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Toxicological evaluation of exosomes derived from human adipose tissue-derived mesenchymal stem/stromal cells

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ABSTRACT

Several studies report that the therapeutic mechanism of action of mesenchymal stem/stromal cells (MSCs) is mainly mediated by paracrine factors that are released from MSCs such as exosomes. Exosomes are nano-sized extracellular vesicles that are transferred to target cells for cell-to-cell communication. Although MSC-derived exosomes (MSC-exosomes) are suggested as novel cell-free therapeutics for various human diseases, evaluation studies for the safety and toxicity of MSC-exosomes are limited. The purpose of our study was to evaluate the toxicological profile, including skin sensitization, photosensitization, eye and skin irritation, and acute oral toxicity using exosomes derived from human adipose tissue-derived MSCs (ASC-exosomes) in accordance with the OECD guidelines and the principles of Good Laboratory Practice. The ASC-exosomes were classified as a potential non-sensitizer in the skin sensitization test, UN GHS no category in the eye irritation test, and as a skin non-irritant in the skin irritation test, and did not induce any toxicity in the phototoxicity test or in acute oral toxicity testing. Our findings are the first to suggest that ASC-exosomes are safe for use as a topical treatment, with no adverse effects in toxicological testing, and have potential application as a therapeutic agent, cosmetic ingredient, or for other biological uses.

1. Introduction

Mesenchymal stem/stromal cells (MSCs) have self-renewal potential for generation of their progenies as well as anti-inflammatory and immunomodulating capabilities. As stem cells, MSCs can differentiate into adipocytes, osteoblasts, chondrocytes, and other cell types (Phinney and Prockop, 2007). Therefore, MSCs have emerged as a promising cell source for cell-based therapies and are explored in clinical trials for various incurable human diseases (Squillaroet al., 2016). More recently, several studies report that the therapeutic mechanism of MSCs is mediated by paracrine effects, but the mechanism may not apply in cell replacement or differentiation in damaged cells or tissues (Speeset al., 2016; Teixeira et al., 2013). Secretomes, including proteins, hormones, and exosomes, released from MSCs play a crucial role in paracrine cell signaling.

Exosomes are membrane-bound vesicles, 30–200 nm in diameter, that are released by the fusion of a multi-vesicular body and the plasma membrane (Properziet al., 2013). Exosomes contain a variety of proteins, nucleic acids, and lipids depending on their cell type and are involved in cell-to-cell communications by delivering their cargo to other cells (Properziet al., 2013). Therefore, exosomes are being investigated as biomarkers, in clinical diagnoses, as therapeutics and for drug delivery (Properziet al., 2013; Nooshabadiet al., 2018; Keshtkaret al., 2018). Analyses show that MSC-exosomes recapitulate MSC functions such as repair/regeneration, anti-inflammatory properties, and immune modulation in animal disease models (Yamashita

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et al., 2018). In fact, applications of MSC-exosomes suggest use in novel cell-free therapeutic strategies in regenerative medicine for immune diseases, cancers, and neurological, cardiovascular, and respiratory diseases (Nooshabadiet al., 2018). For example, the therapeutic benefits of MSC-exosomes are demonstrated in myocardial infarction (Kang et al., 2015), drug-induced acute liver injury (Tan et al., 2014), liver fibrosis (Li et al., 2012), and atopic dermatitis (Choet al., 2018). The use of MSC-exosomes is safe regarding adverse effects of MSC therapy, which may include consequences such as potential tumorigenesis by cell transplantation and occlusion in the distal vasculature by intravascular administration. Advantages are that MSC-exosomes can be mass produced (Yeoet al., 2013) and sterilized by filtration, and have a long shelf-life, but these properties do not extend to MSCs themselves (Choet al., 2018). In addition, because stem cell-derived exosomes are involved in skin repair and rejuvenation (FerreiraGomes, 2018), they are used in cosmetic products.

Although MSC-exosomes are generally considered relatively safe and less toxic (Cheng, 2017), an overall understanding of the toxicity of MSC-exosomes has not been fully elucidated. To evaluate the toxicological profile of exosome derived from human adipose tissue-derived MSCs (ASC-exosomes), we carried out a series of toxicity tests including acute oral toxicity, the local lymph node assay (LLNA) for skin sensitization, the *in vitro* photosensitization assay, and evaluation of skin and eye irritation.

2. Materials and methods

2.1. Cells, chemicals and reagents

Cells, chemicals, and reagents used in this study were sourced and purchased from the following companies: propylene glycol, olive oil, acetone, α-hexyl cinnamic aldehyde (HCA), 5-bromo-2′-deoxyuridine (BrdU), deionized water, ethyl alcohol (Pure), fluorescein, sodium dodecyl sulfate (SDS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), chlorpromazine hydrochloride, lipopolysaccharides from *E. Coli* (LPS), and dexamethasone from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS), penicillin-streptomycin, 200 mM L-glutamine, 1 M HEPES, Dulbecco's Modified Eagle's Medium (DMEM), non-enzymatic cell dissociation buffer, and Normal Human Epidermal Keratinocytes (NHEK) from ThermoFisher Scientific (Waltham, MA, USA). Eagle's Minimum Essential Medium (EMEM), and Hanks' Balanced Salt buffer were obtained from WELGENE (Gyeongsan-si, Gyeongsangbuk-do, Republic of Korea). Human dermal fibroblasts (HDF) and human fibroblast growth medium with supplements were from CEFO (Seoul, Republic of Korea). The murine macrophage RAW 264.7 cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Phosphate buffered saline was purchased from Lonza (Basel, Switzerland).

2.2. Isolation of exosomes

Human ASCs from a healthy donor were collected by liposuction and immediately transferred into the cell culture facility (CEFO Co., Ltd.,Seoul, Republic of Korea). Donor eligibility and the quality of adipose tissue were assessed according to the guideline of Korea Minister of Food and Drug Safety (MFDS). After isolating ASCs from adipose tissue of a healthy donor, cell stocks were made and stored in liquid nitrogen. The quality of ASCs was maintained by assessing quality via a sterility test, a mycoplasma test, determination of cell viability, and virus tests. The cell surface markers for ASCs were determined by flow cytometry including cluster of differentiation (CD) markers, including CD31, CD73, CD105, and CD146. Adipogenic and osteogenic differentiation potencies of ASCs were also measured. The ASC-exosomes (ASCE, ExoCoBio Inc., Seoul, Republic of Korea) were isolated from human ASC conditioned media by ExoSCRT™ technology. Briefly, ASCs at passage 5 were plated at a density of 12,000 cells/cm^2 and cultured with CEFOgro™ ADMSC Media (CEFO Co., Ltd.,Seoul, Republic of Korea) in a humidified atmosphere of 5% CO_2 in air at 37 °C. Twenty-four hours later, the cells were washed with PBS three times and supplemented with serum-free & Xeno-free CEFOgro™ XF-MSC Media (CEFO Co., Ltd., Seoul, Republic of Korea). The cells were further cultured for 24 h. The culture media were collected and centrifuged at 1500 rpm for 5 min to obtain the conditioned media (CM). To obtain 1 L of CM, 4.2×10^6 cells were used. The CM were filtered through a 0.22-μm polyether sulfone membrane filter (Merck Millipore, Billerica, MA, USA) to remove non-exosomal particles such as cells, cell debris, microvesicles, and apoptotic bodies. The CM were concentrated by tangential-flow filtration with a 500 kDa molecular weight cut-off filter membrane cartridge (GE Healthcare, Chicago, IL, USA), and the buffer exchange was performed by diafiltration with PBS. Isolated ASC-exosomes were aliquoted into polypropylene disposable tubes and stored at −80 °C until use. Before use, frozen ASC-exosomes were allowed to thaw at 4 °C without additional freeze-thaw cycles.

2.3. Nanoparticle tracking analysis (NTA)

Quantification of exosomes was performed by Nanoparticles 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 each at camera level 16. Videos were analyzed by NTA 3.2 software and all settings were kept constant. To provide a representative result, at least 5 videos were captured and > 2000 validated tracks were analyzed for each individual sample. The NTA instrument was regularly checked with 100 nm-sized standard beads (ThermoFisher Scientific, Waltham, MA, USA). To provide a representative size distribution of exosomes, the size distribution profiles from each video replicate were averaged.

2.4. Protein quantification

Protein quantification of exosomes was performed using the Micro BCA protein assay kit (ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol.

2.5. Western blot analysis

Exosome surface markers such as CD9, CD63, and CD81 and the negative marker calnexin were characterized by western blotting. Exosomes were lysed with RIPA buffer (Cell Signaling Technology, Danvers, MA) containing the protease inhibitor cocktail (Roche, Mannheim, Germany). The protein concentration was determined by BCA protein assay (ThermoFisher Scientific, Waltham, MA, USA). Exosomal proteins were resolved by electrophoresis on a 10 or 15% polyacrylamide gel and subsequently transferred to PVDF membranes. Membranes were blocked with 5% skim milk (BD Biosciences, Franklin, NJ, USA) in PBS-T (0.1% Tween 20 in Tris-buffered saline; 137 mmol/L NaCl and 20 mmol/L Tris/HCl, pH 7.4) for 1 h at room temperature. The following antibodies were used: anti-CD9 (Abcam, Cambridge, MA, USA); anti-CD63 and anti-CD81 (System Biosciences, Palo Alto, CA, USA); and anti-calnexin (Cell Signaling Technology). Antibodies were diluted 1:1000–1:5000 in 5% skim milk (BD Biosciences, Franklin, NJ, USA) in TBS-T and then incubated overnight at 4 °C on a rocking platform. After incubation, membranes were washed three times with PBS-T and incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA) for 1 h at room temperature. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL) detection reagents (DoGenBio, Seoul, Republic of Korea) and exposed in an Amersham Imager 680 (GE Healthcare, Chicago, IL, USA).

2.6. Cellular uptake assay

Normal human epidermal keratinocytes (NHEKs) were cultured in DMEM supplemented with 10% FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere of 5% CO_2 in air at 37 ˚C. The exosomes were labeled with PKH67 (Sigma-Aldrich, St. Louis, MO, USA) and labeled exosomes were purified by MW3000 Exosome Spin Column (ThermoFisher Scientific, Waltham, MA, USA). Labeled exosomes were added to NHEKs and incubated for up to 48 h in a 5% CO_2 atmosphere at 37 ˚C. The cells were fixed with 10% formalin (Sigma-Aldrich, St. Louis, MO, USA) and counter-stained with CellMask™ (Thermo Fisher Scientific, Waltham, MA, USA) and Hoechst 33258 (Sigma-Aldrich, St. Louis, MO, USA). The uptake of exosomes was visualized using a CELENA S Imaging System (Logos Biosystems, Anyang-si, Gyeonggi-do, Republic of Korea).

2.7. Estimation of extracellular matrix proteins

The HDF cells were plated onto 24-well plates at a density of 5.0×10^4 cells/well and cultured in Human Fibroblast Growth Medium with supplements in humidified atmosphere of 5% CO_2 at 37 ˚C. After 24 h incubation, the cells were incubated with supplement-free medium for 24 h, followed by treatment with ASC-exosomes. The culture medium was collected after 24 h of exosome treatment for collagen and after 72 h of exosome treatment for elastin. The amounts of procollagen type I and elastin proteins were measured according to the manufacturer's protocol using Procollagen Type I C-Peptide (PIP) EIA Kit (Takara Bio, Inc., Otsu, Japan) and Human Elastin ELISA Kit (CUSABIO, Wuhan China), respectively. The absorbance (450 nm, PIP; 450 nm, elastin) was measured by using a SpectraMax i3x Multi-Mode Plate Reader (Molecular Devices, San Jose, CA, USA).

2.8. Anti-inflammation assay

The RAW 264.7 cells were plated onto 96-well plates at a density of 2.5×10^4 cells/well and cultured in DMEM supplemented with 10% FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin in humidified atmosphere of 5% CO_2 at 37 ˚C. The cells were treated with ASC-exosomes for 24 h prior to 100 ng/mL LPS stimulation. Subsequently, the cell culture supernatants were harvested after 24 h of LPS stimulation and inflammatory cytokines were analyzed using the LEGENDplex™ Mouse Inflammation Panel (BioLegend, San Diego, CA, USA) according to the manufacturer's protocol. The data were acquired on a NovoCyte Flow Cytometer System (ACEA Biosciences, Inc., San Diego, CA) and analyzed using LEGENDplex 8.0 software (BioLegend, San Diego, CA, USA).

2.9. In vitro tests of sterility, endotoxin, mycoplasma, and adventitious viruses

2.9.1. Sterility test

The sterility test for exosomes was carried out according to the Microbiological Control for Cellular Products, Eu. Ph 2.6.1 (European Pharmacopoeia Secretariat, 2016a).

2.9.2. Bacterial endotoxins test

The bacterial endotoxin test for exosomes was performed according to the Eu. Ph. 2.6.14 (European Pharmacopoeia Secretariat, 2016b) using the Pyrogent™ Gel Clot Limulus Amebocyte Lysate (LAL) assay (Lonza, Morristown, NJ, USA).

2.9.3. Mycoplasma test

The mycoplasma test for exosomes was conducted according to the Eu. Ph 2.6.7 (European Pharmacopoeia Secretariat, 2016c) using the e-Myco™ Mycoplasma PCR Detection Kit (iNtRON Biotechnology, Seongnam-si, Gyeonggi-do, Republic of Korea).

2.9.4. Adventitious virus test

The adventitious virus *in vitro* test for exosomes was carried out according to the USP41 (United States Pharmacopeia Convention, 2016) by cytopathic effects (CPE), hemadsorption (HAD), and hemagglutination (HA) assay.

2.10. Animals

Specific pathogen free (SPF) female Sprague-Dawley (SD) rats for acute oral toxicity testing (acute toxic class method) were obtained from Samtako Bio Inc. (Osan-si, Gyeonggi-do, Republic of Korea) and SPF female mice for LLNA-BrdU-ELISA were obtained from Koatech Co. Ltd (Pyeongtaek-si, Gyeonggi-do, Republic of Korea). In accordance with the Guide for the Care and Use of Laboratory Animals, 8th edition (The National Research Council of the National Academies, 2010), animals were kept under environmental conditions that remained constant (temperature, 23 ± 3 ˚C; humidity, 55 ± 15%; ventilation, 10–20 air changes/hour; and luminous intensity, 150–300 Lux) in the experimental animal facility at the Nonclinical Research Institute (#001333), ChemOn Inc. or the Korea Testing & Research Institute (#001637) accredited by AAALAC International. Throughout the study period, the temperature and humidity of the animal room were measured every hour with a computer-based automatic sensor, and the environmental conditions such as ventilation frequency and luminous intensity were monitored on a regular basis. Food and water were provided *ad libitum* with a 12-h light-dark cycle. All procedures and protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Nonclinical Research Institute, ChemOn Inc. (Yongin-si, Gyeonggi-do, Republic of Korea) or the Korea Testing & Research Institute (Hwasoon-gun, Jeollanam-do, Republic of Korea) and performed in accordance with the guideline published by the OECD (OECD, 1997) as well as the GLP regulations for Nonclinical Laboratory Studies by the Minister of Food and Drug Safety (MFDS, Republic of Korea) (MFDS, 2017).

2.11. Culture of cells and tissues

The BALB/C 3T3-A31 fibroblast cell line was obtained from ATCC and maintained according to the manufacturer's recommendations. Briefly, the cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 4.5 mM HEPES, 0.17 M sodium bicarbonate, 100 IU/mL penicillin, and 100 μg/mL streptomycin, in a humidified atmosphere of 5% CO_2 at 37 ˚C. Cells were removed from the culture flasks using a trypsin (0.025%)/EDTA (0.02%) solution. The SKINETHIC™, Reconstructed Human Epidermis (RHE) model was obtained from EPISKIN (Lyon, France). Tissues were cultured in growth media in accordance with the manufacturer's instructions.

2.12. Evaluation of skin toxicity

2.12.1. Skin sensitization, LLNA-BrdU-ELISA

The LLNA-BrdU-ELISA for skin sensitization was performed according to the OECD TG 442B (OECD, 2010) under GLP regulations, and the LLNA-BrdU-ELISA was as described previously (Ávilaet al., 2017). Briefly, five animals per dose group were randomly assigned as follows: G1, propylene glycol; G2, 20% (v/v) olive oil in acetone; G3 – G5, ASC-Exo (1.07×10^{10}, 1.61×10^{10} and 2.14×10^{10} particles/mL, respectively); and G6, 25% α-hexyl cinnamic aldehyde (HCA) in 20% olive oil in acetone. The animals were exposed via ear to test articles (25 μL/ear), once daily for three consecutive days. On day 5, the mice were intraperitoneally injected with 0.5 mM bromodeoxyuridine (BrdU; 5 mg/mouse) for incorporation into proliferating lymph node cells. After 24 h, the mice were euthanized, and the auricular lymph nodes were collected on an individual animal basis. From each mouse, a single-cell suspension of lymph node excised bilaterally was prepared by gentle mechanical disaggregation through a glass homogenizer used

for shearing cells. Cell proliferation was evaluated by ELISA using the Cell Proliferation ELISA, BrdU (colorimetric) kit (Roche Applied Science, Mannheim, Germany) in accordance with the manufacturer's instructions. Absorbance was measured using a spectrophotometer at 370 and 492 nm to obtain the BrdU labeling index (LI) using the following equation:

BrdU LI = [(Abs370 nm) – (Abs370 nm blank)] – [(Abs492 nm) – (Abs492 nm blank)]

The stimulation index (SI) was calculated as the ratio of the BrdU LI for each treatment group versus that of the vehicle control group (propylene glycol for ASC-Exo and 20% (v/v) olive oil in acetone for HCA, respectively). The test article was considered potentially sensitizing if the measured value of SI ≥ 1.6.

2.12.2. Photosensitization, in vitro 3T3 Neutral Red Uptake (NRU) phototoxicity test

The *in vitro* phototoxicity test was performed according to the OECD TG 432 (OECD, 2018; ICCVAM, 2006) under GLP regulations. The 3T3 NRU phototoxicity test was as described previously (Ávilaet al., 2017). The 3T3 cells were cultured in 96-well plates for 24 h and then exposed to DMEM as vehicle control, or eight different concentrations of ASC-exosomes (1.7×10^8, 3.3×10^8, 6.7×10^8, 1.3×10^9, 2.7×10^9, 5.4×10^9, 1.07×10^{10} and 2.14×10^{10} particles/mL) and chlorpromazine hydrochloride was used as a positive control and cells were exposed for 1 h to UVA (+Irr, 1.74 mW/cm^2, 5 J/cm^2). A parallel culture with ASC-exosomes and chlorpromazine hydrochloride was protected from UVA exposure by wrapping with aluminum foil (-Irr). After irradiation for 48 min, all solutions were washed away, and the cells were supplemented with fresh media and incubated overnight. To measure the cell viability, the cells were incubated in the culture medium containing neutral red for 3 h. The optical density of neutral red at 540 nm was measured with a Synergy HT microplate reader (BioTek Instruments, Winoosk, VT, USA). Given the measured optical density, photo irradiation factor (PIF) and mean photo effect (MPE) were calculated with Phototox version 2.0 software (ZEBET at BfR, Berlin, Germany) (OECD, 2018). According to these parameters, the test article was classified as non-phototoxic (PIF < 2 or MPE < 0.1), probable phototoxic (2 < PIF < 5 or 0.1 < MPE < 0.15), and phototoxic (PIF > 5 or MPE > 0.15).

2.13. Evaluation of eye irritation: bovine corneal opacity and permeability (BCOP) assay

Evaluation of eye irritation was performed according to the OECD TG 437 (OECD, 2017) under GLP regulations. The BCOP assay was described previously (Ávilaet al., 2017). Fresh bovine eyes were provided by a local abattoir (Hwa Jung Food Co., Ltd, Nonsan-si, Chungcheongnam-do, Republic of Korea) and eyes in HBSS on ice packs were transported to the laboratory. Eye corneas with no corneal damage or abnormalities were excised. Isolated corneas (n = 3/group) were mounted in the holders and the two chambers were filled with pre-warmed complete MEM and incubated at 32 ± 1 °C for 1 h. Opacity was determined for each cornea using an opacitometer (OP 3.0, BASF, Ludwigshafen, Germany) and corneas with opacity values greater than 7 were discarded. Selected corneas were exposed to 750 μL of ASC-exosomes (1.61×10^{10} particles/750 μL), vehicle control or with ethyl alcohol as a positive control at 32 °C for 10 min. After the exposure period, substances were removed from the anterior chamber and corneas were washed at least three times with complete MEM. The anterior chambers were refilled with complete MEM without phenol red, and final opacity measurements were performed and values were used to obtain the corneal opacity. The corneas were further incubated for 2 h under the same conditions. Afterward, the medium was removed from both chambers. The posterior chamber of each holder was refilled with complete MEM, and one mL of fluorescein solution (4 mg/mL) was added to the anterior chamber. The corneas were incubated in a horizontal position at 32 °C for 90 min. Aliquots of 300 μL from the posterior chamber were placed on a 96-well plate and the absorbance was determined at 490 nm using a Synergy HT multiplate reader (BioTek Instruments, Winoosk, VT, USA). Using the opacity and permeability values, the *in vitro* irritancy score (IVIS) was calculated as follows:

IVIS = (opacity) + 15 X (permeability)

The IVIS score was used to classify the irritancy level of the test article as follows: The UN Globally Harmonized System of Classification and Labeling of Chemicals (UN GHS) no category (IVIS ≤ 3); UN GHS no prediction can be made (3 < IVIS ≤ 55); UN GHS category 1 (corrosive or severe irritant) (IVIS > 55).

2.14. Evaluation of skin irritation: the SKINETHIC™ RHE model for in vitro evaluation of skin irritation

Evaluation of skin irritation was performed according to the OECD TG 439 (OECD, 2015) under GLP regulations. The SKINETHIC™ RHE consists of normal human keratinocytes cultured on 0.5-cm^2 polycarbonate filter inserts at the air-liquid interface with a chemically defined growth medium (OECD, 2015; Rosdy and Clauss, 1990; Rosdy et al., 1993). Three tissues per group were used for ASC-exosomes, vehicle control, and positive control, respectively. All solutions were uniformly covered on the epidermis surface. Exposure time was 42 min. At the end of the exposure period, all solutions were carefully washed out from the epidermis surface with PBS. The tissue inserts were then transferred into a fresh 6-well plate containing growth medium and incubated for 42 h in a humidified atmosphere of 5% CO_2 at 37 °C. The treated tissues were placed in a 24-well plate containing 300 μL of MTT solution and incubated for 3 h in a humidified atmosphere of 5% CO_2 at 37 °C. The tissues were transferred onto a 24-well plate containing 800 μL of isopropanol and incubated for 2 h at room temperature with gentle agitation for formazan extraction. The concentration of formazan was quantified by measuring the optical density (OD) at 570 nm with a Synergy HT microplate reader (BioTek Instruments, Winoosk, VT, USA). The OD values of the negative control, the positive control and test article treated tissues were corrected by subtracting the blank OD value and corrected OD values were obtained for all controls and treatments. The mean OD values for all tissues were calculated as a percentage of the mean OD value of the negative (PBS-treated) control. When the cell viability value was ≤50%, the test article was classified as an irritant and > 50%, the test article was classified as a non-irritant according to GHS.

2.15. Acute oral toxicity test

The acute oral toxicity test was performed to assess the toxicity of ASC-exosomes in SD rats following a single oral dose using the acute toxic class method according to the OECD TG 423 under GLP regulations (OECD, 1998). In situations where there is little to no information about toxicity or if the test article is expected to be toxic, this test should be performed. Here, a stepwise procedure with two fixed doses (first dose, 6.42×10^9 particles/kg b.w. and second dose, 4.28×10^{10} particles/kg b.w.) was performed. Three female SD rats per dose were treated with ASC-exosomes by oral gavage. Before dosing, animals were fasted overnight and ASC-exosomes were directly administered into stomach using a syringe tube with a feeding needle. Food was given 3 or 4 h after test article administration. All animals were observed for mortality and clinical signs every hour for 6 h after dosing during the first 24 h and then once daily for a total of 14 days. Body weights were recorded on day 1 (prior to test article administration), and on days 2, 4, 8 and 15 days after dosing. At study termination, all animals were euthanized by CO_2 inhalation and macroscopic necropsy tissue examinations were performed.

2.16. Statistical analysis

The data presented are expressed as mean ± standard deviation (SD). Statistical analysis was performed using SPSS Statistics version 19 (IBM SPSS Statistics, Armonk, NY, USA), using parametric multiple comparison or non-parametric multiple comparison for comparisons among groups and the level of significance was considered as p values < 0.05. Body weights and ear thickness were assumed to be normally distributed and were analyzed by parametric one-way analysis of variance (ANOVA). The assumption of homogeneity was tested using Levene's test. If the overall ANOVA was significant and the assumption of homogeneity of variance was met, Duncan's multiple range test was used as a post-hoc test and Scheffe's multiple range test was used as a post-hoc test if the number of samples was different. If the assumption of homogeneity of variance was not met, Dunnett's T3 test was used as a post-hoc test to identify significantly different groups from the control group.

3. Results

3.1. Isolation, characterization, and functional assays of exosomes

The ASC-exosomes were isolated by the ExoSCRT™ technology from ASC conditioned media under serum-free conditions. The mode size and particle concentration of the ASC were 127.3 nm (94.7% of particles were < 200 nm) and 1.42×10^{12} particles/mL, respectively (Fig. 1A). The ratio of particles per protein was estimated as 1.39×10^{9} particles/μg protein. Western blot analysis of ASC-exosomes confirmed the presence of exosome surface markers such as CD9, CD63 and CD81, as well as the absence of calnexin, a negative marker for exosomes, in the isolated exosomes (Fig. 1B). To evaluate the functionality of ASC-exosomes, several assays were performed. First, the cellular uptake assay of PKH-labeled ASC-exosomes was performed and internalization of fluorescence-labeled exosomes was observed in NHEK cells (Fig. 1C). Second, the synthesis of extracellular matrix proteins by ASC-exosomes was determined in HDF cells. Procollagen type I protein (PIP) was significantly increased by 2-fold (8.0×10^{9} particles/mL) and 4.8-fold (4.1×10^{10} particles/mL) by ASC-Exo treatment as compared to the control (Fig. 1D). In addition, the concentration of elastin was significantly increased by 1.7-fold (1.9×10^{10} particles/mL) and 2.4-fold (6.3×10^{10} particles/mL) by ASC-exosomes treatment as compared to the control (Fig. 1E). Third, the two treatments of ASC-exosomes (6.0×10^{9} particles/mL and 2.0×10^{11} particles/mL, respectively) significantly decreased inflammatory cytokines (e.g., interleukin-6 (IL-6), ineterluekin-27 (IL-27), and interferon-beta (IFN-β)) by over 4-fold in the supernatants of LPS-treated RAW 264.7 cells, except for IFN-β at low a concentration of ASC-exosomes (6.0×10^{9} particles/mL; Fig. 1F). These data show that isolated ASC-exosomes have the characteristics of exosomes and biological functions in target cells. The ASC-exosomes were also tested by various *in vitro* safety tests and no evidence of bacterial, fungal (data not shown), mycoplasma (Table 1), or adventitious viral (Table 2) contamination was found.

3.2. No skin sensitization by ASC-exosomes

Regarding skin sensitization, the murine LLNA can be used to identify test articles that may cause skin sensitization and allergic dermatitis and is the preferred method for sensitization testing (Hou et al., 2015; Ulker et al., 2014b; Kimber et al., 1994). To check skin sensitization, ASC-exosomes or HCA were applied to the ears of mice. Clinical signs, body weights, skin reactions, ear thickness and skin stimulation index (SI) were evaluated. As shown in Tables 3 and 4, no changes in ear thickness or skin response as clinical signs were observed in the animals exposed to various concentration of ASC-exosomes, as they were similar values to that of the vehicle control. We chose 25% HCA as a positive control, as it does not cause excessive skin irritation or systemic toxicity and its SI value is over 1.6 according to the OECD TG442B guideline. As expected, no changes in ear thickness and skin response were observed in animals treated with 25% HCA. In Fig. 2, the data demonstrate that the results from mouse exposure to skin sensitizer HCA for three days had a SI value of 2.4. In contrast, exposure to ASC-exosomes at the highest dose of 2.14×10^{10} particles/mL had a value of 1.5. According to the OECD TG 442B, the test article is considered potentially sensitizing if its SI value is ≥ 1.6, therefore, ASC-Exo were classified as a potential non-sensitizer in LLNA-BrdU-ELISA under our experimental conditions.

3.3. No phototoxicity by ASC-exosomes

Considering the phototoxicity endpoint, the 3T3 Neutral Red Uptake (NRU) phototoxicity assay can be utilized to identify the photoallergic potential of the test article induced by the combination of light (Spielmann et al., 1998; Spielmann et al., 1998b). The 3T3 NRU phototoxicity assesses the cytotoxic effect of the test article after exposure to a non-cytotoxic dose of UVA/VIS light compared to the absence of exposure. Under these experimental conditions, IC_{50}, PIF and MPE values were obtained from various concentrations of ASC-exosomes (Table 5). For chlorpromazine hydrochloride, the values were 1.232 μg/mL with irradiation (IC_{50} value), 28.45 μg/mL without irradiation (IC_{50} value), 23.286 (PIF) and 0.435 (MEF), similar to those described in the OECD (Spielmann et al., 1998b) and acceptably classified as phototoxic (PIF > 5 or MPE > 0.15). The values for ASC-exosomes were $> 2.14 \times 10^{10}$ with or without irradiation (IC_{50} value), none (PIF) and −0.081 (MPE), and are classified as non-phototoxic potential in the 3T3 NRU assay. Therefore, ASC-exosomes did not induce phototoxicity in the BALB/C 3T3 clone A31 cell culture system.

3.4. No effect on eye irritation by ASC-exosomes

For the eye irritation assessment, the BCOP assay is widely used and accepted for the identification of corrosive and severe eye irritants (OECD, 2017; Verstraelen et al., 2013). Accordingly, the *in vitro* BCOP assay was performed to evaluate the eye irritation of ASC-exosomes. As shown in Fig. 3, no changes were found in the opacity score (0.1 ± 1.1) and the permeability score (−0.001 ± 0.003) for ASC-exosomes (1.61×10^{10} particles), which were comparable to the values for the vehicle control. With the opacity and permeability values, the *in vitro* irritancy score (IVIS) was calculated as described in section 2.6.1. The IVIS score for ASC-exosomes was 0.1 ± 1.1, which is classified as UN GHS no category (IVIS score ≤ 3), while the IVIS score for ethyl alcohol as a positive control was 36.8 ± 2.3, which is classified as UN GHS no prediction can be made (3 < IVIS ≤ 55) in the BCOP assay. Thus, ASC-exosomes did not promote eye irritation or serious eye damage under these conditions.

3.5. No effect on skin irritation by ASC-exosomes

Skin irritation is one of the toxicological endpoints that is addressed in a biological risk assessment. The SKINETHIC™ RHE model is reconstructed from human primary keratocytes and mimics human epidermal morphology and physiology. This model has been identified as an ideal biological model for screening the safety or efficacy of various substances (Alépée et al., 2010). As shown in Fig. 4, the viability of skin cells treated with ASC-exosomes (2.14×10^{10} particles/mL) was 104.0% ± 1.8%, while that with 5% SDS as a positive control was 1.2% ± 0.1%. Therefore, ASC-exosomes were classified as a skin non-irritant using the RHE SKINETHIC™ model according to the OECD TG 439.

3.6. No acute oral toxicity by ASC-exosomes

The principle of the acute toxic class method is based on a stepwise

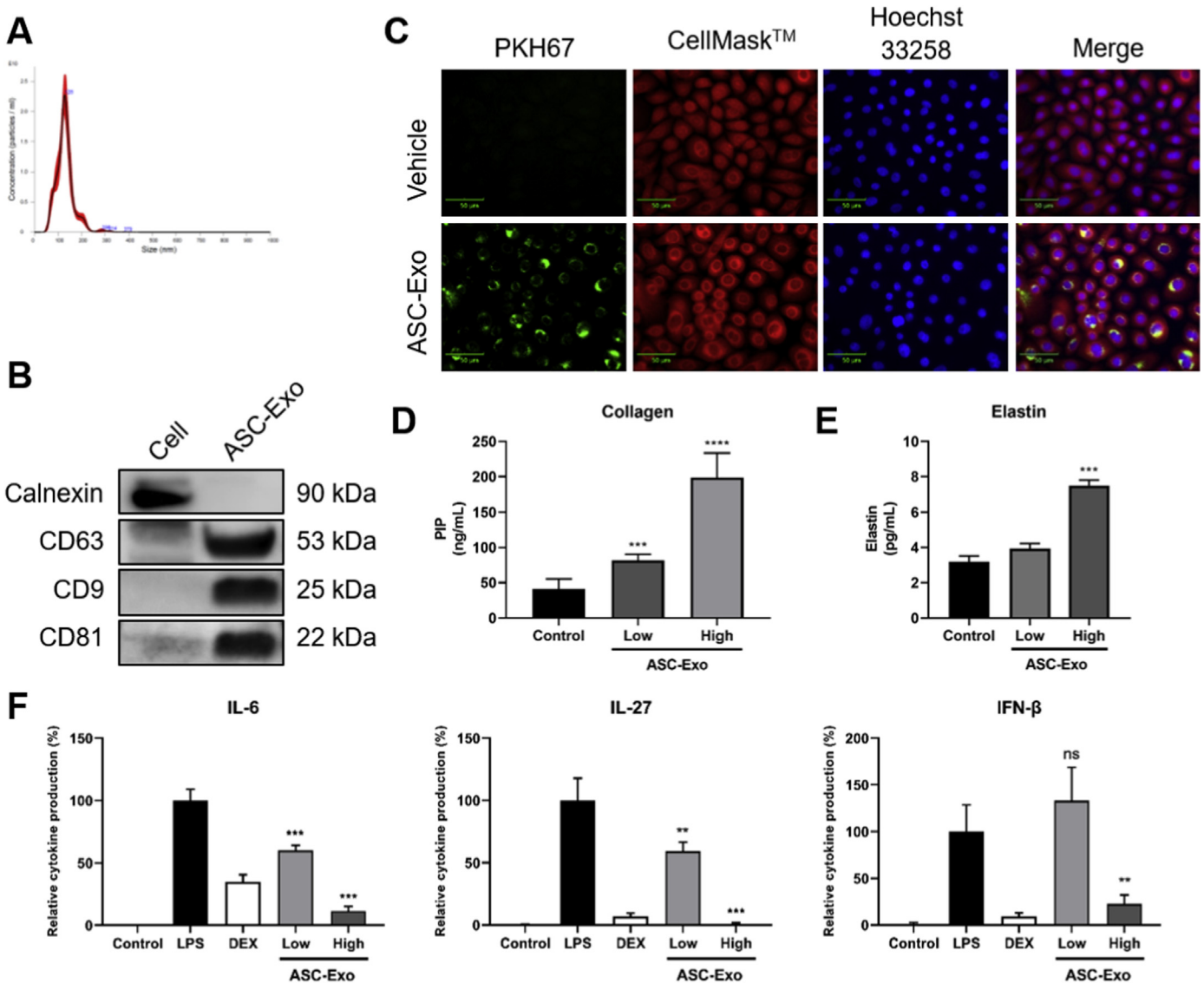

Fig. 1. Characterization of ASC-Exo. (A) Size distribution of ASC-Exo. (B) Western blot analyses of ASC-Exo. CD63, CD9, and CD81 are for exosome surface markers, and Calnexin is for a negative marker. (C) Cellular uptake of ASC-Exo (1.92×10^{11} particles/mL). PKH67 (green), CellMask™ (red), and Hoechst 33258 (blue) were used to visualize exosomes, cell membrane, and nuclei, respectively. (D) Effects of ASC-Exo on procollagen type I protein synthesis in HDF cells. ASC-Exo were treated at low (8.0×10^9 particles/mL) or high (4.1×10^{10} particles/mL) concentration. (E) Effects of ASC-Exo on elastin protein synthesis in HDF cells. ASC-Exo were treated at low (6.0×10^9 particles/mL), med (1.9×10^{10} particles/mL), or high (6.3×10^{10} particles/mL) concentration. (F) Anti-inflammatory effects of ASC-Exo in RAW 264.7 cells. ASC-Exo were treated at low (6.0×10^9 particles/mL) or high (2.0×10^{11} particles/mL) concentration. Representative results are presented as MEAN ± SD from multiple independent experiments performed in triplicate. $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ vs. control group; ns, not significant. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1
In vitro test result for the presence of mycoplasma.

Group		Culture Days		
		3 days	7 days	14 days
Positive Control	M. pneumonia(Aerobic mycoplasma)	+	+	+
	M. orale(Anaerobic mycoplasma)	+	+	+
Negative Control	Culture Media	–	–	–
ASC-Exo		–	–	–

Abbreviations: (+), growth; (−), no growth.

procedure using a minimum number of animals per step, and as such, sufficient information is obtained on the acute toxicity of test articles to enable classification (Schlede et al., 2005). Under the conditions of the present study, there were no mortalities, no clinical signs, no changes in body weight and no gross abnormal findings on necropsy at the concentration of 6.42×10^9 particles/kg and 4.28×10^{10} particles/kg (Tables 6–9). Based on these results, when ASC-exosomes were dosed to SD rats by the acute toxic class method, the test article fell in the GHS category 5 or unclassified according to Annex I. Additionally, the LD_{50} (median lethal dose) cut-off value was determined as over 1×10^{11} particles/kg.

4. Discussion

The MSC-exosomes are currently in the spotlight because of their advantages as compared to other cell types. First, recent studies report that the therapeutic mechanism of MSCs is mainly mediated by paracrine effects and not by MSCs themselves (Speeset al., 2016) and exosomes are a key factor in this paracrine-mediated effect (Keshtkaret al.,

Table 2
In vitro test result for the presence of adventitious virus.

Group	Cytopathic Effect			Hemagglutination Test	Hemadsorption Test
Cell line	MRC-5	Vero	Production Cell Line (ASC)	Production Cell Line (ASC)	Production Cell Line (ASC)
Positive Control	(+)Varicella Zoster Virus	(+)Measles Virus	(+)Measles Virus	(+)Measles Virus	(+)Measles Virus
Negative Control	(−)Culture Media	(−)Culture Media	(−)Culture Media	(−)Culture media	(−)Culture media
ASC-Exo	(−)	(−)	(−)	(−)	(−)

Abbreviations: (+), detected; (−), not detected.

Table 3
Evaluation of ear thickness.

Group		Dose	Sex	Number of Animals	Mean Ear Thickness (mm)			Change (%)
					Day 1(Pre-dosing)	Day 3(48 Hours)	Day 6(In-life)	Day 6 – Day 1
VC	G1	–	Female	5	0.18	0.18	0.18	0.00
	G2	–	Female	5	0.18	0.18	0.18	0.00
TS	G3	50%(1.07×10^{10} particles/mL)	Female	5	0.18	0.18	0.18	0.00
	G4	75%(1.61×10^{10} particles/mL)	Female	5	0.18	0.18	0.18	0.00
	G5	100% (2.14×10^{10} particles/mL)	Female	5	0.18	0.18	0.18	0.00
PC	G6	25%	Female	5	0.18	0.18	0.18	0.00

Abbreviations: , Not applicable; G1, Propylene Glycol; G2, 20% (v/v) Olive oil in Acetone; PC, Positive control (α-Hexyl cinnamic aldehyde); TS, Test substance (ASC-Exo); VC, Vehicle control.

Table 4
Evaluation of skin response.

Group		Dose	Sex	Score	Number of Animals	Day(s)Number of Ears with Score 0											
						1[a]		2[b]		3[c]		4		5[d]		6	
						L	R	L	R	L	R	L	R	L	R	L	R
VC	G1	–	Female	0	5	5	5	5	5	5	5	5	5	5	5	5	5
	G2	–	Female	0	5	5	5	5	5	5	5	5	5	5	5	5	5
TS	G3	50%(1.07×10^{10} particles/mL)	Female	0	5	5	5	5	5	5	5	5	5	5	5	5	5
	G4	75%(1.61×10^{10} particles/mL)	Female	0	5	5	5	5	5	5	5	5	5	5	5	5	5
	G5	100%(2.14×10^{10} particles/mL)	Female	0	5	5	5	5	5	5	5	5	5	5	5	5	5
PC	G6	25%	Female	0	5	5	5	5	5	5	5	5	5	5	5	5	5

Abbreviations: , Not applicable; G1, Propylene Glycol; G2, 20% (v/v) Olive oil in Acetone; L, Left; PC, Positive control (25% α-hexyl cinnamic aldehyde in 20% (v/v) Olive oil in Acetone); R, Right; Score (0): No erythema; TS, Test substance (ASC-Exo); VC, Vehicle control.
[a] 1st treatment.
[b] 2nd treatment.
[c] 3rd treatment.
[d] BrdU injection.

2018). Second, MSC-exosomes are free from the problems that cell therapies may have such as tumorigenicity, occlusion of distal vascular system, and short shelf life, etc. (Choet al., 2018). Third, exosomes are naturally present in most body fluids in humans, so there is less concern about unexpected side effects. Therefore, MSC-exosomes are applied for treatment of incurable diseases as therapeutics or as drug delivery vehicles. Accordingly, studies on functions and efficacy of MSC-exosomes are greatly increasing. Despite the increased interest, the safety and toxicity studies of MSC-exosomes are rare. To date, Maji and colleagues evaluated genotoxic, hematological, and immunological effects, and the endotoxin amount of MSC-exosomes at two doses (10^9 and 10^{12} particles) and provided an *in vitro* safety profile of MSC-exosomes (Majiet al., 2016).

Here, we performed several toxicity tests of ASC-exosomes for *ex vivo* eye irritation, *in vitro* skin toxicity and *in vivo* acute animal toxicity. Currently, no recommended dose of exosomes for human or animal is available. A study suggested dose estimation for human based on the number of exosomes from defined number of MSCs (Kodelas et al., 2014). According to the results, 4 x 10^7 MSCs produced 1.3–3.5 x 10^{10} particles for 48 h. This means 3.3–8.8 x 10^8 particles are produced by 1 x 10^6 MSCs for 48 h. Since 0.4–9.0 x 10^6 MSCs/kg b.w. usually administered to GvHD patients (Kodelas et al., 2014), the number of exosomes achievable from these number of cells is calculated as 0.13–7.9 x 10^9 particles/kg b.w. The animal equivalent dose for rat can be calculated as 0.5–4.9 x 10^{10} particles/kg b.w. according to body surface area (Nair and Jacob, 2016). Based on this, we selected the maximum dose for rat as 4.28 x 10^{10} particles/kg b.w. In addition, we used single batch of exosomes for all other toxicological tests in this study to keep the use of same material in accordance with details for each test in the guidelines.

First, ASC-exosomes were classified as a potential non-sensitizer by LLNA as a stand-alone test for skin sensitization. No changes in ear thickness and skin response were observed as a result of ASC-exosomes application at a maximum concentration of 2.14×10^{10} particles/mL. In addition, stimulation indices of ASC-exosomes at all doses resulted in values less than 1.6, the cutoff value of a potential sensitizer, whereas HCA as a positive control had a value of 2.4. The ASC-exosomes did not induce phototoxicity in the BALB/C 3T3 clone A31 cell culture system. The IC_{50} value of ASC-exosomes was $> 2.14 \times 10^{10}$ particles/mL with or without UV-irradiation, PIF value of ASC-exosomes was not calculated and the MPE value of ASC-exosomes was −0.081 and ASC-exosomes were classified as non-phototoxic. The ASC-exosomes were

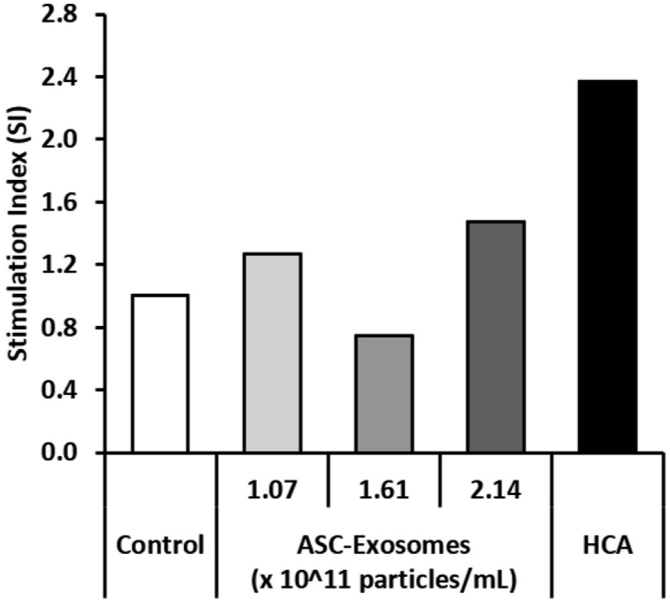

Fig. 2. Evaluation of skin sensitization of ASC-Exo. LLNA-BrdU-ELISA was performed according to the OECD TG 422B. HCA was used as a positive control.

Table 5
Calculation of IC_{50}, PIF and MPE in 3T3 NRU phototoxicity.

Test articles	IC_{50}		PIF	MPE
	UV (-Irr)	UV (+Irr)		
ASC-Exo	$> 2.14 \times 10^{10}$ (particles/mL)	$> 2.14 \times 10^{10}$ (particles/mL)	no PIF	−0.081
Chlorpromazine hydrochloride	28.45 (μg/mL)	1.232 (μg/mL)	23.286	0.435

Abbreviations: IC_{50}, (50% inhibitory concentration); MPE, Mean photo effect; PIF, Photo irradiation factor.

classified as UN GHS no category (IVIS ≤ 3) in the BCOP assay. The IVIS value of ASC-exosomes (1.61×10^{10} particles) was −0.1 ± 1.1 and no changes were found in the opacity score (0.1 ± 1.1) and permeability score (−0.001 ± 0.003). Our results found that ASC-exosomes were classified as a skin non-irritant in the RHE SKINETHIC™ model, which is a reliable prediction of skin irritation potential when compared to *in vivo* rabbit data. Cell viability for the ASC-exosomes-treated group (2.14×10^{10} particles/mL) was 104.0% ± 1.8%, while cell viability for 5% SDS-treated group was 1.2% ± 0.1%. Finally, in the acute oral toxicity test using the acute toxic class method in SD rat,

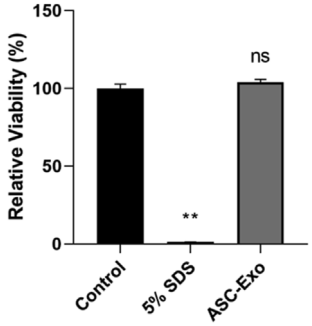

Fig. 4. Evaluation of skin irritation. MTT assay was performed in SKINETHIC™ RHE model, and relative viability was calculated. SDS was used as a positive control. Results are presented as MEAN ± SD. $^{**}P < 0.01$ vs. control group; ns, not significant.

ASC-exosomes fell in the GHS category 5 or unclassified. No mortalities or clinical signs, changes in body weight, and no abnormal findings on gross necropsy were observed resulting from ASC-exosomes administration at the two different doses of 6.42×10^9 particles/kg and 4.28×10^{10} particles/kg. In conclusion, ASC-exosomes were neither skin sensitizing, nor eye irritants, as a phototoxic substance or as a toxic substance that induces acute animal toxicity, with alternative approaches for toxicological screening.

Apart from the toxicological studies discussed above, ASC-exosomes showed the ability to protect cells from damaging by UV irradiation. In the 3T3 NRU assay, ASC-exosomes treatment increased cell viability as compared to the control group under UV irradiation conditions (Fig. 5), and no significant change in cell viability was observed as compared to the control group under UV non-irradiation conditions (data not shown). Although the UVA dose used in this study (5 J/cm^2) is known as insufficient to exhibit severe cytotoxicity, treatment with 5 J/cm^2 UVA induced the decrease of collagen type I alpha 1 (COL1A1) and an increase in matrix metallopeptidase 1 (MMP1), with slight cytotoxicity as noted in normal human face-derived dermal fibroblasts (Shim, 2017). The UVA induces photo-aging of human skin including wrinkle formation, reduction of skin elasticity, and delayed wound healing. These results suggest that ASC-exosomes are capable of protecting cells from stressful environments, such as UV irradiation and are consistent with previous results that human ASC secretomes have a protective effect and reduce age-related damage caused by UV irradiation in human dermal fibroblasts (Sonet al., 2015; Wanget al., 2015).

5. Conclusion

In the present study, we evaluated the toxicological profile of ASC-exosomes such as skin sensitization, *in vitro* photosensitization, eye and

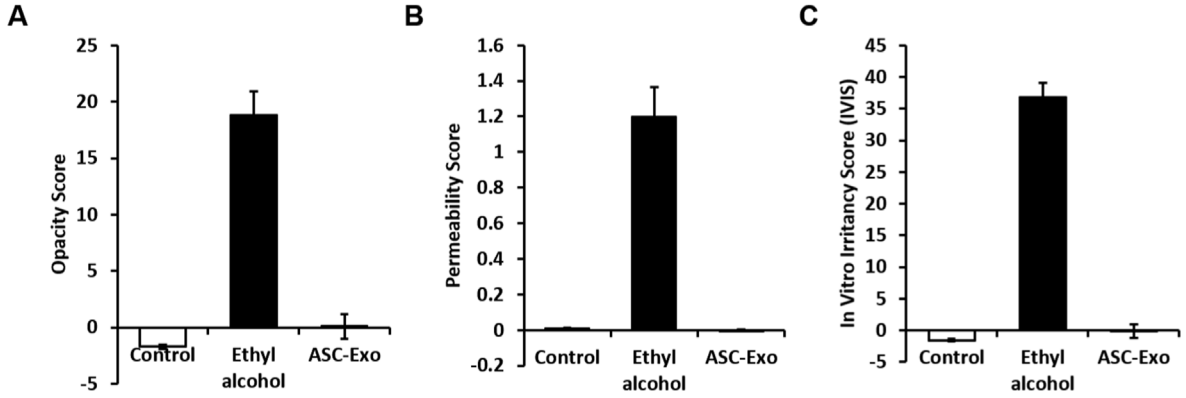

Fig. 3. Evaluation of eye irritation of ASC-Exo. BCOP assay was performed according to the OECD TG 437. (A) opacity, (B) permeability, and (C) *in vitro* irritancy score (IVIS). Ethyl alcohol was used as a positive control.

Table 6
Mortalities.

Groups(Particles/kg b.w.)	No. Dead/No. Dosed	Days after Dose Mortalities at Each Day									LD_{50} Cut-off Value
		1	2	3	4	5	6	7	8	9–15	
G1 (6.42×10^9)	0/3	0	0	0	0	0	0	0	0	0	
G2 (6.42×10^9)	0/3	0	0	0	0	0	0	0	0	0	
G3 (4.28×10^{10})	0/3	0	0	0	0	0	0	0	0	0	
G4 (4.28×10^{10})	0/3	0	0	0	0	0	0	0	0	0	$> 1 \times 10^{11}$ particles/kg b.w.

LD_{50}, Median Lethal Dose.

Table 7
Clinical signs.

Clinical Signs					
Days	Signs	Groups(particles/kg b.w.)Number of Animals with the Sign/Number of Animals Examined.			
		G1(6.42×10^9)	G2(6.42×10^9)	G3(4.28×10^{10})	G4(4.28×10^{10})
1–14	Normal	3/3	3/3	3/3	3/3
15	Normal	3/3	3/3	3/3	3/3
	Terminal Euthanized	3/3	3/3	3/3	3/3

The day of administration was designated Day 1.

Table 8
Body weights.

Body Weights (g)				
Days	Groups(particles/kg b.w.)			
	G1 (6.42×10^9)	G2(6.42×10^9)	G3(4.28×10^{10})	G4(4.28×10^{10})
1	171.59 ± 2.71	171.84 ± 3.72	174.68 ± 1.40	172.86 ± 2.63
2	190.87 ± 1.88	186.89 ± 7.04	194.77 ± 2.98	191.89 ± 2.44
4	190.25 ± 2.74	195.43 ± 2.02	195.07 ± 2.28	194.41 ± 3.33
8	198.88 ± 7.14	202.76 ± 5.42	206.96 ± 8.04	205.95 ± 3.64
15	205.07 ± 12.16	215.19 ± 4.04	214.01 ± 14.32	217.48 ± 9.66
Gain	33.48 ± 11.25	43.35 ± 5.34	39.34 ± 15.45	44.62 ± 7.04
N	3	3	3	3

The day of administration was designated Day 1.
Data are expressed as Mean ± SD.
(Gain) = (body weight on Day 15) – (body weight on Day 1).

Table 9
Necropsy findings.

Necropsy Findings					
Organs	Findings	Groups(particles/kg b.w.)			
		G1(6.42×10^9)	G2(6.42×10^9)	G3(4.28×10^{10})	G4(4.28×10^{10})
No gross findings	3	3	3	3	
N	3	3	3	3	

skin irritation, and acute oral toxicity in accordance with the OECD guidelines and the principles of GLP. Our results suggest that the ASC-exosomes are safe with no adverse effects. Given the potential of exosomes as therapeutics and as drug delivery vehicles and their potential high industrial value, various investigations not only on the mode of action and product release criteria, but also further studies of toxicity profile, biodistribution and pharmacokinetics are required.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: BSC and YWY are founders and stockholders of ExoCoBio Inc. DHH, JHL, SRP, JY, SHL, JEK, JL, BSC, and YWY are employees of ExoCoBio Inc. SDK and HL are employees of ChemOn Inc. Other authors declare no conflict of interest.

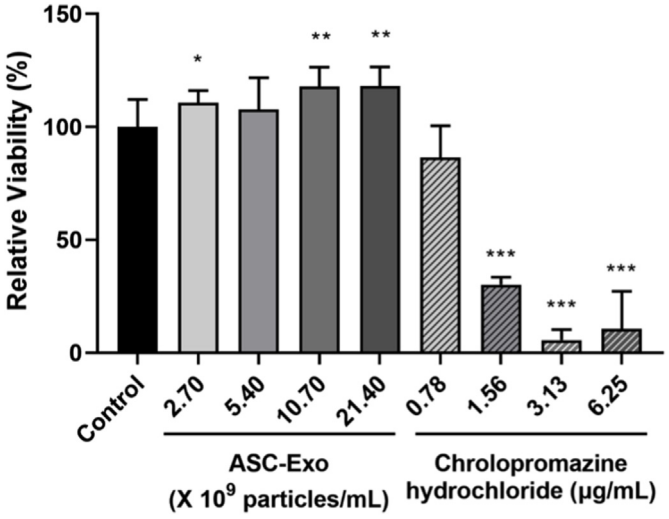

Fig. 5. Cell protection ability of ASC-Exo from UV irradiation. Neutral Red Uptake (NRU) phototoxicity assay was performed in 3T3 A31 cell line, and relative viability was calculated. Chlorpromazine hydrochloride was used as a positive control. Results are presented as MEAN ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs. control group.

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