Isolated Mitochondrial Transplantation as a Novel Treatment for Corneal Chemical Burns

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Purpose. This study aimed to investigate the therapeutic potential of isolated mitochondrial transplantation for the restoration of corneal surface injury in mice after corneal chemical burn.

Methods. Mitochondria were isolated from mesenchymal stem cells via ultracentrifugation, followed by an assessment of their purity and viability. The internalization of mitochondria by human corneal epithelial cells was tracked using a live fluorescence imaging system. Apoptosis-related markers and mitochondrial function were measured by Western blotting and flow cytometry, respectively. Mitochondrial morphology was examined using confocal laser scanning microscopy. The therapeutic effects of subconjunctival administration of isolated mitochondria in vivo were evaluated by fluorescein sodium staining and histopathological examination of the corneas.

Results. Our study demonstrates that corneal epithelial cells possess the capacity to internalize isolated exogenous mitochondria in vitro. Under oxidative stress conditions, recipient cells exhibited an enhanced uptake of exogenous mitochondria. We observed a decrease in apoptosis and a reduction in oxidative stress levels within the recipient cells, as well as a partial restoration of mitochondrial function. Notably, after a single subconjunctival injection, corneal epithelial cells were able to use isolated mitochondria to enhance the repair process in a mouse model of corneal acid burn.

Conclusions. Subconjunctival injection of isolated mitochondria promoted the repair of acute corneal burns in mice. The results of our investigation using injection of isolated mitochondria as a treatment modality for corneal chemical burn offers a novel approach to the treatment of ocular disorders associated with mitochondrial dysfunction.

Keywords: mitochondrial transplantation, corneal acid burns, stem cell therapy, mitochondrial dysfunction, ocular surface repair

Corneal chemical burns represent a common and sight-threatening ophthalmic emergency, necessitating immediate assessment and intervention to prevent blindness. Severe corneal chemical burns result in corneal inflammation, ulceration, neovascularization, conjunctivalization, limbal stem cell deficiency, and stromal scarring, all of which may lead to blindness.[1] It is crucial to initiate timely and appropriate treatment after a chemical injury to facilitate corneal healing and minimize corneal haze.

Mitochondrial dysfunction is a pivotal factor contributing to ocular chemical injury. Previous research provides strong evidence that the acute phase of a chemical injury promotes accumulation of reactive oxygen species (ROS), which can lead to mitochondrial dysfunction in the cornea.[2–4] Inadequate scavenging of ROS leads to an imbalance of antioxidants and pro-oxidants within corneal cells and is a significant mechanism underlying corneal inflammation, scarring, and neovascularization.[5,6] Additionally, the augmentation of oxidative stress disrupts mitochondrial membrane permeability, disturbs electron transfer, and damages cell membranes with consequent cell death.[7,8] It has been shown that inhibiting alkaline-induced oxidative stress alleviates corneal inflammation, inhibits the formation of corneal neovessels, and promotes corneal healing.[9] These observations imply that suppressing oxidative stress to enhance mitochondrial capacity may be a poten-

tial therapeutic means to ameliorate corneal chemical burns.

The primary objectives of preliminary phase treatment are to reduce inflammation and facilitate re-epithelialization.[10] Biological fluids, including autologous serum, platelet-rich plasma, amniotic membrane, and limbal tissue, are commonly used as auxiliary means to promote epithelial healing.[11] Numerous studies have demonstrated that mesenchymal stem cells (MSCs) have a significant impact on the improvement of corneal epithelium recovery and reduction of inflammation and oxidative damage in skin chemical burns and corneal alkali burns.[12–18] Our previous research has highlighted the significant role of mitochondrial transfer in promoting the repair process by MSCs of corneal chemical injury.[19] Nonetheless, ethical concerns have arisen from sourcing issues and production challenges as well as the potential for tumorigenicity. The clinical application of cell-based mitochondrial transfer, therefore, remains limited. There is an ongoing urgency to explore more effective treatment modalities and their practical application in the treatment of ocular diseases. Isolated mitochondrial transplantation, a technique involving the replacement or supplementation of damaged mitochondria with healthy mitochondria isolated from living tissue, has been effective in various diseases.[20–24] This innovative approach has emerged as a promising treatment for mitochondria-related diseases. This study aimed to explore the use of exogenous isolated mitochondria to restore cell function and assess the therapeutic impact of mitochondrial transplantation in the treatment of corneal acid burns in a mouse model.

Methods

Cell Culture

Corneoscleral tissue was procured from the Wenzhou Medical University Eye Bank in Zhejiang Province, China, and originated from donors aged 18 to 75 years. Primary human corneal epithelial cells (pHCECs) were cultivated from limbal explants using a method previously outlined by Zheng et al.[25] The explants were cultured in supplemented hormonal epithelial medium comprising Dulbecco's modified Eagle's medium/F12 medium, 5% fetal bovine serum, 10 ng/mL human epidermal growth factor, 5 µg/mL insulin-transferrin-selenium, 50 µg/mL gentamicin, 1.25 µg/mL amphotericin B, 0.5% dimethyl sulfoxide, 0.5 µg/mL hydrocortisone, and 30 ng/mL choleratoxin. Human MSCs were obtained from Nuwacell (Nuwacell, Cat#RC02003, Hefei, China). The culture medium for human MSCs comprised Dulbecco's modified medium/F12 medium supplemented with 5% fetal bovine serum. Cell cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2, and the medium was changed every 2 to 3 days.

Mitochondrial Isolation

Mitochondrial extraction from MSCs was conducted using a Mitochondria Isolation Kit for Cultured Cells (Invitrogen #89874; Waltham, MA, USA) in conjunction with a Dounce homogenizer (Wheaton #357538, Millville, NJ, USA). The isolation process adhered to the manufacturer's instructions. After extracting mitochondria, a 10 µm cell filter (pluriStainer, 43-10010-50; PluriSelect, Leipzig, Germany) should be used to purify the preparation. Isolated mitochondria were visualized under an inverted phase contrast microscope (Axio Observer 3, Zeiss, Jena, Germany).

Characterization of Isolated Mitochondria

To assess mitochondrial purity, mitochondrial markers TOM20 (1:2000, #11802-1-AP, Proteintech, Rosemont, IL, USA) and cytochrome C oxidase (1:2000, #11967, Cell Signaling Technology, Danvers, MA, USA) and cytoplasmic markers Tubulin (1:2000, # 2128T, Cell Signaling Technology) and GAPDH (1:2000, # AF1186, Beyotime, Jiangsu, China) were analyzed by Western blotting. The completeness of isolated mitochondria was assessed by Western blot analysis of respiratory chain complex (NDUFV2 [1:2000, #15301-1-AP, Proteintech], SDHB [1:2000, # 10620-1-AP, Proteintech], UQCRC2 (1:2000, #14742-1-AP, Proteintech], COX IV (1:2000, #11967, Cell Signaling Technology], and Anti-ATPB [1:1000, # 14884-1-AP, Abcam, Cambridge, UK]). To measure the activity of isolated mitochondria, we used MitoTracker Green FM (1 µM, #M7512, Thermo Fisher Scientific, Waltham, MA, USA) and MitoTracker Red CMXRos (1 µM, #M7514, Thermo Fisher Scientific) for a co-incubation period of 30 minutes. These dyes were used to label all mitochondria and active mitochondria, respectively. After three washes with phosphate-buffered saline (PBS), the samples were observed under an inverted phase contrast microscope (Axio Observer 3, Zeiss). After staining the samples with the Mitochondrial Membrane Potential Detection Kit (C2001S, Beyotime) following the provided instructions, a fluorescent microplate reader was used to measure the average fluorescence intensity and gather statistical data. The functional status of mitochondria was assessed using an ATP Assay Kit (S0027, Beyotime) following the manufacturer's instructions.

Establishment of an In Vitro Mitochondrial Injury Model

Rotenone (rot) (Sigma, Cat# R8875, St. Louis, MO, USA) was added at concentrations of 0, 0.5, 1, 5, 10, 20, 50, 100, 200, and 500 µM for a duration of 2 hours. Afterward, cells were washed three times with PBS and incubated in normal medium for 24 hours. This experiment aimed to investigate the inhibitory effect of rot on mitochondrial function using CCK-8. Subsequently, 10 µM rot was used to induce the mitochondrial injury model.

Observation of MSC-Mito Internalization in pHCECs

The MSCs were labeled with green signals by transfection with Mito-COX8-GFP lentivirus (SBI, Cat# Cyto102-PA-1). pHCECs were seeded onto cell culture slides in a 24-well plate. Mitos-GFP was seeded in the same 24-well plate and co-cultured with pHCECs for 24 hours. The pHCECs were fixed with 4% paraformaldehyde after three PBS washes. Phalloidin (1:400, Cat# A22287, Thermo Fisher Scientific), a high-affinity F-actin probe, was used for F-actin staining of fixed cells. Diamidino-2-phenylindole (DAPI) (#S36938, Thermo Fisher Scientific) was used to label the nucleus. Finally, stained samples were photographed under a confocal microscope (LSM800, Zeiss).

Live cell imaging was used to record the real-time uptake of Mitos-GFP by pHCECs. pHCECs were implanted onto a Laser Confocal Petri Dish (JingAn Biological, Shanghai, China) for 24 hours. Mitos-GFP was then seeded on the laser confocal petri dish. A high-resolution microscope (Elyra7, Zeiss) was used to view the culture plate, and the cell behavior was recorded with Harmony software using Brightfield

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and Alexa 488 channels. Recordings were conducted continuously for 24 hours with an interval of 10 minutes.

Analysis of the mitochondrial transplantation rate was performed through flow cytometry using BD Accuri C6 Plus analyzers (BD Accuri C6 Plus Personal Cytometer; BD Biosciences, Franklin Lakes, NJ, USA). Only active cells were analyzed and mitochondrial engraftment was determined by assessing the proportion of fluorescein isothiocyanate–positive cells.

Evaluation of Cell Proliferation and Apoptosis

The clonogenic assay was used to evaluate in vitro cell survival following treatment with rot. The pHCECs were seeded on 12-well plates for 24 hours and treated with rot for 2 hours before the addition of isolated mitochondria. Cells were subsequently stained 24 hours later with a crystal violet staining solution (Beyotime Institute of Biotechnology).

An Annexin V Apoptosis Detection Kit (#559763, BD Biosciences) was used for the detection of apoptotic cells. Annexin V and PI were stained for 15 min at room temperature as directed by the manufacturer. Flow cytometry was performed using the Attune NxT, and data was analyzed using the FlowJo 10.4.2 program (FlowJo). Cells that were PE-Annexin V positive and 7AAD negative were identified as apoptotic. The protein expression of Bax (1:1000, ab32503, Abcam), Bcl-2 (1:1000, ab182858, Abcam), and GAPDH (1:2000, # AF1186, Beyotime) was analyzed by Western blotting.

Evaluation of Oxidative Stress

The ROS level in pHCECs was measured using CM-H2DCFDA (#C6827, Invitrogen) following the manufacturer's protocol. Cells were washed three times with PBS and incubated in the dark with 5 µM H2DCFDA at 37°C for 30 minutes. ROS production was analyzed by a CytoFLEX flow cytometer (BD Accuri C6 Plus Personal Cytometer; BD Biosciences). FlowJo was used to analyze the mean fluorescence intensity.

Measurement of Mitochondrial Membrane Potential (ΔΨm)

The MitoProbe JC-1 Assay Kit (#M34152, Invitrogen) was used to measure the mitochondrial membrane potential. Cells were incubated with JC-1 working solution at 37°C for 20 minutes, followed by washing with JC-1 staining buffer. The green (JC-1 monomer) to red (JC-1 aggregates) fluorescence ratio was then measured using flow cytometry.

Mitochondrial Superoxide Measurement

A MitoSOX Red Mitochondrial Superoxide Indicator dye (#M36008, Invitrogen) was used to detect MitoSOX. The pHCECs were washed with PBS and incubated in the dark with a 5-µM MitoSOX working solution at 37°C for 20 minutes. The fluorescence intensity of the propidium iodide channel was measured using a flow cytometer (BD Accuri C6 Plus Personal Cytometer; BD Biosciences). Fluorescence intensity was quantified using FlowJo.

Analysis of Mitochondrial Morphology

Analysis of mitochondrial morphology was performed using MitoTracker green dye. The nucleus was stained with DAPI (#S36938,Thermo Fisher Scientific). Microscopy was performed with a confocal microscope (LSM800, Zeiss). Mitochondrial morphology analysis was performed with Mitochondrial Network Analysis Tool (MINA) from ImageJ.

Analysis of Mitochondrial Proteins

The expression level of respiratory chain complexes was determined by Western blotting as described previously.

Animals

Corneal acid burn studies used female C57BL/6 mice aged 6 to 8 weeks. All mouse experiments were performed according to the regulations of Wenzhou Medical University. In this study, the Laboratory Animal Ethics Committee at Wenzhou Medical University and the Laboratory Animal Centre at Wenzhou Medical University (Wenzhou, Zhejiang, China) provided consent and approval.

Establishment of a Mouse Model of Acid Burn–induced Corneal Injury and Treatment With Isolated Mitochondria

An acid burn model in mice was created following systemic administration of 2,2,2-Tribromoethanol (T48402, sigma) and topical Alcaine proparacaine hydrochloride ophthalmic solution (Alcaine, Alcon, Ft Wort, TX, USA) for anesthesia, and corneal acid burn created on the super-central cornea by placement for 20 seconds with a circular 2-mm filter paper saturated with 2 N HCL.[26,27] The corneal surface was then immediately rinsed with saline for 5 minutes. With the assistance of a surgical microscope (Leica, Wetzler, Germany), subconjunctival injection was carried out with a 33G Hamilton needle (Hamilton Company, Waltham, USA). The concentration of mitochondria injected subconjunctivally was 10^7/mL.

Corneal Fluorescein Staining

For assessing corneal epithelial defects on days 1, 2, and 3, sodium fluorescein staining was performed. The corneal fluorescein stain was performed by applying 0.5 µL of 0.1% fluorescein into the inferior conjunctival sac of the eye and the corneal opening.

Hematoxylin and Eosin Staining

Mice were executed and the corneas removed and fixed overnight with 4% paraformaldehyde. The corneas were then embedded in paraffin and cut into 5-µm-thick sections. After deparaffinization with xylene and rehydration with graded alcohol solutions, the sections were stained with hematoxylin and eosin. Corneal tissue was examined under a light microscope (Nikon, Tokyo, Japan) to observe morphological changes.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism v7.0 (GraphPad Software, La Jolla, CA, USA). Quantitative data are presented as mean ± SD, representing the central tendency and dispersion of data, respectively. Comparisons between two groups were performed by Student's unpaired two-tailed *t* test, and comparison of differences in mean among multiple groups by one-way ANOVA. A *P* value

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of less than 0.05 was considered significant (*$P < 0.05$; **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$); $P > 0.05$, nonsignificant (NS).

Results

Isolation of Intact Mitochondria From MSCs

Intact mitochondria were isolated from MSCs by differential centrifugation (Fig. 1A). Purity and quality of isolated mitochondria were evaluated. Images of the extracted mitochondria captured using an inverted phase-contrast microscope revealed uniform mitochondria with a size of approximately 1 to 2 µm (Fig. 1B). The isolated mitochondria were rich in Translocase of the outer mitochondrial membrane 20 (Tomm20) and mitochondrial protein cytochrome C oxidase, whereas cytosolic proteins GAPDH and tubulin were removed completely (Fig. 1C), indicating a high degree of purification. To examine the integrity of the purified mitochondria, protein expression of five respiratory chain complexes (oxidative phosphorylation complexes) was determined (Fig. 1D). The expression of these respiratory chain complexes in the purified mitochondria was found to be consistent with that of MSCs, suggesting that the mitochondria remained intact following the isolation process.

CCCP is an oxidative phosphorylation uncoupler that can inactivate purified mitochondria. Assays were performed on purified mitochondria using MitoTracker Green FM and MitoTracker Red CMXRos, which stain mitochondria, regard-

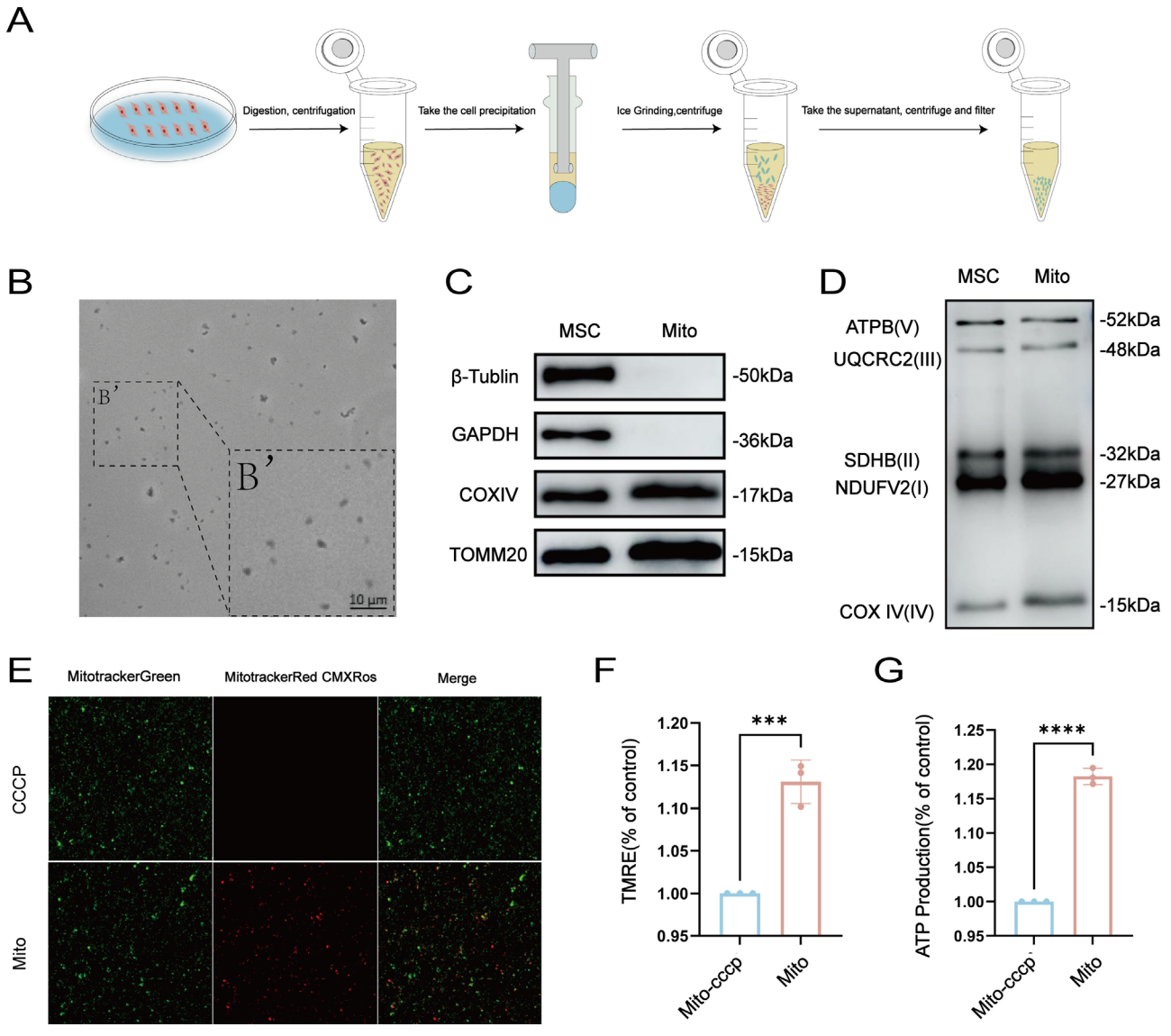

Figure 1. Preparation, identification, and function of MSC-Mitos. (**A**) Process of isolating mitochondria. (**B**) Isolated mitochondria are shown under phase contrast illumination (bright field [BF]). (**C**) Purity of mitochondrial isolation by Western blot. Organelles measured: cytoskeleton (beta-tubulin), cytoplasm (GAPDH), mitochondria (Tom20 and COX IV). (**D**) The oxidative phosphorylation (OXPHOS) complexes include: complex I (NDUFV2), complex II (SDHB), complex III (UQCRC2), complex IV (COX IV), and complex V (ATPB). (**E**) Purified mitochondria were stained with MitoTracker Red CMXRos (MTR) (*red*), a marker for active mitochondria (with appropriate membrane potential), and with MitoTracker Green FM (MTG) (*green*), a marker for pan mitochondria. Mito-CCCP were stained only with MTG, whereas mitochondria were stained with both dyes. (**F**) Mean fluorescence intensity of membrane potential ($n = 3$). (**G**) ATP levels ($n = 3$). ***$P < 0.001$, ****$P < 0.0001$.

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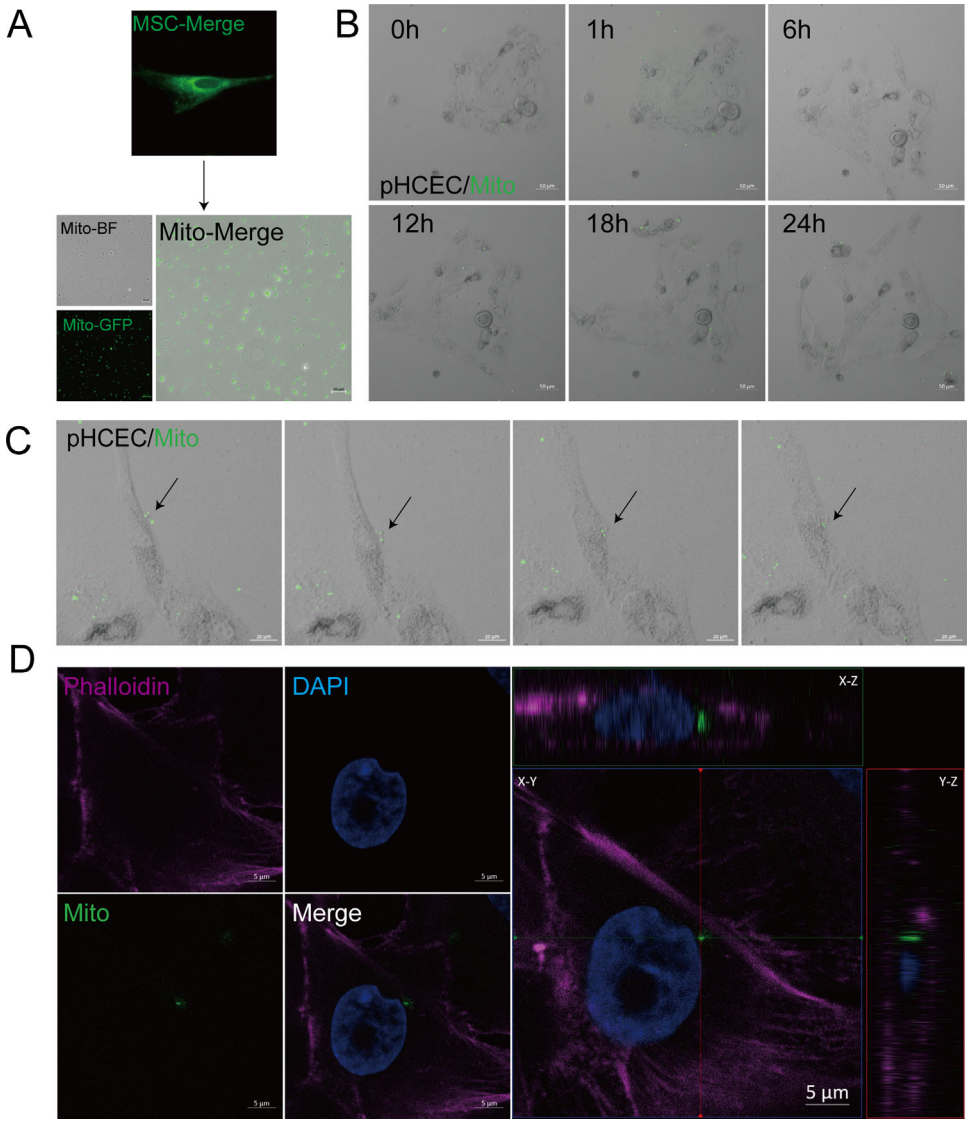

Figure 2. Transplantation of isolated mitochondria into pHCECs. (**A**) The mitochondria of MSCs were labeled using Mito-COX8-GFP lentivirus. (**B**) Internalization of GFP-Mito by pHCECs at 0, 1, 6, 12, 18, and 24 hours. Scale bar, 50 µm. (**C**) pHCEC internalizes the GFP-Mito process. *Black arrow*: GFP-Mito. Scale bar, 20 µm. (**D**) The position of endocytosed mitochondria through the *Z*-axis was observed. The cytoskeleton is marked with phalloidin (*purple*), the nucleus with DAPI (*blue*), and GFP-Mito (*green*) was used to label the exogenous mitochondria. Scale bar, 5 µm.

less of their membrane potential. As shown in Fig. 1E, the purified mitochondria damaged by CCCP (Mito-CCCP) were stained exclusively with MitoTracker Green FM. Conversely, the purified mitochondria were stained with both dyes, indicating the presence of an adequate membrane potential,[28] a crucial indicator of mitochondrial bioactivity. To further confirm the biological activity of purified mitochondria, flow cytometry was used to assess the membrane potential of both the Mito-CCCP group and the mitochondria group, and the average fluorescence intensity was calculated (Fig. 1F). The results revealed a significant statistical difference between the two groups. Production of adenosine triphosphate (ATP) is one of the function of mitochondria. Comparison of ATP production between the Mito-CCCP and mitochondria groups revealed that it was significantly higher in the latter (Fig. 1G), demonstrating the successful isolation of pure and active mitochondria.

Mitochondria Were Internalized by Corneal Epithelial Cells In Vitro

To confirm the intracellular transmission of mitochondria, mitochondria from MSCs were labeled with Mito-COX8-GFP (Fig. 2A). Dynamic mitochondrial internalization over 24 hours was observed using a live fluorescence imaging system (Supplementary Movie S1). Within the first hour of adding isolated mitochondria, internalization of exogenous mitochondria was observed (Fig. 2B). Initially, cells on the periphery of the cell cluster began internalizing isolated mitochondria. As the cells migrated, those in the center of the cluster moved towards the periphery and internalized nearby isolated mitochondria (Supplementary Movie S2, Fig. 2B). We also determined that isolated mitochondria first made contact with the cell membrane and were then taken up by the cell at the appropriate time (Fig. 2C).

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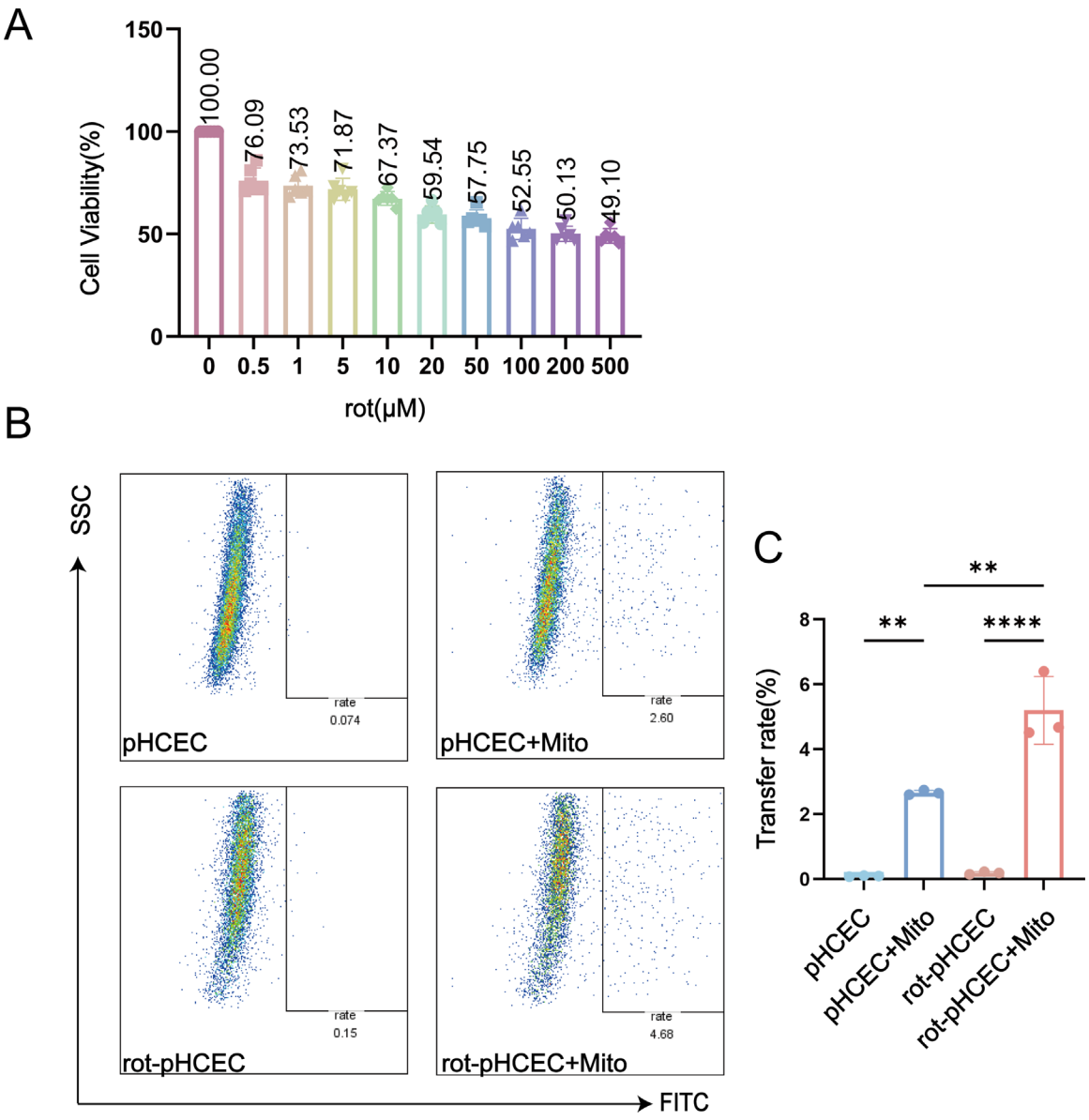

Figure 3. Impact of rot exposure on the uptake of exogenous mitochondria by pHCEC cells. (**A**) pHCEC cells were exposed to various concentrations of rot for 2 hours and then incubated for 24 hours. Cell viability and cytotoxicity were assessed by CCK-8 assay ($n = 6$). (**B and C**) Representative dot plots and statistics for monitoring the frequency of GFP-positive pHCECs determined by flow cytometry ($n = 3$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

Three-dimensional imaging using a microscope revealed that isolated mitochondria were enveloped by the cells, confirming that mitochondria had entered epithelial cells rather than being anchored to the cell membrane (Fig. 2D). Based on these findings, it was evident that corneal epithelial cells could endocytose exogenous mitochondria.

Mitochondrial Dysfunction Enhanced Mitochondrial Uptake of pHCECs

Next, we examined whether moderate oxidative stress could facilitate mitochondrial uptake in pHCECs. Mitochondrial dysfunction was induced in pHCECs by rot, an electron transport chain complex I inhibitor. CCK-8 was used to determine cell activity and the impact of different concentrations of rot. Ultimately, a concentration of 10 μM was determined to be the damaging concentration (Fig. 3A). Quantitative analysis of flow cytometry results revealed presence of isolated mitochondria in both groups after mitochondrial transplantation (Fig. 3B). Nonetheless, compared with the pHCEC + mitochondria group, the rate of mitochondria transplantation in the rot-pHCEC + mitochondria group was approximately double (Fig. 3C), indicating that the transplantation rate of mitochondria was increased effectively in the mitochondria-damaged cells.

The Effect of Exogenous Mitochondria on Cell Viability and Apoptosis in pHCECs

To investigate the impact of exogenous mitochondria on corneal epithelial cell function, we assessed cell activity using CCK-8 (Fig. 4A). The addition of exogenous mitochondria increased cell viability in both the control group and the rot-induced group. Nonetheless, when the concen-

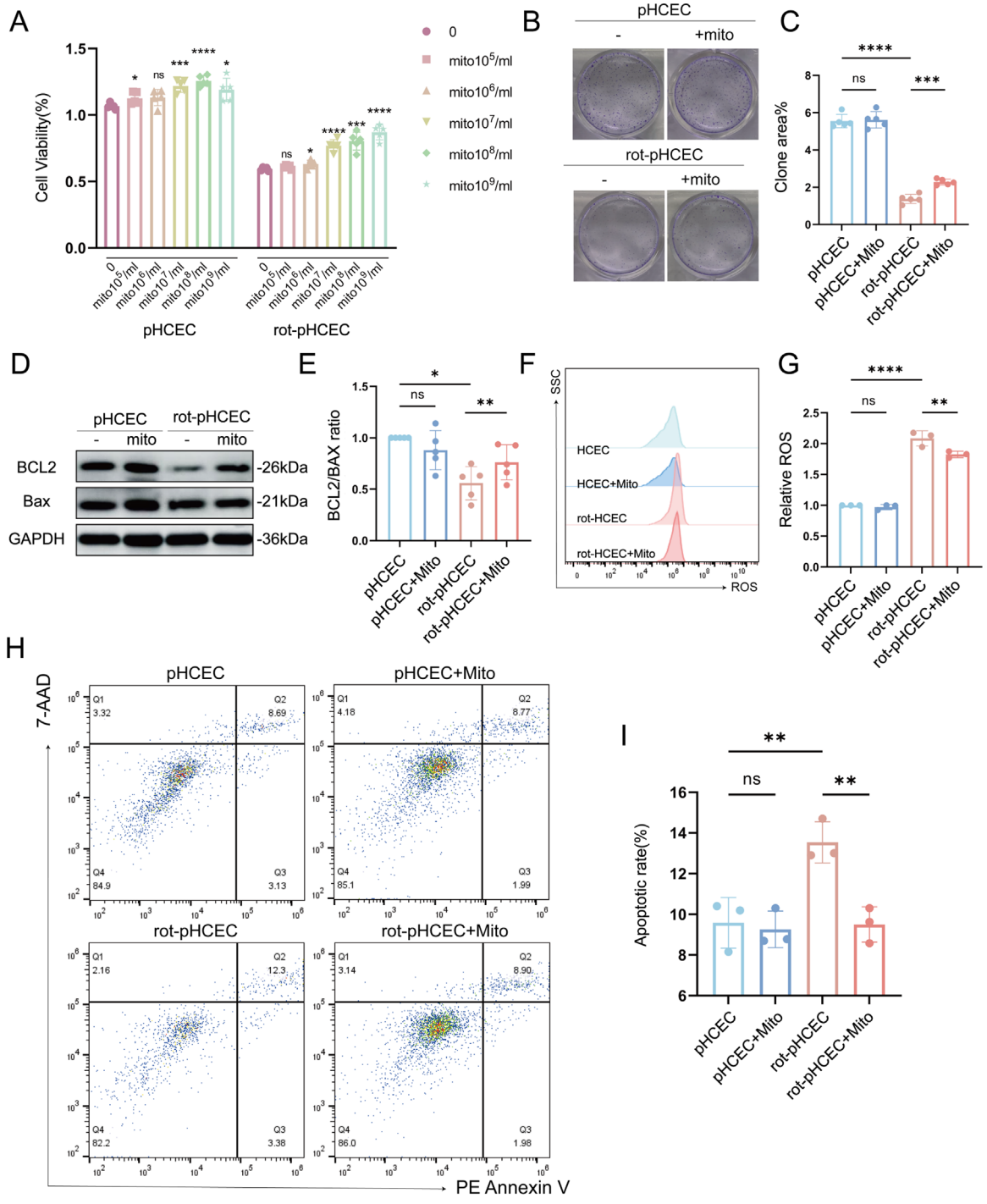

Figure 4. Mitochondrial transplantation reduced apoptosis and oxidative stress. (**A**) CCK-8 assay assessed cell viability ($n = 5$). (**B and C**) Cells were stained using crystal violet ($n = 5$). (**D and E**) Western blotting was used to detect the expression of apoptosis-associated proteins (BCL2 and BAX) and the loading control protein GAPDH ($n = 5$). (**F**) Intracellular ROS level was detected by flow cytometry. (**G**) Histogram analysis showing the intracellular ROS levels ($n = 3$). (**H**) Flow cytometric analysis of cell apoptosis. (**I**) Quantification of cell apoptosis ($n = 3$). Ns, nonsignificant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

tration of exogenous mitochondria reached 10^9/mL, cell viability decreased in the normal group, suggesting that an excessive number of exogenous mitochondria negatively impacted cells with normal function. Based on the CCK-8 results, a concentration of 10^7/mL was selected for subsequent experiments with exogenous mitochondria. Additionally, the crystal violet results showed a significant decrease in cell death rate in the rot-induced group following the addition of exogenous mitochondria (Figs. 4B, 4C). To further investigate the hypothesis that exogenous mitochondria reduce apoptosis, we examined the level of apoptosis-related proteins using Western blotting (Figs. 4D, 4E), as well as the proportion of apoptotic cells using flow cytometry (Figs. 4H, 4I). Although there was no significant change to the apoptosis rate in the normal group after the addition of exogenous mitochondria, exogenous mitochon-

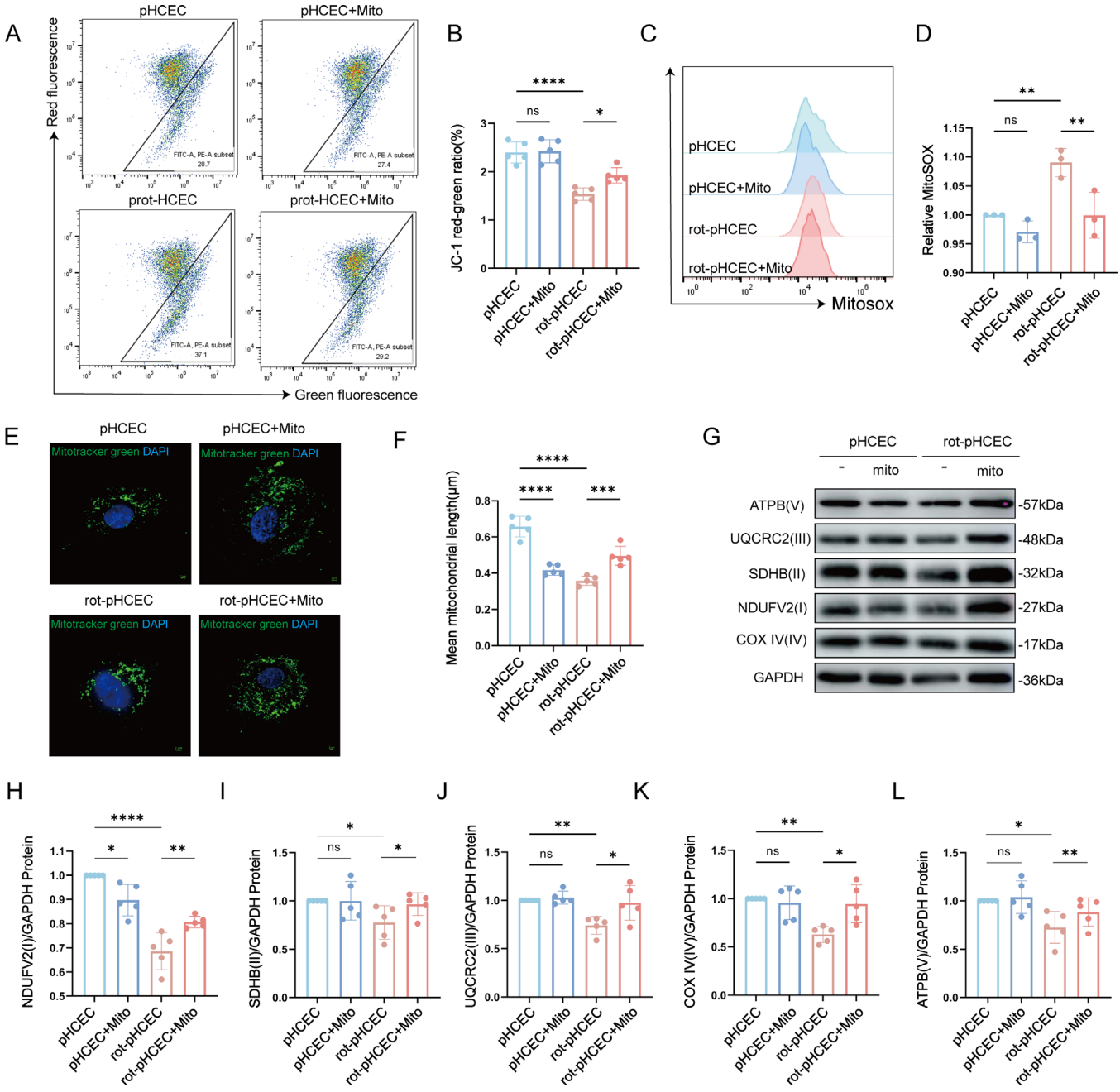

Figure 5. Improvement of mitochondrial function in pHCECs by mitochondrial transplantation. (**A**) Flow cytometric analysis was performed on cells stained with JC-1, with the horizontal axis representing JC-1 green and the vertical axis JC-1 red. *Triangular box*, the rate of JC-1 *green*. (**B**) Quantification of the JC-1 fluorescence ratio ($n = 5$). (**C**) Representative flow cytometry plots of MitoSOXRed straining. (**D**) Quantification of MitoSOXRed fluorescence ($n = 3$). (**E**) Representative confocal images of the mitochondrial morphology of pHCECs stained with MitoTrackerGreen, DAPI labeled. (**F**) Quantification of the mean mitochondrial length ($n = 5$). (**G–L**) WB and WB quantification of respiratory chain protein levels ($n = 5$). ns, nonsignificant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

dria notably reduced the number of apoptotic rot-induced cells.

ROS are considered a significant product of oxidative stress. Interestingly, the introduction of external mitochondria did not induce any oxidative stimulation in the cells of the control group. On the contrary, it reduced the level of ROS in cells affected by rot (Figs. 4F, 4G). Collectively, these findings suggested that exogenous mitochondria could restore the functionality of corneal epithelial cells damaged by oxidative stress effectively.

Restoration of Mitochondrial Morphology and Function After Mitochondrial Uptake by pHCECs

To examine the influence of exogenous mitochondria on restoring mitochondrial function in pHCECs, mitochondrial activity was assessed by measurement of mitochondrial membrane potential (Figs. 5A, 5B). Mitochondrial function of the normal group was not affected by exogenous mitochondria, but membrane potential of the rot-induced group increased. Mitochondrial ROS are generated primarily within

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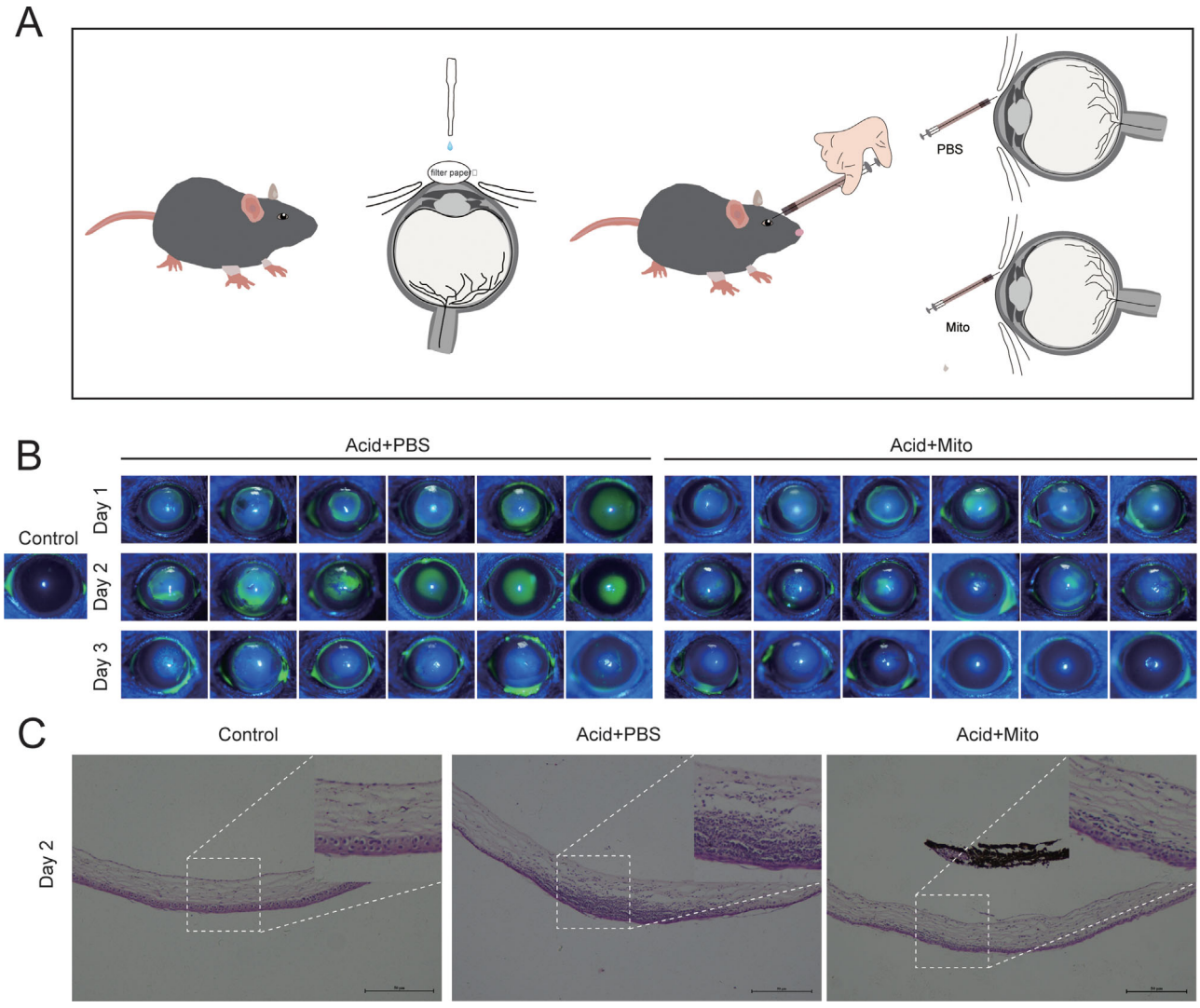

Figure 6. Mitochondrial transplantation promotes corneal epithelial repair in acid-burned mice. (**A**) Simple procedures for animal experiments. (**B**) The dynamic changes to corneal defects in three groups at 1, 2, and 3 days after epithelial abrasion were observed by fluorescein sodium staining ($n = 6$). (**C**) Hematoxylin and eosin (H&E) staining of the cornea ($n = 5$). The control group comprises mice that received no treatment. The acid + PBS group comprises mice that were injected with PBS after the acid burn. The acid + mitochondria group comprises mice that were injected with isolated mitochondria after the acid burn.

the mitochondria and contribute significantly to the cellular ROS level. Previous results have demonstrated that the introduction of exogenous mitochondria can effectively decrease cellular ROS. To verify the role of exogenous mitochondria in this process, flow cytometric analysis was performed to detect mitochondria-derived ROS (Figs. 5C, 5D). Results confirmed that the addition of exogenous mitochondria reduced mitochondrial ROS. To investigate the impact of exogenous mitochondria on recipient cell mitochondria further, Mitotracker green staining was applied and the corresponding images captured (Figs. 5E, 5F). Under the induction of rot, cell mitochondria shrank and became shorter. In contrast, on addition of exogenous mitochondria, cell mitochondria partially restored their length. This result suggested that the addition of exogenous mitochondria restored mitochondrial morphology and function.[29]

We then assessed the level of respiratory chain proteins (Figs. 5G–L). There was a decrease in cellular respiratory chain protein levels induced by rot, but they were restored partially after the addition of isolated mitochondria.

Mitochondrial Transplantation Promotes the Repair of Corneal Acid Burn In Vivo

Corneal acid burns were induced in mice by administration of hydrochloric acid. Subsequently, newly extracted mitochondria were injected under the bulbar conjunctiva to investigate their therapeutic potential (Fig. 6A). The ocular surface was stained with fluorescein and examined using slit-lamp microscopy to evaluate the effect of isolated mitochondria on wound healing of the corneal epithelium. In Fig. 6B, a green disc-shaped burn area can be observed in the center of the cornea. However, by day 2, the mitochondria-treated group showed improved healing, with nearly complete coverage of the wound by the epithelium (Fig. 6B). This finding indicates that isolated mitochondria may help to promote the regeneration of corneal epithelium. To examine detailed structural repair differences between the two groups, a histological analysis was performed. As shown in Figure 6C and Supplementary Figure S1, the control group still exhibited a disordered lamellar squamous

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epithelial structure on the second day after burns, with a large number of inflammatory cells infiltrating the stromal layer. In contrast, the mitochondria-treated group showed relatively complete and well-defined epithelial cells. These findings demonstrate the potential of transferred mitochondria to promote the repair of corneal acid burns.

Discussion

This study reveals that mitochondrial transplantation can mitigate corneal epithelial cell damage effectively both in vivo and in vitro by enhancing mitochondrial function, which decreases oxidative stress-induced corneal epithelial damage in chemical burns. Moreover, our findings indicate that corneal epithelial cells can internalize fluorescently labeled exogenous mitochondria in vitro, with the uptake being enhanced particularly under oxidative stress conditions. Furthermore, the internalization of exogenous mitochondria by these cells leads to a decrease in apoptosis and oxidative stress, as well as a partial restoration of mitochondrial function in corneal epithelial cells. Importantly, subconjunctival injection of exogenous mitochondria in mice resulted in reduced inflammatory cell infiltration induced by acid burn and accelerated the corneal repair process. These results strongly support mitochondrial transplantation as a promising strategy to treat chemical injuries to the cornea by providing cellular protection.

Our results clearly demonstrated that corneal epithelial cells could uptake and internalize exogenous mitochondria from the culture medium by co-cultivation, with cells at the cluster center migrating towards the periphery to actively engage with nearby mitochondria. This mitochondrial uptake was further augmented under rot-induced mitochondrial damage. Similarly, Cowan et al.[30] observed enhanced mitochondrial uptake in ischemic myocardial regions, which may be a response to cardiomyocyte swelling and compromised cellular integrity. Aharoni-Simon et al.[28] also noted a concentration-dependent effect on mitochondrial uptake of retinal ganglion precursor cells, with higher H_2O_2 concentrations significantly enhancing internalization.

Recently, Lin et al. proposed a strategy for artificially transplanting mitochondria that temporarily enhances the bioenergetics of vessel endothelial cells and enables the formation of functional vessels in ischemic tissues without relying on MSC support.[29] Collectively, our study and the above studies suggest that the uptake of exogenous mitochondria is a cell-intrinsic behavior, potentially triggered or enhanced by cellular injury, which may have significant implications for therapeutic interventions aimed at mitigating tissue damage and promoting repair.

Mitochondrial transplantation emerges as a promising strategy to increase cellular energy production, thereby enhancing the cellular response to stress.[31–33] Our findings reveal a significant restoration in the protein expression levels of different respiratory chain complexes subsequent to mitochondrial transplantation. This finding aligns with previous research where the administration of extracted mitochondria into ischemic hearts was found to boost tissue ATP levels and ameliorate cardiac performance.[34,35] Additionally, an intriguing study implemented mitochondrial enhancement by transplanting healthy mitochondria obtained from the child's mother into the child's $CD34^+$ hematopoietic stem cells, followed by reinfusion of these stem cells into the child. Notably, this intervention led to a sustained increase in mitochondrial DNA content within peripheral blood cells among all treated children over a 6- to 12-month period.[36] Rossi et al.[37] further investigated the underlying mechanism of this enhancement, revealing that mitochondrial transplantation in an ischemia–reperfusion injury model resulted in the restoration of TCA cycle enzymes such as citrate synthase, α-ketoglutarate, succinate, and malate dehydrogenase. The activation of these enzymes along with those of the electron transport chain ultimately led to an elevation in intracellular ATP level comparable to nonischemic controls. Our results align with previous findings on the potential of mitochondrial transplantation to enhance cellular energy production and stress response, and they specifically underscore the role of mitochondrial transplantation in modulating the expression of respiratory chain complex proteins, suggesting its potential as a therapeutic intervention for conditions associated with mitochondrial dysfunction.

Our study demonstrates that, in a mouse model of acid burn, using mitochondria to enhance repair led to a significant reduction in inflammatory cell infiltration and a clear speeding up of the healing process for the epithelial layer. In our previous research on intercellular mitochondrial transport, we found that MSCs could transfer their mitochondria to the corneal epithelium in rabbit with corneal alkali burns.[19] The effectiveness of stem cell-mediated mitochondrial transfer has been substantiated by earlier studies.[38–40] However, it's important to note that stem cell therapy has not been extensively advocated over the last two decades, possibly due to ethical concerns about their source as well as the risk of tumorigenesis.[41] Nussbaum et al.[42] demonstrated that transplantation of undifferentiated mouse embryonic stem cells into the heart led to teratoma formation. Encouragingly, a clinical study has effectively exhibited the preliminary clinical application of autologous mitochondrial transplantation for the purpose of myocardial recovery in pediatric patients undergoing extracorporeal membrane oxygenation support for ischemia–reperfusion injury.[43] This clinical study confirmed the safety and efficacy of autologous mitochondrial therapy, aligning with our own findings and reinforcing the concept that mitochondrial transplantation may represent a pivotal shift from cell therapy to organelle therapy as a novel treatment modality.

Conclusions

Our findings indicate that the administration of isolated mitochondria facilitates the repair of corneal acid burns in a mouse model. The results of our investigation using isolated mitochondria as a treatment modality for corneal acid burns provide a novel approach to managing ocular disorders associated with mitochondrial dysfunction.

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cultured the cells, and critically reviewed the manuscript. Dan Jiang, Qizhou Lian: writing- reviewing and Editing. Wei Chen, Supervision. All authors read and approved the final manuscript.

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Supplementary Material

Supplementary Movie S1. Dynamics of mitochondrial internalization in 24h Co-Cultured Cells.
Supplementary Movie S2. Mitochondrial internalization during cell cluster migration.

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