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Basic Science

Therapeutic effects of cell therapy with neonatal human dermal fibroblasts and rabbit dermal fibroblasts on disc degeneration and inflammation

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Abstract

BACKGROUND CONTEXT: Increasing evidence suggests transplanting viable cells into the degenerating intervertebral disc (IVD) may be effective in treating disc degeneration and back pain. Clinical studies utilizing autologous or allogeneic mesenchymal stem cells to treat patients with back pain have reported some encouraging results. Animal studies have shown that cells injected into the disc can survive for months and have regenerative effects. Studies to determine the advantages and disadvantages of cell types and sources for therapy are needed.

PURPOSE: The objective of this study is to determine the impact of donor source on the therapeutic effects of dermal fibroblast treatment on disc degeneration and inflammation.

STUDY DESIGN: Using the rabbit disc degeneration model, we compared transplantation of neonatal human dermal fibroblasts (nHDFs) and rabbit dermal fibroblasts (RDFs) into rabbit degenerated discs on host immune response, disc height, and IVD composition.

METHODS: New Zealand white rabbits received an annular puncture using an 18-guage needle to induce disc degeneration. Four weeks after injury, rabbit IVDs were treated with 5×10^6 nHDFs, RDFs, or saline. At eight weeks post-treatment, animals were sacrificed. X-ray images were obtained. IVDs were isolated for inflammatory and collagen gene expression analysis using real-time polymerase chain reaction and biochemical analysis of proteoglycan contents using

Abbreviations: AF, annulus fibrosus; CCL, chemokine (C-C motif) ligand; COL1, collagen type I; COL2, collagen type II; DAB, 3,3′-diaminobenzidine; DHI, disc height index; DMEM, Dulbecco Modified Eagle medium; DMMB, dimethylmethylene blue; DNA, deoxyribonucleic acid; FDA, Food and Drug Administration; HCl, hydrochloric acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPRT, hypoxanthine phosphoribosyltransferase; IL-8, interleukin-8; IVD, intervertebral disc; MRI, magnetic resonance imaging; mRNA, messenger ribonucleic acid; MSC, mesenchymal stem cell; nHDF, neonatal human dermal fibroblast; NP, nucleus pulposus; PBS, phosphate buffered saline; PCR, polymerase chain reaction, qPCR, quantitative polymerase chain reaction; RDF, rabbit dermal fibroblast; RNA, ribonucleic acid; rRNA, ribosomal ribonucleic acid; U, unit

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dimethylmethylene blue assay. These studies were funded by a research grant from SpinalCyte, LLC ($414,431).

RESULTS: Eight weeks after treatment, disc height indexes of discs treated with nHDF increased significantly by 7.8% ($p<.01$), whereas those treated with saline or RDF increased by 1.5% and 2.0%, respectively. Gene expression analysis showed that discs transplanted with nHDFs and RDFs displayed similar inflammatory responses ($p=.2$ to .8). Compared to intact discs, expression of both collagen types I and II increased significantly in nHDF-treated discs ($p<.05$), trending to significant in RDF-treated discs, and not significantly in saline treated discs. The ratio of collagen type II/collagen type I was higher in the IVDs treated with nHDFs (1.26) than those treated with RDFs (0.81) or saline (0.59) and intact discs (1.00). Last, proteoglycan contents increased significantly in discs treated with nHDF ($p<.05$) and were trending toward significance in the RDF-treated discs compared to those treated with saline.

CONCLUSIONS: This study showed that cell transplantation with nHDF into degenerated IVDs can significantly increase markers of disc regeneration (disc height, collagen type I and II gene expression, and proteoglycan contents). Transplantation with RDFs showed similar regenerative trends, but these trends were not significant. This study also showed that the human cells transplanted into the rabbit discs did not induce a higher immune response than the rabbit cells. These results support that the IVD is immune privileged and would tolerate allogeneic or xenogeneic grafts.

Keywords: Allogeneic; Animal model; Cell therapy; Disc degeneration; Fibroblast; Xenogeneic.

Introduction

Cell therapy provides a promising treatment option for a wide range of diseases which cannot be remedied with pharmaceutical drugs, gene or protein therapies. For decades, hematopoietic cell transplantations have been successful in replacing the blood and immune systems of patients who have undergone whole body irradiation or chemotherapy. The Food and Drug Administration (FDA) has approved the use of autologous fibroblasts for wrinkles, allogeneic keratinocytes, and fibroblasts in artificial skin for wounds, autologous chondrocytes for cartilage repair of the knee, and T cell and other immune cell therapies for leukemia, lymphoma, and certain prostate cancers.

Utilizing cells, biomaterials, and proteins to facilitate the regeneration of musculoskeletal tissues has been studied and tested in the field of orthopedics along with other regenerative medicine fields. Spine surgeons often use iliac crest autograft containing bone marrow cells to induce spine fusions. Also, osteochondral autografts or allografts are considered necessary to help repair large chondral or cartilage defects in knee surgeries.

The potential advantages of cellular therapies for disc degeneration are transplanted cells may provide growth signals, anti-inflammatories, and other unknown factors, and repopulate the tissue to create a microenvironment of regeneration. Preclinical studies on rats [1–3], rabbits [4–10], pigs [11,12], dogs [13–16], goats [17], and sheep [18,19] have shown that intradiscal injection of cells can reduce disc degeneration and promote regeneration. Clinical studies utilizing autologous or allogeneic cell therapy to treat patients with back pain have reported some encouraging results in pain score reduction, although neither changes in disc height nor magnetic resonance imaging (MRI) grades were evident.

More studies are needed to determine cell types and sources that would maximize therapeutic effects and minimize concerns. Some advantages of using autologous cells for treatment are reduced risks in host immune response and disease transmission. On the other hand, an allogeneic cell source would not require an additional procedure for harvesting cells and may have further benefits when obtained from a younger donor. Dermal fibroblasts can easily be obtained from patients themselves without significant donor site morbidity or from donated neonatal human foreskins. They have been shown to transdifferentiate into fat-, cartilage-, and bone-like cells in vitro [20]. Our previous pilot study showed that neonatal human dermal fibroblasts (nHDFs) treatment into rabbit degenerated discs successfully increased disc height and collagen II/collagen I gene expression [10]. Although these results were promising, it was unclear whether an allogeneic or autologous cell source would provide better therapeutic outcomes than xenogeneic cells. In this study, we compared the effects of xenogeneic human dermal fibroblast with allogeneic rabbit dermal fibroblast (RDFs) treatments on host immune response, disc height, and intervertebral disc (IVD) composition in the rabbit disc degeneration model.

Materials and methods

Primary cells and cell culture

Neonatal human dermal fibroblasts isolated from human foreskin were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Rabbit dermal fibroblasts were isolated from adult New Zealand White rabbit skins as

described in "Establishment of Fibroblast Cultures" in *Current Protocols in Cell Biology* [21]. Briefly, to separate the dermis layer from the epidermis layer, the skin samples were incubated in 0.3% trypsin in phosphate buffered saline for 60 minutes at 37°C, and the epidermis layer was mechanically removed. The dermal layer was cut into 2 mm pieces and digested with 0.3% trypsin or 1000 U/mL of collagenase. Fibroblast suspensions were filtered through a 70-μm filter.

Rabbit dermal fibroblasts and nHDFs were expanded and cultured in monolayers for up to seven passages in Dulbecco Modified Eagle medium (high glucose), 1% penicillin-streptomycin, 25 mM HEPES, (all from Invitrogen Life Technologies), and 10% fetal bovine serum. Two to three days before injection, cells were stained with CellVue NIR815 Fluorescent dye (LICOR-Biosciences, Lincoln, NE, USA) based on manufacturer's instructions.

Surgical methods for rabbit disc degeneration and cell treatment

New Zealand white rabbits (Harlan Laboratories, Indianapolis, IN, USA) weighing about 2.5 to 3 kg were used in this study (n=38 total). The rabbits were cared for and maintained in accordance with National Institute of Health guidelines. All studies were approved by Rush University Medical Center's Institutional Animal Care and Use Committee. Under general anesthesia and using aseptic conditions, a left abdominal incision was made and the ventral surface four consecutive lumbar IVDs (L2/L3, L3/L4, L4/L5, and L5/L6) were exposed. Using an 18-gauge needle with a stopper device that allows the needle to go to a depth of 5 mm, the annulus fibrosus was punctured in the ventral aspect into the nucleus pulposus (NP) at all four levels. Suction was applied for the removal of NP tissues. A vascular staple and a suture were placed on the psoas muscle at the L3/L4 level as a marker. The surgical wound created was repaired in layers. The skin was closed using either subcutaneous suture or staples, in accordance with the skin condition of each rabbit. Meloxicam (1.5 mg) was also given orally (1 day before surgery and 2–3 days after the operation). An analgesic (buprenorphine HCl 0.01–0.03 mg/kg) was given up to twice daily for 2 to 3 days, when needed, in consultation with the veterinary staff. After recovery from anesthesia, the rabbits returned to their cages and mobilized ad libitum.

Four weeks postinjury, X-rays were taken to confirm degeneration of discs. Then, a right abdominal incision was made, and ventral surfaces of the L2/L3, L3/L4, L4/L5, and L5/L6 IVDs were exposed. For the dose determination studies (n=6), treatments (volume 8 μl) were randomized between the injured discs of each rabbit, which would include the following: nHDFs (1×10^7, 5×10^7, and 1×10^8 cells/mL) or RDFs (1×10^7 cells/mL). For the remaining studies (n=32), treatments (volume 8 μl) were randomized between saline (Sodium Chloride 0.9%, Baxter, Deerfield, IL, USA), nHDFs (5×10^7 cells/mL) or RDFs (5×10^7 cells/mL) for each rabbit. Postoperative procedures are described above.

Cell tracking

Cells were stained with CellVue NIR815 Fluorescent dye 2 to 3 days before injection. Using this kit, NIR815 near infrared fluorescent dye was stably incorporated unto the lipid membranes of cells, and cells were detected in the 800-nm wavelength channel using a near infrared imager (LI-COR Biosciences, Lincoln, NE, USA). Although this wavelength can penetrate through disc tissues, it cannot be detected through the skin and muscles of the rabbit. Therefore, cell tracking was performed ex vivo. Spine segments and individual discs were isolated and scanned with the near infrared imager to detect signals at the 700 and 800 nm wavelengths. Near infrared fluorescence intensities counts per mm^3 of the individual discs were determined using the imaging software and exported to Microsoft Excel. The near infrared fluorescence intensities between the two time points were compared using the paired *t* test analysis.

Isolation of total RNA and measurement of mRNA levels with real-time polymerase chain reaction

Total RNA was processed for each IVD. Real-time polymerase chain reaction analysis was performed as described in previous studies [22]. Taqman gene expression assays were used for the analysis or normalization of the following genes: rabbit collagen types I and II, IL-8, HPRT1, and 18 S rRNA (Oc03396113_m1, Oc03396134_m1, Oc03397860_m1, Oc03399461_m1, Hs99999901_s1; Applied Biosystems, Foster City, CA, USA). PrimeTime Mini qPCR Assay was designed for rabbit chemokine (C-C motif) ligand (CCL) 2 [Primers: 5′-TCT TGT CCA GGTTGG CAATG-3′ and 5′-CCC AAA GAA GCT GTG ATC TTC A-3′; Probe: 5′-/56-FAM/CCA AGC AGA /ZEN/AGT GGGTCC AGG ATG /3IABkFQ/-3′], CCL3 [Primers: 5′-TCA GCA CCC AGG TCT TCT-3′ and 5′-AGT CGG CTA TGA ATT TGT AGG G-3′; Probe: 5′-/56-FAM/CGC CTG CTG /ZEN/CTT CTC CTA CAT CTC /3IABkFQ/-3′], and CCL5 [Primers: 5′-CAC ACC TGG CGG TTC TT-3′ and 5′-CCC ACG TCA CGG AGT ATT T-3′; Probe: 5′-/56-FAM/CTA TAC CAG /ZEN/CGG CAA GTG CTC CTT/3IABkFQ/-3′] (Integrated DNA Technologies, Coralville, IA, USA). DataAssist Software was used to calculate the relative gene expression using the comparative CT ($\Delta\Delta$CT) method.

Immunostaining for infiltrating macrophages

Intervertebral disc segments were decalcified, fixed with 10% formaldehyde, embedded in paraffin, and sectioned (5 μm). Sections were deparaffinized, treated with Proteinase K, incubated with 0.5% hydrogen peroxide, and

blocked with 5% horse serum before incubating overnight with mouse IgG antibody recognizing a cytoplasmic antigen in rabbit macrophages (1:50) (clone RAM11, Dako, Denmark). Sections underwent secondary antibody incubation with biotinylated horse antimouse IgG (1:400). All antibodies were diluted in phosphate buffered saline with 3% horse serum. Vectastain ABC kit (Vector Labs, Burlingame, CA, USA) and DAB (3,3′-diaminobenzidine) were used to develop the immunostaining. Sections were counterstained with hematoxylin solution (VWR, Radnor, PA, USA). Negative controls were performed by omitting primary antibodies.

Radiographic analysis

X-ray images of sedated rabbits were obtained before the surgery, 4 weeks after disc injury, and 8 weeks post-treatment. Disc height indexes (DHI) (n=21–27) were calculated by three orthopedic researchers who were blinded to the treatment groups using the method of Lu et al. [23] with a slight modification (DHI=IVD height/adjacent IVD body height). The average IVD height 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. Changes in the DHI of injected discs were expressed as %DHI and normalized to the measured preoperative IVD height (%DHI=postoperative DHI/preoperative DHI×100) as previously described [24]. Disc height indices were compared between the two time points using the paired *t* test analysis and between all treatment groups using one-way analysis of variance with posthoc Tukey HSD tests.

Biochemical analysis

Accurate wet and dry weights of disc tissues were obtained using a balance with a readability of 1 μg (Mettler-Toledo, Columbus, OH, USA). Tissues were digested with papain, and the contents of DNA and proteoglycan were determined as we described previously [25,26]. Briefly, the content of DNA in the digest was analyzed by a fluorometric DNA assay using the bisbenzimidozole fluorescent dye method (Hoechst 33258; Polysciences, Inc., Warrington, PA, USA). Total sulfated proteoglycans were analyzed using the dimethylmethylene blue (DMMB, Polysciences, Inc.) dye binding method (n=24–27).

Statistical analysis

The significance of differences among means of data was analyzed using SPSS Statistics (IBM, Armonk, NY, USA). Disc height indices, biochemical parameters, and gene expression analysis were analyzed between all groups using one-way analysis of variance (ANOVA) with post-hoc Tukey HSD tests and between two groups with the independent sample *t* test with a two-tailed test of significance. Disc height indices and near infrared fluorescence intensities between two time points were analyzed using the paired samples *t* test with a two-tailed test of significance. All data were expressed as the mean±standard error. The differences were considered significant when the p value was below 0.05.

Results

Optimal dosage concentration of nHDFs was determined to be 5×10^7 cells/mL

In the first set of studies, we determined the optimal dosage of nHDFs to deliver in vivo using the rabbit disc degeneration model. Cell tracking was performed using near infrared imaging in order to quantitate transplanted cells in the whole disc. Near infrared wavelengths (700–800 nm) can penetrate through disc tissues. Other methods of tracking cells such as overexpression of fluorescent proteins or β-galactosidase have been used in our laboratory and others but are not typically used to quantitate the number of remaining cells in the whole disc [22]. Fluorescent proteins are usually detected in the 500 to 600 nm wavelength and cannot penetrate through the disc tissues, whereas β-galactosidase is usually detected using a colorimetric assay and not quantitated. Both nHDFs and RDFs were labeled with near infrared dye before transplantation. The nHDFs were resuspended into three different concentrations (1×10^7, 5×10^7, and 1×10^8 cells/mL), whereas RDFs were resuspended at a concentration of 1×10^7 cells/mL. Four weeks after injury, treatments were randomized between the four injured discs within each rabbit. At 2, 4, and 8 weeks post-transplantation, the rabbits were euthanized (n=6 rabbits). The spines and individual discs underwent near infrared imaging with the LICOR imaging system. Representative near infrared images are shown in the upper panels of Fig. 1. Individual discs were scanned for the presence of near infrared dye labeled transplanted cells. The rabbit spine and disc contours were detected in the 700-nm wavelength channel (Fig. 1, represented in red). The injected cells were detected in the 800-nm wavelength channel and represented in green when detected alone. When the 800-nm signal of the cells overlapped with the 700-nm signal of the disc and spine, the overlapping signals were represented in yellow. The average of the signal intensities is represented in the lower panel of Fig. 1. At 2 weeks post-transplantation, the average signal intensities from the discs transplanted with 8 μl of 5×10^7 and 1×10^8 nHDFs/mL were about 2,400 counts/mm^3, whereas those transplanted with the lower concentration of cells were about 600 counts/mm^3. At 4 and 8 weeks post-transplantation, the average near infrared signal intensities from the transplanted cells were still detectable yet decreased (Fig. 1). The loss in signal intensity may be due to cell death or normal decay of the half-lives of the molecules.

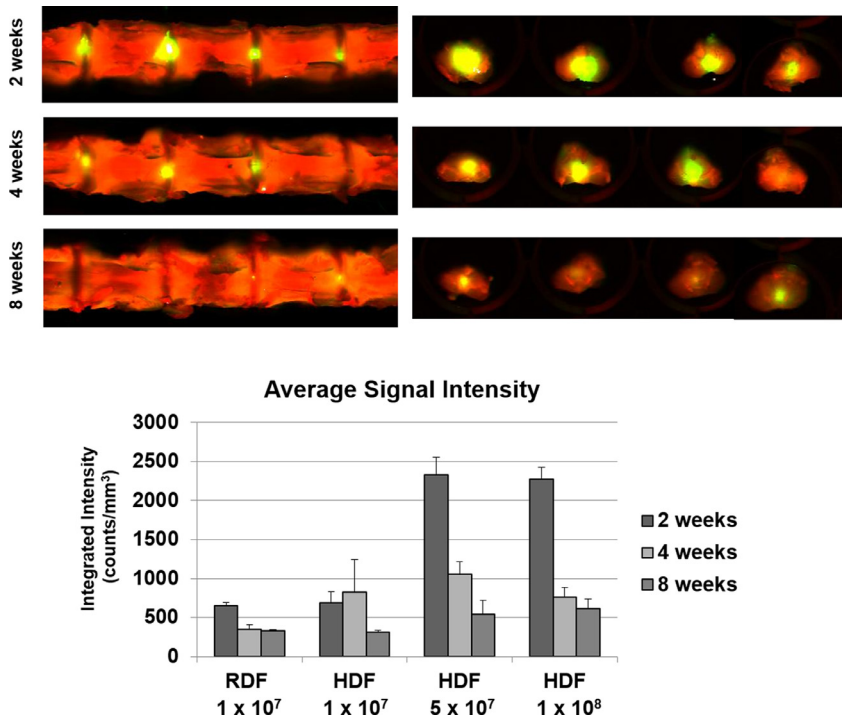
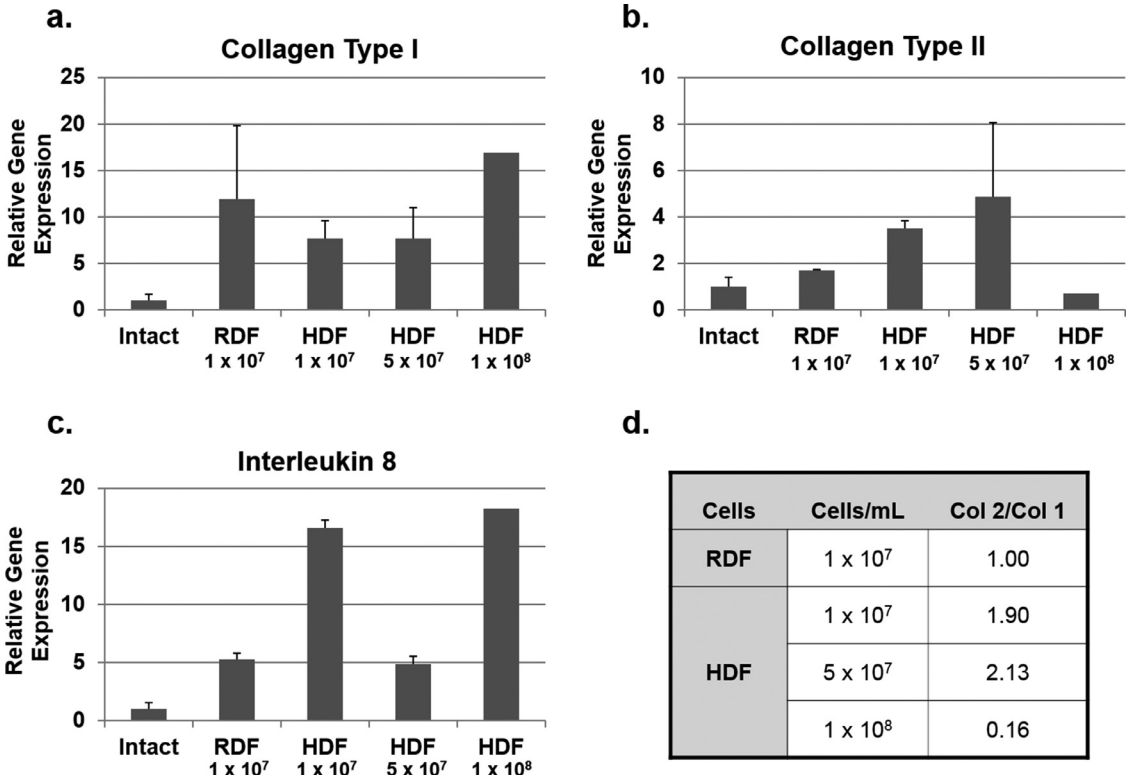


Fig. 1. Cell tracking after injection into degenerating rabbit IVDs in vivo. Upper panels: Representative near infrared images of rabbit spines and individual discs (red) with different concentrations of human dermal fibroblast cells or rabbit dermal fibroblast cells (green and yellow). Lower panel: Average signal intensities of near infrared-dye labeled cells remaining in the rabbit IVDs at 2, 4, and 8 weeks after transplantation.



d.

Cells	Cells/mL	Col 2/Col 1
RDF	1 x 10^7	1.00
HDF	1 x 10^7	1.90
	5 x 10^7	2.13
	1 x 10^8	0.16

Fig. 2. Gene expression analysis of phenotypic intervertebral disc genes. Eight weeks after treatment with rabbit dermal fibroblasts (RDF, 1×10^7 cells/mL) or different concentrations of neonatal human dermal fibroblasts (nHDFs: 1×10^7, 5×10^7, 1×10^8 cells/mL), RNA was isolated from disc tissues for real-time PCR analysis of phenotypic intervertebral disc genes: (a) collagen type I, (b) collagen type II, and (c) interleukin-8. (d) The ratio of collagen type I over collagen type II gene expression was calculated and normalized to the RDF-treated sample.

Table
Inflammatory gene expression analysis of IVDs transplanted with allogeneic and xenogeneic cells. After eight weeks of cell transplantation with RDF (allogeneic) or nHDF (xenogeneic), RNA was isolated from IVD tissues for real-time PCR analysis of inflammatory genes: chemokine C-C motif ligand (CCL)2, CCL3, CCL5 and interleukin8 (IL8). Expression levels were normalized to that of the RDF-treated discs +/- the standard error of the mean.

Gene	Group	Average expression (normalized to RDF)	p Value (Student *t* test)
CCL2	RDF (n=4)	1.00±0.42	.53
	nHDF (n=5)	0.74±0.13	
CCL3	RDF (n=4)	1.00±0.47	.20
	nHDF (n=5)	1.68±0.21	
CCL5	RDF (n=4)	1.00±0.25	.43
	nHDF (n=5)	1.32±0.28	
IL8	RDF (n=4)	1.00±0.26	.80
	nHDF (n=5)	1.12±0.35	

Gene expression analysis was employed on the rabbit IVDs at the 8-week time point (Fig. 2). We analyzed the gene expression of rabbit collagen type I (COL1), collagen type II (COL2), and interleukin 8 (IL-8). Rabbit discs that were injected with the highest concentration of nHDFs (1×10^8 cells/mL) expressed higher levels of COL1 than COL2 and had increased levels of IL-8 (Fig. 2a–c). These discs display a gene expression pattern that may indicate higher amounts of fibrosus tissue and higher inflammation. Discs that were injected with the lower concentrations of nHDFs (1×10^7 and 5×10^7 cells/mL) expressed lower levels of COL1 than COL2 and lower levels of IL-8 (Fig. 2a and b). These discs display a gene expression pattern that may indicate higher cartilaginous tissue and less inflammation. Discs treated with 5×10^7 cells/mL of nHDFs expressed lower levels of IL-8 indicating a less inflammatory environment, and had a higher ratio of COL2/COL1 (Fig. 2d). Based on these studies, the concentration of nHDF (5×10^7 cells/mL) was more optimal than the others and used in the remaining of the studies.

Disc transplanted with nHDFs and RDFs displayed similar inflammatory responses

The IVDs are known to be avascular organs and considered to be immune privileged. Xenogeneic and allogeneic cell transplantation studies into the disc by our group and other research groups have not detected host rejection. In this study, we wanted to do a side by side comparison of the inflammatory and immune response in degenerative discs that have undergone nHDF (xenogeneic) and RDF (allogeneic) cell treatments. After 8 weeks of treatment, the rabbits were euthanized and disc tissues were isolated for RNA analysis and for the presence of immune cells. The average gene expression levels of CCL3 and CCL5 were slightly higher in discs treated with nHDF (n=5) than those treated with RDF (n=4), but this difference was not significant (Table; p>.05). There was not much difference in CCL2 and IL-8 gene expression detected between the two treatments (Table; p>.05).

Macrophages detected at the disc injury site

Both animal and clinical studies have shown that macrophages are detected in the disc space of degenerated discs. To determine whether transplantation with different cells

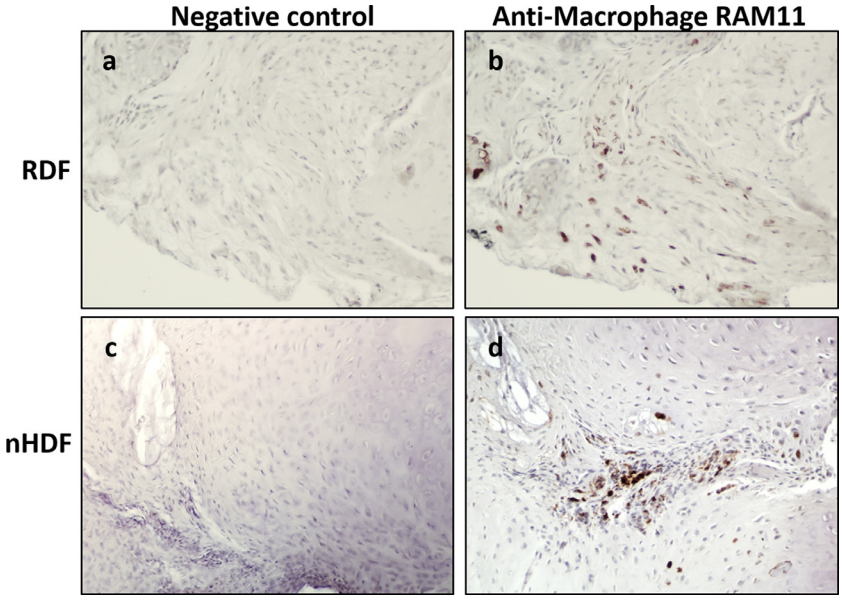


Fig. 3. Representative images of immunohistochemical staining for rabbit macrophages. Rabbit macrophage staining in osteophyte bump tissues of discs treated with (b) rabbit dermal fibroblasts (RDF) or (d) neonatal human dermal fibroblasts (nHDF) using monoclonal antibody against rabbit macrophage, clone RAM11, and (a, c) their respective negative controls (magnification ×200). Negative controls had the primary antibodies omitted. Rabbit macrophage staining was detected from very low levels to undetectable in the disc tissues (data not shown).

Disc Height Index (Relative to Pre-Injury)

	Pre-Injury	Disc injury	Treatment	P-value*
Intact (n=21)	100%	96.8% ± 2.2%	96.8% ± 1.7%	0.99
Saline (n=27)	100%	73.7% ± 1.0%	75.2% ± 1.3%	0.13
nHDF (n=27)	100%	72.9% ± 1.2%	80.8% ± 1.2%	<0.01*
RDF (n=27)	100%	75.0% ± 1.3%	77.0% ± 1.4%	0.06

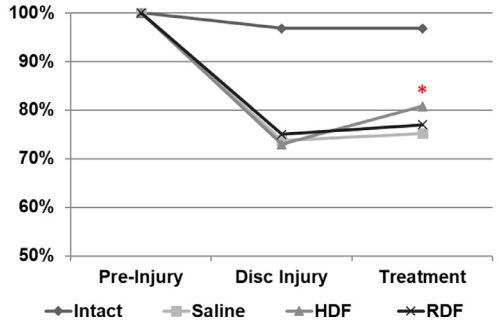


Fig. 4. Changes in the disc height index. Average disc height indexes were calculated preinjury, 4 weeks after injury, and 8 weeks after treatment in discs that were intact or injured and treated with saline, neonatal human dermal fibroblasts (nHDFs) or rabbit dermal fibroblasts (RDFs). The average disc height index was normalized to the pre-injury disc height index. The p values were calculated by comparing disc height indexes before and after treatment. Upper panel: The table shows the average disc height indexes and p values. Lower panel: Graph represents the average disc height indexes. Significant difference between the two time points: 4 weeks after injury and 8 weeks after nHDF treatment (*p<.05).

may increase macrophage recruitment, immunohistochemical analysis of a rabbit macrophage marker was performed on tissue sections of the disc. There was little to no macrophage staining in the annulus fibrosus and NP of the discs treated with saline, nHDFs, or RDFs. However, macrophage staining was observed in the osteophyte bumps of discs of all three treatment groups. Osteophyte bumps are usually formed at the entry site of the needle as part of the wound healing process and contain a mixture of cartilage, bone, and immune cells. Transplantation of xenogeneic dermal fibroblasts, nHDFs, did not increase the presence of these immune cells in these tissues compared to allogeneic dermal fibroblasts, RDFs (Fig. 3). These results suggest that the IVDs may not exhibit an increased immune response when exposed to xenografts.

Disc transplanted with nHDF showed increases in disc height indexes

To determine whether the nHDF or RDF transplantation could help regenerate the disc and increase the disc height, X-rays were taken before injury, 4 weeks after injury, and 8 weeks after transplantation (n=21–27). Changes in disc height indexes were determined for each rabbit at these time points. Average disc height indexes for each group—(1) uninjured and untreated, (2) injured and saline-treated, (3) injured and nHDF-treated, and (4) injured and RDF-treated—were compared (Fig. 4). Four weeks after disc injury, disc height indexes decreased as expected to an average of 72.9% to 75.0% of the original preinjured levels. Eight weeks after treatment, disc height indices of discs treated with nHDF increased significantly from 72.9% to 80.8% (7.9%, p<.01), whereas those treated with saline increased from 73.7% to 75.2% (2.5%, p=.13) or RDF increased from 75.0% to 77.0% (2.0%, p=.06). Also, at the 8-week post-treatment time point, there was a significant difference when comparing the means of the disc height indices between the three treatment groups using the one-way analysis of variance analysis (p<.01) and between the saline and nHDF treatment groups using the posthoc Tukey HSD test (p<.01).

Disc transplanted with nHDF showed increases in collagen gene expression

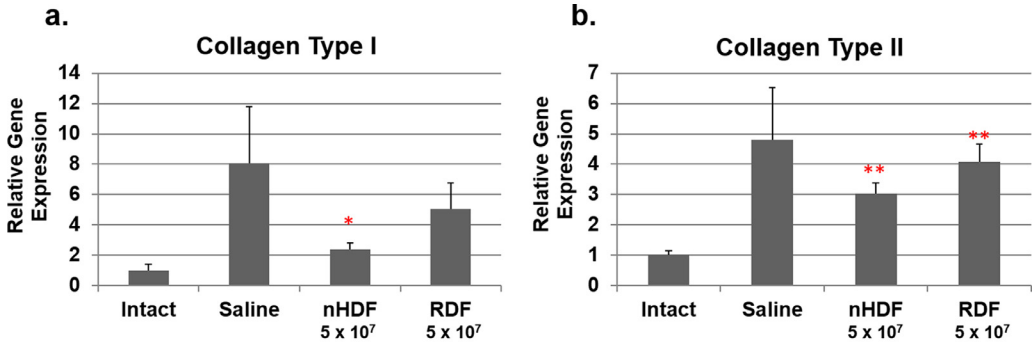
After treatment, transplanted cells can proliferate and help regenerate disc tissues. We tested molecular changes in the disc using RNA and biochemical analyses. To determine the extent of regeneration, gene expression of collagen types I and II and the biochemical make-up of the discs were analyzed. Compared to intact discs, gene expression of collagen types I and II in rabbit IVDs treated with nHDFs, RDFs, and saline increased (n=4–5, Fig. 5a and b). The increases were significant in nHDF-treated discs (p<.01 or p<.05), trending or significant in RDF-treated discs (p<.01 or p<.07), and not significant in saline treated discs (Fig. 5a and b). Compared to the saline treated discs, there was no significant difference in collagen gene expression in nHDF or RDF treated discs. The ratio of collagen II and collagen I gene expression was calculated and normalized to those of the intact discs. The ratio of collagen type II/collagen type I was higher in the IVDs treated with nHDFs (1.26) than those treated with RDFs (0.81) or saline (0.59) and intact discs (1.00) (Fig. 5c).

Disc transplanted with nHDF showed increases in proteoglycan contents

Disc tissues were isolated and processed for biochemical assays to determine DNA and proteoglycan contents (n=24–27). There was an increase in proteoglycan contents normalized to the DNA contents in disc tissues that were treated with nHDF and RDF compared to those treated with saline (Fig. 6). The increases were significant in the nHDF-treated discs (p<.05) and trending in the RDF-treated discs (p<.06) (Fig. 6, Upper panel). These results suggest that transplantation with nHDF into the degenerating discs can increase production of matrix proteins to help restore disc structure.

Discussion

This study demonstrated that cell transplantation with nHDF into degenerated IVDs significantly increased disc



c.

Treatment	Cells/mL	Col 2/Col 1
Intact (n=4)	-	1.00
Saline (n=5)	-	0.59
nHDF (n=5)	5 x 10^7	1.26
RDF (n=4)	5 x 10^7	0.81

Fig. 5. Expression analysis of collagen types I and II genes using real-time PCR. Gene expression levels of injured rabbit IVDs treated with nHDFs, RDFs, or saline, and from intact control discs were measured. At 8 weeks after treatment, expression of collagen type I (a) and collagen type II (b) genes was analyzed. The ratio of collagen II/collagen I gene expression (c) was calculated and normalized to that of the intact discs. Error bars represent the standard error of the mean. Significant difference between intact and nHDF (*p<.05). Significant difference between intact and nHDF or RDF (**p<.01).

Proteoglycan contents

Treatments (5 x 10^7 cells/mL)	Average ratio Proteoglycan/DNA (μg/μg)	P-value Compared to Saline
Intact (n=24)	2,223 ± 158	0.003**
Saline (n=27)	1,625 ± 106	
nHDF (n=27)	1,949 ± 117	0.045*
RDF (n=27)	1,949 ± 125	0.053

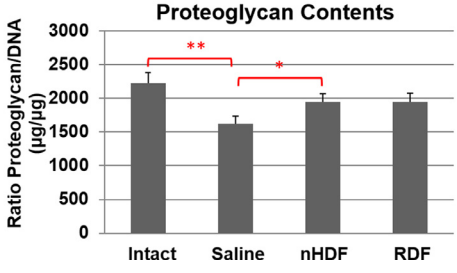


Fig. 6. Biochemical analysis of disc tissues. Eight weeks after treatment, disc tissues were isolated and processed for biochemical assays to determine DNA and proteoglycan contents. Upper panel: The table shows the average proteoglycan contents and p-values compared to saline-treated discs. Lower panel: Graph represents the average proteoglycan contents. There was a significant increase in proteoglycan contents in the discs treated with nHDFs compared to the saline treated discs (*p<.05) and between intact discs and saline treated discs (**p<.01). Error bars represent the standard error of the mean.

height, collagen types I and II gene expression, and proteoglycan contents. Transplantation with RDFs showed similar regenerative trends but these trends were not significant. One difference between these cells other than species origin is that RDFs in this study were from adult rabbit tissue, whereas the HDFs were from neonatal tissue. It has been well documented that fetal wound healing is without scar formation, more rapid and complete than adults suggesting younger donor tissue has some advantages in tissue regeneration [27–29]. In an in vitro study, Mateu et al. found that neonatal human fibroblasts differentiated into cells with adipogenic or chondrogenic markers with more ease than adult fibroblasts [30]. Using an Achilles tendon injury model in mice, Tang et al. found that tendons repaired with fetal fibroblast cell sheets had better biomechanical properties, histological morphologies and larger collagen fibrils than adult fibroblast cell sheets [31]. These studies support that fetal and neonatal fibroblasts have greater regenerative capacities than adult fibroblasts. The mechanisms that give cells from younger donors an advantage over older donors is still unclear. Some possibilities may be that cells from younger donors contain more stem-like cells that have not terminally differentiated and are therefore more plastic. Another advantage may be that younger donor cells have longer telomeres and therefore have a larger capacity to proliferate and divide. Lastly, neonatal cells may be less antigenic and therefore less inflammatory.

In this study, human and rabbit cells transplanted into the rabbit discs induced similar inflammatory responses. Rabbit discs from both cell treatment groups had similar cytokine and chemokine gene profiles. Also, macrophages were present in the osteophyte bump due to needle injury in all groups and not in the disc spaces where the cells or saline were injected. These results support that the IVDs may be immune privileged and tolerate xenogeneic grafts. Other animal studies have also found that xenogeneic cells transplanted into the discs did not cause an immune response. Iwashina *et al.* transplanted human immortalized NP cells into rabbit degenerated discs and did not detect CD4+ T cells or CD58+ antigen presenting macrophages in the transplanted area for up to 24 weeks [32]. When transplanting human mesenchymal stem cells (MSCs) into different animal disc degeneration models, human MSC survival was detected in the porcine injured disc for up to 6 months [11] and in the canine injured disc for up to 20 weeks [33]. Several mechanisms may contribute to the disc's immune privileged status. Avascularity of the disc does not allow the majority of immune cells access to this organ. Secondly, Fas ligand has been detected in the disc and can induce invading macrophages or T cells to undergo apoptosis
[34–38].

In the current study, cell therapy with nHDF in the rabbit degenerated disc showed significant regenerative effects such as increases in disc height, collagen II/collagen I gene expression, and proteoglycan contents. Other animal studies have also reported that intradiscal cell therapy can increase regeneration or reduce degeneration. Rabbit discs treated with rabbit MSCs or NP cells two weeks after injury showed increases in disc height, proteoglycan contents, and collagen II gene expression compared to the injured untreated controls [4,39]. Rabbit discs that were treated with a combination of cells (MSCs or human umbilical cord derived cells), different carriers, scaffolds or proteins resulted in higher MRI signals had lesser signs of disc degeneration than the injured untreated groups [7,40,41]. In the canine disc degeneration model, treatment with MSCs, Wharton Jelly cells or activated NP cells resulted in less disc degeneration (disc height and MRI changes) than injured untreated discs [15,33,42,43]. These studies suggest that cell therapy can prevent degeneration and promote regeneration of the disc. It is still unclear whether the majority of transplanted cells survive and engraft in the disc or whether they provide paracrine effects on resident cells to stimulate growth.

Clinical studies utilizing autologous or allogeneic MSCs to treat patients with back pain have reported encouraging results. Ceteno et al. collected data from 33 patients who received autologous MSC treatment for degenerative disc disease and found that these patients self-reported improvements in pain and function [44]. Noriega et al. conducted a Phase I-II trial with 24 patients with disc degenerative disease and found the cohort that received intradiscal allogeneic MSC treatment improved in pain and disability scores, whereas the scores of control treated cohorts did not [45]. Last, DePalma *et al.* directed a prospective, multicenter, randomized, double-blind, controlled Phase II study with 100 patients with disc degeneration associated chronic low back pain. At the 24-month follow-up, the cohort that received 6 million allogeneic MSCs showed significant improvements in pain and functional scores when compared to the cohort that received the saline placebo treatment [46]. Together, these studies support that autologous and allogeneic cell therapy for disc degeneration and back pain is feasible, well tolerated, does not cause an immune reaction and has some clinical efficacy on patients with back pain. Phase III studies are currently underway.

Cellular therapies that incorporate HDFs have been FDA approved for multiple conditions. Apligraf, Orcel, and Gintuit are products composed of bovine collagen scaffolds seeded with allogeneic neonatal HDFs and keratinocytes co-cultures and have been approved to treat venous ulcers, diabetic foot ulcers, burn victim wounds, or oral soft gum tissues. Dermagraft, a similar skin substitute product composed of only allogeneic neonatal fibroblasts, is also approved for treating diabetic foot ulcers. Last, an injectable product composed of autologous fibroblasts, LAVIV, gained FDA approval to treat nasolabial fold wrinkles in 2011. The approval of these products suggests that dermal fibroblast treatments are safe, tolerable, and can provide therapeutic and regenerative effects.

Dermal fibroblasts are composed of a heterogeneous mixture of cells. Based on the current definition of MSCs by the International Society for Cell Therapy, researchers have found that MSCs are not distinguishable from fibroblasts [47]. Human dermal fibroblasts express the same cell surface markers as MSCs [48,49]. Fibroblasts have demonstrated multipotency and can be induced to differentiate into neuronal [50], adipogenic [20,49,51], osteogenic [20,49,51], myogenic [51], and chondrogenic [20] cells. Also, similar to MSCs, dermal fibroblasts can also prevent T cell activation and proliferation and inhibit in vitro graft versus host reaction [48]. Our results suggest that the therapeutic effects of nHDF in the rabbit degenerated discs are similar to those reported in animal studies that tested MSC intradiscal treatment.

Conclusions

This study supports that the IVD is immune privileged and would tolerate allogeneic or xenogeneic grafts. Also, cells obtained from younger donors may have some advantages over those obtained from patient themselves. To better understand the impact of donor age on cell therapy on the degenerated discs, future studies that compare neonatal with adult rabbit fibroblast treatments in the rabbit disc degeneration model will be needed. Since dermal fibroblast therapies have been found to be safe and well-tolerated in clinical studies and can provide both therapeutic and

regenerative outcomes to diseased tissues, HDFs may be a viable cell source option to treat disc degeneration and back pain.

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