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Effects of increased proliferation of human adipose tissue-derived mesenchymal stem cells by sphingosylphosphorylcholine on the survival of cryopreserved fat grafts

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Abstract: The use of cryopreserved adipose tissue for soft-tissue augmentation is common, but the unpredictability of fat graft viability remains a limitation. Human adipose-derived stem cells (hADSC) have been introduced to enhance viability and improve the survival of transplanted fat tissue. Sphingosylphosphorylcholine (SPC) is a bioactive lipid molecule involved in various cellular responses. SPC stimulates the proliferation of various cell types such as hADSC. We demonstrated the effects of hADSC and SPC on the survival of cryopreserved fat grafts in nude mice. The cryopreserved fat grafts were treated with hADSC or hADSC+SPC, and a normal saline (control) mixture in BALB/c male nude mice. We examined the weight and volume of the mice in each group (n=11) at 8 weeks after transplantation to evaluate the survival of the fat tissue. The hADSC group showed increased weight and volume compared with the control group. The hADSC+SPC group showed a higher survival rate in terms of weight and volume than the control or hADSC group. In addition, the hADSC+SPC treatment significantly increased the expression of angiogenic factors. These results suggest the potential clinical utility of hADSC+SPC.

Key words: human adipose-derived stem cell (hADSC); sphingosylphosphorylcholine (SPC); cryopreservation; fat; angiogenesis

INTRODUCTION

Autologous fat transplantation is a common and ideal technique for soft tissue augmentation and for filling soft tissue defects due to trauma or aging [1]. Although aspirated fat tissue is safe for use as a natural filler because of its natural texture, softness and contours, its use is associated with some problems, including unpredictability and low graft survival rate, which leads to partial necrosis [2]. Cryopreserved fat is frequently used for soft tissue augmentation. However, cryopreserved fat grafts have lower adipocyte-specific enzymatic activity than fresh grafts [3] and the unpredictability of fat graft viability remains a limitation. Many clinical methods have been developed to enhance the viability and improve the survival of transplanted fat tissue [4-7]. Recently, we reported that hADSC (human adipose-derived stem cell) supplementation improved the quality of transplanted fat grafts [8].

SPC (sphingosylphosphorylcholine) is a bioactive lipid molecule involved in various cellular responses, including proliferation, smooth-muscle contraction, wound healing, and angiogenesis [9]. SPC has been reported to stimulate the proliferation of different cell types such as fibroblasts, endothelial cells, keratinocytes, vascular smooth muscle cells and hADSC [10-14].

Thus, in this study, we aimed to examine the effects of SPC on hADSC proliferation and demonstrate the effect of hADSC+SPC treatment on the survival of cryopreserved fat grafts in nude mice.

MATERIALS AND METHODS

The present study was approved by the Animal Experiment Ethics Committee of Pusan National University Hospital (PNUH 2012-21).

Reagents

SPC (99% purity, as verified by using thin-layer chromatography) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Pertussis toxin (PTX) and VPC23019 were obtained from Enzo Life Sciences Inc. (Farmingdale, NY, USA); 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) and modified Hank's balanced salt solution (HBSS) were obtained from Sigma-Aldrich (St. Louis, MO, USA). α-Modified Eagle's medium (α-MEM; GIBCO) and fetal bovine serum (FBS) were purchased from HyClone (Logan, UT, USA). Unless otherwise specified, all the other reagents were purchased from Sigma-Aldrich.

Human fat tissue

Adipose tissue was obtained through elective surgeries with the patient's written informed consent as approved by the Institutional Review Board of Pusan National University Hospital. Adipose tissue was obtained from a 45-year-old healthy woman through suction-assisted lipectomy under general anesthesia. For aspiration, the areas were injected with a local anesthesia solution containing lidocaine (0.05%) and adrenaline (1:1000000) before start of the procedure. The fat was aspirated by a sterile 10 mL syringe and a 14-gauge cannula with a blunt tip. The aspirated fat was processed under sterile conditions by two centrifugation sessions of 4 min each at 1200 *g*. The aspirated fat tissue was frozen in a domestic refrigerator at -20°C for 8 weeks until use in the transplantation experiments. The fat was thawed for 1 h in a 37°C water bath and then transplanted.

hADSC isolation

hADSC were isolated and characterized according to the methods described in our previous studies [8,15]. To isolate hADSC, adipose tissues were washed with phosphate-buffered saline (PBS) and digested at 37°C for 30 min with 0.075% type I collagenase. The enzyme activity was neutralized with α-MEM containing 10% FBS. The samples (each sample contained 0.4 mL of fat) were centrifuged at 1200 *g* for 10 min. The pellet harvested from fat tissue was filtered through a 100-μm nylon mesh to remove cellular debris and incubated overnight at 37°C/5% CO_2 in a control medium (α-MEM, 10% FBS, 100-U/mL penicillin, and 100-μg/mL streptomycin). Following incubation, the plate was washed extensively with PBS to remove residual nonadherent cells. One week later, when a monolayer of adherent cells reached confluency, the cells were trypsinized (0.05% trypsin-EDTA; Sigma-Aldrich) and resuspended into the medium and the total number of cells was measured by trypan blue exclusion with a hemocytometer. For *in vivo* experiments, the prepared hADSC were preserved according to a cryopreservation protocol at -80°C for 7 weeks. Cell viability evaluated by trypan blue exclusion before transplantation was >95%. Cells between the 3rd and 5th passages were used for *in vitro* experiments.

Cell culture

The hADSC were maintained at 37°C in a humidified atmosphere containing 5% CO_2 in α-MEM containing 10% FBS. The adherent cell monolayer was trypsinized at 90% confluence (0.25% trypsin; Sigma-Aldrich) and the cells were resuspended in α-MEM containing 10% FBS and sub-cultured at a concentration of 2000 cells/cm^2. Cells between the 3rd and 4th passages were used in all further experiments.

MTT assay

The hADSC were plated at a density of 2×10^4 cells/well in 24-well plates, serum-starved for 24 h, and then treated with or without different reagents (SPC [0.1, 0.5, 1, 3, 5, 10, and 15 μM], PTX [0, 1, 5, 10, 50, 100, 150, and 200 nM], or VPC23019 [0, 1, 2, 3, 5, and 10 μM]) for the indicated times (1, 2, and 3 days). After the cells were washed, the culture medium containing MTT 0.5 mg/mL was added to each well. The cells were incubated for 2 h at 37°C, the supernatant was removed and the formazan crystals that formed in the viable cells were solubilized with 200 μL of DMSO. A 100-μL aliquot of each sample was then transferred to a well of a 96-well plate, and the absorbance of each

was measured at 560 nm using a microplate reader (Sunrise Reader/serial No. 909004125) with XFLUOR4 software version 4.51 (Tecan, Männedorf, Switzerland). This experiment was repeated four times.

Western blot

The cells were washed twice in cold PBS and lysed in radioimmunoprecipitation assay buffer (Sigma-Aldrich). Proteins were loaded on a 10% sodium dodecyl sulfate polyacrylamide gel, electrotransferred to nitrocellulose membranes (Hybond-ECL; Amersham Pharmacia Biotech, Piscataway, NJ, USA), and probed with the following monoclonal antibodies: glyceraldehyde 3-phosphate dehydrogenase, total p44/42 Map kinase [Erk1/Erk2], and antiphospho-Erk1/2 [Thr202/204]; Cell Signaling Technology, Danvers, MA, USA, Ki-67; Abcam, Cambridge, UK). Immunoreactive bands were detected using anti-mouse and anti-rabbit peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech) and visualized by enhanced chemiluminescence (ECL detection kit; Amersham Pharmacia Biotech).

Animal model and fat transplantation

The animal experiment used 33 six-week-old BALB/c male nude mice weighing 20-30 g each (Biogenomics, Seoul, Korea). The mice were used previously to study fat grafts, providing the possibility to observe the use of human fat in an animal model [16,17]. The mice were divided into hADSC, hADSC+SPC and control (normal saline) groups, with 11 mice in each group. The mice in the hADSC group received a combination of 0.4 mL of cryopreserved fat and 0.04 mL of hADSC (obtained from 0.4 mL of fat). The mice in the hADSC+SPC group received a combination of 0.4 mL of cryopreserved fat, 0.02 mL of ADSC (obtained from 0.4 mL of fat), and 0.02 mL 3 μM SPC. The mice in the control group received a combination of 0.4 mL of fat and 0.04 mL of normal saline. Fat was injected subcutaneously in the back of each mouse using a sharp 16-gauge needle (Coleman injection cannula; Mentor Corp., Santa Barbara, CA, USA). The experimental protocol was approved by the Guide for Care and Animal Experiment of Pusan National University.

Follow-up and data collection

The animals were harvested at 8 weeks after the fat transplantation and the grafted fat was dissected. The weight and volume of the fat were measured. The volume was determined by the liquid overflow method [18].

Real-time polymerase chain reaction (PCR)

Total RNA was isolated from the hADSC or grafted fat using TRIzol Reagent (Invitrogen; Thermo Fisher Scientific, Waltham, MA, USA). The total RNA (2 μg) was then reverse transcribed to cDNA with the reverse transcriptase M-MLV (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol. The internal control for expression analysis was β-actin. The primer sequences used in the experiment were as follows: β-actin: 5′-CTG GTG CCT GGG GCG-3′, 5′-AGC CTC GCC TTT GCC GA-3′; human growth factor (HGF): 5′-CCT ATG CAG AGG GAC AAA GG-3′, 5′-TGC TAT TGA AGG GGA ACC AG-3′; interleukin-6 (IL-6): 5′-AAA GAG GCA CTG GCA GAA AA-3′, 5′-CAG GGG TGG TTA TTG CAT CT-3′; tumor necrosis factor-α (TNF-α): 5′-GAC AAG CCT GTA GCC CAT GT-3′, 5′-TTG ATG GCA GAG AGG TT-3′; vascular endothelial growth factor (VEGF): 5′-AAG GAG GGC AGA ATC AT-3′, 5′-ATC TGC ATG GTG ATG TTG GA-3′; matrix metallopeptidase-9 (MMP-9): 5′-ACC TCG AAC TTT GAC AGC GAC A-3′, 5′-GAT GCC ATT CAC GTC CTT A-3′; granulocyte chemotactic protein-2 (GCP2): 5′-GTC CTT CGG GCT CCT TGT-3′, 5′-AAC TTG CTT CCC GTT CTT CA-3′ (Bioneer, Deajeon, South Korea). All the primer sequences were determined using established GenBank sequences. Real-time quantitation was conducted using a Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) on the ABI 7500 Instrument (Applied Biosystems). ABI 7500 Instrument version 2.0.1 software (Applied Biosystems) was used to analyze the kinetics of PCR and to calculate the quantitative data. All experiments were performed three times, and negative and positive controls were included in all the experiments. β-actin mRNA was amplified as an internal control. For each sample, copy numbers of the target gene mRNA were divided by those of β-actin mRNA to normalize for target gene mRNA expression and to avoid inter-sample differences in RNA quantity.

Statistical analysis

All results are presented as the mean±SEM. Comparisons between two groups were analyzed by the Student's *t* test. Multiple group comparisons were conducted by one-way analysis of variance with Tukey adjustments. Probability values of $P<0.05$ were considered statistically significant. Statistical analysis of differences in fat graft weight and volume between the three groups was performed by the Kruskal-Wallis test with SPSS version 17.0 for Windows (SPSS, Inc., Chicago, IL, USA). The differences were considered significant at $P<0.05$.

RESULTS

Effect of SPC on hADSC proliferation

To investigate the effect of SPC on hADSC proliferation, the cells were exposed to the indicated concentration of SPC for 48 h, and hADSC proliferation was measured by the MTT assay. Exposure of hADSC to SPC resulted in a dose-dependent increase in cell proliferation with a maximal peak at 5 and 10 μM (Fig. 1A). The treatment of hADSC with 5 or 10 μM of SPC increased the cell number in a time-dependent manner (Fig. 1B) and did not affect cell morphology (Fig. 1C). Treatment with 5 or 10 μM SPC did not affect cell survival, but treatment with 15 μM SPC lowered it. Therefore, we used the 5 μM SPC treatment for other experiments. SPC has been reported to induce activation of the Erk1/2 pathway in different cell types [20,21], and it can contribute to cell proliferation. To understand the signaling mechanisms, we determined whether SPC activated the Erk1/2 pathway by Western blotting. Erk1/2 phosphorylation was increased in the SPC-treated hADSC (Fig. 1D). The SPC treatment increased the expression level of the proliferating cell marker, Ki-67 (Fig. 1D). We suggest that SPC increased hADSC proliferation.

To confirm the effect of PTX, an antagonist of SPC, on the proliferation of SPC-treated hADSC, hADSC were treated with PTX and then measured by the MTT assay. PTX exhibited a cytotoxic effect (Fig. 2A), whereas VPC23019, an inhibitor of the downstream SPC mediator sphingosine-1-phosphate, had no effect on hADSC proliferation (Fig. 2B).

As shown in Fig. 2C and D, pretreatment of hADSC with 5 μM VPC23019 completely blocked the SPC-induced increase in hADSC proliferation. These results indicate that the SPC treatment affected hADSC proliferation without affecting their morphological changes.

Effect of SPC on angiogenesis of hADSC

To determine the effect of SPC on the angiogenesis potential of hADSC, we performed real-time PCR. The 5-μM SPC treatment increased the expression levels of angiogenesis-related genes such as IL-6, TNF-α, and MMP9 as compared to the control group (Fig. 3). These results indicate that the treatment with 5 μM increased hADSC proliferation and the angiogenesis potential.

Effect of SPC-treated hADSC on cryopreserved fat tissue angiogenesis potential

To examine the effect of SPC-treated hADSC on cryopreserved fat tissue angiogenesis potential, we determined the expression of angiogenesis-related genes by real-time PCR. The results showed increased expression of angiogenesis-related genes (HGF, IL-6, GCP2, VEGF, and MMP9; $P<0.005$) in the hADSC group compared with the control group. Moreover, the SPC-treated hADSC group had significantly higher expression levels of many angiogenesis-related genes (HGF, GCP2, VEGF, and MMP9; $P<0.005$) than the other groups (Fig. 4).

Effect of SPC-treated hADSC on cryopreserved fat tissue survival

To determine the effect of SPC-induced hADSC proliferation on the survival of fat graft tissues, we conducted a fat transplantation experiment. The weight and volume of the surviving fat graft tissues were determined at 8 weeks after cryopreserved fat transplantation (Fig. 5). The hADSC group showed significant increases in weight and volume as compared to the control group ($P<0.002$). The SPC-treated hADSC (hADSC+SPC) group exhibited a higher survival rate in terms of weight and volume than the control group ($P<0.002$) or the hADSC group ($P<0.002$; Table 1).

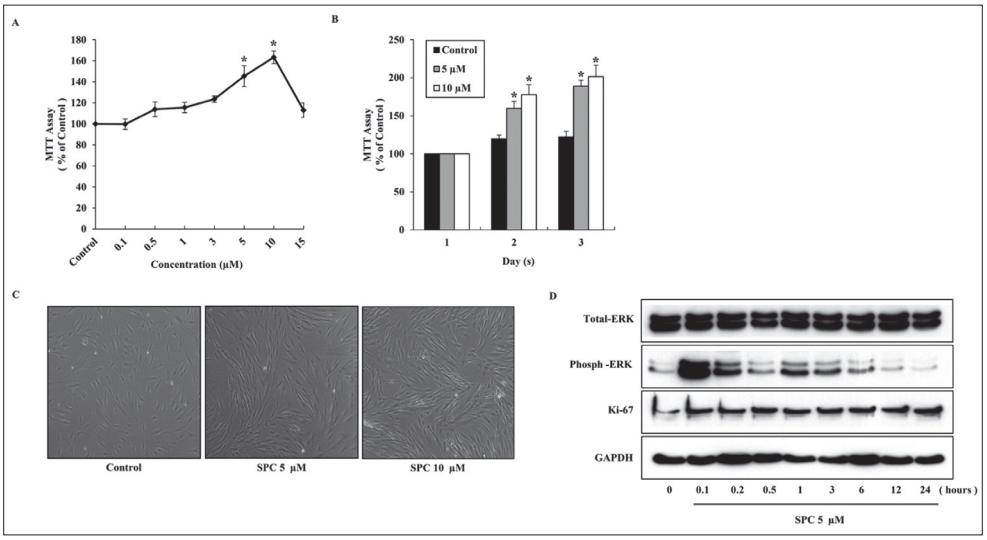

Fig. 1. The effect of SPC treatment on hADSC proliferation. **A**, **B** – hADSC seeded in 24-well culture plates at a density of 2×10^4 cells/well in a growth medium and cultured under a serum-starved condition for 24 h before SPC treatment. The cells treated with the indicated concentrations of SPC for 48 h (**A**) and the proliferation ability of the hADSC treated with vehicle (control), or 5 or 10 μM SPC for the indicated times (**B**). The number of cells was determined by the MTT assay, and the results shown are the percentages of the control. The data are shown as mean±SEM (n=4). **P*<0.05, compared with the control. **C** – The SPC treatment did not affect cell morphology, as shown in the representative phase-contrast photographs of the vehicle (control), 5 μM SPC- and 10 μM SPC-treated hADSC (Olympus IX70). Original magnification: ×100. **D** – ERK activation and Ki67 expression by SPC in hADSC. Serum-starved hADSC were treated with 5 μM SPC for the indicated periods. ERK phosphorylation was determined by Western blotting of the cell lysates with anti-phospho-Erk1/2 antibody, and the amounts of ERK was assessed with an anti-ERK antibody to confirm equal loading. The expression levels of Ki67 were determined by Western blotting with anti-Ki67 antibody. To confirm equal protein loading, the quantities of GAPDH were determined using anti-GAPDH antibody.

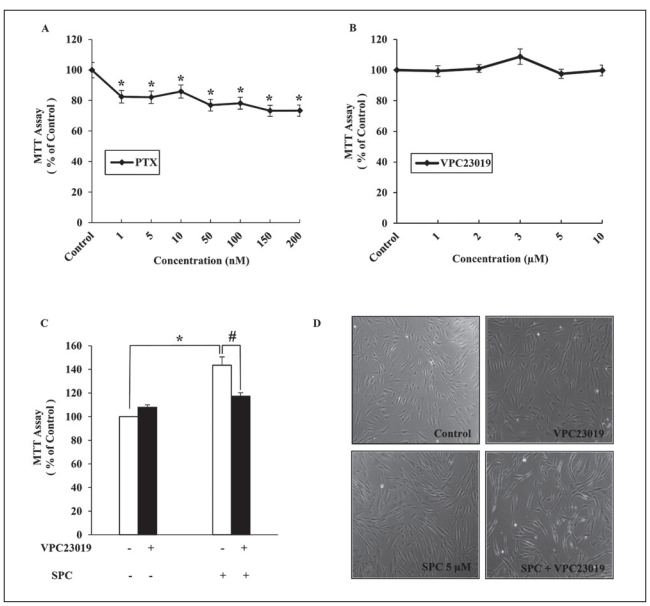

Fig. 2. Effect of SPC treatment on hADSC proliferation. **A**, **B** – hADSC plated at 2×10^4 cells/well in a 24-well plate and serum-starved hADSC, followed by treatment with different concentrations of (**A**) PTX or (**B**) VPC23019 for 48 h. The number of cells was measured by the MTT assay and the results are shown as percentages of the controls. The data are shown as mean±SEM (n = 4). **P*<0.05, compared with the control. **C** – Serum-starved hADSC pretreated with vehicle or 5 μM VPC23019 for 2 h, after which they were treated with vehicle or 5 μM SPC for 48 h. The number of cells was measured by the MTT assay and the results are shown as percentages of the control (without SPC and VPC23019). The data are shown as mean±SEM (n = 4). **P*<0.05, compared with the control; $^{\#}P$<0.05, compared with only the 5 μM SPC-treated group. **D** – Phase-contrast images of hADSC incubated under the indicated conditions were obtained with a digital camera equipped with an inverted microscopy. Original magnification, ×100.

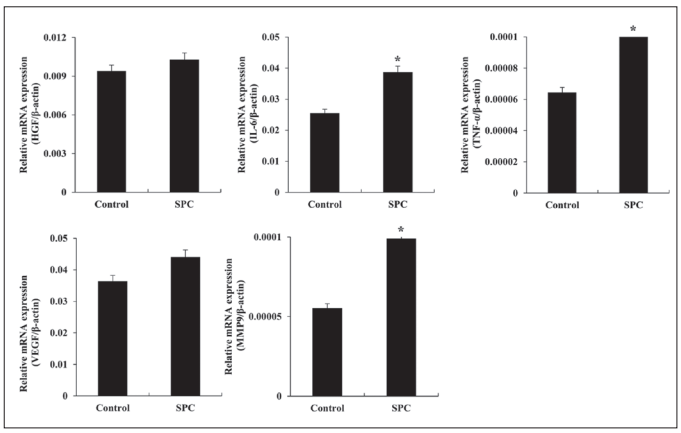

Fig. 3. Effect of SPC treatment on hADSC angiogenesis. Serum-starved hADSC were treated with vehicle (control) or 5 μM SPC (SPC) for 48 h. Total RNA was isolated from the cells, and angiogenesis-related gene expression was analyzed by real-time PCR. Experimental data are expressed as the relative ratio to β-actin levels of the corresponding samples. The data are shown as mean±SEM (n = 3) **P*<0.05, compared with the control.

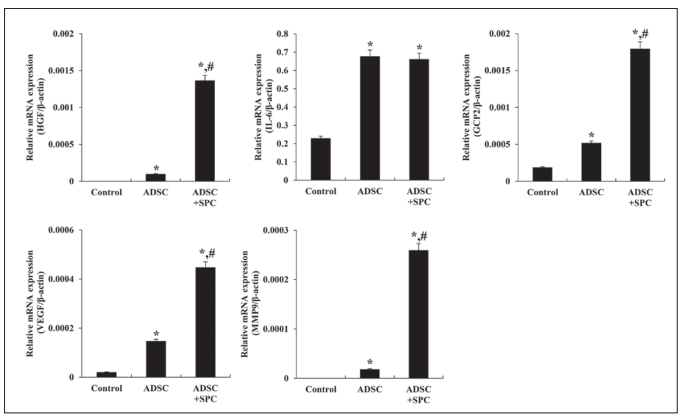

Fig. 4. The effect of hADSC or hADSC+SPC treatment on angiogenesis-related gene expression in the fat grafts. Total RNA was isolated from control fat tissue or hADSC- or hADSC+SPC-treated fat tissue. Angiogenesis-related gene expression was analyzed by real-time PCR. Experimental data are expressed as the relative ratio to β-actin levels of the corresponding samples. The data are shown as mean±SEM (n = 3). **P*<0.05, compared with the control; #*P*<0.05, compared with the hADSC (hADSC-treated fat).

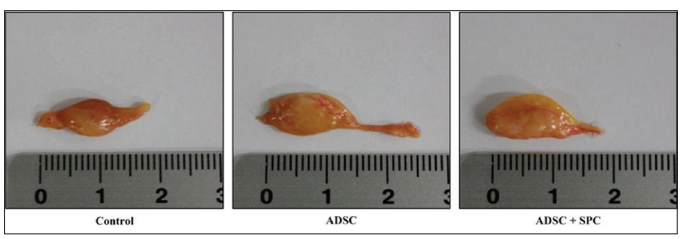

Fig. 5. Fat grafts harvested from mice 8 weeks after transplantation. The features of the fat grafts obtained (from left to right): a representative small fat graft from a normal saline-treated fat graft (control), an intermediate-size hADSC-treated fat graft (hADSC) and a large SPC mixed hADSC-treated fat graft (hADSC+SPC).

Table 1. Effects of hADSC and hADSC+ SPC treatments on fat graft weight and volume

Group	**Weight** (Median, 25^{th}-75^{th})	**Volume** (Median, 25^{th}-75^{th})
Control (n=11)	0.17 (0.15-0.19)	0.19 (0.17-0.21)
hADSC (n=11)	0.20 (0.18-0.21)*	0.22 (0.20-0.23)*
hADSC+SPC (n=11)	0.22 (0.20-0.24)*,#	0.24 (0.23-0.26)*,#

**P*<0.002 (vs the control group); #*P*<0.002 (vs the hADSC group), Kruskal Wallis test
Data are given as medians and 25^{th} to 75^{th} percentile.

DISCUSSION

Cryopreserved fat is frequently used for soft tissue augmentation to avoid repeated harvesting of fresh fat tissues. However, cryopreserved fat grafts have lower adipocyte-specific enzymatic activity than fresh grafts [3] and the unpredictability of fat graft viability remains a limitation. Various studies have been conducted to develop strategies for increasing the survival rate of transplanted fat tissues [4-7]. In one of these studies, we reported that supplementation of hADSC to cryopreserved fat grafts can improve both the survival and quality of the transplanted fat [8].

SPC is reportedly associated with various cellular functions of the cardiovascular system, skin, neurons and immune cells [9,12,17,19]. In addition, the biphasic effect of SPC on proliferation has been observed in other cell types. We recently reported that SPC treatment of cryopreserved fat graft tissue improved fat survival and peaked when the fat was supplemented with 3 μM SPC [22]. Based on the results of the previous study, 3 μM SPC was used in the present animal study. Previous studies reported that SPC can stimulate hADSC proliferation [14,23]. In this study, we demonstrate that treatment with 5 or 10 μM SPC induced proliferation and angiogenesis of hADSC. These results indicate that SPC increased the proliferation of hADSC without affecting their morphological changes. In an *in vivo* experiment, hADSC supplementation improved the survival of the fat graft tissue. However, the combined use of hADSC and SPC significantly increased fat graft survival and that the expression levels of angiogenesis-related genes (HGF, GCP2, VEGF, and MMP9) that were increased in hADSC+SPC-treated

fat tissue. We suggest that the increased fat survival rates in the hADSC and SPC group are caused by the SPC-induced hADSC proliferation and angiogenesis. Therefore, the hADSC+SPC treatment of cryopreserved fat tissue grafts may provide a new approach for improving fat cell survival.

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Author contributions: Yong Chan Bae, Ji Sun Song and Su Bong Nam designed and performed the experiments. Yong Chan Bae, Kyeong Wook Nam and Joo Hyoung Kim analyzed the data. Ji Sun Song and Su Bong Nam wrote the manuscript. All authors read and approved the manuscript.

Conflict of interest disclosure: The authors report no conflicts of interest in this work.

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