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Effect of Cryopreservation on Canine and Human Activated Nucleus Pulposus Cells: A Feasibility Study for Cell Therapy of the Intervertebral Disc

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Abstract

It has been shown that coculture of bone marrow–derived stromal cells (BMSCs) with intervertebral disc (IVD) nucleus pulposus (NP) cells significantly activates the biological characteristics of NP cells in animal models and in humans. We therefore predicted that activated NP cells would be a useful graft source for cellular transplantation therapy in the treatment of degenerative IVDs. However, the activation protocol is based on fresh isolation and activation of NP cells, which limits the timing of clinical application. Cell transplantation therapy could be offered to more patients than is now possible if activated NP cells could be transplanted as and when required by the condition of the patient. No study has investigated the effect of cryopreservation on NP cells after enzymatic isolation. We investigated the effects of cryopreservation of canine and human NP cells in both cell and tissue form before coculture with autologous BMSCs. Cell viability, proliferation, glycosaminoglycan production, aggrecan transcriptional activity, colony generation, and gene expression profile of the cells after cryopreservation and subsequent coculture were analyzed. The influence of cryopreservation on cell chromosomal abnormalities and tumorigenesis was also studied. The results showed that there were no clear differences between the noncryopreserved and cryopreserved cells in terms of cell viability, proliferation capacity, and capacity to synthesize extracellular matrix. Furthermore, the cells showed no apparent chromosomal abnormalities or tumorigenic ability and exhibited similar patterns of gene expression. These findings suggest that by using cryopreservation, it may be possible to transplant activated NP cells upon request for patients' needs.

Key words: cryopreservation; intervertebral disc; nucleus pulposus; stem cells

Introduction

Back pain is one of the most common health care problems globally and is associated with degenerative disc disease, but the details of the mechanism of intervertebral disc (IVD) degeneration have not been clearly identified.[1] Experimental regenerative medicine techniques for treating IVD degeneration include the intra-intervertebral disc injection of cytokines and growth factors,[2–6] gene delivery to IVD cells,[7–9] creation of artificial IVDs using tissue engineering,[10] and cell transplantation.[11–14]

We have primarily focused our research efforts on disc regeneration, specifically on the repair of the nucleus pulposus (NP) by using cell therapy. Nishimura and Mochida[15] reported that reinsertion of autologous NP tissues decelerated disc degeneration. Okuma et al.[16] found the biological viability of NP cells to be activated by using a system of coculture with annulus fibrosus cells. However, to achieve effective results, further upregulation of the biologic and metabolic viabilities of NP cells is necessary due to the low cellular yields and low proliferative activity of NP cells in earlier phases of primary culture. To overcome this problem, Yamamoto et al.[17] cocultured NP cells with bone marrow–derived stromal cells (BMSCs) in direct cell-to-cell contact. This coculture system significantly elevated biological properties of NP cells including cell proliferation, DNA synthesis, and glycosaminoglycan (GAG) synthesis. Furthermore, Watanabe et al.[18] reported that coculture of human NP cells in direct cell-to-cell contact with BMSCs upregulated the activation of the NP cells. Our research group is currently undertaking a clinical trial of autologous transplantation of these activated NP cells.

Once their safety has been established, cryopreserved and activated autologous NP cells could in the future be offered to

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patients as and when their condition required, unconstrained by the requirement for culture immediately after harvest. This would mean that the scope of the cells' applications could be expanded to cover a variety of degenerative diseases that could be treated by IVD regeneration. Therefore, in this study, we investigated the effects of cryopreservation and thawing on the viability of activated NP cells obtained by coculture with BMSCs by comparing cryopreserved and thawed cells with noncryopreserved cells. In addition, we compared the capacity of cryopreserved and noncryopreserved cells to synthesize extracellular matrix (ECM) and proliferate. Young canines, large animals that are unlikely to exhibit IVD degeneration, were used. In addition, NP tissue and BMSCs harvested from patients during surgery for diseases such as lumbar disc herniation were activated after cryopreservation and thawing. As with the canine cells, the results were compared with noncryopreserved cells. The safety of the procedure was evaluated by chromosome banding and *in vivo* testing to rule out tumorigenic transformation of the cells.

Materials and Methods

Animal study

The study was conducted in accordance with protocols approved by the Animal Experimentation Ethics Committee of the Tokai University School of Medicine. Mature canines (age: 3–4 years; $n=8$; weight: $\sim$10 kg; Nosan Beagle, Nosan Corp., Kanagawa, Japan) were used. In each set of results, n varied (cell viability, $n=6$; 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide [MTT] assay, $n=3$; GAG/DNA, $n=3$; aggrecan transcriptional activity, $n=5$; colony generation, $n=4$).

Clinical study

After informed consent was obtained, samples from 10 patients were obtained during surgery for lumbar disc herniation and were registered to this study. The patients' ages ranged from 20 to 40 years. The degree of IVD degeneration in each sample was classified according to Pfirrmann's classification system[19,20] by using magnetic resonance imaging (MRI; Table 1). In each set of results, n varied (cell viability, $n=8$; MTT assay, $n=4$; GAG/DNA, $n=4$; colony generation, $n=5$).

Table 1. Clinical Cases

Case	*Age*	*Sex*	*Disease*	*Operation*	*Disc level*	*MRI disc grade*
1	33	M	LDH	Herniotomy	L5–S1	2
2	29	M	LDH	PLIF	L4–L5	3
3	26	F	LDH	Herniotomy	L5–S1	2
4	29	M	LDH	Herniotomy	L5–S1	3
5	33	F	LDH	Herniotomy	L4–L5	3
6	25	M	LDH	PLIF	L4–L5	3
7	40	M	LDH	PLIF	L5–S1	2
8	33	M	LDH	Herniotomy	L5–S1	3
9	21	M	LDH	Herniotomy	L5–S1	3
10	31	M	LDH	Herniotomy	L4–L5	2

LDH, lumbar disc herniation; PLIF, posterior lumbar interbody fusion; MRI, magnetic resonance imaging.

Cell isolation

All thoracic and lumbar discs were harvested from the canines, and IVD lesion sites were harvested from the humans. The NP and annulus fibrosus were carefully separated, and only the NP tissue was isolated under a dissecting microscope. The NP tissue was sliced into 1-mm-diameter segments, and separated into three groups: Group A, noncryopreservation; Group B, cell cryopreservation; and Group C, tissue cryopreservation. The NP tissue of Groups A and B was treated with enzymes in accordance with protocols currently used in our clinical studies. Briefly, enzymatic treatment was performed using 50% TrypLE Express (Gibco, Grand Island, NY) for 1 h at 37°C, and then Type I collagenase (Worthington Biochemical Corp., Lakewood, NJ) for 2 h at 37°C. After the treated cells were washed twice in normal saline solution, they were centrifuged at 500 g for 5 min. Group A, B, and C cells were cultured according to the protocol described in Fig. 1A. In groups B and C, recovered cells were stored in cryotube at a cell density of 1.0×10^5 cells/mL) and cryopreserved in 70% Dulbecco's modified Eagle's medium (DMEM)/F12 (Gibco), 20% fetal bovine serum (FBS; Gibco) and 10% dimethylsulfoxide (DMSO; Wako, Osaka, Japan). DMSO was added slowly drop by drop to avoid producing heat. Next, the samples were cryopreserved in stages to -80°C by using a controlled-rate cryopreservation device (Bicell, Nihon Freezer, Tokyo, Japan). The next day, the samples were stored in a liquid nitrogen container at -196°C (Fig. 1B). The cryopreservation time was 2 weeks. After this, the samples were rapidly thawed in a 37°C bath, washed twice with normal saline solution, then cultured at 37°C in 5% CO_2, as for Group A.

Bone marrow aspirate (50 mL) collected from the ilium was separated by specific gravity using saline–5% dextran, and the mononuclear cells were isolated. Culture medium containing these mononuclear cells was divided equally into three groups. As with the NP tissue, the groups were Group A (noncryopreservation) and Groups B and C (cryopreservation). The mononuclear cells of Group A were cultivated in a 100-cm^2 culture flask using DMEM/F12 culture medium containing 10% FBS. Adherent cells were considered to be BMSCs. The mononuclear cells in Groups B and C were cryopreserved using the same procedure as for the NP. As with the NPs, the cryopreservation time was 2 weeks. Thawing was accomplished rapidly in a 37°C bath, and afterwards, the culture was performed in the same way as for Group A (Fig. 1A and 1B).

Cell culture method

A monolayer of NP cells was cultured in a six-well plate (Becton Dickinson; 3.0×10^4 cells/mL per well) for 4 days at 37°C in 5% CO_2. A monolayer of mononuclear cells that were isolated from the bone marrow was also cultured in a 100-cm^2 culture flask for 4 days at 37°C and 5% CO_2. A six-well plate and inserts (Becton Dickinson) were used for coculture. After the NP cells were removed using TrypLE Express, the single-cell suspension was seeded into wells at 3.0×10^4 cells/mL per well and cultured in DMEM/F12 containing 10% FBS. In addition, a single-cell suspension of BMSCs was also cultured on the reverse side of the inserts at 3.0×10^4 cells/mL per well, and the cells were cocultured in direct cell-to-cell contact for 3 days at 37°C in 5% CO_2.

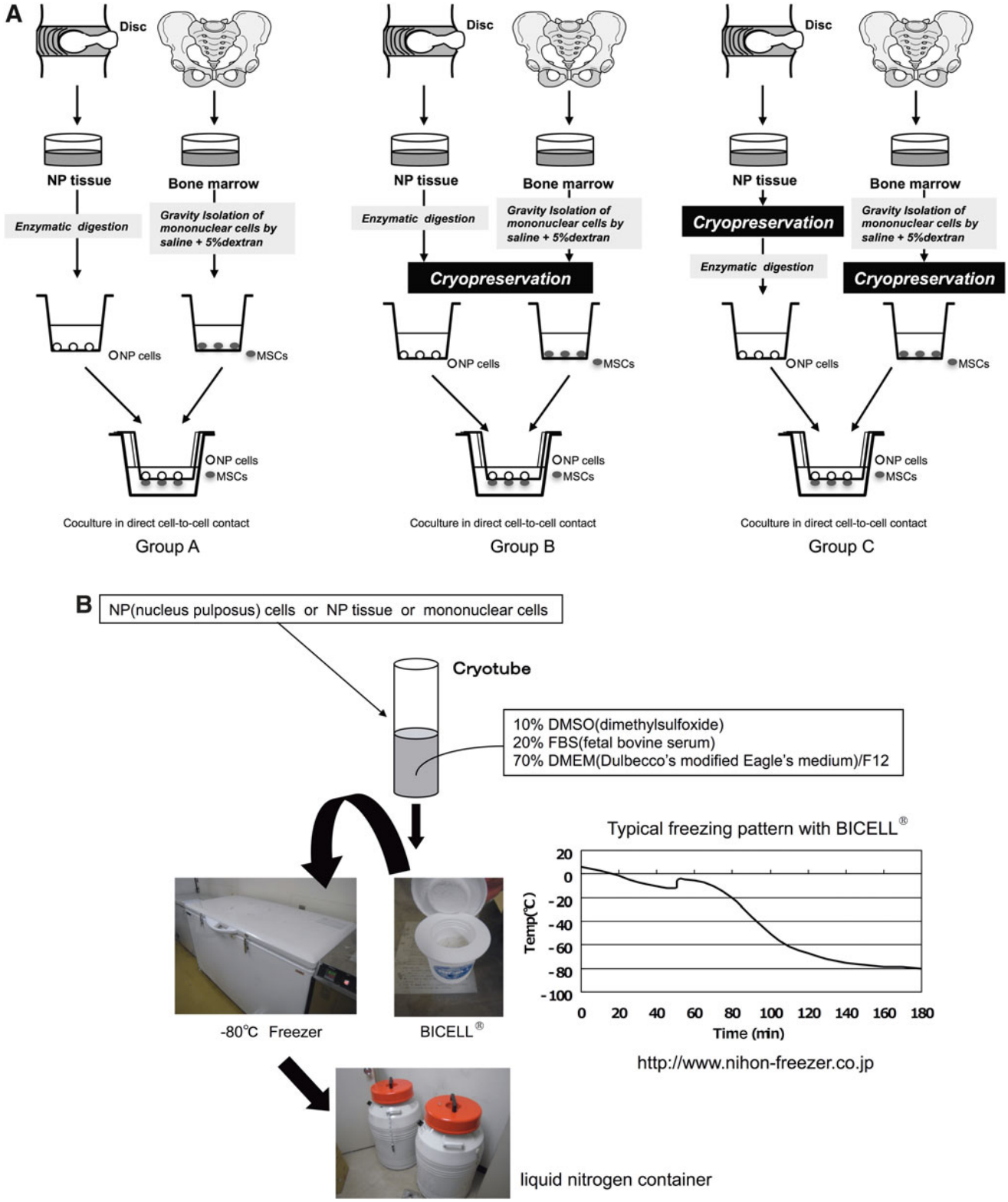

FIG. 1. **(A)** The harvested nucleus pulposus (NP) tissue was separated into three groups (Group A, noncryopreservation group; Group B, cryopreservation cell group; Group C, cryopreservation tissue group). The NP tissue of Groups A and B was enzymatically digested immediately. In Group A, NP cells were cocultured in accordance with the protocols used in a pilot clinical study currently being conducted by our research group as shown. In Group B, cells were cryopreserved for 2 weeks and were cocultured after thawing. The NP tissue of Group C was sliced finely and cryopreserved for 2 weeks in thin tissue sections. After the samples were thawed, they were treated with enzymes and cocultured in accordance with the culture protocols used in Groups A and B.**(B)** NP cells or tissue or the mononuclear cells were preserved in 70% Dulbecco's modified Eagle's medium (DMEM)/F12, 20% fetal bovine serum (FBS), and 10% dimethylsulfoxide in a cryotube. Next, the samples were cryopreserved in stages to −80°C using a controlled-rate cryopreservation device. The next day, the samples were stored in a liquid nitrogen container at −196°C.

Evaluation

To compare the condition of each of the cryopreserved groups when thawed with that of the noncryopreserved group after the samples had been cultured for 7 days, a viability assay was performed and then the adherent cells were recovered and evaluated as follows: cell proliferation (24, 48, and 96 h), GAG/DNA assay, aggrecan transcriptional activity, and colony generation. For the human cells, after they were cultured for 7 days and in addition to the above tests, the adherent cells were recovered and each of the three groups was compared for the presence of NP cell gene expression using reverse-transcription polymerase chain reaction (RT-PCR) and evaluated for the presence of chromosomal abnormalities and tumorigenesis.

Measurement of cell viability

Cell viability was measured using Trypan Blue staining and counting living cells. After the control group was treated with enzymes, the cryopreservation group was cryopreserved for 2 weeks and cell viability was measured after thawing.

Measurement of cell proliferation

After each of the three groups had been cultured for 7 days, the adherent cells were removed using TrypLE Express and plated in 200 μL of culture medium at 3.0×10^3 cells/mL per well in a 96-well plate. DMEM/F12 culture medium containing 10% FBS was used. Twenty-four, 48, and 96 h after the cells were transferred, the culture medium was replaced with 100 μL of 0.5 mg/mL MTT solution (Dojindo, Kumamoto, Japan) dissolved in serum-free phenol red–free DMEM and cells were incubated for 2 h at 37°C in 5% CO_2. A solubilizing solution (80% isopropanol, 20% DMSO, 4% Tween 20) was then added to each well. After mixing, cell proliferation was measured by using a spectrophotometer with its wavelength set to 562 nm, and the cell count was determined using a calibration curve.

Measurement of GAG/DNA

GAG was measured using a sulfated GAG assay kit (Seikagaku Biobusiness, Tokyo, Japan). Approximately 5×10^5 activated NP cells that had been cultured for 7 days were harvested by using a cell scraper. One hundred fifty microliters of protease solution was added, the solution was agitated for 10 sec and then treated for 2 h at 55°C. Afterward, the solution was boiled for 10 min and restored to room temperature. The pretreated sample fluid was stained for 5 min in 1,9-dimethylmethylene blue liquid dye, a chromogenic reaction was produced at room temperature, and the sulfated GAG concentration was calculated by determining the absorbance at 530 nm. Shark cartilage–derived GAG was used as the GAG standard solution and formed the basis for producing the calibration curve. DNA was assayed by fluorescence using 4′,6-diamino-2-phenylindole (DAPI). As with GAG, the pretreated sample fluid was added to a 96-well plate and stained with DAPI, a chromogenic reaction was produced at room temperature, and fluorescence was assayed using a luminescence counter (Wallac 1420 ARVO MX/Light Luminescence Counter, PerkinElmer, Yokohama, Japan). Salmon sperm DNA (Invitrogen, Carlsbad, CA) was used as the DNA standard solution and formed the basis for producing the calibration curve. The result was calculated as the GAG/DNA ratio.

Measurement of aggrecan transcriptional activity

To investigate the capacity of the cells to synthesize ECM, we investigated the transcriptional activity of the gene that encodes aggrecan core protein, a typical component of the extracellular matrix of NP cells. Two days after coculture in six-well plates at a density of 3×10^4 cells/well, activated NP cells were transiently transfected with the aggrecan reporter plasmid (Agg-luc), provided by Dr. Michael C. Naski (University of Texas Health Science Center at San Antonio). As an internal transfection control, we used the empty vector pGL4.74 (Promega, Madison, WI) containing the *Renilla reniformis* luciferase gene. Lipofectamine 2000 (Invitrogen) was used as the transfection reagent. For each transfection, plasmids were premixed with the transfection reagent. Twenty-four hours after the initial transfection, the cells were harvested and a Dual-Luciferase reporter assay system (Promega) was used for the sequential measurements of the firefly and Renilla luciferase activities. Transfection efficiency for activated NP cells was about 60%–70%. The luciferase activities and calculation of the relative ratios were quantified using a luminometer (TD-20/20, Turner Designs, Sunnyvale, CA).

Measurement of colony generation

After the activated NP cells were cultured for 7 days, 500 cells/mL were plated in 1 mL of MethoCult (STEMCELL Technologies, Vancouver, BC, Canada) and incubated at 37°C in 5% CO_2. For testing colony formation, a single-cell suspension of 1.0×10^3 NP cells was seeded into 35-mm diameter dishes and cultured in 1 mL of Methocult H4230 methylcellulose medium (STEMCELL Technologies) for 10 days. Colonies (>10 cells) were counted using an inverted microscope as previously described.[21]

Gene expression profiling

Expression of genes related to human NP cell proliferation and metabolism was evaluated. The mRNA levels of activated NP cells from the noncryopreservation and cryopreservation groups were measured using RT-PCR and compared. After being cultured for 7 days, total RNA was isolated from the activated NP cells using the Total RNA Isolation System (Promega), reverse transcribed into cDNA using a High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA) and amplified by PCR using specific primers for each gene (Table 2). PCR products were electrophoresed, stained with ethylene bromide, and visualized under UV light. Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard.

Scanning microscopic evaluation of chromosomes of activated human NP cells

After the activated human NP cells were cultured for 7 days, they were transferred into separate culture flasks containing DMEM/F12 supplemented with 10% FBS. One percent colchicine was then added to the medium to inhibit mitotic activity, and the samples were fixed in acetic acid/methanol solution. The processed cells were examined by optical and fluorescence microscopy. Ten mitotic cells were

Table 2. Oligonucleotide Primers Used for RT-PCR

Gene	*Primer*
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	F: 5′-TGA TGA CAT CAA GAA GGT GGT GAA G-3′R: 5′-TCC TTG GAG GCC ATG TGG GCC AT-3′
Aggrecan	F: 5′-TGA CCA CCG CCC CTT CTA C-3′R: 5′-AGG CTC TGG ACT TCC AAG GT-3′
Transforming growth factor beta-1 (TGF-β1)	F: 5′-GGG ACT ATC CAC CTG CAA GA-3′R: 5′-CGG AGC TCT GAT GTG TTG AA-3′
Versican	F: 5′-TGA TCC CTA AAA TGG CGA ACA-3′R: 5′-CAC GGC AAC CCA AAA TGA C-3′
Sickle tail (SKT)	F: 5′-ACC TCC CTA ATC CAC CTG CT-3′R: 5′-TTG ACT CTG CGG TGA GAA TG-3′

chosen randomly from each sample for the G-banding procedure. We looked for chromosomal abnormalities including but not limited to trisomy, translocations, and defects.

Tumorigenic activity of activated human NP cells

The activated human NP cells were transferred into separate flasks containing DMEM/F12 supplemented with 10% FBS and cultured. Afterward, 1.0×10^6 cells were used to evaluate tumorigenicity. After the cells were washed twice in normal saline solution, they were suspended in 500 μL of phosphate-buffered saline (PBS). Nine-week-old female NOD-SCID mice ($n = 12$) were used as recipients for determining whether the cells were neoplastic/tumorigenic. The cells suspended in PBS were injected subcutaneously into the mice. After 8 weeks, the mice were euthanized, and the subcutaneous sections containing the transferred cells were fixed in 4% formalin for 1 day. The paraffin sections were then stained with hematoxylin–eosin and safranin O and checked by a pathologist for neoplasm or tumorigenesis.

Statistical analysis

Results are expressed as the mean ± standard deviation. The results for the noncryopreservation and cryopreservation groups were compared using one-way analysis of variance with the Mann–Whitney *U* test. Differences were considered significant at $p < 0.05$.

Results

Cell viability

The percentages of Trypan Blue–positive cells in the noncryopreservation group (Group A), cryopreservation cell group (Group B), and cryopreservation tissue group (Group C) of the canine NP cells after culture are shown in Figure 2A (Group A, 80.7% ± 6.56%; Group B, 77.5% ± 7.37%; Group C, 69.0% ± 6.03%). The percentages of Trypan Blue–positive cells for the three groups in human cells are shown in Figure 2B (Group A, 74.3% ± 10.0%; Group B, 64.5% ± 7.51%; Group C, 54.5% ± 8.99%). In both the canine and human cells, the percentage of Trypan Blue–positive cells for Group A was higher than for Groups B and C, but there was no significant difference compared with Group B. The percentage of Trypan Blue–positive cells for Group A was significantly higher than for Group C (canine, $p < 0.05$; human, $p < 0.05$).

Cell proliferation

The cell proliferation rates on day 5 for Groups A, B, and C of canine cells relative to day 1 (expressed as 1) are shown in Figure 3A (Group A, 2.84 ± 0.03 [day 3], 4.52 ± 1.11 [day 5]; Group B, 2.33 ± 0.86 [day 3], 3.94 ± 0.54 [day 5]; Group C, 2.37 ± 0.6 [day 3], 3.25 ± 0.7 [day 5]). The cell proliferation rates for human cells are shown in Figure 3B (Group A,

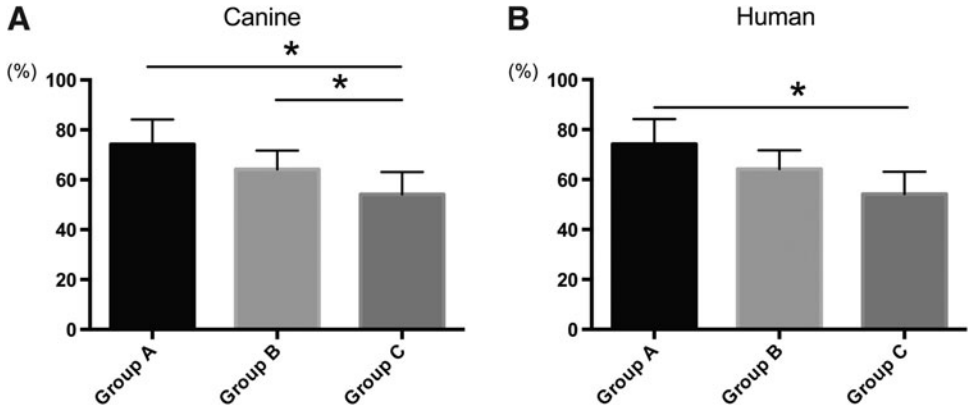

FIG. 2. In both the canines and humans, the percentages of Trypan Blue–positive cells in Group A were higher than for Groups B and C; however, there was no significant difference compared with Group B. The percentages of Trypan Blue–positive cells in Group A was significantly (canine, $p < 0.05$; human, $p < 0.05$) higher than for Group C. **(A)** Canine Group A, 80.7% ± 6.56%; Group B, 77.5% ± 7.37 %; Group C, 69.0% ± 6.03%. **(B)** human Group A, 74.3% ± 10.0%; Group B, 64.5% ± 7.51%; Group C, 54.5% ± 8.99%. Data are represented as mean ± SD.

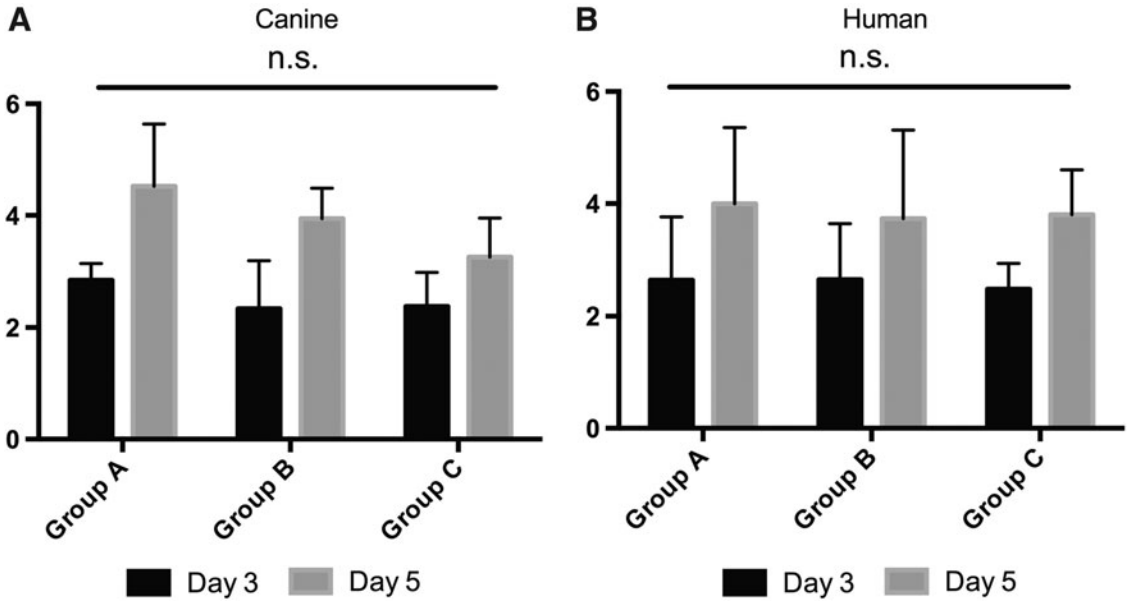

FIG. 3. Result of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay in both the canines and humans showed that there were no significant differences in proliferation rates (expressed as relative value to day 1) between the three groups on days 3 and 5. **(A)** Canine Group A, 2.84 ± 0.03 (day 3), 4.52 ± 1.11 (day 5); Group B, 2.33 ± 0.86 (day 3), 3.94 ± 0.54 (day 5); Group C, 2.37 ± 0.6 (day 3), 3.25 ± 0.7 (day 5). **(B)** Human Group A, 2.63 ± 1.13 (day 3), 4.00 ± 1.36 (day 5); Group B, 2.64 ± 1.0 (day 3), 3.73 ± 1.58 (day 5); Group C: 2.47 ± 0.46 (day 3), 3.80 ± 0.5 (day 5). Data are represented as mean ± SD.

2.63 ± 1.13 [day 3], 4.00 ± 1.36 [day 5]; Group B, 2.64 ± 1.0 [day 3], 3.73 ± 1.58 [day 5]; Group C, 2.47 ± 0.46 [day 3], 3.80 ± 0.5 [day 5]). In both the canine and human cells, there were no significant differences in proliferation rates between the three groups on days 3 and 5.

GAG/DNA synthesis

The GAG/DNA ratios for Groups A, B, and C of the canine cells are shown in Fig. 4A (Group A, 0.26 ± 0.04; Group B, 0.22 ± 0.05; Group C, 0.19 ± 0.06). The GAG/DNA ratios for human cells are shown in Figure 4B (Group A, 0.12 ± 0.05; Group B, 0.13 ± 0.05; Group C, 0.12 ± 0.06). There were no significant differences between the three groups in terms of GAG/DNA, which indicates an unimpaired capacity to synthesize ECM in both canine and human cryopreserved cells.

Aggrecan gene transcription activity

The values for aggrecan gene transcription in canine cells, which indicate the capacity of cells to synthesize ECM, expressed relative to the transfection control, are shown in Figure 5 (Group A, 0.68 ± 0.13; Group B, 0.68 ± 0.14; Group C, 0.56 ± 0.06). There were no clear differences between the three groups in aggrecan gene transcription activity, which together with the results for GAG/DNA indicates the

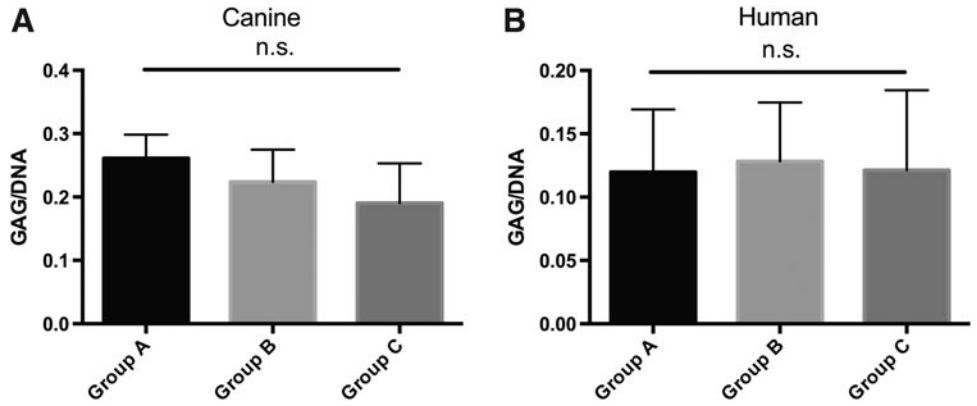

FIG. 4. After each of the three groups of cells had been cultured for 7 days, glycosaminoglycan (GAG) and DNA were measured. There were no significant differences between the three groups of both canine and human cells in the GAG/DNA ratio, which is a marker of extracellular matrix (ECM) synthesis capacity (canine Group A, 0.26 ± 0.04; Group B, 0.22 ± 0.05; Group C, 0.19 ± 0.06; human Group A, 0.12 ± 0.05; Group B, 0.13 ± 0.05; Group C, 0.12 ± 0.06). Data are represented as mean ± SD.

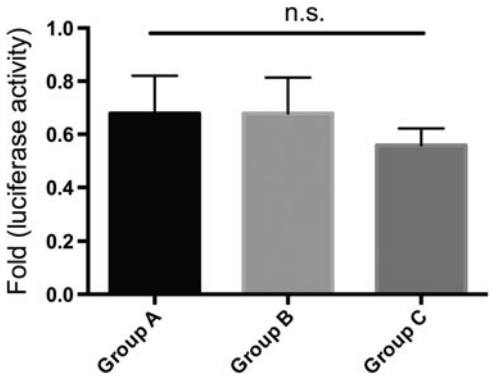

FIG. 5. Transcriptional activity of aggrecan was measured relative to the transfection control. There were no significant differences between the three groups for aggrecan expression, which, together with GAG/DNA, is an indicator of ECM synthesis capacity (Group A, 0.68 ± 0.13; Group B, 0.68 ± 0.14; Group C, 0.56 ± 0.06). Data are represented as mean ± SD.

cryopreserved canine cells have an unimpaired capacity for ECM synthesis.

Colony generation

Colony generation, which indicates self-replication capacity, is shown for the three groups of canine cells in Figure 6A (Group A, 156 ± 43 colonies/5×10^2 cells; Group B, 197 ± 24.5 colonies/5×10^2 cells; Group C, 222 ± 37.9 colonies/5×10^2 cells). Colony generation in the three groups of human cells is shown in Figure 6B (Group A, 121 ± 38.7 colonies/5×10^2 cells; Group B, 137 ± 25.2 colonies/5×10^2 cells; Group C, 173 ± 47.7 colonies/5×10^2 cells). There were no significant differences between the three groups of either canine or human cells in terms of the number of colonies generated.

Gene expression profiling

The RT-PCR results for the genes evaluated in the human cells showed that the same genes were expressed by all three groups, and no effects of cryopreservation were observed (Fig. 7).

Scanning microscopy of chromosomes

We checked the three groups of activated human NP cells for the presence of chromosomal abnormalities. There was no evidence of chromosomal abnormalities in any culture of either the noncryopreservation group or the cryopreservation group. There were also no negative effects on the cell cycle of activated NP cells after cryopreservation and thawing (Fig. 8).

Tumorigenic capacity of activated NP cells

Activated human NP cells from the three groups were suspended in PBS and subcutaneously injected into NOD-SCID mice. After 8 weeks, no noticeable tuberculate appearance under the skin of the mice was observed. The membranes of the transplanted areas were harvested and stained with hematoxylin–eosin and safranin O. The cell nuclei were aligned regularly, and the cell size was uniform. Furthermore, no clearly abnormal findings in parameters indicative of ECM synthesis, such as GAG, were observed and there was no evidence indicating tumorigenesis by activated NP cells (Fig. 9).

Discussion

Disc regeneration therapy by allogeneic or autologous cell transplantation in degenerative IVDs has been reported using various animal models.[11–16] The procedures in our previous *in vitro* study using human surgical specimens were designed for clinical feasibility.[18] From the point of view of safety, it is important for coculture to be performed only for short time periods because coculture for long periods may induce nuclear division and nuclear change, causing tumorigenesis. Therefore, culture times have been set at 7 days in the pilot clinical study currently being conducted by our research group. In this study, if MRI shows moderate IVD degeneration in adjacent IVDs in patients in their 20s undergoing spinal fusion surgery, the cells are isolated from the NP obtained

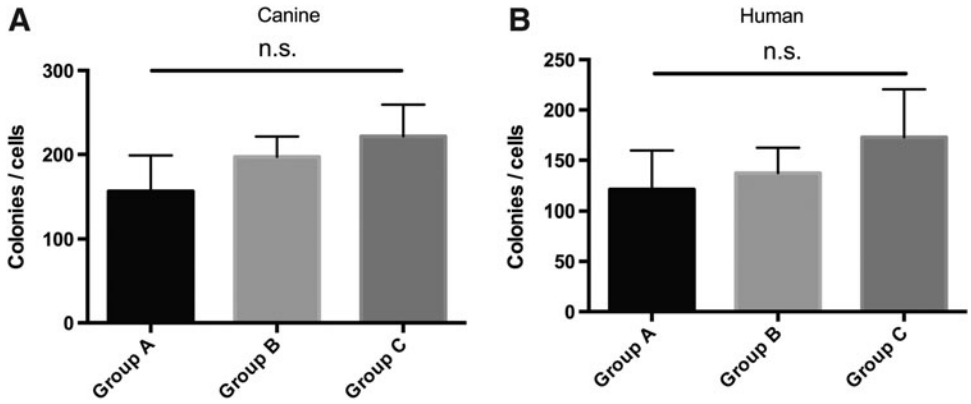

FIG. 6. After each of the three groups of cells had been cultured for 7 days, the number of colonies generated, which is an indicator of self-replication capacity, was measured. There were no significant differences between the three groups in either canine or human cells. **(A)** Canine Group A, 156 ± 43 colonies/5×10^2 cells; Group B, 197 ± 24.5 colonies/5×10^2 cells; Group C, 222 ± 37.9 colonies/5×10^2 cells. **(B)** Human Group A, 121 ± 38.7 colonies/5×10^2 cells; Group B, 137 ± 25.2 colonies/5×10^2 cells; Group C, 173 ± 47.7 colonies/5×10^2 cells. Data are represented as mean ± SD.

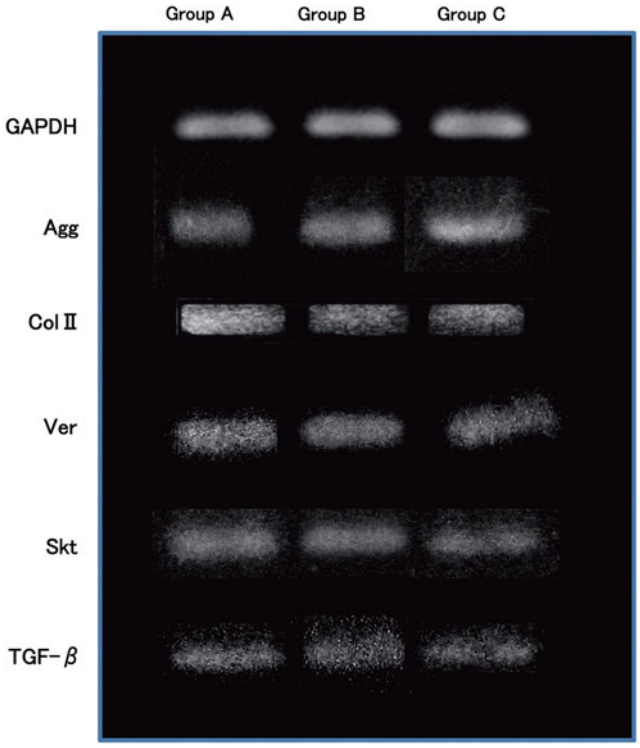

FIG. 7. The mRNA expression of activated NP cells of the noncryopreservation group and cryopreservation group of human cells were compared using reverse-transcription polymerase chain reaction (RT-PCR). After cells were cultured for 7 days, total RNA was isolated from the activated NP cells using the Total RNA Isolation System, reverse transcribed to cDNA using a High Capacity RNA-to-cDNA Kit, and amplified by PCR using specific primers for each gene. The RT-PCR results for all the evaluated genes were the same for all three groups, and no effects of cryopreservation were observed.

from the fused segments, and after coculture with BMSCs for 4 days, transplanted under fluoroscopic guidance into adjacent IVDs. The fact that the culture time is fixed means that the scope of application of these cell transplants is limited. However, if IVDs could be cryopreserved after removal, and then thawed and cultured when required by the patient's condition and extent of disc degeneration, the scope of application would be unconstrained by the short culture time and could be expanded to cover a variety of degenerative diseases that could be treated by IVD regeneration. Therefore, we investigated the effect of cryopreservation and thawing on activated NP cells obtained by coculture of NP cells with autologous BMSCs. The results of this study showed that there were no significant differences in viability or cell proliferation capacity between the cryopreservation cell group and the noncryopreservation cell group. Furthermore, there were no significant differences in terms of GAG/DNA synthesis or aggrecan gene transcription activity, markers of ECM synthesis capacity. The cryopreservation cell group exhibited the same pattern and level of mRNA expression as the noncryopreservation group, and we confirmed that the NP phenotype was maintained. These results indicate the feasibility of cryopreservation of activated NP cells.

From the perspective of therapeutic application of activated NP cells obtained through coculture after cryopreservation and thawing, the safety of the procedure must be established. In this study, we were able to confirm the safety of cryopreservation through the results of chromosome banding and *in vivo* testing to rule out tumorigenic transformation. With regard to other *in vitro* tests for detection of abnormality, tumorigenesis cannot be ruled out using any *in vitro* assays. Therefore, we focused mainly on the subcutaneous transplantation assay because incidence for tumorigenesis is better assessed by in vivo analysis.

Previous studies of the cryopreservation of IVD tissue have been reported from the perspective of allogeneic IVD tissue transplantation.[22–26] Chan et al.[26] reported the usefulness of allogeneic transplantation in a clinical environment by the allogeneic transplantation of cryopreserved IVDs and emphasized that this would be practical because of the demand for size matching. Thus, the safety of tissue cryopreservation has been demonstrated, although only in the context of tissue cryopreservation and allogeneic transplantation. Influenced by these tissue cryopreservation studies, we attempted to investigate the usefulness not only of cell cryopreservation, but also of tissue cryopreservation, although our perspective was autologous cell transplantation therapy. The results of tissue cryopreservation in our study showed that viability after thawing was low compared with the noncryopreservation group. However, the capacity for cell proliferation and ECM synthesis was maintained, and there were no significant differences in colony generation, a gauge of self-renewal capacity. In previous reports, IVD tissue of canines has been cryopreserved using DMSO as a cryoprotectant, and a loss

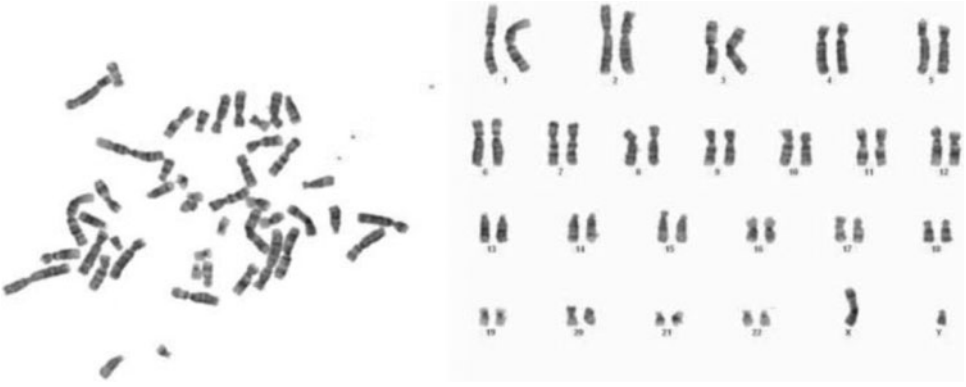

FIG. 8. The activated human NP cells from the three groups were checked for presence of chromosomal abnormalities. There was no evidence of chromosomal abnormalities in any culture. There were no negative effects on the cell cycle of activated NP cells after cryopreservation and thawing.

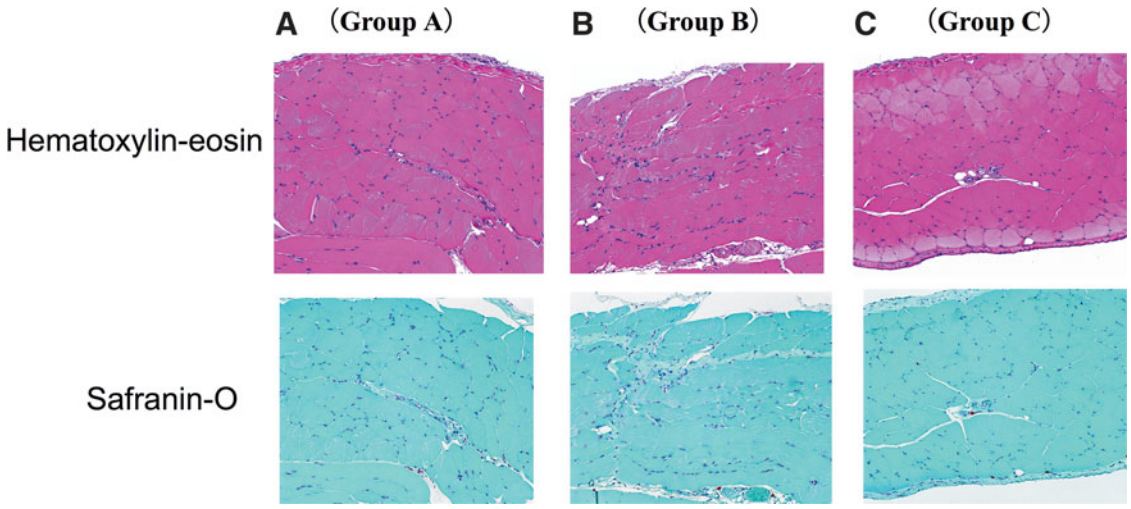

FIG. 9. Activated human NP cells from the three groups were suspended in PBS and subcutaneously injected into NOD-SCID mice. After 8 weeks, no noticeable tuberculate appearance under the skin of the mice was observed. Membranes of the transplanted portions were harvested and stained with hematoxylin–eosin and safranin O. The nuclei were regularly aligned and the cell size was uniform. There was no evidence indicating tumorigenesis of activated NP cells (Group A, non-cryopreservation group; Group B, cell cryopreservation group; Group C, tissue cryopreservation group). Magnifications 40×.

of IVD NP cell metabolic activity has been observed.[24,26] In tissue cryopreservation studies, including ours, the decline in viability after tissues are cryopreserved and thawed is probably the effect of tissue and cell membrane damage incurred during the process. However, the damage to enzyme systems in the cells was kept to a moderate level and in subsequent culturing the tissues tended to recover, which is probably why no significant differences with the noncryopreservation group were observed. Of course, from the perspective of autologous cell transplantation, the significant decline in the cell count and viability compared with the noncryopreservation group is a major disadvantage. Chan et al.[26] investigated and reported the optimal cooling rate, incubation time and cryoprotectant agent concentration for tissue cryopreservation, and the NP metabolic activity they reported in cryopreserved cells was approximately 70% of that in non-cryopreserved cells, virtually the same as our results. From the perspective of autologous IVD cell transplantation, the results of this study highlight the difficulties associated with cryopreservation and thawing of tissue.

Furthermore, the result of the current study opens expectation for use of cryopreservation for allogenic activated NP cell transplantation. If allogenic cell transplantation proves to be effective in humans, in the future, combination of cryopreservation and activation process will expand this treatment to a patient as and when their condition required. In a preliminary analysis, we analyzed expression of MHC class I and class II and no apparent expression of these antigens were detected in the activation and cryopreservation process.

In conclusion, the cell viability, proliferation capacity, and ECM synthesis capacity of activated NP cells obtained by coculture after cryopreservation and thawing were maintained in both canine and human cells. This suggests that the transplantation of activated NP cells when it is required by a patient's condition may be possible. Furthermore, it may be possible in the future to expand the scope of application of activated NP cell transplantation to cover a range of IVD degenerative diseases and to transplant activated NP cells from a cryopreserved cell bank. This illustrates that further research is required to accomplish the successful transplantation of activated NP cells obtained by coculture after tissue cryopreservation and thawing.

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Disclosure Statement

The authors declare no competing financial interests.

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