Special Issue Research Article

Intra-articular autologous uncultured adipose-derived stromal cell transplantation inhibited the progression of cartilage degeneration†

Running title: Effects of autologous UADSC transplantation

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†This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: [10.1002/jor.24174]

Received 31 May 2018; Revised 30 September 2018; Accepted 22 October 2018

Journal of Orthopaedic Research®

DOI 10.1002/jor.24174

Author Contributions Statement

All authors have made substantial contributions to (1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data; (2) drafting the article or

revising it critically for important intellectual content; and (3) final approval of the version to be submitted. The specific contributions of the authors are as follows:

(1) Conception and design of the study: TM, YK.

(2) Analysis and interpretation of the data: all authors

(3) Drafting of the article: TM, YK.

Abstract

The role of uncultured adipose-derived stromal cells for osteoarthritis treatment remains unclear despite sporadic reports supporting their use in clinical settings. This study aimed to evaluate the therapeutic effects of autologous uncultured adipose-derived stromal cell transplantation in a rabbit osteoarthritis model. Uncultured adipose-derived stromal cells isolated from rabbits were administered via intra-articular injection into the knees after osteoarthritis onset. Animals were sacrificed at 8 and 12 weeks after osteoarthritis onset to compare the macroscopic, histological, and immunohistochemical characteristics between the uncultured adipose-derived stromal cell and control groups. Co-culture assay was also performed. The chondrocytes isolated from the model were co-cultured with adipose-derived stromal cells. The cell viability of chondrocytes and expression of chondrocyte-specific genes in the co-culture (uncultured adipose-derived stromal cell) group were compared with the mono-culture (control; chondrocytes only) group. In macroscopic and histological analyses, the uncultured adipose-derived stromal cell group showed less damage to the cartilage surface than the control group at 8 and 12 weeks after osteoarthritis onset. In immunohistochemical and co-culture assay, the uncultured adipose-derived stromal cell group showed higher expression of collagen type Ⅱ and SRY box-9 and lower expression of matrix metalloproteinase-13 than the control group. The cell viability of chondrocytes in the uncultured adipose-derived stromal cell group was higher than that in the control group. Intra-articular autologous uncultured adipose-derived stromal cell transplantation inhibited the progression of cartilage degeneration in a rabbit osteoarthritis model by regulating chondrocyte viability and secreting chondrocyte-protecting cytokines or growth factors, which promote anabolic factors and inhibit catabolic factors. This article is protected by copyright. All rights reserved

Keywords: uncultured adipose-derived stromal cells; stem cell; osteoarthritis;

chondrocyte

Introduction

Osteoarthritis (OA) is characterized by inflammation, bone remodeling, and progressive destruction of the articular cartilage components with functional disability[1]. Established therapies for OA mainly include preventive measures, such as weight control, exercise, and pharmacologic approaches, which usually consist of analgesic therapy, including acetaminophen, salicylates, and non-steroidal anti-inflammatory drugs[2-4]. Intra-articular injections of hyaluronic acid, platelet-rich plasma, hypertonic dextrose prolotherapy, and anabolic cartilaginous agents have been studied as potential therapies[5-7]. However, only few effective therapies to date alter the pathobiologic course of the disease[8].

Mesenchymal stem cells (MSCs), with the capacity to differentiate into a variety of cells including osteoblasts, myocytes, adipocytes, and chondrocytes[9,10], are used in cellular therapy for a broad spectrum of diseases[11]. With regard to therapies for OA, intra-articular injection of MSCs is an increasingly common adjuvant therapy that has shown promising results[12]. A recent study has demonstrated that adipose tissues host multipotent stem cells[13]. These cells, called adipose tissue-derived stromal cells or adipose-derived stromal stem cells (ADSC), were first reported in 2001 by Zuk et al[14]. ADSCs can easily be obtained from liposuction waste in large quantities with little donor site morbidity. They have been gaining attention as a promising source of undifferentiated MSCs[14-16]. Furthermore, De Ugarte et al. reported that uncultured adipose-derived stromal cells (UADSCs) have multilineage potential equivalent to MSCs without the need for culture and expansion[17]. UADSCs are the nonbuoyant cellular fraction containing several types of stem and regenerative cells, including ADSCs, vessel-forming cells, such as endothelial and smooth muscle cells, and

preadipocytes[18]. In the previous clinical study, UADSC has been reported to be safe, feasible and advantageous, as it can secure a sufficient cell count without culture or multiple passages, compared with ADSC[19].

There is insufficient evidence supporting the effectiveness of UADSC for OA despite the general perception regarding their potential for becoming an alternative cell-based treatment modality. Recent studies have reported that the paracrine effect of ADSCs on chondrocytes is the key contributor for the suppressive effects of OA development in a rabbit model[20,21]. Therefore, the primary aim of this study was to evaluate the therapeutic effects of autologous UADSC administration into the knee joint in a rabbit OA model. The secondary aim was to investigate the mechanism underlying the paracrine effects of UADSCs on chondrocytes *in vitro*.

Intra-articular injected UADSCs were hypothesized to promote cartilage repair and prevent or slow articular cartilage degeneration during OA development in a rabbit OA model. This study is first to demonstrate the effects of autologous UADSCs in the OA setting using a rabbit OA model.

Methods

In vivo

Rabbit OA model

Skeletally mature male New Zealand rabbits (age: 6 months old, body weight: 3.5±0.5 kg) were used for this experiment. The Institutional Animal Care and Use Committee of our institution approved this study.

OA was induced surgically by anterior cruciate ligament transection (ACLT)[22,23]. For the ACLT procedure, a 2 cm skin and capsular incision was carried out, and ACL

was exposed through a medial parapatellar approach. To achieve optimal visualization of the ACL, the patellar bone was dislocated laterally, and the knee was placed in full flexion. To avoid spontaneous reattachment, a small fragment of tissue between the two ligament stumps was removed as possible. An anterior drawing test was performed gently to confirm that the ACL was transected completely. The joint was irrigated with sterile saline and closed. All surgical procedures were performed under general anesthesia and sterile conditions.

Postoperatively, ACLT rabbits were permitted activity in a cage. The rabbits were closely monitored for infections and other complications. According to a previous report, the rabbits that passed 8 weeks after ACLT were defined as OA model[22,24]. Table 1 shows the number of rabbits in the experimental study.

UADSCs preparation

UADSCs were isolated by a previously established method[14,25]. Adipose tissues (1.5 g) of male rabbits were harvested from the adipose tissue pouch in the interscapular region located along the dorsomedial line, nearly 5 cm from the skull in the craniocaudal direction. Subsequently, the tissue was washed with phosphate-buffered saline (PBS) solution (Wako, Osaka, Japan) and cut into strips over a period of 5 minutes. Collagenase D (Roche, Basel, Switzerland) was dissolved in PBS to a final concentration of 0.12% in 20 ml and then used to digest adipose tissues at 37℃ for 45 minutes in a water bath. The mixture was shaken every 15 minutes during the digestion period. Immediately after the reaction was completed, 20 ml of Dulbecco's modified Eagle's medium (DMEM) (Sigma Aldrich, MO, USA) was added to neutralize collagenase activity. The resulting solution was filtered with a cell strainer (BD Falcon™, Aichi, Japan). The filtrate was centrifuged at 1,300 rpm for 6 minutes at 25℃,

and the supernatant was removed. After counting the cell population in the pellet dissolved by 4% rabbit serum albumin (RSA) (Sigma Aldrich), a total of 1×10^5 autologous UADSCs in 4% RSA were prepared under sterile condition in 1-ml syringes.

UADSC administration

As an autologous implantation, a total of 1×10^5 UADSCs were re-suspended in 1ml of 4% RSA and administered via an intra-articular knee injection of the same rabbit using a 23-gauge needle after OA onset, 8 weeks from ACLT. Four percent RSA (1ml) was used as the control group. The needle was inserted into the knee joint posterior to the lateral edge of the patella at the junction of the femur and tibia to avoid damage to the articular cartilage. The sample was injected into the joint capsule, and the knee was flexed. The rabbit was held in this position for a few minutes. Animals were sacrificed at 8 and 12 week follow-ups from OA onset to investigate the effect of UADSCs on cartilage.

Macroscopic analysis

The femoral condyles in the UADSC and control groups were dissected and stained with India ink (American MasterTech, CA, USA). Macroscopic pictures were taken using an EOS Kiss X7 camera (Canon, Tokyo, Japan). Cartilage lesions were classified and scored as described in a previous report[26]. A blind assessment was then independently performed by 2 individual examiners (TM, YK), and the scores from the 2 examiners were averaged to obtain an overall score.

Histological analysis

The femoral condyles in the 2 groups were placed in 10% neutral buffered formalin. The osteochondral specimens were decalcified in 4% ethylenediamine tetraacetic acid solution for 3 weeks at room temperature. Subsequently, the specimens

were paraffin embedded, and thin sections (5 μm) were prepared by cutting along the sagittal plane.

Safranin-O/Fast Green (Sigma Aldrich) staining was used to assess general morphology and proteoglycan content in cartilaginous tissues. Histological sections were visualized using a BZ-X700 microscope (Keyence, Osaka, Japan), assessed in a blinded manner by two individual examiners (TM, YK), and quantified according to the OA cartilage histopathology assessment system of the Osteoarthritis Research Society International (OARSI)[26].

Immunohistochemical analysis

Immunohistochemical examinations in the 2 groups were performed as follows. In brief, after deparaffinization, sections were incubated with 3% hydrogen peroxide (Wako Pure Chemical, Osaka, Japan) for 30 min to block endogenous peroxidase activity. The sections were incubated with collagen type II (collagen II) mouse monoclonal antibody (1:25; Novus Biological, CO, USA), matrix metalloproteinase (MMP)-13 mouse monoclonal antibody (1:25; Thermo Fisher Scientific, MA, USA), or SRY box (SOX)-9 mouse monoclonal antibody (1:50; Santa Cruz Biotechnology, CA, USA). All antibody dilutions were made in PBS.

After an overnight reaction with the primary antibody at 4°C, the sections were incubated with peroxidase-labeled anti-mouse immunoglobulin (Histofine Simple Stain MAXPO; Nichirei Bioscience, Tokyo, Japan) at room temperature for 30 min. The signals were developed as brown-reaction products using a 3,3′-diaminobenzidine (Histofine Simple Stain DAB solution; Nichirei Bioscience), and nuclei were counterstained with hematoxylin.

Six microscopic fields (100× magnification) relative to the anterior, central, and posterior regions in the cartilage tissue were used to perform a semi-quantitative analysis of immunohistochemistry. A semi-quantitative method that assigns immunohistochemistry values as a percentage of positive cells (MMP-13 and SOX-9) or positive area (collagen II) was performed for a complete assessment of protein expression, with 100% being the maximum score. The analysis was performed by 2 blinded investigators (TM and YK).

In vitro

Isolation and primary culture of chondrocyte

Primary culture of chondrocytes was performed using articular cartilage tissues of the knee harvested from a rabbit OA model, 8 weeks from ACLT. After harvesting, the joint was irrigated with sterile saline and closed. The surgical procedures were performed under general anesthesia and sterile conditions.

Briefly, thinly sliced cartilage tissues were stripped, diced, and digested in DMEM with 0.2% collagenase D for 2 h and 30 min. Undigested cartilage was removed using a 70-μm nylon filter. The filtrate was centrifuged at 1,200 rpm for 5 minutes at 25°C, and the supernatant was removed. The pellet was resuspended in DMEM containing 10% fetal bovine serum (FBS) supplemented with 100 units/ml penicillin (Sigma-Aldrich), 100 mg/ml streptomycin solution (Sigma-Aldrich), and 0.1 mM dexamethasone (Sigma-Aldrich). The pellet was then cultured in an incubator at 37°C in a humidified atmosphere of 5% CO_2/95% air. Chondrocytes were grown in monolayer cultures and passaged when confluence was reached. Cells at the second passage were used for the assay.

Co-culture assay

To confirm the paracrine effect of UADSCs on chondrocytes, we used the co-culture system. UADSCs were isolated from the rabbit from which the cartilage was previously harvested. Chondrocytes (1×10^4 cells/well) in 12-well plates (Cell Culture Insert Companion Plates, BD Falcon™) were co-cultured with UADSCs (1×10^4 cells/well) in cell culture inserts (0.4 μm pores; BD Falcon™) in DMEM containing 10% FBS (UADSC group). UADSCs themselves could not pass the cell culture inserts, but secreted factors from UADSCs could pass the cell inserts and affect the chondrocytes. The control group was established between no cells in the cell culture inserts and chondrocytes only in the 12-well plates (1×10^4 cells/well) in DMEM containing 10% FBS.

After 48 hours, the cell viability of the 2 groups was assessed using a cell counting kit-8. The absorbance (450 nm) in the UADSC group was measured using the model 680 microplate reader and compared with that of the control group.

Subsequently, the mRNA expression levels of anabolic collagen II and SRY box-9 (SOX-9) and catabolic MMP-13 in the 2 groups were measured with real-time reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from chondrocytes after being cultured with 2 different conditions with QIA shredder and RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The relative mRNA expressions relative to glyceraldehyde 3-phosphate dehydrogenase were assessed with real-time RT-PCR using SYBR Green fluorescent dye. Measurements were performed in duplicate using the Applied Biosystems 7500 real-time PCR system. The primer sequences were purchased from Thermo Fisher Scientific Inc., as listed in Table 2. Melting curve analysis was performed using the Dissociation

Curves software to ensure the amplification of only a single product. Relative mRNA expression was analyzed using the $2^{-\Delta\Delta Ct}$ method. The value of the control group was set as 1.

Separated pellet culture

UADSCs and OA chondrocytes were separated with sterilized culture plate insert (Millicell; Millipore, MA, USA). Pellets of OA chondrocytes (1×10^5 cells) were made in the medium at the bottom of a 15-ml tube, as previously described[27]. The membrane plate was inserted into the 15-ml tube so that the UADSC pellets (1×10^5 cells) were placed within the membrane plate (UADSC group). The control group was established between no cells in the membrane plate and OA chondrocytes only in the 15-ml tube (1×10^5 cells/well). Pellets were made in 1 ml of chondrogenic medium: standard DMEM with 15% FBS (Sigma-Aldrich), 100 units/ml penicillin (Sigma-Aldrich), 100 mg/ml streptomycin solution (Sigma-Aldrich), 0.1 mM dexamethasone (Sigma-Aldrich), 50 mM ascorbate-2-phosphate (Sigma-Aldrich), 0.4 mM proline (Sigma-Aldrich), and 1% insulin-transferrin-sodium selenite (Sigma-Aldrich), with 10 ng/ml transforming growth factor-b3 (R&D Systems, Minnesota, USA) and 500 ng/ml bone morphogenetic protein-6 (Sigma-Aldrich). The pellets were incubated at 37°C in 5% CO_2, and the medium was changed every 2-3 days. Pellets were harvested after 21 days in culture.

Statistical analysis

Statistical analyses were performed using SPSS version 19.0 (SPSS Inc, Chicago, Ill). The results are shown as mean±standard deviation. The Mann-Whitney U test was used for comparisons between the 2 groups. A $p<0.05$ was considered significant.

Results

In vivo

Macroscopic analysis

At 8 weeks after OA onset, severe fibrillation in the cartilage was observed in the control group and mild fibrillation in the UADSC group. At 12 weeks after OA onset, severe fibrillation to moderate erosion in the cartilage was observed in the control group, and moderate fibrillation was noted in the UADSC group (Figure 1A).

The mean macroscopic OA scores for the control group at 8 and 12 weeks after OA onset were 3.0±0.8 and 4.4±1.6, respectively. The UADSC group had mean scores of 1.3±0.5 and 2.3±0.9 at 8 and 12 weeks, respectively, after OA onset. The lower score for the UADSC group indicates less damage to the cartilage surface. Statistically significant differences were found at both 8 and 12 weeks after OA onset (p=0.032, p=0.033) (Figure 1B, C).

Histological analysis

Histological evaluation of articular cartilage showed the same trend as macroscopic evaluation. Histological findings in the control group demonstrated the progression of osteoarthritic changes, including the loss of proteoglycan in the superficial and middle zones of the cartilage layers and decreased chondrocyte density, while the cartilage matrix was predominantly retained in the UADSC group at 8 and 12 weeks after OA onset (Figure 2A).

The mean total OARSI scores for the control group at 8 and 12 weeks after OA onset were 10.7±1.9 and 14.7±2.4, respectively. On the other hand, the UADSC group had mean scores of 4.2±1.2 and 5.7±1.8 at 8 and 12 weeks, respectively, after OA onset. Statistically significant differences were noted at both 8 and 12 weeks after OA onset

(p=0.046, p=0.046) (Figure 2B, C).

Immunohistochemical analysis

UADSC treatment showed an evidence of a chondro-protective effect, promoting the expression of a great amount of collagen II in the cartilage tissue with regard to the control group at 8 and 12 weeks after OA onset (Figure 3A). In particular, significantly higher percentages of positive areas in the UADSC group than in the control group were detected (55.2±6.9% vs 26.5±9.5%, p=0.002; 48.3±13.8% vs 21.3±6.9%, p=0.007) (Figure 3B, C).

With regard to MMP-13, a moderate expression was observed in the control group at 8 and 12 weeks after OA onset. On the other hand, the UADSC group showed a lower expression for MMP-13 than the control group at 8 and 12 weeks (Figure 4A). The proportion of MMP-13-positive cells was significantly lower in the UADSC group than in the control group (18.2±2.6% vs 60.5±5.7%, p<0.001; 36.0±7.0% vs 69.8±8.6%, p<0.001) (Figure 4B, C)

For SOX-9, a higher expression in the cartilage tissue was observed in the UADSC group than in the control group at 8 and 12 weeks after OA onset (Figure 5A). The proportion of SOX-9-positive cells was significantly higher in the UADSC group than in the control group (68.6±6.6% vs 30.2±6.5%, p<0.001; 64.9±6.9% vs 29.6±5.0%, p<0.001) (Figure 5B, C).

In vitro

Co-culture assay

To confirm the paracrine effect of UADSCs on chondrocytes, we used the co-culture system (Figure 6A). The cell viability of chondrocytes co-cultured with UADSCs was higher than that of chondrocytes cultured alone (0.42±0.14 vs 0.06±0.05, $p<0.001$) (Figure 6B).

Collagen II and SOX-9 mRNA expression as an anabolic factor and MMP-13 as a catabolic factor in the co-culture and control groups were evaluated with real-time PCR. The averaged collagen II and SOX-9 mRNA expression in the co-culture group were significantly higher than those in the control group ($p=0.002$, $p=0.004$). On the contrary, the mean MMP-13 mRNA expression in the co-culture group was significantly lower than that in the control group ($p=0.004$) (Figure 6C). These results indicate that UADSC increases the expression of anabolic factors and decreases the expression of catabolic factors by the paracrine effect on OA chondrocytes.

Separated pellet culture

To assess the proliferation of OA chondrocytes cultured in the presence of factors released by UADSCs, we performed micromass pellet co-culture (Figure 7A). Pellet size analysis showed that OA chondrocytes co-cultured with UADSCs formed significantly larger pellets than the control group (2.6±0.5 mm vs 1.7±0.1 mm, $p=0.009$) (Figure 7B). All pellets from the 2 groups showed hyaline extracellular matrix that stained positively for Safranin-O and contained round chondrocyte cells (Figure 7C).

Discussion

The most important finding of the present study was that intra-articular injected UADSCs inhibited cartilage degeneration progression in a rabbit OA model. Furthermore, we focused on the paracrine effects of UADSCs. Intra-articular injected UADSCs could secrete a liquid factor having chondro-protective effects, such as increasing chondrocyte proliferation and anabolic factors and decreasing chondrocyte catabolic factors.

ADSCs and UADSCs have several advantages as sources of tissue stem cells. First, autologous ADSCs and UADSCs can be easily isolated in large amounts from abundant and accessible subcutaneous adipose tissues. Furthermore, harvesting of ADSCs and UADSCs is less invasive than that of bone marrow-derived stem cells and other stem cells, and numerous stem cells can be harvested at a time[28]. Adipose tissues yield approximately 500-fold more stem cells than the same amount of adult bone marrow[17,28]. In the orthopedic field, several studies have reported the effectiveness of ADSCs for OA[20,24]. Regarding UADSC, a case report on efficacy for OA has also been published[29]. For these reasons, we considered that UADSCs also have a potentially efficient source for clinical applications in inhibiting cartilage degeneration progression for OA.

This study aimed to determine the role of autologous UADSCs in the OA setting and their behavior on inflammatory environment within the affected articular joint using a rabbit OA model. The proposed ACLT model is widely validated in investigating OA disease, because it determines biomechanical and pathological changes similar to those observed in humans[23]. Using the same model, Desando et al. reported that an intra-articular injection of ADSCs increases cartilage thickness compared with no

administration[24]. In the current study, OA progression was evidently milder in the UADSC-injected knees than in the control knees at 8 and 12 weeks after OA onset in both macroscopic and histological analyses.

Immunohistochemically, a previous report described that autologous ADSCs provide satisfactory results showing a high expression of collagen II in the cartilage matrix using a rabbit OA model[24]. The present study also showed a higher expression of collagen II in the UADSC-injected group than in the control group at 8 and 12 weeks after OA onset. Furthermore, a higher expression of SOX-9 in the cartilage tissue was observed in the UADSC group than in the control group. Sox-9, a chondrogenic transcription factor, has a key role in increasing the levels of chondrogenesis[30], particularly by activating the co-expression of collagen II[31,32]. It has also been reported that the Sox-9 gene may upregulate aggrecan secretion[33]. These findings suggest that intra-articular injected UADSCs may promote secretion of anabolic factors in the chondrocytes. MMP-13 is speculated to cause damage to cartilage in OA, and this may be mediated by chondrocytes in an autocrine or paracrine manner[34]. Kuroda et al. showed that the production of MMP-13 in articular chondrocytes is reduced when they are treated with injected autologous ADSCs using a rabbit OA model *in vivo*[20]. In this study, the autologous UADSC-injected knees also showed a low expression for MMP-13 compared with the knees without UADSCs at 8 and 12 weeks after OA onset. This previous report and our findings suggest that injected autologous UADSCs, like ADSCs, may inhibit secretion of catabolic factors in OA chondrocytes.

Despite the results showing the chondro-protective effects of ADSCs, the mechanism remains unclear. Toghraie et al. reported that the injected ADSCs may regenerate degenerative tissue after directly filling the lesion[10], but the evidence is

inadequate. Other reports have suggested that that injected ADSCs indirectly stimulate the secretion of bioactive factors such as cytokines and growth factors[11,24,35,36]. Previous studies investigated the paracrine effects of ADSCs on chondrocytes using a co-culture system. Hildner et al. reported increased cartilage formation in the co-culture of chondrocytes with ADSCs[37]. Kuroda et al. indicated that the paracrine effects of ADSCs include regulation of chondrocyte viability in OA[20]. In this study, the cell proliferation of chondrocytes co-cultured with UADSCs was higher than that of chondrocytes cultured alone in co-culture and separated pellet culture assay. In a previous *in vitro* experiment, the MMP-13 concentration of culture fluid is reduced when co-cultured with ADSCs[20]. The present study also showed that the mean MMP-13 mRNA expression in the co-culture (UADSC) group was significantly lower than that in the mono-culture group. Shi et al. confirmed that ADSCs can promote the release of collagen II and SOX-9 expression in chondrocytes by co-culture assay[21]. In this study, UADSCs also confirmed similar results.

Taken together, our findings in the *in vivo* and *in vitro* experiments suggest that UADSCs do not only promote proliferation of cartilage cells but also secrete chondrocyte-protecting cytokines or growth factors. Thus, intra-articular injected UADSCs can be potentially used for OA treatment as an alternative to other MSCs.

Our study had several limitations. First, the present study compared the UADSCs with a control group without UADSCs, but not other cell-based treatment methods. The previous study reported that ADSC may have an inferior potential for chondrogenesis compared with bone marrow-derived MSC[38]. Therefore, further study should be required to evaluate the comparison between UADSCs and the other cell-derived stem cell, including bone marrow-derived MSC. Second, rabbit OA models were used for this

experiment. Rabbits are quadruped animals. Rabbit OA models may not translate to human models due to differences in posture and gait dynamics. Furthermore, New Zealand rabbits used in this study are 6 months old. Usual life span for a New Zealand rabbit is approximately 5 years. Thus, these rabbits may not be actually corresponding the middle or older age human with OA changes. Finally, the histological and immunohistochemical assessments presented in this study were mainly semi-quantitative analysis. More objective and precise quantitative methods are needed for more accurate evaluations.

In conclusion, intra-articular injected autologous UADSCs inhibited cartilage degeneration progression in a rabbit OA model by regulating chondrocyte viability and secreting chondrocyte-protecting cytokines or growth factors, which promote anabolic factors and inhibit catabolic factors.

Acknowledgments

The authors thank Ms Kyoko Tanaka, Ms Minako Nagata, and Ms Maya Yasuda for their technical assistance. We would like to thank Editage (www.editage.jp) for English language editing.

References

1. Goldring MB, Goldring SR. 2007. Osteoarthritis. J Cell Physiol 213:626-634.
2. American College of Rheumatology Subcommittee. 2000. Recommendations for the medical management of osteoarthritis of the hip and knee: 2000 update. American College of Rheumatology Subcommittee on Osteoarthritis Guidelines. Arthritis Rheum 43:1905-1915.
3. Gibofsky A, Barkin RL. 2008. Chronic pain of osteoarthritis: considerations for selecting an extended-release opioid analgesic. Am J Ther 15:241-255.
4. Zhang W, Moskowitz RW, Nuki G, et al. 2007. OARSI recommendations for the management of hip and knee osteoarthritis, part I: critical appraisal of existing treatment guidelines and systematic review of current research evidence. Osteoarthritis Cartilage 15:981-1000.
5. Patel S, Dhillon MS, Aggarwal S, et al. 2013. Treatment with platelet-rich plasma is more effective than placebo for knee osteoarthritis: a prospective, double-blind, randomized trial. Am J Sports Med 41:356-364.
6. Rabago D, Patterson JJ, Mundt M, et al. 2013. Dextrose prolotherapy for knee osteoarthritis: a randomized controlled trial. Ann Fam Med 11:229-237.
7. Wang CT, Lin J, Chang CJ, et al. 2004. Therapeutic effects of hyaluronic acid on osteoarthritis of the knee. A meta-analysis of randomized controlled trials. J Bone Joint Surg Am 86-A:538-545.
8. Wakitani S, Goto T, Pineda SJ, et al. 1994. Mesenchymal cell-based repair of large, full-thickness defects of articular cartilage. J Bone Joint Surg Am 76:579-592.

9. Murphy JM, Fink DJ, Hunziker EB, et al. 2003. Stem cell therapy in a caprine model of osteoarthritis. Arthritis Rheum 48:3464-3474.

10. Toghraie FS, Chenari N, Gholipour MA, et al. 2011. Treatment of osteoarthritis with infrapatellar fat pad derived mesenchymal stem cells in Rabbit. Knee 18:71-75.

11. ter Huurne M, Schelbergen R, Blattes R, et al. 2012. Antiinflammatory and chondroprotective effects of intraarticular injection of adipose-derived stem cells in experimental osteoarthritis. Arthritis Rheum 64:3604-3613.

12. Jevotovsky DS, Alfonso AR, Einhorn TA, et al. 2018. Osteoarthritis and stem cell therapy in humans: a systematic review. Osteoarthritis Cartilage.

13. Puissant B, Barreau C, Bourin P, et al. 2005. Immunomodulatory effect of human adipose tissue-derived adult stem cells: comparison with bone marrow mesenchymal stem cells. Br J Haematol 129:118-129.

14. Zuk PA, Zhu M, Mizuno H, et al. 2001. Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng 7:211-228.

15. Erickson GR, Gimble JM, Franklin DM, et al. 2002. Chondrogenic potential of adipose tissue-derived stromal cells in vitro and in vivo. Biochem Biophys Res Commun 290:763-769.

16. Estes BT, Wu AW, Guilak F. 2006. Potent induction of chondrocytic differentiation of human adipose-derived adult stem cells by bone morphogenetic protein 6. Arthritis Rheum 54:1222-1232.

17. De Ugarte DA, Morizono K, Elbarbary A, et al. 2003. Comparison of multi-lineage cells from human adipose tissue and bone marrow. Cells Tissues Organs 174:101-109.

18. Zhu M, Zhou Z, Chen Y, et al. 2010. Supplementation of fat grafts with adipose-derived regenerative cells improves long-term graft retention. Ann Plast Surg 64:222-228.

19. Mizushima T, Takahashi H, Takeyama H, et al. 2016. A clinical trial of autologous adipose-derived regenerative cell transplantation for a postoperative enterocutaneous fistula. Surg Today 46:835-842.

20. Kuroda K, Kabata T, Hayashi K, et al. 2015. The paracrine effect of adipose-derived stem cells inhibits osteoarthritis progression. BMC Musculoskelet Disord 16:236.

21. Shi J, Liang J, Guo B, et al. 2017. Adipose-Derived Stem Cells Cocultured with Chondrocytes Promote the Proliferation of Chondrocytes. Stem Cells Int 2017:1709582.

22. Grigolo B, Lisignoli G, Desando G, et al. 2009. Osteoarthritis treated with mesenchymal stem cells on hyaluronan-based scaffold in rabbit. Tissue Eng Part C Methods 15:647-658.

23. Vignon E, Bejui J, Mathieu P, et al. 1987. Histological cartilage changes in a rabbit model of osteoarthritis. J Rheumatol 14 Spec No:104-106.

24. Desando G, Cavallo C, Sartoni F, et al. 2013. Intra-articular delivery of adipose derived stromal cells attenuates osteoarthritis progression in an experimental rabbit model. Arthritis Res Ther 15:R22.

25. Kosaka M, Nakase J, Hayashi K, et al. 2016. Adipose-Derived Regenerative Cells Promote Tendon-Bone Healing in a Rabbit Model. Arthroscopy 32:851-859.

26. Laverty S, Girard CA, Williams JM, et al. 2010. The OARSI histopathology

initiative - recommendations for histological assessments of osteoarthritis in the rabbit. Osteoarthritis Cartilage 18 Suppl 3:S53-65.

27. Jadlowiec J, Koch H, Zhang X, et al. 2004. Phosphophoryn regulates the gene expression and differentiation of NIH3T3, MC3T3-E1, and human mesenchymal stem cells via the integrin/MAPK signaling pathway. J Biol Chem 279:53323-53330.

28. Fraser JK, Schreiber R, Strem B, et al. 2006. Plasticity of human adipose stem cells toward endothelial cells and cardiomyocytes. Nat Clin Pract Cardiovasc Med 3 Suppl 1:S33-37.

29. Coughlin RP, Oldweiler A, Mickelson DT, et al. 2017. Adipose-Derived Stem Cell Transplant Technique for Degenerative Joint Disease. Arthrosc Tech 6:e1761-e1766.

30. Shi S, Wang C, Acton AJ, et al. 2015. Role of sox9 in growth factor regulation of articular chondrocytes. J Cell Biochem 116:1391-1400.

31. Davies SR, Chang LW, Patra D, et al. 2007. Computational identification and functional validation of regulatory motifs in cartilage-expressed genes. Genome Res 17:1438-1447.

32. Li Y, Tew SR, Russell AM, et al. 2004. Transduction of passaged human articular chondrocytes with adenoviral, retroviral, and lentiviral vectors and the effects of enhanced expression of SOX9. Tissue Eng 10:575-584.

33. Zamani S, Hashemibeni B, Esfandiari E, et al. 2014. Assessment of TGF-beta3 on production of aggrecan by human articular chondrocytes in pellet culture system. Adv Biomed Res 3:54.

34. Shirai T, Kobayashi M, Nishitani K, et al. 2011. Chondroprotective effect of

alendronate in a rabbit model of osteoarthritis. J Orthop Res 29:1572-1577.

35. Frisbie DD, Kisiday JD, Kawcak CE, et al. 2009. Evaluation of adipose-derived stromal vascular fraction or bone marrow-derived mesenchymal stem cells for treatment of osteoarthritis. J Orthop Res 27:1675-1680.

36. Koh YG, Jo SB, Kwon OR, et al. 2013. Mesenchymal stem cell injections improve symptoms of knee osteoarthritis. Arthroscopy 29:748-755.

37. Hildner F, Concaro S, Peterbauer A, et al. 2009. Human adipose-derived stem cells contribute to chondrogenesis in coculture with human articular chondrocytes. Tissue Eng Part A 15:3961-3969.

38. Im G-I, Shin Y-W, Lee K-B. 2005. Do adipose tissue-derived mesenchymal stem cells have the same osteogenic and chondrogenic potential as bone marrow-derived cells? Osteoarthritis and cartilage 13:845-853.

Table 1. Number of rabbits in the experimental study.

	Control group		UADSC group	
	8 weeks model	12 weeks model	8 weeks model	12 weeks model
Macroscopic analysis	**6**	**6**	**6**	**6**
Histological analysis	**5**	**5**	**5**	**5**
Immunohistochemical analysis	**5**	**5**	**5**	**5**
	OA onset model		**OA onset model**	
Co-culture assay	**6**		**6**	
Pellet culture	**6**		**6**	

8 weeks model; Rabbit model 8 weeks after OA onset, 12 weeks model; Rabbit model 12 weeks after OA onset

Table 2. Gene specific primer sequences for real-time PCR.

Gene	Primer sequences
GAPDH	Forward: 5'-CTCTGCTCCTCCTGTTCGAC-3'
	Reverse: 5'-GCGCCCAATACGACCAAATC-3'
collagen II	Forward: 5'-TGGACGATCAGGCGAAACC-3'
	Reverse: 5'-GCTGCGGATGCTCTCAATCT-3'
MMP-13	Forward: 5'-ACTGAGAGGCTCCGAGAAATG-3'
	Reverse: 5'-GAACCCCGCATCTTGGCTT-3'
SOX-9	Forward: 5'-AAGCTCTGGAGACTTCTGAACG-3'
	Reverse: 5'-CGTTCTTCACCGACTTCCTCC-3'

GAPDH; glyceraldehyde 3-phosphate dehydrogenase, collagen II; collagen type II, MMP-13; matrix metalloproteinase-13, SOX-9; SRY box-9

Figure.1

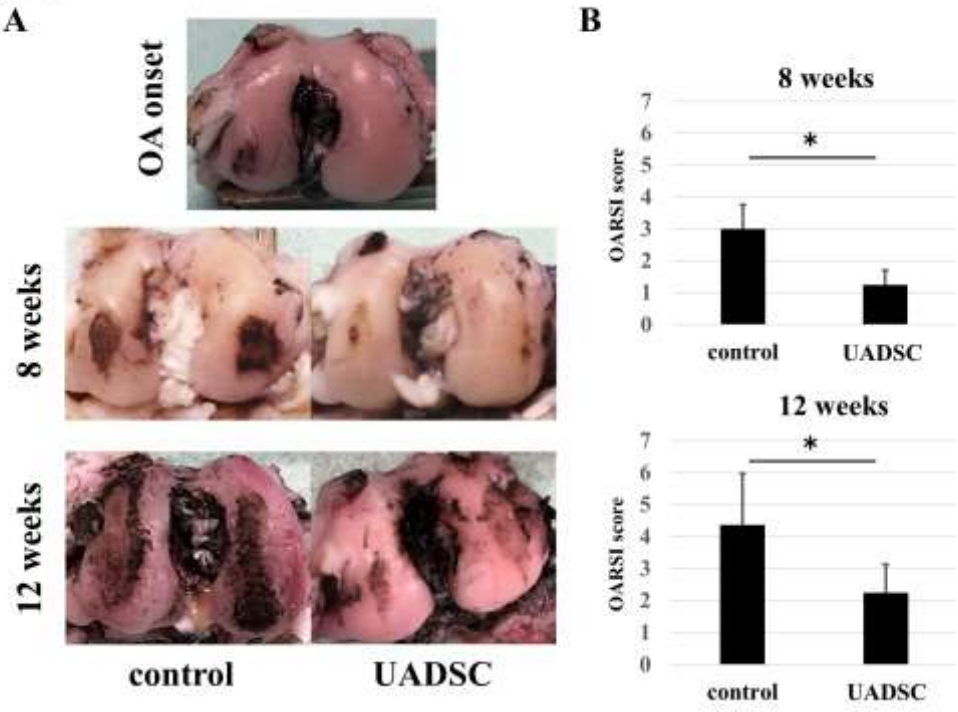


Figure.2

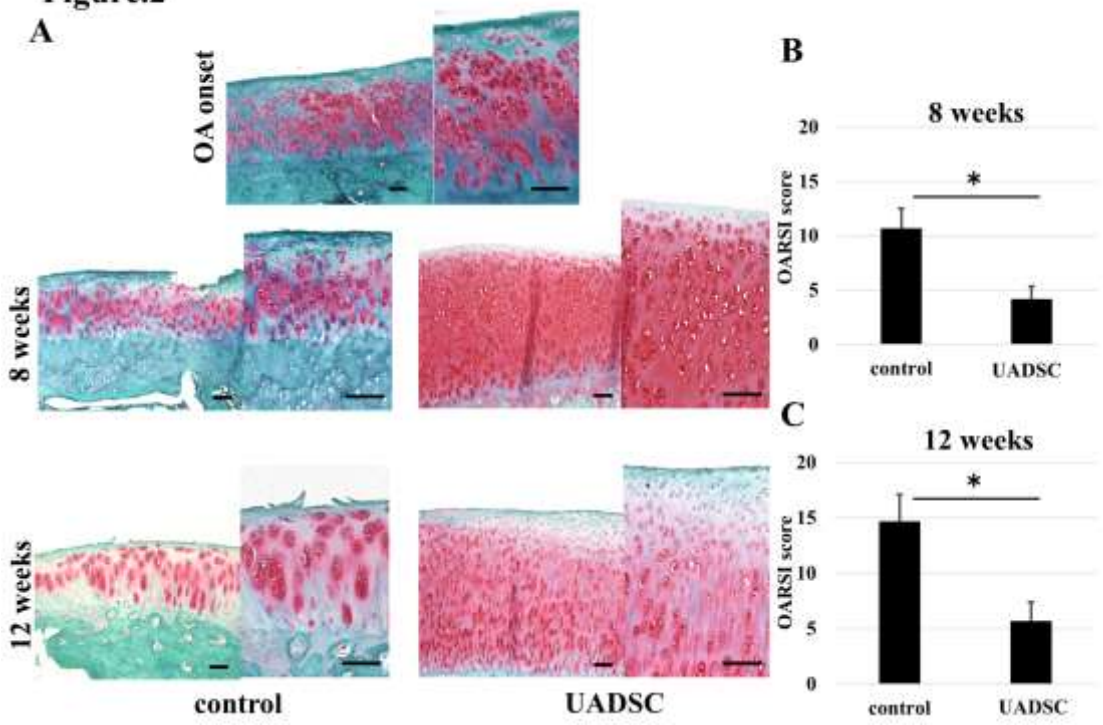


Figure.3

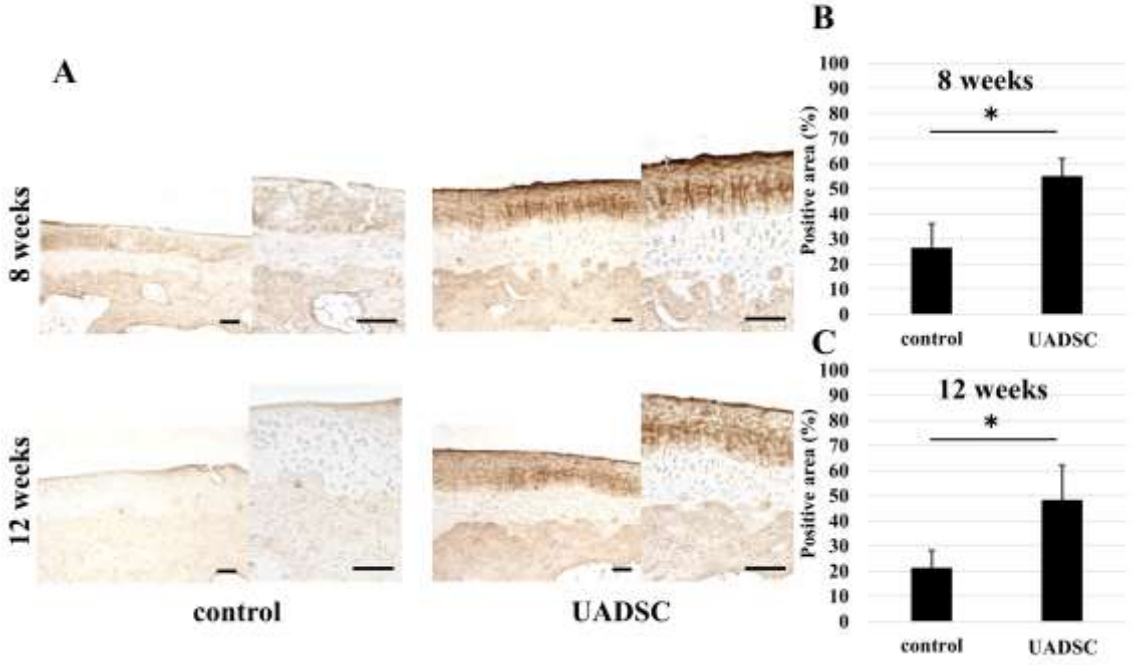


Figure.4

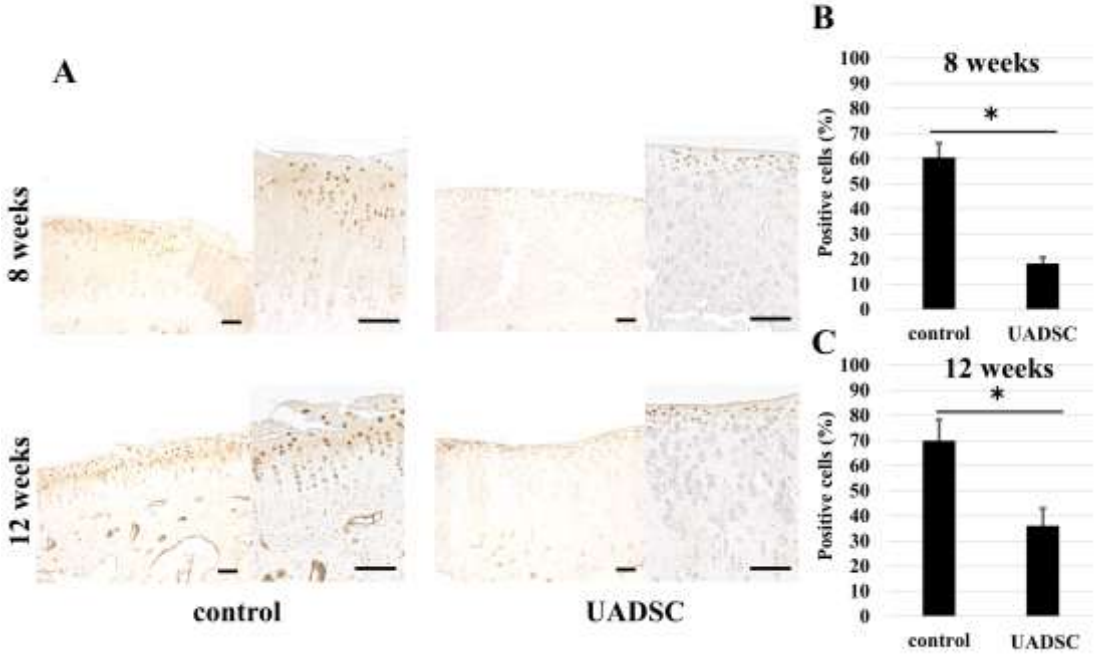


Figure.5

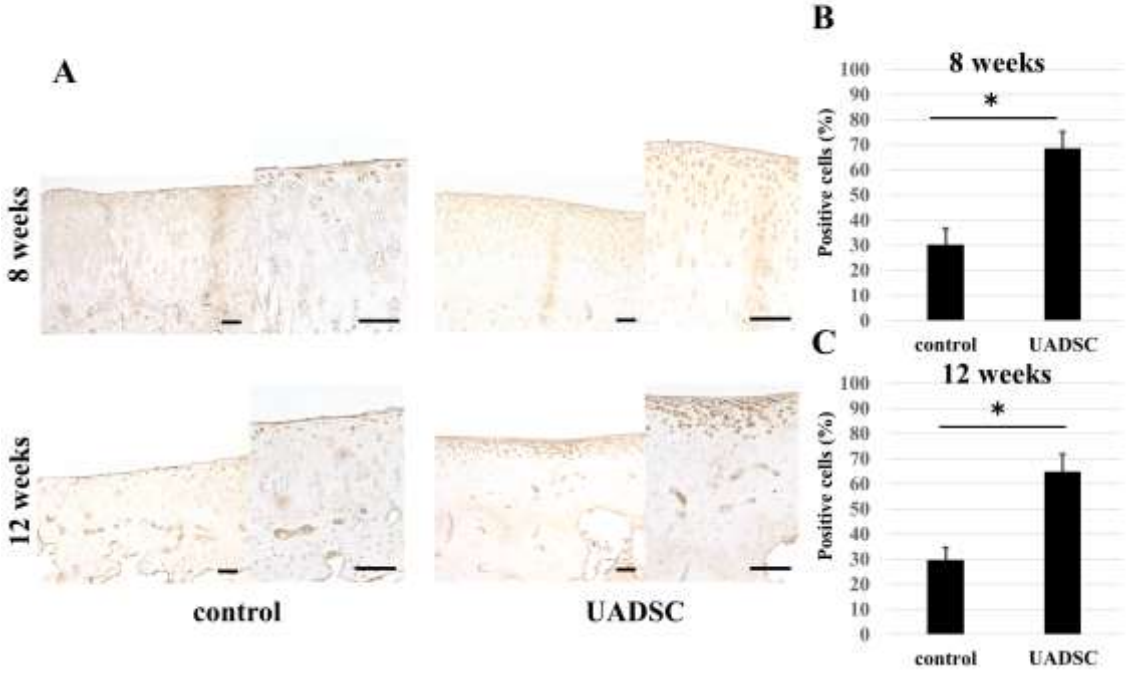
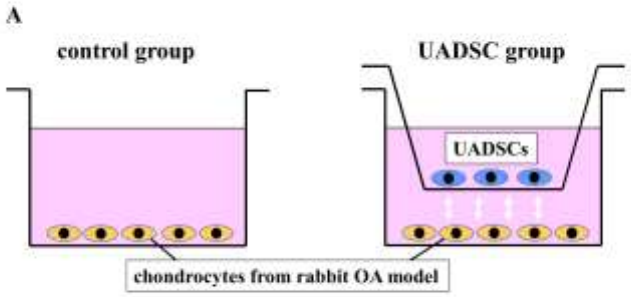
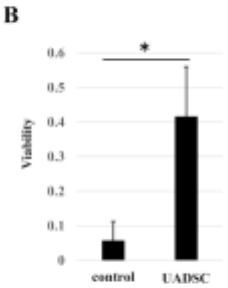


Figure.6





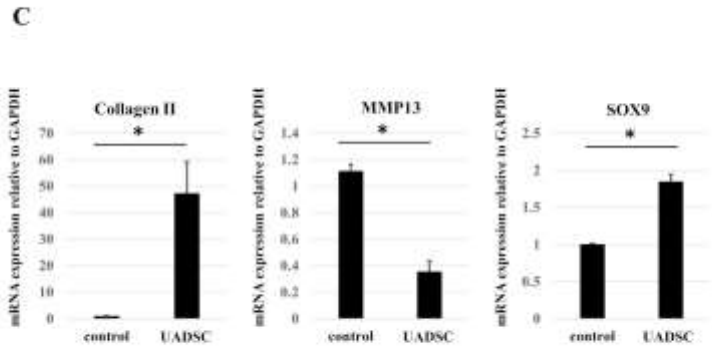


Figure.7