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Report

Generation of Mitochondrial-Activated Amnion-Derived Mesenchymal Stromal Cells via a Mitochondrial Coenzyme Q_{10} Delivery Strategy

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Mesenchymal stromal cell transplantation therapy has been widely studied in regenerative medicine for the treatment of various diseases. We previously developed a mitochondria-targeted drug delivery system, termed the MITO-Porter, which enables large-scale production of coenzyme Q_{10} (CoQ_{10})-encapsulated carriers. This study aimed to generate mitochondrial-activated amnion-derived mesenchymal stromal cells (AMSCs) by delivering CoQ_{10} to mitochondria using the MITO-Porter system, with the potential to improve AMSC transplantation outcomes. We treated AMSCs with CoQ_{10}-encapsulated MITO-Porter and evaluated cellular uptake, co-localization with mitochondria, changes in mitochondrial respiratory capacity, and antioxidant capacity. CoQ_{10}-encapsulated MITO-Porter was internalized by AMSCs and co-localized with mitochondria. Treatment of AMSCs with CoQ_{10}-encapsulated MITO-Porter significantly increased the mitochondrial oxygen consumption rate and decreased superoxide levels. Mitochondrial delivery of CoQ_{10} successfully enhanced the mitochondrial respiratory capacity and antioxidant ability of AMSCs. These mitochondrial-activated AMSCs, newly generated using the MITO-Porter system, represent a promising cell source for improving MSC-based transplantation therapy.

Key words amnion-derived mesenchymal stromal cells, coenzyme Q_{10}, mitochondrial delivery, drug delivery system, lipid nanoparticles, microfluidics

INTRODUCTION

Mesenchymal stromal cells (MSCs) are multipotent cells with the ability to differentiate into various cell lineages, including bone, cartilage, fat tissue, tendons, and muscles.[1-3] They can be isolated from diverse tissues such as bone marrow, adipose tissue, and amnion.[1, 4, 5] MSCs also possess anti-inflammatory properties through their interaction with immune cells mediated by cytokine release.[6, 7] MSC-based transplantation therapy has been widely studied in regenerative medicine for the treatment of various diseases.[8, 9] Beyond their differentiation and immunomodulatory properties, emerging evidence suggests that mitochondrial function critically influences the therapeutic efficacy of MSCs through mechanisms such as intercellular mitochondrial transfer and enhanced mitochondrial quality control in injured tissues (e.g., enhancing ATP production, regulating oxidative stress, and improving cellular bioenergetics).[10]

We previously developed a drug delivery system targeting mitochondria, named MITO-Porter, which facilitates the transport of various molecules to the mitochondria through membrane fusion.[11-14] Furthermore, we successfully established a method for the large-scale production of MITO-Porter encapsulating Coenzyme Q_{10} (CoQ_{10}), a key component of the mitochondrial electron transport chain that also possesses antioxidant properties, utilizing a microfluidic device, herein referred to as MP-(CoQ_{10}).[15, 16] This development enabled the sufficient supply of MITO-Porter as a drug for potential clinical applications. By treating cardiosphere-derived cells (CDCs) with MITO-Porter encapsulating mitochondrial functional molecules, including resveratrol or CoQ_{10}, we successfully generated mitochondrial-activated human CDCs, hereafter referred to as MITO cells, and we demonstrated the preventative and therapeutic efficacy of MITO cell transplantation in a myocardial ischemia-reperfusion model using mice and rats, through both intramyocardial injection and intravenous administration.[17-19]

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Furthermore, in our previous study, we successfully activated the mitochondrial function of human bone marrow-derived mesenchymal stromal cells by using MP-(CoQ_{10}), which has the potential to enhance MSC transplantation therapy.[20]

Recently, the amnion, which is conventionally discarded as medical waste after delivery, has been recognized as a valuable source of MSCs.[21, 22] In animal studies, the transplantation of amnion-derived mesenchymal stromal cells (AMSCs) has been reported to be effective in various diseases, including graft-versus-host disease,[23] myocarditis,[24] glomerulonephritis,[25] pancreatitis,[22] Duchenne muscular dystrophy,[26] and spinal cord injury.[27] Furthermore, clinical trials evaluating the efficacy of AMSC transplantation for Duchenne muscular dystrophy and acute spinal cord injury are currently in progress.

The current study aimed to develop mitochondrial-activated AMSCs by utilizing the MITO-Porter system in AMSCs, which is under development for clinical applications, with the potential to improve the overall outcomes of AMSC transplantation. We treated AMSCs with MP-(CoQ_{10}) and assessed cellular uptake, mitochondrial delivery, changes in mitochondrial respiratory capacity, and antioxidant capacity.

MATERIALS AND METHODS

Preparation of Human AMSCs Human fetal membranes were collected under sterile conditions from women undergoing cesarean section after obtaining informed consent. Isolation and expansion of human AMSCs were performed as previously described.[26, 28, 29] After culture, the cells were harvested and cryopreserved in liquid nitrogen until use. Human AMSCs were obtained at passage 2 and cultured in AMSC medium provided by Kaneka Corporation. The medium was replaced two or three times per week, and AMSCs from passages 5 to 8 were used in the experiments. AMSCs were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Preparation of MITO-Porter Encapsulated with CoQ_{10} We prepared MP-(CoQ_{10}) using a microfluidic device system, as previously reported.[15] The particle properties of MP-(CoQ_{10}) were evaluated using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, U.K.). The lipid content of the MP-(CoQ_{10}) was estimated by measuring the fluorescence intensities of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI)-labeled liposomes with a Varioskan LUX multimode microplate reader (Thermo Fisher Scientific Life Sciences, Waltham, MA, USA). The CoQ_{10} content in the MP-(CoQ_{10}) was assessed by high-performance liquid chromatography (HPLC) using an Arc™ HPLC system (Waters Corporation, Milford, MA, USA), as previously reported.[15]

Intracellular Dynamics of MP-(CoQ_{10}) in AMSCs Cellular uptake of MP-(CoQ_{10}) was evaluated by measuring the fluorescence intensity of DiI-labeled MP-(CoQ_{10}) internalized by AMSCs using flow cytometry. AMSCs were treated with MP-(CoQ_{10}) for 1, 3, 6, 12, or 24 h, and the extent of cellular uptake was evaluated at each time point.

Confocal laser scanning microscopy (CLSM) was employed to analyze the intracellular localization of DiI-labeled MP-(CoQ_{10}) (shown in green pseudocolor) and mitochondria labeled with MitoTracker Deep Red (shown in red pseudocolor) in AMSCs. AMSCs were treated with MP-(CoQ_{10}) for 24 h, and its intracellular localization was observed.

Evaluation of Mitochondrial Respiration and Antioxidant Capacity of AMSCs upon MP-(CoQ_{10}) Treatment The mitochondrial oxygen consumption rate (OCR) was evaluated using the Seahorse XFp Analyzer (Agilent Technologies, Santa Clara, CA, USA), an extracellular flux analyzer. Basal respiration, maximal respiration, and ATP-linked respiration were calculated through the sequential administration of oligomycin, a Complex V inhibitor; carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), a mitochondrial oxidative phosphorylation uncoupler that enhances mitochondrial respiratory capacity; and a combination of rotenone, a Complex I inhibitor, with antimycin A, a Complex III inhibitor.

The antioxidant capacity was assessed using the mtSOX Deep Red reagent (Dojindo Laboratories, Kumamoto, Japan), which detects mitochondrial superoxide. Fluorescence intensity of mtSOX Deep Red was measured using a Varioskan LUX multimode microplate reader (Thermo Fisher Scientific Life Sciences, Waltham, MA, USA). Antimycin A was administered at a final concentration of 10 μM to induce mitochondrial superoxide production.

Cytotoxicity Evaluation of MP-(CoQ_{10}) by WST-1 Assay The cytotoxicity of MP-(CoQ_{10}) in AMSCs was evaluated by measuring cell viability using Cell Proliferation Reagent WST-1 (Takara Bio Inc., Shiga, Japan) and Varioskan LUX multimode microplate reader (Thermo Fisher Scientific Life Sciences, Waltham, MA, USA). 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.

Additional methodological details and materials are summarized in the Supplementary Materials.

Statistical Analysis Data are presented as the mean ± standard error of the mean (SEM) for the specified number of experiments. The number of independent experiments (n) is indicated in each figure legend. Differences between two groups were analyzed using Student's *t*-test, while differences among multiple groups were analyzed using one-way ANOVA followed by Student-Newman-Keuls (SNK) test or Dunnett's multiple comparisons test, as appropriate. The level of significance was set at $p < 0.05$. Statistical analyses were performed in Prism 10 software (GraphPad Software Inc., Boston, MA, USA).

RESULTS

Preparation of MITO-Porter Encapsulated with CoQ_{10} To enhance the mitochondrial respiratory capacity of AMSCs, we formulated the MITO-Porter encapsulating CoQ_{10} using a microfluidic device system. The average particle size, polydispersity index (PDI), which reflects the distribution of particle sizes, and ζ-potential of MP-(CoQ_{10}) were 47 ± 2.9 nm, 0.193 ± 0.009, and 19 ± 1.5 mV, respectively, as presented in Table 1. The MP-(CoQ_{10}) exhibited a positive surface charge as a result of surface modification with Stearylated octaarginine (STR-R8). The nanoscale size and positive surface charge of MP-(CoQ_{10}) were considered favorable for cellular uptake by AMSCs.

The recovery rates of lipid and CoQ_{10} in MP-(CoQ_{10}) were 79 ± 2.8% and 72 ± 1.4%, respectively, as shown in Table 1. The drug-to-lipid ratio (w/w) of MP-(CoQ_{10}) was 0.17 ± 0.006. These values were consistent with those reported in our previous studies [15, 20].

Cellular Uptake of MP-(CoQ_{10}) Evaluated by Flow Cytometry The uptake of MP-(CoQ_{10}) by AMSCs was eval-

Table 1. Particle Properties of Coenzyme Q_{10}-Encapsulated MITO-Porter [MP-(CoQ_{10})]

	Initial amount of CoQ_{10} for preparation (mM)	Size (nm)	PDI	ζ-potential (mV)	Lipid recovery rate (%)	CoQ_{10} recovery rate (%)	Drug:lipid ratio (w:w)
MP-(CoQ_{10})	0.7	47 ± 2.9	0.193 ± 0.009	19 ± 1.5	79 ± 2.8	72 ± 1.4	0.17 ± 0.006

MITO-Porter encapsulating Coenzyme Q_{10} [MP-(CoQ_{10})] was constructed using ethanol suspension containing 4.2 mM lipids (DOPE:SM:DMG-PEG2000:STR-R8 [9:2:0.33:1.1, molar ratio]) and ethanol suspension containing 0.7 mM CoQ_{10}. Average particle size, polydispersity index (PDI), ζ-potential, lipid and CoQ_{10} recovery rates, and drug: lipid ratio (w:w) represent the physicochemical properties of MP-(CoQ_{10}). Data are expressed as mean ± SEM (n = 6). DOPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; SM, sphingomyelin; DMG-PEG2000, 1,2-dimyristoyl-sn-glycerol-methoxy polyethylene glycol 2000; STR-R8, stearylated octaarginine.

uated using flow cytometry. The fluorescence intensity, reflecting the uptake of MP-(CoQ_{10}) by AMSCs, was measured after exposing the cells to MP-(CoQ_{10}) labeled with the fluorescent lipid marker DiI for 1, 3, 6, 12, or 24 h, at a final lipid concentration of 20 μM (Fig. 1).

The intracellular uptake of MP-(CoQ_{10}) increased over time (Fig. 1a). A significant increase in the uptake of MP-(CoQ_{10}) was observed at 6 h or longer compared to the non-treatment group (Fig. 1b). These results suggest that MP-(CoQ_{10}) was internalized by AMSCs, with its uptake influenced by the duration of MP-(CoQ_{10}) exposure.

Intracellular Observation of MP-(CoQ_{10}) Evaluated by CLSM The intracellular localization and dynamics of DiI-labeled MP-(CoQ_{10}) were evaluated by detecting fluorescence shown in green pseudocolor using CLSM. AMSCs, with the mitochondria labeled by fluorescence shown in red pseudocolor using MitoTracker Deep Red, were treated with MP-(CoQ_{10}) for 24 h at a final lipid concentration of 20 μM (Fig. 2).

The cellular uptake of MP-(CoQ_{10}) was observed through the appearance of green dots in AMSCs, demonstrating that MP-(CoQ_{10}) was internalized by the cells. Additionally, co-localization between red-fluorescent mitochondria and green-fluorescent MP-(CoQ_{10}) was observed, as evidenced by the presence of yellow signals.

Evaluation of Mitochondrial Respiration of AMSCs Following MP-(CoQ_{10}) treatment The mitochondrial respiratory capacity of AMSCs was evaluated through extracellular flux analysis by measuring the mitochondrial OCR. The OCR was measured in the non-treatment group, AMSCs treated with ethanol suspension containing CoQ_{10} (naked CoQ_{10} group) for 24 h at a final CoQ_{10} concentration of 3 μM, and AMSCs treated with MP-(CoQ_{10}) for 24 h at a final lipid concentration of 20 μM, with a final CoQ_{10} concentration of 3 ± 0.1 μM (Fig. 3a).

The treatment with naked CoQ_{10} did not result in any significant changes in either basal or maximal respiration (Fig. 3b, 3c). In contrast, the treatment with MP-(CoQ_{10}) led to a significant increase in both basal and maximal respiration (Fig. 3b, 3c). Furthermore, ATP-linked respiration was significantly increased in the MP-(CoQ_{10}) group compared with both the non-treatment and naked CoQ_{10} groups (Fig. 3d). These results indicated that MP-(CoQ_{10}) increased mitochondrial respiratory parameters, including ATP-linked respiration, in AMSCs.

Evaluation of Antioxidant Capacity of AMSCs Following MP-(CoQ_{10}) Treatment The antioxidant capacity was assessed by detecting the levels of mitochondrial superoxide using the mtSOX Deep Red reagent (Dojindo Laboratories, Kumamoto, Japan). Fluorescence intensity of mtSOX Deep Red was measured in the non-treatment group and in AMSCs treated with MP-(CoQ_{10}) (final lipid concentration of approximately 20 μM), for 24 h (Fig. 4). Mitochondrial superoxide production was induced by administering Antimycin A at a final concentration of 10 μM.

Under conditions without Antimycin A, the treatment with MP-(CoQ_{10}) showed a tendency for decreased fluorescence intensity compared to the non-treatment group, although the difference was not statistically significant. Under conditions with Antimycin A, an increase in fluorescence intensity was observed in both the non-treatment group and MP-(CoQ_{10})-treated groups. However, the increase in fluorescence intensity was smaller in the MP-(CoQ_{10})-treated group compared to the non-treatment group, resulting in a significant decrease in fluorescence intensity in the MP-(CoQ_{10}) group relative to the non-treatment group. These results indicated that treatment with MP-(CoQ_{10}) suppressed mitochondrial superoxide generation in AMSCs, particularly under stressed conditions.

Evaluation of Cytotoxicity of MP-(CoQ_{10}) in AMSCs by WST-1 Assay The cytotoxicity of MP-(CoQ_{10}) in AMSCs was evaluated by measuring cell viability employing WST-1 reagent. The viability of AMSCs treated with MP-(CoQ_{10}) for 24 h was compared with that of the non-treatment group.

Treatment with MP-(CoQ_{10}) at final lipid concentration of 10 and 20 μM did not significantly affect cell viability compared with the non-treatment group. In contrast, cell viability was significantly decreased at final lipid concentration of 40 μM or higher (Fig. 5). These results suggest that the final lipid concentration used in the present study (20 μM) did not exert detectable cytotoxic effects on AMSCs.

DISCUSSION

In this study, MSCs isolated from the amnion were treated with CoQ_{10}-encapsulated MITO-Porter, which increased their OCR. Furthermore, the treatment with MP-(CoQ_{10}) led to a reduction in mitochondrial superoxide levels. These results suggest that treating AMSCs with MP-(CoQ_{10}) enhanced mitochondrial function and antioxidant capacity.

We previously reported that the treatment of CDCs with MP-(CoQ_{10}) resulted in an increase in OCR, with the maximal respiration being approximately two-fold higher compared to the non-treatment group.[19] In contrast, in the current study, while treatment with MP-(CoQ_{10}) resulted in a significant increase in the maximal respiration, the increase was approximately 1.2-fold higher compared to the non-treatment group. Similarly, in rapidly expanding clones (RECs) of human bone marrow-derived MSCs, treatment with CoQ_{10}-encapsulated MITO-Porter resulted in a significant increase in maximal respiration; however, the extent of the increase

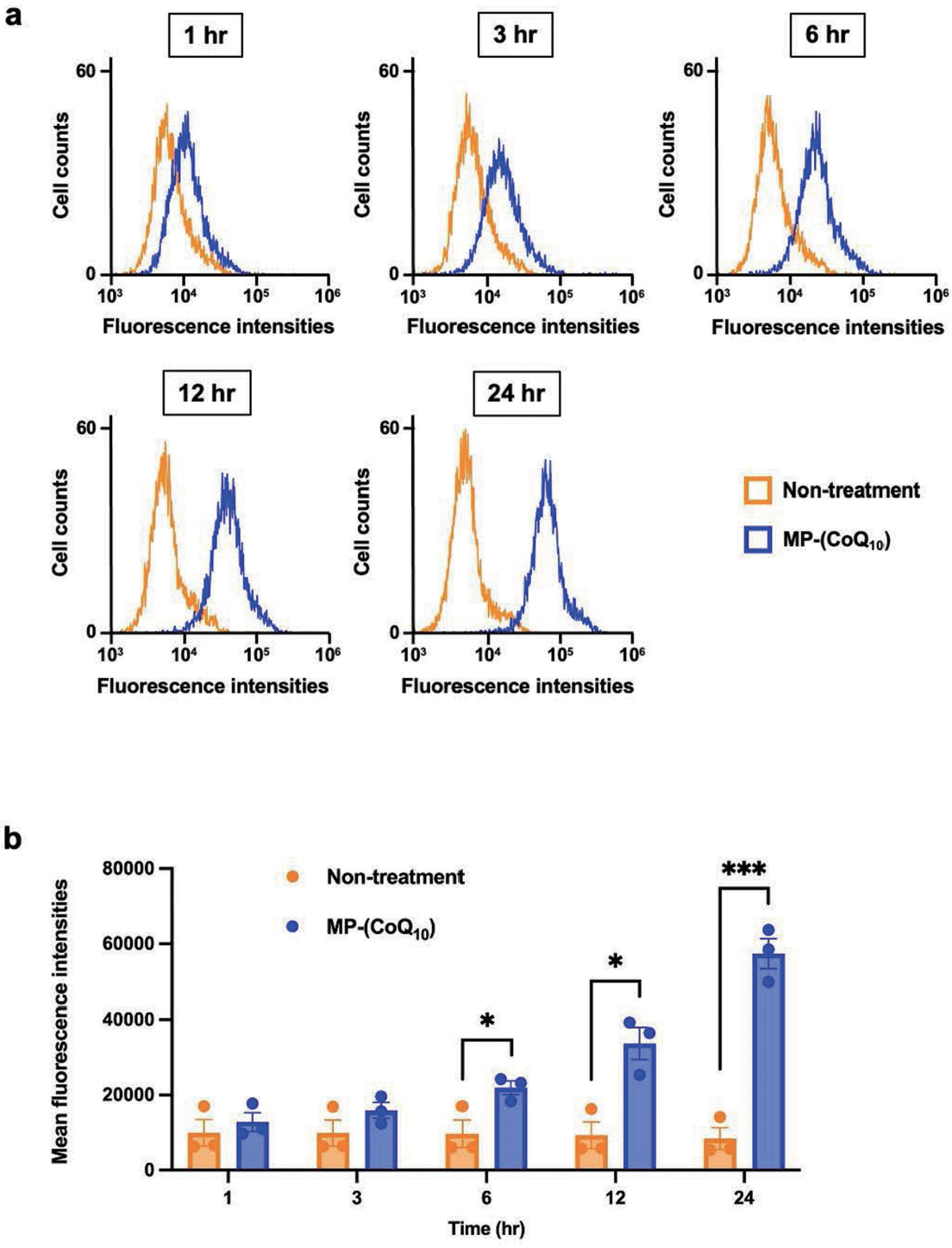

Fig. 1. Cellular Uptake of MP-(CoQ_{10}) Assessed by Flow Cytometry

(a) Histogram showing fluorescence intensities of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) taken up by AMSCs after treatment with DiI-labeled MP-(CoQ_{10}) at a final lipid concentration of 20 μM for 1, 3, 6, 12, and 24 h. (b) Cellular internalization expressed as mean fluorescence intensities. Data expressed as mean ± SEM (n = 3). Significant differences (*p<0.05, ***p<0.001) were calculated by an unpaired t-test.

was smaller compared to that observed in CDCs.[19, 20] MSCs are known to express high levels of glycolytic enzymes, while the expression of enzymes involved in oxidative phosphorylation in mitochondria is relatively low, suggesting a dependence on glycolysis.[30] In fact, it has been reported that oxidative metabolism accounts for more than 90% of the total energy in cardiomyocytes,[31-33] while ATP production via oxidative phosphorylation accounted for 78% of the total energy production in AMSCs (Fig. S1), and 56% of total energy production in RECs.[20] Therefore, in MSCs, there may have been a limit to the increase in OCR induced by MP-(CoQ_{10}). Importantly, even within the metabolic constraints of MSCs, mitochondria-targeted delivery of CoQ_{10} achieved a relative activation of mitochondrial respiration, highlighting the utility of this approach as a platform for functional tuning rather than metabolic reprogramming.

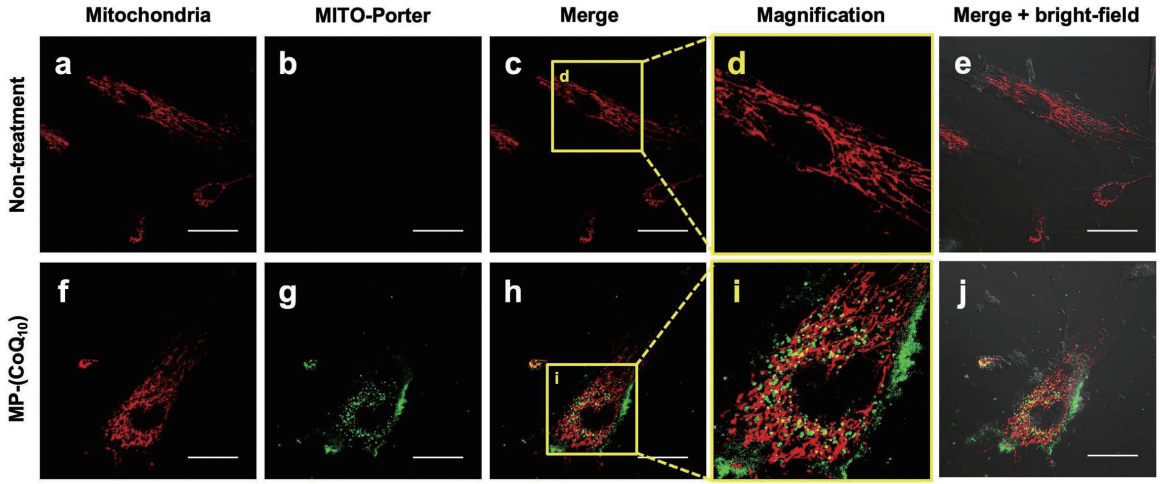

Fig. 2. Intracellular Observation of MP-(CoQ_{10}) by Confocal Laser Scanning Microscopy

Co-localization between mitochondria (red fluorescence) (a, f) and MITO-Porter (green fluorescence) (b, g) was visualized, as indicated by yellow signals in the merged images (c, d, e, h, i, j). Scale bars, 50 μm.

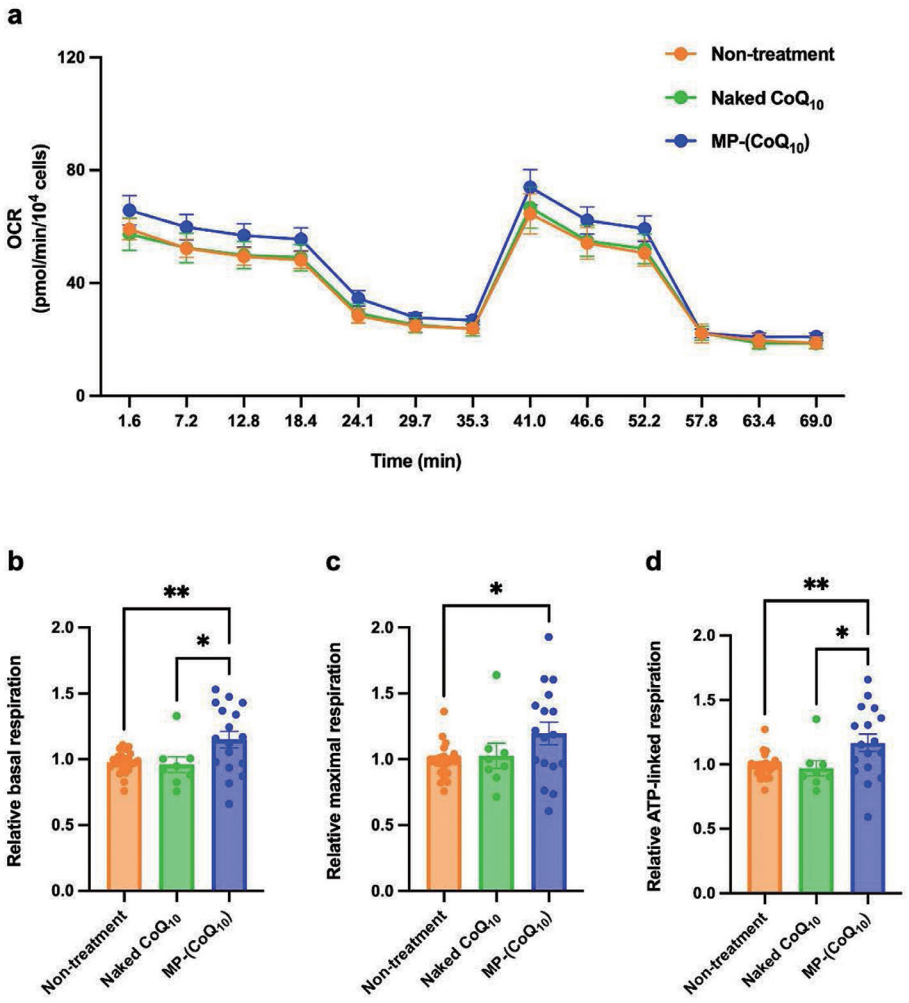

Fig. 3. Evaluation of Mitochondrial Respiratory Function of AMSCs Following Treatment with MP-(CoQ_{10}) by Extracellular Flux Analysis

(a) OCR was evaluated following treatment with phosphate-buffered saline (non-treatment group), ethanol suspension containing CoQ_{10} (naked CoQ_{10} group) for 24 h at a final CoQ_{10} concentration of 3 μM, and AMSCs treated with MP-(CoQ_{10}) for 24 h at a final lipid concentration of 20 μM, with a final CoQ_{10} concentration of 3 ± 0.1 μM. (b) Relative basal respiration, (c) Relative maximal respiration, and (d) Relative ATP-linked respiration were normalized to the values of the non-treatment group. Data are expressed as mean ± SEM (non-treatment, n = 25; naked CoQ_{10}, n = 8; MP-(CoQ_{10}), n = 17). Significant difference (*$p<0.05$, **$p<0.01$) was calculated by one-way ANOVA followed by Student-Newman-Keuls test.

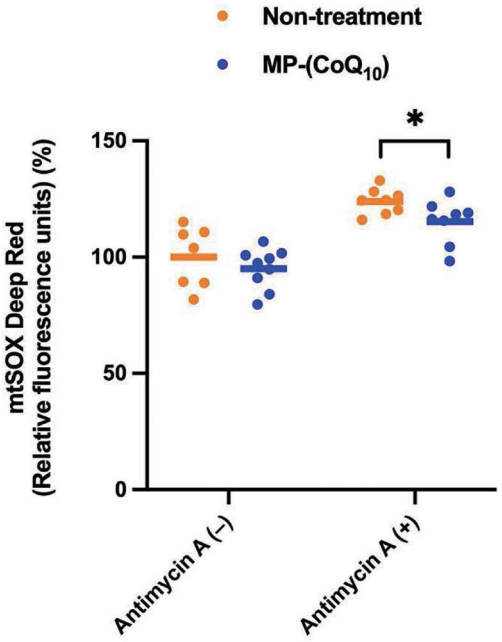

Fig. 4. Evaluation of Antioxidant Capacity of AMSCs Following MP-(CoQ_{10}) Treatment

Antioxidant capacity was evaluated by detecting mitochondrial superoxide levels using the mtSOX Deep Red reagent. Mitochondrial superoxide production was induced by administering Antimycin A at a final concentration of 10 μM. Under conditions with Antimycin A, a significant decrease in fluorescence intensity was observed in the MP-(CoQ_{10}) group compared to the non-treatment group. Each dot represents an individual sample. The horizontal line represents the mean (non-treatment without antimycin A, n = 7; MP-(CoQ_{10}) without antimycin A, n = 8; non-treatment with antimycin A, n = 9; MP-(CoQ_{10}) with antimycin A, n = 8). Significant difference (*p<0.05) was calculated by an unpaired t-test.

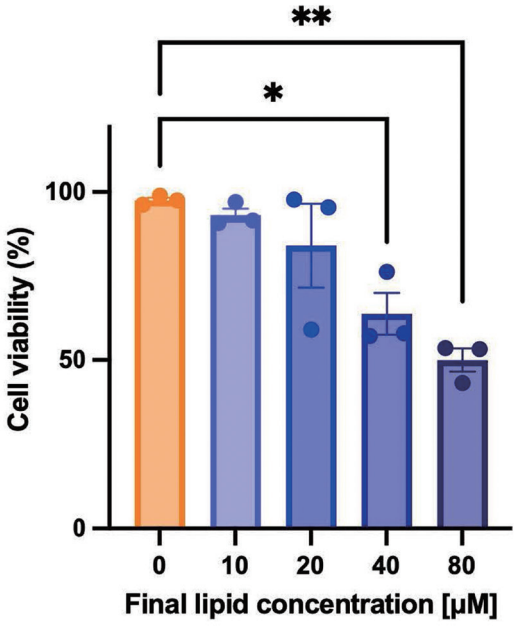

Fig. 5. Evaluation of the Cytotoxicity of MP-(CoQ_{10}) in AMSCs by WST-1 Assay

AMSCs were treated with MP-(CoQ_{10}) for 24 h at final lipid concentrations of 10, 20, 40, or 80 μM. Cell viability was evaluated using a WST-1 assay and expressed relative to the non-treatment group (0 μM). Data are presented as the mean ± SEM (n = 3). Statistical significance was determined by one-way ANOVA followed by Dunnett's multiple comparisons test (*p < 0.05, **p < 0.01 vs. non-treatment group).

CoQ_{10} functions as a key component of the mitochondrial electron transport chain and plays a crucial role in ATP production.[34] Consequently, it is found in higher concentrations in metabolically active tissues, such as heart and skeletal muscles.[34, 35] Furthermore, CoQ_{10} exhibits antioxidant properties, and its reduced form is capable of neutralizing free radicals.[34, 36] Consistent with these roles, treatment with MP-(CoQ_{10}) significantly increased ATP-linked respiration in AMSCs, indicating an enhancement of mitochondrial respiration coupled to ATP synthesis. In fact, in the present study, the delivery of CoQ_{10} to the mitochondria of AMSCs led to an increase in OCR and a reduction in superoxide levels, suggesting that the delivered CoQ_{10} exerted its effect. An increase in OCR does not necessarily lead to higher ROS generation, because mitochondrial ROS largely arises from electron leak under conditions of upstream over-reduction.[37] Mitochondrial delivery of CoQ_{10} may improve electron transfer from complexes I/II to complex III by restoring the CoQ_{10} pool, thereby increasing respiratory flux while reducing electron leak and superoxide production, particularly under stress conditions. In the context of transplantation of AMSCs, if the transplantation site is an aerobic environment such as heart or muscles, pre-supplementing with CoQ_{10} may facilitate adaptation to the environment, potentially promoting cell survival. In future studies, we will evaluate the efficacy of AMSCs with modified mitochondrial function in animal models.

This study has several limitations. First, although cellular uptake of the MP-(CoQ_{10}) was confirmed by flow cytometry and CLSM, mitochondrial delivery of CoQ_{10} itself was not directly quantified. Therefore, the relationship between CoQ_{10} delivery efficiency and the observed functional improvements remains to be clarified. Second, although ATP-linked respiration was significantly increased following MP-(CoQ_{10}) treatment, intracellular ATP levels were not directly quantified. Therefore, whether the enhanced mitochondrial respiration translates into increased ATP production requires further investigation through direct ATP quantification in future studies. Third, an Empty MITO-Porter (MITO-Porter without CoQ_{10} encapsulation) control was not included in the evaluation of mitochondrial respiration. Therefore, the effects of MITO-Porter itself on mitochondrial function in AMSCs could not be assessed, and the possibility that the observed changes were partially influenced by the carrier itself cannot be completely excluded. Future studies should include an Empty MITO-Porter control to distinguish the effects of the carrier from those of mitochondria targeted CoQ_{10} delivery. Fourth, although MP-(CoQ_{10}) significantly reduced mitochondrial superoxide levels under antimycin A-induced oxidative stress, a naked CoQ_{10} control was not included in this experiment. Therefore, the relative contribution of mitochondrial-targeted delivery versus CoQ_{10} itself to the observed antioxidant effect could not be directly evaluated. Finally, the present study was performed using healthy AMSCs and did not investigate the effects of MP-(CoQ_{10}) in MSCs with impaired mitochondrial function, such as senescent or oxidative stress-exposed MSCs. In addition, this study focused on mitochondrial activation and did not investigate whether these changes translate into enhanced therapeutic functions, such as immunomodulatory

capacity, paracrine activity, migration, or tissue repair. Therefore, further studies using disease-relevant or mitochondria-impaired MSC models, together with functional and *in vivo* evaluations, are warranted to determine the therapeutic potential of mitochondrial-activated AMSCs.

In conclusion, the mitochondrial delivery of CoQ_{10} successfully enhanced the mitochondrial respiratory capacity and antioxidant ability of AMSCs. These mitochondrial-activated AMSCs, newly generated by utilizing the MITO-Porter system, may represent a promising material for improving MSC-based cell transplantation therapy.

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Conflict of Interest AMSCs were provided by Kaneka Corporation. M.H. is an employee of Kaneka Corporation. The other authors declare no conflict of interest.

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Author contributions Conceptualization, Y.Y.; methodology, Y.M., J.A., A.T., and M.H.; validation, Y.M., J.A., and Y.Y.; formal analysis, Y.M. and Y.Y.; investigation, Y.M.; writing—original draft preparation, Y.M.; writing—review and editing, J.A. and Y.Y.; supervision, A.T. and Y.Y.; project administration, Y.Y.; funding acquisition, Y.M. and Y.Y.; resources, M.H. and S.O. All authors have read and agreed to the published version of the manuscript.

Supplementary Materials This article contains supplementary materials.

REFERENCES

1) Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. Multilineage potential of adult human mesenchymal stem cells. *Science*, **284**, 143–147 (1999).
2) Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benhaim P, Lorenz HP, Hedrick MH. Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng.*, **7**, 211–228 (2001).
3) Samsonraj RM, Raghunath M, Nurcombe V, Hui JH, van Wijnen AJ, Cool SM. Concise Review: Multifaceted Characterization of Human Mesenchymal Stem Cells for Use in Regenerative Medicine. *Stem Cells Transl. Med.*, **6**, 2173–2185 (2017).
4) Scassiotti RF, de Paula Coutinho M, Pinto Santos SI, Ferreira Pinto PA, Ferreira de Almeida M, Karam RG, Maria da Silva Rosa P, Martins DDS, Coelho da Silveira J, Ambrósio CE. Adipose and amnion-derived mesenchymal stem cells: extracellular vesicles characterization and implication for reproductive biotechnology. *Theriogenology*, **198**, 264–272 (2023).
5) Tago Y, Kobayashi C, Ogura M, Wada J, Yamaguchi S, Yamaguchi T, Hayashi M, Nakaishi T, Kubo H, Ueda Y. Human amnion-derived mesenchymal stem cells attenuate xenogeneic graft-versus-host disease by preventing T cell activation and proliferation. *Sci. Rep.*, **11**, 2406 (2021).
6) Uccelli A, Moretta L, Pistoia V. Mesenchymal stem cells in health and disease. *Nat. Rev. Immunol.*, **8**, 726–736 (2008).
7) Shi Y, Hu G, Su J, Li W, Chen Q, Shou P, Xu C, Chen X, Huang Y, Zhu Z, Huang X, Han X, Xie N, Ren G. Mesenchymal stem cells: a new strategy for immunosuppression and tissue repair. *Cell Res.*, **20**, 510–518 (2010).
8) Squillaro T, Peluso G, Galderisi U. Clinical Trials With Mesenchymal Stem Cells: an Update. *Cell Transplant.*, **25**, 829–848 (2016).
9) Han Y, Li X, Zhang Y, Han Y, Chang F, Ding J. Mesenchymal Stem Cells for Regenerative Medicine. *Cells*, **8**, (2019).
10) Mukkala AN, Jerkic M, Khan Z, Szaszi K, Kapus A, Rotstein O. Therapeutic Effects of Mesenchymal Stromal Cells Require Mitochondrial Transfer and Quality Control. *Int. J. Mol. Sci.*, **24**, (2023).
11) Yamada Y, Akita H, Kamiya H, Kogure K, Yamamoto T, Shinohara Y, Yamashita K, Kobayashi H, Kikuchi H, Harashima H. MITO-Porter: A liposome-based carrier system for delivery of macromolecules into mitochondria via membrane fusion. *Biochim. Biophys. Acta*, **1778**, 423–432 (2008).
12) Yamada Y. Satrialdi, Hibino M, Sasaki D, Abe J, Harashima H: power of mitochondrial drug delivery systems to produce innovative nanomedicines. *Adv. Drug Deliv. Rev.*, **154-155**, 187–209 (2020).
13) Yamada Y, Ishimaru T, Ikeda K, Harashima H. Validation of the Mitochondrial Delivery of Vitamin B(1) to Enhance ATP Production Using SH-SY5Y Cells, a Model Neuroblast. *J. Pharm. Sci.*, **111**, 432–439 (2022).
14) Yamada Y, Munechika R. Satrialdi, Kubota F, Sato Y, Sakurai Y, Harashima H: Mitochondrial Delivery of an Anticancer Drug Via Systemic Administration Using a Mitochondrial Delivery System That Inhibits the Growth of Drug-Resistant Cancer Engrafted on Mice. *J. Pharm. Sci.*, **109**, 2493–2500 (2020).
15) 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).
16) Hibino M, Maeki M, Tokeshi M, Ishitsuka Y, Harashima H, Yamada Y. A system that delivers an antioxidant to mitochondria for the treatment of drug-induced liver injury. *Sci. Rep.*, **13**, 6961 (2023).
17) Abe J, Yamada Y, Takeda A, Harashima H. Cardiac progenitor cells activated by mitochondrial delivery of resveratrol enhance the survival of a doxorubicin-induced cardiomyopathy mouse model via the mitochondrial activation of a damaged myocardium. *J. Control. Release*, **269**, 177–188 (2018).
18) Sasaki D, Abe J, Takeda A, Harashima H, Yamada Y. Transplantation of MITO cells, mitochondria activated cardiac progenitor cells, to the ischemic myocardium of mouse enhances the therapeutic effect. *Sci. Rep.*, **12**, 4344 (2022).
19) Shiraishi M, Sasaki D, Hibino M, Takeda A, Harashima H, Yamada Y. Human cardiosphere-derived cells with activated mitochondria for better myocardial regenerative therapy. *J. Control. Release*, **367**, 486–499 (2024).
20) Maruo Y, Shiraishi M, Hibino M, Abe J, Takeda A, Yamada Y. Activation of Mitochondria in Mesenchymal Stem Cells by Mitochondrial Delivery of Coenzyme Q(10). *Biol. Pharm. Bull.*, **47**, 1415–1421 (2024).
21) Alviano F, Fossati V, Marchionni C, Arpinati M, Bonsi L, Franchina M, Lanzoni G, Cantoni S, Cavallini C, Bianchi F, Tazzari PL, Pasquinelli G, Foroni L, Ventura C, Grossi A, Bagnara GP. Term Amniotic membrane is a high throughput source for multipotent Mes-

enchymal Stem Cells with the ability to differentiate into endothelial cells *in vitro*. *BMC Dev. Biol.*, **7**, 11 (2007).
22) Kawakubo K, Ohnishi S, Fujita H, Kuwatani M, Onishi R, Masamune A, Takeda H, Sakamoto N. Effect of Fetal Membrane-Derived Mesenchymal Stem Cell Transplantation in Rats With Acute and Chronic Pancreatitis. *Pancreas*, **45**, 707–713 (2016).
23) Yamahara K, Harada K, Ohshima M, Ishikane S, Ohnishi S, Tsuda H, Otani K, Taguchi A, Soma T, Ogawa H, Katsuragi S, Yoshimatsu J, Harada-Shiba M, Kangawa K, Ikeda T. Comparison of angiogenic, cytoprotective, and immunosuppressive properties of human amnion- and chorion-derived mesenchymal stem cells. *PLoS One*, **9**, e88319 (2014).
24) Ohshima M, Yamahara K, Ishikane S, Harada K, Tsuda H, Otani K, Taguchi A, Miyazato M, Katsuragi S, Yoshimatsu J, Kodama M, Kangawa K, Ikeda T. Systemic transplantation of allogenic fetal membrane-derived mesenchymal stem cells suppresses Th1 and Th17 T cell responses in experimental autoimmune myocarditis. *J. Mol. Cell. Cardiol.*, **53**, 420–428 (2012).
25) Tsuda H, Yamahara K, Ishikane S, Otani K, Nakamura A, Sawai K, Ichimaru N, Sada M, Taguchi A, Hosoda H, Tsuji M, Kawachi H, Horio M, Isaka Y, Kangawa K, Takahara S, Ikeda T. Allogenic fetal membrane-derived mesenchymal stem cells contribute to renal repair in experimental glomerulonephritis. *Am. J. Physiol. Renal Physiol.*, **299**, F1004–F1013 (2010).
26) Nitahara-Kasahara Y, Nakayama S, Kimura K, Yamaguchi S, Kakiuchi Y, Nito C, Hayashi M, Nakaishi T, Ueda Y, Okada T. Immunomodulatory amnion-derived mesenchymal stromal cells preserve muscle function in a mouse model of Duchenne muscular dystrophy. *Stem Cell Res. Ther.*, **14**, 108 (2023).
27) Takamiya S, Kawabori M, Yamazaki K, Yamaguchi S, Tanimori A, Yamamoto K, Ohnishi S, Seki T, Konno K, Tha KK, Hashimoto D, Watanabe M, Houkin K, Fujimura M. Intravenous transplantation of amnion-derived mesenchymal stem cells promotes functional recovery and alleviates intestinal dysfunction after spinal cord injury. *PLoS One*, **17**, e0270606 (2022).
28) Kobayashi K, Ichihara Y, Sato N, Umeda N, Fields L, Fukumitsu M, Tago Y, Ito T, Kainuma S, Podaru M, Lewis-McDougall F, Yamahara K, Uppal R, Suzuki K. On-site fabrication of Bi-layered adhesive mesenchymal stromal cell-dressings for the treatment of heart failure. *Biomaterials*, **209**, 41–53 (2019).
29) Bieback K, Hecker A, Kocaömer A, Lannert H, Schallmoser K, Strunk D, Klüter H. Human alternatives to fetal bovine serum for the expansion of mesenchymal stromal cells from bone marrow. *Stem Cells*, **27**, 2331–2341 (2009).
30) Chen CT, Shih YR, Kuo TK, Lee OK, Wei YH. Coordinated changes of mitochondrial biogenesis and antioxidant enzymes during osteogenic differentiation of human mesenchymal stem cells. *Stem Cells*, **26**, 960–968 (2008).
31) Da Dalt L, Cabodevilla AG, Goldberg IJ, Norata GD. Cardiac lipid metabolism, mitochondrial function, and heart failure. *Cardiovasc. Res.*, **119**, 1905–1914 (2023).
32) Allard MF, Schönekess BO, Henning SL, English DR, Lopaschuk GD. Contribution of oxidative metabolism and glycolysis to ATP production in hypertrophied hearts. *Am. J. Physiol.*, **267**, H742–H750 (1994).
33) Menendez-Montes I, Garry DJ, Zhang JJ, Sadek HA. Metabolic Control of Cardiomyocyte Cell Cycle. *Methodist DeBakey Cardiovasc. J.*, **19**, 26–36 (2023).
34) Arenas-Jal M, Suñé-Negre JM, García-Montoya E. Coenzyme Q10 supplementation: Efficacy, safety, and formulation challenges. *Compr. Rev. Food Sci. Food Saf.*, **19**, 574–594 (2020).
35) Pravst I, Zmitek K, Zmitek J. Coenzyme Q10 contents in foods and fortification strategies. *Crit. Rev. Food Sci. Nutr.*, **50**, 269–280 (2010).
36) Ouchi A, Nagaoka S, Mukai K. Tunneling effect in regeneration reaction of vitamin E by ubiquinol. *J. Phys. Chem. B*, **114**, 6601–6607 (2010).
37) Murphy MP. How mitochondria produce reactive oxygen species. *Biochem. J.*, **417**, 1–13 (2009).