平成22年度厚生労働科学研究・再生医療実用化研究事業
「自家骨髄間葉系幹細胞により活性化された椎間板髄核細胞を用いた椎間板再生研究
における細胞、組織の安全性、品質確保に関する技術開発」
分担研究報告書

分担研究課題：椎間板髄核細胞と骨髄間葉系幹細胞の管理（品質管理）に関する研究

研究分担者　安藤　潔　東海大学医学部内科学系血液・腫瘍内科学・教授

研究要旨：
平成22度までに臨床応用された9例の活性化髄核細胞の安全性を評価するために、最終産物の感染症検査（各種ウィルス、マイコプラズマ、細菌）、細胞生存率、細胞数を評価した。

A. 研究目的

本研究は、自家骨髄間葉系細胞と椎間板髄核細胞を共培養して髄核細胞を活性化した後に患者椎間板に移入することにより、椎間板変性を防止することが目的である.

自家移植ではあるが、培養期間中の感染、細胞の変化が移植結果に影響する可能性があり、移植直前の細胞の安全性評価が重要な課題である。本研究課題では、そのための標準手順書を作成し、それに則り実際の臨床例に関して安全性を評価することを目的とする。

B. 研究方法

DNA ウィルス（HBV, ParvovirusB19NS1, ParvovirusB19VP2, HSV, VZV, CMV, EBV, HHV6, HHV7, HHV8）は最終産物より抽出したDNA、RNAウィルス(HCV, HIV-1, HTLV-1）は最終産物より抽出したRNA由来cDNAをtemplateとしてPCRによりウィルス検出を行った。細菌は培養液の好気性培養、嫌気性培養により検出した。またマイコプラズマについてもPCR法を併用した。マイコプラズマは更に培養法により確認を行った。

C. 研究結果とD. 考察

結果は次ページの表で示す。培養期間中にDNAウィルス（HBV, ParvovirusB19NS1, ParvovirusB19VP2, HSV, VZV, CMV, EBV, HHV6, HHV7, HHV8）, RNAウィルス(HCV, HIV-1, HTLV-1)、マイコプラズマ、細菌の感染は検出されなかった。また培養後の細胞増幅率は4.5-6.3倍であり、細胞生存率は90.0-99.0%であった。

E. 結論

以上の結果から、本研究における細胞プロセス工程は無菌的に安定した結果を得られることが確認された。

G. 研究発表

1. 論文発表

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B-cell non-Hodgkin lymphoma and mantle cell lymphoma. Cancer Science 101, 2054-2058, 2010
3) Tobinai K, Ogura M,[s1] Maruyama D, Uchida T, Uike N, Choi I, Ishizawa K, Itoh K, Ando K, Taniwaki M, Shimada N, Kobayashi K. Phase I and pharmacokinetic study of everolimus (RAD001) in patients with relapsed or refractory non-Hodgkin lymphoma. Int J Hematol 92, 563-570, 2010.
4) Muguruma Y, Matsushita H, Yahata T, Yumino S, Tanaka Y, Miyachi H, Ogawa Y, Kawada H, Ito M and Ando K. Establishment of xenograft model of human myelodysplastic syndromes. Hematologica, 2011, in press
5) Yahata T, Takanashi T, Muguruma Y, Matsuzawa H, Uno T, Sheng Y, Onizuka M, Ito M, Kato S, Ando K. Accumulation of oxidative DNA damage restricts the self-renewal capacity of human hematopoietic stem cells. Blood in press, 2011

H. 知的財産権の出願・登録状況
なし

ID	1	2	3	4	5	6	7	8	9
検査項目	2009.02.10	2009.02.17	2009.04.21	2009.06.23	2009.07.07	2009.08.18	2010.3.2	2010.6.21	2010.8.3
HBV	-	-	-	-	-	-	-	-	-
HIV-1	-	-	-	-	-	-	-	-	-
HTLV-1	-	-	-	-	-	-	-	-	-
Parvo.B19NS1	-	-	-	-	-	-	-	-	-
Parvo.B19VP2	-	-	-	-	-	-	-	-	-
HSV	-	-	-	-	-	-	-	-	-
VZV	-	-	-	-	-	-	-	-	-
CMV	-	-	-	-	-	-	-	-	-
EBV	-	-	-	-	-	-	-	-	-
HHV6	-	-	-	-	-	-	-	-	-
HHV7	-	-	-	-	-	-	-	-	-
HHV8	-	-	-	-	-	-	-	-	-
HCV	-	-	-	-	-	-	-	-	-
HIV-1(RNA)	-	-	-	-	-	-	-	-	-
HTLV-1(RNA)	-	-	-	-	-	-	-	-	-
結果受け取り日	2009/2/18	2009/2/18	009.04.27	2009/7/6	2009/7/8	2009/8/19	2010/3/8	2010/6/21	2010/8/7
エンドトキシン		0.1716pg/ml 以下							
	(0.004865EU/ml 未満)	(0.000858EU/ml 未満)	0.004491EU/ml 未満	0.003725EU/ml 未満	0.00319EU/ml 未満	0.01324EU/ml	0.004366EU/ml 未満	0.001875EU/ml 未満	0.003854EU/ml 未満
結果受け取り日	2009/2/10	2009.02.18	2009.04.21	2009.06.26	2009.07.08	2009.08.18	2010.3.2	2010.6.21	2010.8.9
マイコプラズマ（RT-PCR 法）	-	-	-	-	-	-	-	-	-
結果受け取り日	2009/2/10	2009.02.18	2009.04.21	2009.06.26	2009.07.08	2009.8.19	2010.3.3	2010.6.21	2010.8.7
マイコプラズマ（nested）	-	-	-	-	-	-	-	-	-
結果受け取り日	2009/3/12	2009.03.17	2009.05.21	2009.06.26	2009.07.08	2009.08.19	2010.3.3	2010.6.21	2010.8.11
マイコプラズマ（培養法）	-	-	-	-	-	-	-	-	-
結果受け取り日	2009/3/12	2009.03.17	2009.05.21	2009.07.21	2009.08.04	2009.08.21	2010.3.30	2010.6.21	2010.9.8
細菌培養（好気性）	-	-	-	-	-	-	-	-	-
細菌培養（嫌気性）	-	-	-	-	-	-	-	-	-
結果受け取り日	2009/2/17	2009.02.25	2009.04.30	2009.07.01	2009.07.14	2009/9/15	2010.3.3	2010.7.11	2010.8.11

平成22年度厚生労働科学研究・再生医療実用化研究事業
「自家骨髄間葉系幹細胞により活性化された椎間板髄核細胞を用いた椎間板再生研究における細胞、組織の安全性、品質確保に関する技術開発」
分担研究報告書

分担研究課題：活性化ヒト髄核細胞の腫瘍原性に関する研究

研究分担者　　中村　雅登　　東海大学医学部基盤診療学系再生医療科学・教授

研究要旨：
前年度に確立された活性化髄核細胞の腫瘍原性を否定するための安全性確認システム：超免疫不全NOGマウス皮下に再生治療に供された活性化髄核細胞の移植実験を実施した。6週間の観察期間中に腫瘍形成は確認できなかった。

A. 研究目的

活性化髄核細胞の腫瘍原性を否定するための超免疫不全NOGマウスを用いたin vivo安全性試験系により移植活性化髄核細胞の非腫瘍原性を確認する。

B. 研究方法

超免疫不全NOGマウスの皮下に移植再生医療に用いる活性化髄核細胞を移植し、6週間観察し腫瘍形成の有無を組織学的に確認する。本年度は実際に再生治療に供された9症例の内、十分細胞数の確保が可能であった7症例についてNOGマウス皮下への移植実験を行った。

C. 研究結果

ヒト脊索腫細胞株を $5x10^5$ 個/頭で皮下に移植した場合、6週間で腫瘍形成が確認できる。再生治療実施に供した活性化髄核細胞については更に感度をあげて 10^6 個/頭の細胞を皮下移植し観察を行った。結果：移植7症例についてはいずれも腫瘍形成を認め無かった。

D. 考察

低悪性度のヒト脊索腫細胞株でも腫瘍形成の確認できる感度の高いＮＯＧマウス皮下移植、6週間観察でも、ヒト髄核再生治療実施活性化髄核細胞では腫瘍形成が確認できなかった。現時点ではこの条件で腫瘍形成能を判定するのが、最も高感度の腫瘍形成能確認試験と考えられるが、それによってもヒト活性化髄核細胞の腫瘍原性にかかる安全性が確認できたと考えられる。

E. 結論

平成２２年度からの再生医療に用いられた活性化髄核細胞の移植実験、腫瘍原性否定―安全性確認システムによって実地再生医療用活性化髄核細胞の安全性が確認できた。

G. 研究発表

1.　論文発表
無し
2.　学会発表
無し

H. 知的財産権の出願・登録状況

1. 特許取得
無し
2. 実用新案登録
無し
3. その他
特に無し

厚生労働科学研究費補助金(再生医療実用化研究事業)
自家骨髄間葉系幹細胞により活性化された椎間板髄核細胞を用いた椎間板再生研究における細胞、組織の安全性、品質確保に関する技術開発
分担研究報告書

分担研究課題：活性化椎間板髄核細胞の変性椎間板への移植術に関する研究

研究分担者　酒井大輔　東海大学医学部外科学系整形外科学　講師
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渡辺拓也　東海大学医学部外科学系整形外科学　助教

研究要旨：
20 歳以上 30 歳未満の腰椎椎間板ヘルニア、分離症あるいは椎間板症で椎体間固定術を行う症例の内、その頭側あるいは尾側の隣接椎間板に中等度の変性を持つ症例に対して、2009 年度の 7 例に加え、2010 年度は 2 例で活性化髄核細胞の移植術を施行した。椎間板組織からの髄核組織の分離、腸骨からの骨髄液採取の手技、培養用末梢血採取、cell processing center への移送は安全かつ確実に行われた。活性化が終了した髄核細胞の移植用キット（注射針セット）への注入、手術場への搬送も支障なく実施された。骨髄間葉系幹細胞との共培養によって活性化された髄核細胞の中等度変性隣接椎間板内への経皮的移植は、全例で安全、確実に実施できた。骨髄液採取部や活性化髄核細胞移植部に新たな愁訴は認められなかった。

A. 研究目的
自家骨髄間葉系幹細胞との細胞間接着を伴う共培養で活性化された自家椎間板髄核細胞移植術の全過程を検証し、安全、確実な移植術が実施可能かについて検討する。

B. 研究方法
椎体間固定時に採取された椎間板髄核組織と腸骨より採取された骨髄液の状態を術中に評価し cell processing center への移送の可否を検討する。最終製品として得られた活性化椎間板髄核細胞を、細胞判定委員会の議に従って受け取り、移植術が基準通りに実施されることを検証する。

C.研究結果ならびに D.考察
Cell processing center に移送する椎間板髄核組織ならびに骨髄液は、研究実施計画通りの方法で採取され、その過程に一切の問題点はなかった。髄核組織、骨髄液の質、量ともに研究計画の基準を満たしていた。最終製品として得られた活性化髄核細胞の変性椎間板移植キットへの注入も、当該手術室内で安全に実施された。局所麻酔下で実施された活性化椎間板髄核細胞の移植術は、平均 30 分間で実施され、当該移植部の疼痛、下肢痛発生などの合併症は一切認められなかった。2009 年度実施の 7 例と 2010 年度実施の 2 例を合わせて、細胞、組織採取から活性化椎間板髄核細胞移植術実施の手術過程は、基準通りに実施可能な定型手術であると評価できた。

E.結論
活性化椎間板髄核細胞移植術に関わる手術室におけるすべての工程は、安全、確実に実施できることが確認された。

G. 研究発表　なし

H. 知的財産権の出願・登録状況　なし

厚生労働科学研究費補助金（再生医療実用化研究事業）
自家骨髄間葉系幹細胞により活性化された椎間板髄核細胞を用いた椎間板再生研究における細胞、組織の安全性、品質確保に関する技術開発
分担研究報告書

分担研究課題：適応患者選択ならびに細胞の均一化に関わる外部評価に関する研究

研究分担者　　波呂　浩孝　山梨大学大学院医学工学総合研究部整形外科学・教授

研究要旨：
2009 年以降、東海大学医学部附属病院における活性化椎間板髄核細胞移植症例の外部評価を行った。臨床研究参加者（患者）の資料、すなわち年齢、性別、既往歴、単純X線画像と MRI の画像所見は、症例ごとに東海大学における適応決定前の段階で送付された。2009 年度の 7 例に加え、2010 年度に 2 症例について評価を行い、全て本臨床研究の適応基準に合致していることを評価決定し、書面にて報告を行った。なお、移植された活性化椎間板髄核細胞の細胞処理の均一化については、東海大学において予定されている全 10 例の臨床研究が終了した時点で、全例のデーターを比較検討し、外部評価者としての見解を報告する。

A. 研究目的
骨髄間葉系幹細胞による細胞間接着を伴う共培養で椎間板髄核細胞の活性化を行う本プロジェクトに関して、研究計画の順守、安全性と有効性に関して科学的に検証する。

B. 研究方法
東海大学医学部整形外科学では椎間板の再生に関する基礎的研究を行い、その結果を踏まえて、活性化椎間板髄核細胞を 7 日後に椎体間固定術を施行した部分の隣接椎間で、中等度の変性を持ち、年齢や臨床症状、および画像上の基準に合致する症例に移植するプロジェクトを継続している。

外部評価者としての研究方法は、ヒト幹細胞臨床研究に関する審査委員会で承認された適応基準に患者が合致していることを事前に検討し、また細胞処理が的確に実施されているかについての評価を実施することである。

C. 研究結果
2009 年以降、東海大学医学部附属病院における活性化髄核細胞移植症例の外部評価を行った。2010 年度は計 2 症例の評価を実施し、術前に患者さんの年齢と性別、症状、神経所見、既往歴、レントゲンと MRI の画像所見について適応を検討した。症例基準は、20 才代腰椎椎間板ヘルニア症例で後方椎体間固定術が適応であり、これに隣接した椎間が椎間板変性を有するが不安定性がみられず活性化髄核細胞移植部位として適当であるかについて、検討を行った。2010 年度に提示された 2 症例は 2009 年度の 7 例と同様に全て臨床研究の適応基準に合致していることを術前に評価を行い、書面にて報告を行った。

なお、活性化椎間板髄核の細胞処理の均一化については、全 10 例の臨床研究が終了した時点で、データーを比較検討し、外部評価者としての評価を行う。

E. 結論
2009 年度以来 2010 年度までに東海大学において選択された 9 症例はすべて適応基準に合致しており、外部評価者として適応可との判定を行った。

G. 研究発表　なし

H. 知的財産権の出願・登録状況　なし

III.　研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Serigano K, Sakai D, Hiyama A, Tamura F, Tanaka M, Mochida J	Effect of cell number on mesenchymal stem cell transplantation in a canine disc degeneration model	J Orthop Res	28	1267-1275	2010
Hiyama A, Sakai D, Risbud MV, Tanaka M, Arai F, Abe K, Mochida J	Enhancement of intervertebral disc cell senescence by WNT/β-catenin signaling-induced matrix metalloproteinase expression	Arthritis and Rheumatism	62	3036-3047	2010
Murai K, Sakai D, Nakamura Y, Nakai T, Igarashi T, Seo N, Murakami T, Kobayashi E, Mochida J	Primary immune system responders to nucleus pulposus cells: evidence for immune response in disc herniation.	Eur Cell Mater	19	13-21	2010
Hiyama A, Sakai D, Tanaka M, Arai F, Nakajima D, Abe K, Mochida J	The relationship between the Wnt/β-catenin and TGF-β/BMP signals in the intervertebral disc cell	J Cell Physiol	226	1139-1148	2011
持田讓治	自家骨髄間葉系幹細胞により活性化された椎間板髄核細胞を用いた椎間板再生研究	日本再生医療学会雑誌	9	428-432	2010
持田讓治	椎間板再生の展望	からだの科学	266	126-129	2010

IV.　研究成果の刊行物・別刷

Effect of Cell Number on Mesenchymal Stem Cell Transplantation in a Canine Disc Degeneration Model

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ABSTRACT: Transplantation of mesenchymal stem cells (MSCs) inhibits the progression of disc degeneration in animal models. We know of no study to determine the optimal number of cells to transplant into the degenerated intervertebral disc (IVD). To determine the optimal donor cell number for maximum benefit, we conducted an in vivo study using a canine disc degeneration model. Autologous MSCs were transplanted into degenerative discs at 10^5, 10^6, or 10^7 cells per disc. The MSC-transplanted discs were evaluated for 12 weeks using plain radiography, magnetic resonance imaging, and gross and microscopic evaluation. Preservation of the disc height, annular structure was seen in MSC-transplantation groups compared to the operated control group with no MSC transplantation. Result of the number of remaining transplanted MSCs, the survival rate of NP cells, and apoptosis of NP cells in transplanted discs showed both structural microenvironment and abundant extracellular matrix maintained in 10^6 MSCs transplanted disc, while less viable cells were detected in 10^5 MSCs transplanted and more apoptotic cells in 10^7 MSCs transplanted discs. The results of this study demonstrate that the number of cells transplanted affects the regenerative capability of MSC transplants in experimentally induced degenerating canine discs. It is suggested that maintenance of extracellular matrix by its production from transplanted cells and/or resident cells is important for checking the progression of structural disruption that leads to disc degeneration. © 2010 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 28: 1267–1275, 2010

Keywords: intervertebral disc; mesenchymal stem cells; regenerative medicine; disc degeneration; nucleus pulposus

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Degenerative spinal disease not only causes lumbar pain in individuals, but also has a major socioeconomic impact from stress on the medical system and loss of productivity from occupational disease.[1,2] While intervertebral disc (IVD) degeneration has been implicated as one of the major causes of degenerative spinal disease, other factors thought to be involved including age, trauma, genetic predisposition, and lifestyle factors, such as obesity, smoking, occupation, and stress.[3–5]

Because IVD degeneration is an irreversible progressive disease, various treatments are currently being developed to repair and regenerate damaged IVDs. Experimental regenerative medicine techniques for IVD degeneration include the intradiscal injection of cytokines and growth factors,[6–10] gene delivery to IVD cells,[11–13] creation of artificial IVDs using tissue engineering,[14] and cell transplantation.[15–18] In vitro and in vivo studies using these techniques have reported increased IVD cell activity, the stimulation of extracellular matrix synthesis, and the suppression of IVD degeneration.

Recent advances in molecular biology and regenerative medicine have drawn an attention to mesenchymal stem cells (MSCs), because these immature cells remain pluripotent, capable of differentiating into bone, cartilage, or fat cells depending on their treatment. Undifferentiated MSCs are also immune tolerant. Autologous MSC transplantation has become an important treatment for various diseases.[19,20] Facilitating clinical applications, MSCs are easily harvested, and extracorporeal isolation and culture are relatively simple, requiring no highly specialized procedures. A number of basic studies have been performed using MSC transplantation into IVDs. Sakai et al.[15] performed autologous MSC transplantation using a rabbit IVD degeneration model, showing by radiology, histology, and biochemistry that this procedure suppressed IVD degeneration. They also analyzed the differentiation of transplanted MSCs into nucleus pulposus cells, establishing that transplanted MSCs expressed markers for the resident nucleus pulposus cells, indicating that the differentiation of MSCs is dependent on their environment.[16,17] Hiyama et al.[18] examined the immune privilege of IVD tissue, particularly the nucleus pulposus, including the anatomical characteristics separating disc tissue from the host immune system. Using a canine IVD degeneration model, these authors transplanted MSCs and measured changes in the expression of the Fas/Fas-ligand (FasL) system in addition to ascertaining any suppressive effects of the transplant on disc degeneration. In their study, the transplantation of MSCs suppressed IVD degeneration as assessed by radiological, histological, and biochemical findings. Their results also suggest that MSC transplantation could be effective in maintaining the immune privilege of IVDs.

Based on these studies and others, MSC transplantation into degenerated IVDs offers a promising new interventional technique in animal models.[21,22] Although the efficacy of MSC transplantation has been documented, some questions remain unanswered. For

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example, the optimal cell number for achieving IVD regeneration from MSC transplantation have not been elucidated. Using a canine model of disc degeneration, the present study examined the dosage of autologous MSCs to transplant, investigated the inhibitory effects of the MSC transplant on disc degeneration, and determined the viability of transplanted MSCs.

MATERIALS AND METHODS

Animal experiments were carried out with IACUC approval. A total of 30 beagle dogs, 12- to 18-month old, weighing approximately 10 kg, (Nosan Beagle; Nosan Corporation, Kanagawa, Japan) were divided equally into five groups of six. The N group was the unoperated control group. Three groups with induced disc degeneration were given subsequent MSC transplants of 10^5, 10^6, or 10^7 cells per disc. The D group of operated controls had induction of disc degeneration without MSC transplants. Lateral radiographs and magnetic resonance imaging (MRI) were obtained for every animal before experimental use to confirm the absence of abnormalities. At 12 weeks after the first operation, final radiological and MRI assessments were obtained and all dogs were euthanized by a lethal dose of 120 mg/kg sodium pentobarbital (Abbott Laboratories, Abbott Park, IL). The L3/4, L4/5, and L5/6 discs were isolated with the vertebral bodies attached.

Disc Degeneration Model

Four weeks before the initial MSC transplantation, disc degeneration was induced in L3/4, L4/5, and L5/6 IVDs by NP aspiration according to the method of Hiyama et al.[18] Briefly, an 18-gauge needle was inserted at the center of the disc through the AF into the NP. To induce disc degeneration, the NP was aspirated using a 10-ml syringe, as previously described. The mean mass of the nucleus pulposus aspirated from each disc was 13.4 ± 4.7 mg.

Bone Marrow Collection, Analyses, and Transplantation of MSCs

Autologous MSCs were obtained from the iliac crest from each animal receiving autologous transplants according to the method of Hiyama et al.[18] To measure survival rate of MSCs transplanted into the NP cavity, the MSCs were infected with AcGFP1 (Takara, Japan), a retrovirus vector expressing the green fluorescent protein (GFP) gene (conditioning studies were performed, data not shown). Vector incorporation was more than 90% (data not shown) using FACS analysis. Following the second passage in culture, adherent autologous MSCs were enzymatically released from monolayer culture and the designated cell number was transplanted percutaneously into IVDs in which degeneration had been induced (L3/4, L4/5, and L5/6) 4 weeks before. Transplants were done in the three lumbar discs in a randomized sequence using a discogram needle (25-gauge) guided by fluoroscopic imaging.

EVALUATIONS

Radiological Assessment

Lateral radiographs were taken under general inhalation anesthesia in all groups at 0, 4, 8, and 12 weeks after induction of degeneration. A fluoroscopic imaging intensifier (70 kV, 10 mA, distance 100 cm) was used. Vertebral body heights and disc heights were measured using image J software (Free soft, Image Processing and Analysis, rsb.info.nih.gov/ijl). The data were then transferred to the Excel software program (Microsoft Excel, 2003) and the disc height index (DHI) was calculated, using the method of Masuda et al.[25] Changes in the DHI were expressed as %DHI and normalized to the measured preoperative IVD height by the following equation [DHI = (post-operative DHI/preoperative DHI) × 100].

MRI Assessment

MRI images were also taken to evaluate signal changes in T2-weighted images at 0, 4, 8, and 12 weeks after the first operation in all groups. All MRIs were obtained using a spine coil (1.5T, Gyroscan, ACS-NT, Powertrak6000, Philips, Amsterdam, the Netherlands) under anesthesia. T2-weighted sections in the sagittal plane were obtained using a fast spin echo sequence with time-to-repetition (TR) of 4,000 ms and time-to-echo (TE) of 150 ms; interslice gap of 0.3 mm; matrix at 512 × 512; the field-of-view (FOV) was 200 mm × 200 mm; the number of excitations was 4 and TSE echo spacing was at 18.8. At 12 weeks after the induction of degeneration, the signal intensity of the T2-weighted image of each disc was evaluated using the Pfirmann classification.[26,27]

Gross Anatomical Findings

The spinal segments from five dogs from each group ($n = 25$ IVDs) were fixed in 10% formalin neutral buffer solution (Wako, Osaka, Japan) and decalcified in Plank–Rychlo solution (Decalcifying Solution A; Wako, Tokyo, Japan). Specimens were cut longitudinally through the center of the disc for macroscopic evaluation.

Histological Examination

Discs L3/4, L4/ 5, and L5/6 were excised from the lumbar spine of 10 dogs from each group ($n = 50$ IVDs). Each IVD was fixed in 10% formalin, decalcified, and embedded in paraffin. The paraffin blocks were stained with hematoxylin and eosin or Safranin-O, and evaluated by histology and immunostaining. The hematoxylin and eosin stain evaluated degenerative changes in annulus fibrosus using the disc degeneration grading system of Nishimura and Mochida.[28] Two observers familiar with human and animal IVD specimens and blinded to this study evaluated the sections. The intraobserver error was very small. The kappa value for grading scale was 0.90, showing an excellent agreement. For immunohistochemistry, primary mouse monoclonal antibody (collagen type II, Daiichi Fine Chemical Co., Toyama, Japan) was used with standard protocol.

Apoptotic Cell Counts

The DeadEnd™ Colorimetric TUNEL System (Promega, Madison, WI) was used according to the manufacturer's instructions to detect apoptotic cells in the NP. End labeling was performed on formalin-fixed sections from lumbar spine of five dogs from MSC-transplantation groups. Positive control sections were pretreated with DNase I (Promega). The terminal deoxynucleotidyl transferase (TdT) was replaced by

PBS for the negative controls. Two pathologists counted the cells in the nucleus pulposus area in 10 random high power fields (HPFs; magnification ×400). The percent of TUNEL positive cells of the total nucleus pulposus cells was calculated.

Viability of Nucleus Pulposus Cells after Transplantation

The viability of cells recovered from nucleus pulposus samples of five dogs from each group ($n = 25$ IVDs) were analyzed at 12 weeks after induction of IVD degeneration. Specimens were weighed, then digested in Dulbecco's modified Eagle medium (DMEM; Gibco, Invitrogen Corporation, Carlsbad, CA) containing 0.4% (w/v) actinase E (Kaken Pharmaceutical Inc., Tokyo, Japan) for 1 h, followed by 2 h in DMEM containing 0.016% (w/v) bacterial collagenase P (Roche Diagnostics GmbH, Mannheim, Germany). The digested tissue was passed through a 70 μm pore size cell filter (Becton Dickinson Labware Co. Ltd, Franklin Lakes, NJ). Cells were washed, filtered (Falcon cell strainer, 100 μm), seeded in 96-well microtiter plates (100 μl, 20,000 cells/well, four-wells/disc), and incubated for 20 min to allow settling. Then, 50 μl of ethidium homodimer-1 (EthD-1, 2 μmol/L)/calcein AM (1.6 μmol/L) solution (Live/Dead® cell viability kit; Invitrogen, Basel, Switzerland) were added to each well and incubated for 20 min. Methanol-treated cells (100 μl/150 μl cell suspension) were used as negative controls. Cell fluorescence was observed using an inverse microscope (Leica, DM IL; filters: I3 S 450–490 nm and N2.1 S 515–560 nm). High-resolution digital photos of three visual fields/well were taken using both filters at 25×. Image analysis software (Quantity One®; Bio-Rad, Hercules, CA) was used to quantify cell numbers.

Evaluation of the Survival of Transplanted MSCs

Formalin-fixed sections from lumbar spine of five dogs from the GFP-positive MSC-transplantation group ($n = 15$ IVDs) were analyzed at 12 weeks after induction of IVD degeneration. At 12 weeks post-degeneration induction were embedded in paraffin blocks and sectioned at about 2 μm. Standard procedures were applied for fluorescent immunohistochemistry with primary antibody (Anti-Green Fluorescent Protein; 1:50; Medical&Biological Laboratories, Nagoya, Japan). Alexa 488 (1:100; Molecular Probes, Eugene, OR) was used as a second antibody. Tissues were mounted on slides using VECTASHIELD Mounting medium with 4′,6′-diamino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA). Two pathologists counted the cells in the nucleus pulposus area in 10 random HPFs (magnification ×400). The percent of GFP-positive MSCs of the total nucleus pulposus cells was calculated.

Statistical Analysis

The significance of differences among means of data on radiograph measurements, TUNEL staining for apoptotic cell counts, Live/Dead® for viability of nucleus pulposus cells after transplantation, and the survival rate of the GFP-positive transplanted MSCs was performed using a repeated measure ANOVA and Fisher's PLSD post hoc test. Statistical significance was accepted at $p < 0.05$. The Kruskal–Wallis test and Mann–Whitney U-test were used to analyze the non-parametric data from MRI and histology grading. The Statview program was used for the statistical analyses. Error bars were set to represent 1 SD unit.

RESULTS

Radiographic and MRI Findings

Radiographs showed significant ($p < 0.01$) narrowing of the disc space at 4 weeks after induction of IVD degeneration in the operated control D group and the MSCs transplant groups. The mean %DHI in the D group continued to decrease until 8 weeks post-induction, then plateaued. The mean %DHI of the N group was nearly 100% throughout the study. In contrast, at 4, 8, and 12 weeks after MSC transplantation the mean %DHI of the MSC-transplanted disc groups was higher than the pre-transplant index. At 4, 8, and 12 weeks after the first operation, the mean %DHI increased significantly ($p < 0.01$) in the transplant groups compared to the D group. No significant differences were seen among the three MSC-transplant groups (Fig. 1). At 12 weeks post-induction, the results of MRI (T2-weighted) signal intensity measurement of discs from the D group was less than all other groups. The MSC-transplant groups showed a significant increase in the disc signal intensity. The use of Pfirrmann's classification revealed a significant delay in the progression of disc degeneration in the transplant groups, suggesting an increase in water content in MSC-transplanted discs.[27] The signal intensity level of the N group was constant throughout the study period. Again, no significant differences were detected among the three MSC-transplant groups (Fig. 2).

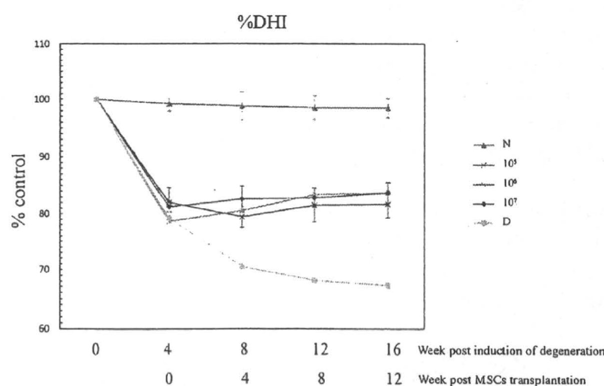

Figure 1. Radiological assessment of mesenchymal stem cell (MSC) transplantation using a canine disc degeneration model. Changes in average %DHI and reductions in %DHI 4 weeks after transplantation of 10^5, 10^6, or 10^7 MSCs. All transplant groups showed significantly ($p < 0.01$) smaller changes than the operated control (D) group (N: 4 w, 98.8 ± 2.5%; 8 w, 98.5 ± 2.1%; 12 w, 98.5 ± 1.7%. 10^7: 4 w, 82.6 ± 2.3%; 8 w, 82.8 ± 1.7%, 12 w, 83.7 ± 1.8%. 10^6: 4 w, 80.5 ± 0.7%; 8 w, 83.4 ± 2.0%, 12 w, 83.7 ± 1.9%. 10^5: 4 w, 79.4 ± 1.9%; 8 w, 81.5 ± 3.0%, 12 w, 81.7 ± 2.4%. D: 4 w, 70.5 ± 1.9%; 8 w, 68.1 ± 2.1%, 12 w, 67.2 ± 3.0%.). No significant differences were seen among the MSC-transplanted groups.

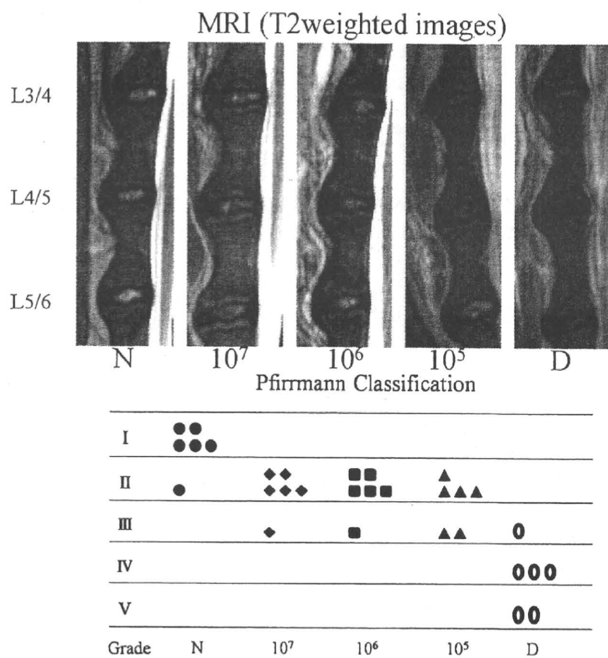

Figure 2. MRI analysis signal changes in T2-weighted images at 12 weeks after induction of degeneration, using the Pfirrmann classification grades 1–5 based on changes in the degree and area of signal intensity. Changes in MRI signal intensity over time showing the signal intensity of the nucleus pulposus was lower ($p < 0.01$) at 12 weeks after transplantation for the operated control D group, but was relatively well maintained for the MSCs transplant groups. No significant differences existed among the three MSCs transplantation groups (N: 1.16 ± 0.4; 10^7: 2.16 ± 0.4; 10^6: 2.16 ± 0.4; 10^5: 2.33 ± 0.5; D: 4.16 ± 0.7).

Macroscopic Findings

The gross appearance of the dissected spines at 12 weeks post-induction of degeneration showed more apparent disc space narrowing and connective tissue invasion of the nucleus cavity in the D group than the other groups. The 10^6 and 10^7 MSC-transplant groups appeared similar to the N group, but the discs from the 10^5 cell transplant group appeared more similar to the connective tissue invasion seen in the D group. Overall, the disc structure was maintained better in the 10^6 and 10^7 cell transplant discs (Fig. 3).

Histology

The histological analysis also showed noteworthy regenerative effects of the MSC transplants. Hematoxylin and eosin staining of N group discs showed a relatively well preserved oval-shaped nucleus with no collapse of inner and outer annular structures. The 10^6 and 10^7 cell groups also showed a relatively well preserved inner annulus structure similar to the N group, but discs from the 10^5 cell group had less preservation of the annular structure (Fig. 4a,c). Safranin-O staining of NPs from the 10^6 and 10^7 groups showed relatively dark staining of nucleus tissue, similar to the N group, while discs from the 10^5 and D groups showed lighter staining of the nucleus and inner annulus (Fig. 4b). The D group stained poorly for collagen type II compared with the other groups (Fig. 5).

Detection of Apoptosis in MSC-Transplanted Discs

The results of TUNEL staining revealed that N group discs had low numbers of apoptotic cells, while D group discs showed significantly higher numbers ($p < 0.01$) of dying and dead cells compared to the other groups. Among the three MSC-transplant groups, the ratio of apoptotic cells was significantly lower for the 10^6 group than the 10^5 and 10^7 groups. No significant difference existed between 10^5 and 10^7 groups ($p = 0.25$; Fig. 6a). There was not a specific region for TUNEL positive cells. It stained scattered in the NP cavity (Fig. 6b).

Assessment of Viability of Nucleus Pulposus Cells after MSC Transplantation

The dual-fluorescence Live/Dead® cell viability assay was used to label viable nucleus pulposus cells 16 weeks after induction of degeneration in the D and MSC-transplant groups. Live cells were stained green with Calcein-AM and dead cells were stained red with Ethidium homodimer-1. The N group had more live cells, while the D group showed significantly ($p < 0.01$) less than the other groups. Among the three MSC-transplant groups, the number of live cells in the 10^6 and 10^7 groups was significantly ($p < 0.01$) greater than the 10^5 group. The 10^7 group had smaller number of live cells than the 10^6 group, although the difference was not statistically significant ($p = 0.20$; Fig. 7).

Survival of Transplanted MSCs in the Nucleus Pulposus

GFP-positive MSCs were detected using an FITC filter. GFP-positive MSCs were seen primarily in the central region of the nucleus pulposus. Only cells that stained with DAPI to show a clearly visible nucleus were counted. These cells were consecutively counted

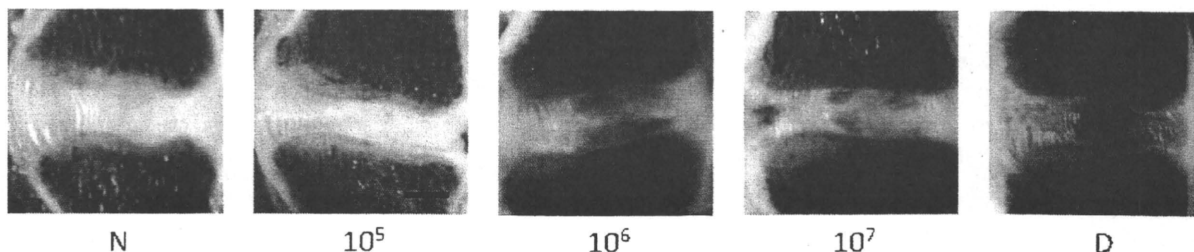

Figure 3. Gross anatomical findings greater intervertebral disc degeneration was seen at 12 weeks after transplantation for the operated control D group when compared to the other groups. Among the transplant groups, the morphology of the MSC 10^6 and 10^7 groups resemble that of the N group, while fewer and less severe morphological changes were seen in 10^5 group. Bar = 5 mm.

a

40X

b

N 10^7 10^6 10^5 D

c

Degenerative changes in the annulus fibrosus

Grade	N	10^7	10^6	10^5	D
0	●●● ●●●				
1		◆◆◆	■■		
2		◆◆◆	■ ■■■	▲	
3				▲▲	
4				▲▲	000
5				▲	000

- Grade 0: normal structure
- Grade 1: mildly serpentine with rupture
- Grade 2: moderately serpentine with rupture
- Grade 3: severely serpentine with mildly reversed contour
- Grade 4: severely reversed contour
- Grade 5: indistinct

Nishimura and Mochida 1998

Figure 4. Histology. (a) The structures of degenerative discs receiving mesenchymal stem cell (MSC) transplantation at MSC 10^6 and MSC 10^7 are near normal, but partial disruption of the annulus fibrosus is seen in discs from the 10^5 group. (Hematoxylin and eosin staining, original magnification ×40). (b) Safranin-O staining is stronger in the transplantation group discs compared to the D group. (Original magnification ×40). (c) Histological analysis using the disc degeneration grading system of Nishimura and Mochida, which measures morphological changes in the annular structure, 12 weeks after induction of degeneration, annular structures were significantly ($p < 0.01$) better maintained in the transplant groups than group D (Mann–Whitney test). Bar = 500 μm.

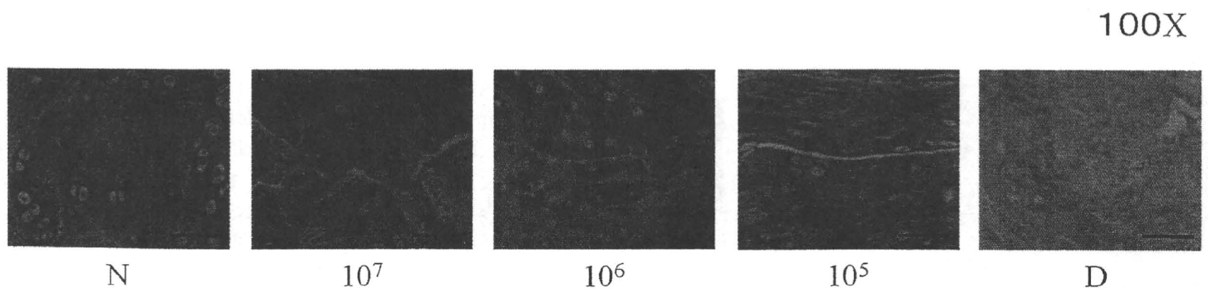

Figure 5. Immunohistochemical staining. Staining of the nucleus pulposus for collagen type II for the operated control D group was weaker than for the other groups. (Original magnification ×100). Bar = 100 μm.

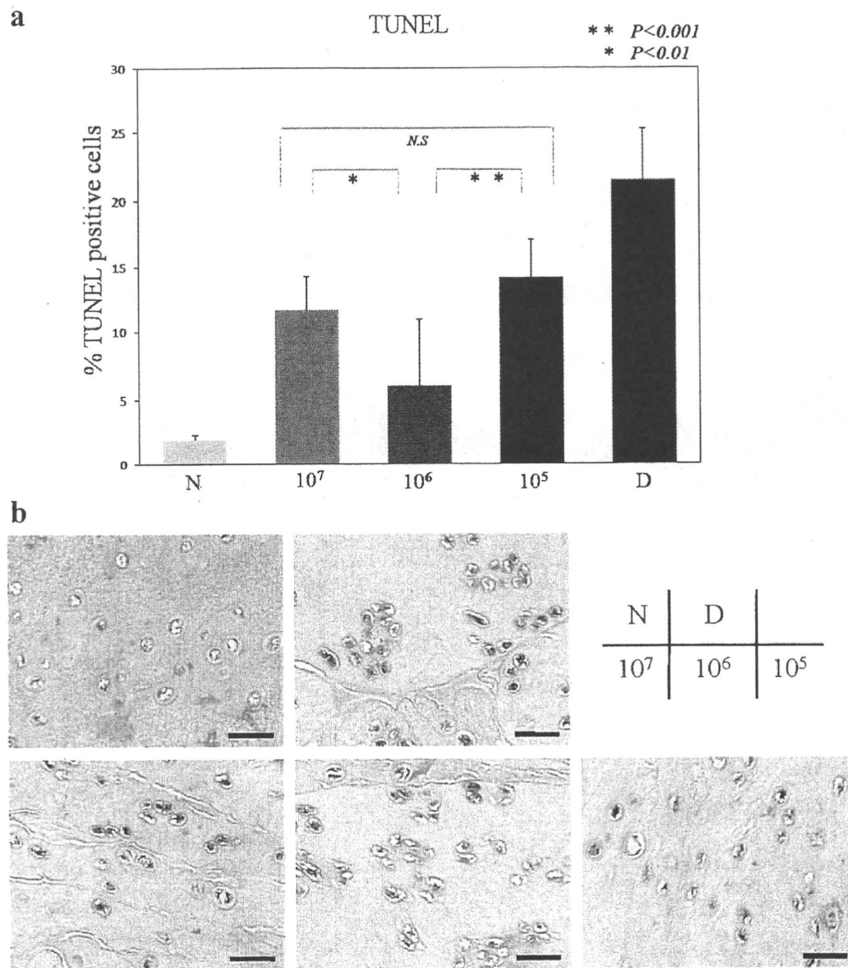

Figure 6. TUNEL staining. (a)The TUNEL staining revealed the N group discs did not show relevant number of apoptotic cells, while the D group discs showed significant increase compared to the other groups. Among the three MSCs transplanted groups, compared to the 10^5 and 10^7 groups, the ratio of apoptotic cells was significantly lower for the 10^6 group. No significant difference existed between 10^5 and 10^7 groups (N: $1.9 \pm 0.4\%$; 10^7: $11.7 \pm 2.6\%$; 10^6: $6.0 \pm 4.9\%$; 10^5: $14.2 \pm 2.9\%$; D: $21.5 \pm 3.8\%$; $p = 0.25$). (b) TUNEL staining of the NP cavity formed at 12 weeks after first operation. Bar = 100 μm.

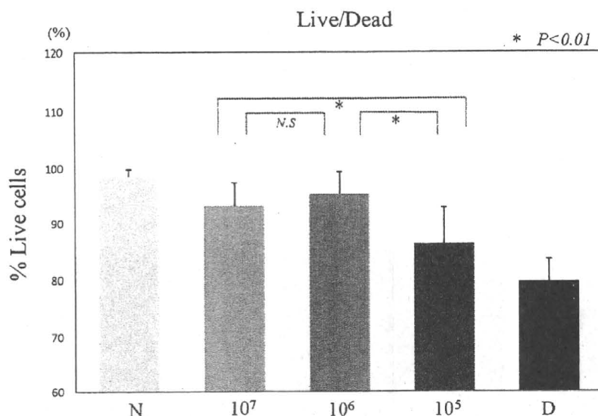

Figure 7. LIVE/DEAD cell viability assay. The LIVE/DEAD assay of vertebral tissues 12 weeks after transplantation shows that the number of living NP cells in the 10^6 and 10^7 groups was significantly (*$p < 0.01$) higher than that of the 10^5 group (N: $98.1 \pm 1.1\%$; 10^7: $91.7 \pm 2.4\%$; 10^6: $94.5 \pm 3.5\%$; 10^5: $85.7 \pm 5.8\%$; D: $79.4 \pm 3.4\%$).

in 10 randomly selected visual fields of nucleus pulposus area and averaged after costaining with GFP to detect the labeled transplanted MSCs. Percentages of GFP-positive cells for the 10^6 cell group was 62.8 ± 12.4, and the 10^7 group $75.2 \pm 16.1\%$, both were significantly ($p < 0.01$) higher than the $15.3 \pm 9.2\%$ found in the 10^5 group (Fig. 8a). There was no specific region for its distribution and they were scattered in NP cavity (Fig. 8b).

DISCUSSION

In order to evaluate the effect of cell doses in MSCs transplantation for experimental disc degeneration, changes in disc height, as assessed by radiographic %DHI, signal, and in relative signal intensity as classified by Pfirrman et al. of the nucleus pulposus on MRI T2-weighted imaging were determined. We found that the increase of %DHI in the MSC-transplant groups compared to the D group decreased beginning at 4 weeks after transplantation. On T2-weighted imaging, MSCs transplanted discs showed less degeneration

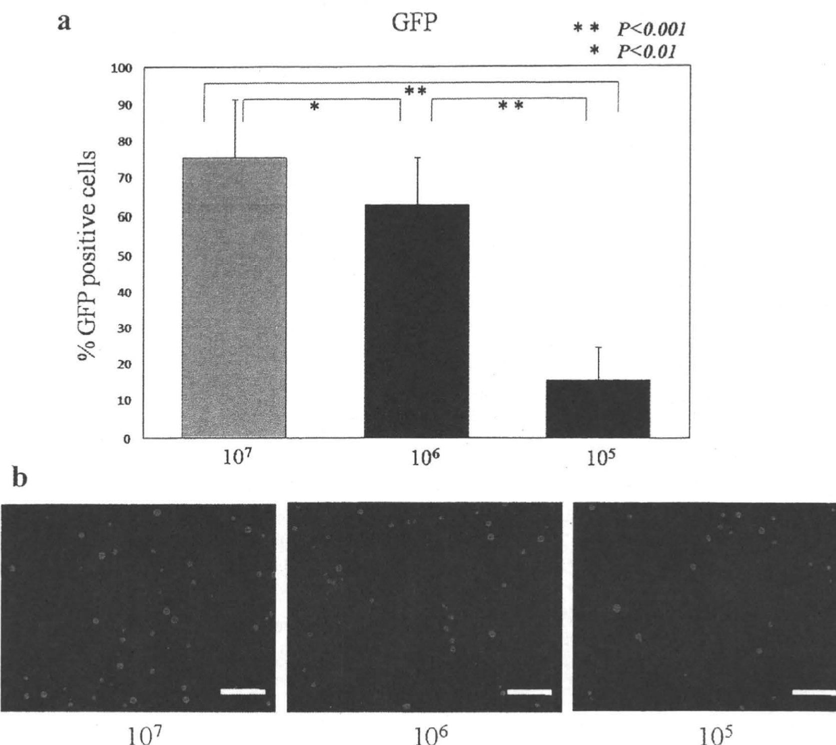

Figure 8. Green fluorescent protein (GFP) positive cells. (a) The number of GFP-positive MSCs in the nucleus pulposus at 12 weeks after transplantation was significantly (**$p < 0.001$) higher for the 10^6 and 10^7 groups than for the 10^5 group. (b) GFP-positive cells image of the NP cavity formed at 12 weeks after first operation. Bar = 100 μm.

in Pfirrman classification. Diagnostic imaging results showed that the progression of IVD degeneration was inhibited in all three MSC-transplanted groups, with no significant differences among the three groups. Evaluation of the histology of the experimental discs indicated that disc degeneration was limited in the MSC-transplant groups. The annulus fibrosus structures were maintained at near normal status in the MSC 10^6 and 10^7 groups, but not the 10^5 group. In the NP of the MSC 10^6 and 10^7 groups, proteoglycan, type II collagen, and other extracellular matrix components were maintained, indicating that IVD degeneration was suppressed. There were no significant differences in imaging results between 10^5 versus 10^6 and 10^7 groups in MSC-transplanted group discs despite the fact that there were significant differences in histological analysis. This may be resulting from the fact that disc height and T2-weighted signal on MRI would not correlate directly to histological disc degeneration in short observation time. In the previous literature, Ho et al.[22] suggested that differences between imaging and histological findings are probably due to differences in sensitivity between diagnostic imaging and histological analysis; however, differences in follow-up time intervals may also contribute. Further studies utilizing longer follow-up times are needed to resolve the differences between imaging and histological findings.

The three parameters used to assess post-transplant cell activity in discs were the number of remaining transplanted MSCs, the survival rate of NP cells, and apoptosis of NP cells in transplanted discs. For the MSC 10^5 group, the numbers of transplanted MSCs remaining and viable cells in the nucleus pulposus tissue were low. An insufficient cell number may not be capable of reconstructing the matrix and microenvironment of degenerating discs, resulting in increased death of both resident and transplanted cells. As seen in the MSC 10^7 group, when too many cells were transplanted into the limited space of the IVD, an imbalance between the relatively slow rate of diffusion of nutrients and cell number may have occurred, inducing apoptosis of both transplanted MSCs and resident cells; this needs to be verified in longer-term studies. These findings from the transplantation of 10^5, 10^6, and 10^7 MSCs suggest that quantity of the transplanted cells are important to optimize the inhibition of progression of degeneration and to regenerate damaged IVDs and that there most likely is a minimal number that affects its therapeutic effect. However, there is still much more parameters to discuss such as evaluation of adequate nutrition for the number of MSCs transplanted which may also be important for maintaining the microenvironment of the disc or differentiation status of the transplanted MSCs. Inducing MSCs towards NP in vitro may most likely affect the therapeutic outcome as well.

Animal models for studies of cell transplantation in IVD degeneration have employed rabbits, rats, and larger animals, such as beagles.[23,24,29] Several techniques have been developed to produce disc degeneration, but an ideal animal model for the human

condition has not been established.[30–32] It has been well known that notochord-like cells are present in the nucleus pulposus of rats and young rabbits, whereas only chondrocyte-like cells constitute the nucleus pulposus in humans. Small animals may therefore be unsuitable for use as models of the human disease. We use beagles as an alternative animal model because we believe the discs of this chondrodystrophic breed more closely approximate the anatomy of human discs. In addition, beagle IVD degeneration and herniation are likely to occur spontaneously, and beagle nucleus pulposus cells are chondrocyte-like. Beagles are sometimes being used to study IVD degeneration since their disc cells share similarities with human discs.[33–37]. Degeneration is produced by nucleotomy in the model used in the present study, and thus may have differences from naturally occurring IVD degeneration in humans. Although this may be considered a limitation of this model, no animal model that completely mimics human IVD degeneration exists at this time. The nucleotomy model may well have usefulness for studying disc degeneration progression following disc surgery.

In the human NP, the average total cell density of the disc is about $4–6 \times 10^3$ cells/mm^3.[38,39] Human discs are rich in extracellular matrix and possess immune privilege. For clinical application, the optimal number of MSCs transplanted to restore these characteristics in damaged IVDs must be determined. In a supplementary experiment, we investigated 20 IVD specimens from 10 beagles, 10 of which were normal IVDs taken from beagles sacrificed at a very similar age and from the same disc level of animals used in the study and 10 of which same procedure was performed as the initial study for induction of disc degeneration. Results demonstrated that average volume of NP tissue in single IVDs of beagles used in the study was 107.18 ± 1.03 mg (wet weight) and the average viable cell number was $1.03 \pm 0.19 \times 10^6$ cells. For the IVDs, where induction of disc degeneration was performed, average volume of NP tissue at the time of injection was 87.38 ± 4.23 mg (wet weight) and the average viable cell number was $1.36 \pm 0.15 \times 10^5$ cells. These data indicates that there is not truly a large difference in number of viable cells among the IVDs used in the study. This also justifies the rational of injecting equal numbers of MSCs in the discs. Furthermore, a base line of 10^6 viable cells may be the adequate number to maintain disc homeostasis, which also is understandable in interpreting the result of initial study where injection of 10^5 cells was not fully sufficient. Previous animal model studies of cell transplantation to IVDs have used 10^6 cells/disc for transplants,[15–18] therefore, we transplanted 10^5, 10^6, or 10^7 cells per disc in the present study, establishing three transplant groups with the number of cells transplanted increasing at 10- to 100-fold. Although actual clinical situation for translation of this procedure will not be clarified without clinical trials, we believe that grade 2 or 3 in Pfirrman classification, where disc degeneration is mild-to-moderate with less nutritional problem may be one candidate.[40]

In the present study, the effect of the number of MSCs transplanted into a degenerative disc was investigated using a canine disc degeneration model. From the perspective of clinical application, the optimal number of transplanted cells may also be affected by the severity of recipient disc degeneration.[22] However, in the current limited model, the transplantation of 10^6 MSCs, when compared to 10^5 or 10^7, produced the best maintenance of the structure of IVDs and best inhibited IVD degeneration. Because results from a study such as ours may vary in other animal models and in humans, care must be taken in the clinical application of our findings.

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