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Effects of Age and Gender on the Viability and Stem Cell Markers, mRNA, and Protein Expression of Bone Marrow–Derived Stem Cells Cultured in Growth Media

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Purpose: Bone marrow has long been a source of primary cells. This study was performed to evaluate the effects of age and sex on the cellular viability and expression of stem cell markers of mRNA and on the protein expression of bone marrow stem cells (BMSCs) derived from healthy donors.

Materials and Methods: Stem cells were isolated from human bone marrow and plated in culture plates. The shape of the BMSCs was observed under inverted microscope. Quantitative cellular viability was evaluated using a Cell-Counting Kit-8 assay. The expression of stem cell surface markers was tested and a series of quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot was performed to evaluate the expression in each group.

Result: The shapes of the cells at 20s, 30s, and 50s were similar to each other. No significant changes in cellular viability were noted among different age groups or sex groups. The BMSCs expressed CD44, CD73, and CD90 surface markers but did not express CD14 and CD34. There were no noticeable differences in CD surface markers among the different age groups. The expressions of CD surface markers were similar between men and women. No significant differences in the secretion of vascular endothelial growth factors (VEGFs) were noted at Day 3 between different age groups. qRT-PCR regarding the expression showed differences between the age groups. However, Western blot analysis showed a decrease in expression but did not reach statistical significance ($P>0.05$).

Conclusion: This study clearly showed no significant differences in shape, cell viability, expression of stem cell surface markers, or secretion of human VEGF among different age groups. However, western blot analysis showed a tendency of age-related decrease which did not reach statistical significance. Collectively, autologous or allogeneic BMSCs should be meticulously applied to obtain optimal results regarding age and sex.

Key Words: Age factors; Bone marrow; Cell survival; Sex; Stem cells

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Introduction

Bone marrow has been long been used as primary cell sources[1]. Bone marrow stroma consists of a unique cell population, *i.e.*, mesenchymal stem cells, which are able to differentiate into diverse mesenchymal cell lineages[2]. Bon e marrow-derived stem cells can differentiate into skeletal tissue part such as bone, fat, cartilage and the stroma[3]. Biological and possible clinical uses of mesenchymal stem cells have been reported, and mesenchymal stem cells have been applied in the field of transplantation with respect to immunoregulation, hematopoietic support, and transplant facilitation[4].

In a previous report, cultured adherent cells from marrow was suggested to serve as abiding precursor cells for bone and cartilage in animal models[5]. However, bone marrow-derived stem cells show variations in yield, growth, and differentiation[2]. It was shown that the number of mesenchymal stem cells and differentiation potential decrease with increasing age[6]. The objective of this study is to evaluate the effects of age and sex on cellular viability, the expression of stem cell markers and mRNA, and the protein expression of bone marrow stem cells (BMSCs) derived from healthy donors.

Materials and Methods

1. Stem Cells Isolated from Human Bone Marrow

Human bone marrow-derived mesenchymal stem cells (Catholic MASTER Cells) were gathered from the Catholic Institute of Cell Therapy (CIC, Seoul, Korea). The Institutional Review Board reviewed and approved this study (KC17SNSI0606), and informed written consent from the participants was obtained. All of the methods used in this study were conducted in accordance with the relevant guidelines and regulations. Cells that did not adhere to the culture dish were cleared away. The media was changed every 2 to 3 days, and the cells were incubated in an incubator with 5% CO_2 and 95% O_2 at 37°C.

2. Evaluation of Cellular Morphology

The cells were loaded at a density of 2.0×10^3 cells/well in 96-well plates and cultured in growth media (an alpha-minimal essential medium [α-MEM]; Gibco, Grand Island, NY, USA) consisting of 200 mM of L-glutamine (Sigma-Aldrich, St. Louis, MO, USA), 10 mM of ascorbic acid 2-phosphate (Sigma-Aldrich), 15% fetal bovine serum (Gibco), 100 U/ml of penicillin, and 100 μg/ml of streptomycin (Sigma-Aldrich). The shape of the tested BMSCs was assessed with inverted microscopy (CKX41SF; Olympus, Tokyo, Japan) on Days 1 and 3, and the images were saved as JPG files.

3. Determination of Cell Viability

The viability of the BMSCs was qualitatively tested with a Live/Dead Kit assay (Molecular Probes, Eugene, OR, USA). The assay is built on the principle that the activity of intracellular esterase causes non-fluorescent, cell-permeable calcein acetoxymethyl ester to become intensely fluorescent, producing viable stem cells a green fluorescence. Ethidium homodimer penetrates into the damaged cell membrane and then binds to nucleic acids and produces a red fluorescence.

Cultured stem-cells were washed twice with the growth media and then suspended in 1 ml of α-MEM consisting of 2 μl of 50 mM calcein acetoxymethyl ester working solution and 4 μl of 2 mM ethidium homodimer-1 for 30 minutes at room temperature. The BMSCs were stained with calcein acetoxymethyl ester and ethidium homodimer-1 and observed under a fluorescence microscope (Axiovert 200; Zeiss, Göttingen, Germany) on Day 4.

On Days 1 and 3, we performed a cell-viability analysis. WST-8 (Cell Counting Kit-8; Dojindo, Tokyo, Japan) was loaded to the cultures and the cells were incubated for 1 hour at 37°C in the incubator. Mitochondrial dehydrogenases oxidize WST-8 into

a formazan product. Microplate reader (BioTek, Winooski, VT, USA) was used to measure the spectrophotometric absorbance of the samples at 450 nm.

4. Flow Cytometric Analysis

Approximately 3×10^5 BMSCs were incubated with specific fluorescein isothiocyanate-conjugated mouse monoclonal antibodies for human CD44, CD73, CD90, CD14, and CD34 (11-0441-81, 11-0739-42, 11-0909-42, 11-0149-42, 11-0349-42; eBioscience, San Diego, CA, USA). Flow cytometric analysis was performed with a flow cytometer (FACS Canto II; BD Biosciences, San Jose, CA, USA) and the FACSDiva program (BD Biosciences).

5. Immunofluorescence

Human SSEA-4 antibody (mab1435; R&D Systems, Minneapolis, MN, USA) was used for the immunofluorescent assay performed on Day 3. The tested cells were fixed, permeabilized, blocked, and incubated with primary antibodies of SSEA-4. The cultures were incubated with the secondary antibody conjugated with fluorescein isothiocyanate (F2761; Abcam, Cambridge, UK) and then incubated with 4',6-diamidino-2-phenylindole. The cells were then observed under a fluorescence microscope (Axiovert 200) for the immunofluorescence analyses.

6. Secretion of Human Vascular Endothelial Growth Factor (VEGF) for Paracrine Effect

Human VEGF was determined in three-dimensional systems using a commercially available kit (DVE00, Quantikine® ELISA; R&D Systems, Minneapolis, MN, USA). All reagents and samples were prepared according to the manufacturers' recommendations. The absorbance levels at 450, 540, and 570 nm were measured, and the differences were used as the value.

7. Total RNA Extraction and Quantification by Real-Time Polymerase Chain Reaction

BMSCs were harvested on Days 2 and 3. Total RNA was obtained using a GeneJET RNA Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA); 1 ng of total RNA was used as a template for reverse transcription using SuperiorScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA), and quantities were resolved by spectrophotometer (ND-2000; Thermo Fisher Scientific) with proportions of absorbance at 260 nm and 280 nm for Days 2 and 3, respectively.

mRNA expression was detected by quantitative real-time polymerase chain reaction (qRT-PCR) using SYBR Green Real-Time PCR Master Mixes (Enzynomics, Daejeon, Korea) as stated in the manufacturer's protocol. The primers used for the qRT-PCR were designed based on GeneBank: Runx-2 forward 5′-AAT GAT GGT GTT GAC GCT GA-3′; reverse 5′-TTG ATA CGT GTG GGA TGT GG-3′; Collagen I forward 5′-TCA TGG CCC TCC AGC CCC CAT3′; and reverse 5′-ATG CCT CTT GTC CTT GGG GTT C-3′. Reference gene for normalization was β-actin as a housekeeping gene.

8. Western Blot Analysis

The samples were washed twice with ice-cold phosphate-buffered saline and lysed in RIPA lysis buffer (Thermo Fisher Scientific) with protease inhibitors (PPI1015; Quartett, Bern, Germany) on Day 2 or 3 for 30 minutes. The lysates were centrifuged at 13,000 rpm for 10 minutes at 4°C. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (Mini-PROTEAN® TGX™ Precast Gels; Bio-Rad, Hercules, CA, USA) was used for the separation of the samples. Then the samples were transblotted to polyvinylidene difluoride membranes (IB24002, Immun-Blot®; Bio-Rad) using a transfer apparatus (iBlot® 2 Transfer Stacks; Bio-Rad), and immunoblotted with the corresponding antibodies and enhanced chemiluminescent detection kits. Primary antibodies

against RUNX2 (ab76956) and GAPDH (ab9485) and secondary antibodies (goat anti-mouse IgG F(ab')2, polyclonal antibody [ADI-SAB-100-J], and goat anti-rabbit IgG, polyclonal antibody [ADI-SAB-300-J]) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), Thermo Pierce™ (Rockford, IL, USA), Cell Signaling Technology (Danvers, MA, USA), and Enzo Life Sciences (Farmingdale,

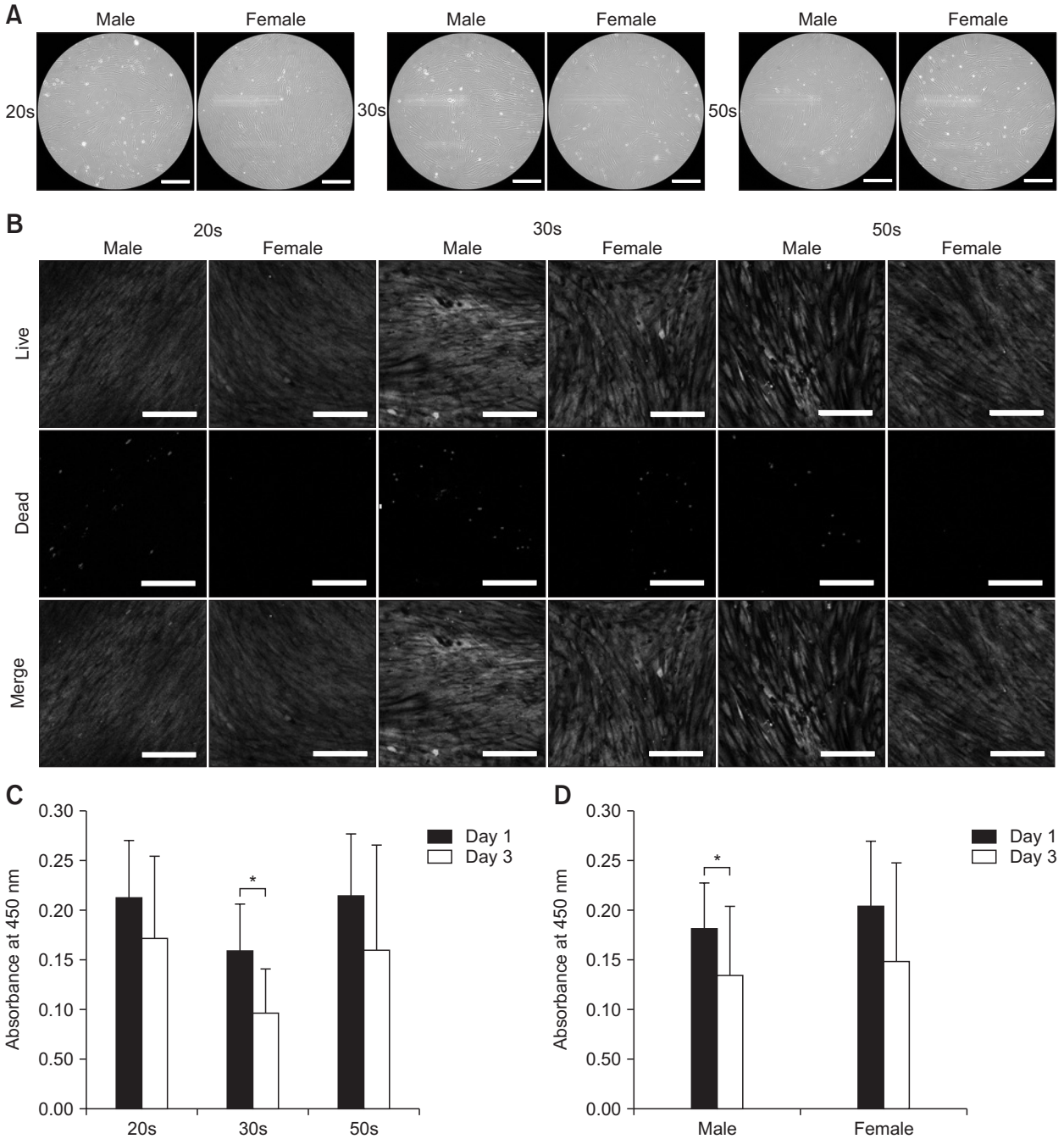

Fig. 1. (A) Evaluation of bone marrow stem cells (BMSCs)'s shape on Day 3 in growth media (×100). Scale bars=400 μm. (B) Qualitative cellular viability results under a confocal microscope on Day 4. Live images, dead images, merged images, and central images are provided. (C) Cellular viability using CCK-8 assay at Days 1 and 3 among different age groups. *Statistically significant differences were noted when compared with the results of the 30s group on Day 1. (D) Cellular viability using CCK-8 assay at Days 1 and 3 in males and females. *Statistically significant differences were noted when compared with the results of males on Day 1.

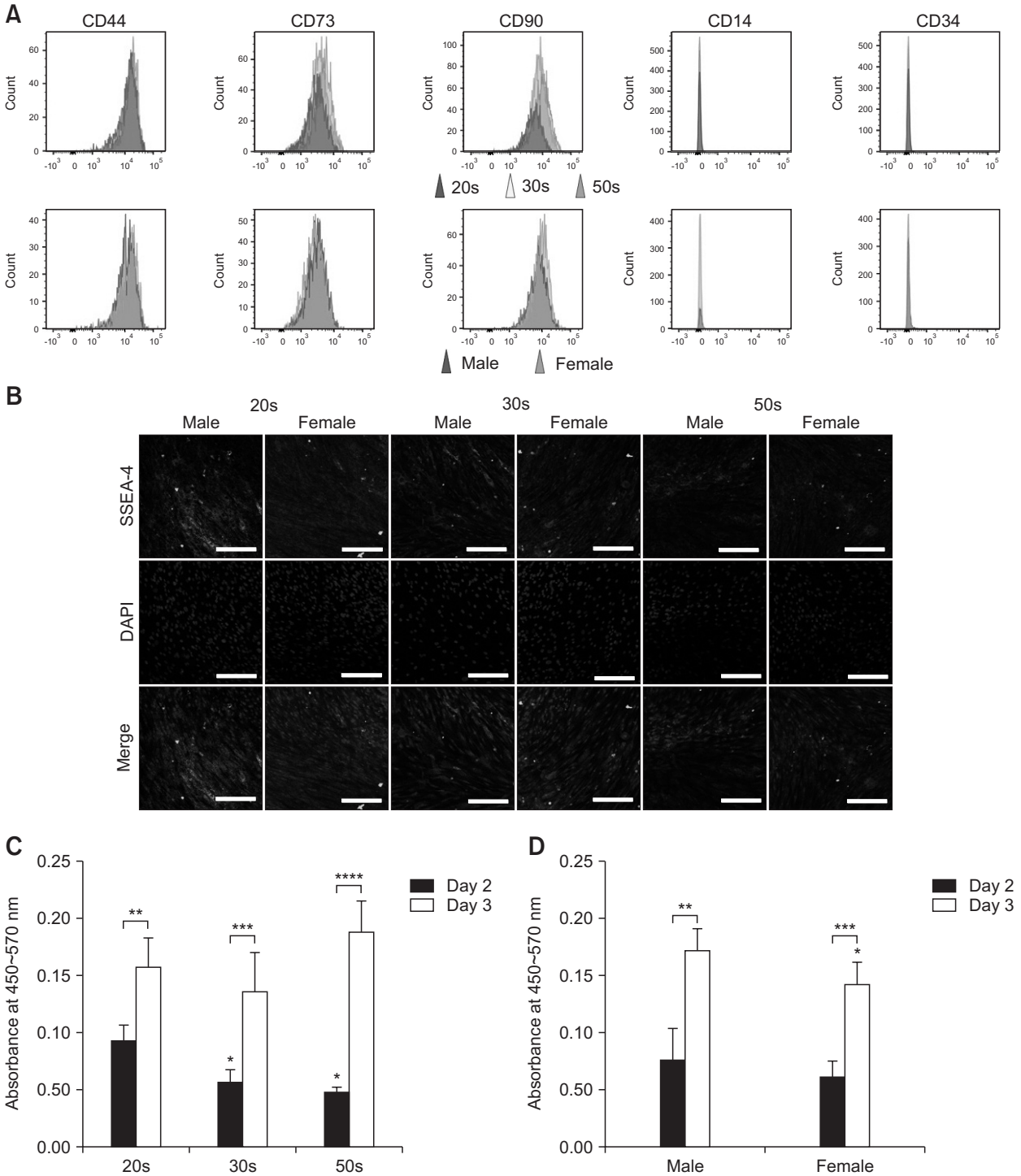

Fig. 2. (A) Expression of stem cell immunophenotype observed with flow cytometry. The bone marrow stem cells (BMSCs) in all groups expressed CD44, CD73, and CD90 surface antigens but did not express CD14 or CD34. (B) Expression of the stem cell markers SSEA-4. (C) Secretion of vascular endothelial growth factors from BMSCs among different age groups. *Statistically significant differences were noted when compared with the results of the 20s group on Day 2. **Statistically significant differences were noted when compared with the results of the 20s group on Day 2. ***Statistically significant differences were noted when compared with the results of 30s group on Day 2. ****Statistically significant differences were noted when compared with the results of the 30s group on Day 2. (D) Secretion of vascular endothelial growth factors in males and females. *Statistically significant differences were noted when compared with the results of males on Day 3. **Statistically significant differences were noted when compared with the results of males on Day 2. ***Statistically significant differences were noted when compared with the results of females on Day 2.

NY, USA). Expresion of RUNX2 and GAPDH was quantitatively done with image processing and analysis software (ImageJ; National Institutes of Health, Bethesda, MD, USA).

9. Statistical Analysis

The results were represented as means±standard deviations of the experiments. A normality test was performed with Shapiro-Wilk test. Student's t-test or a two-way analysis of variance with post hoc Tukey test was performed to determine the differences between the groups using statistical package for the social sciences (SPSS 12 for Windows; SPSS, Chicago, IL, USA). The level of significance was set at 0.05.

Result

1. Evaluation of Cell Morphology and Cellular Viability

The BMSC groups showed normal fibroblast morphology on Day 3 (Fig. 1A). The shapes of the cells in their 20s, 30s, and 50s were similar to each other. The cellular viability results determined at Day 4 via live and dead assays using confocal microscope are shown in Fig. 1B. Most of the cells emitted green fluorescence. The shape of the BMSCs was long and thin, and noticeable differences among age groups were noted. The CCK-8 results on Days 1 and 3 are shown in Fig. 1C. No significant changes in cellular viability were noted among the different age groups or sex groups (Fig. 1C, D). However, significant decreases in cellular viability were noted in the 30s group when comparing Day 1 with Day 3 ($P<0.05$).

2. Flow Cytometric Analysis and Immunofluorescence

The BMSCs expressed CD44, CD73 and CD90 surface markers but did not express CD14 or CD34 (Fig. 2A). The expression percentages were ≤1% for the negative surface markers and >95% for the positive surface markers. There were no noticeable differences among the CD surface markers by age. The expressions of CD surface markers were similar between males and females. Fig. 2B shows the results of the BMSCs stained with the stem cell markers SSEA-4. All of the age groups were well stained with the stem cell markers. There were no noticeable differences between the male and female groups.

3. Secretion of Human VEGF from BMSCs

Secretions of VEGFs from the BMSCs were seen in all groups for Day 3 (Fig. 2C, D). Observable secretion of the VEGF was seen on the second day. Moreover, the results showed a stable production of VEGF during the culture period. No significant differences in VEGF secretion were noted at Day 3 among the age groups. However, significant differences were noted between males and females at Day 3 ($P<0.05$).

4. Validation of mRNA Expression by RT-PCR

qRT-PCR was performed to detect the mRNA expression of Runx2 and collagen I. mRNA levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels and expressed as a fold change (Fig. 3). First, when comparing the expression levels of Runx2 among different age groups, a significant increase was noted in the 30s groups (Fig. 3A). However, no differences in Runx2 expression were noted between males and females (Fig. 3B). Similar trends were seen with collagen I expression (Fig. 3C, D). No statistical differences were noted between males and females in collagen I expression.

5. Western Blot

The protein expression of Runx2 and collagen I expression are shown in Fig. 4. The relative expression of Runx2 for the 20s, 30s, and 50s groups were 1.000±0.711, 0.804±0.383, and 0.478±0.159, respectively (Fig. 4B). There were no significant differences in Runx 2 and collagen I expression among the different age groups (Fig. 4C). The relative expression of Runx2

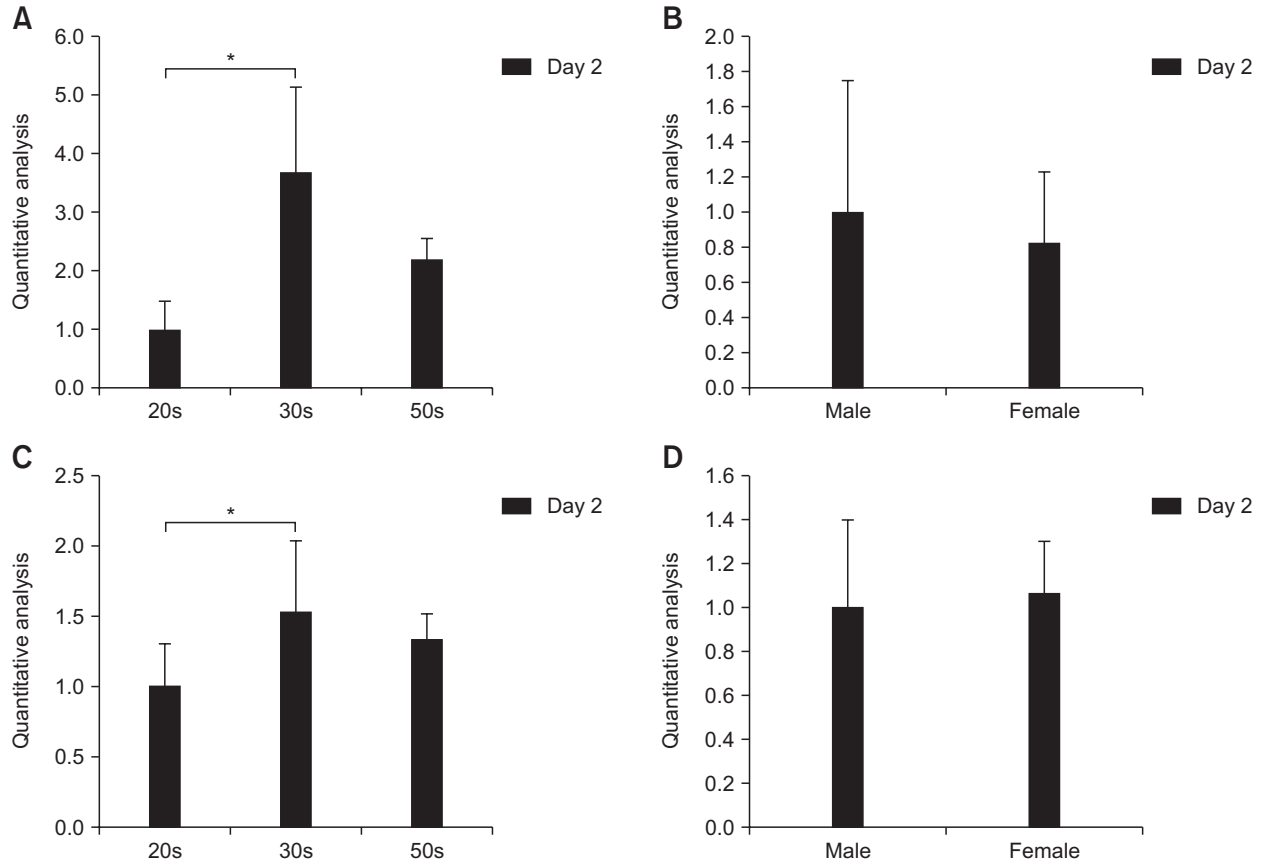

Fig. 3. (A) Runx2 gene expression of human bone marrow-derived stem cells (hBMSCs) according to age. *Statistically significant differences were noted when compared with the results of the 20s group on Day 2. (B) Runx2 gene expression of hBMSCs according to sex. (C) Collagen I gene expression of hBMSCs according to age. *Statistically significant differences were noted when compared with the results of the 20s group on Day 2. (D) Collagen I gene expression of hBMSCs according to sex.

for the 20s, 30s, and 50s groups were 1.000±0.069, 0.614±0.068, and 0.497±0.057, respectively (Fig. 4D).

Discussion

This report discusses the effects of age and sex on cellular viability and expression of stem cell markers and expression of mRNA and the protein of BMSCs derived from healthy donors.

Previously, comparative analyses of human mesenchymal stem cells from adipose tissue, bone marrow, and umbilical cord blood as sources of cell therapy have shown differing proliferation capacity and possible culturing periods[6,7]. In another report, the effects of aging on bone marrow-derived stem cells were tested by isolating cells from short-, medium-, or long-lived mouse strains; mesenchymal stem cells derived from the numerous mouse strains acted equivalently *in vitro* regarding growth rate[8]. In this report, no significant differences were noted in shape or cell viability among the different age groups.

Several things need to be considered for the interpretation of the data. Many apparent inconsistencies are due to different methods of extracting and isolating mesenchymal stem cells, which comprise various subsets of adult stem cells that vary not only in their differentiation capability but also in their susceptibility to senescence, ranging from a quasi-somatic lifespan to perennial vigor[9]. In a previous report, researchers tested the cellular milieu *in vivo* and reported considerable differences among various mouse strains; they suggested that

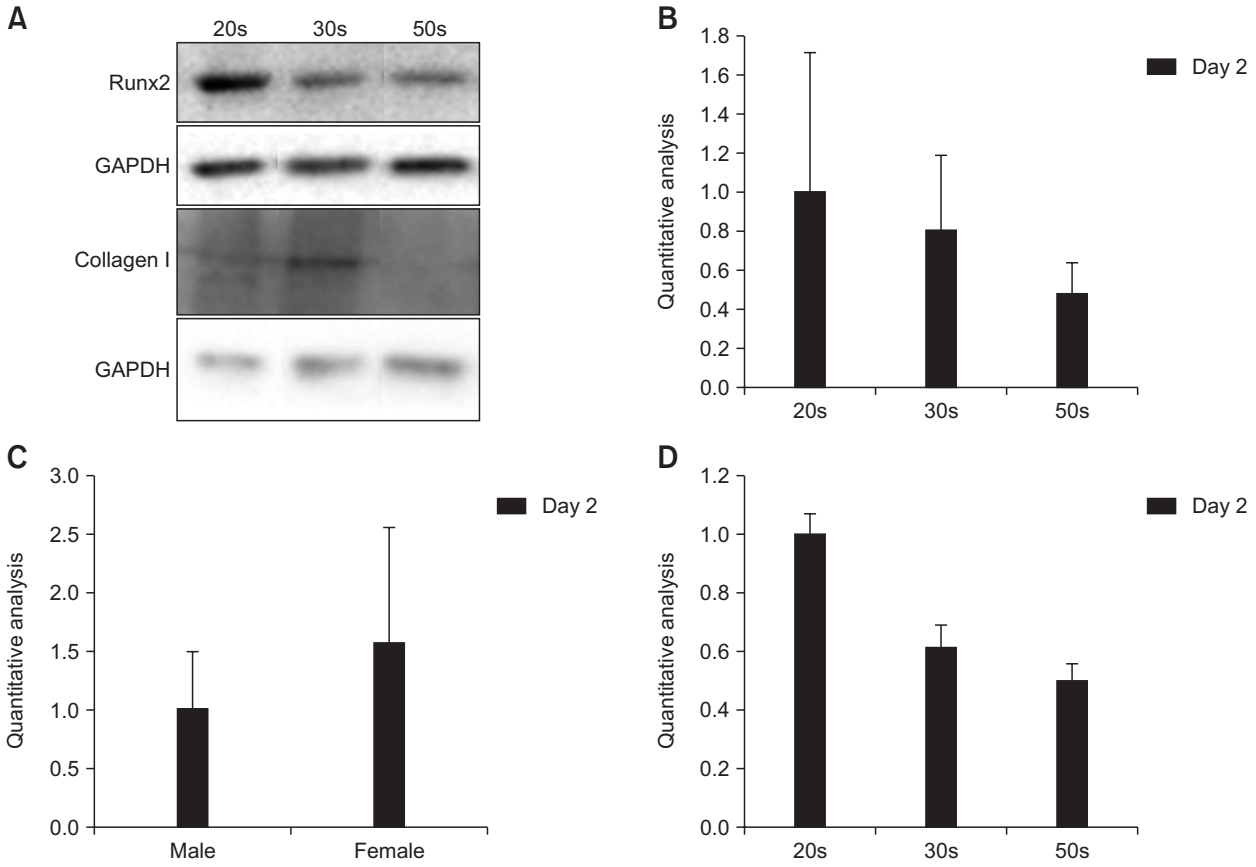

Fig. 4. (A) Western blot analysis to detect the protein expressions of Runx2, collagen I and GAPDH. (B) Quantitative analysis was performed to show levels of Runx2 according to age. (C) Runx2 expression according to sex. (D) Quantitative analysis of collagen I expression according to age.

the primary stem cells are highly affected by their molecular circumstance[8]. Additionally, the number of mesenchymal stem cells obtained by marrow aspiration declines with age[10]. A previous report evaluated the aging process in human bone marrow-derived stromal cells and found that mesenchymal stem cells gradually disappear in aging *in vitro* after cloning experiments[11]. This study shows that the secretion of human VEGF and protein expression differed among age groups. There may be an age-related decline in characteristics of bone marrow derived-stem cells, which may lead to issues when using autologous mesenchymal stem cells for cell therapy[10].

Conclusion

This study showed no significant differences in shape, cell viability, expression of stem cell surface markers, or secretion of human VEGF among age groups. Western blot analysis regarding Runx2 and collagen I expression showed a tendency of age-related decrease which did not reach statistical significance. Collectively, autologous or allogeneic BMSCs should be meticulously applied to obtain optimal results regarding age and sex.

Conflict of Interest

No potential conflict of interest relevant to this article was reported.

Acknowledgement

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References

1. Peptanariu D, Zlei M, Negură A, Carasevici E. Optimization of culture conditions for bone marrow stromal cells in RPMI-1640 medium. Rev Med Chir Soc Med Nat Iasi. 2012; 116: 222-7.
2. Phinney DG, Kopen G, Isaacson RL, Prockop DJ. Plastic adherent stromal cells from the bone marrow of commonly used strains of inbred mice: variations in yield, growth, and differentiation. J Cell Biochem. 1999; 72: 570-85.
3. Bianco P, Riminucci M, Gronthos S, Robey PG. Bone marrow stromal stem cells: nature, biology, and potential applications. Stem Cells. 2001; 19: 180-92.
4. Deans RJ, Moseley AB. Mesenchymal stem cells: biology and potential clinical uses. Exp Hematol. 2000; 28: 875-84.
5. Pereira RF, Halford KW, O'Hara MD, Leeper DB, Sokolov BP, Pollard MD, Bagasra O, Prockop DJ. Cultured adherent cells from marrow can serve as long-lasting precursor cells for bone, cartilage, and lung in irradiated mice. Proc Natl Acad Sci U S A. 1995; 92: 4857-61.
6. 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; 24: 1294-301.
7. Jin HJ, Bae YK, Kim M, Kwon SJ, Jeon HB, Choi SJ, Kim SW, Yang YS, Oh W, Chang JW. Comparative analysis of human mesenchymal stem cells from bone marrow, adipose tissue, and umbilical cord blood as sources of cell therapy. Int J Mol Sci. 2013; 14: 17986-8001.
8. Fehrer C, Laschober G, Lepperdinger G. Aging of murine mesenchymal stem cells. Ann N Y Acad Sci. 2006; 1067: 235-42.
9. Sethe S, Scutt A, Stolzing A. Aging of mesenchymal stem cells. Ageing Res Rev. 2006; 5: 91-116.
10. Stolzing A, Jones E, McGonagle D, Scutt A. Age-related changes in human bone marrow-derived mesenchymal stem cells: consequences for cell therapies. Mech Ageing Dev. 2008; 129: 163-73.
11. Mets T, Verdonk G. In vitro aging of human bone marrow derived stromal cells. Mech Ageing Dev. 1981; 16: 81-9.