

Original Article

The use of allogenic adipose-derived stem cells in combination with platelet-rich fibrin for the treatment of cartilage defects in rabbit ear

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Abstract: The current study aims to assess the efficacy of allogenic adipose-derived stem cells (ADSCs) together with platelet-rich fibrin (PRF) for the treatment of rabbit ear cartilage defects. For this study, 12 New Zealand white rabbits were randomly allocated into 4 groups. Two full-thickness cartilage defects were created in the rabbit ears. Group 1 was left untreated; Group 2 was treated with allogenic ADSCs, Group 3 was treated with PRF; Group 4 was treated with allogenic ADSCs and PRF. Macroscopic observation, hematoxylin and eosin staining, and Alcian blue staining after 3 months suggested that the allogenic ADSCs/PRF significantly accelerated cartilage regeneration compared to other groups and this was associated with increased expression of collagen II relative to the other groups. Expression of genes associated with immune response such as cluster of differentiation 4 and 8 (CD4, CD8), and interleukin 2 and 4 (IL-2, IL-4) displayed no significant statistical difference compared to Group 1. In conclusion, these results suggest that allogenic ADSCs in combination with PRF can accelerate regeneration in full-thickness cartilage defects in the rabbit ear model without causing a significant immune response. The results suggest that allogenic ADSCs with PRF could successfully be used for cartilage regeneration.

Keywords: Adipose-derived stem cells, platelet-rich fibrin, cartilage defects, immune response

Introduction

Cartilage injuries take a long time to repair because the cartilage tissue is avascular and comprises few cells with low mitotic activity [1, 2]. Cartilage injuries are treated mainly through the use of drugs, cells, and joint distraction. However, these methods suffer from several drawbacks. None of the drugs have been formally approved for use in patients for cartilage repair. The use of cells as a treatment modality is not effective on its own as structural properties also need to be considered. Furthermore, most studies on joint distraction focus on the ankle and tend to cause joint motion [3]. The most promising approach to treat cartilage defects is tissue engineering [2, 4]. Adipose-derived stem cells (ADSCs) are readily available and can be expanded compared to stem cells

derived from other sources, making them an ideal candidate for the repair of cartilage defects [5, 6]. Moreover, since the cartilage tissue is avascular, it is possible to treat these tissues with allogenic ADSCs. Tissue repair and regeneration are complex processes that require the participation of cells, but also need additional growth factors [7]. Studies have demonstrated that a variety of growth factors are associated with cartilage repair, including transforming growth factor β [8], insulin-like growth factor [9], and also platelet-rich plasma (PRP) [10, 11]. Platelet-rich fibrin (PRF) is a second-generation preparation of platelet concentrates with many advantages over the PRP. These include their simple preparation, growth factor abundance, and their slow release [12-14]. These advantages have resulted in the widespread use and application of the PRP for research.

Table 1. List of primers used for quantitative real-time PCR

Gene	Primers Sequences
GAPDH forward	5'-AGACACGATGGTGAAGGTCG-3'
GAPDH reverse	5'-TGCCGTGGGTGGAATCATAC-3'
Collagen II forward	5'-GCACCCATGGACATTGGAGG-3'
Collagen II reverse	5'-AGCCCCGCACGGTCTTGCTT-3'

We have previously demonstrated that PRF can decrease adipose implantation resorption rates from 49.39% to 36.41%. PRF, together with autologous ADSCs, also improved angiogenesis and remodeling [15, 16]. In the event of salivary gland injury, we found that autologous ADSCs also protected the salivary gland against damage [17]. Above studies focused on the role of soft tissues for autologous ADSCs, but autologous ADSCs can be difficult to isolate thus inhibiting its utility. In the current study, we hypothesized that we could promote the repair of full-thickness cartilage defects using allogenic ADSCs/PRF, thus shortening the duration between isolation and application. In order to facilitate direct observation, we generated a cartilage defect model in rabbit's ear cartilage not in the knee. The efficacy of allogenic ADSCs/PRF in stimulating cartilage regeneration was evaluated by macroscopic and microscopic analysis and the immune response was assessed using markers such as cluster of differentiation 4 and 8 (CD4, CD8), and interleukin 2 and 4 (IL-2, IL-4).

Materials and methods

All experimental protocols using animals were approved by the Institutional Animal Care and Use Committee at the Fourth Military Medical University, Xi'an, China.

Isolation and expansion of rabbit ADSCs

ADSCs were isolated from rabbits as previously reported [18]. Briefly, New Zealand white rabbits weighing 2 to 3 kg each and between 4 and 6 months of age were housed in the Fourth Military Medical University of Stomatology animal center. After performing general anesthesia, the scapular area of adipose tissue was removed, rinsed with phosphate buffered saline (PBS), followed by removal of the fat capsule and small blood vessels and chopping of the fat to a size of about 1 mm³. An equal vol-

ume of 1% type I collagenase was added and digestion was performed on a thermostat shaker (37°C, 180 rpm, 40 min). The resulting digest was then centrifuged (1000 r/min, 5 min) and then filtered through a 200 µm mesh screen filter and then centrifuged again. Dulbecco's Modified Eagle's Medium (DMEM)/F12 medium containing 100 µg/ml penicillin/streptomycin and 10% FBS was used to resuspend the cells after adjusting the cell concentration to 1 × 10⁵ cells/ml. Cells were then inoculated in a 25 cm² flask and cultured under a humid atmosphere of 95% air, 5% CO₂ at 37°C. The medium was changed every three days, until the cells reached 80-90% confluence. Cells were then passaged using a 0.25%/0.02% trypsin/EDTA solution.

ADSCs from passage 3 were characterized according to their adipogenic and osteogenic differentiation properties in vitro by staining with Oil Red O, Alizarin red, and Alkaline phosphatase.

Allogenic ADSCs/PRF were implanted directly into normal cartilage

PRF was prepared by centrifugation as previously described [19]. Briefly, the blood was isolated from rabbits and centrifuged immediately (3000 rpm, 10 min). The yellow segment in the third layer is the PRF. A total of 1-2 × 10⁶ ADSCs from passage 3 were labeled with fluorescent Dil dye (Sigma, USA) was mixed with PRF (0.3 ml) and injected into the rabbit ear cartilage area. Euthanasia was performed 1 month after implantation for histological research.

Allogenic ADSCs/PRF repair of rabbit ear cartilage defects

Twelve rabbits were randomly divided into three pairs. Eight ears of each pair were subjected to full-thickness cartilage defects (5 × 5 × 1 mm). Each of ears were treated and assigned to the following groups: Control group (G1), Allogenic ADSCs group (G2), PRF group (G3), Allogenic ADSCs/PRF group (G4). The rabbits were sacrificed using an intravenous overdose of pentobarbital and the bilateral defective ears were harvested and subjected to digital photographs (Nikon-D7000, Japan), hematoxylin and eosin (HE) staining and Alcian blue staining. To test the immune response of the introduced allogenic ADSCs, 2 ml of blood before and after

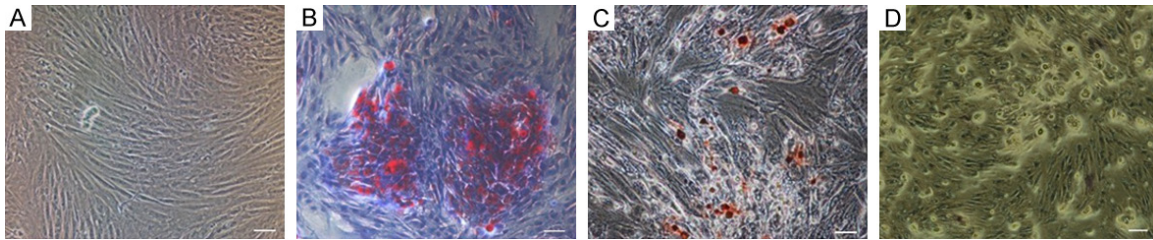


Figure 1. Identification of rabbit ADSCs (A) phase contrast imaging of living ADSCs, which exhibited fibroblast-like morphology. (B) Lipid drops were present in ADSCs after 2 weeks of adipogenic induction as identified by staining with Oil red O. (C) Calcium nodules were formed after 4 weeks of osteogenic induction as indicated by Alizarin red staining. (D) Alkaline phosphatase positive cells were observed after 1 week of osteogenic induction.

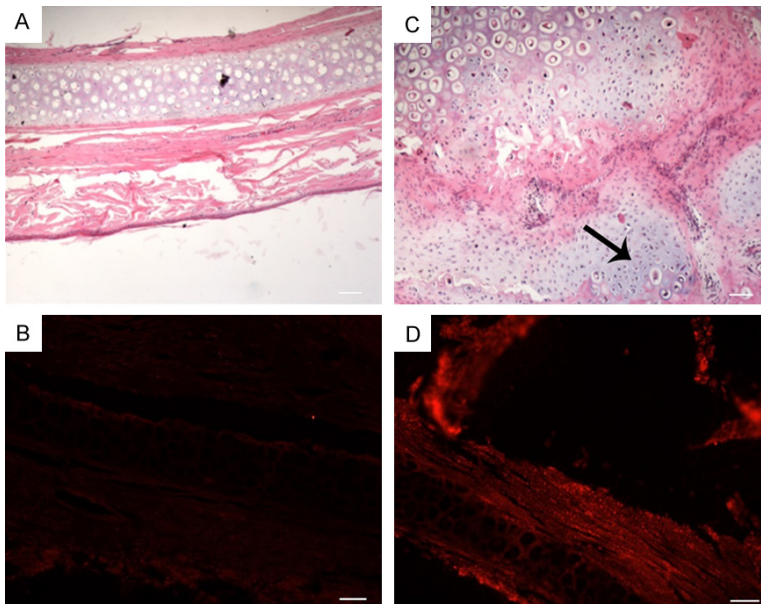


Figure 2. Growth and differentiation of ADSCs/PRF in the normal ear cartilage. A, B. Control group, HE and DiI labeled, respectively; C, D. HE and DiI labeled tissue at 1 month post-implantation showed new cartilage region (indicated by the arrow) Scale bar = 100 μ m.

sacrifice from the superficial ear vein of each rabbit was saved 1, 2, and 3 months after surgery for the determination of CD4/CD8 and IL-2 and IL-4 levels.

Quantitative RT-PCR

For evaluation of collagen II (col-II) levels in cartilage repair region, total RNA was extracted using real-time PCR (Takara, Japan) and processed according to the manufacturer's instructions. The Primer sequences for GAPDH and col II are shown in **Table 1**.

Western blot analysis

After 3 months, total protein was collected and quantified according to the manufacturer's

instructions using the bicinchoninic acid (BCA) Protein Assay kit (Thermo Fisher Scientific) and the expression levels of col-II in the repaired area was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Statistical analysis

All quantitative data were expressed as mean \pm standard deviation (S.D.). To compare the different groups undergoing repair, testing was performed using one-way analysis of variance (ANOVA) and then further analyzed by using Newman-Keuls method (SNK-q). Image-Pro Plus 6.0 (USA) was used to analyze the HE images to compute defect repair rate and graphs were

generated using GraphPad Prism 6.0 (USA). The significance threshold was set at $P < 0.05$.

Results

ADSCs were isolated and expanded from rabbit scapular adipose tissue. ADSCs exhibited fibroblast-like morphology in culture (**Figure 1A**). We have previously characterized the phenotype of ADSCs in culture extensively [15]. At passage 3, the differentiation capacity of rabbit ADSCs was confirmed by staining with Oil Red O, Alizarin red, and Alkaline phosphatase (**Figure 1B-D**).

ADSCs at passage 3 were labeled with the fluorescent dye DiI and mixed with PRF. This mixture was injected into normal ear cartilage area

Tissue engineering for the treatment of cartilage defects in rabbit ear



Figure 3. Stepwise procedure for repair of rabbit ear cartilage by ADSCs/PRF. A. G1, Control group; G2, allogenic ADSCs group; G3, PRF; G4, allogenic ADSCs/PRF group. B. Full-thickness cartilage defect (5 × 5 × 1 mm). C. Allogenic ADSCs/PRF (0.3 ml).

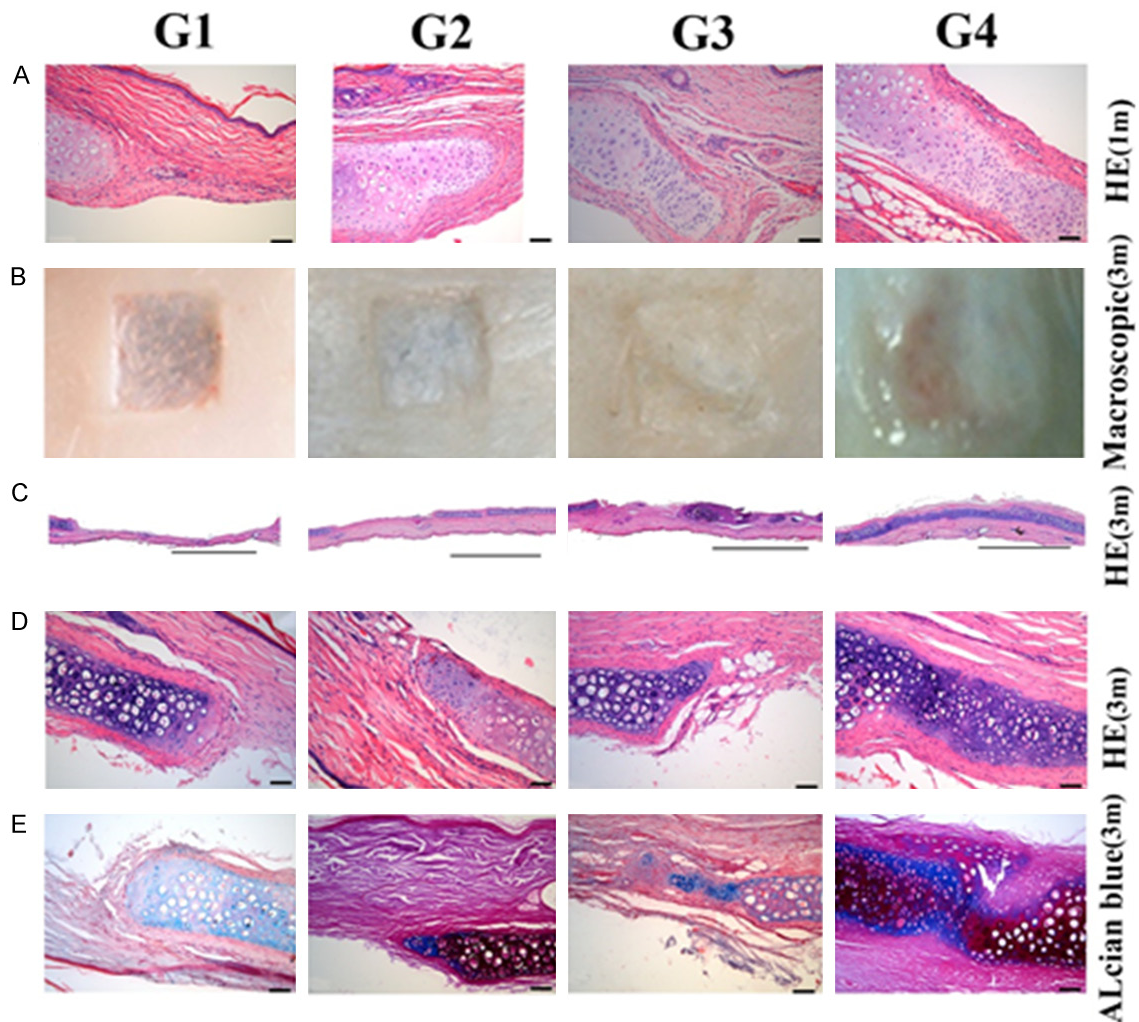


Figure 4. Cartilage defect after injection of cells. A. HE images after the operation 1 month later (Scale bar = 100 mm). B. Representative macroscopic images 3 months after implantation. C. Overall HE across 4 groups (Scale bar = 2 mm). D. HE of sections (Scale bar = 100 mm) used to determine the cartilage defect repair rate (%). E. Alcian blue images of sections (Scale bar = 100 mm). (G1 was untreated; G2 was treated with allogenic ADSCs, G3 was treated with PRF; G4 was treated with allogenic ADSCs/PRF).

for 1 month. The results demonstrated that compared to the controls (Figure 2A and 2B), a

new cartilage region can be observed in the injection zone (Figure 2C) and expressed red

Table 2. Cartilage defect repair rate (%)

Group	Post-implantation		
	1 M	2 M	3 M
G1	0	1.27±0.40	1.68±0.17
G2	7.47±0.25	9.3±0.36	15.4±0.91
G3	19.1±0.44	28.6±0.8	32.0±2.76
G4	36.85±0.84 ^a	62.7±0.53 ^b	89.37±0.79 ^c

a = statistically significant difference between groups after 1 month surgery ($P < 0.01$). b = statistically significant difference between groups after 1 month surgery ($P < 0.01$). c = statistically significant difference between groups after 1 month surgery ($P < 0.001$). All statistical analyses were performed by one-way ANOVA and data are represented as mean ± standard error of mean (SEM).

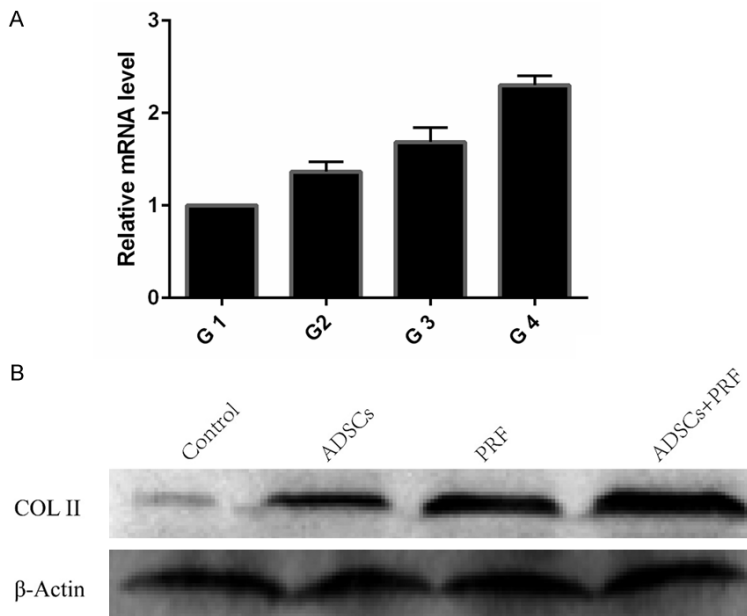


Figure 5. The expression of col-II in new cartilage. A. Total RNA was extracted from repaired tissues at 3 months post-injection. In group G4, expression of col-II was significantly higher than in the other groups. Expression in group G3 was higher than in G2. B. Western blot analysis demonstrated that the protein expression of col-II was significantly increased in the G4 group, compared with the other groups. (G1 was untreated; G2 was treated with allogenic ADSCs, G3 was treated with PRF; G4 was treated with allogenic ADSCs/PRF).

fluorescence (Figure 2D), thus suggesting that the ADSCs can be directly induced to form cartilage in vivo in the presence of PRF.

To study cartilage regeneration following ear defect, we followed the process delineated in Figure 3. After 1, 2, and 3 months, four rabbits each were sacrificed for general observation, and the samples were stained using HE and Alcian blue.

In the G1 control group, cartilage defect area can be identified as a significant depression

with neat margins and clear cartilage tissue boundaries with little new cartilage tissue formation. The G2 group treated with allogenic ADSCs alone showed a thin layer of membrane-like tissue covering. The G3 group treated with PRF alone displayed blurry defect edges with a small amount of tissue-like newborn cartilage. The G4 group showed that the defective edge no longer exists and newborn cartilage tissue filled defect area (Figure 4B).

Histological analysis of HE stained tissues showed that G2 and G4 displayed no obvious inflammatory cell recruitment compared to G1 at the boundary between the wound area and the normal tissue after 1 month (Figure 4A). G2 and G4 also showed no significant inflammatory response at the margin of the defect as compared to G1 after 3 months (Figure 4D). These observations were validated by HE staining (Figure 4C). Table 2 shows the rate of cartilage repair in the defect area. G4 group had the best rate of repair at all observation points and the repair rate was 90% greater than other groups at 3 months. There were significant statistical differences between the G1, G2, and G3 groups at several time points ($P < 0.001$).

Alcian blue staining, which marks naïve chondrocytes, was observed in higher numbers within groups G2 and G3 compared to group G1 at the junction (Figure 4E). The cartilage defect area in G4 was almost completely filled by naïve chondrocytes. The above results that allogenic ADSCs or PRF alone can repair cartilage defects to a certain degree, but allogenic ADSCs together with PRF repairs cartilage defects much more efficiently.

Quantitative real-time PCR was performed for detecting the expression of the col-II gene in

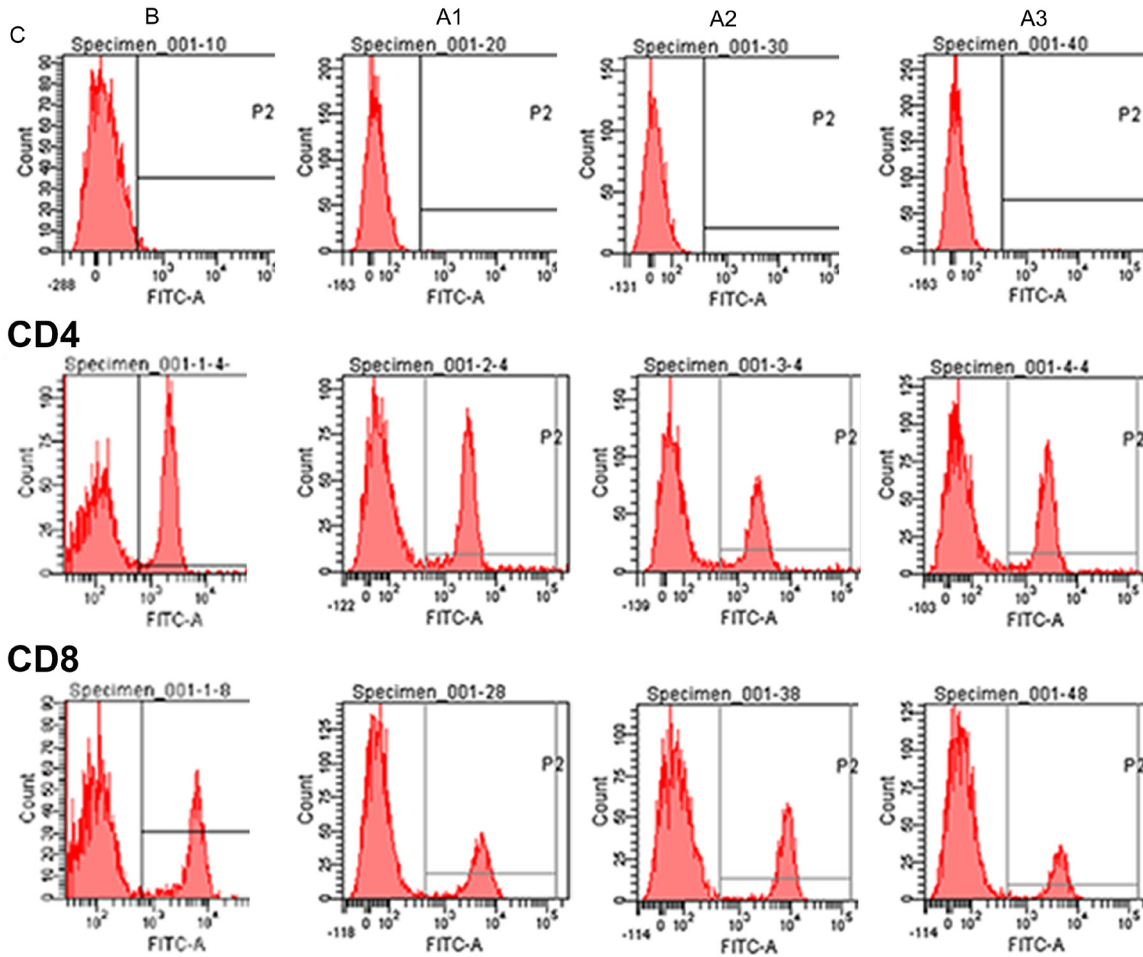


Figure 6. The results of CD4/CD8, IL-2 and IL-4 staining before and after allogeneic ADSC implantation. B = before implantation, A1 = 1 month after implantation, A2 = 2 months after implantation, A3 = 3 months after implantation. C = Control group.

Table 3. The results of CD4/CD8, IL-2, and IL-4 staining before and after allogeneic ADSC implantation

	CD4/CD8	IL-2 (pg/ml)	IL-4 (pg/ml)
B	1.80±0.072*	169.7±9.31*	63.56±7.35*
A _{1m}	1.93±0.145	168.5±10.52	64.45±5.34
A _{2m}	1.82±0.124	170.9±8.47	65.67±6.42
A _{3m}	1.94±0.124	169.6±10.35	65.69±6.34

**P* > 0.05, B = before implantation, A = after implantation. Data are represented as mean ± standard error of mean (SEM).

the new cartilage isolated from the different groups after 3 months. Col-II expression levels in G4 were significantly higher compared to G1, G2, and G3. Col-II expression levels in G3 were higher than in G2 (**Figure 5A**). Western blot results also suggest that PRF significantly

increases col-II expression at the protein level (**Figure 5B**).

In order to further test whether there was an immune response to the exogenous introduction of ADSCs and PRF, we tested the relevant markers. The results of CD4, CD8, CD4/CD8, and IL-2 and IL-4 before and after allogeneic ADSC implantation were compared with their levels at G1. No significant statistical differences were observed (**Figure 6** and **Table 3**). These results indicate that allogeneic ADSC transplantation does not induce a significant immune response.

Discussion

Mesenchymal stem cells have recently become a popular source of cells in the field of tissue engineering due to their proliferation and differ-

entiation ability. ADSCs and bone marrow stem cells (BMSCs) are the most common mesenchymal stem cells used for this purpose. BMSCs are superior to ADSCs in terms of their osseointegration. However, ADSCs are more readily available, can be expanded in vitro, and are comparable to BMSCs in the context of allogenic transplantation [6, 16, 18]. Cartilage damage occurs mostly due to tumors, infections, and degenerative diseases. Cartilage tissue derives most of its nutrition from the surrounding tissues because it is avascular [1]. ADSCs are implanted directly into the cartilage defect area, often differentiating into osteogenic lineages rather than chondrogenic lineages, possibly due to the length of the blood vessels and endochondral ossification [1]. In this study however, allogenic ADSCs seeded with PRF differentiate into cartilage. This may suggest that PRF can regulate lineage decisions of ADSCs within the cartilage environment. PRF contains high levels of growth factors such as vascular platelet-derived growth factor (PDGF) and transforming growth factor- β 1 (TGF- β 1), which favor cartilage regeneration [8, 20]. Col-II is an important indicator of cartilage regeneration [21]. The expression of the col-II gene was significantly increased in G3 compared to G2, suggesting that growth factors in the PRF can promote cartilage regeneration. Western blot results also demonstrated that PRF significantly increases col-II protein expression. This result is consistent with other studies about the effects of growth factors on cartilage differentiation [22, 23]. After 3 months, the defects were repaired in the group where ADSCs were administered together with PRF compared to incomplete repair in other groups. Our study also showed that allogenic ADSCs and allogenic ADSCs/PRF repaired cartilage defects without mounting an immune response (Table 3), consistent with previous reports [24].

In conclusion, allogenic ADSCs/PRF can not only repair the cartilage, but also not induce an immune response. Despite our research findings on the advantages of ADSCs and PRF in promoting soft tissue regeneration, there is a need to study how PRF modulates the specific mechanisms by which allogenic ADSCs are transformed into the chondrogenic lineage. We believe that our method will help develop an allogenic ADSC bank that can be used for the regeneration of soft tissue.

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Disclosure of conflict of interest

None.

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