http://dx.doi.org/10.2106/JBJS.20.00606

The following content was supplied by the authors as supporting material and has not been copy-edited or verified by JBJS.

Appendix

Materials and Methods

Induction of IVD Degeneration

The rabbits received general anesthesia with an injection of ketamine (10 mg/kg) and xylazine (3 mg/kg) intravenously and O_2 and air (3.0 L/min) mixed with sevoflurane (2-3%) in spontaneous ventilation. To induce IVD degeneration, AF puncture was performed percutaneously using an 18-gauge needle through a precut dilator (METRx; Medtronic Sofamor Danek) under fluorography guidance with a small skin incision[16-18].

Collecting Bone Marrow and Preparation of Autogenic BMSCs

In the BMSCs-UPAL group, 10 mL of bone marrow was collected from the iliac crests at the same time as AF puncture. Anticoagulated bone marrow with 4000 U of heparin was mixed with 10 mL of culture medium containing Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Nichirei Bioscience), 1% penicillin/streptomycin, and 1.25 mg/mL fungizone (Life Technologies) after filtration through a cell strainer for excluding blood clot and debris. Mononuclear cells were isolated by centrifugation at 700 ×g for 5 min. The obtained cells were resuspended with medium, expanded in culture dishes, and cultured at 37°C with 20% O_2 and 5% CO_2 in a humidified atmosphere[14,24,30]. BMSCs grew as colonies[31], and the medium was changed twice a week. The BMSCs were expanded to passage 2.

Prior to implantation, the BMSCs were labeled with 20 μM of 5,6-caboxyfluorescein diactetate succinimidyl ester (CFDA-SE; CFDA-SE Cell Proliferation Assay Kit; BIO RAD) according to the manufacturer's instructions[17,32]. In addition, we prepared a 2% w/v UPAL (Sea Matrix; Mochida Pharmaceutical Co. Ltd.) solution as previously described[2,17]. The UPAL (1700 kDa of molecular weight) was packaged in sterilized vials after filtration thorough a 0.22-mm-pore size filter. The material was dissolved in phosphate-buffered saline (Wako Pure Chemical Industries) before use. Then, the fluorescently labeled BMSCs were encapsulated in the 2% UPAL solution[17].

Preparation of BMAC

The BMAC for the BMAC-UPAL group was prepared using the BioCUE Bone Marrow Aspiration Concentration System (ZIMMER BIOMET) in accordance with the manufacturer's instructions. A total of 30 mL of anticoagulated bone marrow aspirate (10 mL of heparin with 20 mL of bone marrow) was collected from the iliac crests by aspiration under general anesthesia, and transferred to BioCUE kit. Approximately 2 mL of BMAC was obtained after centrifugation at 3200 rpm for 15 minutes. The BMAC was then mixed with an equal volume of 4% UPAL solution to prepare the BMAC-UPAL mixture. We confirmed that the BMAC-UPAL mixture, as well as the UPAL solution alone and the BMSCs-UPAL mixture, gelated upon adding 102 mM $CaCl_2$ solution.

Unconfined Compression Tests

The disk-shaped gels (diameter, 4.5 mm; thickness, 2 mm) were compressed at a constant speed of 0.5 mm/min, and the Young's moduli were calculated from the linear regions between 10% and 20% compression strain of the stress/strain curves (n = 4 gels per group)[2].

http://dx.doi.org/10.2106/JBJS.20.00606

Implantation to Degenerated IVDs After Discectomy

Four weeks after IVD puncture, discectomy and implantation at L2/3 and L4/5 were performed under general anesthesia using an antero-lateral retroperitoneal approach[2,17]. In all groups, 10 mg wet weight (per IVD) of degenerated NP tissues were removed at L2/3 and L4/5 IVDs to create an IVD cavity using a pair of micro ear forceps (Nagashima Medical Instruments Co. Ltd.) after making a hole by puncture with an 18-gauge needle[17]. L3/4 IVDs were left intact as controls. In the UPAL group, the IVD defects were filled with 2% UPAL solution (20 μL per IVD) with a 27-gauge needle and a microsyringe (Hamilton Medical), and in the BMSCs-UPAL group, the IVD defects were filled with the same amount of BMSCs-UPAL suspension. In the BMAC-UPAL group, the IVD defects were filled with 20 μL UPAL solution mixed with BMAC. Then, the $CaCl_2$ solution was injected on the top of the implanted UPAL solution for gelation. Five minutes later, we washed the operative wound with normal saline and closed after confirming gelation. At 4 and 12 weeks after surgery, the treated rabbits were euthanized by pentobarbital overdose intravenously for qualitative evaluation of IVD degeneration.

Evaluation of Viability of the Implanted Cells

We evaluated whether the cells implanted in the treated IVDs were viable at 4 and 12 weeks after implantation by qualitatively assessing the intensity of the CFDA-SE fluorescent label[17,21,22]. In the Intact control, BMSCs-UPAL, and BMAC-UPAL groups, the excised IVDs were embedded with optimum cutting temperature (OCT) compound (Sakura Finetek) and frozen in liquid nitrogen after horizontal crosscutting in halves. The half-cut IVDs were sectioned into 5-μm slices and counterstained with 4',6-diamino-2-phenylindole (DAPI; P36935, Invitrogen).

MRI Analysis

The MR images were acquired using a 7.0-T MR scanner (Varian Unity Inova; Varian Medical Systems)[2,17,18]. The target IVDs were graded for IVD degeneration based on the Pfirrmann classification system[2,17,18]. To analyze quantitatively, the MRI index was also calculated using the Analyze software version 12.0 (AnalyzeDirect). The MRI index is the product of the NP area and the average signal intensity. The index was compared with that of the intact control IVD. In addition, we calculated the relative MRI index as the percentage relative to the value of the intact controls as previously described[2,16-18,23].

Histological Analysis

After the decalcification procedure, 5-μm-thick midsagittal sections were stained by hematoxylin and eosin (H&E) and safranin O-fast green to evaluate the expression of proteoglycan, as previously described[2,17,18]. Semiquantitative analysis was carried out using a histological grading system focusing on structural changes in the inner AF[24,25].

Immunohistochemical Analysis

We used type I collagen (Sigma-Aldrich; C2456) and type II collagen (Kyowa Pharma Chemical; F-57) mouse monoclonal antibodies as primary antibodies. Midsagittal sections were pretreated with proteinase K (Dako, Agilent Technologies; S3020) for 15 minutes before washing with PBS, then treated with 1% H_2O_2 in methanol for 30 minutes, and finally incubated with the primary antibody overnight at 4°C (for type I collagen) or for 60 minutes

http://dx.doi.org/10.2106/JBJS.20.00606

at room temperature (for type II collagen). Subsequently, the sections were incubated in peroxidase (EnVision + System Kit; Dako) for 30 minutes. As a counterstain, staining was developed using 3,3'-diaminobenzidine hydrochloride (Dako) and Mayer's hematoxylin (Merck).

Results

Evaluation of Water Content by MRI

Table. Pfirrmann grades and Relative MRI index

	Pfirrmann grades		Relative MRI index	
Group	4 weeks	12 weeks	4 weeks	12 weeks
Intact control	1.13 ± 0.35 (0.88 to 1.37)	1.13 ± 0.35 (0.88 to 1.37)	-	-
Discectomy	3.25 ± 0.71 (2.76 to 3.74)	4.25 ± 0.89 (3.63 to 4.86)	21.1 ± 2.5 (19.3 to 22.8)	7.7 ± 7.2 (2.8 to 12.7)
UPAL	2.50 ± 0.76 (1.98 to 3.02)	3.13 ± 0.53 (2.75 to 3.50)	31.6 ± 16.7 (20.0 to 43.1)	25.4 ± 9.2 (19.0 to 31.7)
BMSCs-UPAL	2.13 ± 0.35 (1.88 to 2.37)	2.00 ± 0.76 (1.48 to 2.52)	38.5 ± 14.5 (28.5 to 48.6)	42.8 ± 14.4 (32.8 to 52.7)
BMAC-UPAL	2.38 ± 0.74 (1.86 to 2.89)	2.25 ± 0.71 (1.76 to 2.74)	34.9 ± 11.3 (27.1 to 42.7)	34.8 ± 12.1 (26.4 to 43.1)

All data expressed as means ± SD, with the 95% confidence interval in parentheses.

http://dx.doi.org/10.2106/JBJS.20.00606

Histological Evaluation

Table. Histological grades

	Histological grades	
Group	4 weeks	12 weeks
Intact control	0.25 ± 0.46 (0 to 0.57)	0.25 ± 0.46 (0 to 0.57)
Discectomy	4.13 ± 0.64 (3.68 to 4.57)	4.75 ± 0.46 (4.43 to 5.07)
UPAL	3.38 ± 0.74 (2.86 to 3.89)	3.63 ± 0.52 (3.27 to 3.98)
BMSCs-UPAL	2.25 ± 0.71 (1.76 to 2.74)	2.50 ± 0.53 (2.13 to 2.87)
BMAC-UPAL	2.75 ± 0.64 (2.31 to 3.20)	2.75 ± 0.64 (2.31 to 3.20)

All data expressed as means ± SD, with the 95% confidence interval in parentheses.

http://dx.doi.org/10.2106/JBJS.20.00606

Extracellular Matrix Production in the Implanted IVDs

Table. Percentage of type II collagen-positive cells

	Percentage of type II collagen	
Group	4 weeks	12 weeks
Intact control	73.3 ± 2.5 (71.6 to 75.0)	70.0 ± 2.3 (68.4 to 71.6)
Discectomy	27.1 ± 6.8 (22.4 to 31.8)	21.8 ± 3.3 (19.5 to 24.0)
UPAL	46.9 ± 10.4 (39.7 to 54.1)	39.0 ± 4.3 (36.0 to 41.9)
BMSCs-UPAL	57.1 ± 4.8 (53.8 to 60.4)	59.8 ± 4.2 (56.9 to 62.7)
BMAC-UPAL	49.8 ± 4.2 (46.9 to 52.7)	50.0 ± 3.4 (47.6 to 52.3)

All data expressed as means ± SD, with the 95% confidence interval in parentheses.

http://dx.doi.org/10.2106/JBJS.20.00606

Table. Percentage of type I collagen-positive cells

	Percentage of type I collagen	
Group	4 weeks	12 weeks
Intact control	26.6 ± 4.0 (23.8 to 29.4)	27.3 ± 3.6 (24.8 to 29.8)
Discectomy	75.3 ± 3.1 (73.2 to 77.5)	78.4 ± 5.0 (75.0 to 81.9)
UPAL	60.4 ± 2.8 (58.5 to 62.3)	61.7 ± 2.8 (59.8 to 63.7)
BMSCs-UPAL	45.3 ± 3.9 (42.6 to 47.9)	46.1 ± 2.5 (44.4 to 47.8)
BMAC-UPAL	50.6 ± 4.7 (47.4 to 53.9)	51.0 ± 3.2 (48.8 to 53.3)

All data expressed as means ± SD, with the 95% confidence interval in parentheses.

http://dx.doi.org/10.2106/JBJS.20.00606

References

1. Oehme D, Ghosh P, Shimmon S, Wu J, McDonald C, Troupis JM, Goldschlager T, Rosenfeld JV, Jenkin G. Mesenchymal progenitor cells combined with pentosan polysulfate mediating disc regeneration at the time of microdiscectomy: a preliminary study in an ovine model. J Neurosurg Spine. 2014;20(6):657-69.
2. Tsujimoto T, Sudo H, Todoh M, Yamada K, Iwasaki K, Ohnishi T, Hirohama N, Nonoyama T, Ukeba D, Ura K, Ito YM, Iwasaki N. An acellular bioresorbable ultra-purified alginate gel promotes intervertebral disc repair: a preclinical proof-of-concept study. EBioMedicine. 2018;37:521-34.
3. Noriega DC, Ardura F, Hernández-Ramajo R, Martín-Ferrero MÁ, Sánchez-Lite I, Toribio B, Alberca M, García V, Moraleda JM, Sánchez A, García-Sancho J. Intervertebral disc repair by allogeneic mesenchymal bone marrow cells: a randomized controlled trial. Transplantation. 2017;101(8):1945-51.
4. Orozco L, Soler R, Morera C, Alberca M, Sánchez A, García-Sancho J. Intervertebral disc repair by autologous mesenchymal bone marrow cells: a pilot study. Transplantation. 2011;92(7):822-8.
5. Chu CR, Rodeo S, Bhutani N, Goodrich LR, Huard J, Irrgang J, LaPrade RF, Lattermann C, Lu Y, Mandelbaum B, Mao J, McIntyre L, Mishra A, Muschler GF, Piuzzi NS, Potter H, Spindler K, Tokish JM, Tuan R, Zaslav K, Maloney W. Optimizing Clinical Use of Biologics in Orthopaedic Surgery: Consensus Recommendations From the 2018 AAOS/NIH U-13 Conference. J Am Acad Orthop Surg. 2019;27(2):e50-e63.
6. Piuzzi NS, Dominici M, Long M, Pascual-Garrido C, Rodeo S, Huard J, Guicheux J, McFarland R, Goodrich LR, Maddens S, Robey PG, Bauer TW, Barrett J, Barry F, Karli D, Chu CR, Weiss DJ, Martin I, Jorgensen C, Muschler GF. Proceedings of the Signature Series Symposium "Cellular Therapies for Orthopaedics and Musculoskeletal Disease Proven and Unproven Therapies-Promise, Facts and Fantasy," International Society for Cellular Therapies, Montreal, Canada, May 2, 2018. Cytotherapy. 2018 Nov;20(11):1381-1400.
7. Centeno C, Pitts J, Al-Sayegh H, Freeman M. Efficacy of autologous bone marrow concentrate for knee osteoarthritis with and without adipose graft. Biomed Res Int. 2014;2014:370621.
8. Mautner K, Bowers R, Easley K, Fausel Z, Robinson R. Functional outcomes following microfragmented adipose tissue versus bone marrow aspirate concentrate injections for symptomatic knee osteoarthritis. Stem Cells Transl Med. 2019;8(11):1149-56.
9. Pascual-Garrido C, Rolón A, Makino A. Treatment of chronic patellar tendinopathy with autologous bone marrow stem cells: a 5-year-followup. Stem Cells Int. 2012;2012:953510.
10. Pettine KA, Suzuki RK, Sand TT, Murphy MB. Autologous bone marrow concentrate intradiscal injection for the treatment of degenerative disc disease with three-year follow-up. Int Orthop. 2017;41(10):2097-103.
11. Shapiro SA, Kazmerchak SE, Heckman MG, Zubair AC, O'Connor MI. A prospective, single-blind, placebo-controlled trial of bone marrow aspirate concentrate for knee osteoarthritis. Am J Sports Med. 2017;45(1):82-90.
12. Gobbi A, Whyte GP. Long-term clinical outcomes of one-stage cartilage repair in the knee with hyaluronic acid-based scaffold embedded with mesenchymal stem cells sourced from bone marrow aspirate concentrate. Am J Sports Med. 2019;47(7):1621-8.
13. Pettine KA, Murphy MB, Suzuki RK, Sand TT. Percutaneous injection of autologous

http://dx.doi.org/10.2106/JBJS.20.00606

bone marrow concentrate cells significantly reduces lumbar discogenic pain through 12 months. Stem Cells. 2015;33(1):146-56.
14. Li YY, Diao HJ, Chik TK, Chow CT, An XM, Leung V, Cheung KM, Chan BP. Delivering mesenchymal stem cells in collagen microsphere carriers to rabbit degenerative disc: reduced risk of osteophyte formation. Tissue Eng Part A. 2014;20(9-10):1379-91.
15. Lei T, Zhang Y, Zhou Q, Luo X, Tang K, Chen R, Yu C, Quan Z. A novel approach for the annulus needle puncture model of intervertebral disc degeneration in rabbits. Am J Transl Res. 2017;9(3):900-9.
16. Sudo H, Minami A. Caspase 3 as a therapeutic target for regulation of intervertebral disc degeneration in rabbits. Arthritis Rheum. 2011;63(6):1648-57.
17. Ukeba D, Sudo H, Tsujimoto T, Ura K, Yamada K, Iwasaki N. Bone marrow mesenchymal stem cells combined with ultra-purified alginate gel as a regenerative therapeutic strategy after discectomy for degenerated intervertebral discs. EBioMedicine. 2020;53:102698.
18. Ura K, Sudo H, Iwasaki K, Tsujimoto T, Ukeba D, Iwasaki N. Effects of intradiscal injection of local anesthetics on intervertebral disc degeneration in rabbit degenerated intervertebral disc. J Orthop Res. 2019;37(9):1963-71.
19. Gaul F, Bugbee WD, Hoenecke HR Jr, D'Lima DD. A review of commercially available point-of-care devices to concentrate bone marrow for the treatment of osteoarthritis and focal cartilage lesions. Cartilage. 2019;10(4):387-94.
20. Hegde V, Shonuga O, Ellis S, Fragomen A, Kennedy J, Kudryashov V, Lane JM. A prospective comparison of 3 approved systems for autologous bone marrow concentration demonstrated nonequivalency in progenitor cell number and concentration. J Orthop Trauma. 2014;28(10):591-8.
21. Polzer H, Volkmer E, Saller MM, Prall WC, Haasters F, Drosse I, Anz D, Mutschler W, Schieker M. Long-term detection of fluorescently labeled human mesenchymal stem cell in vitro and in vivo by semi-automated microscopy. Tissue Eng Part C Methods. 2012;18(2):156-65.
22. Wang H, Zhou Y, Huang B, Liu LT, Liu MH, Wang J, Li CQ, Zhang ZF, Chu TW, Xiong CJ. Utilization of stem cells in alginate for nucleus pulposus tissue engineering. Tissue Eng Part A. 2014;20(5-6):908-20.
23. Yamada K, Sudo H, Iwasaki K, Sasaki N, Higashi H, Kameda Y, Ito M, Takahata M, Abumi K, Minami A, Iwasaki N. Caspase 3 silencing inhibits biomechanical overload-induced intervertebral disk degeneration. Am J Pathol. 2014;184(3):753-64.
24. Sakai D, Mochida J, Iwashina T, Hiyama A, Omi H, Imai M, Nakai T, Ando K, Hotta T. Regenerative effects of transplanting mesenchymal stem cells embedded in atelocollagen to the degenerated intervertebral disc. Biomaterials. 2006;27(3):335-45.
25. Nishimura K, Mochida J. Percutaneous reinsertion of the nucleus pulposus: an experimental study. Spine (Phila Pa 1976). 1998;23(14):1531-8.
26. Iatridis JC, Weidenbaum M, Setton LA, Mow VC. Is the nucleus pulposus a solid or a fluid? Mechanical behaviors of the nucleus pulposus of the human intervertebral disc. Spine (Phila Pa 1976). 1996;21(10):1174-84.
27. Zeng Y, Chen C, Liu W, Fu Q, Han Z, Li Y, Feng S, Li X, Qi C, Wu J, Wang D, Corbett C, Chan BP, Ruan D, Du Y. Injectable microcryogels reinforced alginate encapsulation of mesenchymal stromal cells for leak-proof delivery and alleviation of canine disc degeneration. Biomaterials. 2015;59:53-65.

http://dx.doi.org/10.2106/JBJS.20.00606

28. Ha CW, Park YB. Editorial commentary: considering clinical application of bone marrow aspirate concentrate for restoration of cartilage defects in the knee? Is it a kind of stem cell therapy? Arthroscopy. 2019 Jun;35(6):1878-9.
29. Leonardo Cavinatto, Betina B Hinckel, Ryan E Tomlinson, Sunny Gupta, Jack Farr, Arthur R Bartolozzi. The role of bone marrow aspirate concentrate for the treatment of focal chondral lesions of the knee: A systematic review and critical analysis of animal and clinical studies. Arthroscopy. 2019;35(6):1860-77.
30. Cai F, Wu XT, Xie XH, Wang F, Hong X, Zhuang SY, Zhu L, Rui YF, Shi R. Evaluation of intervertebral disc regeneration with implantation of bone marrow mesenchymal stem cells (BMSCs) using quantitative T2 mapping: a study in rabbits. Int Orthop. 2015;39(1):149-59.
31. Ho G, Leung VY, Cheung KM, Chan D. Effect of severity of intervertebral disc injury on mesenchymal stem cell-based regeneration. Connect Tissue Res. 2008;49(1):15-21.
32. Sato M, Uchida K, Nakajima H, Miyazaki T, Guerrero AR, Watanabe S, Roberts S, Baba H. Direct transplantation of mesenchymal stem cells into the knee joints of Hartley strain guinea pigs with spontaneous osteoarthritis. Arthritis Res Ther. 2012;14(1):R31.