Supplementary Materials

Materials

1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and sphingomyelin (SM) were purchased from Avanti Polar Lipids (Alabaster, AK, USA). 1,2-dimyristoyl-sn-glycerol-methoxy polyethylene glycol 2000 (DMG-PEG 2000) was obtained from the NOF Corporation (Tokyo, Japan), and stearylated octaarginine (STR-R8) was obtained from Toray Industries, Inc. (Tokyo, Japan). Coenzyme Q_{10} (CoQ_{10}) and Antimycin A were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine Perchlorate (DiI), MitoTracker Deep Red FM, and 1% penicillin streptomycin were purchased from Thermo Fisher Scientific Life Sciences (Waltham, MA, USA). mtSOX Deep Red was obtained from Dojindo Laboratories (Kumamoto, Japan). All other chemicals used were commercially available reagent-grade products.

Preparation of MITO-Porter encapsulated with CoQ_{10}

An ethanol (EtOH) suspension containing 4.2 mM lipids (DOPE:SM:DMG-PEG2000:STR-R8) [9:2:0.33:1.1, molar ratio] and EtOH suspensions containing 0.7 mM CoQ_{10} were used to prepare MP-(CoQ_{10}). For fluorescence labeling, DiI was added to the EtOH suspension at a concentration of either 0.1 mol% or 0.5 mol% relative to the total lipid, depending on the experiment. MITO-Porter was prepared by mixing lipids and CoQ_{10} in EtOH, and phosphate-buffered saline (PBS) using a microfluidic device system at a total flow rate of 500 μL/min. The lipid phase (20% EtOH concentration) was introduced at a flow rate of 100 μL/min, while PBS was supplied at 400 μL/min. The flow rate was controlled using syringe pumps (Harvard Apparatus, Hollison, MA, USA). The obtained suspension was subsequently dialyzed against PBS for 2 hours using Spectra/Por 4 dialysis membranes (molecular weight cutoff 12–14 kDa; Spectrum Laboratories, Rancho Dominguez, CA, USA).

The average particle size and polydispersity index (PDI) were measured using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK), with particle diameter values presented as volume distribution.

The estimated lipid amount was determined by measuring the fluorescence intensities of DiI-labeled liposomes using a Varioskan LUX multimode microplate reader (Thermo Fisher Scientific Life Sciences, Waltham, MA, USA), with excitation at 549 nm and emission at 565 nm. To qualify the amount of CoQ_{10} encapsulated in MITO-Porter, the CoQ_{10} content was measured using high-performance liquid chromatography (HPLC) with an Arc™ HPLC system, as described in our previous report [1]. The recovery rate was calculated using the following formula: lipid or CoQ_{10} amount of sample/initial lipid or CoQ_{10} amount × 100. To determine the drug-to-lipid content ratio, the drug-to-lipid ratio (w/w) was calculated as follows: final amount of CoQ_{10} (μg)/final amount of total lipid (μg).

Cellular internalization analysis by flow cytometry

Cellular uptake of MP-(CoQ_{10}) was assessed by measuring the fluorescence intensity of DiI-labeled MP-(CoQ_{10}) internalized by AMSCs using flow cytometry.

AMSCs (1.0×10^5 cells/well) were seeded in 6-well plates (Corning, Corning, NY, USA) and incubated with AMSC culture medium at 37°C in a 5% CO_2 atmosphere for 24 hours. The cells were treated with MP-(CoQ_{10}) labeled with 0.5 mol% DiI at a final lipid concentration of 20 μM in the wells, or an equal volume of PBS (non-treatment group), and incubated for 1, 3, 6, 12, or 24 hours at 37°C in a 5% CO_2 atmosphere. After washing with 1 mL of PBS and twice with 500 μL of PBS containing heparin (20 U/mL), the cells were trypsinized and resuspended in AMSC culture medium. After centrifugation (700 × g, 4°C, 4 min), the supernatant was removed, and the pellet was resuspended in PBS containing 0.5% bovine serum albumin and 0.1% sodium azide. The cell suspension was filtered through a nylon mesh and then analyzed using flow

cytometry with a CytoFLEX (Beckman Coulter, Pasadena, CA, USA). The results were analyzed using Kaluza software (Beckman Coulter).

A standard gating strategy was applied. Cells were first gated on a forward scatter area (FSC-A) versus side scatter area (SSC-A) plot to exclude debris and select the main cell population. DiI fluorescence was measured in the PE channel (PE-A). A total of 10,000 events were collected per sample, and cellular uptake was quantified as mean fluorescence intensity (MFI).

DiI was excited with 561 nm light, and fluorescence was detected using a 585/42 nm bandpass filter (BP). Cellular uptake was quantified as the mean fluorescence intensity.

Intracellular observation by confocal laser scanning microscopy (CLSM)

Confocal laser scanning microscopy was employed to observe the intracellular localization of DiI-labeled MITO-Porter (shown in green pseudocolor) and mitochondria labeled with MitoTracker Deep Red (shown in red pseudocolor) in AMSCs.

AMSCs (2.5×10^4 cells/well) were seeded in 35 mm glass-bottom dishes (Iwaki, Tokyo, Japan) and incubated with AMSC culture medium at 37°C in a 5% CO_2 atmosphere for 24 hours. The cells were treated with MP-(CoQ_{10}) labeled with 0.1 mol% DiI, at a final lipid concentration of 20 μM in the wells, or with an equal volume of PBS (non-treatment group), and incubated for 24 hours at 37°C in a 5% CO_2 atmosphere. The mitochondria were stained with MitoTracker Deep Red (final concentration, 60 nM) for 30 min. The cells were then washed twice with AMSC culture medium, after which the medium was replaced with fresh AMSC culture medium. Intracellular trafficking was observed using CLSM with an A1 R series (Nikon, Tokyo, Japan) equipped with an ECLIPSE Ti microscope, a water-immersion objective lens (Plan Apo VC 60x/1.20 PFS WI), and a dichroic mirror (DM405/488/561/640). The cells were excited with light at 561 nm to detect DiI-labeled MP-(CoQ_{10}) and at 638 nm to detect mitochondria stained with MitoTracker Deep Red. The two fluorescence detection channels (Ch) were configured with the following filters:

Ch1: 595/50 BP (green) for DiI, and Ch2: 700/75 BP (red) for MitoTracker Deep Red. The data were analyzed using NIS-Elements software (Nikon, Tokyo, Japan).

Evaluation of the mitochondrial respiratory capacity of AMSCs by extracellular flux analysis

AMSCs ($5.0 \times 10^3 - 8.0 \times 10^3$ cells/well) were seeded in 8-well plates (Agilent Technologies) and incubated with AMSC culture medium for 24 hours. The initial seeding density was adjusted within this range according to differences in proliferation rates, which varied among different lots of the culture medium, to achieve comparable cell confluency at the time of analysis. The cells were then treated with MP-(CoQ_{10}) at a final lipid concentration of 20 μM (with an estimated final CoQ_{10} concentration of 3 ± 0.1 μM), an ethanol suspension containing CoQ_{10} (final CoQ_{10} concentration 3 μM) (naked CoQ_{10} group), or PBS (non-treatment group), and incubated for 24 hours at 37°C in a 5% CO_2 atmosphere. Prior to analysis, the cells were incubated with phenol red-free DMEM containing glucose, pyruvate, and glutamine (final concentrations of 5.5, 1.25, and 4.0 mM, respectively) at 37°C in a CO_2-free environment for 1 hour. Oxygen consumption rate (OCR) was measured using a Seahorse XFp Analyzer (Agilent Technologies, Santa Clara, CA, USA), an extracellular flux analyzer. The measurements were taken following the sequential addition of oligomycin, carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), and rotenone with antimycin A, at a final concentration of 2.0, 1.5, and 0.5 μM, respectively. To prevent pyruvate depletion, pyruvate was added at the time of FCCP addition, with a final concentration of 15.0 mM. Basal respiration, maximal respiration, and ATP-linked respiration were calculated from the OCR data.

Following OCR measurement, the cells in each well were collected after trypsinization and counted using a CellDrop automated cell counter (DeNovix, Wilmington, DE, USA). The obtained OCR was normalized to the cell number and expressed as pmol/min/10^4 cells. Relative values of basal respiration, maximal respiration, and ATP-linked respiration were calculated with respect to those of the non-treatment group.

The characteristics of the MP-(CoQ_{10}) formulation were as follows: the lipid concentration was 446 ± 18 μM, and the CoQ_{10} concentration was 74 ± 2 μM. For all cell treatment experiments, the MP-(CoQ_{10}) formulation was diluted to achieve a final lipid concentration of 20 μM. At this dilution, the corresponding final CoQ_{10} concentration was approximately 3 μM. Therefore, naked CoQ_{10} was used at the same final concentration (3 μM) for comparison. Because the lipid-to-CoQ_{10} ratio varied slightly among independently prepared batches of MP-(CoQ_{10}), the exact final CoQ_{10} concentration also varied slightly. For the batch used in the mitochondrial respiration experiments, the final CoQ_{10} concentration at a lipid concentration of 20 μM was 3 ± 0.1 μM.

Evaluation of the energy metabolism of AMSCs by extracellular flux analysis

AMSCs (8.0×10^3 cells/well) were seeded in 8-well plates (Agilent Technologies) and incubated with AMSC culture medium for 48 hours. Prior to the analysis, the cells were incubated with phenol red-free DMEM containing glucose, pyruvate, and glutamine (final concentrations, 5.5, 1.25, and 4.0 mM, respectively) at 37°C, CO_2-free for 1 hour. ATP production rate (OCR) was measured using Seahorse XFp Analyzer (Agilent Technologies, Santa Clara, CA, USA), an extracellular flux analyzer, with the sequential addition of oligomycin, and rotenone with antimycin A (final concentrations, 2.0 and 0.5 μM, respectively) (Figure S1).

Evaluation of antioxidant capacity

AMSCs (5.0×10^3 cells/well) were seeded in 96-well black/clear-bottom plates (Corning, Corning, NY, USA), and incubated with AMSC culture medium for 24 hours. The cells were then treated with PBS (non-treatment group) or MP-(CoQ_{10}) to constitute 4.5% of the total volume (the estimated final lipid concentration of MP-(CoQ_{10}) was approximately 20 μM), and incubated for 24 hours at 37°C, 5% CO_2. After washing the cells with 100 μL of PBS, the cells were incubated with AMSC culture medium including mtSOX Deep Red reagent (Dojindo Laboratories,

Kumamoto, Japan) for 30 min at 37°C, 5% CO_2. mtSOX Deep Red reagent was used to detect mitochondrial superoxide. The estimated amount of mitochondrial superoxide was measured by determining the fluorescence intensities of the wells using a Varioskan LUX multimode microplate reader (Thermo Fisher Scientific Life Sciences, Waltham, MA, USA) (excitation at 540 nm and emission at 670 nm). Antimycin A was added to a final concentration of 10 μM to promote mitochondrial superoxide production. The relative fluorescence units (RFU) of mtSOX Deep Red were calculated relative to the RFU of the non-treatment group.

For the mitochondrial superoxide assay, DiI-free MP-(CoQ_{10}) was used because the fluorescence spectra of DiI overlap with those of mtSOX Deep Red, which could interfere with fluorescence measurements. The lipid concentration of the MP-(CoQ_{10}) formulation was 446 ± 18 μM. Therefore, 4.5% (v/v) of the DiI-free MP-(CoQ_{10}) formulation was added, corresponding to an estimated final lipid concentration of approximately 20 μM, which matched the lipid concentration used in the other experiments.

Cytotoxicity evaluation by WST-1 assay

AMSCs (1.0×10^4 cells/well) were seeded in 48-well plates (Corning, Corning, NY, USA) and cultured in AMSC culture medium at 37°C in a 5% CO_2 atmosphere for 24 hours. The cells were then treated with PBS (non-treatment group, 0 μM) or MP-(CoQ_{10}) at a final lipid concentration of 10, 20, 40, or 80 μM at 37°C in a 5% CO_2 atmosphere for 24 hours. After treatment, the cells were washed with 250 μL of PBS and incubated with AMSC culture medium containing Cell Proliferation Reagent WST-1 (Takara Bio Inc., Shiga, Japan) for 2 hours at 37°C in a 5% CO_2 atmosphere. Absorbance was measured using a Varioskan LUX multimode microplate reader (Thermo Fisher Scientific Life Sciences, Waltham, MA, USA) according to the manufacturer's instructions. Cell viability was calculated as follows: Cell viability (%) = $V_S/V_U \times 100$, where V_S and V_U represent the absorbance values of the treated samples and untreated cells, respectively.

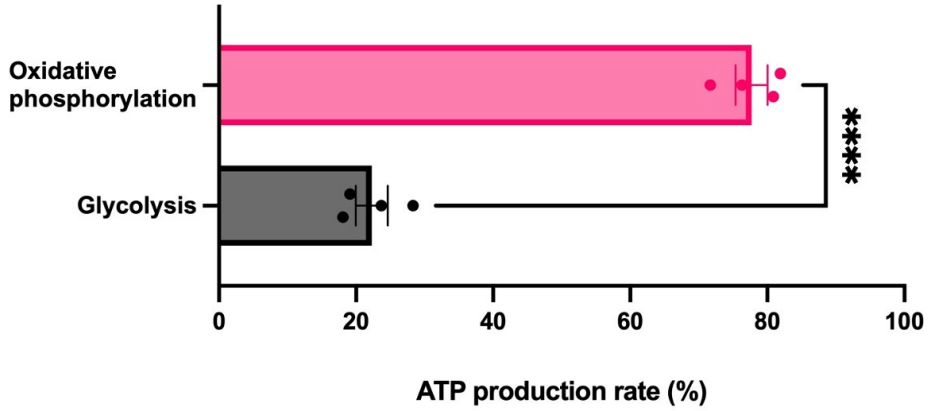

Figure S1. Evaluation of energy metabolism in amnion-derived mesenchymal stromal cells (AMSCs) using an extracellular flux analyzer.

ATP production rates of glycolysis and oxidative phosphorylation were evaluated using a Seahorse XFp Analyzer (Agilent Technologies) according to the manufacturer's instructions. The ATP production rate was significantly elevated during oxidative phosphorylation, but glycolysis accounted for 22% of the energy metabolism in AMSCs. Data are expressed as mean ± SEM (n = 4). Significant difference (****$p<0.0001$) calculated by unpaired t-test.

References

1) Hibino M, Yamada Y, Fujishita N, Sato Y, Maeki M, Tokeshi M, Harashima H: The Use of a Microfluidic Device to Encapsulate a Poorly Water-Soluble Drug CoQ(10) in Lipid Nanoparticles and an Attempt to Regulate Intracellular Trafficking to Reach Mitochondria. *J Pharm Sci,* **108**, 2668-2676 (2019).