Comparative characteristics of mesenchymal stem cells derived from human various sources and optimization of *in vitro* storage conditions for clinical transplantation

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Comparative characteristics of mesenchymal stem cells derived from human various sources and optimization of *in vitro* storage conditions for clinical transplantation

Directed by Professor Hyun Ok Kim

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This certifies that the Doctoral Dissertation of Hoon Sang Sohn is approved.

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Finally, I would like to dedicate this thesis, the fruits of my efforts and time so far, to my wife who has lived for7 years with me, my eldest daughter and the valuable baby to be born soon.

<TABLE OF CONTENTS>

LIST OF FIGURES

LIST OF TABLES

<ABSTRACT>

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Mesenchymal stem cells (MSCs), which are multipotent stromal cells, have the ability to self-renew and differentiate into various cell types. Their plasticity and availability make them promising candidates for regenerative medicine. Since bone marrow-derived MSCs were first discovered, MSCs have been isolated from various tissues of the body, each of which has demonstrated slightly different properties. These differences may be due to different isolation protocols or the heterogeneity of MSCs. In this study, we undertook a comparative analysis of the characteristics of MSCs derived from various human tissues to define their differences and find effective cell sources for cell therapy. Our results showed that the morphology of MSCs, including human dermal fibroblasts, did not differ. Immunophenotyping and analysis of pluripotency markers revealed similar expression patterns in all cell types, but the multilineage differentiation potential of

the cells *in vitro* was different depending on cell origin. All MSCs other than fibroblasts had tri-lineage differentiation capacity. Further, we attempted to identify specific markers to distinguish one type of MSC from the others. We also compared expression profiles of neurotrophic and anti-inflammatory factors, but there were no significant differences in our experiments, despite utilizing a variety of approaches. These results demonstrated that all MSCs with features of stem cells, such as differentiation capacity, from a variety of tissues are promising tools for cell-based therapies.

For successful clinical application, the viability and potency of *in vitro*-expanded MSCs need to be maintained during preparation and transportation prior to transplantation. However, the stability and potency of MSCs under these conditions have not been thoroughly examined. Another goal of this study was to standardize MSC preparation and storage prior to clinical application to ensure reproducible quality and potency for the intended clinical purpose. We examined the viability and potency of MSCs after short-term *in vitro* storage in saline or dextrose solution at 4℃ and room temperature (RT). We then analyzed cell viability, proliferation capacity, and differentiation potential. MSCs harvested and suspended in saline for 1~2 h showed greater than 90% viability regardless of storage temperature. When cells were stored for longer than 2 h in saline, however, their viability decreased gradually over time, whereas the viability of cells stored in dextrose deteriorated rapidly. MSCs lost their colony-forming unit (CFU) and differentiation capacities rapidly as storage time increased. Altogether, we found that a storage period greater than 2 h resulted in a significant decrease in cell viability, proliferation capacity, and differentiation potency. Therefore, storage of culture-harvested MSCs for longer than 2 h is likely to result in suboptimal MSC-mediated tissue regeneration due to decreased cell viability and differentiation capacity.

Key words : mesenchymal stem/stromal cells, differentiation, *in vitro* storage, cell therapy

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I. INTRODUCTION

Mesenchymal stem/stromal cells (MSCs) have high potential for regenerative medicine and cell therapy due to their accessibility, expandability, and multipotentiality. MSCs are defined by their plastic-adherent growth and by *in vitro* and *in vivo* differentiation potential.[1] They are of intense therapeutic interest because they have curative potential, as they can differentiate into mesodermal lineage cells, such as osteoblasts, adipocytes, and chondrocytes, as well as non-mesodermal cells, such as neural cells and hepatocytes.[2-5] MSCs have several advantages over other stem cells. First, their use evokes few or no ethical issues that are typically associated with the use of embryonic stem (ES) cells. Second, the relative ease with which they can be obtained and expanded *in vitro* makes MSCs

particularly attractive candidates for cell therapy in the regenerative medicine field. Third, MSCs are known to have immunosuppressive capacity, and thus, are likely beneficial for allogeneic transplantation because the risks of rejection and other complications are reduced. As interest in MSCs has increased, MSCs have been isolated from a variety of tissues, and they are extensively studied.[6-9] However, the characteristics and definition of MSCs described in these studies slightly differ. One major obstacle in the MSC field is the lack of standardized methods for MSC isolation and characterization for both research and clinical applications.

In 2006, the International Society for Cellular Therapy (ISCT) defined MSCs as cells with the following features: 1) plastic-adherent when maintained in standard culture conditions, 2) express CD105, CD73, and CD90 and lack expression of CD45, CD34, CD14 or CD11b, CD79α or CD19, and HLA-DR surface molecules, and 3) differentiate into osteoblasts, adipocytes, and chondroblasts *in vitro*.[10] Despite this definition, however, MSCs described in the literature often do not satisfy these criteria. In addition, the characteristics of MSCs can differ substantially depending on the specific protocol and tissue culture environment. Thus, it is important to clarify the features of MSCs in greater detail to support further developments in stem cell research.

It is currently thought that bone marrow is the most common tissue source of MSCs, but MSCs that are referred to as pericytes reside within the connective tissue of virtually all organs and tissues.[11] For example, isolation of MSCs is possible from adipose, placental, umbilical cord blood, umbilical cord, amniotic fluid, amnion, blood, liver, spleen, and lung tissues. MSCs derived from various human fetal and adult tissues display similar phenotypes and multilineage potential. Previously, it was reported that many genes were differentially expressed in MSCs isolated from different sources as determined by global gene expression profiles.[12] Even phenotypically identical MSCs exhibit fundamental differences in gene expression. Based on the results of global gene expression profile studies, MSCs derived from different sources might have different biological activities. Significant differences

have been also identified in the capacities of MSCs that are derived from different sources.[13] Thus, defining the various features of MSCs as well as suitable cell sources is necessary for clinical application of MSCs.

Growing knowledge of their differentiation potency and properties has intensified research on MSCs for clinical application to treat diseases such as myocardial infarction, ischemic stroke, Parkinson's disease, spinal cord injury, and renal failure.[14] In addition, MSCs possess immunomodulatory capacity and are thus being exploited to prevent or treat immunologic disorders, such as graft-versus-host disease (GvHD) and various autoimmune diseases.[15] Experimental studies have provided evidence that human MSCs can reduce the severity of a disease and/or enhance recovery.[16]

Given that MSC treatment of various degenerative diseases requires a large quantity of autologous or allogeneic MSCs for multiple injection/transplantation events, Good Manufacturing Practice (GMP)-compliant, large-scale *ex vivo* expansion is an essential component of obtaining cell products for therapeutic purposes.[17] In addition, the harvesting and subsequent storage of culture-expanded cells need to be standardized to ensure the suitability of cellular products for their clinically intended purpose and to reduce any significant adverse effects related to the storage process.[18]

Therefore, in this study, we first compared the morphology, immunophenotype, *in vitro* differentiation capacity, and gene expression profiles of pluripotency, anti-inflammatory, and neurotrophic factors of MSCs derived from adult human bone marrow, cord blood, placenta, and adipose tissues. We also evaluated the viability and potency of culture-expanded MSCs during *in vitro* storage to define the acceptable duration of cell storage prior to cell transplantation using the most distinguished source among the MSCs derived from various sources according to our data.

II. MATERIALS AND METHODS

1. Mesenchymal stem cell derivation from various sources

Healthy human donors undergoing bone marrow harvesting for allogeneic bone marrow transplantation donated 5–10 mL of bone marrow concentrate after providing informed consent according to institutional guidelines under an approved protocol (IRB 4-2008-0643).[19] Briefly, mononuclear cells (MNCs) from the bone marrow concentrates were separated by centrifugation over a Ficoll-Hypaque gradient (GE Healthcare, Uppsala, Sweden), suspended in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), L-glutamine, and penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA), plated in 75-cm^2 culture flasks (Nunc, Roskilde, Denmark), and incubated at 37℃ and 5% humidified CO_2. The next day, non-adherent cells were removed by washing with phosphate-buffered saline (PBS; Invitrogen), and monolayers of adherent cells were cultured until they reached 70% confluence. Cells were then trypsinized (0.05% trypsin with 0.1% EDTA; Invitrogen) and sub-cultured at a 1:3 ratio.

Cord blood samples from full-term deliveries, with the respective woman's informed consent, were collected, and the MNCs were separated within 6 h of collection using density gradient centrifugation. MNCs were washed three times with PBS and cultured at a density of $3x10^6$ cells/cm^2 in 6-well plates (Nunc) coated with fibronectin (Sigma-Aldrich, St. Louis, MO, USA) in endothelial growth medium-2 (EGM-2; Cambrex, Lonza, USA). The medium was changed every three days. After colony formation, between days 14 and 20, cells were harvested using 0.05% trypsin-EDTA solution (Invitrogen) and cultured thereafter in DMEM containing 10% FBS.

Human dermal fibroblasts were provided by Dr. Dong-Wook Kim (Yonsei University). Placenta (P) and adipose (A) tissue-derived MSCs were kindly provided by Dr. Ja Young Kwon (Yonsei University) and Dr. Kyoung Sik Kim (Yonsei University), respectively. Bone marrow (BM)-, cord blood (CB)-, and P-

MSCs were cultured and maintained in DMEM with 10% FBS and 1% penicillin/streptomycin. Fibroblasts were cultured in Medium 106 (Invitrogen). A-MSCs were cultured in DMEM with 10% FBS, 1% penicillin/streptomycin, 10 μg/mL fibronectin (Sigma-Aldrich), 10 ng/mL basic fibroblast growth factor (bFGF; Peprotech, London, UK), and 10 ng/mL epidermal growth factor (EGF; Peprotech). All cells were cultured at 37℃ with 5% CO_2, and media was replaced every three or four days. When cells reached a density of 80~90%, they were photographed with an inverted phase microscope (Olympus IX-71; Olympus Corporation, Japan) and sub-cultured. Fibroblasts were used as a negative control.

2. FACS analysis

Cells were stained with the following fluorochrome-conjugated antibodies: anti-CD14-FITC, anti-CD29-FITC, anti-CD31-PE, anti-CD34-FITC, anti-CD44-PE, anti-CD45-PE, anti-CD73-PE, anti-CD90-FITC, anti-CD105-PE, anti-CD106-FITC, and anti-STRO-1-PE (Beckman Coulter, Brea, California, USA). The corresponding isotype controls were also used. Anti-*TM4SF1* antibody was generously provided by Dr. Hoeon Kim (Biotherapeutic Division Aprogen, Inc., Daejon, Korea). First, the cells were stained with the anti-*TM4SF1* antibody for 20 min at 4℃. Next, anti-mouse FITC was added for 1 h at RT. The remaining antibodies were then added for 20 min at 4℃. The stained cells were washed with PBS and fixed with 1% paraformaldehyde (Biosesang, Seongnam-si, Korea). Finally, the labeled cells were analyzed using a Cytomics^{TM} Flow Cytometer (Beckman Coulter).

3. Differentiation assay

To induce osteogenic, adipogenic, and chondrogenic differentiation, the cells derived from each tissue were seeded simultaneously in differentiation media (Cambrex, Lonza). The cells were cultured for three weeks, changing the media every three or four days. Whenever the medium was changed for chondrogenesis, 10 ng/mL TGF-β3 was added to the cells. After three weeks, cells were analyzed for

osteogenesis, adipogenesis, and chondrogenesis by von Kossa staining, oil red O staining, and Safranin-O staining, respectively. The stained cells were photographed under a phase microscope (Olympus IX-71).

After detachment of MSCs from the plate, we evaluated storage conditions and duration in different media (Cambrex, Lonza) at 4℃ versus RT and the subsequent capacity of MSCs to differentiate into osteogenic, adipogenic, or chondrogenic lineages as described above.

4. Reverse transcription polymerase chain reaction (RT-PCR)

We performed PCR analysis to examine MSCs derived from various adult tissues and their pluripotent potency as compared to ES cells. We used cells derived from three different donors for our differentiation assay to account for donor-to-donor variation. Total RNA was extracted using RiboEx^{TM} (GeneAll, Seoul, Korea) reagent. Standard reverse transcription was carried out using Transcriptase II (Invitrogen). RT-PCR was performed with commercially available PCR primers (Bioneer, Daejeon, Korea) under the conditions listed in Table 1. As an internal control, *GAPDH* levels were used. In addition, cDNA from human embryonic stem cells (hESCs) was provided by Dr. Dong-Wook Kim (Yonsei University) and used as a control.

5. Cell viability test

Cultured BM-MSCs were harvested and suspended in 0.9% saline (JW Pharmaceutical, Seoul, Korea) or 0.5% dextrose (Sigma-Aldrich) prior to analysis. The same number of cells was subjected to storage for different durations of time (1, 2, 4, 6, and 8 h) and at two different temperatures (4℃ and RT). At the end of each time point, the viability of the stored cells was analyzed using the Annexin V-FITC/propidium iodide (PI) apoptosis detection kit (BioBud, Seoul, Korea) according to the manufacturer's instructions. Briefly, cells were stained with Annexin V-FITC for 15 min at RT in the dark. After washing, PI was added to cells,

and cell viability was analyzed with a Cytomics™ flow cytometer (Beckman Coulter).

6. Colony-forming unit (CFU) assay

To assess the capacity of cells to self-renew after *in vitro* short-term storage at 4℃ or RT, 1000 cells were seeded per 100-mm tissue culture plate (Corning, Corning, NY, USA). Following expansion for 10 days at 37℃ in a 5% humidified CO_2 atmosphere, cells were washed with PBS and stained with 0.5% crystal violet (Sigma-Aldrich) in methanol for 5 min at RT. The plates were then washed twice with PBS and photographed. Stained colonies with more than 50 cells were scored as colony-forming unit-fibroblasts (CFU-F) and were counted.

7. Statistical analysis

Results of replicate experiments are presented as mean ± standard deviation (SD). Statistical significance was assessed by comparing mean values (±SD) using the *t*-test and were considered statistically significant when $p < 0.05$.

Table 1. Primer sequences

Gene	Primer sequence	Annealing temperature (℃)	Product size (bp)
Oct4	GACAACAATGAGAACCTTCAGGAGA TTCTGGCGCCGGTTACAGAACCA	62	218
Sox2	AACCAAGACGCTCATGAAGAAG GCGAGTAGGACATGCTGTAGGT	62	341
c-Myc	TCGGATTCTCTGCTCTCCTC CGCCTCTTGACATTCTCCTC	62	413
Klf4	ATTCTCTCCAATTCGCTGACCC TTCAGCACGAACTTGCCCAT	62	376
Nanog	ATAGCAATGGTGTGACGCAG GATTGTTCCAGGATTGGGTG	62	219
Lin28	GCTCCGTGTCCAACCAGCAG TTTCCTTTTGGCCGCCTCTC	58	376
ActivinA	GATGTACCCAACTCTCAGCCA GCCGATGTCCTTGAAACTGAC	55	866
REX1	CTGAAGAAACGGGCAAAGAC GAACATTCAAGGGAGCTTGC	58	344
Runx2	GACCAGTCTTACCCCTCCTACC CTGCCTGGCTCTTCTTACTGAG	58	190
PPARG	TCTCTCCGTAATGGAAGACC GCATTATGAGACATCCCCAC	55	474
C/EBPA	CCAAGAAGTCGGTGGACAAGAA TCATTGTCACTGGTCAGCTCCA	62	145
BMP7	CCAACGTCATCCTGAAGAAATAC GCTTGTAGGATCTTGTTCATTGG	60	271
Sox9	GGTTGTTGGAGCTTTCCTCA TAGCCTCCCTCACTCCAAGA	61	400
SLC2A1	TCACTGTGCTCCTGGTTCTG CCTGTGCTCCTGAGAGATCC	60	230
PTGES	CTTCCTTTTCCTGGGCTTCG GAAGACCAGGAAGTGCATCCA	60	77
MFGE8	GCCCTGGATATCTGTTCCAA GCTCGACACATTTCGTCTCA	58	151
PPAP2B	CGACTTCGGTTACTGCCTTC GCTTCTCTGGCTCCTTCTGA	60	204
CD146	CATCCAGCTCCGCGTCTACA ACCAGCTGTGTGCGGTTCAG	52	888
SLIT3	GCGCCTGAACAAGAATAAGC CCAGTTGCAGGTTCTTCACA	58	152

LTBP2	GCCAAGGAGTAGCAGTCAGG AGCTGGGAGAGATGAAAGCA	58	157
CDH11	CTGTGTCTGGCGTTCTCAAG TGTCCAGAGTTTAAGGCGAAA	58	391
NGF	GACAGTGTCAGCGTGTGGGTT CCCAACACCATCACCTCCTT	57	75
BDNF	AACCATAAGGACGCGGACTTG TGTTTGCGGCATCCAGGTA	55	117
NT-3	AAACGTCGCAAACCTACGTCC TGACAAGGCACACACACAGGA	55	100
TGS-6	GGTGTGTACCACAGAGAAGCA GGGTTGTAGCAATAGGCATCC	60	284
TGF-β1	GAGGTGACCTGGCCACCATT TCCGCAAGGACCTCGGCTGG	55	194
GAPDH	GTGGTCTCCTCTGACTTCAACA CTCTTCCTCTTGTGCTCTTGCT	62	22

III. RESULTS

1. Morphology of MSCs from different human adult tissues

MSCs were obtained and cultured from human bone marrow (BM), umbilical cord blood (CB), placenta (P), and adipose (A) tissue. Human fibroblasts were isolated from dermis. All MSCs, including fibroblasts, displayed a spindle-shaped morphology and no differences in morphology were obvious (Figure 1).

2. Immunophenotyping

All types of MSCs displayed very similar immunophenotype patterns by flow cytometric analysis (Figure 2). The cells were negative for CD14, CD31, CD34, CD45, and CD106 (known markers of hematopoietic and endothelial cells), while they were strongly positive for CD29, CD44, CD73, CD90, and CD105 (well-known markers of MSCs). Expression of CD90 in P-MSCs was less obvious compared to the other cells. Most markers were expressed in all types of MSCs, including fibroblasts. These results indicated that the different MSC types are indistinguishable by surface molecule expression. By flow cytometric analysis using anti-*TM4SF1* antibody, which was reported to be a specific marker for MSCs by Sohyun Bae,[20] only BM- and CB-MSCs were positive for *TM4SF1*, whereas cells derived from other sources were negative. The expression of Stro-1, which is the best-known MSC marker,[21] was very low in our experiments. This may be the result of our experimental conditions and/or the use of a different antibody. Taken together, no significant differences in the expression of these surface antigens, including *TM4SF1* and Stro-1, could be observed. These surface markers are therefore insufficient for the identification or distinction of MSCs derived from various tissues.

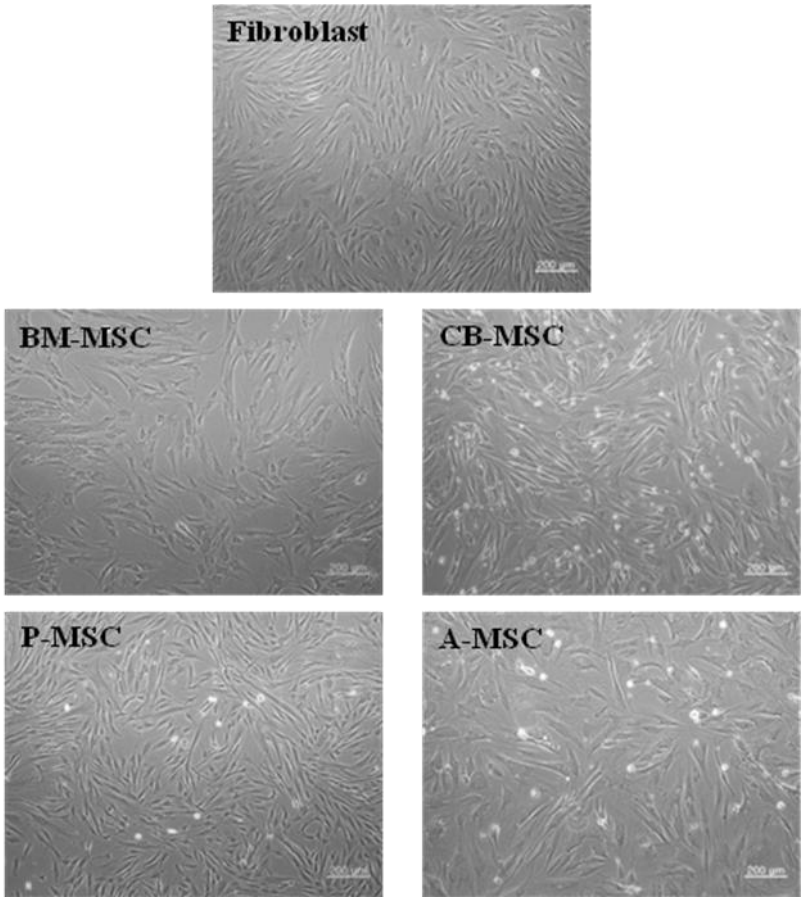

Figure 1. Morphology of mesenchymal stem cells (MSCs) derived from various human adult tissues was taken picture by phase microscope (x100). (BM:Bone Marrow, CB:Cord Blood, P:Placenta, A:Adipose tissue)

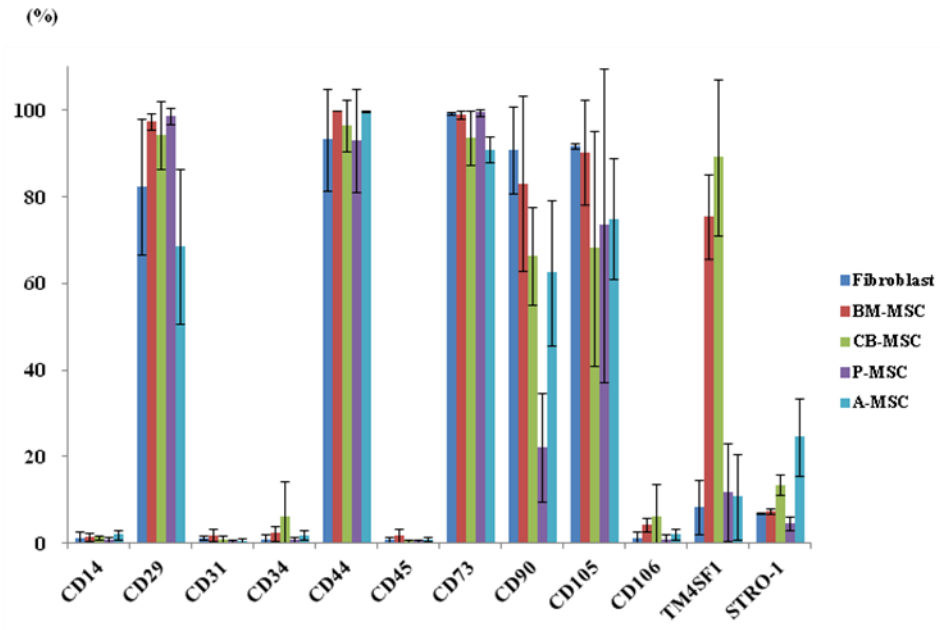

Figure 2. Immunophenotyping of MSCs derived from different sources by flow cytometry (n=3). (BM:Bone Marrow, CB:Cord Blood, P:Placenta, A:Adipose tissue)

3. Differentiation capacity of MSCs *in vitro*

Next, we performed differentiation assays with all MSC types to identify any differences. Calcium deposition was verified in all BM- and A-MSCs, whereas other MSCs either did or did not differentiate into osteoblasts when cultured in osteogenic differentiation medium (Figure 3). Fibroblasts were negative for osteogenesis, despite long-term induction. Unlike osteogenic differentiation, lipid

vacuoles were observed in all cells by oil red O staining after adipogenic differentiation (Figure 4). Human fibroblasts also differentiated into adipocytes, which is in accordance with previous results (Figure 4).[22-23] All cells were positive for adipogenesis, but BM- and A-MSCs, as observed for osteogenesis, were strongly positive, and many lipid vacuoles were observed in these cells after adipogenic differentiation. Chondrogenesis was next verified by Safranin-O staining after induction for three weeks. After two weeks, a cartilage-like morphology appeared, all MSC types were positive by Safranin-O staining, and significant no differences were observed (Figure 5). All MSCs, except for fibroblasts, displayed multipotency into three lineages. BM- and A-MSCs were completely and strongly differentiated into osteoblasts, adipocytes, and chondrocytes, whereas CB- and P-MSCs showed limited capacity, differentiating into one or two lineages depending on the donor. Analysis of surface proteins revealed that fibroblasts displayed very similar expression patterns as MSCs. Furthermore, fibroblasts, used as a negative control, differentiated into adipocytes and chondrocytes, but they did not differentiate into osteoblasts. These results indicated that the *in vitro* differentiation assay could be a useful tool for discriminating MSCs from other cell types. We also showed that BM- and A-MSCs had stronger differentiation capacity compared to the other MSC types, suggesting that BM- and A-MSCs may be more attractive options for tissue engineering. We did not find any correlation between immunophenotype and differentiation capacity among MSCs. As shown in Figure 6, all cells similarly expressed osteoblast-, adipocyte-, and chondrocyte-related genes; however, despite these PCR results, the cells showed variable differentiation in *in vitro* tests.

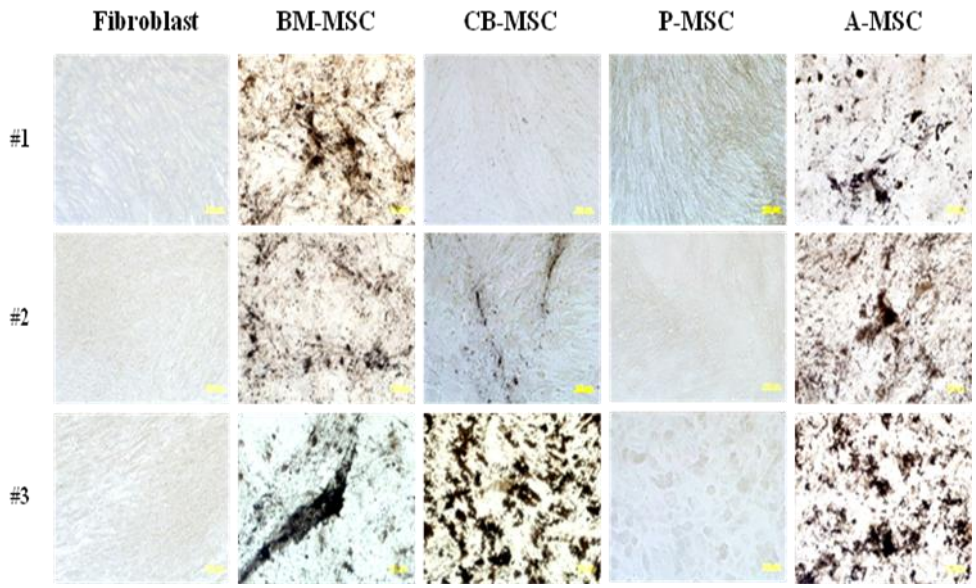

Figure 3. Osteogenesis. Fibroblast, Bone Marrow (BM)-, Cord Blood (CB)-, Placenta (P)-, and Adipose (A) tissue-derived MSCs were evaluated by von Kossa staining after osteogenic induction (x200).

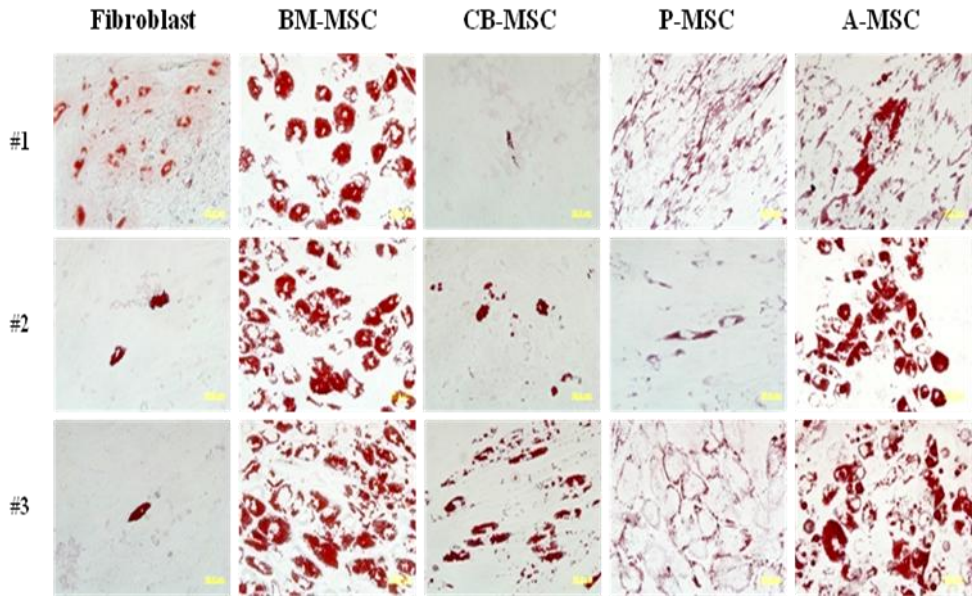

Figure 4. Adipogenesis. Fibroblast, Bone Marrow (BM)-, Cord Blood (CB)-, Placenta (P)-, Adipose (A) tissue-derived MSCs were stained by Oil red O after adipogenic induction (x400).

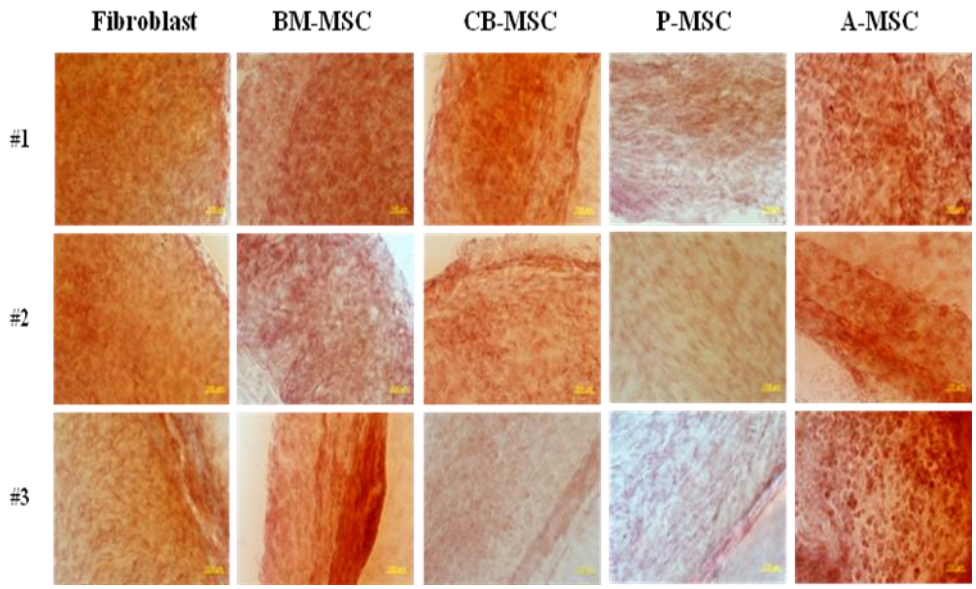

Figure 5 Chondrogenesis. Fibroblast, Bone marrow (BM)- Cord Blood (CB)-, Placenta (P)-, Adipose (A) tissue-derived MSCs were induced and analyzed by Safranin-O staining (x200).

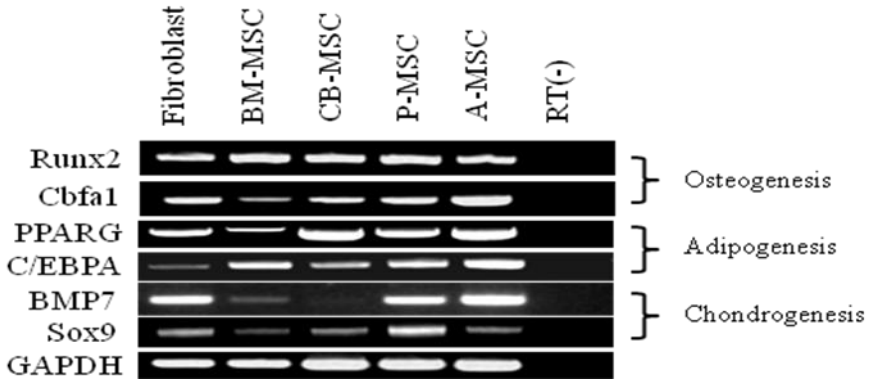

Figure 6 Osteogenesis-, Adipogenesis-, and Chondrogenesis-associated markers were examined by RT-PCR.

4. Expression of pluripotency-associated markers in MSCs

We also analyzed pluripotency markers of MSCs compared to ES cells by PCR. Pluripotency markers, such as *Oct4*, *Sox2*, *c-Myc*, *Klf4*, *Nanog*, *Lin28*, *Activin A*, and *REX1*, were expressed in ES cells, whereas MSCs, including fibroblasts, did not express those genes or expressed them differently depending on the cell source (Figure 7). No MSCs with similar expression patterns as ES cells were observed, indicating that MSCs possess limited differentiation potential.

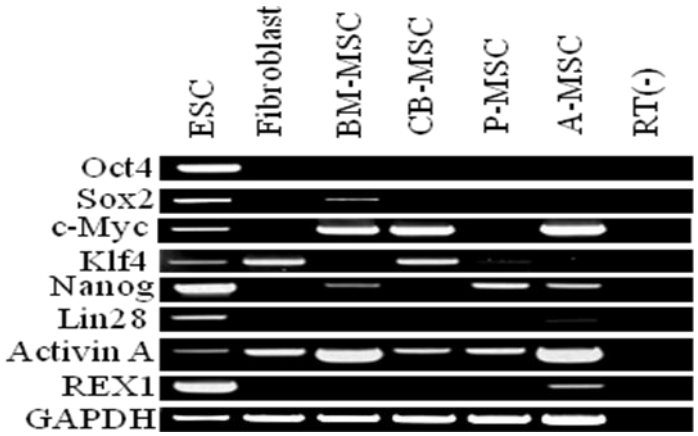

Figure 7. Gene expression of pluripotency markers was analyzed by RT-PCR. ES

cells were used as a positive control.

5. Gene expression analysis to identify a specific marker of MSCs

We performed PCR analysis to examine the expression of CD146, which was reported to be a marker for primitive MSCs.[24] The results showed that CD146 was strongly expressed by all MSC types, indicating that it could be a marker restricted to primitive MSCs (Figure 8). Next, we tried to identify a candidate surface marker that could distinguish MSCs from fibroblasts, in response to previous results.[20] We detected up-regulation of *SLC2A1*, *PTGES*, *MFGE8*, *PPAP2B*, and *ANKH* genes in MSCs relative to the other cell populations.[20] *PTGES* and *MFGE8* were slightly differently expressed, but this difference was not significant. In addition, we evaluated *SLIT3*, *LTBP2*, and *CDH11* genes, which are reportedly involved in stemness regulation, to further investigate a discriminating marker in response to a previous dataset of 190 transcripts.[25] However, all MSCs, including fibroblasts, expressed the selected markers similarly. Taken together, many of the genes we selected as candidate, novel, specific markers were similarly expressed by all cells, and both fibroblasts and MSCs exhibited similar expression levels for these genes.

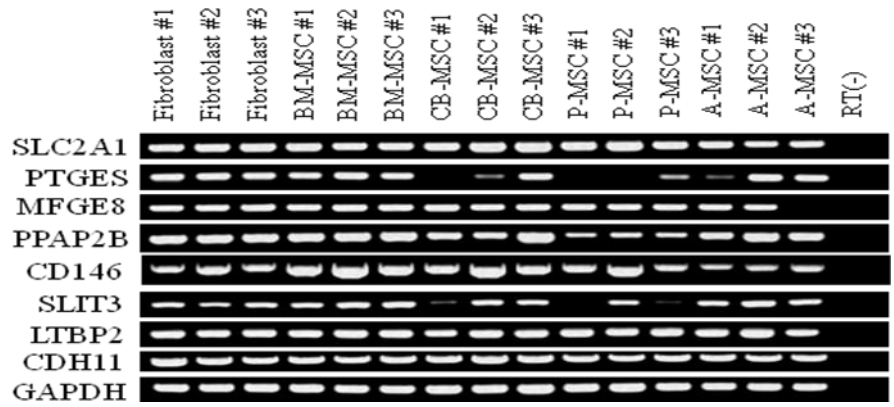

Figure 8. PCR analysis was performed to identify a specific marker among various sources.

6. Expression of neurotrophic and anti-inflammatory factors in MSCs derived from various tissues

MSCs release neurotrophic factors, including glial-derived neurotrophic factor (GDNF).[26] To further compare the characteristics of MSCs derived from different tissues, we carried out PCR analysis to examine the expression of various neurotrophic factors. The results showed little difference in BDNF (brain-derived neurotrophic factor) expression, but NGF (nerve growth factor) and NT-3 (Neurotrophin-3) displayed similar expression patterns in all MSC types (Figure 9). It is also known that MSCs secrete anti-inflammatory cytokines and growth factors.[27] We next studied the expression by MSCs of the paracrine effectors TSG-6 (TNF-α-stimulated gene 6) and TGF (transforming growth factor)-β1. The results showed that all MSCs, including fibroblasts, expressed both TSG-6 and TGF-β1. The markers analyzed herein are well-known, representative neurotrophic and anti-inflammatory factors of MSCs; however, strangely, these factors were expressed equally in fibroblasts. This suggests that fibroblasts have considerably similar properties to MSCs.

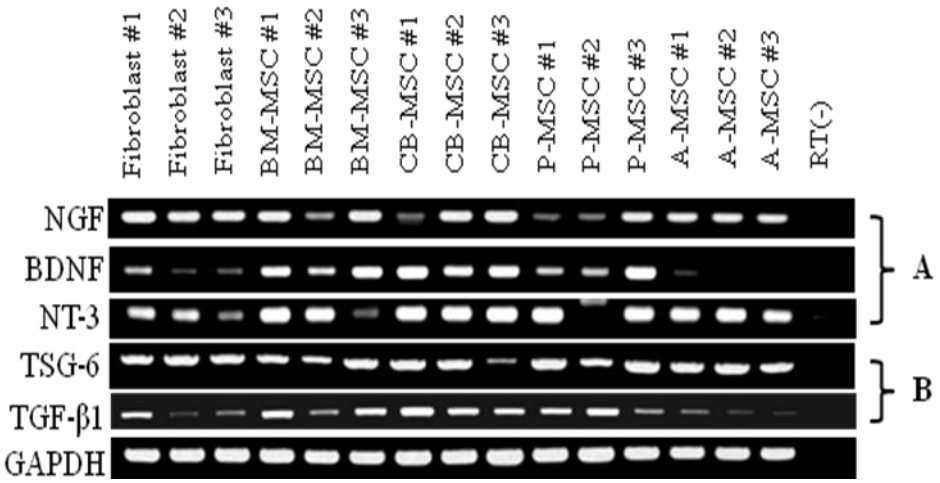

Figure 9. (A) Expression of neurotrophic factor in cell from various sources was examined by RT-PCR. (B) Expression of anti-inflammatory genes in diverse cells

by RT-PCR.

7. Effect of culture medium and *in vitro* storage duration on cell viability

When differentiation capacity and pluripotency associated markers were compared among MSCs derived from various tissue sources, BM- and A-MSCs showed similar characteristics. Therefore, the effects of storage duration, culture medium type, and temperature on harvested MSCs with regard to their subsequent viability and potency for cellular therapeutic purposes were examined using BM-MSCs.

After storage for 1, 2, 4, 6, or 8 h at 4℃ or RT in 0.9% saline solution or 5% dextrose solution, cell viability was measured by Annexin V-FITC/PI staining. Cell viability decreased gradually when cells were retrieved from culture plates with trypsin-EDTA. MSCs stored in saline for 1 or 2 h at either 4℃ or RT displayed >90% viability, while MSCs stored for 6 h at either 4℃ or RT routinely displayed >85% viability (Figure 10). When cells were stored in dextrose, however, their viability decreased significantly, even after 1 h. To confirm their viability, cells stored in saline or dextrose for 2 h were re-plated on tissue culture plates with complete culture medium and incubated overnight, and cell adhesion and proliferation were monitored. Compared to cells stored in saline (>80% viability), which showed viability and proliferation characteristics of normal MSCs, cells maintained in dextrose failed to bind to the culture plate (<5%), indicating a loss of viability (Figure 11). While the viability of MSCs stored for 2 h in dextrose solution was approximately 80% as determined by flow cytometric analysis, they were not viable, as verified by typical *in vitro* culture conditions. Together, these results showed that the viability of MSCs stored in saline solution decreased gradually over time regardless of the storage temperature, while that of MSCs kept in dextrose decreased dramatically over a short period of time.

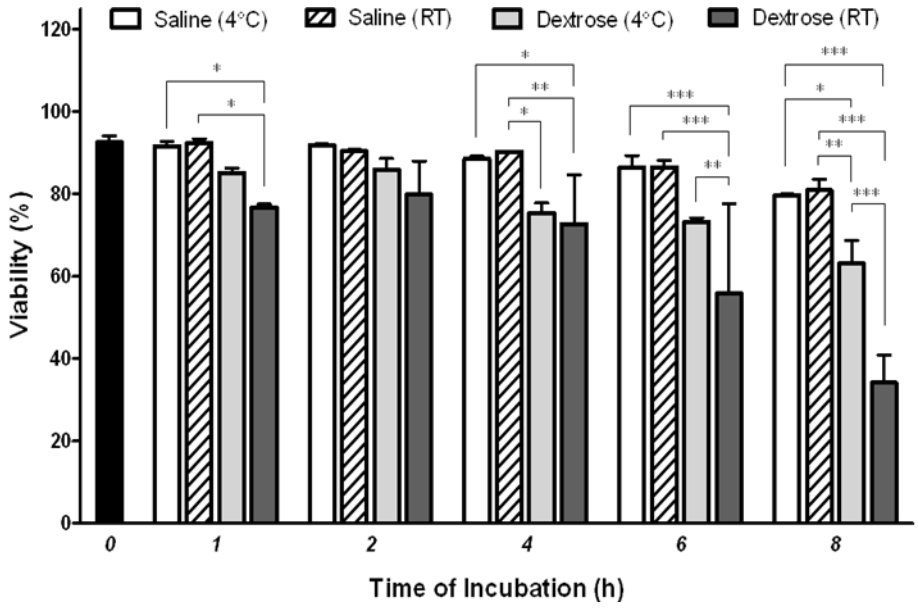

Figure 10. Effects of storage duration, media, and temperature on the viability of MSCs. MSCs were stored in saline or dextrose at 4℃ or RT from 0 h to 8 h. The data represent the mean ± SD of three experiments. The asterisk (*) indicates a significant difference at the 95% level between the control (0 h) and *in vitro*-stored samples.

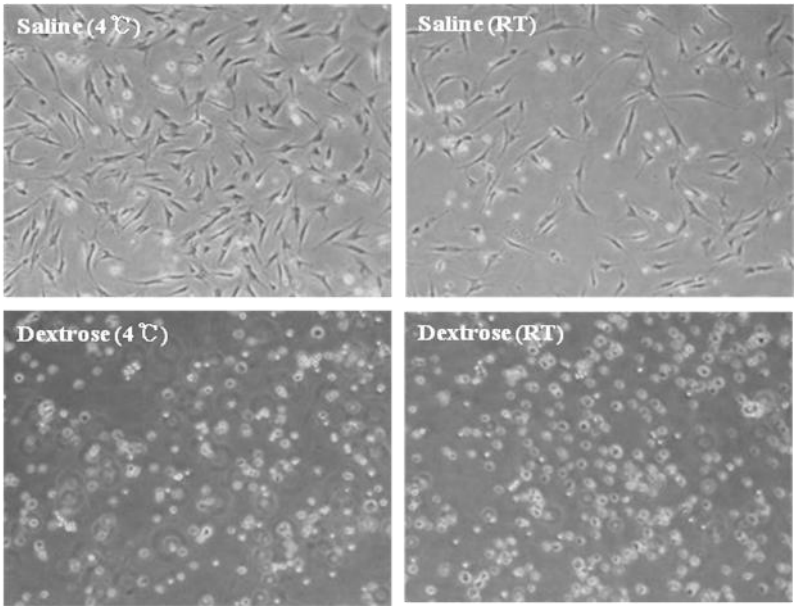

Figure 11. Morphology of MSCs replated on culture plates after storage in different types of media for 2 h at 4℃ and RT. MSCs stored in dextrose were much less viable than cells stored in saline solution. Magnification, 100 x.

8. Effect of short-term *in vitro* storage on self-renewal capacity

We measured the CFU-F capacity of MSCs stored *in vitro* for short periods of time at 4℃ and RT. While cells stored for 2 h *in vitro* in saline solution showed greater than 90% viability, colony frequency decreased rapidly even after 1 h storage at either 4℃ or RT (Figure 12). Furthermore, cells stored for 2 h experienced a ~50% loss in total colony frequency compared to cells freshly harvested from culture. Cells stored for 4 h had approximately 1/5 of the CFU-F ability of control cells. Similar to the findings of the cell viability test, storage temperature did not significantly affect the self-renewal capacity of the cells. These results indicated that the viability of MSCs in saline did not necessarily reflect self-renewal capacity or cell proliferation capacity as shown in the CFU-F assay.

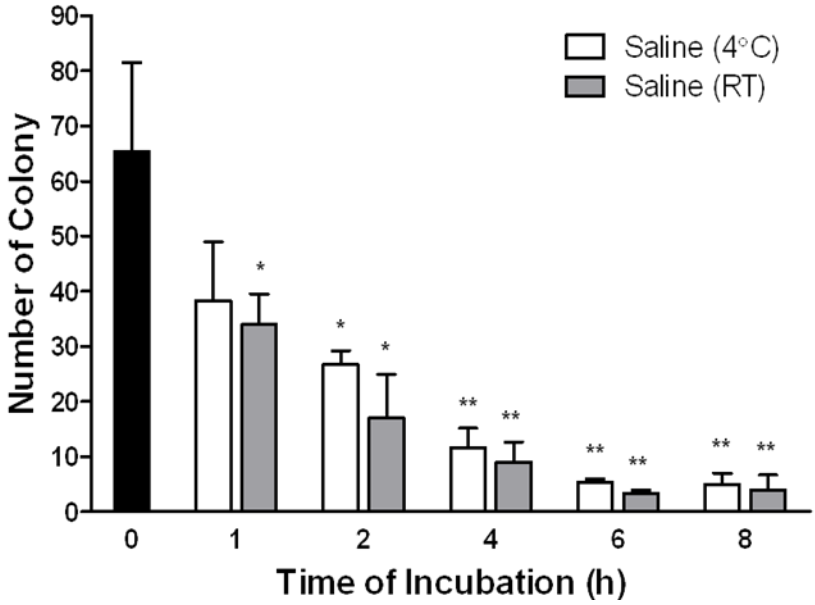

Figure 12. CFU-F assay of MSCs after *in vitro* short-term storage. Representative images of 10 day CFU-F. The number of colonies (>50 cells) that formed per 1000 cells was enumerated.

9. Effect of short-term *in vitro* storage on differentiation potency

After storage for 2 or 6 h in saline solution, cells were cultured in complete differentiation medium until they reached a confluence level appropriate for the different differentiation assays described in detail in Materials and Methods. As shown in Figure 13, the differentiation potential of MSCs gradually decreased over time, regardless of storage temperature, when compared to freshly harvested MSCs. Cells stored for 6 h *in vitro* exhibited a much weaker differentiation potential than control cells, implying that a substantial fraction of the cells lost their differentiation capacity during *in vitro* storage. In contrast, cells stored for 2 h at either 4℃ or RT

exhibited a slightly reduced differentiation potential compared to that of control cells. These results strongly suggest that *in vitro* storage of MSCs for longer than 2 h can significantly affect both their self-renewal capacity and multipotency.

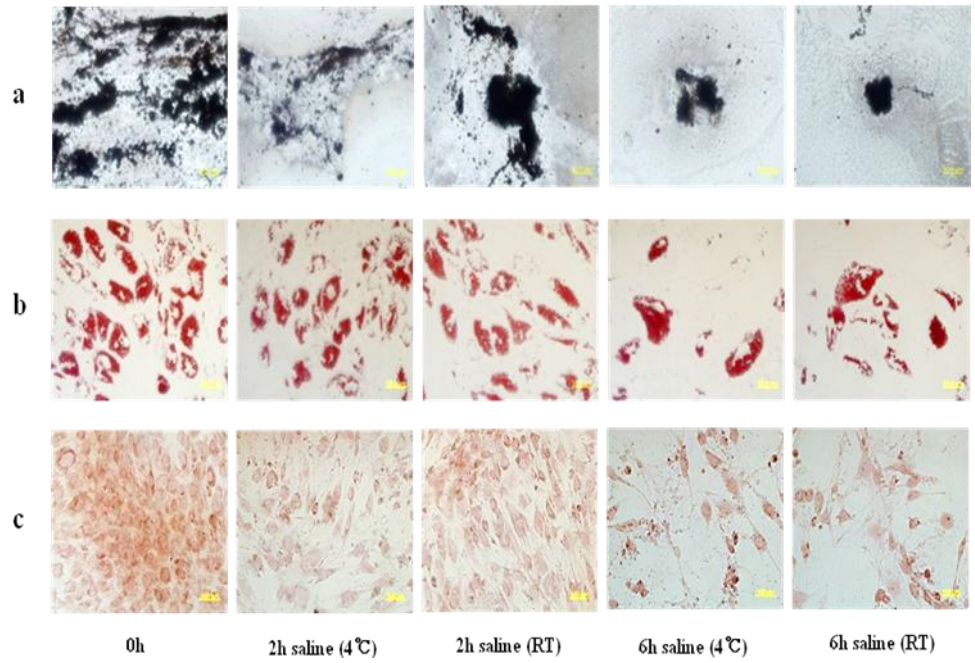

Figure 13. Differentiation capacity of MSCs upon short-term *in vitro* storage in saline solution at 4℃ or RT. Osteogenic (a), adipogenic (b) and chondrogenic (C) differentiation of MSCs were slightly and drastically reduced after 2 h and 6 h of *in vitro* storage, respectively. These results are representative of three independent experiments.

IV. DISCUSSION

Since it was first discovered that a variety of MSC-like cells existed in various tissues of the human body, many researchers have isolated MSC-like cells from these tissues. Protocols for the expansion, characterization, and functional analysis of MSCs vary widely. To resolve this issue, in 2006 the ISCT offered classification criteria of MSCs to clarify their characteristics. Cells with the following features were defined as MSCs: 1) expression of specific surface markers, including CD73, CD105, CD29, CD44, and CD90, 2) adherence to plastic surfaces, and 3) the capacity to differentiate into osteoblasts, adipocytes, and chondrocytes. Despite these criteria, cells with few of the defined surface markers or with partial differentiation potential have been referred to as MSCs in published studies.

Here, we tried to examine and compare the characteristics of cells referred to as MSCs derived from a variety of sources. We prepared BM-, CB-, P-, and A-MSCs for study, and fibroblasts were used as a negative control. Morphology, analysis of surface proteins, and differentiation tests were used to define MSC characteristics. By morphological observation, all MSCs derived from the sources examined displayed fibroblastic, spindle-like shapes under a phase microscope, and there were no significant differences in growth rate. In addition, all MSC types similarly expressed most of the surface proteins commonly known as MSC markers. We also analyzed the expression of *TM4SF1* (transmembrane 4 L6 family), which is known to induce formation of membrane microdomains and play diverse roles in various blood cells. It was also recently reported to be a specific marker of MSCs.[20] By flow cytometric analysis, all MSCs, including fibroblasts, showed identical expression patterns of *TM4SF1*, but fibroblasts, P-, and A-MSCs were negative for *TM4SF1* expression, whereas BM- and CB-MSCs strongly expressed *TM4SF1*. Since only BM- and CB-MSCs expressed *TM4SF1*, we thought it might be an indicator of differentiation potential. It was previously reported that *TM4SF1*-positive cells could be induced to differentiate along an osteogenic lineage *in vivo*.[20] Thus, we

performed a differentiation assay to evaluate the differentiation capacity of these cells. We found that all MSCs had the potential to differentiate into mesenchymal lineage cells, such as osteoblasts, adipocytes, and chondrocytes, regardless of *TM4SF1* expression. It was observed that BM- and A-MSCs had the strongest differentiation capacity. In 2007, Lysy *et al.* demonstrated that human dermal-derived fibroblasts had mesodermal stem cell characteristics.[28] In our experiments, fibroblasts differentiated into adipocytes and chondrocytes, but they did not develop into osteoblasts, even after long periods of culture. To further investigate differentiation potential, we examined the expressions of mesenchymal lineage-associated genes, such as *Runx2*, *Cbfa1*, *PPARG*, *C/EBPA*, *BMP7*, and *Sox9*, by PCR analysis. According to the results, all cells expressed these genes similarly, and we did not find any differences based on MSC source. Taken together, we could not distinguish MSCs from fibroblasts, because both cell types had similar expression patterns of surface proteins. Although fibroblasts differentiated into adipocytes and chondrocytes, they did not show complete differentiation capacity, unlike MSCs, indicating that the *in vitro* differentiation assay may be an important tool to discern MSCs.

MSCs as adult stem cells have self-renewal and multipotent properties. These features are important for tissue engineering and clinical applications. We examined which cells had more pluripotent capacity among MSCs derived from different tissues, compared to ES cells. It is known that *Oct4*, *Sox2*, and *Nanog* play key roles in maintaining stem cells in an undifferentiated state, indicating that those genes have vital functions for stem cells. Thus, we performed PCR analysis to analyze the expressions of pluripotency markers, including *Oct4*, *Sox2*, *c-Myc*, *Klf4*, *Nanog*, *Lin28*, *Activin A*, and *REX1*. The results showed that MSCs, including fibroblasts, expressed a portion of these genes compared to ES cells.

Previously, it was reported that the neural ganglioside, GD2, is a novel surface marker of MSCs.[29] We carried out PCR analysis to identify specific markers of MSCs derived from different tissues. We selected genes that were up-regulated in

MSCs and were related to stemness, and compared their relative expressions. Unfortunately, no specific marker was detected by the PCR results. This may be due to our gene selection or to heterogeneity of MSCs. As it is known that MSCs secrete molecules with potentially critical roles in regenerative medicine, we performed PCR analysis to investigate the expression of neurotrophic and anti-inflammatory factors in the cells derived from diverse sources. As with the previous PCR results, however, we did not observe differences. All MSCs, including fibroblasts, expressed these factors similarly.

Herein, we analyzed MSCs derived from bone marrow, cord blood, placental, and adipose tissues to compare their features *in vitro* as well as their gene expression levels and basic MSC characteristics. The cells from different tissues displayed similar morphologies and immunophenotypes, and there were no differences in gene expression levels, pluripotent or multipotent capacity, or expression of neurotrophic and anti-inflammatory factors. We also attempted to identify a specific marker of MSCs, but failed to do so based on the results of our PCR analysis.

Our study had several limitations. For example, a wide range of available analytical tools was not used. Certainly, results may have showed differences due to individual variations and/or use of different protocols. In addition, this study was only performed *in vitro*; results may be different *in vivo*. In our analysis, fibroblasts were similar to MSCs in all aspects, except that we could distinguish MSCs from fibroblasts by *in vitro* differentiation assay. It was difficult to identify multipotency-associated factors among MSCs from various sources. This may have been due to the heterogeneous population of MSCs. More detailed approaches, such as single-cell assays, are needed.

To ensure the safety and potency of a cell therapy product for transplantation, special care should be taken to standardize the cell processing conditions, including culture, storage, and transportation, as these conditions can significantly affect the viability and subsequent functionality of the final product. While there is a general

consensus from the majority of cell processing facilities that *in vitro* storage for hematopoietic stem cells/hematopoietic progenitor cells (HSC/HPC) should be limited to less than 2 h for ensuring the integrity of cell therapy products,[30] no guidelines for MSCs or other cellular products with regard to short-term storage are available. In addition, there is very limited information about the optimal media for storage and transportation of MSCs. In our study, only BM- and A-MSCs completely and strongly differentiated into osteoblasts, adipocytes, and chondrocytes. We investigated the effect of the storage medium, storage duration, and storage temperature on the viability and multipotency of cultured BM-MSCs after harvesting, as cell viability and multipotency are critical determinants of whether the MSCs will be clinically effective upon transplantation. Our results showed that MSCs stored in 0.9% saline maintained their viability (>85%) after 6 h. When cells were stored for 2 h or less, their self-renewal capacity and differentiation potency were slightly decreased; however, when cells were stored for longer than 2 h, their self-renewal capacity and differentiation potency decreased markedly. In contrast, Pal *et al.*[31] recently reported that holding (storing) MSCs for less than 8 h at 4℃ in 5% dextrose solution or up to 6 h at 4℃ in saline was permissible for clinical transplantation, as assessed by their viability and osteogenic/adiopogenic differentiation assays. When dextrose was used as a storage solution in the current study, the viability of cells decreased rapidly and cells failed to adhere and proliferate even after only 2 h of *in vitro* storage at 4℃ or RT. This is in agreement with recent reports, which state that exposing MSCs to high levels of glucose reduced their colony-forming activity and induced premature senescence.[32-33] Li *et al.*[34] also reported that 4-day exposure to a high concentration of glucose significantly decreased the proliferation of MSCs. However, the precise mechanism by which dextrose could affect cell quality during short-term storage is not clear.

Storage time after harvest from culture vessels is a critical factor for maintaining viability of human MSCs in clinical settings. While Lee *et al.* reported that MSCs maintained viability up to 6 h after harvesting by flow cytometric

analysis, ultra-structural morphological analysis, gene expression profiling, proliferative capacity, and differentiation potency after 6- and 12-h of storage in PBS have not been determined.[35] Muraki *et al.*[14] reported that human MSCs can be stored for up to 24 h in PBS without a significant loss of viability (>85%) and while preserving osteogenic potency. These discrepancies with our results may be due to differences in media (PBS versus saline) and/or which differentiation assay was utilized. PBS is known to possess stronger pH buffering capacity than saline. While short-term stored MSCs in our hands also displayed differentiation capacity, it was far less efficient than that of freshly prepared MSCs. The storage temperature (4℃ versus RT) did not significantly affect cell quality. Although cell viability is the most critical issue in clinical application of cell products, as the absolute number of viable cells is the most important index of transplantation efficacy, our data strongly suggest that other measures addressing self-renewal capacity and differentiation capacity should be considered for determining MSC suitability for clinical transplantation. For example, MSCs stored for 4 h exhibited over 90% viability, which was comparable to that of cells stored for 1 and 2 h. However, the self-renewal capacity of these cells (as shown by CFU-F) was severely reduced, implying that gross cell viability is not a credible indicator of the quality of clinical cell products.

These results indicate that MSCs may lose their potency under conventional conditions in a very short period of time; we therefore strongly recommend the development of improved storage and transportation media and/or strategies to preserve the quality of MSCs. This study confirms the critical importance of prompt usage of cell therapy products after cell processing. We anticipate that the findings presented here will facilitate safe and efficient storage of stem cell therapeutics for clinical applications.

REFERENCES

1. Javazon EH, Beggs KJ, Flake AW. Mesenchymal stem cells: paradoxes of passaging. Exp Hematol 2004 May;32(5):414-25.
2. Prockop DJ, Gregory CA, Spees JL. One strategy for cell and gene therapy: harnessing the power of adult stem cells to repair tissues. Proc Natl Acad Sci U S A 2003 Sep;30:100.
3. Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, et al. Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 2002 Jul;418(6893):41-9.
4. Petersen BE, Bowen WC, Patrene KD, Mars WM, Sullivan AK, Murase N, et al. Bone marrow as a potential source of hepatic oval cells. Science 1999 May;284(5417):1168-70.
5. Schwartz RE, Reyes M, Koodie L, Jiang Y, Blackstad M, Lund T, et al. Multipotent adult progenitor cells from bone marrow differentiate into functional hepatocyte-like cells. J Clin Invest 2002 May;109(10):1291-302.
6. Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, et al. Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng 2001 Apr;7(2):211-28.
7. Toma JG, Akhavan M, Fernandes KJ, Barnabe-Heider F, Sadikot A, Kaplan DR, et al. Isolation of multipotent adult stem cells from the dermis of mammalian skin. Nat Cell Biol 2001 Sep;3(9):778-84.
8. Campagnoli C, Roberts IA, Kumar S, Bennett PR, Bellantuono I, Fisk NM. Identification of mesenchymal stem/progenitor cells in human first-trimester fetal blood, liver, and bone marrow. Blood 2001 Oct;98(8):2396-402.
9. Young HE, Steele TA, Bray RA, Hudson J, Floyd JA, Hawkins K, et al. Human reserve pluripotent mesenchymal stem cells are present in the connective tissues of skeletal muscle and dermis derived from fetal, adult, and geriatric donors. Anat Rec 2001 Sep;264(1):51-62.

10. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 2006;8(4):315-7.
11. da Silva Meirelles L, Chagastelles PC, Nardi NB. Mesenchymal stem cells reside in virtually all post-natal organs and tissues. J Cell Sci 2006 Jun 1;1(11):2204-13.
12. Wagner W, Wein F, Seckinger A, Frankhauser M, Wirkner U, Krause U, et al. Comparative characteristics of mesenchymal stem cells from human bone marrow, adipose tissue, and umbilical cord blood. Exp Hematol 2005 Nov;33(11):1402-16.
13. Sakaguchi Y, Sekiya I, Yagishita K, Muneta T. Comparison of human stem cells derived from various mesenchymal tissues: superiority of synovium as a cell source. Arthritis Rheum 2005 Aug;52(8):2521-9.
14. Salem HK, Thiemermann C. Mesenchymal stromal cells: current understanding and clinical status. Stem Cells. 2010;28:585-96.
15. Kode JA, Mukherjee S, Joglekar MV, Hardikar AA. Mesenchymal stem cells: immunobiology and role in immunomodulation and tissue regeneration. Cytotherapy. 2009;11:377-91.
16. Satija NK, Singh VK, Verma YK, Gupta P, Sharma S, Afrin F, et al. Mesenchymal stem cell-based therapy: a new paradigm in regenerative medicine. J Cell Mol Med. 2009;13:4385-402.
17. Sotiropoulou PA, Perez SA, Salagianni M, Baxevanis CN, Papamichail M. Characterization of the optimal culture conditions for clinical scale production of human mesenchymal stem cells. Stem Cells. 2006;24:462-71.
18. Ginis I, Grinblat B, Shirvan MH. Evaluation of bone marrow-derived mesenchymal stem cells after cryopreservation and hypothermic storage in clinically safe medium. Tissue Eng Part C Methods. 2012;18:453-63.
19. Kim HS, Choi DY, Yun SJ, Choi SM, Kang JW, Jung JW, et al. Proteomic

analysis of microvesicles derived from human mesenchymal stem cells. J Proteome Res. 2012;11:839-49.

20. Bae S, Shim SH, Park CW, Son HK, Lee HJ, Son JY, et al. Combined omics analysis identifies transmembrane 4 L6 family member 1 as a surface protein marker specific to human mesenchymal stem cells. Stem Cells Dev 2011 Feb;20(2):197-203.
21. Ning H, Lin G, Lue TF, Lin CS. Mesenchymal stem cell marker Stro-1 is a 75 kd endothelial antigen. Biochem Biophys Res Commun 2011 Sep;413(2):353-7.
22. Kern S, Eichler H, Stoeve J, Klüter H, Bieback K. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. Stem Cells 2006 May;24(5):1294-301.
23. Jääger K, Neuman T. Human dermal fibroblasts exhibit delayed adipogenic differentiation compared with mesenchymal stem cells. Stem Cells Dev 2011 Aug;20(8):1327-36.
24. Baksh D, Yao R, Tuan RS. Comparison of proliferative and multilineage differentiation potential of human mesenchymal stem cells derived from umbilical cord and bone marrow. Stem Cells 2007 Jun;25(6):1384-92.
25. Saulnier N, Puglisi MA, Lattanzi W, Castellini L, Pani G, Leone G, et al. Gene profiling of bone marrow- and adipose tissue-derived stromal cells: a key role of Kruppel-like factor 4 in cell fate regulation. Cytotherapy 2011 Mar;13(3):329-40.
26. Whone AL, Kemp K, Sun M, Wilkins A, Scolding NJ. Human bone marrow mesenchymal stem cells protect catecholaminergic and serotonergic neuronal perikarya and transporter function from oxidative stress by the secretion of glial-derived neurotrophic factor. Brain Res 2012 Jan;11(1431):86-96.
27. van Buul GM, Villafuertes E, Bos PK, Waarsing JH, Kops N, Weinans H, et al. Mesenchymal stem cells secrete factors that inhibit inflammatory processes in short-term osteoarthritic synovium and cartilage explants culture. Osteoarthritis Cartilage 2012 Oct;20(10):1186-96.
28. Lysy PA, Smets F, Sibille C, Najimi M, Sokal EM. Human skin fibroblasts:

From mesodermal to hepatocyte-like differentiation. Hepatology 2007 Nov;46(5):1574-85.

29. Martinez C, Hofmann TJ, Marino R, Dominci M, Horzitz EM. Human bone marrow mesenchymal stromal cells express the neural ganglioside GD2: a novel surface marker for the identification of MSCs. Blood 2007 May;109(10):4245-8.
30. Pamphilon DH, Selogie E, Szczepiorkowski ZM, BEST Collaborative Cellular Therapies team. Transportation of cellular therapy products: report of a survey by the cellular therapies team of the Biomedical Excellence for Safer Transfusion (BEST) collaborative. Vox Sang. 2010;99:168-73.
31. Pal R, Hanwate M, Totey SM. Effect of holding time, temperature and different parenteral solutions on viability and functionality of adult bone marrow-derived mesenchymal stem cells before transplantation. J Tissue Eng Regen Med. 2008;7:436-44.
32. Stolzing A, Coleman N, Scutt A. Glucose-induced replicative senescence in mesenchymal stem cells. Rejuvenation Res. 2006;9:31-5.
33. Choudhery MS, Khan M, Mahmood R, Mohsin S, Akhtar S, Ali F, et al. Mesenchymal stem cells conditioned with glucose depletion augments their ability to repair infracted myocardium. J Cell Mol Med. 2012. March 21. [Epub ahead of pub].
34. Li YM, Schillng T, Benisch P, Zeck S, Meissner-Weigl J, Schneider D, et al. Effects of high glucose on mesenchymal stem cell proliferation and differentiation. Biochem Biophys Res Commun. 2007;363:209-15.
35. Lee KA, Shim W, Paik MJ, Lee SC, Shin JY, Ahn YH, et al. Analysis of changes in the viability and gene expression profiles of human mesenchymal stromal cells over time. Cytotherapy. 2009;11:688-97.

< ABSTRACT (IN KOREAN) >

다양한 인간 조직에서 유래한 중간엽 줄기세포의 특성 비교 및 이식을 위한 최적의 *in vitro* 보관 조건 분석

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손훈상

중배엽 유래의 중간엽 줄기세포는 자가재생을 하며 특정한 조건에서 배양할 경우 골아세포, 지방세포 및 연골세포로 분화하는 특성을 가지는 대표적인 성체 줄기세포의 하나이다. 골수에서 중간엽 줄기세포가 처음 발견된 이래로 여러 조직에서 중간엽 줄기세포가 분리되고 있으며 다양한 난치성 질환을 대상으로 줄기세포 치료제로서 현재까지 유일하게 임상적용이 상용화되어 있다.

그러나 최근 다양한 조직으로부터 중간엽줄기세포의 특성을 만족하는 중간엽 줄기세포의 분리율이 증가하면서 각 유래된 조직에 따라서 그 성상에 차이가 있음이 보고되고 있다. 이러한 차이점들은 기본적으로 조직에 따른 특성의 차이, 실험실들의 분리 배양 방법에서 다른 프로토콜 사용 등 많은 원인이 있겠으나 아직 중간엽 줄기세포에 대한 확실한 성상 규명이 이루어지지 못한 중간엽 줄기세포가 갖는 heterogenecity가 그 결과에 큰 차이를 나타내는 것으로 보고되고 있

다.

이번 연구에서 본 연구자는 다양한 인간 조직 (골수, 제대혈, 태반, 지방조직)에서 유래한 중간엽 줄기세포들에 대해 유세포 분석기법, 분화실험, PCR 기법들을 동원하여 세포들의 특성을 비교 분석하고자 하였다. 중간엽 줄기세포와 비슷한 특성을 보이는 섬유아세포를 대조로 하여 먼저 세포들의 morphology를 확인하였다. 모든 세포들은 방추형의 전형적인 섬유아세포의 모양을 보였으며 현미경상의 모양 관찰에는 차이가 없었다. 다음으로 유세포 분석기법을 이용하여 CD14, CD29, CD31, CD34, CD44, CD45, CD73, CD90, CD105, CD106들의 surface markers의 발현율을 분석하였다. 중간엽 줄기세포에서 강하게 발현하는 makers로 알려진 CD29, CD44, CD73, CD90, CD105들이 모든 세포에서 비슷하게 발현이 되었으며, 섬유아세포에서도 큰 차이 없이 비슷한 양상으로 발현하였다. 그러나 분화실험에서는 공통적으로 모두 골아세포, 지방세포, 연골세포로 분화가 되었지만 여러 세포들 중에서도 골수 및 지방조직에서 유래된 중간엽 줄기세포가 가장 강한 분화력을 보였다. 섬유아세포도 중간엽 줄기세포와 비슷한 분화양상을 보이기는 했지만 골아세포로는 전혀 분화가 되지 않았다.

Surface protein 분석 및 분화실험과 더불어 우리는 여러 조직유래의 세포들 중 특이적인 marker를 발현하는 세포를 찾고자 PCR 기법을 이용하여 중간엽 줄기세포에서 발현율이 높은 그리고 줄기세포의 자가재생 능력과 관련된 유전자들을 선별하여 발현 정도를 비교하였다. 그러나 모든 중간엽 줄기세포는 선택한 markers을 유사하게 발현하고 있었으며, 섬유아세포도 큰 차이 없이 동일한 양상으로 관찰되었다.

또 다른 중간엽 줄기세포의 특성 중에 하나는 중간엽 줄기세포에서 분비하는 neutrophic factors 및 anti-inlfammatory factors에 의한 paracine effect로서, 중간엽 줄기세포 치료에서 손상된 조직의 기능회복에 큰 역할을 하는 것으로 알려져 있다. 따라서 다양한 조직에서 유래된 세포들에서 이러한 기능이 더 우수한 세포가 있는지 알아보기 위해 neurotrophic 그리고 anti-inflammatory 인자(factor)들에 관련된 유전자들을 선별하여 비교 분석하였다. 하지만 이 실험에서도 세포들간의 큰 차이는 발견하지 못하였다.

본 연구에서는 여러 인간 조직에서 유래한 중간엽 줄기세포들의 특성의 차이를 찾고자 하였으나 분화실험에서 골수 및 지방조직 유래의 세포들이 가장 강한 분화능력을 보인 것 외에는 유래된 조직에 따라 형태학적, 면역학적, 유전학적으로 큰 특성의 차이를 발견할 수 없었다. 따라서 현재까지는 중간엽 줄기세포를 이용한 임상적용의 경우 비교적 손쉽게 채취할 수 있는 조직을 선택하여 사용하는 것이 조금 더 유용할 수 있을 것이다.

중간엽 줄기세포의 가소성(plasticity)과 비교적 얻기 쉽다는 장점은 재생의학 분야에서 임상적용에 제 1순위 세포치료제로서 받아들여지고 있다. 그러나 안전한 임상적용을 위해서 중간엽 줄기세포는 good manufacturing practice (GMP) 시설에서 많은 양의 세포들을 증식시켜야 한다. 게다가 외부(in vitro)에서 증식된 중간엽 줄기세포는 임상적용에 앞선 준비과정에서 중간엽 줄기세포로서의 고유한 특성, 예를 들어 분화능 및 기능성은 물론 높은 생존율을 유지해야 한다. 하지만 지금까지 중간엽 줄기세포의 임상 전 준비과정에 있어 그들의 안정성 및 특성유지에 대한 분석이 제대로 연구되지 않았다. 따라서 본 연구자는 임상적 사용을

목적으로 한 중간엽 줄기세포의 보관 및 준비과정에 있어 세포의 상태변화를 분석하고 최적의 조건을 찾아 표준화하고자 하였다.

본 연구를 위해 임상에서 주로 사용되는 생리식염수(saline)와 포도당(dextrose)을 보존 용액으로 선택하여 냉장 및 실온에서 보관상태에 따른 중간엽 줄기세포의 생존율과 기능성에 대해 분석하였다. 세포 배양용기에서 탈착 된 중간엽 줄기세포는 생리식염수(saline)에서 1~2시간 정도 보관 되었을 때 온도와 관계없이 약 90% 이상의 생존율을 나타냈다. 그러나 2시간이상 보관 되었을 경우 생존율은 시간에 비례하여 점차적으로 떨어졌으며, 포도당(dextrose)에 보관된 세포들은 급격하게 상태가 나빠졌다. 또한 보관시간이 증가함에 따라 중간엽 줄기세포는 고유한 특성인 colony-forming unit (CFU) 그리고 분화능력을 빠르게 상실하였다. 즉 탈착 된 중간엽 줄기세포는 2시간 이상 보관될 경우 세포생존율, 증식율 그리고 분화능이 급격하게 감소함을 보여주었다. 결론적으로 임상적용을 목적으로 준비된 세포는 가능한 2시간 이내에 사용하는 것이 안전하고 최상의 세포치료의 효과를 기대할 수 있을 것이다.

핵심되는 말: 중간엽 줄기세포, 분화, 체외 보관 조건, 세포치료