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Research article

BMP4 aggravates mitochondrial dysfunction of HRMECs

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ABSTRACT

Mitochondria are important places for the oxidative phosphorylation of glucose and the maintenance of cell oxidation and antioxidant stability. However, mitochondrial dysfunction causes cell dysfunction. Meanwhile, retinal vascular endothelial cell dysfunction may cause vascular inflammation, hemorrhage, angiogenesis, and other manifestations. Our previous studies have shown that Bone morphogenetic protein 4 (BMP4) is an important target for the treatment of retinal neovascularization, but the mechanism remains unclear. Therefore, our study aims to observe the effects of BMP4 on vascular endothelial cells and hopes to provide a new target for diabetic retinopathy. 4-Hydroxynonenal (4HNE), a kind of lipid peroxide, was used to induce the oxidative stress model. Human retinal microvascular endothelial cells (HRMECs) were randomly divided into control, 4HNE, negative control, and siBMP4 groups. *Si*-BMP4 significantly reduced leukocyte adhesion and 4HNE-induced high ROS level and restored the mitochondrial membrane potential and OCR. This indicates that BMP4 plays an important role in inducing leukocyte adhesion, oxidative stress, and mitochondrial dysfunction. The relationship between BMP4 and retinal vascular endothelial cell dysfunction is preliminarily confirmed by this study. Mitochondrial dysfunction and oxidative stress may be involved in BMP4-mediated retinal vascular endothelial cell dysfunction.

1. Introduction

Diabetic retinopathy is a chronic retinal microvascular inflammatory disease, which is the most important disease-causing blindness among the working-age population [1]. The expression of inflammatory factors in diabetic patients is increased, and these inflammatory factors induce leukocyte migration [1,2]. Once leukocytes are interconnected with vascular endothelial cells, these polytypic leukocytes will damage endothelial cells, thus leading to vascular endothelial cell dysfunction. Hence, repeated invasion and obstruction seriously affect the patient's vision [1,2].

Retinal vascular endothelial cell dysfunction may cause vascular inflammation, hemorrhage, angiogenesis, and other

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Abbreviations

RNV	retinal neovascularization
BMP4	Bone morphogenetic protein 4
OIR	Oxygen induced retinopathy
HRMECs	Human retinal microvascular endothelial cells
OCR	oxygen consumption rate
ROS	reactive oxygen species
BS	basal respiration rate
ATP	ATP production
MR	maximal respiration rate
SRC	spare respiratory capacity

manifestations. Mitochondria can supply most of the cellular ATP through oxidative phosphorylation, keep Ca2+ homeostasis, and regulate apoptosis [3]. Therefore, mitochondrial dysfunction will cause cell dysfunction. Mitochondrial dysfunction exists in various diseases and numerous causes can lead to mitochondrial dysfunction such as exposure to harsh environmental conditions and excessive production of reactive free radicals [3]. Meanwhile, oxidative stress is the imbalance between oxidative and antioxidant components in cells, which generally refers to excessive oxidative components. In addition, reactive oxygen species (ROS, broadly refers to free radicals and non-free radicals from oxygen sources; e.g., O_2^-, H_2O_2, OH•, $^1O_2$) refer to chemical reactive chemical substances containing oxygen and are the primary cell oxidants. Mitochondria are an important source of reactive oxygen species. However, mitochondrial dysfunction causes a large number of reactive oxygen species and induces apoptosis. Increased ROS generation by leukocytes at the inflammation site will further aggravate the dysfunction of vascular endothelial cells [4]. Moreover, the large production of ROS will cause the expression of vascular endothelial growth factor (VEGF) and aggravate angiogenesis [5,6].

Bone morphogenic protein4 (BMP4) is a multifunctional growth factor and has been widely studied in cartilage, heart, and nerve. Thus, the role of BMP4 in vascular development has gradually been paid attention to with the in-depth study of BMP4. Furthermore, BMP4 can induce the relocation and activation of VEGFRII on the cell membrane [7]. In addition, BMP4 is closely related to mitochondrial dysfunction/abnormal energy metabolism. In the process of mesoderm differentiation, both pyruvate production and mitochondrial metabolism are essential for BMP4 to induce mesoderm differentiation [8]. Moreover, BMP4 leads to the reduction of mitochondrial volume and the expression of genes as well as oxidative phosphorylase related to mitochondrial biogenesis in brown adipose cells [9]. Therefore, BMP4 plays an important role in angiogenesis and mitochondrial dysfunction. However, there are only a few reports on whether or not BMP4 induces angiogenesis by reducing mitochondrial function.

BMP4 was highly expressed in various models of diabetes both in vivo and in vitro, and the oxidative stress model of retinal vascular endothelial cells was one of them [10]. Thus, in this study, 4HNE, a lipid peroxide, will be used to establish a model of oxidative stress, explore the effects of BMP4 and oxidative stress, and observe the effects of BMP4 on vascular endothelial cells from the perspective of inflammation.

2. Materials and methods

2.1. Human retinal microvascular endothelial cells culture

Herein, HRMECs (Cell Systems Corporation, USA) were grown using ECM plus antibiotics (Lonza) and 10% FBS (Atlanta Biologicals) at 37 °C and 5% CO2. In addition, HRMECs were randomly treated with control (0.01% ethanol), BMP4 (MCE, HY-P7007, 100 ng/mL, 24 h), 4HNE (Sigma, 870608H, 10 μg/mL, 24 h), and siBMP4 (MCE, 10 nmol/L, 24 h), respectively.

2.2. Transfection

Hieff Trans™ In vitro siRNA/miRNA Transfection Reagent was purchased from Yeasen (40806ES03). BMP4 siRNAs were obtained from HIPPO BIOTECHNOLOGY (Zhejiang, China). The sequences were as follows: siBMP4 forward: 5′-AGAUCCACAGCACUGGUCUUGAGUA-3′; reverse: 5′-GGGCUUCCACCGUAUAAACAUUUAU-3′. This was operated by strictly following the instructions. In brief, siBMP4 and transfection reagents were fully mixed in ECM and added to HRMECs. Subsequently, the cell culture medium was changed after 6 h.

2.3. Animal and peripheral blood mononuclear cells (PBMCs) extraction

Healthy male C57 mice aged 8 weeks were purchased from Vital River (Beijing, China). After deep anesthesia, the abdominal cavity of the mice was opened and blood was taken from the apex of the heart. The fresh blood was then slowly spread onto the same volume of ficoll (Solarbio, P4350), and was rinsed with PBS after centrifugation. PBMCs were used for subsequent experiments after centrifugation.

2.4. Endothelial-leukocyte adhesion

In addition, 6×10^5 HRMECs were inoculated into six-well plates and HRMECs in each group were treated with 2 ng/mL human recombinant tumor necrosis factor α (TNF-α, MCE, HY-P70426G) for 6 h at 37 °C. Afterward, peripheral blood mononuclear cells (PBMCs) were obtained from fresh blood. Subsequently, PBMCs in each group were labeled with 2′,7′-bis(2-carboxyethyl)-5 (6)-carboxyfluorescein acetoxymethyl ester (BCECF/AM, 10 μM, Boehringer-Mannheim) in DMEM medium for 45 min. Then, the PBMCs were tiled to HRMECs. TNF-α induced HRMECs were more conducive to the adhesion of PBMC. However, after 6 h, PBMCs that did not adhere to the surface were washed with PBS. Considering that the nucleus of PBMCs is smaller and rounder than that of HRMECs, the number of both PBMCs and HRMECs could be calculated after DAPI staining. Then, BCFCE/AM-labeled PBMCs were detected by flow cytometry analysis of fluorescent signal using a FITC channel.

2.5. ROS production

HRMECs (1.5×10^4) were inoculated into 96-well cell plates with 200 μL culture medium. After the cells reached about 70% confluence, transfection treatment was carried out based on the experimental groups. After 24 h of transfection, the cells were treated with 4-HNE for 24 h. Then, we performed 2,7-dichlorodihydrofluorescein (DCF) diacetate (H2DCFDA) assays (Sigma) to determine intracellular ROS production. Subsequently, cells were incubated for 30 min with 25 μM of DCFH-DA. Moreover, a cell-permeable fluorogenic probe was used to detect ROS. Afterward, cells were washed twice with PBS. A total of 5 μM of mitoSOX was used to measure mitochondrial superoxide following the manufacturer's instructions. Herein, cells were incubated with 5 μM of MitoSOX Red (MCE, HY-D1055) for 10 min at 37 °C and washed with warm PBS thrice. Finally, cells were analyzed using the flow cytometer (CytoFLEX, Beckman).

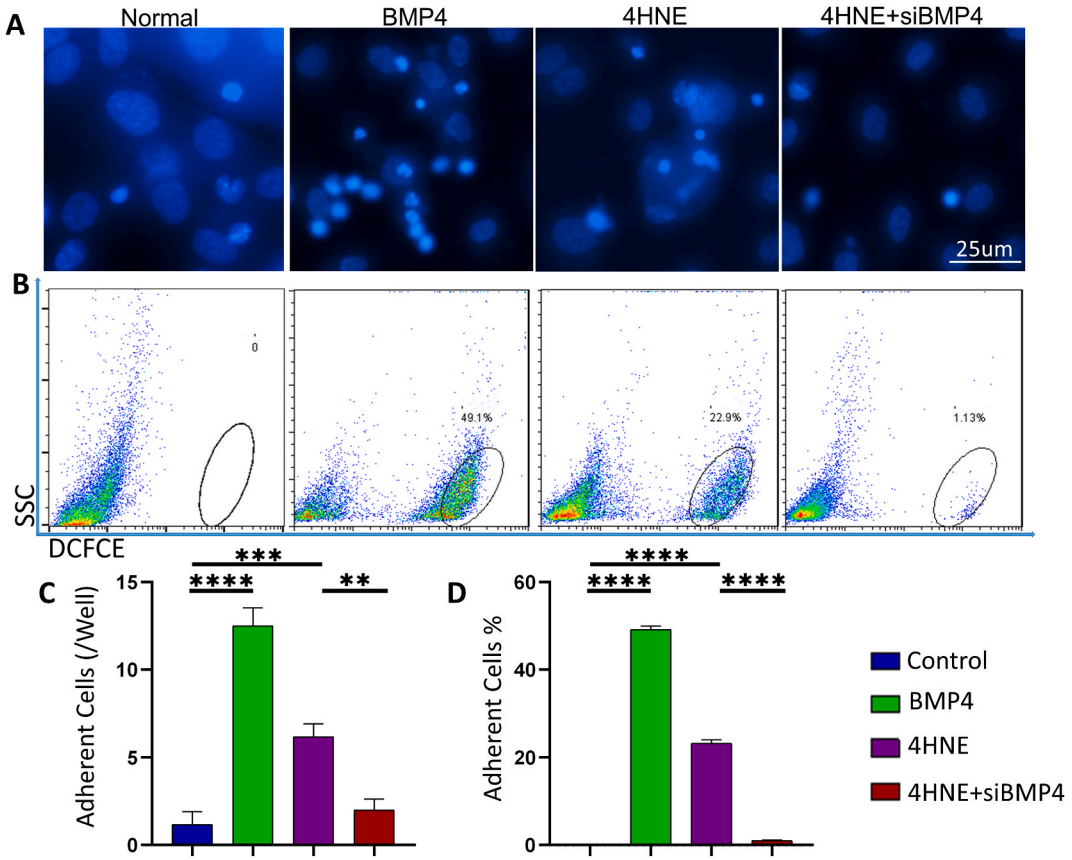

Fig. 1. Impact of BMP4 inhibition on leukocyte adhesion in 4HNE induced human retinal vascular endothelial cells. HRMECs were randomly divided into four groups: control group, BMP4 group, 4HNE group and 4HNE + siBMP4 group. While 0.01% ethanol were done to the control group, the BMP4 group and 4HNE group were treated with BMP4 and 4HNE respectively, and the 4HNE + siBMP4 group was treated with 4HNE and siBMP4. Monocytes were co-cultured with HRMECs, and both the culture conditions and grouping treatment were consistent with those of HRMECs, which were cultured alone. Monocytes adherent to EC were quantified by cell count (A, C) and by flow cytometry (B, D). All experiments were repeated a minimum of three times, and values are represented as mean ± SD, n = 6, **P < 0.01, ****P < 0.0001.

2.6. Mitochondrial membrane potential

Mitochondrial membrane potential (ΔΨm) was estimated using a fluorescent probe JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbo-cyanine iodide) (BD Biosciences). Samples were stained with 2.5 μmol/L JC-1 at 37 °C for 15 min and immediately analyzed by FCM [3]. When the mitochondrial membrane potential is low, JC-1 is a monomer and can produce green fluorescence, thus, the cells with low mitochondrial membrane potential primarily concentrate in quadrant Q3. Similarly, cells with high mitochondrial membrane potential are primarily concentrated in quadrant Q4. Then, the percentage of cells in quadrants Q3 and Q4 is counted.

2.7. Mitochondrial stress test

Herein, HRMECs were measured by Seahorse XFe96 Flux Analyzer® to study the mitochondrial function oxygen consumption rate (OCR). Then, 2×10^4 HRMECs were placed in a 96-well plate. In addition, HRMECs were cultured in an analytical media supplemented with 12 mM glucose, 10 mM HEPES, 1 mM pyruvate, and 2 mM glutamine. Oligomycin (1 μM), FCCP (1 μM), and rotenone/antimycin A (RAA, 2 μM) (Agilent Seahorse XF Cell Mito Stress Test Kit, 103,015–100) were then added successively based on the manufacturer's instructions. When adding FCCP, Hoechst is simultaneously added to the same hole. Cytation I/VI automatically counts cells stained by hoechst for data normalization.

2.8. Statistical analysis

All experiments were performed thrice as a minimum. The quantitative data were expressed as Student's t-test and one-way ANOVA when two groups and when more than two groups are compared, respectively. $P < 0.05$ was considered statistically significant.

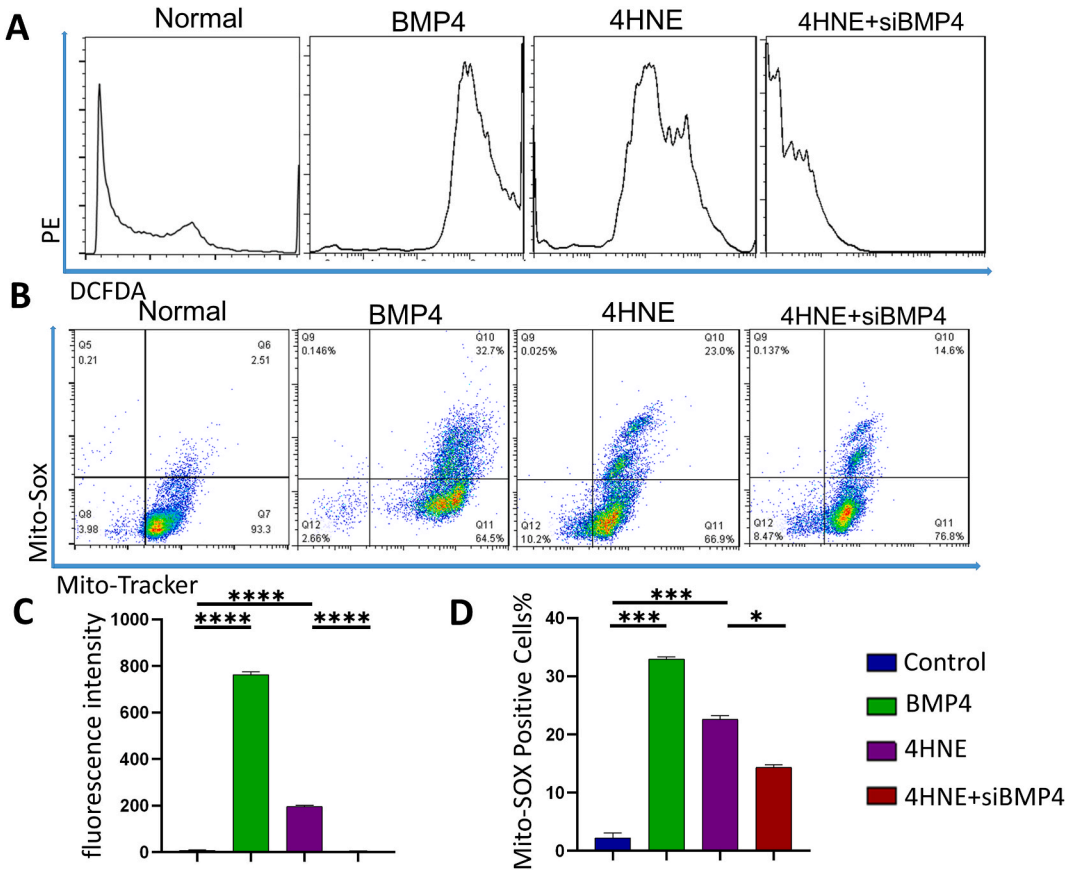

Fig. 2. Impact of BMP4 on ROS production. Histograms for fluorescence were induced by DCFDA upon stimulation of HRMECs for 24 h and its quantification (A, C). The MitoSOX Red Mitochondrial Superoxide Indicator was detected by mitochondrial superoxide probe. Flow cytometry was used to detect superoxide in mitochondria and its quantification (B, D). All experiments were repeated a minimum of three times, and values are represented as mean ± SD, n = 6, ***P < 0.001, ****P < 0.0001.

3. Result

3.1. BMP4 significantly induces the HRMECs-PBMCs adhesion

Leukocyte adhesion to endothelial cells seriously affects the function of endothelial cells, and ultimately leads to the destruction of endothelial cells and the destruction of the vascular barrier [11]. Based on this, we verified the effect of BMP4 on HRMECs-PBMCs adhesion. As shown in Fig. 1A, the nucleus of PBMCs is small and round. The number of PBMCs' nuclei in the BMP4 group was significantly higher than that in the control group, which pointed out that BMP4 induced the HRMECs-PBMCs adhesion (Fig. 1A and C). 4HNE is a product of lipid peroxidation and is often used in intracellular oxidative stress models [12]. The number of PBMCs nuclei in the 4HNE group was significantly higher than that in the control group (Fig. 1A and C), which pointed out that 4HNE-induced oxidative stress significantly promotes the HRMECs-PBMCs adhesion. Interestingly, the number of PBMCs' nuclei in the 4HNE + siBMP4 group was significantly less than that in the 4HNE group (Fig. 1A and C), thereby suggesting that BMP4 mediates the 4HNE-induced HRMECs-PBMCs adhesion. Flow cytometry was used to detect the percentage of labeled PBMCs in total cells. As shown in Fig. 1B, there are almost no PBMCs in the control group, however, the proportion of PBMCs in BMP4 and 4HNE groups is significantly increased, whereas the number of PBMCs in the 4HNE + siBMP4 group is significantly reduced as compared with 4HNE group (Fig. 1B and D). The figure proves again that BMP4 promotes HRMECs-PBMCs adhesion.

3.2. BMP4 significantly induces the level of ROS

Total intracellular ROS was detected to further explain the relationship between BMP4 and oxidative stress. The intracellular ROS in BMP4 and 4HNE groups significantly increased as compared with the control group (Fig. 2A and C), thereby suggesting that BMP4 and 4HNE can induce the production of intracellular ROS. Meanwhile, the intracellular ROS in the 4HNE + siBMP4 group significantly decreased as compared with the 4HNE group (Fig. 2A and C), thereby suggesting that BMP4 participated in the production of intracellular ROS induced by 4HNE. Mitochondria are an important source of active oxygen, and O_2^-, H_2O_2, OH•, and so on, are all products of aerobic metabolism. Mitochondrial superoxide are detected to further explore the effect of BMP4 on cellular reactive oxygen species. BMP4 significantly induces the production of mitochondrial superoxide, which is consistent with that of 4HNE which also induces the production of mitochondrial superoxide, whereas siBMP4 inhibits the rise of 4HNE-induced mitochondrial superoxide (Fig. 2B). The difference was statistically significant (Fig. 2D). Thus, BMP4 can induce the production of intracellular ROS, which may be caused by the increase of mitochondrial superoxide.

3.3. BMP4 significantly reduces mitochondrial membrane potential

The decrease of mitochondrial membrane potential is one of the early event markers of apoptosis [13]. JC-1 is an ideal probe to

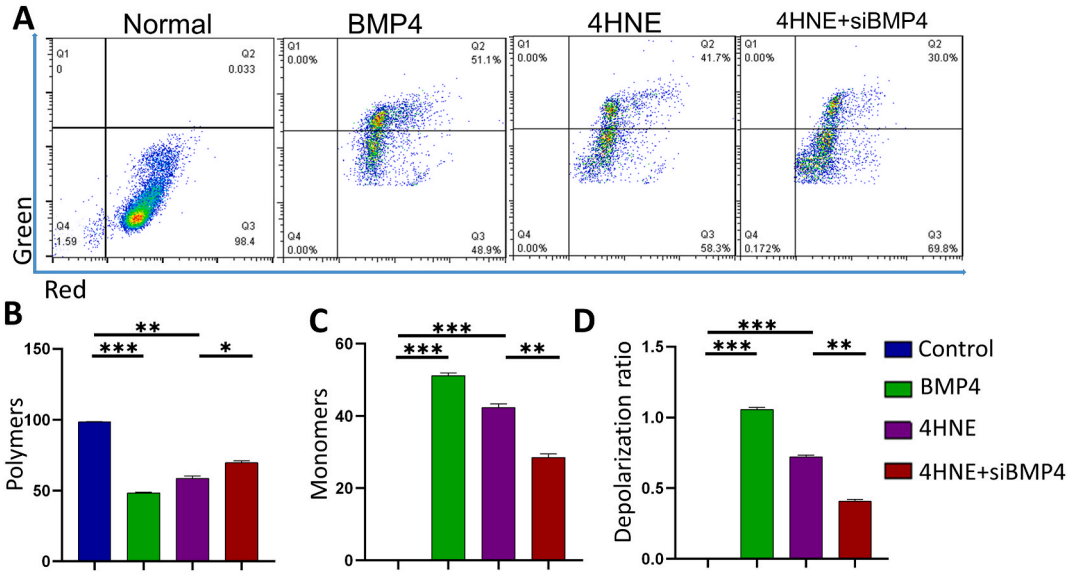

Fig. 3. Impact of BMP4 on mitochondrial membrane potential. JC-1 was used to detect mitochondrial membrane potential. Mitochondria with low membrane potential are monomers, while mitochondria with high membrane potential are polymers. JC-1 dyes the monomer green and the polymer red. The relative ratio of red to green fluorescence is often used to measure mitochondrial depolarization ratio. (A–C) JC-1 fluorescence probe diagram and its polymer and monomer quantification. All experiments were repeated a minimum of three times, and values are represented as mean ± SD, n = 6, ***P < 0.0001, ****P < 0.0001.

detect mitochondrial membrane potential, and the change from red to green through JC-1 represents the decrease of mitochondrial membrane potential. The results showed that the JC-1 polymers in BM4 and 4HNE groups significantly decreased, meanwhile, the JC-1monomers in these groups significantly increased as compared with the control group (Fig. 3A–D), which pointed out that BMP4 and 4HNE reduce mitochondrial membrane potential. However, the JC-1 polymers in the 4HNE + siBMP4 group significantly increased, whereas the JC-1 monomers in these groups significantly decreased (Fig. 3A–D). Thus, BMP4 significantly reduced mitochondrial membrane potential.

3.4. BMP4 inhibited mitochondrial function of HRMECs

Agilent seahorse XF cell mitochondrial stress test is a widely recognized method to determine mitochondrial function. The analysis can report several key parameters, including basal respiration (BS, shows energetic demand of the cell under baseline conditions), ATP production (ATP, shows ATP produced by the mitochondria), maximal respiration (MR, shows the maximum rate of respiration that the cell can achieve), and spare respiratory capacity (SRC, an indicator of cell fitness or flexibility). For data standardization, the OCR value of 2×10^4 cells is obtained after automatic counting by Cytation I/VI. Therefore, we measured OCR in isolated HRMECs using a Seahorse XF Cell Mito Stress Test Kit to further determine the role of BMP4 in regulating mitochondrial function in HRMECs. The results indicated that BMP4 significantly decreased the level of OCR values of BS, ATP, MR, and SRC (Fig. 4B–E), while siBMP4 significantly restored the level of OCR reduced by 4HNE (Fig. 4). Thus, BMP4 induces mitochondrial dysfunction. In addition, our results show that the negative control of siRNA has no effect on leukocyte adhesion, ROS, mitochondrial superoxide, mitochondrial membrane potential and OCR value (Supplementary materials).

4. Discussion

Diabetic retinopathy is one of the microvascular complications of diabetes, which is the most important disease-causing blindness among the working-age population [14]. Oxidative stress often refers to the overproduction of ROS and damage to the antioxidant system, which has been proven to be one of the pathogenesis of diabetic retinopathy. However, excessive ROS leads to changes in the structure and function of endothelial cells, mitochondrial damage, lipid peroxidation, inflammation, and apoptosis [15]. In addition, inflammation induces directed migration and aggregation of leukocytes, and a large amount of ROS produced by leukocytes will further aggravate the dysfunction of endothelial cells. Moreover, the migration of leukocytes out of blood vessels will aggravate the structure between vascular endothelium and the functional damage of endothelial cells [4].

The BMP family has been considered as a member of the TGF family related to fibrosis [16]. With the understanding of the BMPs family, an increasing number of studies have found that BMP4 also plays a very important role in the development of blood vessels

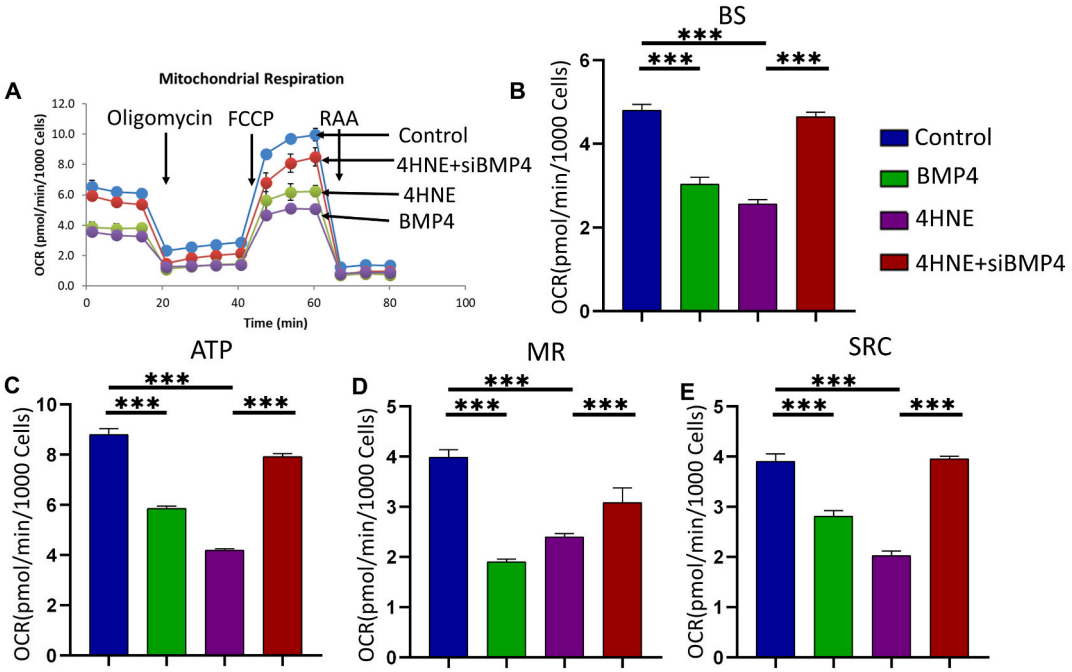

Fig. 4. Impact of BMP4 on mitochondrial oxidation in HRMECs. Normal, BMP4, 4HNE and 4HNE + siBMP4 HRMECs were used for the mitochondrial stress assays using a Seahorse XFe96 Analyzer. (A) Representative traces of oxygen consumption rate (OCR) and (B–E) quantification of basal respiration rate (BS), ATP production (ATP), maximal respiration rate (MR) and spare respiratory capacity (SRC). All experiments were repeated a minimum of three times, and values are represented as mean ± SD, n = 6, **P < 0.001, ***P < 0.001.

[17]. Meanwhile, BMP4 was up-regulated in vascular endothelial cells stimulated by hypoxia, oxidative stress, and AGEs, and promoted the migration of vascular endothelial cells [10]. Jennifer et al. pointed out that BMP4 induced the angiogenesis activity of endothelial cells by regulating the differences between two different microRNAs [18]. Moreover, the destruction of the vascular barrier often causes bleeding [19]. Furthermore, leukocyte adhesion causes the dysfunction of vascular endothelial cells, which is one of the important factors in the destruction of the vascular barrier [20]. BMP4 is an effective activator of inflammation, which promotes the rolling, adhesion, and extravasation of leukocyte subsets by up-regulating the expression levels of *E*-selectin and ICAM-1 [21]. In this study, BMP4 induced an increase in the number of adherent leukocytes, whereas its inhibition significantly reduced the number of adherent leukocytes. However, BMP4 can inhibit cell migration by promoting the expression of *E*-cadherin and claudin [22]. Although the research conclusions of BMP4 on cell migration are not consistent, BMP4 promotes the directional migration of leukocytes in inflammation [21,23].

ROS regulates cell redox balance at the physiological level and plays an important role in cell proliferation, migration, differentiation, and gene expression [24,25]. In addition, ROS-induced ROS is a positive feedback mechanism, which will increase the mitochondrial ROS and lead to mitochondrial and cell damage [26]. The organelle excitability function for electrical and Ca^{2+} signals of mitochondria further amplifies ROS signaling [27]. Thus, ROS-induced ROS release amplifies the ROS signal among each subcellular compartment [28]. Thus, in this study, BMP4 can increase the production of intracellular ROS and cause mitochondrial depolarization, thereby suggesting that it can lead to the destruction of the redox balance of vascular endothelial cells and induce mitochondrial dysfunction.

Mitochondria are the main source of cellular energy, and their functions include the generation and detoxification of ROS, regulation of mitochondrial matrix calcium, synthesis and catabolism of metabolites, and involvement in apoptosis [14]. Normal mitochondrial function is essential for cell survival and is thought to play a central role in microvascular complications of diabetes [14]. Hence, abnormality in any of these processes can be considered as mitochondrial dysfunction [14,29]. Mitochondrial dysfunction leads to the apoptosis of retinal vascular endothelial cells, thus leading to the destruction of the blood-retinal barrier and neovascularization [30]. Bachar et al. demonstrated that endothelial cell dysfunction was induced to develop toward neovascularization by inhibiting mitochondrial respiration-induced endothelial cell metabolism adaptation [31]. Moreover, stabilizing mitochondrial function can significantly inhibit diabetic retinopathy [32,33]. Our previous work studied the role of mitochondrial dysfunction in fundus diseases. We also found that PSF effectively enhanced the function of mitochondria and inhibited the proliferation of retinal endothelial cells in oxygen-induced retinopathy [34]. In this study, BMP4 induced the decline of mitochondrial metabolic function, which further confirmed the effect of BMP4 on mitochondrial dysfunction.

In this study, BMP4 can induce leukocyte adhesion. However, this study has not fully confirmed the me between BMP4 and inflammation. Next, we will further verify the effect of BMP4 on leukocyte adhesion by detecting adhesion molecules, and verify the effect of BMP4 on retinal vascular endothelial cell inflammation by detecting inflammatory factors. The relationship between BMP4 and inflammation will be further explored in the next study.

In conclusion, this study found that BMP4 caused mitochondrial dysfunction by up-regulating mitochondrial ROS and down-regulating mitochondrial membrane potential and mitochondrial OCR. We will further explore the relationship between BMP4 and inflammation in the next study to provide a new target for the treatment of retinal vascular inflammation.

Ethics approval and consent to participate

This study was conformed to international guidelines for the ethical use of experimental animals. This study was approved by the animal ethics committee of the eye hospital of Tianjin Medical University (TJYY20190103002).

Author contribution statement

Yong Wang: Analyzed and interpreted the data; Wrote the paper.
Hui Li: Performed the experiments; Wrote the paper.
Jingjing Cao: Performed the experiments; Analyzed and interpreted the data.
Aihua Liu, Zhenyu Kou, Weiting An: Performed the experiments.
Jingli Liang: Analyzed and interpreted the data.
Xiaomin Zhang, Xiaorong Li: Contributed reagents, materials, analysis tools or data.
Lijie Dong: Conceived and designed the experiments.

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Data availability statement

Data will be made available on request.

Declaration of interest's statement

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e13824.

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