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Research Article

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Mitochondria derived from human embryonic stem cell-derived mesenchymal stem cells alleviate the inflammatory response in human gingival fibroblasts

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Abstract: Periodontitis is a common oral disease caused by bacteria coupled with an excessive host immune response. Stem cell therapy can be a promising treatment strategy for periodontitis, but the relevant mechanism is complicated. This study aimed to explore the therapeutic potential of mitochondria from human embryonic stem cell-derived mesenchymal stem cells (hESC-MSCs) for the treatment of periodontitis. The gingival tissues of periodontitis patients are characterized by abnormal mitochondrial structure. Human gingival fibroblasts (HGFs) were exposed to 5 μg/mL lipopolysaccharide (LPS) for 24 h to establish a cell injury model. When treated with hESC-MSCs or mitochondria derived from hESC-MSCs, HGFs showed reduced expression of inflammatory genes, increased adenosine triphosphate (ATP) level, decreased reactive oxygen species (ROS) production, and enhanced mitochondrial function compared to the control. The average efficiency of isolated mitochondrial transfer by hESC-MSCs was determined to be 8.93%. Besides, a therapy of local mitochondrial injection in mice with LPS-induced periodontitis showed a reduction in inflammatory gene expression, as well as an increase in both the mitochondrial number and the aspect ratio in gingival tissues. In conclusion, our results indicate that mitochondria derived from hESC-MSCs can reduce the inflammatory response and improve mitochondrial function in HGFs, suggesting that the transfer of mitochondria between hESC-MSCs and HGFs serves as a potential mechanism underlying the therapeutic effect of stem cells.

Key words: Human embryonic stem cell-derived mesenchymal stem cells (hESC-MSCs); Mitochondrial transfer; Inflammatory response; Mitochondrial dysfunction; Periodontal disease

1 Introduction

Periodontitis is an inflammatory disease that can damage the soft and hard tissues supporting teeth. Human gingival fibroblasts (HGFs), crucial to the connective tissue of gums, play an important role in the defense against pathogenic bacteria and in restoring tissue health. However, excessive host immune responses can lead to harmful effects, such as tissue destruction and an imbalance in the oxidation-reduction system (Chapple and Matthews, 2007; Liu et al., 2017).

The mechanisms by which mesenchymal stem cells (MSCs) mitigate periodontitis are multifaceted. A particularly noteworthy element is mitochondrial transfer, as mitochondrial dysfunction plays a critical role in the pathogenesis and treatment of periodontitis (Huang et al., 2020; Jiang et al., 2023). Recent research has shown that mitochondrial dysfunction is present in gingival tissues (Sun et al., 2017), and abnormal mitochondrial structures have been observed in gingival tissues and HGFs derived from patients with periodontitis (Liu J et al., 2022, 2023). The above studies underscore the significant role of mitochondria in the pathogenesis of periodontitis and signify mitochondrial dysfunction as a promising therapeutic target.

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The transfer of healthy mitochondria from MSCs to injured target cells is an ongoing research topic (Liu et al., 2021). While mitochondrial transfer has shown promise in alleviating severe inflammatory symptoms under conditions like asthma, stroke, and myocardial infarction (Paliwal et al., 2018), limited research has been conducted on its role in periodontitis.

Human embryonic stem cell-derived MSCs (hESC-MSCs) are a subtype of MSCs derived from pluripotent stem cells (PSC-MSCs). Compared to MSCs derived from somatic tissues (st-MSCs), PSC-MSCs have achieved superior therapeutic effects through mechanisms such as mitochondrial transfer, paracrine signaling, and exosomes (Seo and Jeon, 2022).

This study analyzed the inflammatory response and mitochondrial function after treatment with hESC-MSCs and their derived mitochondria. The objectives were to explore the ability of hESC-MSCs to reduce the inflammatory response and mitochondrial dysfunction in HGFs and to elucidate the role of mitochondria in the rescue process.

2 Results

2.1 Abnormal mitochondrial structure and cell injury observed in the gingival tissues of patients with chronic periodontitis

First, to determine changes in mitochondria, we examined the mitochondria in chronic periodontitis (CP) patients and healthy controls (HCs) by transmission electron microscopy (TEM). We found that the mitochondria in the CP patients had abnormal structures, with swelling due to membrane disruption and the disappearance of cristae. In comparison, the mitochondria in HCs were more oval-shaped, with a larger aspect ratio and clear cristae (Fig. 1a).

Next, we assessed cell injury in the CP patients. The collagen fibers were loosely arranged and disordered, and there was a higher density of inflammatory cells compared to HCs (Fig. 1b). To demonstrate cell injury, DNA breakage was detected by terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL) staining, and more positive points were observed in the nuclei in the CP patients (Fig. 1c).

We also detected the expression levels of certain genes and proteins related to the inflammatory process. The messenger RNA (mRNA) levels of interleukin-6 (*IL-6*), NOD-like receptor family pyrin domain-containing 3 (*NLRP3*), and *IL-1β* in gingival tissues were higher in CP patients than HCs (Fig. 1d), along with higher protein expression of NLRP3, the stimulated form of Caspase-1, and IL-1β (Fig. 1e).

2.2 Mitochondrial dysfunction detected in LPS-induced HGFs

To determine the concentration of lipopolysaccharide (LPS) that induces an inflammatory response and mitochondrial dysfunction, we initially exposed HGFs to various concentrations of LPS (0, 0.1, 0.5, 1.0, 5.0, and 10.0 μg/mL). We found that LPS raised the mRNA expression of inflammatory factors (*IL-6* and *IL-1β*) particularly at 5.0 μg/mL, and the mRNA expression of *NLRP3* was also markedly elevated at 5.0 μg/mL (Fig. 2a). Our data also revealed that cell viability significantly decreased when the LPS concentration exceeded 1.0 μg/mL (Fig. 2b).

When comparing the mitochondrial function of HGFs treated with or without 5.0 μg/mL LPS, we observed a lower fluorescence ratio of aggregates versus monomers in HGFs stimulated by LPS, indicating that LPS treatment at that concentration resulted in a reduced mitochondrial membrane potential (Fig. 2c). LPS-stimulated HGFs generated more reactive oxygen species (ROS) than the control group (Fig. 2d). Regarding mitochondrial respiration, LPS at 5 μg/mL significantly diminished both adenosine triphosphate (ATP) production and basal respiration in HGFs (Fig. 2e).

In summary, we demonstrated that 5 μg/mL LPS induced an inflammatory response and mitochondrial dysfunction in HGFs. Hence, this concentration was chosen for the subsequent experiments.

2.3 Effects of hESC-MSCs on the inflammatory response in HGFs after mitochondrial transfer

As previously mentioned, we constructed a model of mitochondrial dysfunction using 5 μg/mL LPS treatment on HGFs, so as to explore the rescue capability of hESC-MSCs. Three techniques are available to treat injured HGFs with hESC-MSCs: indirect Transwell co-culture (Group IC), direct co-culture (Group DC), and supernatant of hESC-MSC-conditioned medium (Group SUP) (Fig. 3a).

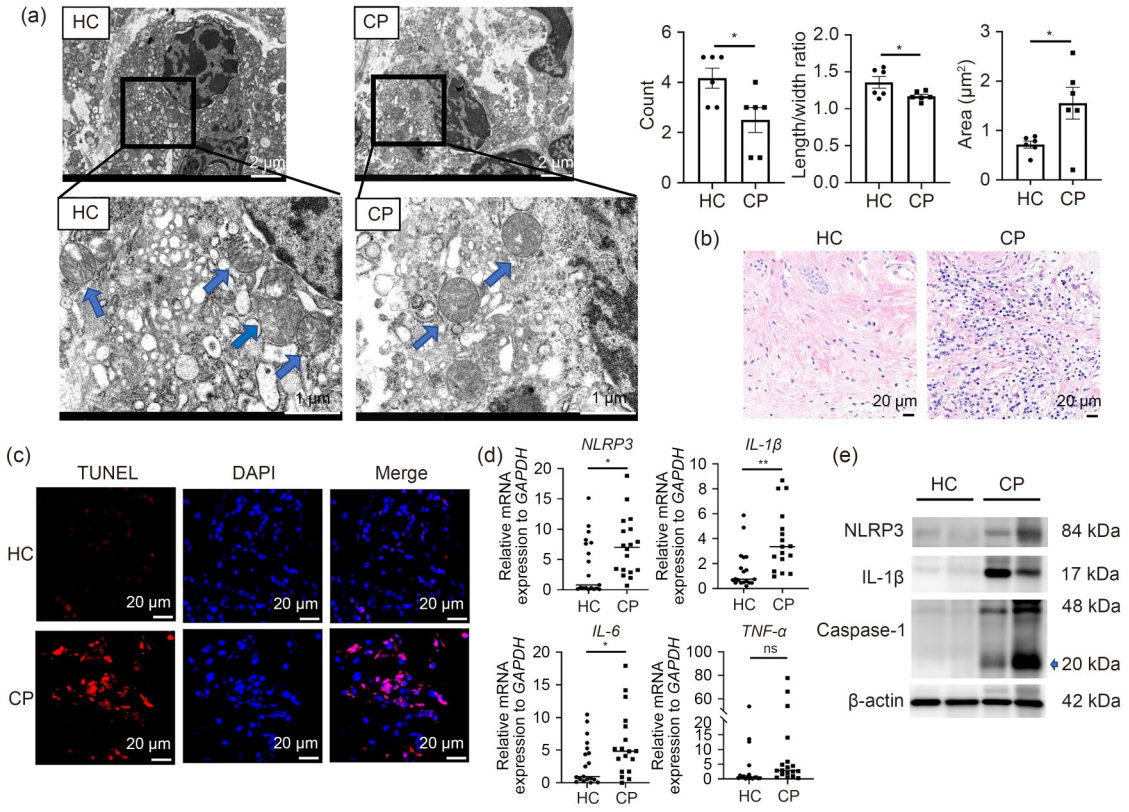

Fig. 1 Abnormal mitochondrial structure and cell injury observed in the gingival tissues of patients with chronic periodontitis. (a) The substructure of mitochondria in gingival tissues was observed by TEM. The arrows indicate mitochondria. The mitochondria in the HC group are normal in size with a larger aspect ratio, and the double membrane structure and cristae are clear. The mitochondria from the CP group show abnormal structure, including swelling, enlargement, and loss of cristae. The mitochondrial count, length/width ratio, and area were calculated (*n*=6). (b) H&E staining shows the structure of gingival tissues from donors with or without chronic periodontitis. (c) Immunofluorescence assay of TUNEL. (d) Relative mRNA expression of *NLRP3*, *IL-1β*, *IL-6*, and *TNF-α* in gingival tissues in the HC (*n*=19) and CP (*n*=17 or *n*=18) groups. (e) The protein expression levels of NLRP3, IL-1β, Caspase-1, and β-actin were measured by western blot. Data were presented as mean±SEM. Unpaired two-tailed Student's *t*-test was conducted. **P*<0.05, ***P*<0.01; ns: not significant. CP: chronic periodontitis; DAPI: 4',6-diamidino-2-phenylindole; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; HC: healthy control; H&E: hematoxylin and eosin; IL: interleukin; mRNA: messenger RNA; NLRP3: NOD-like receptor family pyrin domain-containing 3; SEM: standard error of the mean; TEM: transmission electron microscopy; TNF-α: tumor necrosis factor-α; TUNEL: terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling.

Initially, we performed cell membrane staining for HGFs and mitochondrial staining for hESC-MSCs, followed by 24 h of direct co-culture (Group DC). Notably, we observed mitochondria with green staining within the HGFs, which confirmed the transfer of mitochondria from hESC-MSCs to HGFs (Fig. 3b). Then, we quantified the efficiency of mitochondrial transfer by hESC-MSCs using flow cytometry (Fig. 3c). The average efficiency was determined to be 33.55%.

Next, to further evaluate the rescue effects of hESC-MSCs, we established Groups IC and SUP, and detected the supernatants of Groups DC, IC, and SUP. The results showed that Group DC had the lowest IL-6 concentration in the supernatant among these groups (Fig. 3d). Both Groups IC and SUP showed the ability to alleviate the inflammatory response in HGFs, with reduced mRNA expression levels of inflammatory markers (*IL-6*, *IL-1β*, and tumor necrosis factor-α (*TNF-α*)) and *NLRP3* (Fig. 3e).

A comparison between Groups DC, IC, and SUP suggested that hESC-MSCs alleviated inflammation in various ways. Notably, Group DC exhibited a relatively good effect on reducing IL-6 (Fig. 3d). Meanwhile, Group IC showed better effects than Group SUP on reducing mRNA expression levels of *IL-1β* and *NLRP3* (Fig. 3e), indicating that the existence of hESC-MSCs made a difference and the transfer of mitochondria could be responsible. We

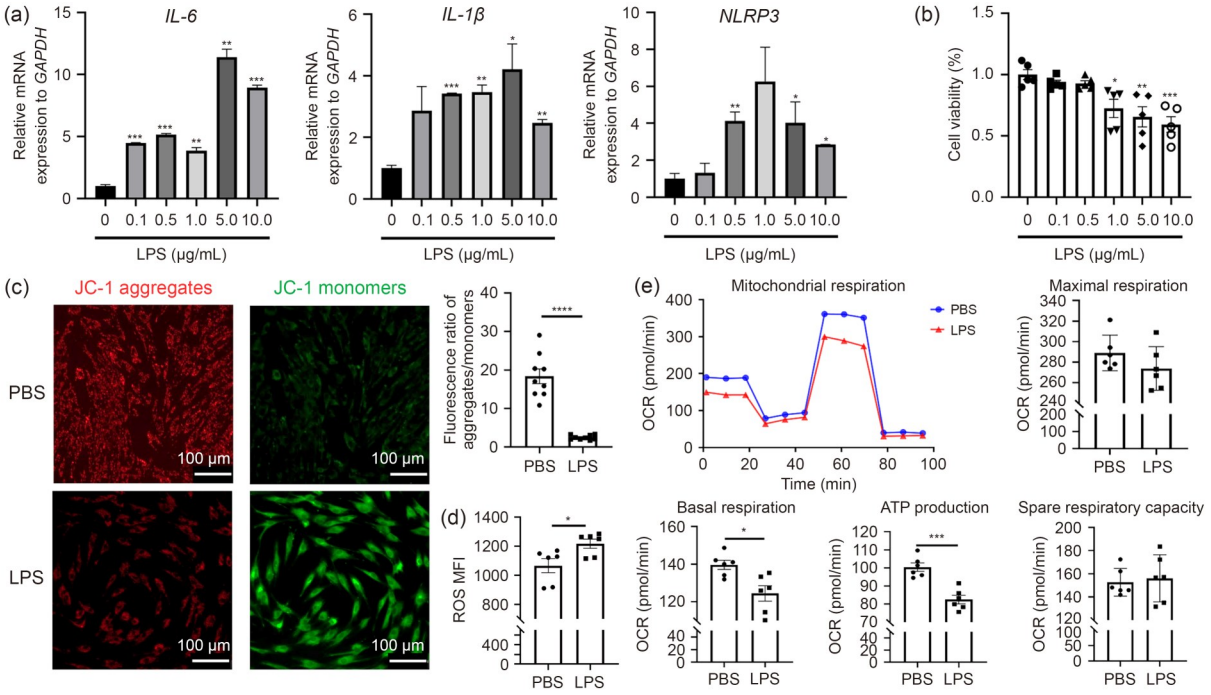

Fig. 2 LPS-induced inflammatory response and mitochondrial dysfunction of HGFs. (a, b) HGFs were treated with different concentrations of LPS (0, 0.1, 0.5, 1.0, 5.0, and 10.0 μg/mL). (a) Relative mRNA expression of *IL-6*, *IL-1β*, and *NLRP3* (*n*=3). (b) Cell viability was determined by CCK8 assays (*n*=5). (c–e) HGFs were treated with or without 5.0 μg/mL LPS. (c) Representative fluorescence images of JC-1 aggregates and monomers. The fluorescence ratio of aggregates to monomers was analyzed (*n*=9). (d) MFI of ROS was analyzed by flow cytometry (*n*=6). (e) Representative Seahorse Mito Stress assay. Calculated values for respiratory parameters: basal respiration, ATP production, maximal respiration, and spare respiratory capacity (*n*=6). Data were presented as mean±SEM; Unpaired two-tailed Student's *t*-test was conducted. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$, compared to Group PBS. ATP: adenosine triphosphate; CCK8: cell counting kit 8; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; HGFs: human gingival fibroblasts; IL: interleukin; JC-1: 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide; LPS: lipopolysaccharide; MFI: median fluorescence intensity; mRNA: messenger RNA; NLRP3: NOD-like receptor family pyrin domain-containing 3; OCR: oxygen consumption rate; PBS: phosphate-buffered saline; ROS: reactive oxygen species; SEM: standard error of the mean.

then performed cell membrane staining for HGFs and mitochondrial staining for hESC-MSCs. Interestingly, we found mitochondria with green staining in the HGFs, indicating the transfer of mitochondria from hESC-MSCs to HGFs through the Transwell assay. To prevent the influence of the dyeing solution, we used the supernatant of the last wash buffer (Fig. 3f).

2.4 Effects of mitochondria derived from hESC-MSCs on the inflammatory response and mitochondrial dysfunction in HGFs

To gather additional evidence regarding the functionality of the transferred mitochondria in HGFs, we isolated mitochondria from hESC-MSCs and co-cultured them with HGFs (Group MITO). HGFs either indirectly cultured with hESC-MSCs or co-cultured with exogenous mitochondria showed elevated mitochondrial membrane potential (Fig. 4a) and diminished ROS production (Fig. 4b). The mitochondrial function of HGFs appeared to be a little stronger in indirect cultures than in co-cultures with mitochondria, while the difference was nonsignificant.

In addition, we found that isolated mitochondria significantly improved mitochondrial respiratory function in HGFs, as indicated by increases in basal respiration, ATP production, maximal respiration, and spare respiratory capacity (Fig. 4c).

To substantiate the occurrence of mitochondrial transfer to HGFs, we performed staining followed by isolating the mitochondria from hESC-MSCs and co-culturing them for 24 h. Staining revealed the presence of green mitochondria within HGFs (Fig. 4d). Additionally, we assessed the efficiency of mitochondrial transfer by flow cytometry (Fig. 4e), with an average efficiency of 8.93%.

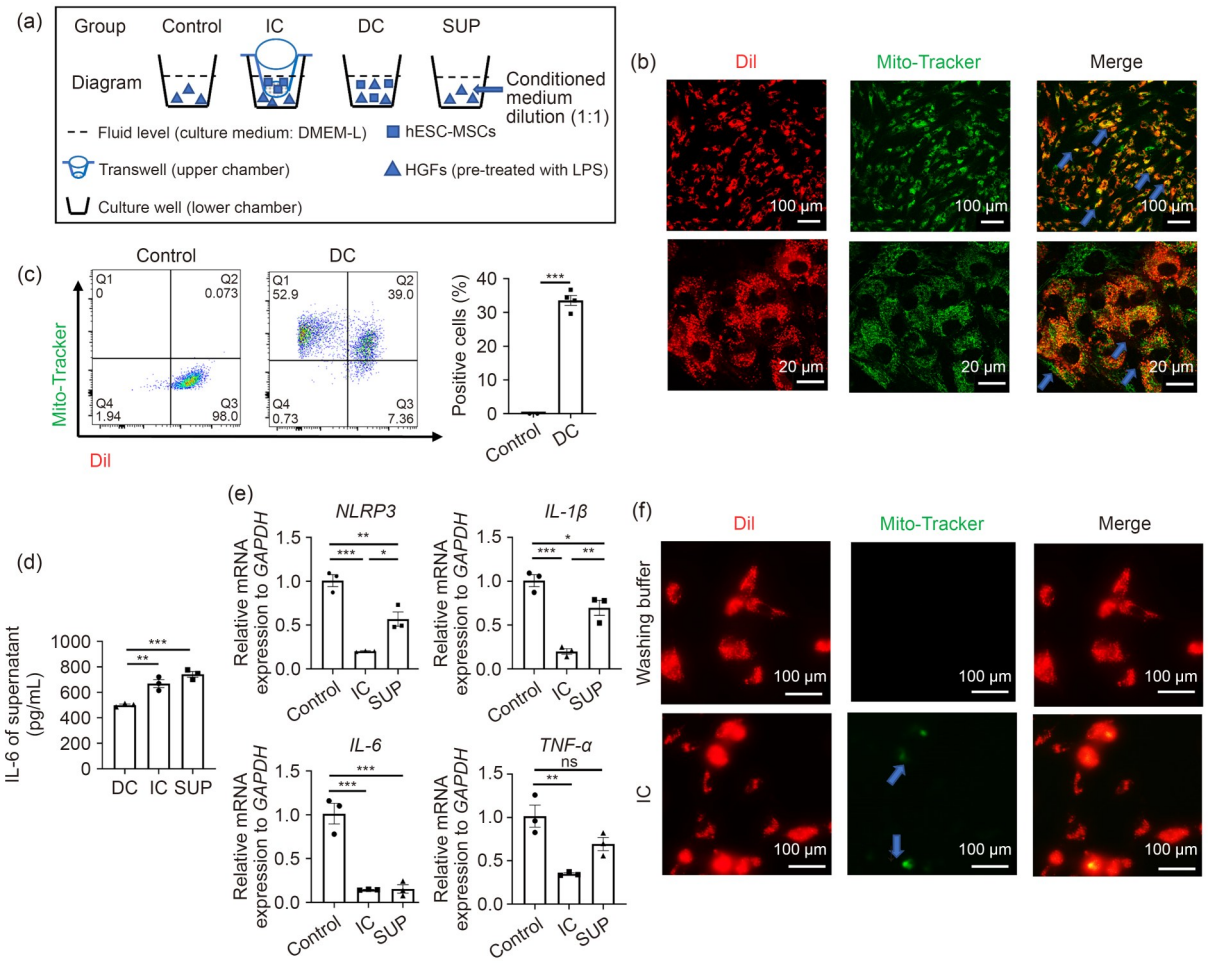

Fig. 3 Effects of hESC-MSCs on the inflammatory response in HGFs through mitochondria. HGFs were pretreated with 5 μg/mL LPS. (a) Grouping diagram: HGFs were cultured alone (Group control), indirectly co-cultured with hESC-MSCs by Transwell (Group IC), directly co-cultured with hESC-MSCs (Group DC), or cultured with supernatant of hESC-MSC-conditioned medium (Group SUP). (b) Confocal fluorescence images of Group DC. Mitochondria from hESC-MSCs were labeled with Mito-Tracker and HGFs were stained with Dil. They were directly co-cultured for 24 h. The arrows show that the mitochondria derived from hESC-MSCs were transferred to HGFs. (c) Representative scatter charts of Groups control and DC. The transfer subset ($FITC^+/PE^+$) cells were detected by flow cytometry (n=4). (d) IL-6 content of supernatant after 24 h of culture (n=3). (e) Relative mRNA expression of *NLRP3*, *IL-1β*, *IL-6*, and *TNF-α* (n=3). (f) Fluorescence images of Group IC. HGFs were cultured with the last washing buffer during Mito-Tracker staining (upper). HGFs were cultured with hESC-MSCs labeled with Mito-Tracker by Transwell (lower). The arrows show mitochondria from hESC-MSCs in HGFs. Data were presented as mean±SEM. Unpaired two-tailed Student's t-test was conducted. $^*P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001; ns: not significant. DMEM-L: Dulbecco's modified Eagle's medium-low glucose; FITC: fluorescein isothiocyanate; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; hESC-MSCs: human embryonic stem cell-derived mesenchymal stem cells; HGFs: human gingival fibroblasts; IL: interleukin; LPS: lipopolysaccharide; NLRP3: NOD-like receptor family pyrin domain-containing 3; PE: phycoerythrin; SEM: standard error of the mean; TNF-α: tumor necrosis factor-α.

Moreover, we observed that HGFs co-cultured with isolated mitochondria displayed lower mRNA expression levels of several inflammatory markers, including *NLRP3*, *IL-1β*, and *IL-6* (Fig. 4f). The protein expression levels of NLRP3, IL-1β, gasdermin-D (GSDMD), and activated Caspase-1 also decreased (Fig. 4g).

2.5 Effects of mitochondria derived from hESC-MSCs on the recovery of experimental periodontitis in mice

We established an experimental periodontitis mouse model to evaluate the therapeutic effects of hESC-MSC-derived mitochondria (Fig. 5a). After

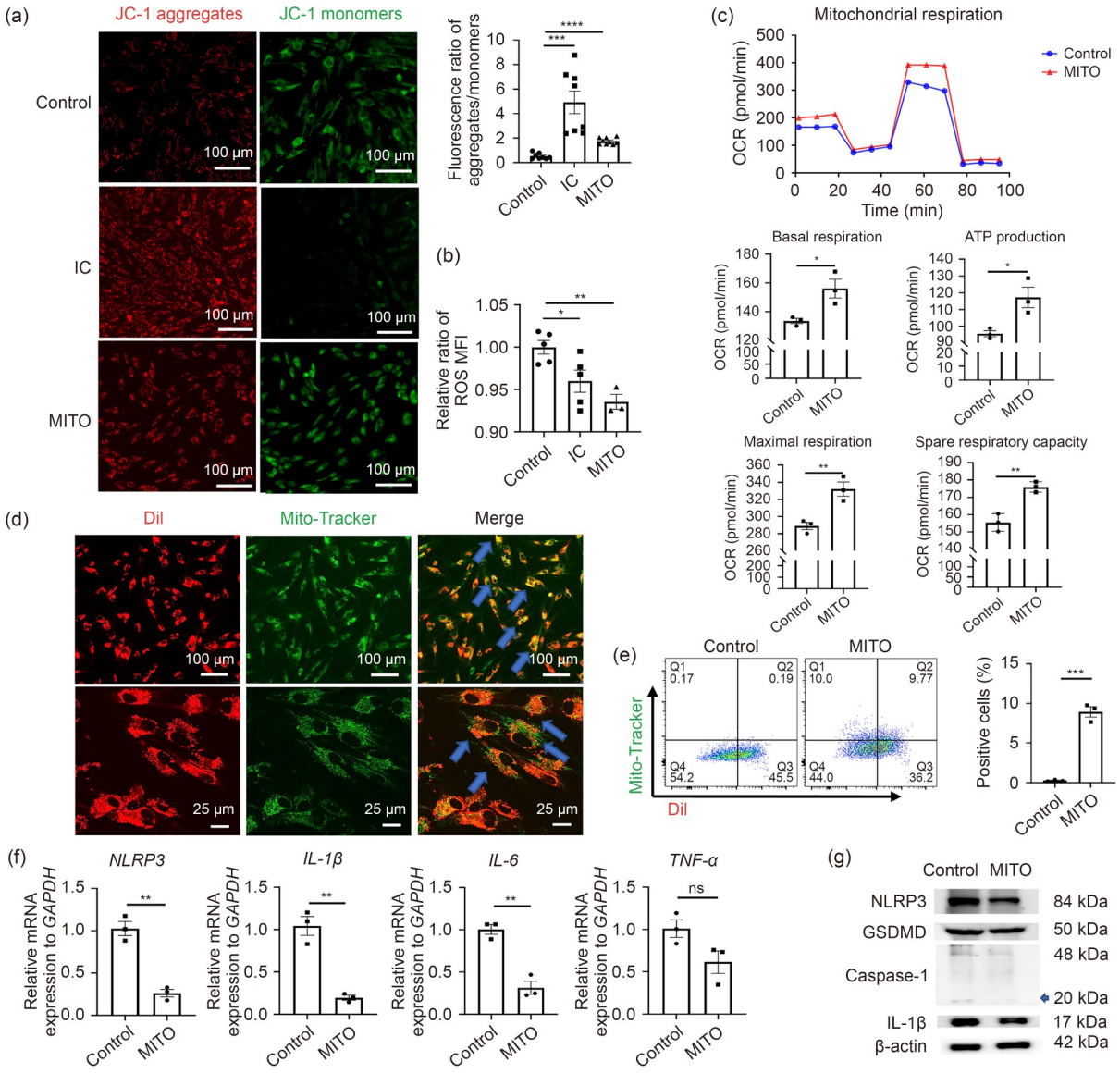

Fig. 4 Effects of mitochondria derived from hESC-MSCs on the inflammatory response and mitochondrial dysfunction in HGFs. HGFs were pretreated with 5 μg/mL LPS. HGFs were cultured with mitochondria derived from hESC-MSCs (Group MITO), indirectly cultured with hESC-MSCs (Group IC), or cultured alone (Group control). (a) Representative fluorescence images of JC-1 aggregates and monomers. The fluorescence ratio of aggregates to monomers was analyzed (*n*=8). (b) The MFI of ROS was analyzed by flow cytometry (*n*=3 or 5). (c) Representative Seahorse Mito Stress assay. Calculated values for respiratory parameters: basal respiration, ATP production, maximal respiration, and spare respiratory capacity (*n*=3). (d) Confocal fluorescence images of Group MITO. Mitochondria derived from hESC-MSCs were labeled with Mito-Tracker and HGFs were stained with Dil, and then co-cultured for 24 h. The arrows indicate that labeled mitochondria from hESC-MSCs appeared in HGFs. (e) Representative scatter charts of Group control and Group MITO. The transfer subset ($FITC^+/PE^+$) cells were detected by flow cytometry (*n*=3). (f) Relative mRNA expression of *NLRP3*, *IL-1β*, *IL-6*, and *TNF-α* (*n*=3). (g) The protein expression levels of NLRP3, GSDMD, Caspase-1, IL-1β, and β-actin were measured by western blot. Data are presented as mean±SEM. Unpaired two-tailed Student's *t*-test was conducted. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$; ns: not significant. ATP: adenosine triphosphate; GSDMD: gasdermin-D; FITC: fluorescein isothiocyanate; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; hESC-MSCs: human embryonic stem cell-derived mesenchymal stem cells; HGFs: human gingival fibroblasts; IL: interleukin; JC-1: 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide; LPS: lipopolysaccharide; MFI: median fluorescence intensity; mRNA: messenger RNA; NLRP3: NOD-like receptor family pyrin domain-containing 3; OCR: oxygen consumption rate; PE: phycoerythrin; ROS: reactive oxygen species; SEM: standard error of the mean; TNF-α: tumor necrosis factor-α.

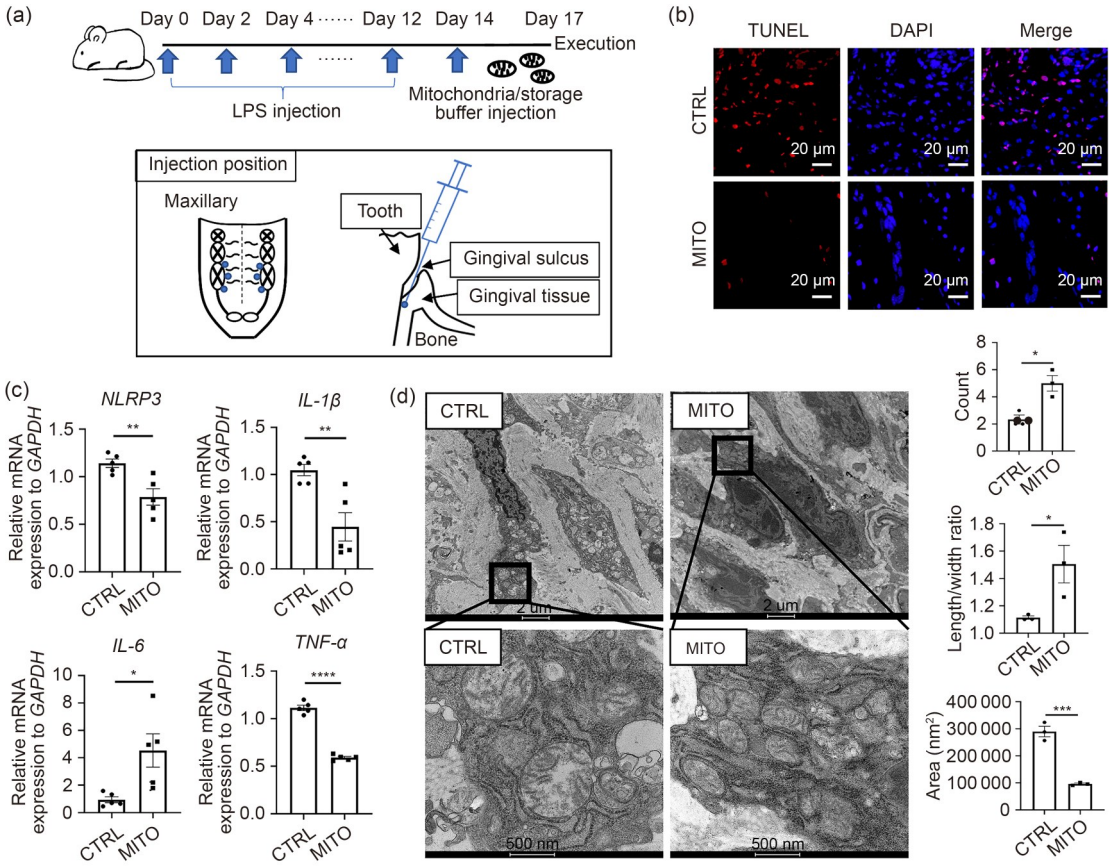

Fig. 5 Effects of mitochondria derived from hESC-MSCs on the recovery of experimental periodontitis in mice. (a) Diagram of the animal experimentation process. The injection position was in the palatal gingival sulcus of the first maxillary molar, which was indicated by the dots. (b) Gingival tissue sections stained with TUNEL and DAPI. (c) Relative mRNA expression of *NLRP3*, *IL-1β*, *IL-6*, and *TNF-α* (n=5). (d) The substructure of mitochondria of gingival tissues observed by TEM. The mitochondria from Group CTRL show swelling, enlargement, and loss of cristae. The mitochondria from Group MITO are smaller and have a larger aspect ratio. The double membrane structure and cristae are clear. The mitochondrial count, length/width ratio, and area were calculated (n=3). Data were presented as mean±SEM. Unpaired two-tailed Student's t-test was conducted. $^{*}P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001, $^{****}P$<0.0001. CTRL: control; DAPI: 4',6-diamidino-2-phenylindole; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; hESC-MSCs: human embryonic stem cell-derived mesenchymal stem cells; IL: interleukin; LPS: lipopolysaccharide; mRNA: messenger RNA; NLRP3: NOD-like receptor family pyrin domain-containing 3; TEM: transmission electron microscopy; SEM: standard error of the mean; TNF-α: tumor necrosis factor-α; TUNEL: terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling.

treatment with mitochondria, fewer injured cells were observed in the gingival tissues (Fig. 5b). We assessed the mRNA expression of inflammatory markers, including *NLRP3*, *IL-1β*, *IL-6*, and *TNF-α*. Intriguingly, the levels of *NLRP3*, *IL-1β*, and *TNF-α* showed a significant reduction after being treated with mitochondria, whereas the *IL-6* levels increased (Fig. 5c). The decreased expression of *NLRP3*, *IL-1β*, and *TNF-α* in Group MITO indicated reduced inflammatory response and enhanced recovery.

To examine the potential changes in mitochondrial morphology within gingival tissues, we analyzed the mitochondrial substructures using TEM. In Group CTRL, numerous swollen and enlarged vacuolar mitochondria were observed within cells. Conversely, the mitochondria in the cells from Group MITO displayed an oval shape with a larger aspect ratio and well-defined cristae (Fig. 5d). These observations provided evidence that mitochondrial health and structure improved in the MITO group.

3 Discussion

In this study, we identified a phenomenon wherein mitochondria from hESC-MSCs could be transferred

into HGFs to mitigate the inflammatory response and ameliorate LPS-induced mitochondrial dysfunction in HGFs. Additionally, mitochondria derived from hESC-MSCs could effectively rescue injured HGFs and facilitate recovery in an experimental mouse model of periodontitis. These findings highlight the therapeutic potential of mitochondrial transfer in periodontal treatment.

Our observations also revealed that the inflammatory response and mitochondrial dysfunction can be ameliorated by MSCs. This concurs with findings of earlier studies that documented the anti-inflammatory effects and modulation of mitochondrial function through MSC therapy (Paknejad et al., 2015; Rodríguez-Fuentes et al., 2021). For instance, MSC-derived exosomes have been reported to improve alveolar-capillary barrier properties by restoring mitochondrial function (Dutra Silva et al., 2021). Studies have also shown that MSCs and their exosomes can counteract cigarette smoke-induced mitochondrial dysfunction in mice (Maremanda et al., 2019; Li et al., 2021), and human umbilical cord-derived MSCs can mitigate age-associated skeletal muscle dysfunction by modulating mitochondrial damage in mice (Piao et al., 2022). Although these investigations were not conducted in the context of periodontal disease, they underscore the potential of MSCs to regulate mitochondrial function in a more general scope.

We observed isolated mitochondria from hESC-MSCs transferred to HGFs and detected an enhanced mitochondrial function and a reduced inflammatory response in damaged cells. These results align with earlier studies showing that transferring healthy mitochondria into damaged cells can improve mitochondrial biosynthesis, aerobic respiration, and ATP production while reducing reactive stress (Levoux et al., 2021). Transferring healthy mitochondria from stem cells to supplant damaged ones in injured cells has emerged as a promising therapeutic strategy for periodontitis. Mitochondrial transfer has been observed in MSCs derived from various tissues, such as bone marrow, umbilical cord, and fat (Shanmughapriya et al., 2020). Some MSC-derived extracellular vesicles (EVs) (MSC-EVs) contain mitochondria that can transfer to human small airway epithelial and pulmonary microvascular endothelial cells (Ren et al., 2020).

On the other hand, treatment with isolated mitochondria did not attain therapeutic advantages over indirect co-culture. This was reasonable because the compounds secreted by MSCs can also boost periodontal function (Chew et al., 2019; Regmi et al., 2019; Nakao et al., 2021). Therefore, mitochondrial transfer is considered to play a role in the hESC-MSC rescue effect.

Tunnelling nanotubes (TNTs), EVs, and free mitochondrial capture are the most popular routes of intercellular mitochondrial transport (Borcherding and Brestoff, 2023). The mechanism of mitochondrial transfer in restoring injured cells remains unclear, but it is always related to cell energy metabolism and mitochondrial quality control. Under pathological conditions, healthy mitochondria are transferred from donors to recipients to replace the damaged ones and thus improve cell respiration recovery and biogenesis and decrease oxidative stress (Liu et al., 2021). Mitochondrial transfer contributes to the classic mitochondrial quality control mechanism, which includes mitophagy, mitochondrial fission/fusion, mitochondrial biogenesis, and proteome quality control. The natural transfer of mitochondria from MSCs and the artificial transplantation of exogenous mitochondria could also trigger mitophagy in recipient cells, resulting in cytoprotective effects, including an enhanced engraftment capability (Lin et al., 2024). In addition, transferred mitochondria can act as stimulants. By transferring a small number of mitochondria, exogenous mitochondrial DNA (mtDNA) can readily replace or integrate with endogenous mtDNA in recipient cells (Liu Y et al., 2023). However, the mechanism of mitochondrial effect after transfer needs further exploration.

The rescue effect of healthy isolated mitochondria may be related to the inhibition of pyroptosis, with ROS overdose being the key signaling event of pyroptosis. Typical pyroptosis occurs through the inflammasome and GSDMD pathways. LPS can induce NLRP3 and Caspase-1, and then stimulate GSDMD. The cleaved N-terminal domain anchors to the cell membrane, causing the leakage of cell contents, such as IL-1β (Broz et al., 2020). In this study, we tested the protein expression of NLRP3, GSDMD, Caspase-1, and IL-1β, and the results showed that mitochondria derived from hESC-MSCs decreased the expression of pyroptosis-related proteins in HGFs stimulated by LPS. The mice treated with mitochondria also presented a significant inhibition on the gene expression of *NLRP3* and *IL-1β*. The mechanism between

mitochondrial transfer and pyroptosis of target cells is an interesting issue for further exploration.

Although no specific report has focused on mitochondrial transfer using hESC-MSCs, several studies have highlighted the benefits of using PSC-MSCs. For example, induced PSC-MSCs (iPSC-MSCs) possess a higher capacity for mitochondrial transfer than bone marrow-derived MSCs (BM-MSCs), effectively mitigating the mitochondrial damage induced by cigarette smoke (Li et al., 2014). This increased efficiency is attributed to the higher levels of intrinsic MIRO1, a mitochondrial Rho-GTPase that regulates intercellular mitochondrial movement, in iPSC-MSCs rather than BM-MSCs (Zhang et al., 2016).

However, we noted a higher mitochondrial transfer efficiency than that reported in other studies, where the percentage of transferred mitochondria averaged around 5% without additional stimulation (Anitua et al., 2013). This discrepancy could be attributed to the centrifugation process applied in this study, which may have enhanced the transfer efficiency (Caicedo et al., 2015). As we observed, the uptake of exogenous mitochondria increased proportionally to the quantity of mitochondria used, and the transfer rate could reach as high as (92.7±5.9)% (Kim et al., 2018).

This study has certain limitations. First, we did not directly evaluate mitochondrial function in HGFs co-cultured with hESC-MSCs. The reason is that although a direct co-culture would offer a more comprehensive investigation of mitochondrial transfer, it could lead to reduced cell quantity and viability. Second, it was difficult to explain the paradoxical trend of changes in IL-6 expression when mice were treated with isolated mitochondria. This observation revealed the complexity of mitochondrial therapy in vivo; it was reported that mitochondrial transfer may trigger classic IL-6 signaling and generate anti-inflammatory functions (Xu et al., 2024). Additionally, our animal models of experimental periodontitis may not fully recapitulate the complexities of chronic periodontitis in humans. Despite these limitations, our findings offer initial evidence of the rescue effect of hESC-MSC-derived mitochondria.

4 Conclusions

In summary, our study demonstrated that hESC-MSCs can reduce the inflammatory response and improve mitochondrial function in HGFs via mitochondrial transfer. Moreover, mitochondria derived from hESC-MSCs maintained their ability to ameliorate damaged HGFs and inflamed gingival tissues. These results highlight the critical role of mitochondria in periodontitis and suggest the promising value of mitochondrial therapy for stem cell-based treatments. However, the regulatory mechanisms underlying intercellular mitochondrial transfer and its functional implications in chronic periodontitis remain to be elucidated.

Materials and methods

The full materials and methods are provided in the electronic supplement of this paper.

Data availability statement

The data presented in this study are available from the corresponding author upon reasonable request.

Acknowledgments

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Author contributions

Bicong GAO contributed to the conception and design, acquisition, analysis, and interpretation, and drafted and critically revised the manuscript. Chenlu SHEN and Kejia LV contributed to the acquisition, and drafted and critically revised the manuscript. Xuehui LI, Yongting ZHANG, and Fan SHI contributed to the analysis. Hongyan DIAO and Hua YAO contributed to the conception, design and interpretation, and drafted and critically revised the manuscript. All authors have read and approved the final manuscript, and therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Bicong GAO, Chenlu SHEN, Kejia LV, Xuehui LI, Yongting ZHANG, Fan SHI, Hongyan DIAO, and Hua YAO declare that they have no conflicts of interest.

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (the Research Ethics Committee of The First Affiliated

Hospital, School of Medicine, Zhejiang University, Hangzhou, China (No. IIT20221030A)) and with the Helsinki Declaration of 1975, as revised in 2013. Informed consent was obtained from all patients for being included in the study. Additional informed consent was obtained from all patients for which identifying information is included in this article. All experiments on animals were approved by the Animal Ethics Committee of The First Affiliated Hospital, School of Medicine, Zhejiang University (No. 2022-1601).

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Supplementary information

Materials and methods