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Differentiation of Mesenchymal Stem Cells towards a Nucleus Pulposus-like Phenotype Utilizing Simulated Microgravity *In Vitro*[*]

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Summary: Mesenchymal stem cells (MSCs) were induced into a nucleus pulposus-like phenotype utilizing simulated microgravity *in vitro* in order to establish a new cell-based tissue engineering treatment for intervertebral disc degeneration. For induction of a nucleus pulposus-like phenotype, MSCs were cultured in simulated microgravity in a chemically defined medium supplemented with 0 (experimental group) and 10 ng/mL (positive control group) of transforming growth factor β1 (TGF-β1). MSCs cultured under conventional condition without TGF-β1 served as blank control group. On the day 3 of culture, cellular proliferation was determined by WST-8 assay. Differentiation markers were evaluated by histology and reverse transcriptase-polymerase chain reaction (RT-PCR). TGF-β1 slightly promoted the proliferation of MSCs. The collagen and proteoglycans were detected in both groups after culture for 7 days. The accumulation of proteoglycans was markedly increased. The RT-PCR revealed that the gene expression of Sox-9, aggrecan and type Ⅱ collagen, which were chondrocyte specific, was increased in MSCs cultured under simulated microgravity for 3 days. The ratio of proteoglycans/collagen in blank control group was 3.4-fold higher than positive control group, which denoted a nucleus pulposus-like phenotype differentiation. Independent, spontaneous differentiation of MSCs towards a nucleus pulposus-like phenotype in simulated microgravity occurred without addition of any external bioactive stimulators, namely factors from TGF-β family, which were previously considered necessary.
Key words: mesenchymal stem cells; simulated microgravity; cell differentiation; transforming growth factor β1

Low back pain affects approximately 80% of the adult population at some point in their lives and has a massive impact on social-economic due to both health care costs and loss of productivity[1]. Although the causes of low back pain are thought to be multi-factorial, in almost all cases there is evidence of intervertebral disc degeneration[2]. An ideal solution to manage disc degeneration would be to repair (or regenerate) the nucleus pulposus (NP), producing a matrix with similar or improved biological and biomechanical properties compared with the original. Cell-based tissue engineering methods are realistic treatments for intervertebral disc degeneration[3]. Nevertheless, acquiring autologous intervertebral disc NP cells[4], as for its limited number and decreased function, remains a significant impediment to successful transplantation therapy for patients with intervertebral disc degeneration. Due to proliferative potential and multi-differentiation capacity[5], mesenchymal stem cells (MSCs)-based culture system has been paid more attention. Numerous experiments were carried out through biochemical and cellular way to induce NP cells. Growth factors, including transforming growth factor β1 (TGF-β1), bone morphogenic protein, and insulin-like growth factor, have been evaluated for the potential to enhance chondrogenesis[6]. Experimental study on 3-D culture resulted in a chondrogenic differentiation[7].

Study by Yamamoto *et al*[8] using co-culture of rabbit NP cells with MSCs showed an increase in cell proliferation and proteoglycan synthesis, is a viable method for generating a population of differentiated cells that could be used in cell-based tissue engineering therapies for regeneration of the degenerate intervertebral disc. Darko *et al*[9] demonstrated a spontaneous chondrogenic differentiation of bovine MSCs in pellet culture. Our early researches[10] verified MSCs differentiation to NP cells, as well as revealed its limitations: including the confined cell function and the consuming inducing time in conventional culture system. To maintain, *in vitro*, the phenotype of highly differentiated cell types and to promote the formation of tissue constructs of dissimilar cells, cells have been cultured utilizing simulated microgravity technology[11]. It is natural to form pellet in simulated microgravity for its low shear, high mass transfer, and three-dimensional growth without sacrificing any other parameters. Furthermore, incubation of MSCs in simulated microgravity may reduce the maturation time by differentiation acceleration *in vitro*[12]. In this study, we evaluated the *in vitro* differentiation capacity of MSCs in

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simulated microgravity and determined the potential application of MSCs in cell-based therapy for disc degeneration. We hypothesized that the simulated microgravity could supplement and replenish the MSCs population as well as enhance the differentiation of MSCs into NP -like cells.

1 MATERIALS AND METHODS

1.1 Materials

FBS, penicillin G, streptomycin, amphotericin B and L-glutamine TGF-β1 were purchased from Sigma Chemical, USA. Histopaque-1077, CCK-8, Collagenase Ⅱ, Trypsin, DMEM/F12, Trizol reagent were procured from Invitrogen, USA. Hematoxylin, Alcian blue, Picrosirius Red were purchased from Dafeng, Co., Wuhan, China. PCR primers, Taq DNA polymerase, DNA ladder were obtained from Sangon, China. New Zealand rabbits were from the Experimental Animal Center of Beijing Medical College, Peking University, Beijing, China. Rotary Cell Culture System was from Synthecon, Inc., USA.

1.2 Cell Culture

Bone marrow MSCs were isolated from New Zealand white rabbits using a Histopaque-1077 density gradient method[6], and cultured in a 75-cm^2 flask with DMEM/F12, supplemented with 10% FBS, 100 U/mL penicillin G, 100 μg/mL streptomycin, 2.5 μg/mL amphotericin B, and 2 mmol/L L-glutamine at 37°C in a humid atmosphere containing 5% CO_2. After 7 days, non-adherent cells were discarded, and adherent cells were cultured to confluence, with medium changed every 3 days. MSCs were used in the experiments only after 2 to 3 expansion passages to ensure depletion of monocytes and macrophages.

1.3 Pellet Culture in Simulated Microgravity

Passage 3 cell suspensions containing 5×10^5 cells were centrifuged at 20 *g* for 5 min in 5 mL growth medium in 15 mL polypropylene conical tubes to form an aggregated cell pellet[13]. After 72 h of incubation, the pellets (group B: 0 ng/mL TGF-β1; group C: 10 ng/mL TGF-β1) were transferred into a rotary cell culture system (RCCS) equipped with high aspect ratio vessels and cultured in the defined medium (DMEM/F12, supplemented with 10% FBS, 100 U/mL penicillin G, 100 μg/mL streptomycin, 2.5 μg/mL amphotericin B, and 2 mmol/L L-glutamine). Group A acted as the control (group A: 0 ng/mL TGF-β1 without RCCS). The RCCS is a relatively new development in bioreactor technology that enables the cultivation of highly differentiated three-dimensional cell aggregates mimicking the structure and function of parental tissue. In the present study, the 10-mL HARV bio-chamber with variable speed power supply was used to simulate microgravity culture and co-culture; the protocol for its use has been previously reported[8]. The bio-chamber was placed in a convention incubator at 37°C with 5% CO_2, and set at the rotational speed of 6.5 r/min. The culture incubation period was 3 days without media change. The MSCs pellets were also cultured in conventional condition as control. The pellets were harvested at the day 0 and 3 for evaluation. All cultures were conducted in triplicate.

1.4 Measurement of Cellular Proliferation

Cellular proliferation was determined by WST-8 assay using Cell Counting Kit-8. The pellets (group B and group C) were treated with 0.25% trypsin with 1 mmol/L ethylenediaminetetraacetic acid (EDTA), and then checked by WST-8 assay. A 96-well plate containing MSCs from each of the 3 groups was inoculated with 10 μL of prepackaged CCK-8 solution. They were incubated at 37°C in a humid atmosphere containing 5% CO_2 for 2 h, and absorbance (*A*) values at 450 nm of the supernatant were measured spectrophotometrically. Cell counts were determined with a calibration curve.

1.5 Histology

Randomly selected pellets (*n*=2 per data point) were fixed overnight at 4°C in 4% paraformaldehyde, paraffin-embedded, and sectioned to 5 μm. Consecutive sections were stained with trichromate stain (hematoxylin, Alcian blue, picrosirius) for collagen red and proteoglycans blue as described by HE Grube[14]; briefly, sections were deparaffinized, hydrated to distilled water and stained in Weigert's hematoxylin for 10 min. After rinsing in tap water for 10 min, the specimens were stained in alcian blue solution, pH 2.5 for 30 min at room temperature. The sample was rinsed in tap water for 2 min and stained in picrosirius red solution for 1 h at room temperature. After rinsing in 0.01 mol/L HCl for 2 min, the specimen was dehydrated, cleared and mounted.

1.6 Measurement of Proteoglycan Synthesis

To assess proteoglycan (PG) accumulation, the sulfated glycosaminoglycan contents in each of the pellets were quantified by dimethylmethylene blue (DMMB, Sigma, USA) dye assay. Briefly, randomly selected pellets (*n*=3 per data point) were digested using 10 times concentrated papain solution (200 mg/mL in 50 mmol/L EDTA, 5 mmol/L L-cysteine) for 24 h in 55°C. Digested samples were mixed with DMMB buffer solution and measured spectrophotometrically, as previously described[26]. DMMB dye reagent (200 mL) with 40 mL papain digested samples was added to wells of a 96-well plate. The plate was read on a microplate reader at 525 nm with the purified nasal septum D1 PG (Sigma, USA) to create a standard curve. An aliquot of papain digests were also used to measure DNA content by uptake of [^{3}H]-thymidine, in order to obtain PG per ng DNA data.

1.7 RT-PCR

The total RNA was extracted from pellets (*n*=3) using TRIzol as the reagent to detect chondrogenic marker genes (Sox 9, collagen II, and aggrecan) of differentiated MSCs. mRNA expression of each target gene was normalized to that of the housekeeping gene GAPDH. The PCR amplification was conducted as follows: 1 cycle at 94°C for 1 min, 30 cycles at 94°C for 30 s, 55°C for 10 s, 72°C for 40 s and 1 cycle at 72°C for 5 min[10]. Primer sequences and lengths of the amplified products were as follows: Sox9 (5'-GACTTCCGCGACGTGGAC-3' 5'-CAGTACCTGCCGCCCAAC-3'); Collagen II (5'-GG CAATAGCAGGTTCACGTACA-3' 5'-CGATAACAGT CTTGCCCCACT-3'); Aggrecan (5'-CGCGAGACCTGG GTGGATGC-3' 5'-GAAGGGGCAGGCTGGATATTGC -3'); GAPDH (5'-TGAAGGTCGGAGTCAACGGATTT GGT-3' 5'-CATGTGGGCCATGAGGTCCACCAC-3'). The RT-PCR products were electrophoresed and quantified by scanning with an optical densitometer. The ratios

of the target genes/GAPDH were calculated and used as indicators of relative levels.

1.8 Statistical Analysis

The experiment was repeated three times. All data were represented as $\bar{x}\pm s$ and statistical analysis was carried out employing the SPSS software package (Version 12.0). Data were analyzed by using the independent-samples *t*-test and ANOVA. $P<0.05$ was considered statistically significant.

2 RESULTS

2.1 Cell Proliferation

WST-8 assay showed the number of cells in groups A, B and C was 7.94×10^5, 43.4×10^5, and 79.0×10^5 respectively. The number of cells in group B was approximately 5 times higher than that of group A, and that in group C was 2 times higher than that of group B. There was a significant difference between the groups (fig. 1).

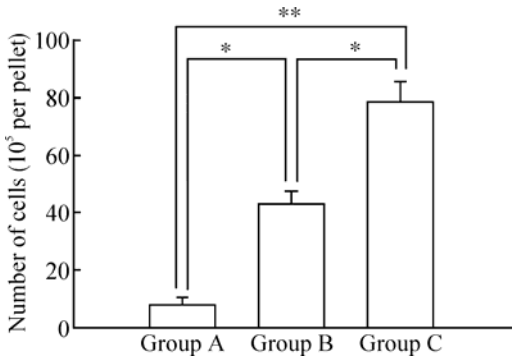

Fig. 1 Effect of simulated microgravity on cell proliferation on the 3rd day
$^*P<0.01$, $^{**}P<0.001$ (n=10)

2.2 Histochemical Staining

Even though all pellets initially contained the same number of cells (5×10^5), the 3-day MSCs pellets cultured in simulated microgravity (groups B and C) were significantly greater in size than those in group A. At the same time, most of pellets in group A attached to the bottom of flask and lost its three-dimensional culture condition (data not shown). Using this staining procedure, collagen was stained as red colour, and sulfated and carboxylated acid mucosubstances (proteoglycans) stained as blue colour. As shown in fig 2A, B and C, there was the significant staining difference of pellets under the different culture conditions. Concentrations of proteoglycans blue were readily discerned, and individual proteoglycans bundles could be seen in areas where the matrix contained less proteoglycans. Accumulation of collagen red was readily seen. In simulated microgravity, dense regions of proteoglycans blue (fig. 2B, group B) were distinguished from those with sparser matrix (fig. 2C, group C). Under the convention culture condition, the focal matrix concentrations that were stained with red/blue colour were markedly reduced (fig. 2A, group A).

2.3 PG Synthesis and Accumulation

PG accumulation, evaluated by measurement of S-GAG content, was significantly increased in the pellets seeded in simulated microgravity after culture for 7 days (group A: 16.5±2.7; group B: 34.4±1.6; and group C: 58.3±3.4 mg/mL, $P<0.001$, fig. 2D). The mean and standard deviations of DNA content after 7 days were as follows: group A, 10.31±0.67; group B, 14.33±0.55, and group C, 7.47±0.37 ng/mL ($P<0.01$, Date not shown). S-GAG/DNA in group C was significantly higher than that in groups A and B at the day 7 (group A: 1.6±0.11, group B: 2.4±0.15, group C: 7.8±0.30, $P<0.001$, fig. 2E).

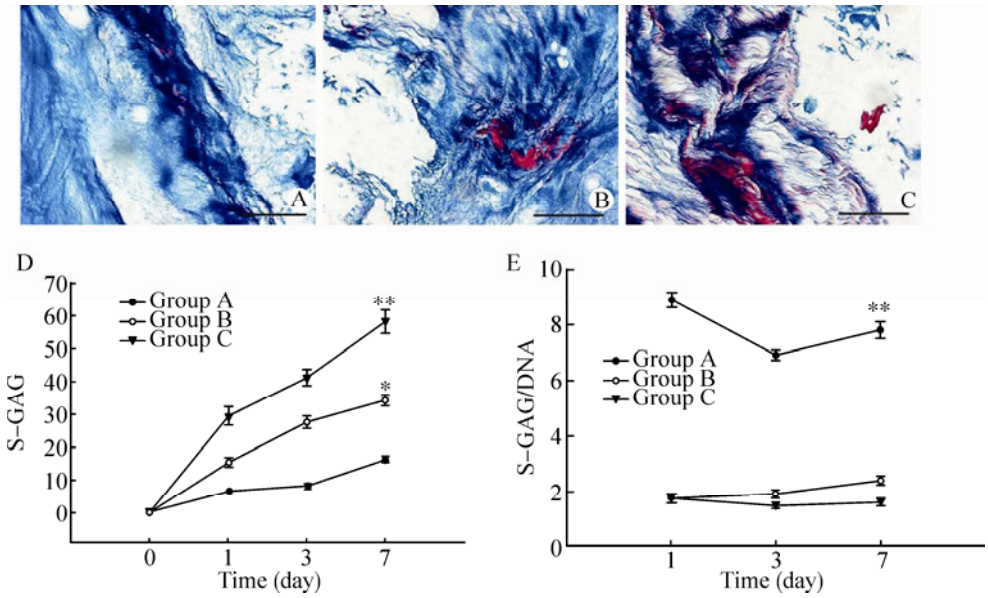

Fig. 2 Biochemical analysis of simulated microgravity on the 7th day
A-C: Histology of pellets specimens in culture of group A (A), group B (B) and group C (C). Sections were made from paraffin-embedded specimens and stained with trichromate stain: hematoxylin, Alcian blue, picrosirius. Collagen was stained with red, and proteoglycans stained with blue. Scale bar=100 μm; D: Chondrogenic differentiation (S-GAG content) of the pellets from three groups; E: Chondrogenic differentiation (DNA content) of the pellets from three groups; F: Chondrogenic differentiation index was shown as the ratio of S-GAG to DNA. $^*P<0.01$, $^{**}P<0.001$ (n=3)

2.4 Relative RNA Expression in MSCs

Semi-quantitative RT-PCR was employed to study the changes in gene expression between control and cultured samples. After culture in simulated microgravity, relative mRNA expression levels of Sox-9, aggrecan, and type Ⅱ collagen were significantly increased, and reached the peak at the 3rd day (P<0.01, fig. 3A). The ratio of aggrecan/collagen was significantly higher in group B than in groups A and C (P<0.01, fig. 3B).

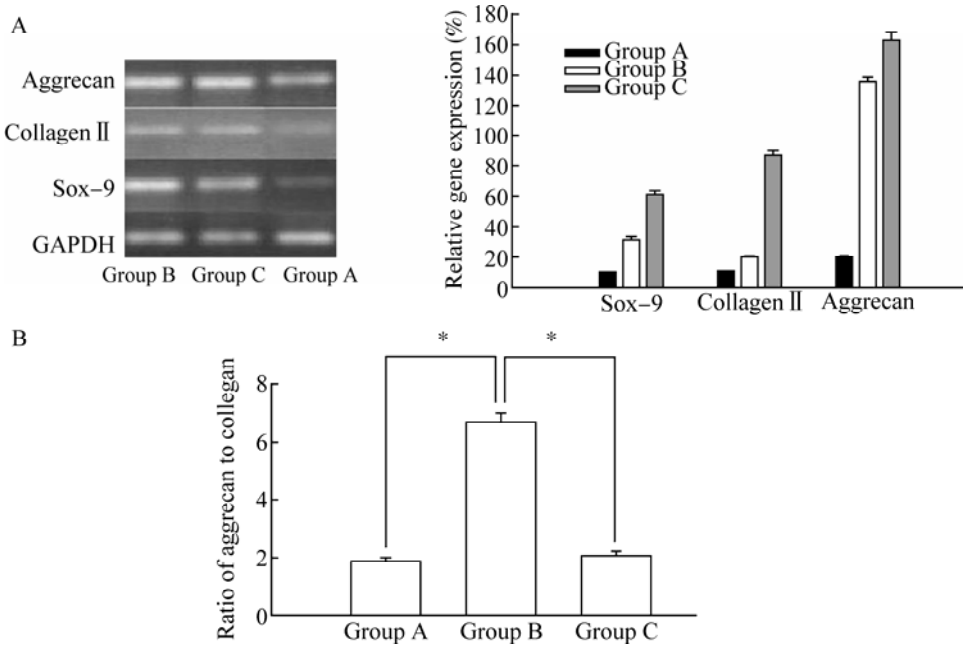

Fig. 3 A: Relative gene expression of S-GAS in each group; B: The ratio of aggrecan/collagen Ⅱ gene expression in each group
*P<0.01 (n=8)

3 DISCUSSION

More and more evidence has shown that the three-dimensional inducing culture environment of MSCs is a viable method for generating differentiated cells that could be used in cell-based tissue engineering therapies for regeneration of the degenerated intervertebral disc. Like chondrocytes, intervertebral disc cells maintained their native morphology and phenotype more readily when seeded in a three-dimensional culture system[15]. Thus, cell-cell interactions and environmental cues seem to be important in modulating the phenotype of these cells grown *in vitro*.

Since the impotence of NP and MSCs co-culture under two-dimensional culture conditions, various materials have been suggested for co-culturing these cells in three-dimensional conditions including alginate, agarose, collagen gel, collagen sponges and fibrin gels[3]. Although such scaffold systems provide an appropriate *in vitro* environment for intervertebral disc cells, it has several major disadvantages. Lack of uniformity of quality and size of the microspheres may occur between experiments and investigators, which makes the comparison of the studies difficult. In addition, *in situ* histology is tedious on alginate beads, as sufficient contrast between cell-produced matrix and the alginate matrix is difficult to achieve. More importantly, it is difficult to transfer the scaffold carrying differentiated cells into intervertebral disc for the *in vivo* experiments. Another factor that influences the inducing culture most is the high cell density, which allows cell-to-cell interaction[16]. Most likely, the condensed culture condition is the first stimulant for inducing further autocrine/paracrine cytokine secretion on a cellular level[17].

A new and technically simple method to culture intervertebral disc cells, in a pellet configuration, was described in 2001 by Lee[18], and was further utilized in co-culture system by Le Visage[19], Vadalà[20], and Chen[21]. This culture system involves the formation of cells aggregates by a simple centrifugation step and provides three-dimensional inducing culture environment as well as the high cell density. Presumably, the resulting aggregate or pellet allows three-dimensional interactions between the neighboring cells, leading to a more favorable synthesis of the extracellular matrix and more convenient cell-to-cell interaction. However, pellets are prone to emerge two-dimensional culture conditions because of its gravity. To overcome those obstacles, Chen *et al* used an orbital shaker to maintain the three-dimension of pellet[21]. In comparison to orbital shaker, simulated microgravity, for its low shear, high mass transfer, and three-dimensional growth without sacrificing any other parameters[12], may solve the gravity-related problem. The RCCS is a zero head space, aqueous medium-filled bioreactor that suspends particles or cell aggregates by rotating the vessel wall and integral gas diffusion membrane around the horizontal axis. The rotation vessel can hold particles/aggregates of up to 1 cm in diameter in orbital suspension, because the sedimentation forces induced by gravity are counterbalanced by the centrifugal force generated by the rotation of the vessel. As the aggregates expand, the rotational speed is increased. Recent literature data suggests two possible

determinants, which may act in concert under our experimental conditions: cellular condensation and cell shape. The latter was only recently identified as a major determinant of cell fate during the differentiation of stem cells, and was further confirmed in co-culture of NP cells and adipose stem cell. Stem cells have better outcomes with a round shape (three-dimensions) than a stretched morphology (two-dimensions) during the differentiation into adipocytes. A similar cell shape effect may be expected for the NP (and stem cells), since adipocytes and chondrocytes share the feature of a round cell shape. Our results conformed the priority of simulated microgravity in inducing culture of MSCs.

Furthermore, the results from this study denoted a NP-like phenotype differentiation of MSCs in simulated microgravity. A recent report indicated that the ratio of proteoglycan to collagen can be used to differentiate NP from cartilage and a 27:1 ratio was found in the NP, but only 2:1 in cartilage, demonstrating the importance of proteoglycans, in particular aggrecan, in the structure and function of the intervertebral disc[22]. Therefore, the increase in aggrecan, particularly in MSCs after 3 days under simulated gravity, suggests that these cells have differentiated to cells with an NP-like phenotype and could potentially be used in therapeutic strategies. Moreover, independent, spontaneous differentiation of MSCs towards a NP-like phenotype in simulated microgravity occurred without addition of any external bioactive stimulators, namely factors from TGF-β family, which were previously considered necessary. The mechanism of this differentiation remains unknown and is the subject of our ongoing research.

In conclusion, we demonstrated that three- dimensional culture environment and high cell density must be considered in inducing MSCs towards a NP-like phenotype and established a new cell-based tissue engineering treatments for intervertebral disc degeneration.

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