

## Comparison of viability and antioxidant capacity between canine adipose-derived mesenchymal stem cells and heme oxygenase-1-overexpressed cells after freeze-thawing

Mijung KIM<sup>1,2)</sup>, Yongsun KIM<sup>1,2)</sup>, Seunghoon LEE<sup>1,2)</sup>, Minyoung KUK<sup>1,2)</sup>, Ah Young KIM<sup>2)</sup>, Wanhee KIM<sup>2)</sup> and Oh-Kyeong KWEON<sup>1,2)\*</sup>

<sup>1)</sup>BK21 PLUS Program for Creative Veterinary Science Research, Research Institute for Veterinary Science and College of Veterinary Medicine, Seoul National University, Seoul, 151–742, Korea

<sup>2)</sup>Department of Veterinary Surgery, College of Veterinary Medicine, Seoul National University, 1, Gwanak-ro, Seoul, 151–742, Korea

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**ABSTRACT.** Allogenic adipose-derived mesenchymal stem cells (Ad-MSCs) are an alternative source for cytotherapy owing to their antioxidant and anti-inflammatory effects. Frozen-thawed allogenic Ad-MSCs can be used instantly for this purpose. However, the viability and function of frozen-thawed Ad-MSCs have not been clearly evaluated. The purpose of this study was to compare the viability and function of Ad-MSCs and heme oxygenase-1 (HO-1)-overexpressed Ad-MSCs *in vitro* after freeze-thawing. The viability, proliferation, antioxidant capacity and mRNA gene expression of growth factors were evaluated. Frozen-thawed cells showed significantly lower viability than fresh cells (77% for Ad-MSCs and 71% for HO-1 Ad-MSCs,  $P<0.01$ ). However, the proliferation rate of frozen-thawed Ad-MSCs increased and did not differ from that of fresh Ad-MSCs after 3 days of culture. In contrast, the proliferation rate of HO-1-overexpressed Ad-MSCs was lower than that of Ad-MSCs. The mRNA expression levels of *TGF- $\beta$* , *HGF* and *VEGF* did not differ between fresh and frozen-thawed Ad-MSCs, but *COX-2* and *IL-6* had significantly higher mRNA expression in frozen cells than fresh cells ( $P<0.05$ ). Fresh Ad-MSCs exhibited higher *HO-1* mRNA expression than frozen-thawed Ad-MSCs, and fresh HO-1-overexpressed Ad-MSCs exhibited higher than fresh Ad-MSCs ( $P<0.05$ ). However, there was no significant difference between fresh and frozen HO-1-overexpressed Ad-MSCs. The antioxidant capacity of HO-1-overexpressed Ad-MSCs was significantly higher than that of Ad-MSCs. Cryopreservation of Ad-MSCs negatively affects viability and antioxidant capacity, and HO-1-overexpressed Ad-MSCs might be useful to maximize the effect of Ad-MSCs for cytotherapy.

**KEY WORDS:** canine, cryopreservation, heme oxygenase-1, mesenchymal stromal cell

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Mesenchymal stem cells (MSCs) are cytotherapeutic agents with great potential in the field of regenerative medicine to repair damaged tissue. Adipose-derived MSCs (Ad-MSCs) exhibit stable growth and proliferation during culture and potential differentiation to a variety of cells, including bone marrow stem cells [9, 40]. Ad-MSCs are used to promote bone regeneration as well as in the treatment of neurological disorders, such as spinal cord injury, stroke and multiple sclerosis [3, 16, 18, 25]. Rather than direct conversion into differentiated cells, the repair mechanism is thought to involve the secretion of growth factors and promotion of the endogenous regenerative process by decreasing cell death and promoting nerve regeneration and revascularization [4–6].

Although allogenic Ad-MSCs have an immunomodulatory effect, which is required for cytotherapy [1, 15, 24],

they do not provide total immune evasion and thus the co-administration of immunosuppressive drugs needs to be considered [2, 11]. However, autologous Ad-MSCs may be able to completely evade a wide range of innate and adaptive immune systems. However, the time required to collect, expand and administer usable cells makes the application of Ad-MSCs difficult in patients with acute injuries. The key to successful clinical application of Ad-MSCs is to provide a sufficient quantity of Ad-MSCs in a timely manner. Frozen-thawed allogenic Ad-MSCs could serve as an alternative to overcome this limitation.

Heme oxygenase-1 (HO-1) is able to control the cell cycle and has cytoprotective, pro-angiogenic and anti-inflammatory properties [10, 27, 34, 35]. The catabolism of heme provides cytoprotection via the induction of ferritin, antioxidative action of biliverdin and bilirubin, and anti-inflammatory effects of carbon monoxide. Methylprednisolone sodium succinate (MPSS) is being widely used as a scavenging agent in clinical treatment of acute spinal cord injury (SCI). Recent retrospective cohort studies have demonstrated the lack of a statistically significant difference in clinical outcomes [12]. MSCs might be used as an alternative agent for the treatment of acute SCI. HO-1 overexpressed MSCs would be more potent for cell therapy. Frozen allogenic MSCs are more convenient to provide a sufficient quantity

\*CORRESPONDENCE TO: KWEON, O.-K., Department of Veterinary Surgery, College of Veterinary Medicine, Seoul National University, 1, Gwanak-ro, Gwanak-gu, Seoul, 151–742, Korea.  
e-mail: ohkweon@snu.ac.kr

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Table 1. Primers used in the PCR to detect mRNA of the canine Ad-MSCs

Target gene	Primer	Sequence	Size
Interleukin-6 (IL-6)	Forward	TCTGTGCACATGAGTACCAAGATCC	124 bp
	Reverse	TCCTGCGACTGCAAGATAGCC	
Cyclooxygenase-2 (COX-2)	Forward	ACATCCTGACCCACTTCAAG	387 bp
	Reverse	CAGGTCCTCGCTTATGATCT	
Transforming growth factor beta (TGF- $\beta$ )	Forward	CTC AGT GCC CAC TGT TCC TG	215 bp
	Reverse	TCC GTG GAG CTG AAG CAG TA	
Hepatocyte growth factor (HGF)	Forward	CCCGACAAGGGCTTTGATGA	873 bp
	Reverse	TCTGTTTCGAGAGGGGAAACAT	
Vascular endothelial growth factor (VEGF)	Forward	CTACCTCCACCATGCCAA	785 bp
	Reverse	CATTGCCCTCAATGACCACT	
Heme oxygenase-1 (HO-1)	Forward	GACAGCATGCCCCAGGAT	879 bp
	Reverse	TCACAGCCTAAGGAGCCAGT	
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	Forward	CATTGCCCTCAATGACCACT	104 bp
	Reverse	TCCTTGGAGGCCATGTAGAC	

of Ad-MSCs in a timely manner until autologous MSCs are prepared. However, it was reported that cryopreservation attenuates activities of immunosuppression and binding and engraftment of MSCs as well as viability [7, 13].

This study was conducted to compare the viability, proliferation, antioxidant capacity and mRNA gene expression levels of growth factors between canine Ad-MSCs and HO-1-overexpressed Ad-MSCs in relation to freeze-thawing.

## MATERIALS AND METHODS

**Isolation and cultivation of canine Ad-MSCs:** Canine Ad-MSCs were obtained according to the method described in our previous paper [28]. Briefly, adipose tissues were aseptically collected from gluteal subcutaneous fat of 2-year-old beagle dogs (4 females). All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Seoul National University (SNU-141210-1), Korea. Adipose tissues (approximately 1 g) were washed extensively with phosphate-buffered saline (PBS), finely cut and digested with collagenase type I (1 mg/ml; Sigma-Aldrich, St. Louis, MO, U.S.A.) for 2 hr at 37°C. The tissue samples were washed with PBS solution and then centrifuged at 300  $\times$ g for 10 min. The stromal vascular fraction pellets were resuspended, filtered through a 100  $\mu$ m nylon mesh and incubated overnight in 10% fetal bovine serum (FBS, Gibco BRL, Grand Island, NY, U.S.A.) and Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Billings, MT, U.S.A.) at 37°C with 5% humidified CO<sub>2</sub>. After 24 hr, unattached cells and residual non-adherent red blood cells were removed by washing with PBS solution. The medium was changed at 2-day intervals until the cells became confluent. After the cells reached 90% confluence, they were subcultured. At passage 3, half of the cells were immediately used for *in vitro* experiments, and the remaining half were stored in a -150°C ultra-low temperature freezer and used after 2 weeks for the evaluation of the frozen-thawed cells.

**Generation of lentivirus containing canine HO-1 and transfection into Ad-MSCs:** We cloned the canine HO-1 gene in the reference to the gene database in the PubMed. pPACK

Packaging Plasmid Mix (System Biosciences, Mountain View, CA, U.S.A.) was used for lentiviral packaging. In brief, the gene encoding Flag-tagged HO-1 was amplified from cDNA of canine peripheral blood using Phusion DNA Polymerases (Thermo Scientific, Pittsburgh, PA, U.S.A.), and a canine HO-1-specific primer set (Table 1) was inserted into a pCDH-EF1-MCS-pA-PGK-copGFP-T2A-Puro vector, with *Eco*RI and *Bam*HI restriction enzymes (System Biosciences). HEK293T cells (Thermo Scientific) were maintained in 10% fetal bovine serum and 1% penicillin/streptomycin in DMEM at 37°C and 5% CO<sub>2</sub>. Twenty-four hours before transfection, 4  $\times$  10<sup>6</sup> HEK293 cells were seeded into a 100 mm dish. The following day, 20  $\mu$ l of lentiviral packaging mix (System Biosciences) encoding viral proteins Gag-Pol, Rev and VSV-G and 2  $\mu$ g lentiviral transgene plasmids were transfected into cells for lentivirus production using Turbofect (Thermo Scientific). Fourteen hours after transfection, the DNA reagent mixture was removed and replaced with 5% FBS in 14 ml of fresh DMEM. At 48 hr post-transfection, lentiviral supernatants were harvested and filtered with 0.45  $\mu$ m filters. One volume of cold (4°C) PEG-it Virus Precipitation Solution (System Biosciences) was added to every 4 volumes of lentiviral particle-containing supernatant. The supernatant/PEG-it mixture was centrifuged at 1,500  $\times$ g for 30 min at 4°C. After the viral pellet was resuspended in 10  $\mu$ l of cold (4°C) DMEM media, 100  $\mu$ l of diluted viral particles (1  $\times$  10<sup>8</sup> TU/ml) was added to the Ad-MSCs for the transfection and incubated in a T75 flask for 72 hr at 37°C; 10 ml of DMEM (10% FBS and 1% penicillin-streptomycin) was added before culture for 48 hr, and the colonies of cells were selected with puromycin (3  $\mu$ g/ml). After culturing to passage 3, half of the cells were immediately used for the cell evaluation experiments, and the other half were cryopreserved and thawed after 2 weeks for evaluation.

**Cryopreservation and thawing:** Cells in a T175 flask were separated using 0.05% trypsin-EDTA (Gibco) and neutralized with DMEM after incubation for 15 min at 37°C, 5% CO<sub>2</sub>. After centrifuging for 5 min at 900  $\times$ g, the supernatant was removed, and 5  $\times$  10<sup>6</sup> cells in cryogenic medium [50% DMEM and 40% FBS containing 10% dimethyl sulfoxide

(DMSO)] were re-suspended. Since then, the cell suspensions were incubated at 4°C for 1 hr, at -20°C for 2 hr and at -80°C overnight [8]. And then, the cryogenic vials were moved to a -150°C ultra-low temperature freezer to store for 2 weeks. The cryopreserved cells were thawed at 37°C for 5 min for the recovery.

**MTS assay:** The cell viability and proliferation rate for fresh canine Ad-MSCs (MSCs), frozen-thawed canine Ad-MSCs (F-MSCs), fresh HO-1-overexpressed Ad-MSCs (HMSCs) and frozen-thawed HO-1-overexpressed Ad-MSCs (F-HMSCs) were compared by measuring 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium using a commercially available kit (CellTiter 96<sup>®</sup> Aqueous, Promega, Madison, WI, U.S.A.). When the cells reached 80% confluence, attached cells were harvested with trypsin-EDTA and then immersed in a 96-well plate at a density of  $1 \times 10^4$  cells/100  $\mu$ l per well, and incubated for 0, 24, 48 and 72 hr. CellTiter 96 Aqueous One Solution Reagent (20  $\mu$ l) was dispensed, and the plate was cultured for 2 hr at 37°C in a humidified 5% CO<sub>2</sub> atmospheric environment. Plates were read on a 680 micro-plate reader (Bio-Rad, Hercules, CA, U.S.A.) at a wavelength of 492 nm.

**Observation of cell morphology:** Cell morphology was observed 3 days after seeding using an inverted microscope (Olympus-ckx41, Tokyo, Japan). At passage three,  $1 \times 10^5$  MSCs, F-MSCs, HMSCs and F-HMSCs were seeded and grown in 6-well plates with DMEM medium containing 10% FBS.

**Reverse transcription polymerase chain reaction:** The total RNA was extracted using a Hybrid-R RNA Extraction Kit (GeneAll, Seoul, Republic of Korea), and the RNA concentrations were determined by measuring the absorbance at 260 nm using ImplenNanoPhotometer (model 1443, Implen GmbH, Munich, Germany). The Takara PrimeScript II First-strand cDNA Synthesis Kit (TaKaRa Korea, Seoul, Republic of Korea) was used to synthesize cDNA based on 1,000 ng total RNA. One microliter of cDNA was amplified for polymerase chain reaction (PCR) analysis. PCR was performed with a final volume of 20  $\mu$ l, which contained 10  $\mu$ l of PCR Premix (EmeraldAmp<sup>™</sup> PCR Master Mix, Takara, Otsu, Japan), 20  $\mu$ M forward primer and 20  $\mu$ M reverse primer (Standard Oligo, Bioneer, Daejeon, Korea), 3  $\mu$ l of DNA (0.1–1.0  $\mu$ g, diluted in TE buffer) and 3  $\mu$ l of distilled water. The primers are shown in Table 1. Gene expression levels were compared after the target genes were normalized to the endogenous reference (GAPDH). cDNA templates were amplified for 27 to 35 cycles and include denaturation at 94°C (30 sec), annealing at 50–59°C (30 sec), 72°C (1 min) and a final extension at 72°C. The PCR reactions were performed using the T3000 Thermocycler (Whatman, Biometra, Biomedizinische Analytik GmbH, Goettingen, Germany). After the reactions, 5  $\mu$ l of PCR product was visualized using a 2% agarose gel (UltraPure<sup>™</sup> Agarose, Invitrogen, Carlsbad, CA, U.S.A.) by electrophoresis. Redsafe<sup>™</sup> Nucleic Acid Staining Solution (iNtRON Biotechnology Inc., Seoul, Korea) was used to stain a 2% agarose gel. The longitudinal sections of the visualized gel were analyzed using ImageJ (version 1.37, National Institutes of Health, Bethesda, MD, U.S.A.).

**Total antioxidant capacity assay:** The Total Antioxidant Capacity (TAC) Assay Kit (Cell Biolabs OxiSelect<sup>™</sup>, San Diego, CA, U.S.A.) was used to measure the antioxidant capacity of the cell extract. Cells were scrapped after washing them 3 times with PBS. The cells were suspended in cold PBS ( $1 \times 10^7$ /ml), sonicated and centrifuged for 10 min at 10,000  $\times$ g and 4°C. Twenty microliters of cell extracts were dispensed to the 96-well microtiter plate, and 180  $\mu$ l of the 1 $\times$  reaction buffer were added to each well and mixed. Fifty microliters of the 1 $\times$  copper ion reagent were dispensed to each well to start the reaction and incubated in a shaker for 5 min. Finally, 50  $\mu$ l of 1 $\times$  stop solution were added to end the reaction. The absorbance values were proportional to the total reductive capacity of the sample. Results are expressed as uric acid equivalents (UAE). A standard curve was used to determine the UAE (mM) of the sample, where  $y=0.462x + 0.061$  ( $R^2=0.9938$ ); the  $y$ -value indicated the absorbance, which was used to obtain the UAE (mM) that provides the same OD at 490 nm.

**Statistical analysis:** The results were expressed as means  $\pm$  SD. The data were analyzed using the SPSS statistical program (version 20.0. IBM, Armonk, NY, U.S.A.). The Kruskal-Wallis test was used to assess differences among the groups. Mann-Whitney tests were carried out for the post-hoc test. A  $P$ -value of less than 0.05 indicated a significant difference between the groups.

## RESULTS

**Viabilities of frozen-thawed Ad-MSCs:** The absorbance of live MSCs was  $0.43 \pm 0.06$ , and that of F-MSCs was  $0.33 \pm 0.06$  ( $n=29$ ), indicating that the viability of F-MSCs was less than that of MSCs (77%,  $P<0.01$ ). The absorbance of live HMSCs was  $0.48 \pm 0.11$ , and that of F-HMSCs was  $0.34 \pm 0.05$  ( $n=19$ ), indicating that the viability of F-HMSCs was less than that of HMSCs (71%,  $P<0.01$ ). Furthermore, there were no significant differences between MSCs and HMSCs or between F-MSCs and F-HMSCs (Fig. 1).

**Proliferation rates after culture of frozen-thawed Ad-MSCs:** The proliferation rate (Fig. 2) of F-MSCs was lower than that of MSCs until 48 hr, after which the rate of F-MSCs increased abruptly and did not differ from that of MSCs at 3 days after culture. The proliferation rates of HMSCs after 3 days of culture were significantly lower than those of MSCs ( $P<0.05$ ).

**Morphology of cells:** The images taken 3 days after seeding (Fig. 3) showed approximately 60–70% confluency in MSCs and F-MSCs, with slim and spindle-shaped cells. Approximately 30–40% confluency was observed in HMSCs and 20–30% in F-HMSCs, showing delayed proliferation with more broad and spindle-shaped cells than were observed for MSCs.

**mRNA expression levels of TGF- $\beta$ , HGF, VEGF, COX-2, IL-6 and HO-1:** The mRNA expression levels of TGF- $\beta$ , HGF and VEGF did not show significant differences among MSCs, F-MSCs, HMSCs and F-HMSCs. However, significantly higher COX-2 and IL-6 mRNA expression levels were observed in frozen-thawed cells including F-MSC

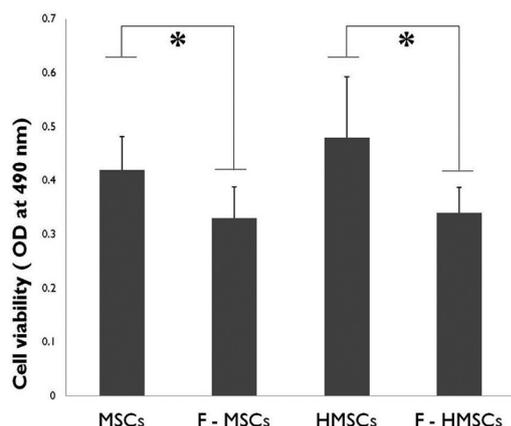


Fig. 1. Comparison of viabilities among fresh and frozen-thawed Ad-MSCs and HO-1-overexpressed Ad-MSCs. The viabilities of frozen MSCs and HMSCs were less than those of fresh MSCs and HMSCs, respectively. All values are expressed as the means  $\pm$  standard deviations, and \* indicates a significant difference between groups ( $P < 0.05$ ). MSCs, fresh canine Ad-MSCs; F-MSCs, frozen-thawed canine Ad-MSCs; HMSCs, fresh HO-1-overexpressed Ad-MSCs; F-HMSCs, frozen-thawed HO-1-overexpressed Ad-MSCs.

and F-HMSCs than in fresh cells ( $P < 0.05$ ). *HO-1* mRNA expression in MSCs was significantly higher than in F-MSCs ( $P < 0.05$ ). *HO-1* mRNA expression in HMSCs was significantly higher than in MSCs ( $P < 0.05$ ), but not higher than F-HMSCs (Fig. 4).

**Analysis of total antioxidant capacity:** HMSCs had higher antioxidant capacity than MSCs ( $P < 0.05$ ). The total antioxidant capacities of F-HMSCs and F-MSCs were significantly lower than those of HMSCs and MSCs, respectively (85% for HMSCs; and 66% for MSCs,  $P < 0.05$ ). The antioxidant capacity of F-MSCs was significantly lower than that of F-HMSCs (44% for F-HMSC,  $P < 0.05$ ) (Fig. 5).

## DISCUSSION

Viability differed between fresh and frozen-thawed cells immediately after thawing. Some studies have shown that freeze-thawing can reduce cell viability [26, 36]. However, other studies have concluded that cryopreservation does not affect viability, morphology or differentiation potency [19, 41]. It has been suggested that the conflicting results reflect the different methods used to measure cell viability. Our study evaluated viability by MTS assay, which measured biological activity, rather than by a simple assessment of cell status (i.e., live or dead). Our findings confirmed that the viability of frozen-thawed cells was about 70% relative to that of fresh cells.

The proliferation rates of fresh and frozen-thawed HO-1-overexpressed Ad-MSCs were lower than those of intact Ad-MSCs after 3 days of culture. HO-1 activity is associated with enhanced cell survival, proliferation and migration, and a reduced inflammatory response [20, 23, 33]. However, it was reported that overexpressed HO-1 activity in vascular

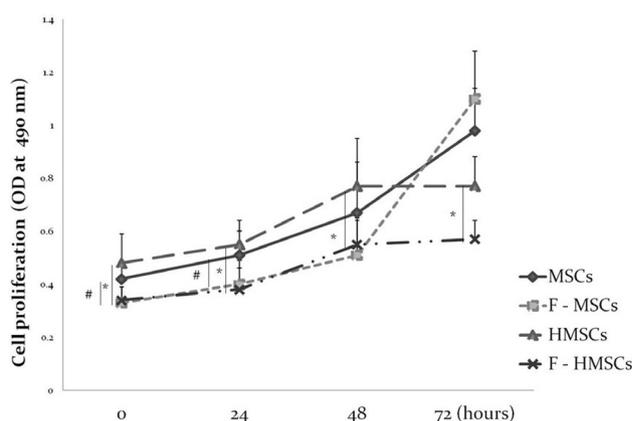


Fig. 2. Changes in proliferation rates of fresh and frozen-thawed Ad-MSCs and HO-1-overexpressed Ad-MSCs. Proliferation rate of F-MSCs did not differ from that of MSCs at 3 days after culture, but that of HMSCs was significantly lower than MSCs. All values are expressed as the means  $\pm$  standard deviations, and #, \* indicates a significant difference between groups ( $P < 0.05$ ). (#; between MSCs and F-MSCs, \*; between HMSCs and F-HMSCs). MSCs, fresh canine Ad-MSCs; F-MSCs, frozen-thawed canine Ad-MSCs; HMSCs, fresh HO-1-overexpressed Ad-MSCs; F-HMSCs, frozen-thawed HO-1-overexpressed Ad-MSCs.

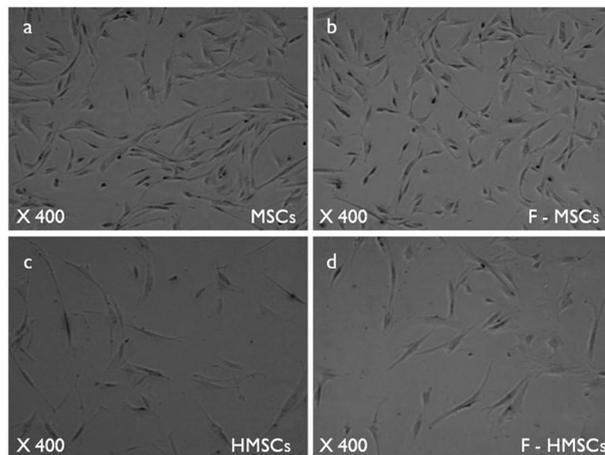


Fig. 3. Morphology of Ad-MSCs 3 days after seeding at passage 3. (400 $\times$  magnification) (A) MSCs, 60–70% confluency, slim, spindle-shaped (B) F-MSCs, 60–70% confluency, slim, spindle-shaped (C) HMSCs, 30–40% confluency, broad, spindle-shaped (D) F-HMSCs, 20–30% confluency, broad, spindle-shaped.

smooth muscle cells results in a slower growth rate than that of wild-type vascular smooth muscle cells [42]. Ninety one percent of human HO-1-transfected cells were in the growth-arrested phase of the cell cycle (G0/G1). Although reduced proliferation of overexpressed-HO-1 Ad-MSCs in the present study was observed, the total antioxidant activity was significantly higher than that of fresh Ad-MSCs as well as frozen Ad-MSCs. If the benefits of Ad-MSC therapy involve indirect environmental modification via anti-oxidation, anti-

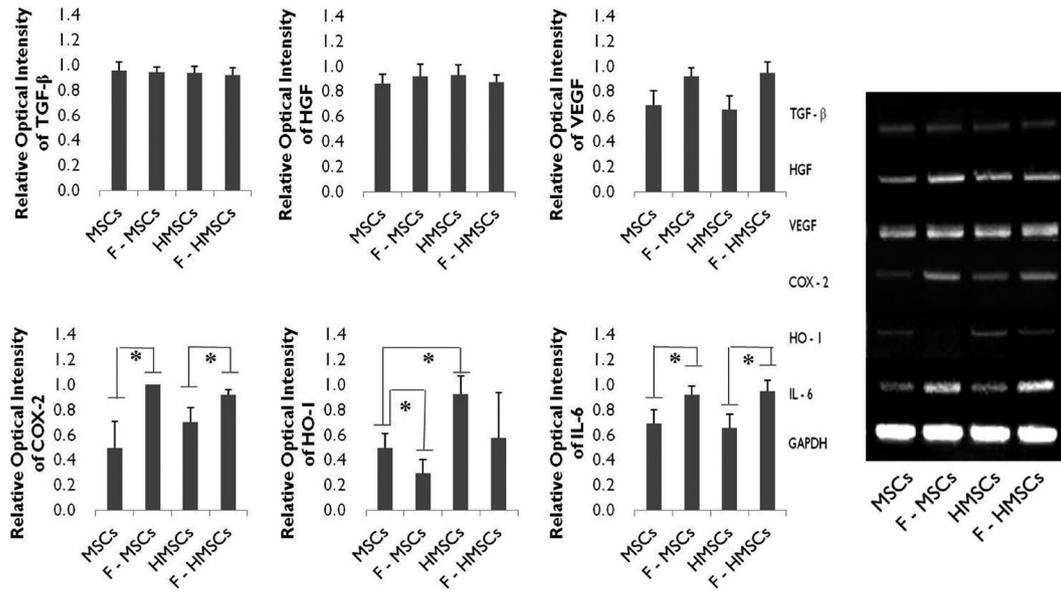


Fig. 4. Comparisons of mRNA expression levels of *TGF-β*, *HGF*, *VEGF*, *COX-2*, *IL-6* and *HO-1* among MSCs, F-MSCs, HMSCs and F-MSCs. The mRNA expressions levels of *TGF-β*, *HGF* and *VEGF* did not show significant differences among groups, but significantly higher *COX-2* and *IL-6* mRNA expression levels were observed in frozen-thawed cells ( $P < 0.05$ ). *HO-1* mRNA expression in MSCs was significantly higher than that in F-MSCs, but not than HMSCs and F-HMSCs. Each measure, expressed as means  $\pm$  standard deviations, is expressed relative to the maximum value among groups. \* $P < 0.05$ .

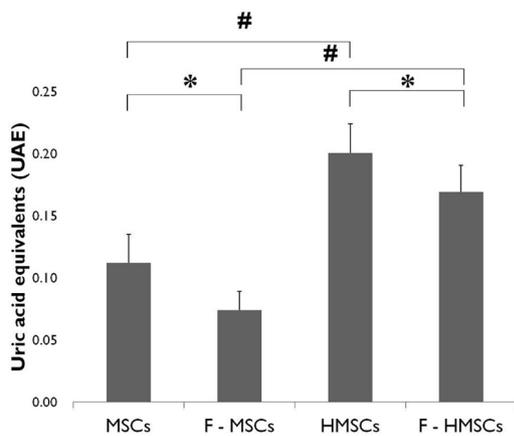


Fig. 5. Total antioxidant capacity (TAC) for MSCs, F-MSCs, HMSCs and F-HMSCs. \*, between MSCs and FMSCs, between HMSCs and FHMSCs; #, between MSCs and HMSCs, between F-MSCs and F-HMSCs ( $P < 0.05$ ).

inflammation and anti-apoptosis effects rather than direct differentiation [29], frozen HO-1-overexpressed Ad-MSCs may be useful.

VEGF secreted by Ad-MSCs is involved in the control of nerve regeneration as well as the maintenance and survival of newly created blood vessels [17, 32]. In addition, VEGF, HGF and other various growth factors behave as neuroprotectors [22, 31]. In this way, growth factors, such as HGF, TGF- $\beta$  and VEGF, are important for healing damaged tissue.

In the present study, there was no detectable difference in the mRNA expression of growth factor genes between MSCs, F-MSCs, HMSCs and F-HMSCs. However, the inflammatory factors, *COX-2* and *IL-6*, in frozen Ad-MSCs have increased expression. Heat stress during the freeze-thaw process promotes *COX-2* expression [30] and can enhance the expression of *IL-6* [14, 39]. Therefore, the inflammatory response is likely to occur when frozen-thawed cells are used.

HO-1 increases the survival of Ad-MSCs in acute myocardial infarction [37] and may control the differentiation of chondrocytes, neurons and osteoblast [21, 38]. In addition, HO-1 has an effect on blood flow recovery and nerve function recovery [34]. Our study showed that HO-1-overexpressed Ad-MSCs have higher antioxidant capacity than Ad-MSCs, regardless of cryopreservation. These results suggested that frozen-thawed HO-1-overexpressed Ad-MSCs are an alternative source for cytotherapy.

In clinical trials, the use of cryopreserved products immediately after thawing fails more often than the use of fresh Ad-MSCs. Challenges in the clinical application are the preservation of the stem cell and the functional impairment for homing [7, 13]. We need early usable and effective MSCs after thawing.

Freezing cells are more convenient, but thawing cells have some disadvantage. In the present study, frozen-thawed Ad-MSCs were limited as a therapeutic tool owing to reduced viability, lower *HO-1* mRNA expression and lower total antioxidant activity relative to fresh cells. However, the meaningfulness of our research is that with the integration of cryopreservation and gene manipulation, we opened pos-

sibilities to make cells that can immediately be connected to clinical application.

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