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Repair of Degenerative Intervertebral Discs in Rabbits by Human Umbilical Cord Mesenchymal Stem Cells Embedded in Type I Collagen Hydrogel

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ABSTRACT

Intervertebral disc regeneration based on stem cell differentiation is an attractive approach towards repairing/regenerating the nucleus pulposus. Here, we attempted to repair degenerative intervertebral discs using human umbilical cord mesenchymal stem cells (hUCMSCs) in combination with type I collagen. The disc degeneration model was established in New Zealand white rabbits by disc puncture. Two weeks after puncture, rabbits showed typical intervertebral disc degeneration, with internal disc disruption; stenosis of the intervertebral space, weakening T2 disc signal, and decreased disc height. Using X-ray and T2-weighted MRI analyses, we demonstrated that transplantation of hUCMSCs embedded in collagen type I hydrogel exhibited a therapeutic effect in repairing degenerative discs, as shown by better disc height index, lower disc degeneration grade, and relatively preserved inner annulus structure with minimal fibrosis in the nucleus region. Similarly, immunohistochemical and spectrophotometry analyses revealed lower intervertebral disc degeneration grading, more nucleus pulposus cells, higher expression of type II collagen and proteoglycan around nucleus pulposus cells in the hUCMSC/collagen treated group. Collectively, our study demonstrates co-transplantation of hUCMSCs and type I collagen has a restorative potential in the treatment of intervertebral disc degeneration.

Keywords: Intervertebral disc degeneration, Tissue engineering repair, Human umbilical cord, Mesenchymal stem cells, Type I collagen, Rabbit

INTRODUCTION

Low-back pain, a prevalent, debilitating and costly condition, is considered to be a public health concern by the global medicine community [1,2]. Among etiological factors, intervertebral disc degeneration (IDD) is implicated as a major cause of low-back pain [3]. The nucleus pulposus (NP) of normal disc includes sparse chondrocytes surrounded by extracellular matrix consisting of type II collagen and proteoglycan. It functions as a shock absorber against mechanical load due to its highly hydrophilic structure [4]. Histopathologically, the IDD shows a decrease in water content associated with reduced proteoglycan content of the NP, resulting in destruction of the annular and

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flattening of the disc [5]. Conventional treatment approaches, based on conservative treatments and operations, can ameliorate the patients' clinical symptoms, but are limited by surgical techniques and the use of novel biological solutions [6-8]. Thus, there is an urgent need for new regenerative therapies that restore native tissue structure and mechanical function in patients with disc degeneration.

Currently, strategies to regenerate the disc have focused primarily on restoring the ability to restore the disc tissue. These include strategies involving cell transplantation therapy, cytokine and growth factor induction, gene therapy, and tissue engineering [9-13]. Multiple sources of donor cells have been used for cell therapy to repair the degenerating intervertebral disc (IVD). Autologous NP cell transplantation has become one of the major techniques to prevent IVD in animal models [13,14]. However, it has been considered clinically difficult for broad application as the procedure requires more cells than can be harvested from a single disc. Some reports have demonstrated that transplantation of bone marrow mesenchymal stem cells (MSCs) delayed degeneration of the nucleus pulposus [15]. MSCs can be isolated from tissues other than bone marrow [16-18]. Some studies have harvested potent mesenchymal stem cell population from the Wharton's jelly of the human umbilical cord, which possess cell markers of multipotent mesenchymal stromal cells and had ability to differentiate to osteogenic, adipogenic and chondrogenic lineages in vitro under defined monolayer or cell mass-based differentiation condition [19,20]. Leckie et al have presented data showing that injecting human umbilical tissue-derived cells into the NP improved the biomechanical properties of the degenerating IVD in vivo [21]. Wang et al showed that the chondrogenic differentiation of hUCMSCs produced more glycosaminoglycan and collagen than bone marrow MSCs, suggesting that hUCMSCs possesses a greater potential for cell-based treatment of IDD [22]. Several studies have focused on the use of hUCMSCs because of its potent repairing effect on degenerative diseases and damaged organs [23,24]. Experimental hUCMSCs transplantation therapies are effective in a variety of diseases including the articular cartilage and myocardium [23,25]. The effect of hUCMSCs used in the nucleus pulposus tissue engineering, however, has not well been fully explored.

Recently, tissue engineering using adult mesenchymal stem cells (MSCs) as a candidate cell type has shown a great potential for cell-based treatment of these spinal problems [26,27]. Many forms of biomaterials have been investigated as scaffolds, such as alginate, chitosan, type I collagen, type II collagen/aggrecan/hyaluronan, fibrin/hyaluronan and PLLA-hyaluronan nanofibres, all of which serve as potential cell carriers for treatment of NP [28-32]. Type I collagen, a natural and frequently-used scaffold material with special biological activity and biocompatibility, is non-immunogenic, biodegradable and can withstand the mechanically loaded environment in the IVD. This scaffold is degraded slowly to allow the seeded cells to differentiate and produce new matrix [23,33]. Bertolo et al. showed that MSCs in collagen matrixes produced more mRNA and proteins of the chondrogenic markers collagen type I, collagen type II (COL2) and aggrecan (ACAN) compared with cells embedded in alginate or chitosan [23]. Proteoglycan accumulation and cell survival were also higher in collagen and gelatin matrixes. Type I collagen is naturally present in the disc without immune rejection and compatibility issues, so it may be a good scaffold material used for NP tissue engineering. It has been shown that transplantation of BM-MSCs embedded in type I collagen into articular cartilage defects improves arthroscopic and histological grading scores. Kuroda et al transplanted collagen gel-embedded BM-MSCs in an athlete with a grade IV cartilage defect and found that the defect was covered with smooth tissues after seven months [26]. Wakitani et al also found that transplanted type I collagen gel embedded autologous BM-MSCs repaired cartilage defects with a fibrocartilage-like tissue after one year [27]. These results suggest that type I collagen is a good scaffold for NP tissue engineering.

Although MSCs used for tissue engineering have shown a great potential in cell-based treatment of degenerative diseases, studies that combine UCMSCs with type I collagen scaffold for NP tissue engineering have not well been reported. In this study, we transplanted type I collagen scaffold-embedded hUCMSCs into degenerative intervertebral discs of rabbit model, and assessed its therapeutic effects using radiography, magnetic resonance imaging (MRI), and immunohistochemistry.

MATERIALS AND METHODS

Isolation and characterization of hUCMSCs

hUCMSCs were supplied by Shenzhen Beike Stem cell Engineering Institute. The passages 2-3 of hUCMSCs were used for this study. The immunophenotype of the culture-expanded hUCMSCs was analyzed by flow cytometry for specific cell surface markers (CD90, CD73 and CD105), hematopoietic cell markers (CD45, CD34, CD14 and CD19) and major histocompatibility elements (HLA-DR). Flow cytometry was performed with the use of FACSCalibur. All antibodies were purchased from BD Biosciences, CA.

Adipogenic, osteogenic and chondrogenic differentiation

The multipotent ability of hUCMSCs at passage 2 was performed by adipogenic, osteogenic and chondrogenic differentiation as previously described previously [34,35]. After induction, cells were stained with the oil red O (Sigma, USA), alizarin red (Sigma, USA) or Alcian Blue (Sigma, USA) to detect the presence of neutral lipid vacuoles in differentiated adipocytes, calcium deposition in osteocytes or proteoglycan in chondrocyte, respectively.

Hydrogel preparation and chondrogenic differentiation

Due to its non-immunogenic and biodegradable feature, we used type I collagen as the supportive material in NP tissue engineering as previously described [36]. Type I collagen was purchased from Shengyou Biotechnology Co. (Hangzhou, China) and dissolved in 0.1% acetic acid with a concentration of 5 mg/mL. For the fabrication of collagen hydrogel, cells were suspended in chondrogenesis-induced medium (high glucose DMEM, 0.1 μmol/L dexamethasone, 50 mg/L ascorbate, 1 mmol/L sodium pyruvate, 40 mg/L L-proline, 6.25 mg/L insulin, 6.25 mg/L transferrin, 6.25 mg/L sodium selenite, 10 μg/L TGF-β1). Immediately before use, 600μl collagen solution was neutralized with 40 μl neutralization buffer consisting of 10x PBS and 0.1M NaOH. The neutralizing hydrogels (1.5, 2.5, and 3.5 mg/ml) were gently mixed with hUCMSCs (7.5, 22.5, 67.5×10^5 cells/ml, respectively), following the orthogonal experiment table design. The culture medium/type I collagen scaffold constructs were used as the control.

All components were gently mixed to avoid air bubbles, and then seeded into 96-wells plate. The plates with hydrogel were incubated at 37 ℃ for 15 min in a 5% CO_2 humidified atmosphere for gel polymerization. After polymerization, 150 μl chondrogenesis-induced medium were added to the plate. Chondrogenic differentiation of hydrogel-embedded hUCMSCs was further examined by histochemistry and immunohistochemistry staining for the expression of type II collagen and GAG using primary mouse anti-type II collagen antibody, rabbit anti-GAG antibody, and an UltraSensitive™ SP (Mouse/Rabbit) IHC Kit (MaiXin BIO, Fuzhou, China), according to the manufacturer's protocol.

In vivo transplantation

Thirty-six New Zealand white rabbits, weighing an average of 2.5 kg, were anesthetized with pentobarbital sodium (1 ml/kg). The rabbits were then placed into a lateral prone position, and the anterior surface of the lumbar spine was exposed through the anterolateral approach, and intervertebral discs (L3 to L4, L4 to L5, and L5 to L6) were identified. Using a number 20-gauge, a 5 mm deep puncture was made into 3 contiguous discs (L3 to L4, L4 to L5, and L5 to L6) through the ventral anulus. Care was taken to avoid excessive exposure of surrounding ligaments and tissues to avoid postoperative spur formation. The disc L6 to L7 intact was used as a normal control. The wound was then thoroughly irrigated with sterile saline and closed with layered sutures. The rabbits were injected with penicillin 3 days after surgery and returned to their cages after a short recovery observation. Two weeks post surgery, disc degeneration was examined with MRI. Rabbits with IDD were randomly divided into 3 groups (12 per group): Control group (CG), Experimental group (EG), and Degeneration group (DG). Animals were treated with collagen type I in the CG group and hUCMSCs/type I collagen hydrogel in the EG group. Animals in the DG group did not receive any treatment as the control. The discs that were not punctured were collected as the normal group (NG). At 4, 8, 12 weeks postoperatively, the pathological changes were evaluated by MRI, X-ray and histological analysis.

Radiographic and MRI analysis

Rabbits were anesthetized with pentobarbital sodium (1 ml/kg). Radiographs were taken using X-ray equipment (55KV, 100mA, 50ms). The IVD height was expressed as the disc height index (DHI) based on the method of Masuda et al. [37]. The average IVD height (DHI) was calculated by averaging the measurements obtained from the anterior, middle, and posterior portions of the IVD and dividing that by the average of adjacent vertebral body heights. Alterations in the DHI of injected discs were expressed as %DHI and normalized to the measured preoperative IVD height (%DHI = postoperative DHI/preoperative DHI × 100).

MR imaging were taken using a Semiens 1.5-T imager. T2-weighted turbo spin-echo images (TE 150 ms, TR 4,300 ms) of the lumbar spine were obtained at each time point. MRI images of each disc section were graded according to the method used by Pfirrmann et al. [38].

Histology and immunohistochemistry

The intervertebral discs including the adjacent vertebral bodies were fixed in 10% neutral-buffered formalin and embedded in paraffin. Midline sagittal sections of the intervertebral discs were stained with Hematoxylin and Eosin. Based on the condition of anulus fibrosus, the border between the anulus fibrosus and nucleus pulposus, the cellularity of the nucleus pulposus, and the matrix of the nucleus pulposus through midsagittal sections, we graded each disc section according to the histological grading scale developed by Masuda et al. [37]. Cells randomly selected from four horizons in each slice were counted under high magnification (× 400).

Proteoglycan analysis

NP tissues of rabbits were collected and examined by phloroglucinol spectrophotometry analysis for the expression of proteoglycan according to the manufacturer's protocol.

Statistical analysis

Data were expressed as the means ± SD. Statistical analysis was performed by SPSS 17.0 software. The Student's t-test was used to compare serum parameters. $P < 0.05$ was considered to indicate a statistically significant result.

RESULTS

Characterization of hUCMSCs

To verify the lineage potential, hUMSCs were differentiated in six-well plates. After 21, 28 and 14 days of culture, cells

were examined for adipogenic, osteogenic and chondrogenic differentiation. Intracytoplasmic lipid droplets stained with oil red O, calcium deposits stained with alizarin red and proteoglycan stained with Alcian Blue were observed in cell (**Figure 1A**), demonstrating the potential of adipogenic, osteogenic and chondrogenic differentiation of the isolated hUCMSCs.

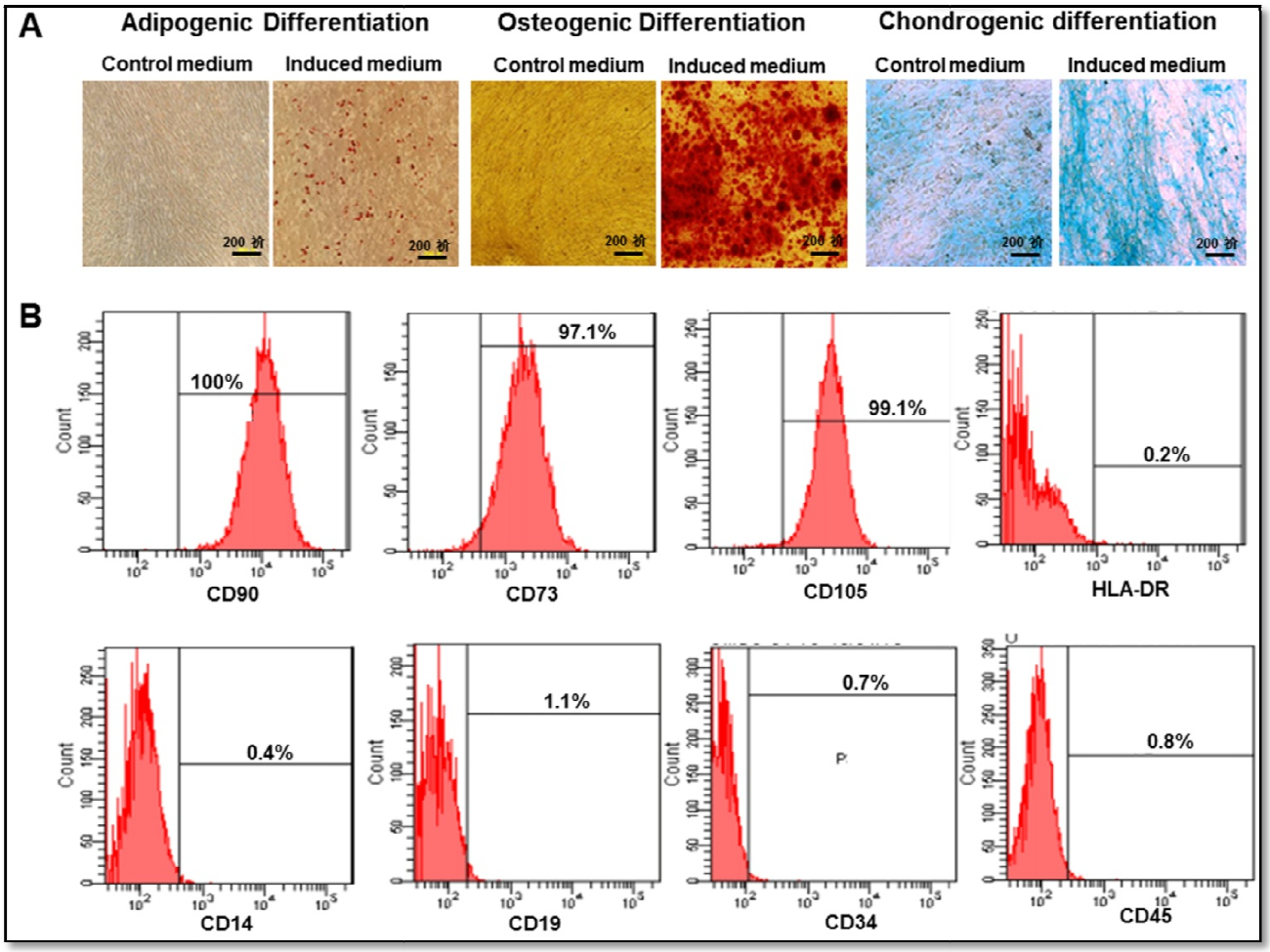

Figure 1. Characterization of hUCMSCs. A. Adipogenic differentiation (×400), Osteogenic differentiation (×100) and Chondrogenic differentiation (×200) of hUCMSCs. **B.** Immunophenotype of hUCMSCs is determined by flow cytometry at passage 2 with the use of labeled antibodies specific for the indicated human surface antigens.

The immunophenotype of the culture-expanded hUCMSCs at passage 2 was analyzed by flow cytometry for specific cell surface markers. As shown in **Figure 1B**, hUMSCs were positive for known MSC markers (CD105, CD73 and CD90) and were negative for hematopoietic markers (CD34, CD45, CD19, CD14 and HLA-DR). After characterization, the hUCMSCs were used for the subsequent study.

Immunohistochemical analyses of chondrogenesis in type I collagen hydrogel

To develop nucleus pulposus-like tissue, variable amounts of hUCMSCs were seeded in scaffold consisting of three levels of type I collagen (1.5, 2.5, and 3.5 mg/mL). The culture medium/type I collagen scaffold constructs were used as the control. After 24 h of cell seeding, the morphology of hUCMSCs encapsulated in collagen I hydrogels were observed. As shown in **Figure 2A**, the hUCMSCs displayed the typical fibroblast-like morphology. After 2 weeks of culture in standard chondrocyte conditioning medium, the complex of hUCMSCs with type I collagen scaffolds was analyzed by immunohistochemistry analyses. We found that hUCMSCs embedded in collagen I hydrogel highly expressed GAG and collagen II after exposed cells-embedded hydrogels (Figure **2B, 2C**). Among the 3x3 experiment design groups, the 7.5×10^5/ml hUMSCs/1.5mg/ml type I collagen group secreted the highest GAG and type II collagen. These data suggest that the collagen hydrogel embedded-MSCs were able to undergo chondrogenic differentiation and surrounded by sulfated proteoglycan-rich extracellular matrix.

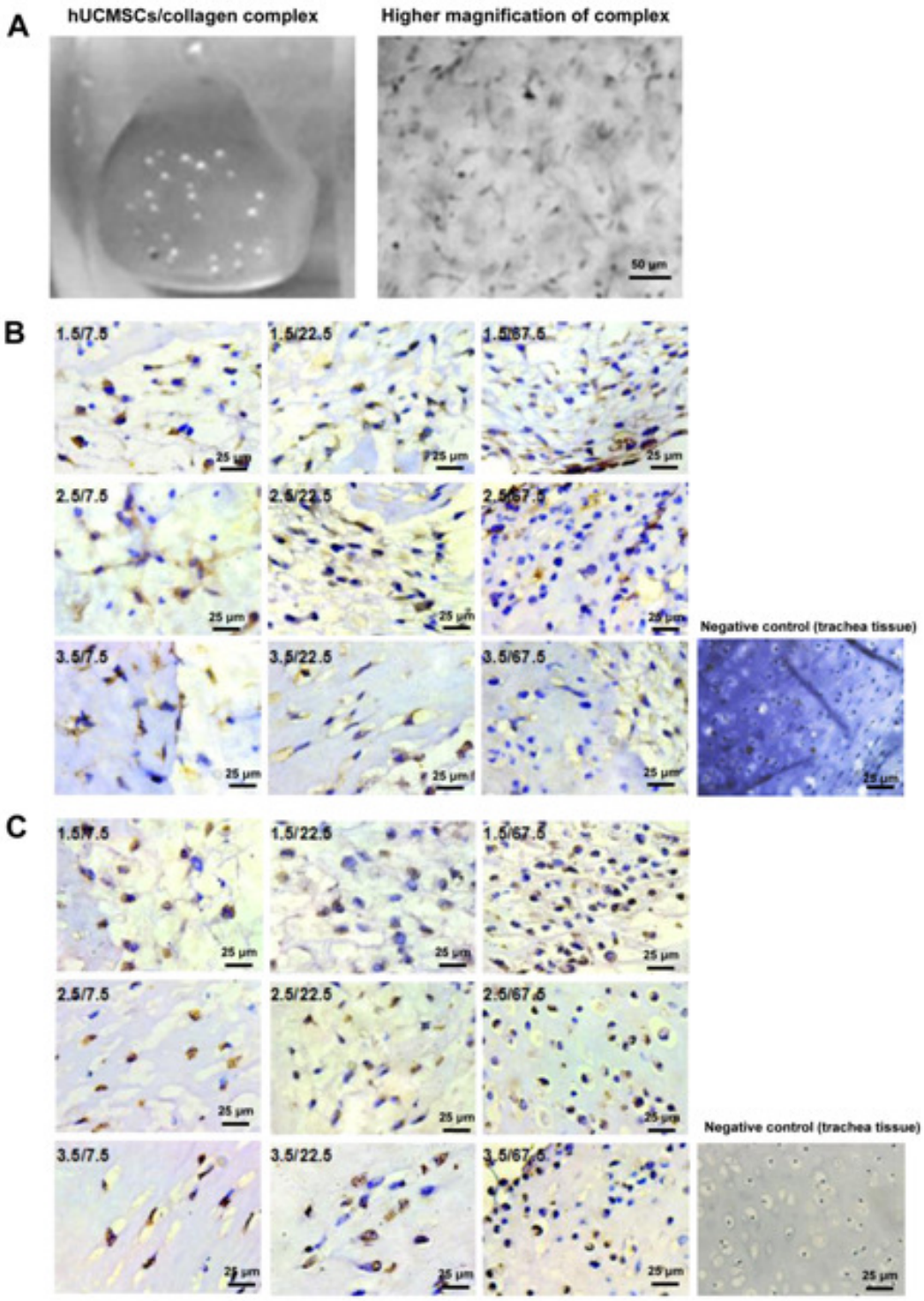

Figure 2. Hydrogel preparation and chondrogenic differentiation induction. A. The morphology of hUCMSCs encapsulated in the collagen I hydrogels. **B** and **C**. Immunohistochemistry analyses for the expression of GAG and type II collagen (×200). The orthogonal experiment table design was used to examine the combination of type I collagen hydrogel (1.5, 2.5, and 3.5 mg/ml) and hUCMSC density (7.5, 22.5, 67.5×10^5 cells/mL). Numbers in the left top: type I collagen hydrogel/ hUCMSC density.

Imaging analyses of degenerative discs

To determine the effect of the hUCMSCs/type I collagen therapy on repairing the degenerative discs, 36 New Zealand white rabbits were punctured to establish animal model of degenerative intervertebral disc (**Figure 3A**). Twenty-four rabbits were treated with culture medium/collagen type I (Control group, CG) and hUCMSCs/type I collagen (Experimental group, EG), and another 12 rabbits were untreated as the "degeneration group" (DG). The animals with discs un-punctured were referred to the "normal group (NG)". Two weeks post puncture, the changes were evaluated by X-ray and MRI analysis. The IVD height was expressed as the disc height index (DHI) based on the method of Masuda et al [37] (**Figure 3B**). Animals showed typical internal disc disruption, including stenosis of the intervertebral space, weakening T2 disc signal and decreased disc height, suggesting that degenerative intervertebral disc rabbit models were successfully established.

After cell transplantation for 4, 8 and 12 weeks, changes in the DHI of injected discs were evaluated. The X-ray analysis showed that the disc height index in the CG and DG group decreased gradually and reached bottom at 12 weeks. However, the % DHI in the treated EG group was statistically higher than that in the CG and DG group at any time points (**Figure 3C**).

T2-weighted MR images showed that the signal intensity of nucleus pulposus in the CG and DG group considerably decreased at 4 weeks and thereafter (**Figure 3D**). Although the intensity in the EG group also gradually reduced compared to that in the NC group, it remained higher than those in the CG and DG groups. The disc degeneration grading of the EG group discs was graded as 2-3 at the most, compared with 4-5 in the CG and DG groups. NC group discs maintained grade 0 throughout the study (**Figure 3E**).

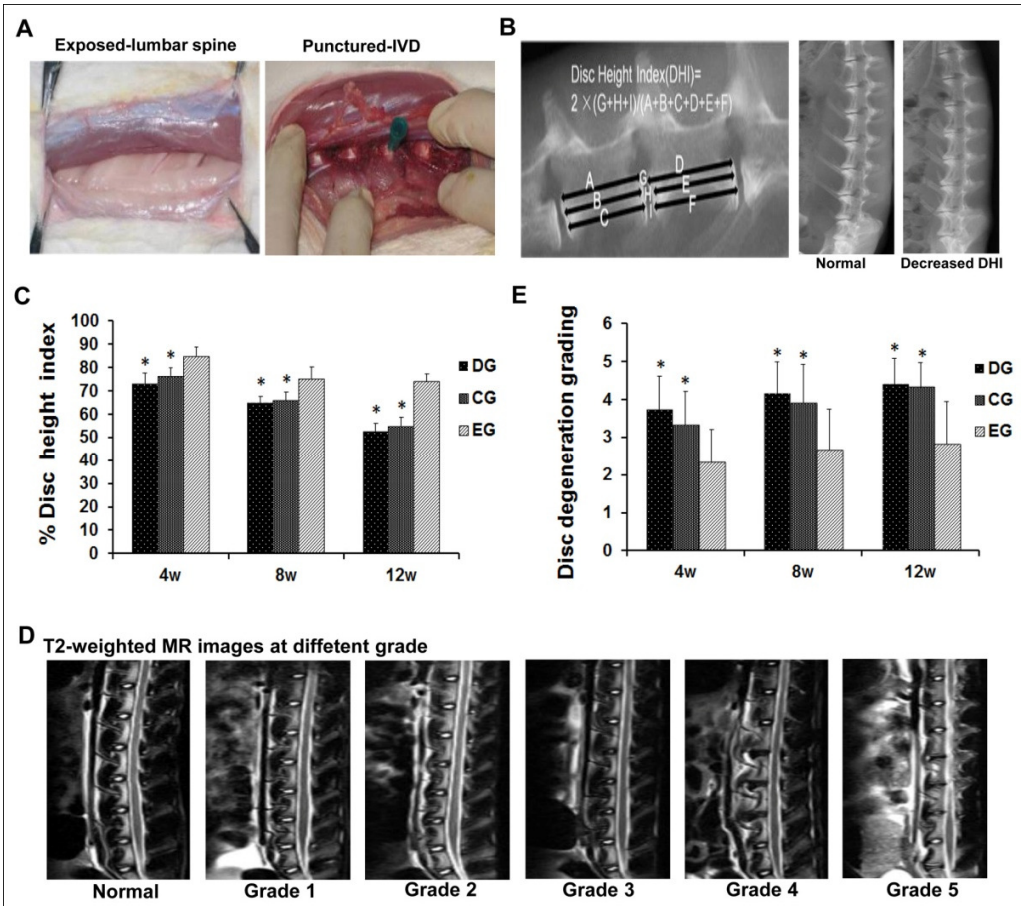

Figure 3. Transplantation of hUCMSCs embedded in type I collagen maintain disc height. A. The lumbar spine of the rabbit was exposed to identify intervertebral discs, which was then punctured to establish the degenerative intervertebral disc model. **B**. X-ray image of rabbit spine for measurement of disc height index. **C**. Sequential changes of disc height index after cotransplantation of hUCMSCs and type I collagen. Average percentages of the value are shown with standard deviations. *$P < 0.05$ compared with the EG group. **D**. T2-weighted MR images of intervertebral discs at different grade. **E**. Representative T2-weighted MR images of intervertebral discs at 4 to 12 weeks after operation. *$P < 0.05$ compared with the EG group.

Transplantation of hUCMSCs/type I collagen repairs the degenerative discs

The intervertebral discs including the adjacent vertebral bodies were fixed in 10% neutral-buffered formalin and embedded in paraffin. Midline sagittal sections of the intervertebral discs were stained with HE. Typical histologic changes of degeneration were shown in **Figure 4A**. The discs in the EG group showed relatively preserved inner annulus structure with minimal fibrosis in the nucleus region. The disc degeneration histopathologic score in the EG group were lower than those in the CG and DG groups. In the degeneration group, the nucleus pulposus could hardly be seen. In the EG group, however, the nucleus pulposus looked comparable to that in the normal group (**Figure 4A**). In high magnified histology, the nucleus pulposus was replaced with fibrous tissue in the CG and DG group but consisted of sparse cells surrounded with matrix in the EG group. The number of nucleus pulposus-like cells in the EG group were more than that in the CG and DG group (**Figure 4B**, $p<0.05$). Interestingly, the nucleus pulposus-like cells was also relatively higher in the EG group than that in the normal group, suggesting active regeneration in the degenerative intervertebral disc following the treatment.

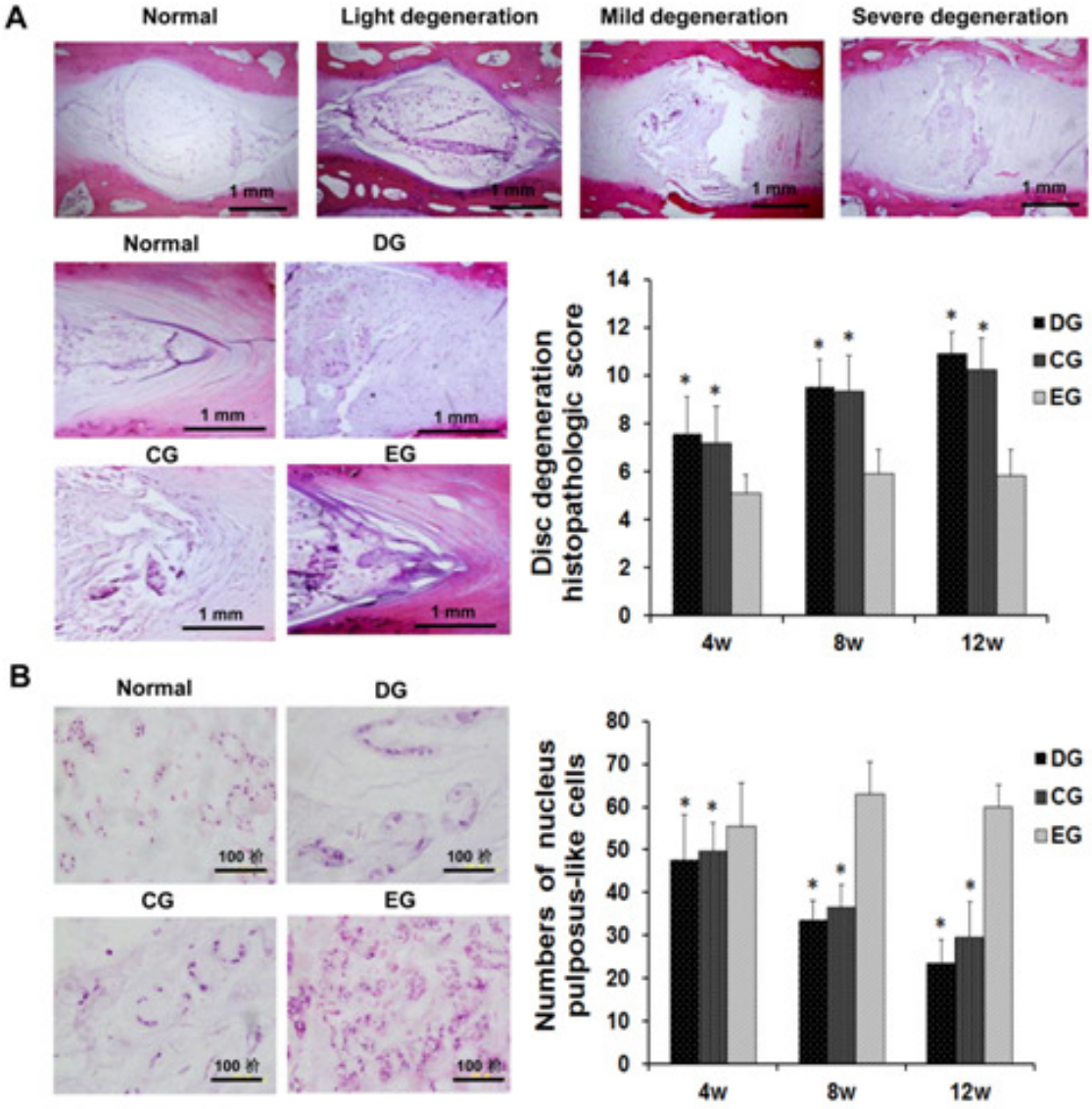

Figure 4. Transplantation of hUCMSCs embedded in type I collagen maintain microstructure of nucleus pulpous. A. Sagittal sections with Hematoxylin-Eosin (HE) staining after operation and disc degeneration histopathologic score. **B**. High magnification of the framed area and nucleus pulposus-like cells counting. *$P < 0.05$ compared with the EG.

Immunohistochemistry analyses and proteoglycan assay revealed significantly high expression of type II collagen and proteoglycan around nucleus pulposus cells in the EG than that in the CG and DG groups (**Figure 5A, 5B**). These results indicate that co-transplantation of hUCMSCs and type I collagen can restore the extracellular matrix in degenerative discs.

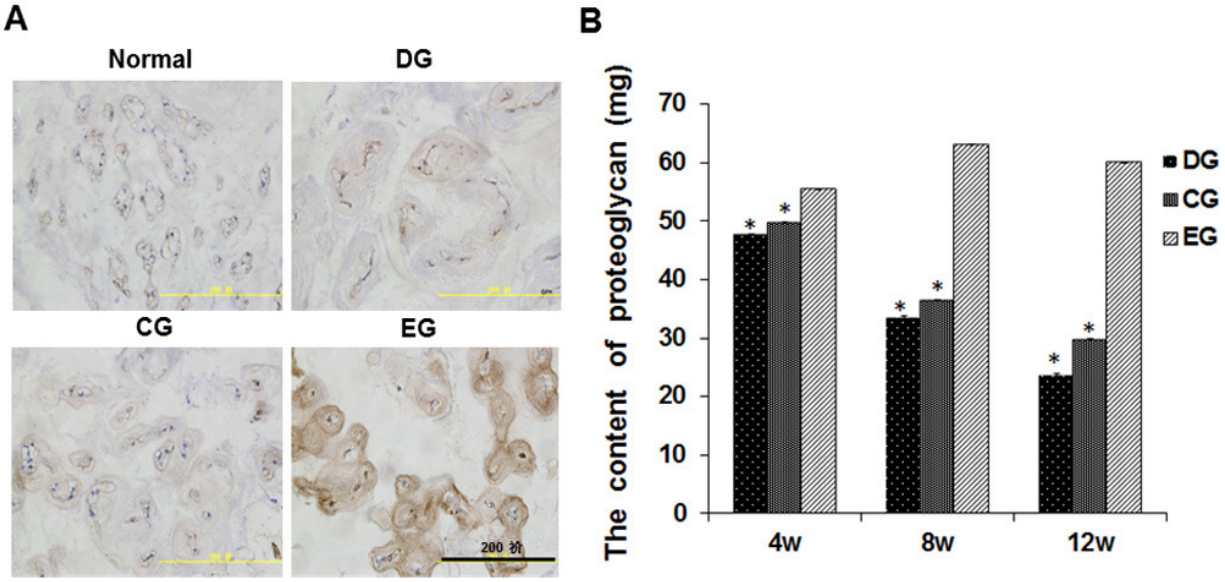

Figure 5. Transplantation of hUCMSCs embedded in type I collagen increases expressions of chondrogenic markers. **A**. Immunohistochemistry analyses for the expression of type II collagen. **B**. Proteoglycan production was detected by spectrophotometry analysis. *$P < 0.05$* compared with the EG.

DISCUSSION

Tissue engineering using adult mesenchymal stem cells (MSCs) has shown a great potential for cell-based treatment of degenerative diseases and damaged organs [26,27]. As an alternative to MSCs, hUCMSCs possess clear advantages such as being obtained from a readily available source as well as having low immunogenic potency and high proliferative activity [19,20]. Several studies have reported the therapeutic effects of hUCMSCs in degenerative full-thickness articular cartilage defects, myocardial infarction, and osteogenesis imperfecta, bone regeneration, liver cirrhosis and acute liver failure models [34,39,40]. Despite such an interest and the growing number of research data, studies directed toward nucleus pulposus tissue engineering to regeneration of IVD have not well been determined. Type I collagen is a protein-based three-dimensional hydrophilic and polymeric networks with high water content facilitating rapid diffusion of nutrients and metabolites. It allows embedded cells to grow in a three-dimensional environment, which is very suitable for disc cells and chondrocytes in vitro [23,33]. In this study, type I collagen was used here as the delivery scaffold to investigate the effect of hUCMSCs on repairing the degenerative discs in a rabbits model.

To determine the effect of type I collagen-embedded hUCMSCs on degenerative discs, hUCMSCs were isolated, cultured and evaluated in vitro for osteogenic, adipogenic and chondrogenic differentiation potential and immunophenotype. We showed that hUCMSCs were differentiated into osteogenic, adipogenic and chondrogenic lineages and possess specific MSC cell surface markers (CD105, CD73 and CD90), suggesting that the isolated cells possessed the properties of MSC. The hUCMSCs were further seeded in collagen I hydrogel to investigate their chondrogenic differentiation potential. Our data showed that hUCMSCs embedded in collagen I hydrogel can undergo chondrogenesis characterized by significantly increased expressions of GAG and collagen II, the main collagenous (about 90% of the collagenous fraction) element within the cartilage, suggesting that hUCMSCs undergo NP-like chondrogenesis in collagen I scaffolds. Our result is consistent with that reported by Chen et al. [23], who showed that hUCMSCs in type I collagen-hydrogel undergo chondrogenic differentiation, indicating that hUCMSCs seeded in type I collagen scaffold was suitable for nucleus pulposus tissue engineering. Thus, we used these hUCMSCs embedded in collagen I for transplantation to investigate its role in degenerative discs of rabbit model induced by puncture.

Restoration of disc height and T2-weighted signal intensity on MRI are two major parameters for evaluating disc degeneration in clinical settings. A high signal intensity of T2-weighted images in MRI is often used indirectly to evaluate water content in the IVD [37,38]. Based on these parameters, the degenerative intervertebral disc rabbit models we established showed typical internal disc

disruption; stenosis of the intervertebral space, weakening T2 disc signal and decreased disc height after two weeks of puncture, suggesting that the degenerative intervertebral disc rabbit model was successfully established. Degenerated IVDs were significantly improved according to X-ray analyses after hUCMSCs-collagen I complex transplantation for 4 weeks. The DHI% in the EG group remained higher than that in the CG and DG group at 4 weeks and thereafter. T2 weighted MRI showed that the disc degeneration grading of EG group discs were graded as 2-3 at the most, compared with 4-5 in the CG and DG group. Immunohistological analyses revealed lower disc degeneration grading and more nucleus pulposus cells in the EG than that of the CG and DG group at 4 weeks and thereafter.

Generally, type II collagen functions as a frame work in the nucleus pulposus [41,42], maintaining disc height and histological features. Proteoglycans are important components of the noncollagenous cartilage matrix responsible for the mechanical properties of cartilage [43]. In our experiments, the increase in the expression of cartilage ECM was detected by immunohistochemical staining and spectrophotometry analysis. We showed higher expression of type II collagen and proteoglycan around nucleus pulposus cells in the EG group than that of the CG and DG group. Thus, these results indicate that cotransplantation of hUCMSCs and type I collagen can restore the extracellular matrix, which may be beneficial for the therapy of intervertebral disc degeneration. Leckie et al have shown that injecting hUCMSCs into the NP improved the biomechanical properties of the degenerating IVD in vivo [21]. Chen et al showed that hUCMSCs embedded in collagen hydrogel can undergo chondrogenesis characterized by significantly increased expressions of chondrogenic markers collagen II, aggrecan, COMP (cartilage oligomeric matrix protein) and sox9 [23]. Taken together, the data indicate that transplantation of hUCMSCs combined with type I collagen is a promising alternative approach in nucleus pulposus tissue engineering.

In summary, our study has demonstrated that cotransplantation of hUCMSCs and type I collagen exert a restorative effect in a degenerative intervertebral disc rabbit model. Thus, chondrogenic differentiation of hUCMSCs in type I collagen-hydrogel for nucleus pulposus tissue engineering may have a potential application in the treatment of human IVD.

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DISCLOSURE

The authors declare no conflict of interest.

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