

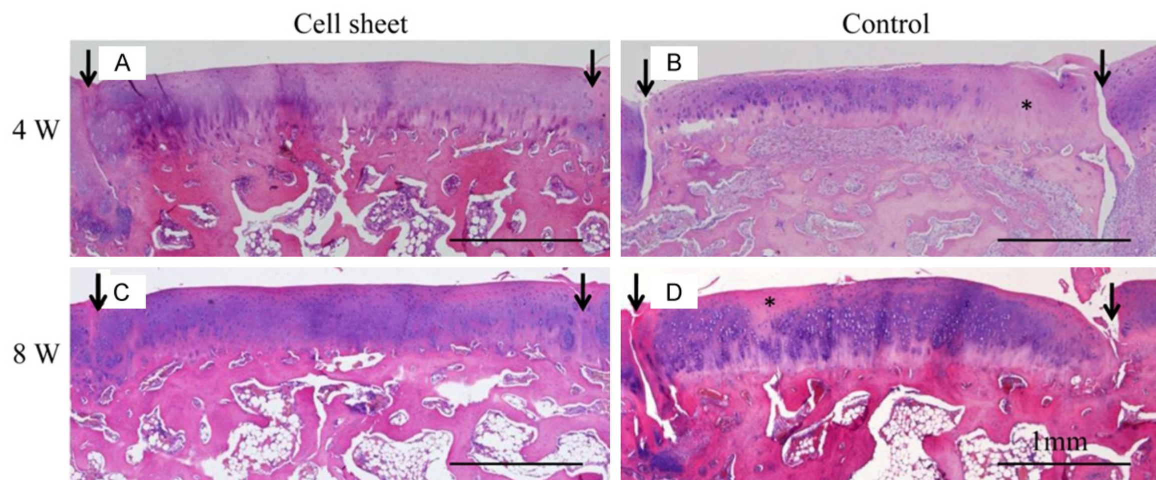


**Figure 3.** Representative H&E staining of the sections. A: No obvious clefts were observed at the defect margins (arrows) 4 weeks postoperation in the cell sheet group. B: H&E staining 4 weeks after surgery revealed clear clefts (arrows) and severe hypocellularity (asterisk), which indicated degeneration of the graft in the control group. C: 8 weeks after surgery in the cell sheet group, the margins (arrows) were barely distinguishable. D: Clefts (arrows), hypocellularity (asterisk), and slight disruption of the cartilage were observed in the control group 8 weeks after operation. Scale bar: 1 mm in all the images.

gaps between the grafts and adjacent cartilage in the cell sheet group, although the demarcations were still clear (**Figure 3A** and **3C**). The surfaces of the grafts were smooth and intact without any delamination or disruption (**Figure 3A** and **3C**). The control group showed clear cracks at the defect margins (**Figure 3B** and **3D**). Obvious hypo-cellularity and delamination of the grafts were also observed in control group (**Figure 3B** and **3D**).

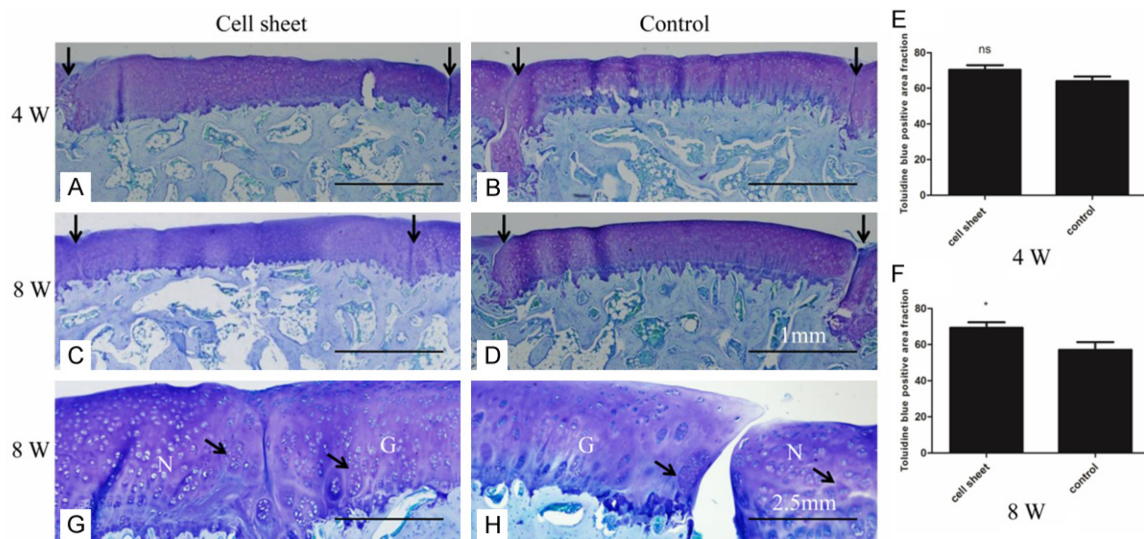


**Figure 4**. Representative toluidine blue staining of the sections. A and C: The grafts and interfaces in the cell sheet group were stained intensely and homogeneously with no clear demarcations. The matrix in control group (B, D) was slightly stained, and the clefts were obvious. E and F: Quantification of toluidine blue staining positive area fraction for the sections. Data represent mean ± standard error of mean (SEM) (n = 6, ns: P > 0.05, *P < 0.05). G: 8 weeks after surgery at higher magnification, staining and cell density were regular at the grafts G and the adjacent cartilage N in the cell sheet group, although a few cell clusters (arrows) were observed. H: Lighter staining and severe hypocellularity was observed at both of the graft G and the native cartilage N in the control group. Many cell clusters (arrows), a sign of cartilage degeneration, were shown in the cartilage of the graft G and the native cartilage N. Scale bar: 1 mm in A-D; 2.5 mm in G and H.

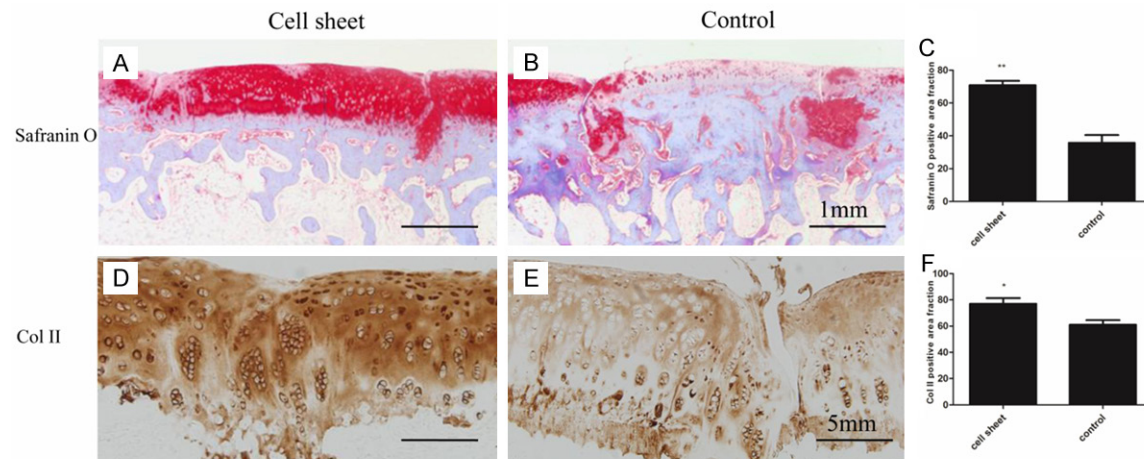


**Figure 5.** Safranin O and type II collagen immunohistochemical staining at 8 weeks after operation. A: Homogeneously intense safranin O staining in the graft and the native tissue in the cell sheet group. B: Most of the ECM in the graft and some ECM in the native tissue of the control group were negatively stained. D: Strong type II collagen staining was observed in the cell sheet group. D: The ECM of the control group was stained slightly. C and F: Quantification of staining positive area fraction for the sections. Data represent mean ± SEM (n = 6, *P < 0.05, **P < 0.01). Scale bar: 1 mm in A and B; 5 mm in D and E.

At 4 and 8 weeks, the proteoglycan staining was greater in the cartilage of the cell sheet group (**Figure 4A** and **4C**) than in the control group (**Figure 4B** and **4D**). Quantification of the staining positive area fraction showed that the staining of cartilage was almost same between the two groups 4 weeks post surgery (**Figure 4E**), while, the proteoglycan staining was lighter in the control group than that in the cell sheet group at 8 weeks postoperation (**Figure 4F**). At higher magnification, the grafts (G) of the cell sheet group (**Figure 4G**) showed nearly normal

**Table 2.** Results of Histological Evaluation at 8 weeks

| Variable | Mean Score ± SD | | P Value | 95% Confidence Interval | |
|---|---|---|---|---|---|
| | Cell sheet | Control | | Lower Limit | Upper Limit |
| Nature of the predominant tissue | 6.83 ± 0.17 | 6.33 ± 0.21 | 0.0924 | -0.10 | 1.10 |
| Cellular morphology | 4.00 ± 0.00 | 4.00 ± 0.00 | 1.0000 | - | - |
| Safranin O staining of the matrix | 2.83 ± 0.17 | 2.33 ± 0.21 | 0.0924 | -0.10 | 1.10 |
| Structure characteristics | 8.00 ± 0.37 | 5.67 ± 0.92 | 0.0400* | 0.13 | 4.54 |
| Surface regularity | 2.67 ± 0.21 | 2.33 ± 0.33 | 0.4178 | -0.55 | 1.21 |
| Structure integrity | 1.83 ± 0.17 | 0.83 ± 0.31 | 0.0169* | 0.22 | 1.78 |
| Thickness | 1.83 ± 0.17 | 1.83 ± 0.17 | 1.0000 | -0.53 | 0.53 |
| Bonding to the adjacent cartilage | 1.67 ± 0.21 | 0.50 ± 0.22 | 0.0035* | 0.48 | 1.85 |
| Freedom from cellular changes of degeneration | 4.17 ± 0.48 | 2.50 ± 0.34 | 0.0176* | 0.36 | 2.97 |
| Hypocellularity | 2.83 ± 0.17 | 2.00 ± 0.26 | 0.0219* | 0.15 | 1.52 |
| Chondrocyte clustering | 1.33 ± 0.33 | 0.50 ± 0.22 | 0.0646 | -0.06 | 1.73 |
| Freedom from degenerative changes in adjacent cartilage | 2.50 ± 0.22 | 1.50 ± 0.22 | 0.0101* | 0.30 | 1.71 |
| Total | 21.50 ± 0.76 | 16.00 ± 1.21 | 0.0033* | 2.31 | 8.69 |

*Statistically significant differrence.

cell distribution and cell density. Few cell clusters were observed. However, severe hypocellularity and a large number of cell clusters were observed in both the grafts (G) and adjacent cartilage (N) in the control group (**Figure 4H**), which indicates degeneration of the cartilage.

*Safranin O and COL II staining of the sections*

At 8 weeks, the staining of the cartilage was more intense and regular than in the control group (**Figure 5A-C**). On immunohistochemical staining of the type II collagen, the cartilage of the cell sheet group (**Figure 5D**) demonstrated stronger staining in both the grafts and adjacent cartilage than that of the control group (**Figure 5E**). The quantification of staining positive area fraction also confirmed this result (**Figure 5F**). These results demonstrated that the cartilage in the control group already had features of degeneration.

*Histological scoring*

At 8 weeks, the overall histological score was significantly higher in the cell sheet group than in the control group (mean, 21.5 ± 0.76 versus 16.0 ± 1.21; $P = 0.003$). The assessment of the nature of the predominant tissue including cellular morphology and toluidine blue staining of the matrix revealed no difference between these two groups. The evaluation of structural characteristics including plug integration (1.67 ± 0.21 vs 0.50 ± 0.22; $P = 0.004$) and structural integrity (1.83 ± 0.17 vs 0.83 ± 0.31; $P = 0.02$) showed that the mean score was higher for the cell sheet group than for the control group (8.0 ± 0.37 vs 5.7 ± 0.92; $P = 0.04$). The assessment of graft degeneration showed that the mean score was also significantly higher for the cell sheet group than for the control group (4.2 ± 0.48 vs 2.5 ± 0.34; $P = 0.02$). Similarly, the adjacent cartilage showed less degenerative changes in the cell sheet group than in the control group (2.5 ± 0.22 vs 1.5 ± 0.22; $P = 0.01$) (**Table 2**).

## Discussion

In the present study, the use of three-layered SMSC sheets significantly improved the integration of the osteochondral grafts at the defect margins and decreased graft degeneration. No adverse events were detected with cell sheet transplantation.

Several possible reasons may lead to the following results. First, the paracrine effects of implanted SMSC sheets may contribute to promote cell survival and attract host chondrocytes and MSCs to the defect margins. Several studies have demonstrated that MSCs can secrete trophic factors including growth factors, cytokines, and chemokines [32-35]. Then,

the paracrine and local environmental inductive effects may induce the progenitor cells in the defect margins into repair tissue. Second, the adhesion and barrier function of SMSC sheets may protect injured cartilage from catabolic factors in the joint fluid. There has been a study that demonstrated the adhesion and barrier function of cell sheet [36]. Similarly, SMSC sheets may intensely adhere to the surface of cartilage defects in the present study. Thus, catabolic factors in the joint fluid cannot contact injured cartilage, and proteoglycan loss may also be avoided. In this way, cartilage degeneration may decrease.

Some similar studies have also been conducted. Smyth *et al.* [19] repaired the interfaces using MSCs that were recruited from the bone marrow by platelet-rich plasma. Leng *et al.* [20] used MSCs to reconstruct the clefts between the mosaic grafts and adjacent cartilage. The cell sheets used in this study have an obvious advantage over isolated MSCs in those studies. The ECM, cell-cell interaction, and cell-matrix connections were all well preserved in the present study. The abundant ECM in the cell sheet can protect cells from joint fluid and promote cell viability (Current progress of cells sheet tissue engineering and future perspective). Thus, the efficiency could be improved using the present method. In this study, SMSCs were used to fabricate cell sheets, as SMSCs were reported to have greater proliferation and chondrogenic ability compared to other tissue-originated MSCs [37]. And, SMSCs that are isolated even from elderly donors exhibit less senescence and maintain high multi-potent capacity [38]. Enzymes were not used to prepare cell sheets, because they can harm the cell-matrix interaction. In the present study, 45 minutes' shaking on horizontal rotators at room temperature was used to detach a cell sheet from culture plate. This study hypothesized that shaking and relative low temperature lead to the detachment. Thus, the present method is simple, effective and less-expensive. However, the present study also observed that cell type may play a pivotal role in getting an intact cell sheet using this method. We failed to use bone marrow derived mesenchymal stem cells (BMSCs) to fabricate a cell sheet in this way, as BMSCs proliferated slowly secreted much less ECM.

This study also had several limitations. First, the SMSCs were not labeled to track the fate of transplanted cells. It was not known if the transplanted cells survived or proliferated in the healing zone. Maybe, the host chondrocytes and MSCs that were recruited to the defect margins by paracrine effects of SMSC sheets played a crucial role in gap reconstruction. Second, the 4-and 8-week outcomes were fairly short. Longer time points may have poorer results. Third, no biomechanical testing was conducted in the present study. So, there was no idea about how well the implants were integrated with the host tissue.

## Conclusion

Poor lateral integration and graft degeneration are the main obstacles that limit autologous OCT clinically used. Our results showed that three-layered SMSC sheets can improve the quality of autologous OCT at a relative early stage. This study indicated that the cell sheet technology may be a possible solution to improve clinical OCT transplantation. As a preliminary study, this study has some limitations. Further researches are needed to confirm this new therapeutic approach for treating of cartilage defects.

## Acknowledgements

This work was supported by National Nature Science Foundation of China (81125013, 81101338) (to DQS and QJ).

## Disclosure of conflict of interest

None.

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