Effects of sphingosylphosphorylcholine on cryopreserved fat tissue graft survival

YONG CHAN BAE[1,2], CHI WON CHOI[1], KYEONG WOOK NAM[1], JI SUN SONG[2] and JAE WOO LEE[1]

[1]Department of Plastic and Reconstructive Surgery, School of Medicine, Pusan National University;
[2]Biomedical Research Institute, Pusan National University Hospital, Busan 602-739, Republic of Korea

Received April 6, 2015; Accepted February 25, 2016

DOI: 10.3892/mmr.2016.5677

Abstract. Autogenous microfat grafting is widely used to augment depressed deformities or for other cosmetic purposes. Since the microfat survival rate is unpredictable due to absorption and calcification, previously cryopreserved fat is widely used for secondary procedures. Sphingosylphosphorylcholine (SPC) is a lysophospholipid, which has a role in several cellular responses, and is known to stimulate DNA synthesis and proliferation. Since endothelial progenitor cells (EPCs) are known to enhance the survival rate of transplanted fat tissue, the present study assessed the effects of SPC on EPCs, in order to verify its positive effects on proliferation. Cryopreserved human fat tissues mixed with various concentrations of SPC were grafted into the nude mouse model. After grafting, the viability of each SPC mixed group was determined and compared with that of the non-SPC group. SPC exhibited a positive effect on EPC proliferation and angiogenic potential over 3 days when used at specific concentrations. The fat grafts of the 3 μM SPC-treated group weighed significantly more and the volume was markedly increased, as compared with the control group. A reverse transcription-quantitative polymerase chain reaction was conducted on the total RNA extracted from SPC-treated fat tissues, which detected increased mRNA expression levels of matrix metallopeptidase-9 and tumor necrosis factor-α compared with in the control group. These results indicate that specific concentrations of SPC may exert favorable effects on grafted cryopreserved human fat tissue, which may be due to the increased mRNA expression levels of genes associated with angiogenesis.

Correspondence to: Professor Jae Woo Lee, Department of Plastic and Reconstructive Surgery, School of Medicine, Pusan National University, 179 Gudeok-ro, Seo-gu, Busan 602-739, Republic of Korea
E-mail: surgeon@pusan.ac.kr

Key words: sphingosinephosphorylcholine, cryopreservation, fat, angiogenesis

Introduction

Autogenous microfat grafting is a popular and relatively simple procedure, which is widely used to augment depressed deformities or for other cosmetic purposes. Various temporary and permanent fillers have been used as alternatives with varying degrees of success (1); however, the autogenous microfat grafting technique is most commonly selected by surgeons because it is easy to perform, produces no ill-effects associated with foreign body reactions, and can compensate for large volume deficits.

Despite numerous advantages associated with the use of microfat grafting for soft tissue recontouring, some problems must be considered with regards to this procedure, particularly with regards to the unpredictability of the final survival rate due to absorption and calcification. In addition, microfat retouching must be frequently performed a few months after the initial procedure using previously cryopreserved fat tissues. However, cryopreserved fat tissues tend to exhibit greater absorption with re-grafting compared with the initial procedure, and increased survival of the cryopreserved fat tissues is indispensable to the final result. To prevent unintended absorption, grafted fat tissues should be revascularized within a short time period (2).

Sphingosylphosphorylcholine (SPC) is a lysophospholipid with a role in several cellular responses, including migration, wound healing and differentiation, which is known to stimulate DNA synthesis and proliferation (3). A strong mitogenic effect of SPC has also been observed in numerous types of cells, including endothelial cells from different vascular beds (4).

It has previously been demonstrated that endothelial progenitor cells (EPCs) can become incorporated into active sites of angiogenesis, and augment collateral vessel growth to ischemic tissues (5). In a previous study, EPCs were reported to enhance the survival rate of transplanted fat tissues, possibly due to the induction of angiogenesis (6). However, the use of EPCs in the fat graft domain is limited because it requires human donors and an *in vitro* culture period of >7 days. Therefore, the present study aimed to determine the effects of SPC on EPCs *in vitro*, in order to confirm the usefulness of employing SPC in cryopreserved fat tissues to improve post-transplantation fat graft survival rates. To verify the effects of SPC on fat tissues *in vivo*, cryopreserved human fat tissues were mixed with various concentrations of SPC and the effects were analyzed.

Materials and methods

Reagents. SPC (99% purity, as verified by thin-layer chromatography) was purchased from Avanti Polar Lipids, Inc. (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 Hanks' balanced salt solution were obtained from Sigma-Aldrich (St. Louis, MO, USA). Endothelial cell basal growth medium-2 (EBM-2) was purchased from Lonza Group AG (Basel, Switzerland), and fetal bovine serum (FBS) was obtained from HyClone; GE Healthcare Life Sciences (Logan, UT, USA). Unless otherwise specified, all other reagents were purchased from Sigma-Aldrich.

Cell culture. The EPCs were obtained from the Korean Cell Line Bank (Seoul, South Korea) and were maintained at 37˚C in an atmosphere containing 5% CO_2, in EBM-2 (CC-3162; Lonza Group AG) supplemented with EGM-2 Bullet kit (CC3156 and CC-4176; Lonza Group AG) and 5% FBS. There cells were then sub-cultured to 90% confluencey for 3-5 days using 0.05% trypsin EDTA. In the experiments of the present study, the cells used were sub-cultured less than eight times.

MTT proliferation assay. EPC proliferation was evaluated using an MTT assay. The cells were plated at a density of $1x10^4$ cells/well in 24-well plates, were serum-starved for 24 h, and were then treated with or without various reagents [SPC (0.1, 0.5, 1, 3, 5, 10 and 15 μM), PTX (1, 5, 10, 50 and 100 nM) or VPC23019 (1, 2, 3, 5, 7 and 10 μM)] for the indicated durations (1, 2, 3 and 4 days). After washing, culture medium containing 0.5 mg/ml MTT was added to each well. The cells were then incubated for 2 h at 37˚C, after which the supernatant was removed and the formazan crystals that had formed in the viable cells were solubilized using 200 μl DMSO. A 100 μl aliquot of each sample was then transferred to a well in a 96-well plate, and the absorbance was measured at 560 nm using a microplate reader and XFLUOR4 software version 4.51 (Tecan Group, Männedorf, Swizerland). This experiment was repeated four times.

In vitro EPC tube formation. The *in vitro* angiogenic potential of SPC was assessed by the ability of the EPCs to form tubes on a basement membrane matrix (BD Matrigel™ Matrix; BD Biosciences, Franklin Lakes, NJ, USA). The EPCs were plated at a density of $1x10^2$ cells/well in a 96-well plate and were treated with 1, 3, 5, 10 and 15 μM SPC. The EPCs were evaluated using a light microscope (Olympus, Tokyo, Japan) under x200 magnification after 12 h of culture (7).

Human fat tissue. Adipose tissue was obtained from an elective surgery. The patient provided written informed consent, and the study was approved by the Institutional Review Board of Pusan National University Hospital (Busan, South Korea). Adipose tissue was obtained through suction-assisted lipectomy from a 22-year-old healthy woman undergoing suction-assisted lipectomy under general anesthesia. The areas were injected with a local anesthetic solution containing lidocaine (0.05%) and adrenaline (1:1,000,000) prior to the start of the procedure. The fat was aspirated using a sterile 10 ml syringe and a 14-gauge cannula with a blunt tip. The aspirated fat was processed under sterile conditions by two-step centrifugation (4 min each; 1,200 x g). The aspirated fat tissue was frozen at -20˚C for 8 weeks until being used in the transplantation experiments. Prior to transplantation, the fat was thawed for 1 h in a 37˚C water bath.

Animal model and fat transplantation. The animal experiment used 48 male Balb/C nude mice (Biogenomics, Inc., Seoul, Korea; age, 6 weeks; weight, 20-30 g). During the study, mice were housed under constant laminar airflow and were fed standard laboratory chow and water. They were kept under an artificial 12 h light/dark cycle at a constant temperature range (24±2˚C) and relative humidity (55±10%). Nude mice have previously been used to study fat grafts and enable the observation of the use of human fat in an animal model (8,9). The mice were divided into 1, 3, 5, 10 and 15 μM SPC-treated groups, and the control group (n=8 mice/group). Mice in the SPC-treated groups received a combination of 0.4 ml cryopreserved fat and 0.02 ml SPC (1, 3, 5, 10 or 15 μM). Mice in the control group received a combination of 0.4 ml fat and 0.02 ml normal saline. Fat was injected subcutaneously into the back of each mouse using a 16-G sharp needle (Coleman injection cannula; Mentor Worldwide LLC, Santa Barbara, CA, USA). The Animal Care and Experiment Committee of Pusan National University (Busan, South Korea) approved the experimental protocol.

Follow-up and data collection. The mice were humanely sacrificed by CO_2 asphyxiation 8 weeks after fat transplantation. The grafted fat was carefully dissected from their backs, and its volume and weight were measured. The volume was determined using the liquid overflow method (10). Fat samples from the six mouse groups [untreated control group, and SPC-treated groups (1, 3, 5 , 10 and 15 μM)] were fixed in 10% formalin and embedded in paraffin. Tissue sections were acquired from the center of the dissected fat biopsy. For CD31 staining, 5-mm sections were permeabilized with 0.3% Triton X-100 for 10 min, then blocked for 1 h with 8% bovine serum albumin (BSA) at room temperature. Primary rabbit anti-CD31 antibodies (cat. no. Ab28364, Abcam Cambridge, MA, USA) were diluted to 1:500 in phosphate-buffered saline (PBS) with 2% BSA, and were incubated with the sections overnight at 4˚C. The biotinylated goat anti-rabbit immunoglobulin G (cat. no. BA-1000, Vector Laboratories: Burlingame, CA, USA) were diluted to 1:100 in PBS and incubated for 1 h at room temperature. Staining was visualized using biotin-avidin-peroxidase complexes (Vector Laboratories) and diaminobenzidine (Vector Laboratories). Images were collected using a Leica TCL-SP2 confocal microscope system at x200 magnification (Leica Microsystems Heidelberg GmbH, Heidelberg, Germany).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total cellular RNA was isolated from the EPCs or homogenized fat using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Waltham, MA, USA). The total RNA (2 μg) was then reverse transcribed into cDNA with the Reverse Transcriptase M-MLV (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol. The primers used in the experiment were as follows: β-actin, forward 5'-CTGGTG

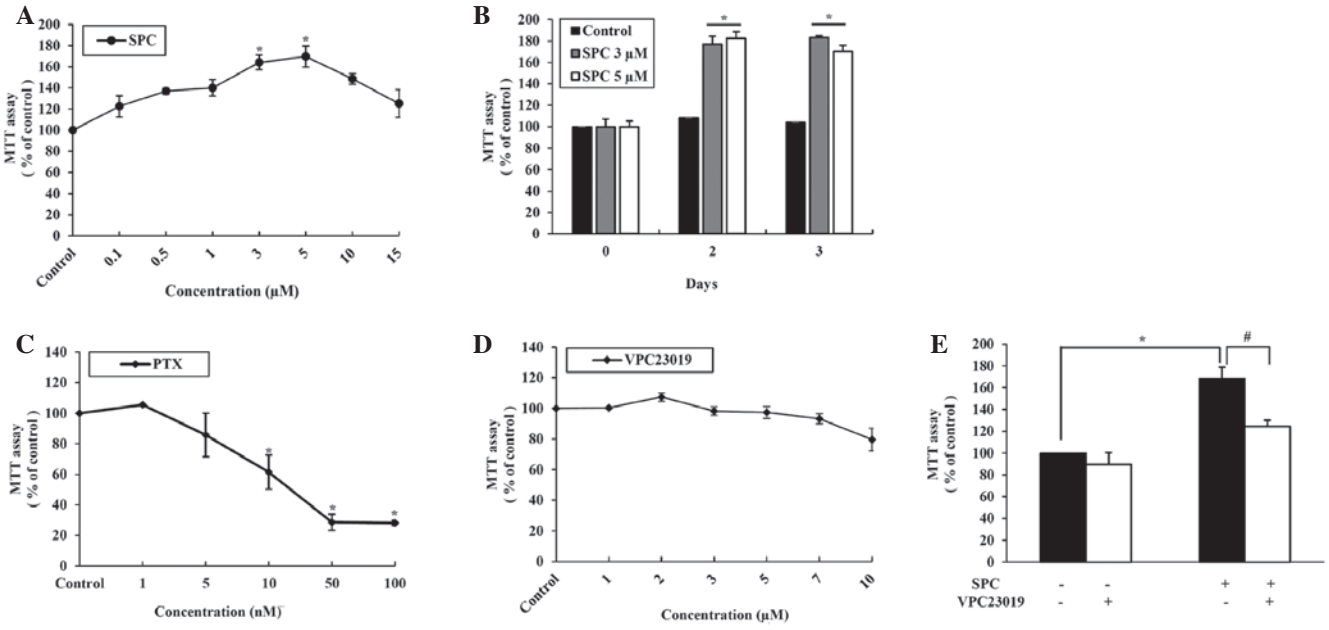

Figure 1. Effects of sphingosylphosphorylcholine (SPC) on the proliferation of endothelial progenitor cells (EPCs). (A) SPC exhibited a maximal effect on EPC proliferation when used at 3 and 5 μM. (B) The positive effects of SPC remained efficacious at day 3. (C) Pertussis toxin (PTX) exhibited a direct cytotoxic dose-dependent effect on EPCs, (D) whereas VPC23019 did not. To confirm the positive effects of SPC on EPC growth, cells were cotreated with SPC and VPC23019. (E) SPC promoted EPC growth, which was suppressed following the addition of VPC23019. Data are presented as the mean ± standard error of the mean. *P<0.05, vs. the control group, #P<0.05, vs. the only SPC treated group. MTT, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

CCTGGGGCG-3', reverse 5'-AGCCTCGCCTTTGCCGA-3'; human growth factor (HGF), forward 5'-CCTATGCAGAGG GACAAAGG-3', reverse 5'-TGCTATTGAAGGGGAACC AG-3'; interleukin-6 (IL-6), forward 5'-AAAGAGGCACTG GCAGAAAA-3', reverse 5'-CAGGGGTGGTTATTGCATCT3 '; tumor necrosis factor-α (TNF-α), forward 5'-GACAAG CCTGTAGCCCATGT-3', reverse 5'TTGATGGCAGAGAGG AGGTT-3'; matrix metallopeptidase (MMP)-2, forward 5'-CAG GTGATCTTGACCAGAAT-3, reverse 5'-CATCATGGATTC GAGAAAAC-3'; MMP-9, forward 5'-ACCTCGAACTTTGAC AGCGACA-3', reverse 5'-GATGCCATTCACGTCGTCCTT A-3'; and vascular endothelial growth factor (VEGF), forward 5'-AAGGAGGAGGGCAGAATCAT-3', and reverse 5'-ATC TGCATGGTGATGTTGGA-3' (Bioneer Corporation, Deajeon, Korea). All of the primer sequences were determined according to established GenBank sequences (http://www.ncbi.nlm.nih.gov/genbank/). RT-qPCR was conducted using a Power SYBR Green PCR Master mix (Applied Biosystems, Warrington, UK) on the ABI 7500 Instrument (Applied Biosystems). The reaction mixture contained 2 μl of 10 ng/μl cDNA, 10 μl SYBR (qPCR master mix), 0.5 μl of 10 pmol forward primer, 0.5 μl of 10 pmol reverse primer and 7 μl nuclease-free water to produce a final volume of 20 μl. The amplification program consisted of one cycle at 95˚C with a 60 sec hold ('hot start') followed by 40 cycles at 95˚C with a 0 sec hold, a 60˚C annealing step with a 5 sec hold, 72˚C with a 12 sec hold, and a 60˚C acquisition step with a 2 sec hold. All experiments were conducted three times, and negative and positive controls were included in all experiments. β-actin mRNA was amplified as an internal control. LightCycler software version 3.3 (Roche Diagnostics) was used to analyze the PCR kinetics and calculate the quantitative data. For each sample, copy numbers of target gene mRNA were divided by those of β-actin mRNA to normalize for target gene mRNA expression and avoid inter-sample differences in RNA quantity.

Statistical analysis. The results are presented as the mean ± standard error of the mean. Comparisons between groups were analyzed using the Student's t-test or one-way analysis of variance for multiple groups. Tukey's adjustment post-hoc test was conducted to determine which means were significantly different. The statistical analysis of differences in fat graft weight and volume among the six groups was performed using Mann Whitney U-test. SPSS for Windows (version 17.0; SPSS, Inc., Chicago, IL, USA) was used to analyze the data. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of SPC on EPC proliferation. SPC exhibited a maximal effect on EPC proliferation when used at 3 (P=0.0013) and 5 μM (P=0.0005; Fig. 1A). The positive effects of SPC were still efficacious at day 3 (Fig. 1B). Conversely, PTX exhibited a direct dose-dependent cytotoxic effect on EPCs (Fig. 1C), whereas VPC23019 had no effect on proliferation (Fig. 1D). To confirm the positive effects of SPC on EPC growth, cells were co-treated with SPC and VPC23019. EPC growth was promoted by treatment with SPC, which was suppressed following the addition of VPC23019 (Fig. 1E).

Effects of SPC on EPC angiogenesis. *In vitro* tube formation was increased following treatment of EPCs with SPC in a dose-dependent manner (Fig. 2A). RT-qPCR was conducted on total RNA extracted from EPCs treated with various concentrations of SPC, which detected increased mRNA expression levels of HGF, IL-6, MMP-2, MMP-9, TNF-α and VEGF,

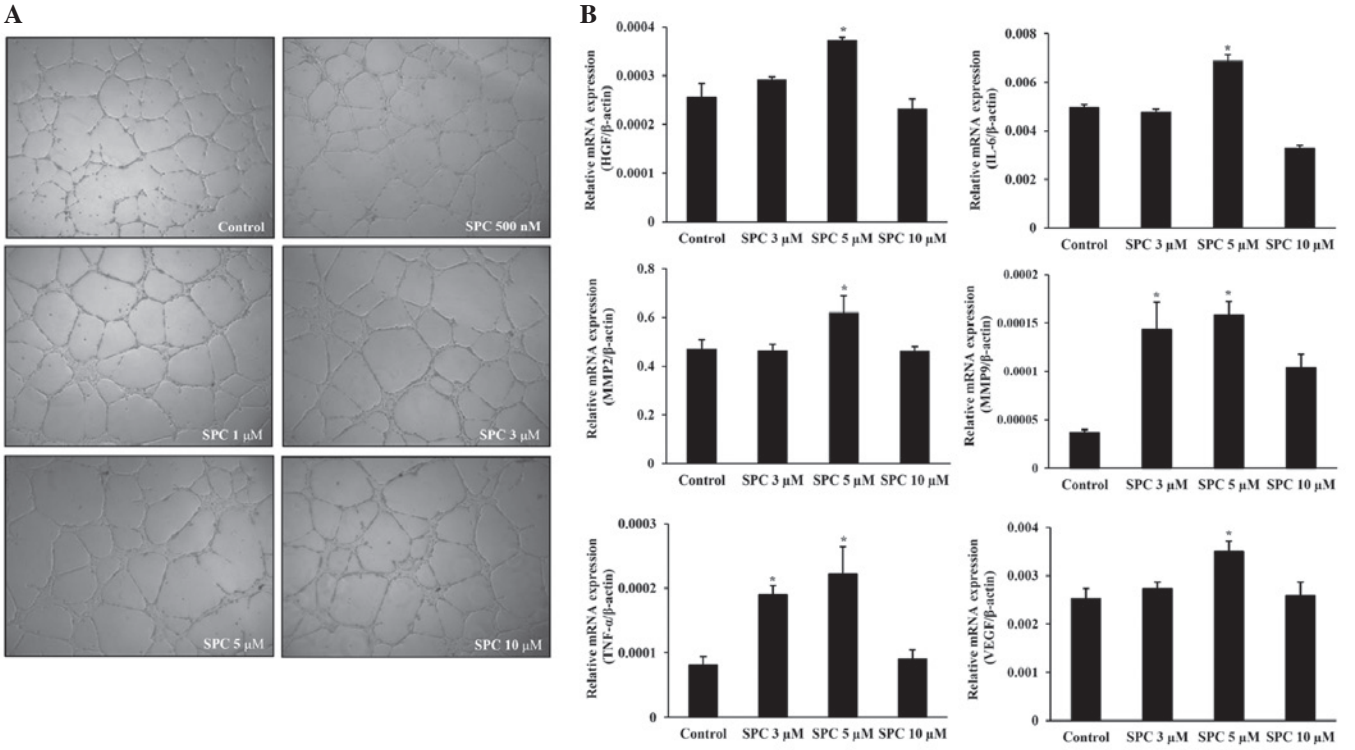

Figure 2. (A) Tube formation assay. Increased tube formation was detected following treatment of endothelial progenitor cells (EPCs) with sphingosylphosphorylcholine (SPC). Magnification, x200. (B) Reverse transcription-quantitative polymerase chain reaction of total RNA extracted from EPCs treated with various concentrations of SPC. Increased mRNA expression levels of genes associated with angiogenesis were detected. Data are presented as the mean ± standard error of the mean. *P<0.05, vs. the control group. HGF, human growth factor; IL-6, interleukin-6; MMP, matrix metallopeptidase; TNF-α. tumour necrosis factor-α; VEGF, vascular endothelial growth factor.

as compared with the control group. HGF (P=0.021), IL-6 (P=0.010), MMP-2 (P=0.047) and VEGF (P=0.026) expression was significantly increased following treatment with 5 μM SPC. MMP-9 (P=0.025/P=0.006) and TNF-α (P=0.016/P=0.036) expression significantly increased at 3 and 5 μM SPC (Fig. 2B).

Effects of SPC on cryopreserved fat tissue survival. Grafted fat was retrieved from the mice and the gross findings exhibited a significant bulk increase in the 3 μM SPC-treated group (Fig. 3). Weight and volume measurements indicated that grafted fat survival was increased when treated with various concentrations of SPC. Statistical analysis was performed and the 3 μM SPC-treated group exhibited a statistically significant increase (P=0.04) in both weight and volume (Table I).

Effects of SPC on cryopreserved fat tissue angiogenesis. Increased angiogenesis of the SPC-treated fat graft tissue of the grafted fat tissue was detected by immunohistochemistry, as evidenced by increased CD31 expression (Fig. 4A). RT-qPCR analysis of total RNA extracted from SPC-treated fat tissue revealed increased mRNA expression levels of MMP9 (P=0.0001) and TNF-α (P=0.0024) compared with the control group (Fig. 4B).

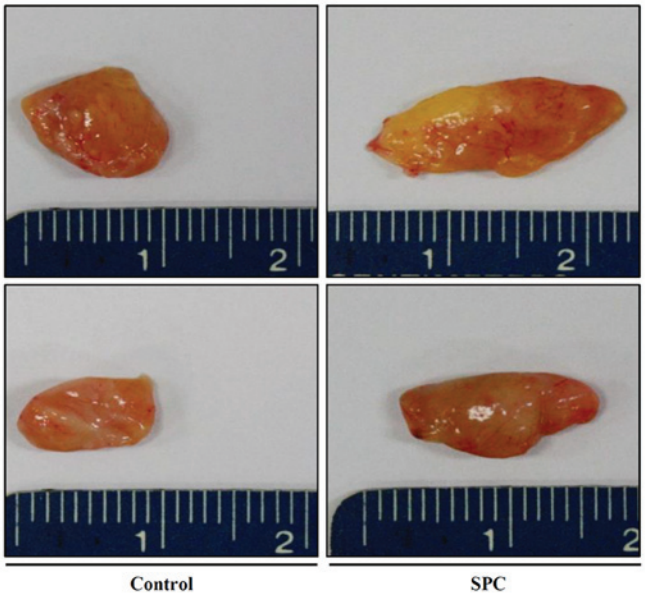

Figure 3. Fat grafts harvested from individual mice at 8 weeks post-transplantation. The features of fat grafts obtained from the control group and the 3 μM SPC-treated group.

Discussion

SPC is reportedly associated with various cellular functions of the cardiovascular system, skin, neurons, and immune cells (3,4,9,11); however, its effects on the survival of grafted fat tissues have yet to be reported.

The present study demonstrated that SPC exerted strong mitogenic effects on endothelial cells, and an *in vitro* study was conducted to elucidate the direct mitogenic effect of

Table I. Effect of SPC on fat graft weight and volume.

Group	Weight (Median, 25th-75th)	P-value (vs. Control)	Volume (Median, 25th-75th)	P-value (vs. Control)
Control (n=8)	0.15 (0.14-0.17)		0.17 (0.15-0.19)	
SPC 1 μM (n=8)	0.16 (0.13-0.19)	0.72	0.18 (0.15-0.20)	0.72
SPC 3 μM (n=8)	0.19 (0.17-0.21)	0.04[a]	0.21 (0.19-0.23)	0.04[a]
SPC 5 μM (n=8)	0.17 (0.15-0.19)	0.20	0.18 (0.17-0.20)	0.33
SPC 10 μM (n=8)	0.15 (0.13-0.19)	0.96	0.16 (0.15-0.20)	0.96
SPC 15 μM (n=8)	0.14 (0.11-0.18)	0.57	0.17 (0.13-0.19)	0.72

[a]P<0.05; Mann Whiney U-test. SPC, sphingosylphosphorylcholine.

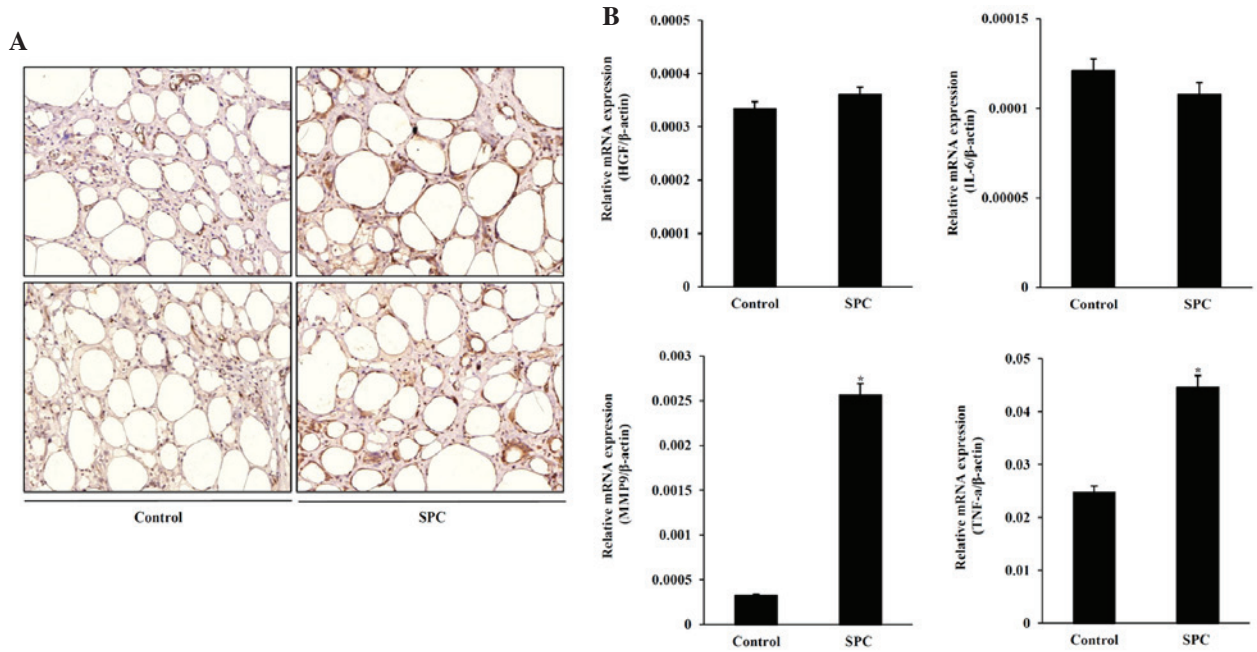

Figure 4. (A) Immunohistochemical analysis detected increased angiogenesis of grafted fat tissue, as evidenced by increased cluster of differentiation 31 expression. Magnification, x200. (B) Reverse transcription-quantitative polymerase chain reaction analysis of total RNA extracted from sphingosylphosphorylcholine (SPC)-treated fat tissue detected increased mRNA expression levels of matrix metallopeptidase-9 (MMP-9) and tumor necrosis factor (TNF)-α compared with the control group. Data are presented as the mean ± standard error of the mean. *P<0.05, vs. the control group. HGF, human growth factor; IL-6, interleukin-6.

SPC on EPCs. EPC proliferation was increased following SPC treatment, and proliferation peaked when the cells were treated with 3 or 5 μM SPC; this effect lasted for 3 days. Co-treatment with VPC23019, an inhibitor of the downstream SPC mediator sphingosine-1-phosphate, confirmed that significant EPC proliferation resulted from SPC treatment. In addition, tube formation assay and RT-qPCR demonstrated that SPC was able to increase the angiogenic potential of EPCs.

An *in vivo* experiment using cryopreserved human fat tissue to conduct a fat graft in mice demonstrated statistically significant differences in weight and volume between the control and 3 μM SPC-treated groups.

With the favorable histological outcome, immunohistochemical result, and RT-qPCR findings of the present study, SPC may increase the survival rate of cryopreserved fat tissue by improving its angiogenic potential.

Previous studies have reported that SPC can stimulate the proliferation of human adipose tissue-derived mesenchymal stem cells (hADSC) (12,13). Therefore, the increased survival of cryopreserved fat detected in the present study may also be caused by SPC-induced hADSC proliferation. These results suggested that with an SPC-induced intensified EPC role in transplanted fat tissue, the cryopreserved fat survival rate could be further increased. In addition, these results indicated that specific concentrations of SPC have a favorable role in grafted cryopreserved human fat tissue, and this positive effect may be promoted by increased angiogenesis-associated mRNA expression.

Acknowledgements

The present study was supported by the Biomedical Research Institute, Pusan National University Hospital (grant no. 2012-12).

References

1. Gamboa GM and Ross WA: Autogenous fat transfer in aesthetic facial recontouring. Ann Plast Surg 70: 513-516, 2013.
2. Baran CN, Celebioğlu S, Sensöz O, Ulusoy G, Civelek B and Ortak T: The behavior of fat grafts in recipient areas with enhanced vascularity. Plast Reconstr Surg 109: 1646-1651, 2002.
3. Meyer zu Heringdorf D, Himmel HM and Jakobs KH: Sphingosylphosphorylcholine-biological functions and mechanisms of action. Biochim Biophys Acta 1582: 178-189, 2002.
4. Sun L, Xu L, Henry FA, Spiegel S and Nielsen TB: A new wound healing agent - sphingosylphosphorylcholine. J Invest Dermatol 106: 232-237, 1996.
5. Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G and Isner JM: Isolation of putative progenitor endothelial cells for angiogenesis. Science 275: 964-967, 1997.
6. Yi C, Pan Y, Zhen Y, Zhang L, Zhang X, Shu M, Han Y and Guo S: Enhancement of viability of fat grafts in nude mice by endothelial progenitor cells. Dermatol Surg 32: 1437-1443, 2006.
7. Bae YH, Park HJ, Kim SR, Kim JY, Kang Y, Kim JA, Wee HJ, Kageyama R, Jung JS, Bae MK and Bae SK: Notch1 mediates visfatin-induced FGF-2 up-regulation and endothelial angiogenesis. Cardiovasc Res 89: 436-445, 2011.
8. Ferguson RE, Cui X, Fink BF, Vasconez HC and Pu LL: The viability of autologous fat grafts harvested with the LipiVage system: A comparative study. Ann Plast Surg 60: 594-597, 2008.
9. Pietrzak WS and Eppley BL: Platelet rich plasma: Biology and new technology. J Craniofac Surg 16: 1043-1054, 2005.
10. Ayhan M, Senen D, Adanali G, Görgü M, Erdoğan B, Albayrak B: Use of beta blockers for increasing survival of free fat grafts. Aesthetic Plast Surg 25: 338-342, 2001.
11. Nixon GF, Mathieson FA and Hunter I: The multi-functional role of sphingosylphosphorylcholine. Prog Lipid Res 47: 62-75, 2008.
12. Jeon ES, Song HY, Kim MR, Moon HJ, Bae YC, Jung JS and Kim JH: Sphingosylphosphorylcholine induces proliferation of human adipose tissue-derived mesenchymal stem cells via activation of JNK. J Lipid Res 47: 653-664, 2006.
13. Moon HJ, Jeon ES, Kim YM, Lee MJ, Oh CK and Kim JH: Sphingosylphosphorylcholine stimulates expression of fibronectin through TGF-beta1-Smad-dependent mechanism in human mesenchymal stem cells. Int J Biochem Cell Biol 39: 1224-1234, 2007.