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Beneficial effects of Trehalose on Mesenchymal Stem Cell-derived Exosomes: A very effective tool in the industrial use of exosomes

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Abstract

In recent years, basic and clinical research on extracellular vesicles, including exosomes, which are natural nanoscale particles, has been intensifying. However, many issues still need to be addressed regarding strategies for the industrial utilization of exosomes. In this study, we investigated the effect of trehalose on the number and the property of exosomes released from human adipose-derived mesenchymal stem cells. We characterized their sizes and surface marker profiles by using flow cytometry analysis.

The addition of trehalose to the culture medium increased the number of exosomes produced. Analysis of the anti-inflammatory function of exosomes induced by lipopolysaccharide in the presence or absence of trehalose suggested that trehalose does not affect the specific activity of the exosomes produced. Purification of exosomes using trehalose as an additive to the buffer solution significantly increased the yield of exosomes attributable to its effect in preventing collapse and aggregation of exosomes during ultracentrifugation and ultrafiltration.

Thus, our findings that trehalose is a suitable additive for exosomes in all of these production, purification, and preservation processes indicate that trehalose is a promising tool for the industrial application of exosomes. Furthermore, the results suggest that the separation and preservation methods presented in this study can facilitate therapeutic applications of exosomes.

Keywords: trehalose; exosomes; human mesenchymal stem cells; exosome yield; aggregation; extracellular vesicles.

Introduction

Extracellular vesicles (EVs) are secreted by almost all cell types, are small (50-1000 nm in diameter) membrane vesicles surrounded by a lipid bilayer and are characterized by their inability to replicate themselves **[1]**. In other words, they are a generic term for particles without a nucleus, known to exist in large quantities in biological fluids and cell culture media. In general, EVs include exosomes derived from endosomes, microvesicles derived from cell membranes, and apoptotic vesicles derived from apoptotic cell membranes, depending on the difference in their formation process.

In recent years, research on EVs, including exosomes, has progressed rapidly. Since the molecules contained in these EVs (proteins, RNA, lipids, metabolites, *etc*.) are passed on to other cells and involved in the exchange of gene expression information and novel intercellular signaling mechanisms, functional analyses and disease relevance are rapidly becoming the focus of research worldwide **[2-5]**. For example, the search for biomarkers for EVs and the development of drug discovery using EVs are becoming increasingly competitive, especially in applications **[6-7]**. Furthermore, natural nanoscale particle exosomes are actively investigated in basic and clinical trials. In particular, mesenchymal stem cells (MSCs)-derived exosomes have shown therapeutic efficacy against various diseases, including tumors, neurodegenerative diseases, and cardiovascular and wound repair, and are being developed as nanotherapeutics **[1, 8-11]**. However, many fundamental issues still need to be solved, such as reliable methods for the production, isolation, purification, analysis, and storage of EVs containing exosomes.

Recently, trehalose has been reported to be helpful in purifying exosomes and improving their stability during freeze-drying and storage **[12]**. In this study, we investigated the beneficial effects of low endotoxin trehalose on exosomes released from MSCs, which have been reported for diverse medical applications, using flow cytometry (FCM) analysis concerning the Minimal Information for Studies of Extracellular Vesicles 2018 (MISEV 2018) guidelines **[13]**.

Materials and Methods

Reagents

Normal human MSCs (adipose-derived, Hispanic 34 years old, female, KW-4109) were purchased from Kurabo Industries, Ltd (Osaka, Japan). Since normal human MSCs are adhesive cells, Stempro™ Accutase™ cell dissociation reagent (ThermoFisher Scientific, Grand Island, NY) was used as a cell detachment agent. Human monocytic cell line, THP-1 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA).

Trehalose used in the entire experiment was manufactured by Hayashibara Co., Ltd. (TREHALOSE SG, Okayama, Japan). Endotoxin content in the TREHALOSE SG was measured according to a protocol described in the Japanese Pharmacopoeia using the Endospecy ES-50M set (Seikagaku Co., Tokyo, Japan). It was found to be less than 0.3 endotoxin units/g. Lipopolysaccharide (LPS; *E. coli* O111B4, Merk, 100 ng/ml) was purchased from Sigma-Aldrich Japan (Tokyo, Japan). FCM for the detection of exosomes was performed with the Gallios AS08042 (Beckman Coulter Inc., Fullerton, CA). Instrument performance for small particle detection was evaluated with a commercially available fluorescein bead mixture (Megamix-Plus FSC 50, BioCytex SARL, Marseille, France) containing four kinds of bead populations ranging from 100 nm to 900 nm in diameter. Human tumor necrosis factor (TNF)-α and monoclonal antibodies (mAb) for human TNF-α enzyme-linked immunosorbent assay (ELISA) were prepared and purified in our laboratories as described **[14-15]**.

Cell culture and exosome induction

Normal human MSCs were cultured in Dulbecco's Modified Eagle Medium (D-MEM; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) replenished with a 10% supplement mix for Mesenchymal Sem Cell Growth Medium DXF (C-28019, PromoCell™ GmbH, Heidelberg, Germany). Cells were cultured under standard conditions in a humidified 5% CO_2 and air mixture at 37°C. After incubation for 24 hrs, the culture supernatant was removed, cells were washed with phosphate-buffered saline <PBS (-)> three times, and trehalose (Hayashibara Co., Ltd.) solution with or without LPS (100 ng/mL) was added to the culture medium and MSCs were incubated further for 48 hrs. The culture supernatant was collected, and EVs containing exosomes were purified for further investigation. MSCs detached with Accutase (250 µL/cm^2) were stained with trypan blue, and the number of cells was counted using a blood cell calculator.

For the determination of TNF-α production experiments, on the other hand, the Human monocytic cell line, THP-1 cells were maintained in a complete medium comprised of RPMI 1640 (Sigma-Aldrich) supplemented with 10% fetal calf serum (FCS, GE Healthcare Life Sciences, South Logan, UT) and 1% Penicillin-Streptomycin (Wako Pure Chemical) in a 5% CO_2 humidified atmosphere at 37°C. THP-1 cells were pe-cultured with 2 mmol/L sodium butyrate for 4 to 5 days at 37°C before assessing anti-inflammatory activities.

Isolation of MSCs-derived exosomes

Ultracentrifugation and ultrafiltration methods were investigated for the purification of MSCs-derived exosomes.

(I) Ultracentrifugation method: MSCs-derived culture supernatant was filtered through a 0.22 µm filter, filled into ultracentrifugation tubes, and they were centrifuged at 10,000 g for 30 min at 4°C using an ultracentrifuge (HIMAC CENTRIFUGE, Eppendorf Himac Technologies Co., Ltd., Hitachinaka). The supernatant was collected and transferred into a new ultracentrifugation tube (Eppendorf Himac Technologies Co., Ltd.) and centrifuged at 100,000 g for 70 min at 4°C using the ultracentrifuge. The supernatant was removed, and the precipitate was collected using 0.22 µm filtered PBS as the exosome fraction.

(II) Ultrafiltration method: MSCs-derived culture supernatant was 0.22 µm filtered, and a portion of supernatant (500 µL) was added to a 10 kDa ultrafiltration membrane (Amicon Ultra) equilibrated with PBS (0.1 µm filtered), and was centrifuged at 9,000 g for 20 min at 4°C. After removing the filtrate from the collection tube, 500 µL of fresh PBS was added, mixed uniformly by pipetting, and centrifuged again at 9,000 g for 20 min at 4°C. Five hundred µL of new PBS was added to the filter cup, mixed uniformly by pipetting, and centrifuged again at 9,000 g for 20 min at 4°C. The concentrated EVs in the filter cup were collected and adjusted to 200 µL using 0.1 µm filtered PBS as the exosome fraction. Protein levels were determined using a Pierce™ bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific Inc., Rockford, IL).

Surface antigen immunostaining of exosomes and FCM analysis

Culture supernatants containing exosomes (approx. 100 µg protein) or exosomes purified from MSCs (Passage 4-10, approx. 150 µg protein) by ultracentrifugation were stained with 0.1 µm pre-filtered PBS containing 1 µL of each primary antibody solution (final concentration 0.4 µg/mL) and placed on ice for 60 min. The reaction solution was passed to a 300 kDa ultrafiltration membrane (Pharma Foods International Co., Ltd, Kyoto) and centrifuged at 4°C and 9,000 g for 10 min. The filtrate was removed from the collection tube, 500 µL of fresh PBS was added, mixed uniformly by pipetting, and centrifuged again at 9,000 g for 10 min at 4°C. Then, 500 µL of new PBS was added to the filter cup, mixed uniformly by pipetting, and centrifuged again at 9,000 g for 20 min 4°C. In addition, 500 µL of staining buffer was added to the filter cup, mixed uniformly by pipetting, collected in a protein-free tube, 1 µL of each secondary antibody solution (final concentration 0.4 µg/mL) was added, and allowed to react for 60 min on ice shielded from light. The reaction solution was added to a 300 kDa ultrafiltration membrane (Pharma Foods International Co., Ltd.) and centrifuged at 9,000 g for 10 min

at 4°C. The filtrate was removed from the collection tube, 500 µL of fresh PBS was added to the filter cup, mixed uniformly by pipetting, and centrifuged again at 9,000 g for 10 min at 4°C, and this procedure was repeated twice. Subsequently, the materials were collected in 0.1 µm filtered PBS and determined for the percentage of positive particles at the gate set at 1% of the particles stained with Isotype control antibody using FCM (Gallios AS08042 <Beckman Coulter Inc., Fullerton, CA>). The number of particles by FCM was determined by mixing 200 µL of the sample with 50 µL of Flow-Count standard particles (Beckman Coulter), measuring the number of particles per 2000 common particles, and calculating from the following formula. The volume of sample taken from each tube was automatically recorded by the software and used to normalize calculations.

$$\text{Particle number (/µL} = \frac{\text{Measured particle number}}{\text{Number of Flow-count Measurements}} \times \text{Flow-count standard particle counts}$$

In this experiment, one of the following antibodies was used as a primary antibody: anti-cluster of differentiation (CD)9 antibody (Cat# sc-13118, 1:1000, Clone: C-4; Santa Cruz Biotechnology), anti-CD63 antibody (Cat# EXOAB-CD63A-1, 1:1000, Clone: C-term; System Biosciences), and anti-CD81 antibody (Cat# EXOAB-CD81A-1, 1:1000, Clone: C-term; System Biosciences). Then, as the secondary antibodies, either an anti-Mouse IgG (H+L) Alexa FluorTM488 (#A11017; 1:1000, Thermo Fisher Scientific Inc., Waltham, MA) or an anti-Rabbit IgG (H+L) Alexa Fluor™488 (#A11070; 1:1000, Thermo Fisher Scientific Inc.) was used, respectively **(Table 1)**. Then, the anti-IgG_{2A} antibody CSF (Clone 133303) was used as a negative (isotype) control.

Table 1: Summary of antibodies used for FCM analysis and their requirement.

Antigen		Antibody name	Species	Maker	Catalog No.	Compatible species
CD9	1st Ab*	CD9 antibody (C-4)	Mouse	Santa Cruz Biotechnology	sc-13118	Human, Mouse, Rat
	2nd Ab*	Anti-mouse IgG(H+L) Alexa Fluor™ 488	Goat	ThermoFisher Scientific	A11017	Mouse
CD63	1st Ab*	CD63 antibody (C-term)	Rabbit	System Biosciences	EXOAB-CD63A-1	Human, Mouse, Rat, Horse
	2nd Ab*	Anti-rabbit IgG(H+L) Alexa Fluor™ 488	Goat	ThermoFisher Scientific	A11070	Rabbit
CD81	1st Ab*	CD61 antibody (C-term)	Rabbit	System Biosciences	EXOAB-CD81A-1	Human, Mouse, Rat
	2nd Ab*	Anti-rabbit IgG(H+L) Alexa Fluor™ 488	Goat	ThermoFisher Scientific	A11070	Rabbit
Isotype control	1st & 2nd Ab*	Anti-IgG_{2A} antibody CSF (Clone 133303)	Mouse	R&D systems	IC0041P	Control

Western blotting analysis of Rab7 and Rab27a

MSCs were seeded in 6 healthy plates at a concentration of $5x10^3$ cells/well/2 mL Stem Life® MSC Comp kit. After overnight preincubation in a 5% CO_2 incubator at 37°C, trehalose or glucose was added at final concentrations of 50 and 100 mmol/L, and the cells were incubated for 48 hrs. After removing the supernatant and washing with 500 µL of PBS, 100 µL of SDS-PAGE sample buffer (1% SDS, 12.5% glycerol, 0.005% bromophenol blue, 2.5% 2-mercaptoethanol, 25 mmol/L Tris-HCl, pH 6.8) was added. Whole-cell lysates were then prepared, and the protein concentrations were determined by Pierce™ BCA protein assay (Cat# 23225, Thermo Fisher Scientific). Subsequently, 20 mg of respective lysate protein was subjected to various Western blotting experiments. Separate membrane was then probed with an anti-Rab7 (D95F2) XP® rabbit mAb (#9367S; 1:1000, Cell Signaling Technology, Danvers, MA), an anti-RAB27A antibody (1G7) (#ab55667; 1:1000, Abcam plc., Cambridge, UK) or an anti-β actin antibody (#ATCBD11B7; 1:5000, Santa Cruz Biotechnology, Inc., Dallas, TX). The respective membrane was incubated with the primary antibody at 4°C overnight. They were washed three times with PBS containing 0.1% Tween 20 (PBS-T) and reacted with horseradish peroxidase (HRP)-conjugated anti-Rabbit IgG (#P0448; 1:2000, Agilent Technologies Inc., Santa Clara, CA), an HRP-conjugated anti-mouse IgG (#P0447; 1:5000, Agilent Technologies Inc., Santa Clara, CA), an anti-Rabbit IgG(H+L) Alexa Fluor™488 (#A11070; 1:1000, Thermo Fisher Scientific Inc.) or an anti-Mouse IgG (H+L) Alexa Fluor™488 (#A11017; 1:1000, Thermo Fisher Scientific Inc.) for 1 hr at ambient temperature, respectively. After washing three times with PBS-T, chemiluminescent protein detection was performed using an ECL™ Prime Western Blotting System (Immobilon Western Chemiluminescent HRP substrate; GE Healthcare, UK) by an Image

Quant™ LAS 500 (GE Healthcare Life Sciences). The relative density of bands was quantified by densitometry using Image Quant TL software (GE Healthcare Life Sciences).

Stimulation of THP-1 macrophages

After a resting period, THP-1 macrophages were washed once with RPMI-1640 medium supplemented with 1% FCS and 1% Penicillin-Streptomycin (conditioned medium) and were incubated with various concentrations of exosome solution. After incubation, THP-1 macrophages were stimulated with 20 ng/mL human interferon (IFN)-γ and 5 mg/mL LPS at 37°C for 24 hrs **[16]**. The culture supernatants were then collected and frozen at -80°C until assay for cytokine content.

Evaluation of TNF-α production by THP-1 cells as exosome activity

Human TNF-α in culture supernatants were measured by a two-site sandwich ELISA. Levels of the human TNF-α were determined by an ELISA system that was developed in our laboratory as described elsewhere **[14-15]**.

Morphological observation of exosomes by phase contrast microscopy and scanning electron microscopy (SEM)

Glutaraldehyde was added to the solution containing exosomes to a final concentration of 2% and fixed at ambient temperature for 10 min. Five hundred µL was added to a 10 kDa ultrafiltration membrane (Amicon Ultra) equilibrated with 0.1 µm filtered PBS and centrifuged at 4°C and 9,000 g for 20 min. The filtrate was removed from the collection tube, 500 µL of the fresh PBS was added, mixed uniformly by pipetting, and centrifuged again at 4°C and 9,000 g for 20 min. Subsequently, 500 µL of ultrapure water was added to the filter cup, mixed uniformly by pipetting, and centrifuged at 4°C and 9,000 g for 20 min. A total of three washes were performed in the same manner. Exosomes concentrated in filter cups were collected, 50 µL were dropped onto glass slides and allowed to air-dry, followed by immersion in 50, 70, 90, and 100% ethanol in that order, dehydrated, and air-dried. The samples were observed under a phase contrast microscope and photographed. Samples for SEM observation were deposited with 15 nm thick gold on glass slides using a JFC-1100 ion sputtering system (1200 V, 10 mA, 3 min). SEM observations were performed in a high vacuum at an acceleration voltage of 15 kV.

Statistical analysis

Data were analyzed by one-way analysis of variance followed by Dunnett's multiple-comparison test. The *t*-test was used for comparison between the two variables. p values < 0.05 were considered statistically significant.

Results

MSCs-derived exosomes could be purified to high quality by ultracentrifugation.

FCM has been utilized as an exosome analytical tool **[17-18]**. Therefore, we performed an FCM-based assay for surface marker analysis and particle counting of MSC-derived exosomes. Under the experimental conditions, the fluorescent beads for FCM standardization (0.1 µm) were visible on the plot, and the Flow-Count™ standard particles for counting (10 µm diameter) were plotted at a position (gate T) where they did not overlap with the exosomes **(Figure 1A and 1B)**. The FL 1, 2, 4, and 6 voltage was adjusted using compensation beads. Under these conditions, the peak of exosomes stained with the positive antibody was well separated from that of the isotype control.

MSCs-derived exosomes purified by ultracentrifugation under the same conditions were plotted close to 0.1 µm fluorescent beads, with forward scatter (FS) and side scatter (SS) of 0.38 ± 0.24 and 1.76 ± 0.62 (Mean ± Standard Deviation < SD>) for Gate U 3211 particles, respectively **(Figure. 1A and 1B)**. Surface marker analysis showed that the positivity rates for three tetraspanin molecules, CD9, CD63, and CD81, which are widely considered canonical EVs markers, were 99.6%, 99.8% and 99.9%, respectively **(Figure 1C)**. Particle size distribution was also confirmed by using dynamic light scattering (DLS), and the cumulative (Z) mean particle size of 74.44 nm (PDI 0.617, intercept 0.921) was close to the values as previously reported (15; 50-80 nm).

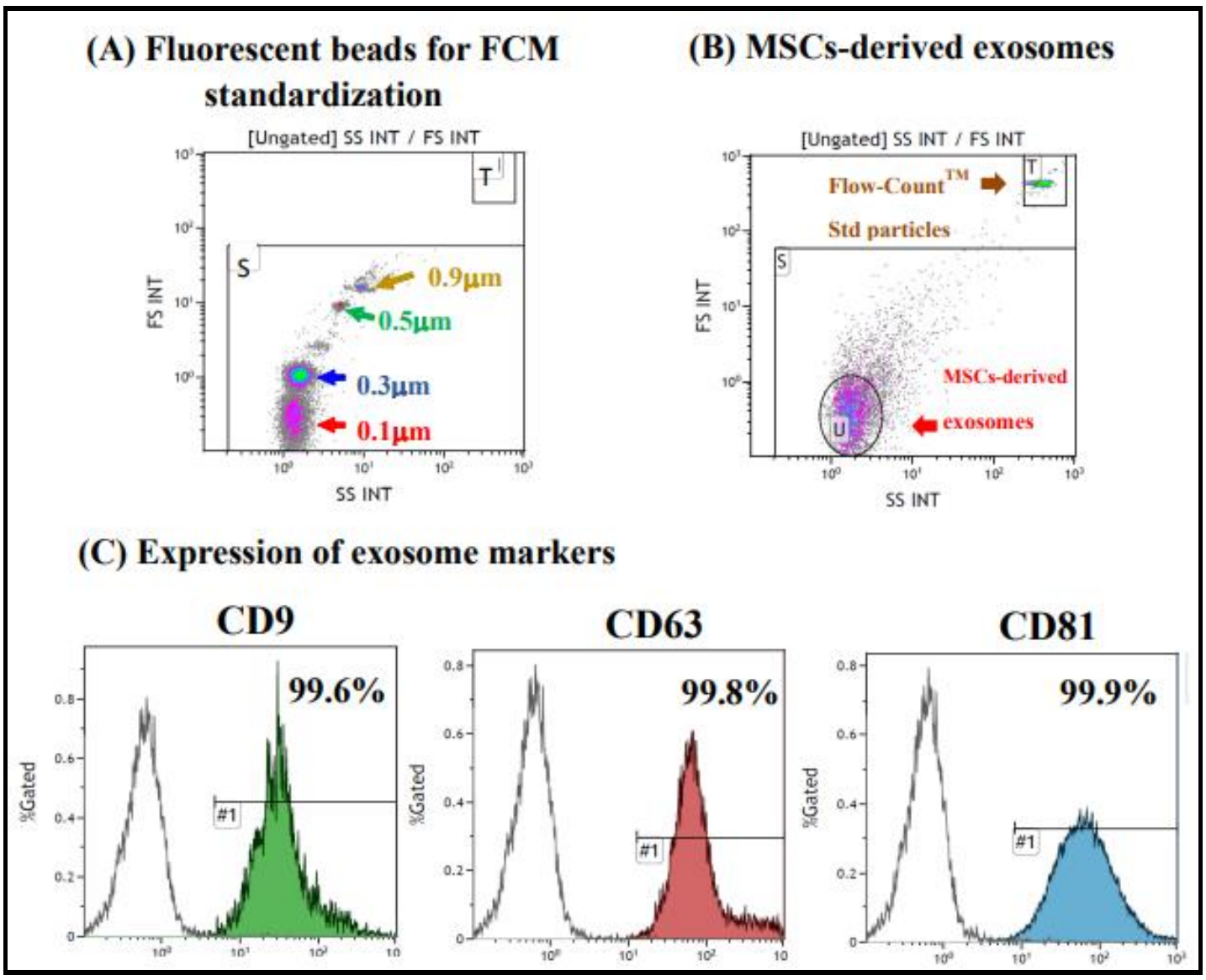

Figure 1: Characterization of MSCs-derived exosomes by FCM analysis.

MSCs were cultured in D-MEM replenished with 10% supplement mix for MSC Growth Medium DXF in 48-well plates at 37°C for 2-3 days. Fluorescent beads for FCM standardization were assessed by FCM (A). MSCs-derived exosomes after ultracentrifugation were determined by FCM (B). Expression of exosome markers such as CD9, CD63 and CD81 was determined as described in Materials and Methods. Results are representative of three independent triplicate experiments with similar results.

Trehalose promotes exosome production from MSCs.

Next, the effect of trehalose on exosome production by MSCs was investigated. As shown in **Figure 2A**, trehalose was added to the MSCs culture medium, and the cell number was counted after 48 hrs incubation. At the time of cell counting, cells had reached confluency, but trehalose at concentrations up to 100 mmol/L did not affect cell number, and no cytotoxicity as assessed by lactate dehydrogenase release was observed (data not shown). Analysis of the number of EVs in cell culture supernatants by FCM revealed that the addition of trehalose resulted in a marked increase in the number of EVs, with statistically significant differences at 50 mmol/L and 100 mmol/L trehalose compared with a control **(Figure 2B)**. The positivity of EVs in MSCs culture supernatants for the exosome markers CD9, CD63 and CD81 was examined by FCM and was 87.3%, 96.9% and 96.8%, respectively, in the absence of trehalose, indicating that the EVs in the culture supernatants are exosomes. Furthermore, the addition of trehalose (100 mmol/L) also showed that EVs were predominantly positive for the three exosome markers, indicating that trehalose promotes the production of exosomes. The reason for the slightly lower CD9 expression is unknown, but it is possible that EV CD9 expression is more susceptible than CD63 or CD81 expression, depending on the cell condition. The number of exosomes after purification by ultracentrifugation was also shown to be increased by the addition of 50 mmol/L and 100 mmol/L trehalose **(Figure 2C)**. These results suggest that the enhancement of EVs containing exosome production may be influenced not only by osmotic pressure but also by the concentration of trehalose. It is interesting to note that cell proliferation almost reached a plateau with the addition of 100 mmol/L trehalose, but the effect on EV production was more sustained.

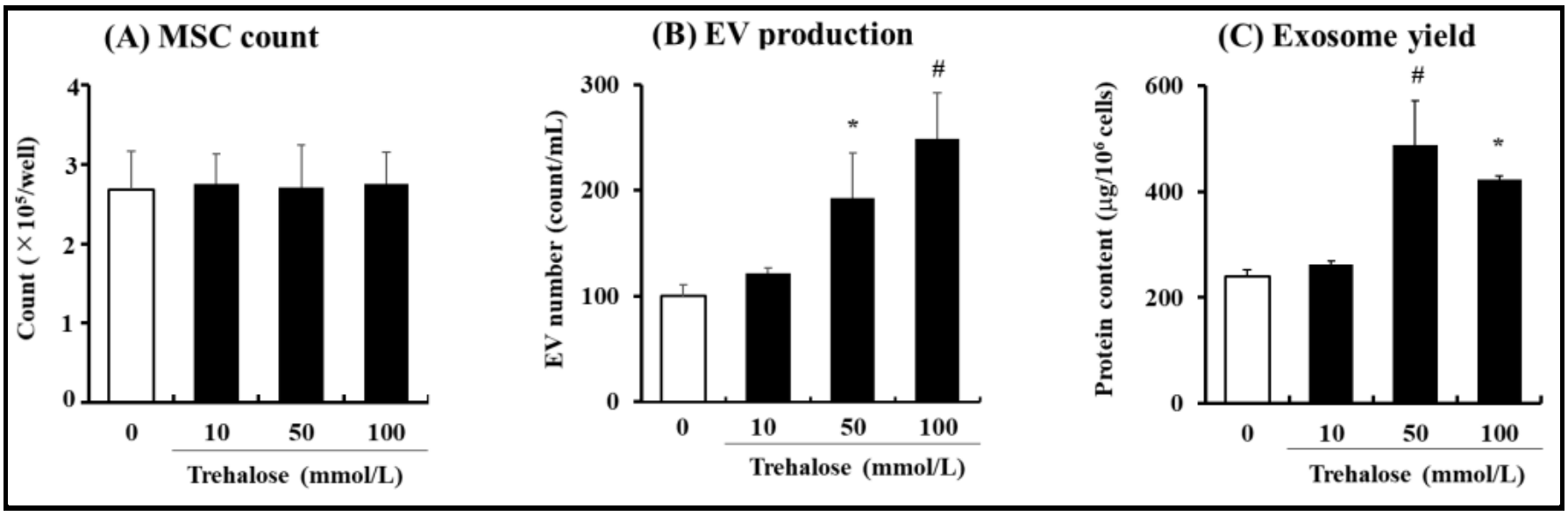

Figure 2: Beneficial effect of trehalose on EVs production (B) and exosome yield (C) on MSCs.

MSCs were cultured under standard conditions in a humidified 5% CO_2 and air mixture at 37°C. After incubation for 24 hrs, the culture supernatants were removed, cells were washed three times with PBS (-) and transferred to culture medium. Fresh culture medium containing 10 - 100 mmol/L trehalose solution was then added with or without LPS and incubated further for 48 hrs. (A) Cells were then collected using Accutase™ and the number of cells was counted. (B) The number of EVs in the culture supernatant of MSCs was counted using FCM. (C) Ultracentrifugation method was examined to isolate and purify the MSCs-derived exosomes. The concentrated EVs in the filter cup were collected and adjusted to 200 μL using 0.1 μm filtered PBS as the exosome fraction. Protein levels were determined using the Pierce™ BCA protein assay kit (C). Results are the means ± S.D. of three consecutive cultures. $^{\#}p < 0.01$ and $^{*}p < 0.05$ compared with the Control cultures (Trehalose 0 mmol/L; □, Trehalose 10~100 mmol/L; ■)

Under these conditions, the effect of trehalose alone on exosome production was examined, and its development was compared with that of glucose. As shown in **Figure 3A**, trehalose increased exosome production at 50 and 100 mmol/L, showing a statistically significant difference from the control cultures (**p<0.01 *vs*. Control). Equimolar glucose also showed a stimulatory effect on exosome production, although the effect was weaker than trehalose, and a statistically significant difference was observed at 100 mmol/L. These results suggest that trehalose alone effectively promotes exosome production and has a higher exosome production-promoting effect than glucose. Furthermore, we examined the effect of an exosome production-promoting agent (EV-Up™) in combination with the saccharides. As shown in **Figure 3A and 3B**, EV-Up™ alone showed a higher exosome production-promoting effect than the Control without adding EV-Up™. When combined with trehalose, synergistically higher exosome production capacity was observed. (**p<0.01 *vs*. Control and EV-Up™ medium, respectively). The addition of glucose and sucrose together with EV-Up™ also tended to increase exosome production compared to EV-Up™ alone, although not statistically significant. However, its effect was lower than trehalose's **(Figure 3B)**. These results indicate that trehalose, alone or in combination with exosome production enhancers, has a higher exosome production enhancing effect than other carbohydrates.

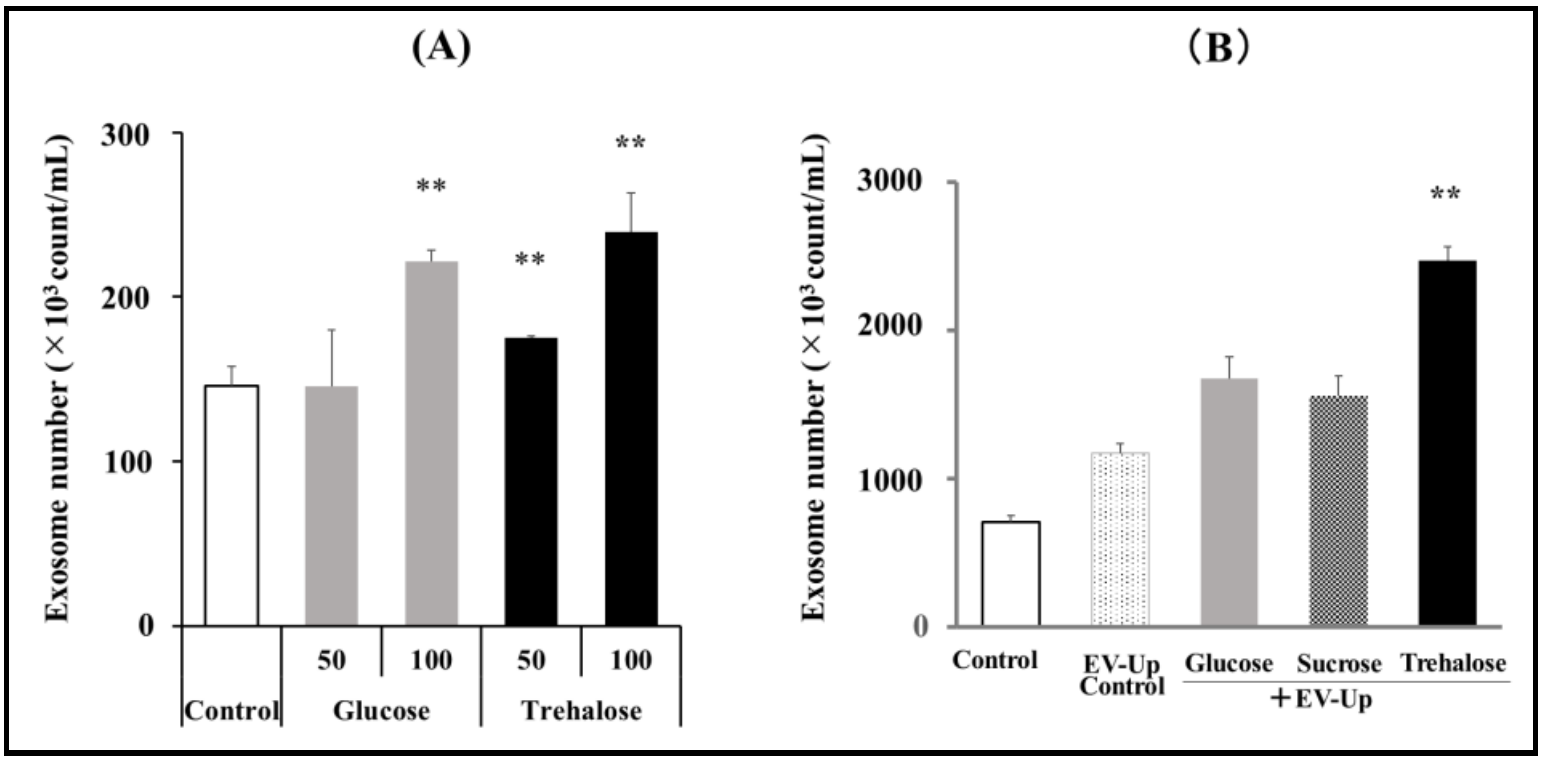

Figure 3: Effect of trehalose and other carbohydrates alone (A) or in combination with exosome production stimulants (B) on exosome production.

Trehalose increased exosome production at 50 and 100 mmol/L on day 3 of addition, showing a statistically significant difference compared to the non-added Control (**p<0.01 vs. Control). Equimolar glucose also stimulated exosome production, although its effect was weaker than that of trehalose, and a statistically significant difference was obtained at 100 mmol/L. The effect of the combination of an exosome production stimulator (EV-Up^{TM}) and trehalose on exosome production was examined. EV-Up^{TM} alone showed a higher stimulating effect than the no-added Control after 3 days of addition. When used in combination with trehalose, they showed synergistically higher exosome production capacity on day 3. In comparisons on day 3, when exosome production was highest, showed that addition of glucose and sucrose also tended to increase exosome production compared to non-addition, but the effect was less pronounced than with trehalose (Figure 3B). Results are the means ± S.D. of three consecutive cultures. **p < 0.01 compared with the Control cultures (Control 0 mmol/L; □, Glucose 50-100 mmol/L; ■, Trehalose 50-100 mmol/L; ■, EV-Up Control; ▒, Glucose + EV-Up; ■, Sucrose + EV-Up; ▓, Trehalose + EV-Up; ■).

Trehalose does not affect exosome function from MSCs.

Exosomes produced by MSCs have been reported to exhibit anti-inflammatory activity by inducing the differentiation of macrophages from M1- to M2-type when induced under inflammatory conditions using LPS and are expected to have applications for wound healing and immunotherapy **[16]**. Therefore, utilizing this characteristic feature of exosomes, we compared the specific activity of MSCs-derived exosomes produced with trehalose to those made without.

The anti-inflammatory activity of LPS-induced MSC-derived exosomes was then evaluated using polarized THP-1 macrophages induced by sodium butyrate, which produces TNF-α in response to 5 mg/mL LPS and 20 ng/mL human IFN-γ **(Figure 4)**. When sodium butyrate-pretreated THP-1 cells were preincubated with LPS-induced exosomes for 6 hrs at 37°C before stimulation with LPS and IFN-γ, TNF-α production was inhibited in a dose-dependent manner, with a statistically significant difference compared to exosomes without LPS above 2.5 µg/mL **(Figure 4A)**. In contrast, exosomes without LPS treatment did not show any inhibitory effect on TNF-α production, but on the contrary, a statistically significant increase in TNF-α production was induced. These results suggest that the mechanism of the anti-inflammatory effect of LPS-induced exosomes is due to the internalization of let-7b via the TLR4/NFκB/STAT3/AKT pathway activated by LPS, as reported previously **[16]**.

When exosome production was induced by LPS in the presence of trehalose, the number of exosomes produced increased (data not shown). Interestingly, however, the anti-inflammatory effect of purified exosomes induced by LPS and trehalose was not altered compared with the purified exosomes induced by LPS only **(Figure 4)**. These results suggest that trehalose promotes exosome production by MSCs without affecting the specific activity of the exosomes produced, *i.e.*, the decreased amount of TNF-α per treated THP-1 cells.

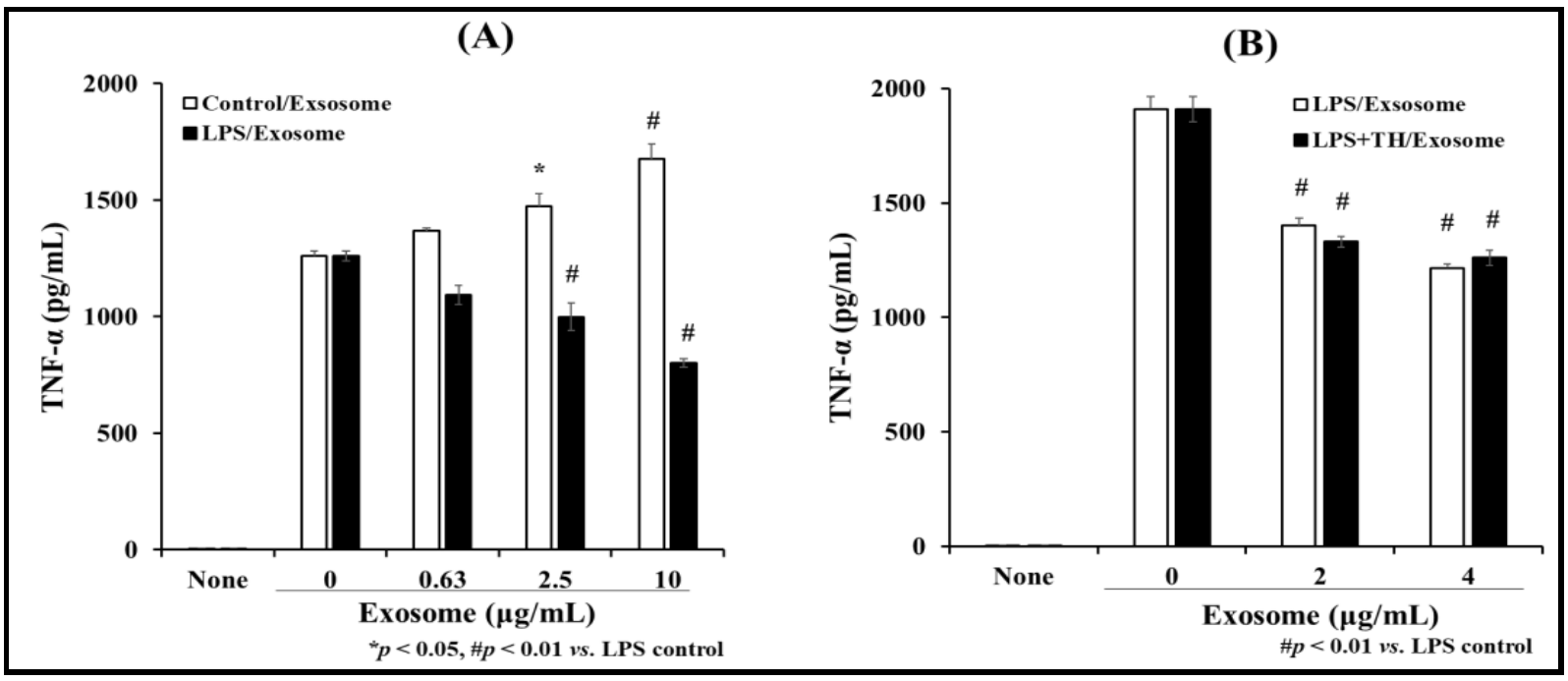

Figure 4: Dose-dependent anti-inflammatory effects by MSCs-derived exosomes induced by LPS (A) and the effect of trehalose on its action (B). THP-1 macrophages were cultured with exosomes at various concentrations with or without LPS at 37°C for 3 days [16]. Sodium butyrate-treated THP-1 macrophages were preincubated with LPS-induced exosomes at various concentrations at 37°C for 6 hrs. THP-1 macrophages were washed once with RPMI-1640 medium supplemented with 1% FCS and 1% Penicillin-Streptomycin (conditioned medium) and were then stimulated with 20 ng/mL human IFN-γ and 5 µg/mL LPS at 37°C for 24 hrs. The culture supernatants were then collected and levels of human TNF-α in culture supernatants were measured by a two-site sandwich ELISA. Results are the means ± S.D. of three consecutive cultures. Results are representative of three independent experiments with similar results. *p < 0.05, #p < 0.01 compared with respective Control. **p < 0.01 compared with Control cultures.

Trehalose promotes MSCs-derived exosome production via Rab7.

The fact that glucose and sucrose were less effective in promoting exosome production suggests that trehalose may exert its effects through a different mechanism than other carbohydrates. Therefore, we examined the effect of trehalose on the expression of two membrane-associated factors reported to play a significant role in exosome production, Rab7 **[19-22]** and Rab27a **[20, 23]**.

Western blotting of the expression of these two factors revealed that the addition of trehalose above 50 mmol/L markedly upregulated the expression of Rab7, and a statistically significant difference was obtained compared to the Control **(Figure 5A and 5B)**. In contrast to the results obtained in Rab7, only a slight increase was observed in Rab27a when treated with 100 mmol/L of trehalose **(Figure 5A and 5C)**. These results suggest that induction of Rab7 expression is involved in the mechanism of exosome production by trehalose.

No significant changes in the levels of either Rab7 or Rab27a were observed when glucose or sucrose was added **(Figure 5A, 5B, and 5C)**. Thus, it is suggested that trehalose induces exosome production from MSCs via the Rab7 pathway, distinct from other sugar molecules.

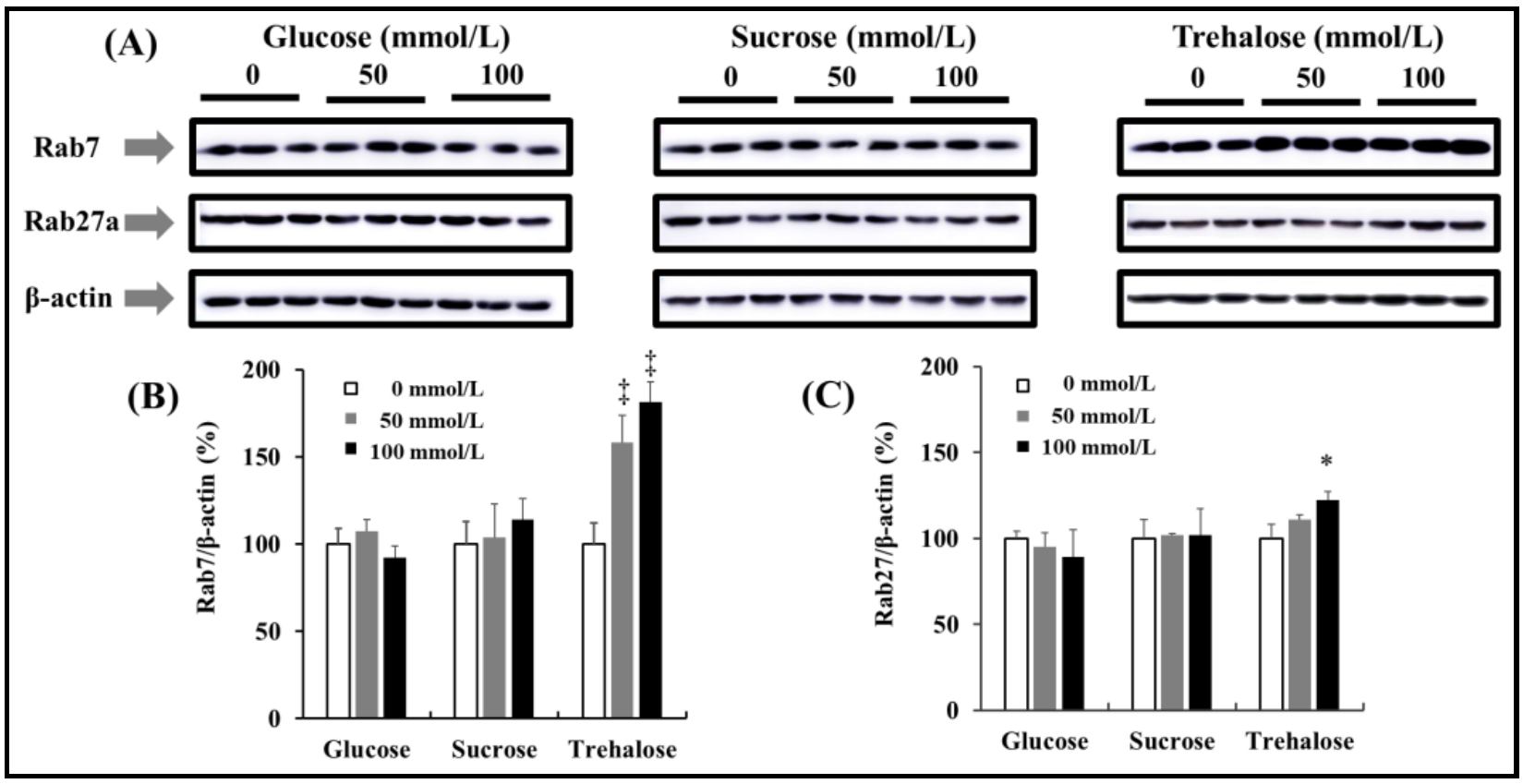

Figure 5: Western blotting analysis of Rab7 (A, B) and Rab27a (A, C) protein expression of MSCs treated with the respective saccharide. MSCs (5×10^3 cells/2 mL of Stem Life® MSC Comp kit) were cultured in 6-well plates at 37°C for overnight. The supernatant was then discarded and 1 μg/mL LPS was added to fresh medium containing trehalose or glucose at the final concentrations of 50 and 100 mmol/L and incubated for 48 hrs. Subsequently, cells were washed with PBS and lysed in 100 μL of SDS sample buffer. After Western blotting, the respective developed film was scanned by ImageQuant™ and the respective intensity of specific bands was calibrated. The relative intensity was evaluated as a percentage of the intensity of the cell lysates grown in D-glucose-containing EV-Up™ medium. Results represent one typical experiment of three similar experiments. (A) Western blot analysis of Rab7, Rab27a and β-actin expression in MSCs cultured with D-glucose (Left), Sucrose (Middle) and Trehalose (Right) of MSCs. Relative intensities of Rab7 (B) and Rab27a (C) protein expression were quantified as the intensity of immunostaining relative to β-actin protein expression as a loading control, respectively. *$p < 0.05$, ‡$p < 0.01$ compared with Control cultures (0 mmol/L).

Morphological observations of exosomes by phase contrast microscopy and SEM

The ultrafiltration method is attracting attention as a new exosome purification method **[24-25]**. Compared to the current ultracentrifugation and chemical methods, the ultrafiltration method causes relatively minor damage to exosomes and is expected to be applied to diagnostics and other applications because it can quickly process multiple specimens with less cost and labor. Therefore, we investigated the effect of trehalose on the purification yield of exosomes by ultrafiltration. The remaining fraction of exosomes after purification by 10 kDa ultrafiltration with trehalose added immediately before purification was evaluated in terms of protein content and particle number **(Figure 6A)**. Exosome yield by ultrafiltration was recovered by the addition of trehalose, and both protein content and particle counts were statistically significantly increased compared to the fraction without trehalose. Microscopic

observation of the state of exosome particles after ultrafiltration showed that the addition of trehalose reduced the number of large-size aggregates in a dose-dependent manner **(Figure 6B)**, whereas particles with non-uniform size due to aggregation were observed in the fraction without trehalose. Similarly, trehalose inhibited exosome aggregation, leaving particles of uniform size **(Figure 6C)**. These results indicate that adding trehalose reduces the damage caused by ultrafiltration during the purification of exosomes and significantly improves particle uniformity and recovery.

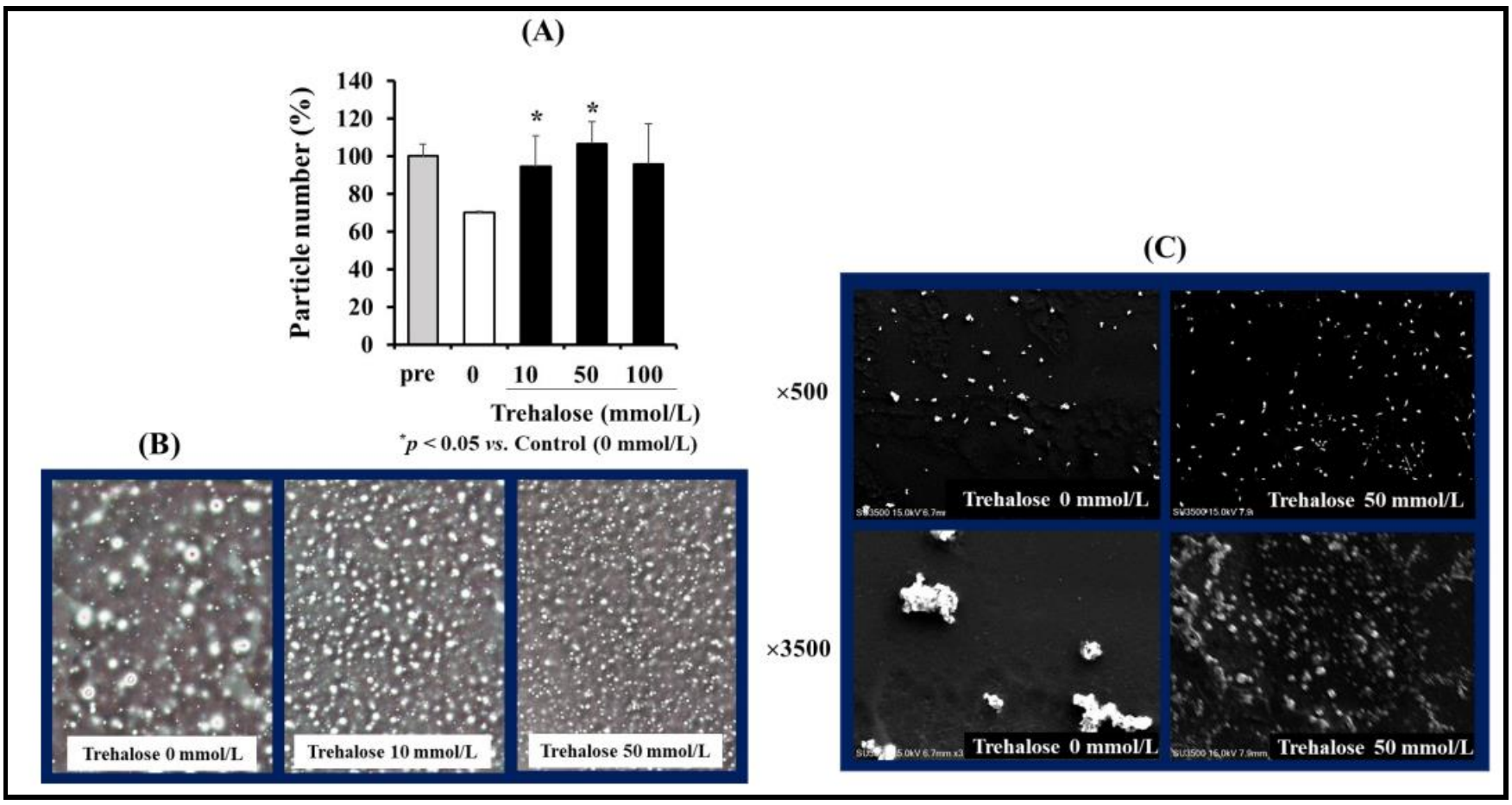

Figure 6: Recovery ratio (A), microscopic images (B) and SEM images (C) of exosomes purified by ultrafiltration. (A) Trehalose increased exosome production at 10 and 50 mmol/L on day 3 of addition, with a statistically significant difference compared to the non-added control ($^{*}P$ <0.05 vs. Control). (B) Microscopic observation of the state of exosome particles after ultrafiltration revealed that particles of non-uniform size due to aggregation were observed in the absence of trehalose, while the addition of trehalose reduced the number of large-sized aggregates in a dose-dependent manner. (C) SEM observations showed similar results, indicating that trehalose inhibited the aggregation of exosomes and that particles of uniform size remained. Results are representative of three independent experiments with similar results.

Discussion

In recent years, exosomes have attracted attention as a new therapeutic tool and are the subject of extensive clinical trials and basic research **[1,8-11]**. Since EVs, including exosomes, are released from cells, there are safety risks, and the mechanism of action is often unknown, making it challenging to identify quality characteristics and evaluate functional equivalence. Therefore, there is concern that treatments may only be implemented with sufficient objective evaluation of the scientific basis for safety and efficacy. Furthermore, methods based on different principles are used to isolate exosomes, such as ultracentrifugation, density gradient ultracentrifugation, and ion chromatography, currently the most used methods. However, each method has advantages and disadvantages, such as methods that can obtain large amounts of exosomes with low purity or techniques that can get highly purified exosomes even with low yields. Thus, it is necessary to select the method according to the purpose.

In the present study, adding trehalose to the exosome induction medium promotes exosome production. However, the evaluation of the anti-inflammatory activity of LPS-induced exosomes showed that adding trehalose did not affect the specific activity of the exosomes produced. Regarding the application of trehalose to the purification process, an inhibition of exosome aggregation and an increase in yield were observed. These results indicate that trehalose provides a very effective strategy for the industrial preparation of exosomes, contributing to the improvement of exosome yield from MSCs during the exosome production and purification processes.

Since FCM is one of the methods used to analyze exosomes **[17-18]**, we analyzed MSC-derived exosomes using FCM and performed surface marker analysis and particle counting of the exosomes. Interestingly, the number of EVs that were highly positive for three exosome markers showed a marked increase with the addition of trehalose. Trehalose did not affect the expression of three exosome markers, indicating that trehalose promotes exosome production. Furthermore, it was fascinating to note that combining exosome production stimulators with trehalose synergistically increased

exosome production. At the same time, the other carbohydrates showed only additive effects **(Figure 3)**. Although Bosch S. *et al.* reported that trehalose induced higher particle counts compared with that of PBS using nanoparticle tracking analysis by MIN6 insulinoma cells **[12]**, we report here for the first time that addition of trehalose significantly enhances the production of MSCs-derived exosomes that are positive for three exosome markers.

Exosomes produced by MSCs have been reported to exhibit anti-inflammatory effects by inducing differentiation of macrophages from M1- to M2-type when added under inflammatory conditions using LPS and are expected to be utilized for wound healing and immunotherapy **[16]**. Therefore, we evaluated the anti-inflammatory effects of MSCs-derived exosomes induced by LPS using TNF-α production by THP-1 macrophages polarized to M1-type as an indicator. Interestingly, MSC-derived exosomes induced by LPS showed a dose-dependent inhibition of TNF-α production, while exosomes derived from the medium alone showed a statistically significant enhancement of TNF-α production. Furthermore, trehalose did not affect the inhibitory effect of MSCs-derived exosomes induced by LPS on TNF-α production, suggesting that trehalose does not affect the desired physiological function of exosomes.

Activation of p38MAPK/p53/TSAP6, which is induced by increased osmolarity, is known to be one of the pathways that may be involved in the enhancement of exosome production by trehalose **[16,26]**. In our present study, 50 mmol/L glucose did not affect the exosome production by MSCs, while adding equimolar amounts of trehalose significantly increased exosome production. These results suggest that mechanisms other than osmolarity are involved in the exosome production-promoting effect of trehalose. We then examined the effect of trehalose on the expression of Rab27a and Rab7, two membrane trafficking factors reported to play a central role in exosome production. Rab7 is a marker of the pre-exosome stage, and it has been reported that suppressing Rab7 significantly reduces exosome development from formation to secretion **[22]**. Rab27a is also a Rab effector molecule that regulates the exocytosis of exosomes, promoting the fusion of the multivesicular body (MVB) to the plasma membrane and releasing exosomes into the extracellular space **[19,21]**. It has also been reported that siRNA-mediated silencing of Rab27a decreases exosome release without altering the internal protein content **[20]**.

Western blotting of the expression of these two factors showed that Rab7 was markedly increased when trehalose was added at 50 mmol/L or more, and a statistically significant difference was obtained compared with the control culture **(Figure 5A and 5B)**. These results suggest that the induction of Rab7 is involved in the mechanism of increased exosome production by trehalose. On the other hand, when glucose was added, no change was observed in the expression levels of Rab7 or Rab27a **(Figure 5B and 5C)**. Thus, it was suggested that the activation of the p38MAPK/p53/TSAP6 pathway induced by osmosis is the main pathway for exosome production in glucose and that trehalose synergistically induces exosome production in MSCs through the combined mechanisms of osmosis-induced pathway and Rab7-induced pathway. The findings that neither glucose nor sucrose increased Rab7 expression further suggest that trehalose plays a distinctive role than other sugar molecules in exosome production in MSCs.

The increase in Rab7 protein levels by trehalose observed in the present study is consistent with reports that trehalose induces an increase in Rab7 protein levels via activation of transcription factor EB (TFEB) without affecting cellular glucose uptake **[27]**. Rab7 levels are also involved in a significant autophagy-inducing pathway, including the expression of autophagy-related proteins, lysosome biogenesis, vacuolation and acidification, and accelerated autophagosome degradation. Moreover, no induction of Rab7 was observed in SMER28, another mTOR-independent autophagy inducer, indicating that this pathway is specific for trehalose. On the other hand, it has also been reported that trehalose does not effect the protein levels of Rab5, which is involved in autophagy and late endosome maturation, Rab11, which is involved in endosome recycling, and Rab3a, which controls endocytosis **[28]**. Since the Rab27a expression was less affected by trehalose and the other carbohydrates tested, it is speculated that Rab27a is unrelated to carbohydrate-mediated exosome induction, but the detailed mechanism is unknown.

As a mechanism for the stabilization of exosomes by trehalose, studies using a membrane model of lipid bilayers have proposed that membrane fusion is suppressed by glass formation due to a decrease in the liquid crystal phase transition temperature (T(m)) **[29]**. It has also been suggested that trehalose may be bound via hydrogen bonds to the phospholipid groups of the lipid bilayer **[30]**. Trehalose may also migrate into the lipid bilayer of exosomes and stabilize the membrane structure via viscosity increase, glass formation, and hydrogen bonding interactions **[31]**, preventing exosome aggregation and collapse.

Conclusions

We demonstrated that adding trehalose during exosome production from MSCs increases the production of exosomes without compromising their function and contributes to the prevention of exosome aggregation and reduction of residual exosomes during purification and storage. As trehalose can be safely used as a component of injectable formulations, exosomes pretreated with trehalose could also be safe. Thus, our results provide a very promising strategy for the resolution of the industrial use of exosomes.

Abbreviations

EVs: extracellular vesicles, MSCs: mesenchymal stem cells, FCM: flow cytometry, MISEV2018: minimal information for studies of extracellular vesicles 2018, LPS: lipopolysaccharide, TNF: tumor necrosis factor, mAb: monoclonal antibody, ELISA: enzyme-linked immunosorbent assay, D-MEM: Dulbecco's modified eagle medium, PBS: phosphate buffered saline (-), FCS: fetal calf serum, BCA: bicinchoninic acid, CD: cluster of differentiation, PBS-T: PBS containing 0.1% Tween-20, HRP: horseradish peroxidase, IFN: interferon, SEM: scanning electron microscopy, FS: forward scatter, SS: side scatter, S.D.: standard deviation, DLS: dynamic light scattering.

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Authors' contributions

HW conceived of the study, participated in the experimental work and data interpretations, and TA drafted the manuscript. KK, S K-M and KH participated in the experimental work. HW, KK, S K-M, HK, TM, YS, and KI reviewed and approved the final manuscript. All authors read and approved the final manuscript.

References

1. Lee Y, Ni J, Beretov J, Wasinger VC, Graham P, Li Y (2023) Recent advances of small extracellular vesicle biomarkers in breast cancer diagnosis and prognosis. Mol Cancer. 22(1): 33-55.
2. Wei W, Ao Q, Wang X, Cao Y, Liu Y, et al. (2021) Mesenchymal Stem Cell Derived Exosomes: A Promising Biological Tool in Nanomedicine. Front Pharmacol. 11: 590470.
3. Armstrong JPK, Holme MN, Stevens MM (2017) Re-engineering extracellular vesicles as smart nanoscale therapeutics. ACS Nano. 11(1): 69-83.
4. Pegtel DM, Gould SJ (2019) Exosomes. Annu Rev Biochem. 88: 487-514.
5. Kalluri R, LeBleu VS (2020) The biology, function, and biomedical applications of exosomes. Science. 367(6478): eaau6977.
6. Shao J, Zaro J, Shen Y (2020) Advances in Exosome-Based Drug Delivery and Tumor Targeting: From Tissue Distribution to Intracellular Fate. Int Nanomedicine. 15: 9355-9371.
7. Srivastava A, Amreddy N, Pareek V, Chinnappan M, Ahmed R, et al. (2020) Progress in extracellular vesicle biology and their application in cancer medicine. WIREs Nanomed Nanobiotechnol. 12(4): e1621.
8. Zhao X, Wu X, Qian M, Song Y, Wu D, et al. (2018) Knockdown of TGF-β1 expression in human umbilical cord mesenchymal stem cells reverts their exosome-mediated EMT promoting effect on lung cancer cells. Cancer Lett. 428: 34-44.
9. Riazifar M, Mohammadi MR, Pone EJ, Yeri A, Lässer C, et al. (2019) Stem cell-derived exosomes as nanotherapeutics for autoimmune and neurodegenerative disorders. ACS Nano. 13(6): 6670-88.
10. Cui GH, Wu J, Mou FF, Xie WH, Wang FB, et al. (2018) Exosomes derived from hypoxia-preconditioned mesenchymal stromal cells ameliorate cognitive decline by rescuing synaptic dysfunction and regulating inflammatory responses in APP/PS1 mic. FASEB J. 32(2): 654-668.
11. Rao F, Zhang D, Fang T, Lu C, Wang B, et al. (2019) Exosomes from human gingiva-derived mesenchymal stem cells combined with biodegradable chitin conduits promote rat sciatic nerve regeneration. Stem Cells Int. 2019: 2546367.
12. Bosch S, de Beaurepaire L, Allard M, Mosser M, Heichette C, et al. (2016) Trehalose prevents aggregation of exosomes and cryodamage. Sci Rep. 6: 36162.
13. Théry C, Witwer KW, Aikawa E, Alcaraz MJ, Anderson JD, et al. (2018) Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. J Extracell Vesicles. 7(1): 1535750.
14. Nagatomo D, Taniai M, Ariyasu H, Taniguchi M, Aga M, et al. (2015) Cholesteryl Pullulan Encapsulated TNF-α Nanoparticles Are an Effective Mucosal Vaccine Adjuvant against Influenza Virus. Biomed Res Int. 2015: 471468.
15. Kohno K, Koya-Miyata S, Harashima A, Tsukuda T, Katakami M, et al. (2021) Inflammatory M1-like macrophages polarized by NK-4 undergo enhanced phenotypic switching to an anti-inflammatory M2-like phenotype upon co-culture with apoptotic cells. J Inflamm. 18(1): 2.
16. Ti D, Hao H, Tong C, Liu J, Dong L, et al. (2015) LPS-preconditioned mesenchymal stromal cells modify macrophage polarization for resolution of chronic inflammation via exosome-shuttled let-7b. J Transl Med. 13: 308.

17. Rad F, Pourfathollah AA, Yari F, Mohammadi S, Kheirandish M (2016) Microvesicles preparation from mesenchymal stem cells. Med J Islam Repub Iran. 30: 398.
18. Robert S, Lacroix R, Poncelet P, Harhouri K, Bouriche T, et al. (2012) High-sensitivity flow cytometry provides access to standardized measurement of small-size microparticles--brief report. Arterioscler Thromb Vasc Biol. 32(4): 1054-8.
19. Jäger S, Bucci C, Tanida I, Ueno T, Kominami E, et al. (2004) Role for Rab7 in maturation of late autophagic vacuoles. J Cell Sci. 117(20): 4837- 4848.
20. Ostrowski M, Carmo NB, Krumeich S, Fanget I, Raposo G, et al. (2010) Rab27a and Rab27b control different steps of the exosome secretion pathway. Nat Cell Biol. 12(1): 1-13.
21. Hyttinen JMT, Niittykoski M, Salminen A, Kaarniranta K (2013) Maturation of autophagosomes and endosomes: A key role for Rab7. Biocheim Biophys Acta. 1833(3): 503-510.
22. Park DJ, Yun WS, Kim WC, Park J-E, Lee SH, et al. (2020) Improvement of stem cell-derived exosome release efciency by surface-modifed nanoparticles. J Nanobiotechnol. 18(1): 178-194.
23. Bobrie A, Krumeich S, Reyal F, Recchi C, Moita LF, et al. (2012) Rab27a supports exosome-dependent and -independent mechanisms that modify the tumor microenvironment and can promote tumor progression. Cancer Res. 72(19): 4920-4930.
24. Hu G-W, Li Q, Niu X, Hu B, Liu J, et al. (2015) Exosomes secreted by human-induced pluripotent stem cell-derived mesenchymal stem cells attenuate limb ischemia by promoting angiogenesis in mice. Stem Cell Res Ther. 6(1): 10.
25. Shu S, Allen CL, Benjamin-Davalos S, Koroleva M, MacFarland D, et al. (2021) A Rapid Exosome Isolation Using Ultrafiltration and Size Exclusion Chromatography (REIUS) Method for Exosome Isolation from Melanoma Cell Lines. Methods Mol Biol. 2265: 289-304.
26. Kishi H, Nakagawa K, Matsumoto M, Suga M, Ando M, et al. (2001) Osmotic shock induces G1 arrest through p53 phosphorylation at Ser33 by activated p38MAPK without phosphorylation at Ser15 and Ser20. H. J. Biol. Chem. 276(42): 39115-22.
27. Rawat P, Hon S, Teodorof-Diedrich C, Spector SA (2020) Trehalose Inhibits Human Immunodeficiency Virus Type 1 Infection in Primary Human Macrophages and $CD4^+$ T Lymphocytes through Two Distinct Mechanisms. J Virol. 94(17): e00237-20.
28. Clark AE, Sabalza M, Gordts PLSM, Spectora DH (2018) Human Cytomegalovirus Replication Is Inhibited by the Autophagy-Inducing Compounds Trehalose and SMER28 through Distinctively Different Mechanisms. J Virol. 92(6): e02015-17.
29. Cacela C, Hincha DK (2006) Low amounts of sucrose are sufficient to depress the phase transition temperature of dry phosphatidylcholine, but not for lyoprotection of liposomes. Biophys J. 90(8): 2831-2842.
30. Strauss G, Schurtenberger P, Hauser H (1986) The interaction of saccharides with lipid bilayer vesicles: stabilization during freeze-thawing and freeze-drying. Biochim Biophys Acta. 858(1): 169-80.
31. Kilburn D, Townrow S, Meunier V, Richardson R, Alam A, et al. (2006) Organization and mobility of water in amorphous and crystalline trehalose. Nat Mater. 5(8): 632-5.