Supplementary material to article by H. H. Kwon et al. "Combination Treatment with Human Adipose Tissue Stem Cell-derived Exosomes and Fractional CO2 Laser for Acne Scars: A 12-week Prospective, Double-blind, Randomized, Split-face Study."

**Appendix S1**

**SUPPLEMENTARY MATERIAL**

**Generation of adipose-derived stem cell-conditioned medium**

Human adipose-derived stem cells (ASC) from a healthy donor were collected by liposuction and immediately transferred into the cell culture facility. Donor eligibility and the quality of adipose tissue were assessed according to the guidelines of the Korean Ministry of Food and Drug Safety (MFDS). After isolating ASCs from the adipose tissue of a healthy donor, ASCs were sub-cultured at a density of 3,000 cells/cm² with 10% foetal bovine serum (FBS) containing Dulbecco’s Modified Eagle’s Medium (DMEM) (Thermo Fisher Scientific, Waltham, MA, USA) in a humidified atmosphere of 5% CO₂ in air at 37°C. Cells were harvested with porcine trypsin-ethylenediaminetetracetic acid (Thermo Fisher Scientific), and washed with Dulbecco’s phosphate-buffered saline (PBS) (Thermo Fisher Scientific). Cell stocks of passage 4 were stored in liquid nitrogen (1,100,000 cells/ml/1 vial). The quality of ASCs was controlled by assessing the sterility test, mycoplasma test, cell viability, endotoxin test, and virus tests. The surface markers for ASCs were also determined by flow cytometry, including positive markers of CD31, CD73, CD105, and CD146. Adipogenic, chondrogenic, and osteogenic differentiation potencies of ASCs were also measured. To generate the ASC-conditioned medium (CM), a cell stock was thawed and sub-cultured until passage 7. ASCs at passage 7 were plated at a density of 6,000 cells/cm² and cultured with 10% FBS containing DMEM in a humidified atmosphere of 5% CO₂ in air at 37°C for 3 days up to 90% confluency. Cells were harvested with a 500 kDa molecular weight cut-off filter membrane cartridge (GE Healthcare, Chicago, IL, USA), and buffer exchange was performed by dialfiltration with PBS. Isolated ASCs were aliquoted into polypropylene disposable tubes and stored at approximately –178°C within the TEM, a side entry Gatan 626 cryo-holder (Gatan, Pleasanton, CA, USA) was used. The grids were then examined with a Tecnai G2 Spirit Twin TEM equipped with anti-contaminator (FEI). A 4K×4K, Ultrascan 4000 CCD camera (Gatan) was used for image recording. A low-dose method (exposures at 1,000 electrons per nm²/s) was used for cryo-TEM.

**Isolation and quantification of adipose tissue stem cell-derived exosomes**

Adipose tissue stem cell-derived exosomes (ASCE) were isolated from the ASC-CM by tangential flow filtration (TFF) as previously described (18, 35). Briefly, the CM was filtrated through a 0.22-μm polyethersulfone membrane filter (Merck Millipore, Billerica, MA, USA) to remove non-exosomal particles, such as cells, cell debris, microvesicles, and apoptotic bodies. The CM was concentrated by TFF with a 500 kDa molecular weight cut-off filter membrane cartridge (GE Healthcare, Chicago, IL, USA), and buffer exchange was performed by dialfiltration with PBS. Isolated ASCE were aliquoted into polypropylene disposable tubes and stored at –80°C until use. Before using, frozen ASCE were left at 4°C until completely thawed and were not frozen again. The purity of isolated exosomes was confirmed by measuring the amounts of negative markers for exosomes. The ASCs and ASCE were lysed with RIPA buffer (Cell Signaling Technology, Danvers, MA, USA), and their protein concentrations were measured by the bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific). Each lysate was analysed using human CANX/calnexin ELISA kit (LifeSpan Bioscience, Seattle, WA, USA) and human cytochrome c ELISA kit (Abcam, Cambridge, MA, USA), respectively, according to the manufacturer’s instruction.

**Nanoparticle tracking analysis**

The characterization of ASCE was further performed according to the criteria suggested by the Minimal Information for Studies of Extracellular Vesicles 2018 (MISEV 2018) and the Korean MFDS. To determine the size distribution and particle concentration, ASCE diluted with PBS were analysed by nanoparticle tracking analysis (NTA) using a NanoSight NS300 (Malvern Panalytical, Amesbury, UK) equipped with a 642-nm laser. Exosomes, diluted with PBS to between 20 and 80 particles per frame, were scattered and illuminated by the laser beam and their movement under Brownian motion was captured for 20 s at a camera level of 16. Videos were analysed by the NTA 3.2 software with constant settings. To provide a representative result, at least 5 videos were captured and >2,000 validated tracks were analysed for each individual sample. The NTA instrument was regularly checked with 100 nm-sized standard beads (Thermo Fisher Scientific). To provide representative size distribution of exosomes, size distribution profiles from each video replicates were averaged. Exosome surface markers were analysed using bead-based flow cytometry. Briefly, ASCE were captured by Exosome Dynabead for human CD9, CD63, or CD81 (Invitrogen, Carlsbad, CA, USA), respectively, according to the manufacturer’s instruction. Captured exosomes were incubated with phycoerythrin-conjugated anti-human CD9, CD63, or CD81, or isotype antibody (BD Biosciences, Franklin Lakes, NJ, USA), respectively.

**Cryo-transmission electron microscopy (cryo-TEM)**

Cryo-TEM images were obtained using a BIO-TEM installed at the Korea Basic Science Institute (Osong, Republic of Korea). The isolated ASCE were applied to Quantifoil grids (Electron Microscopy Sciences, Hatfield, PA, USA) and subsequently blotted using Vitrobot Mark IV (FEI, Hillsboro, OR, USA) with the following settings: 100% humidity; 4°C; blot time of 5 s; blot force of 5; blot total of 1; wait time of 5 s; drain time of 0 s. For maintenance of vitrified sample grids at a temperature of approximately –178°C within the TEM, a side entry Gatan 626 cryo-holder (Gatan, Pleasanton, CA, USA) was used. The grids were then examined with a Tecnai G2 Spirit Twin TEM equipped with anti-contaminator (FEI). A 4K×4K, Ultrascan 4000 CCD camera (Gatan) was used for image recording. A low-dose method (exposures at 1,000 electrons per nm²/s) was used for cryo-TEM.

**In vitro potency assay of adipose tissue stem cell-derived exosomes**

Isolated and quantified ASCE were tested in vitro for their potency in terms of collagen synthesis, inflammatory IL-6 reduction, cell migration, and cell proliferation. Human dermal fibroblasts (HDFs) were obtained from CEFO (Seoul, Republic of Korea) and cultured in Human Fibroblast Growth Medium with 10% supplements (CEF0), 100 IU/ml penicillin, and 100 μg/ml streptomycin in humidified atmosphere of 5% CO₂ at 37°C. For collagen assay, HDF cells were incubated with supplement-free medium for 24 h, followed by ASCE (7.8×10¹¹ particles/ml) treatment. After 24-h incubation, the culture medium was collected and the amount of procollagen type I was measured according to the manufacturer’s protocol using procollagen type I C-peptide EIA kit (Takara Bio, Inc., Otsu, Japan). For cell migration assay, a single wound was created by using IncuCyte® WoundMaker Tool (Essen Bioscience, Hertfordshire, UK) when HDF cells reached 90% confluency. The cells were cultured in supplement-free medium with ASCE (1×10¹¹ particles/ml) for 24 h. The data were acquired and analysed by IncuCyte® S3 Live-Cell Analysis System and software (Essen Bioscience). For cell proliferation assay, HDF cells were plated at a density of 3,000 cells/cm². After 24 h, the culture medium was changed into growth medium with 1% supplement, and ASCE (3.3×10¹¹ particles/ml) was treated. The cell proliferation was measured and analysed using IncuCyte® S3 Live-Cell Analysis System and software (Essen Bioscience). For anti-inflammatory assay, the RAW 264.7 cells (ATCC, Manassas, VA, USA) were cultured in DMEM supplemented with 10% FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin in humidified atmosphere of 5% CO₂ at 37°C. The cells were incubated with ASCE (1.3×10¹¹ particles/ml).
Determination of dose of adipose tissue stem cell-derived exosomes

Currently, no recommended dose of exosomes for human or animal is available (S1). Since this is the first clinical study that uses ASCE for human skin wound repair, we carefully chose the dose to be as high as possible. The median lethal dose cut-off value was higher than $1 \times 10^{11}$ particles/kg in acute oral toxicity test performed in Sprague Dawley (SD) rats (S1). In addition, no observed effect level was calculated as over $1.07 \times 10^{11}$ particles/kg/day in repeated dermal toxicity test performed in SD rats with daily dermal application for 13 weeks. Human equivalent dose (HED) was calculated by the equation of [HED (per kg)]=[animal dose (per kg)/conversion factor] (S2, S3). Since the conversion factor of rat is 6.17, the HED of ASCE (per 50 kg of person) was $(1.07 \times 10^{11} \text{ particles/kg/day})/6.17=8.67 \times 10^{10}$ particles/day. According to the US FDA recommendation, the maximum recommended starting dose (MRSD) was calculated by the equation of [MRSD (per kg)]=(HED (per kg))/10 (S2, S3). Thus, the MRSD was $(8.67 \times 10^{10} \text{ particles/day})/10=8.67 \times 10^{9}$ particles/day. Based on these calculations, we carefully chose the dose of ASCE for the day of fractional CO$_2$ laser (FCL) treatment as $9.78 \times 10^{9}$ particles/ml (within $[8.67 \pm 1.73] \times 10^{9}$ particles/day) with 20% error range in consideration of the loss during the treatment. For days subsequent to FCL treatment, we further reduced the dose of ASCE below 1/5 of MRSD to reduce any adverse effect that may be caused by repeated applications, and it was calculated as $1.63 \times 10^{9}$ particles/ml (less than 20% of $8.67 \times 10^{9}$ particles/day).

Composition of the adipose tissue stem cell-derived exosomes and control gel solution

The ASCE gel solution contained 30% ASCE, 2% 1,2-hexanediol (Cosbon Co., Ltd, Hwaseong, Republic of Korea), 1% glycerin (Procter and Gamble Chemicals, Cincinnati, OH, USA), 0.6% ammonium acryloyldimethyltaurate/VP copolymer (Clariant International Ltd, Muttenz, Switzerland), 0.0045% L-arginine (Daesang, Seoul, Republic of Korea), and 66.3955% water for injection (Dai Han Pharm. Co., Ltd, Seoul, Republic of Korea). Percentages refer to weight per weight. For the control agent, 30% of 0.03% PBS was used in the gel solution in place of the exosomes.

Statistical analysis

Percentage changes from baseline in total échelle d’évaluation clinique des cicatrices d’acné (ECCA) scores and changes in scores of 3 major subtypes (V-, U-, and M-shaped) of atrophic scars were analysed using linear mixed effect models that included treatment group, visit, and their interaction as fixed factors, and a random factor for subjects and crossed random effect terms for treatment group and visit nested within subject. Percentage changes from baseline in atrophic scar volume, mean pore volume, and skin surface roughness were assessed using linear mixed effect models which included treatment group, visit, and their interaction as fixed factors, and a random intercept at subject level. The duration of downtime was evaluated using a generalized linear mixed model with log link function and generalized Poisson family, which included the same fixed and random effects as those in the ECCA analyses. Post-treatment pain, erythema, oedema, and dryness were assessed using cumulative link mixed models with logit link function that included treatment group, post-treatment day, and their interaction as fixed factors, and random intercepts for subject and visit nested within the subject.

**SUPPLEMENTARY REFERENCES**

