• Basic Research •

Ghrelin alleviates high glucose-induced retinal microvascular endothelial cell injury by activating Nrf2/HO-1 pathway to inhibit ferroptosis

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Abstract

● **AIM:** To investigate the protective role of ghrelin against diabetic retinopathy (DR), focusing on its anti-ferroptotic mechanism in high glucose-induced retinal endothelial injury.

● **METHODS:** First, small interfering RNA (siRNA)-mediated interference was conducted to knockdown nuclear factor erythroid 2-related factor 2 (Nrf2). Using reverse transcription-polymerase chain reaction (RT-PCR), the expression level of Nrf2 was determined from human retinal microvascular endothelial cells (HRMECs) transfected with either si-NC or si-Nrf2. After that, cells were treated with 10 nmol/L ghrelin and then cultured in a high glucose (30 mmol/L) environment. EdU assay was utilized to assess cell proliferation, while transmission electron microscopy was employed to observe mitochondrial morphology. Flow cytometry was used to measure the level of intracellular reactive oxygen species (ROS), and biochemical assays were conducted to detect malondialdehyde (MDA), glutathione (GSH), superoxide dismutase (SOD), and ferrous iron (Fe^{2+}). Western blotting was used to identify the presence of ferroptosis-related proteins such as glutathione peroxidase 4 (GPX4), solute carrier family 7 member 11 (SLC7A11), Nrf2, and haem oxygenase-1 (HO-1).

● **RESULTS:** Under a high glucose environment, ghrelin could significantly promote the proliferation of HRMECs and mitochondrial status, remarkably decrease the levels of intracellular ROS and MDA, and up-regulate the level of GSH and SOD. Besides, ghrelin greatly reduced Fe^{2+} level in the cells while increased protein levels of GPX4 and SLC7A11. Subsequently, we found that high glucose induced inactivation of Nrf2/HO-1 axis and the protein expression profile were significantly promoted by ghrelin. Moreover, silencing of Nrf2 by siRNA delivery markedly diminished the changes induced by ghrelin in high glucose-induced HRMECs, shown as reduced cell proliferation and increased mitochondrial malformation, up-regulated ROS, MDA, Fe^{2+}, GPX4 and SLC7A11, as well as down-regulated GSH, SOD, Nrf2 and HO-1.

● **CONCLUSION:** Ghrelin attenuates high glucose-induced injury of retinal endothelial cells *via* inhibiting ferroptosis, and activation of Nrf2/HO-1 pathway may be one of the mechanisms involved in this effect of ghrelin.

● **KEYWORDS:** ghrelin; human retinal microvascular endothelial cells; ferroptosis; nuclear factor erythroid 2-related factor 2; haem oxygenase-1; oxidative stress

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INTRODUCTION

The visual health of the population, from working-age adults to the elderly[1], is gravely endangered by diabetic retinopathy (DR), a microvascular complication of diabetes mellitus. The pathological features of DR consist of endothelial dysfunction, pericyte loss, formation of neovascularization, blood-retinal barrier breakdown and neurovascular unit

destruction[2]. In the development process of DR, many factors can be linked and interact with each other through their own interference mechanisms, mediating a series of cellular reactions, resulting in irreversible malignant consequences. Among them, oxidative stress, inflammation, mitochondrial disorders, and gene epigenetics are the key mechanisms leading to the occurrence of diabetic retinal complications[3-6]. Significant progress has been achieved in the management of DR in recent decades, with the advent of intravitreal anti-vascular endothelial growth factor (VEGF) therapies significantly transforming the way DR is treated[7]. Moreover, new pharmacologic agents targeting other non-VEGF-driven pathways, and novel therapeutic strategies such as gene therapy are being developed for DR[8]. Despite the tremendous progress that the field of DR has already seen, there are still more advances to be made.

Kojima *et al*[9] were the first to uncover ghrelin in 1999, a novel peptide hormone of the brain-intestinal tract in the gastric mucosal endocrine cells and the hypothalamic arcuate nucleus of rats and humans. This protein binds to the growth hormone secretagogue receptor and promotes the secretion of growth factors. Subsequent studies have found that ghrelin can also regulate a series of biological activities in the body, including hormone release, glucose homeostasis, cardiovascular function, anxiety and depression, cell proliferation and survival[10-13]. In recent years, ghrelin has been shown to have a potential protective effect against many complications of diabetes, such as lung disease[14], cardiac dysfunction[15], and glomerular diseases[16]. Therefore, pharmacological targeting of endogenous ghrelin systems has been widely recognized as a valuable approach for the treatment of metabolic complications and may lead to new prevention or early intervention strategies for diabetes and its complications[17]. Our previous study revealed that ghrelin can promote cell viability and inhibit retinal angiogenesis under high glucose conditions *in vitro*[18], however, the research about the effect of ghrelin on DR is not abundant yet.

Iron, as one of the important trace elements, is closely related to the body's oxygen transport, DNA, and adenosine triphosphate (ATP) synthesis, so it is crucial to maintain the iron content and iron homeostasis in the body. In recent years, a unique form of cell death called ferroptosis has been identified, which relies on iron and involves the buildup of lipid reactive oxygen species (ROS) within cells[19]. This process stands in contrast to other mechanisms of cell death. Ferroptosis has become increasingly recognized as an important process that mediates the pathogenesis and progression of neurological diseases, diabetes, tumours, and eye diseases, which has opened up a novel way for clinical research and prevention of these diseases[20]. Moreover, abnormal levels of ferroptosis-related biomarkers are found in DR patients[21]. As more research emerges, it is becoming increasingly clear that various ways in which cells can die, such as ferroptosis, play a role in the death of neurovascular cells in DR. In this study, we sought to determine if the ability of ghrelin to protect retinal endothelial cells from high glucose-induced damage is linked to ferroptosis.

MATERIALS AND METHODS

Cell Culture and Treatment The human retinal microvascular endothelial cells (HRMECs; Shanghai Zhong Qiao Xin Zhou Biotechnology Co., Ltd., China) were cultured in M199 medium (Gibco, USA) with the addition of 10% fetal bovine serum (FBS; Excell Bio, China) and 1% penicillin-streptomycin solution at 37℃ in an environment with 95% humidity and 5% CO_2[18]. The HRMECs were divided into five groups randomly and incubated for 24h. These groups included the control group (cells cultured in M199 medium with 5.5 mmol/L glucose), the high-glucose group (cells cultured in M199 medium with 30 mmol/L glucose), the HG+ghrelin group (cells cultured in M199 medium with 30 mmol/L glucose and 10 nmol/L ghrelin), HG+ghrelin+si-NC group (cells transfected with a nonspecific siRNA sequence for 24h and then cultured in M199 medium with 30 mmol/L glucose and 10 nmol/L ghrelin), and the HG+ghrelin+si-nuclear factor erythroid 2-related factor 2 (Nrf2) group (cells transfected with Nrf2-specific siRNA for 24h and then cultured in M199 medium with 30 mmol/L glucose and 10 nmol/L ghrelin).

siRNA Transfection HRMECs were seeded onto 96-well plates and incubated at 37℃ with 5% CO_2 for the entire night. Two hours before transfection, the medium was changed to serum-free M199 medium. In accordance with the manufacturer's directions, a Nrf2-specific siRNA (20 μmol/L; Tsingke Biotechnology Co., Ltd., China) was administered to HRMECs using Lipofectamine™ 2000 reagent (Invitrogen, USA). Cells transfected with the Nrf2-specific siRNA and cells transfected with the nonspecific siRNA sequence (negative control) were set as the si-Nrf2 group and si-NC group, respectively. The expression of Nrf2 was examined 24h after transfection by using reverse transcription-polymerase chain reaction (RT-PCR). Three si-Nrf2 sequences (591, 1211, 1722) were designed, and the one with the best interference efficiency was screened by RT-PCR, which was used in subsequent experiments. The following sequences of si-Nrf2 were present: si-Nrf2-591 (forward 5'-UGACAGAAGUUGACAAUUATT-3', reverse 5'-UAAUUGUCAACUUCUGUCATT-3'); si-Nrf2-1211 (forward 5'-GAGAAAGAAUUGCCUGUAATT-3', reverse 5'-UUACAGGCAAUUCUUUCUCTT-3'); si-Nrf2-1722 (forward 5'-GUAAGAAGCCAGAUGUUAATT-3', reverse 5'-UUAACAUCUGGCUUCUUACTT-3').

Reverse Transcription-Polymerase Chain Reaction The mRNA level of Nrf2 was determined using RT-PCR. Total RNA was extracted from HRMECs with an RNA extraction kit (Thermo Fisher Scientific, USA) following the manufacturer's protocols. RT-PCR was used to synthesize cDNA from 2 μg of mRNA, using HiScript® II Q RT SuperMix (Vazyme, China). Quantitative real-time PCR was then carried out in a PCR system using SYBR Green Master Mix (Vazyme, China) in a PCR system. The primers (Tsingke Biotechnology Co., Ltd., China) used for target mRNA detection were as follows: Nrf2 (forward 5'-CAACCCTTGTCACCATCTCAG-3', reverse 5'-TTCCGATGACCAGGACTTACA-3'); glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward 5'-TCAAGAAGGTGGTGAAGCAGG-3', reverse 5'-TCAAAGGTGGAGGAGTGGGT-3'). The results are presented as values normalized to GAPDH expression using the $2^{-\Delta\Delta Ct}$ method.

Cell Proliferation To assess cell proliferation, the fixed cell slides were treated with 4% paraformaldehyde for 15min and subsequently rinsed with phosphate buffered saline with tween (PBST). Following this, 1 mL of phosphate buffered saline (PBS) with 0.2% Triton X-100 was applied to each well and left to incubate at room temperature for 5min. Staining was carried out following the guidelines of the BeyoClick™ EdU-594 cell proliferation detection kit (Beyotime, China), and the slides were sealed using a sealing liquid containing anti-fluorescence quench agent. The images were observed and collected under the optical microscope, and the cell proliferation rate was calculated by randomly selecting three fields of view. Cell proliferation rate (%)=number of proliferated cells/total number of cells ×100%.

Transmission Electron Microscopy Using transmission electron microscopy (TEM), we observed the ultrastructure of mitochondria in HRMECs. After collecting cells from each group, they were fixed in 2.5% glutaraldehyde, treated with 1% osmic acid, dehydrated in ethanol, stained with 70% uranium acetate, and finally embedded in epoxy resin. The blocks were cut into 80 nm segments, then treated with a staining mixture comprised of a 0.2% lead citrate solution and 1% uranyl acetate. Subsequently, the cellular ultrastructure was examined and captured via a transmission electron microscope (FEI Tecnai G20 TWIN, USA).

Western Blotting Proteins were extracted from treated cells using radio-immunoprecipitation assay (RIPA) lysis buffer (Beyotime, China) and quantified with a bicinchoninic acid (BCA) protein assay kit (Beyotime, China) for Western Blotting. The samples were then separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. Subsequently, the membranes were treated with primary polyclonal antibodies, specifically anti-glutathione peroxidase 4 (GPX4; 1:5000), anti-solute carrier family 7 member 11 (SLC7A11; 1:1000), anti-Nrf2 (1:2500), and anti-haem oxygenase-1 (HO-1; 1:3000; Proteintech, China). Anti-GAPDH antibodies (dilution 1:1000, Hangzhou Xianzhi Biology Co., Ltd., China) were applied to the membranes and left to incubate overnight at 4°C. Subsequently, the membranes were treated with a secondary antibody conjugated to horseradish peroxidase (dilution 1:10000, Beyotime, China) for 2h at room temperature. The protein bands were detected using enhanced chemiluminescence (ECL) and the intensity of the bands was quantified with Bandscan software 5.0 (Glyko Inc., CA, USA). Using GAPDH as the internal control, the relative expression levels of every target protein were determined, with three biological replicates conducted for each group.

Flow Cytometry Flow Cytometry was employed to assess the ROS concentrations of HRMECs, utilizing a detection kit (Beyotime, China). In brief, cells were placed in 6-well plates for an entire night and then treated in distinct groups. After digestion, cells were collected and then re-suspended with PBS. 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) was diluted in serum-free M199 medium at 1:1000 to a final concentration of 10 mmol/L. Totally 1 mL of diluted DCFH-DA was added to the medium and the cells were incubated at 37°C for 20min. Flow cytometry (BECKMAN, USA) was used to ascertain the mean fluorescence intensity of cells collected after three washings with serum-free medium.

Detection of Oxidative Stress Markers and Fe^{2+} Content Following treatment in various groups, the levels of glutathione (GSH), malonic dialdehyde (MDA), superoxide dismutase (SOD), and ferrous iron (Fe^{2+}) in the cells were assessed using specific kits (Nanjing Jiancheng Bioengineering Research Institute, China), which involved detecting oxidative stress markers and Fe^{2+} content.

Statistical Analysis The statistical analysis was conducted using the software Statistics SPSS 19.0 (IBM, USA). Mean±standard deviation (SD) was used to represent the quantitative data from three separate experiments. Statistical significance of each variable was assessed through one-way analysis of variance (ANOVA) with LSD post hoc test. Results with a *P* value less than 0.05 were deemed statistically significant.

RESULTS

Screening of Nrf2 Interference Sequences To demonstrate the role of ghrelin in the activation of Nrf2, siRNA transfection was conducted using three different siRNA sequences. RT-PCR analysis revealed that Si-Nrf2-1211 had the most effective interference effect, as shown in Figure 1. This siRNA sequence was then utilized in further experiments to successfully silence Nrf2.

Effects of Ghrelin on the Proliferation of HRMECs The results of EdU-based assay of cellular proliferation demonstrated that the proliferation of HRMECs decreased significantly in HG group. While cells were treated with ghrelin in combination with HG, the proliferation rate was increased. When si-Nrf2 was introduced, the promoting effect of ghrelin on cell proliferation was weakened when compared with those in cells treated with ghrelin with or without si-NC (Figure 2). These results suggest that ghrelin protects the proliferation of HRMECs under HG conditions through Nrf2 signalling.

Effects of Ghrelin on Morphological Changes of Mitochondria of HRMECs Mitochondria, as an important organelle of organisms, play critical roles in cell death. The morphological characteristics of cells during ferroptosis are obviously different from other cell death ways, especially the morphological changes of mitochondria. Thus, the morphology of mitochondria was observed. Under TEM, there was no significant change in mitochondria in the control group. HRMECs from HG group showed altered mitochondrial morphology compared to the control group. Specifically, we observed deeply stained and shrunken mitochondria with reduced mitochondrial crista structures and increased mitochondrial membrane density, consistent with the morphological features of mitochondria during ferroptosis reported previously[22]. Compared with HG group, morphological changes of mitochondria in HG+ghrelin group and HG+ghrelin+si-NC group was significantly alleviated, and some of the mitochondria were normal. However, si-Nrf2 treatment aggravated mitochondrial changes, and the cell state was similar to that of HG group (Figure 3).

Effects of Ghrelin on the Protein Expressions of GPX4, SLC7A11, Nrf2 and HO-1 of HRMECs As previously mentioned, GPX4 and SLC7A11 are critical markers of ferroptosis. And Nrf2 and HO-1 are key regulatory proteins in cellular oxidative stress. Western blotting analysis revealed that under high-glucose conditions, the protein levels of GPX4 and SLC7A11 in HRMECs were decreased, indicating that ferroptosis was triggered. However, when ghrelin was added, the levels of these proteins were significantly increased. In addition, this effect of ghrelin was markedly suppressed by knockdown of Nrf2, as shown by the lower expression levels of GPX4 and SLC7A11 proteins (Figure 4). Following a 24-hour period, levels of Nrf-2 and HO-1 were found to be lower in the HG group compared to the control group. However, after ghrelin treatment, the levels of Nrf-2 and HO-1 proteins increased compared to the HG group, but this increase was reversed by the addition of si-Nrf2 (Figure 5). These results indicated that ghrelin can activate Nrf2/HO-1 axis under high glucose stress.

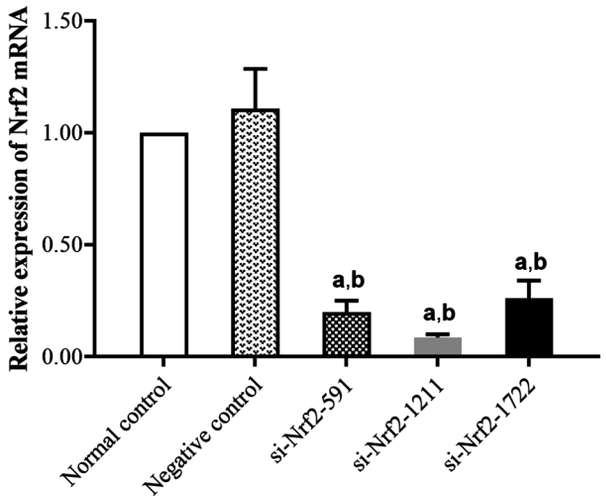

Figure 1 The levels of Nrf2 mRNA expression in HRMECs were analyzed using RT-PCR across various groups, with each group consisting of three samples [a]*P*<0.05 *vs* normal control group; [b]*P*<0.05 *vs* negative control group. Nrf2: Nuclear factor erythroid 2-related factor 2; HRMECs: Human retinal microvascular endothelial cells; RT-PCR: Reverse transcription-polymerase chain reaction.

Effects of Ghrelin on Oxidative Stress Markers and Fe^{2+} Levels of HRMECs Ghrelin's impact on oxidative stress markers and Fe^{2+} levels in HRMECs examines the imbalance between oxidation and antioxidant activity in the body, characterized by excessive production of oxygen free radicals and their byproducts like ROS and MDA, in contrast to antioxidants such as GSH and SOD. As expected, high glucose induced oxidative stress in HRMECs, shown as increased levels of ROS and MDA, and decreased levels of GSH and SOD. Combination with ghrelin treatment reversed these changes. However, when si-Nrf2 was added to treat HRMECs together with HG and ghrelin, the effects of ghrelin were significantly weakened (Figure 6). Similarly, the iron levels in the cells after HG treatment were significantly higher than those in the control group, while ghrelin reduced this level under HG conditions. Moreover, si-Nrf2 increased this level when compared with the ghrelin group (Figure 6). These results indicated that ghrelin can inhibit high glucose-induced oxidative stress through Nrf2.

DISCUSSION

The distribution of ghrelin throughout the human body is widespread, and it can traverse the blood-brain barrier to reach eye tissue. Both anterior and posterior segments have been observed to produce ghrelin[23]. Thus, ghrelin presents as a possible local regulator in the eye, with pathophysiological implications, constituting a target for clinical and therapeutic research and interventions. In the field of eye research, in vitro studies have confirmed that ghrelin has antioxidant and neuroprotective effects on optic nerve damage caused by glaucoma[24-25], and also exerts antioxidant effects on

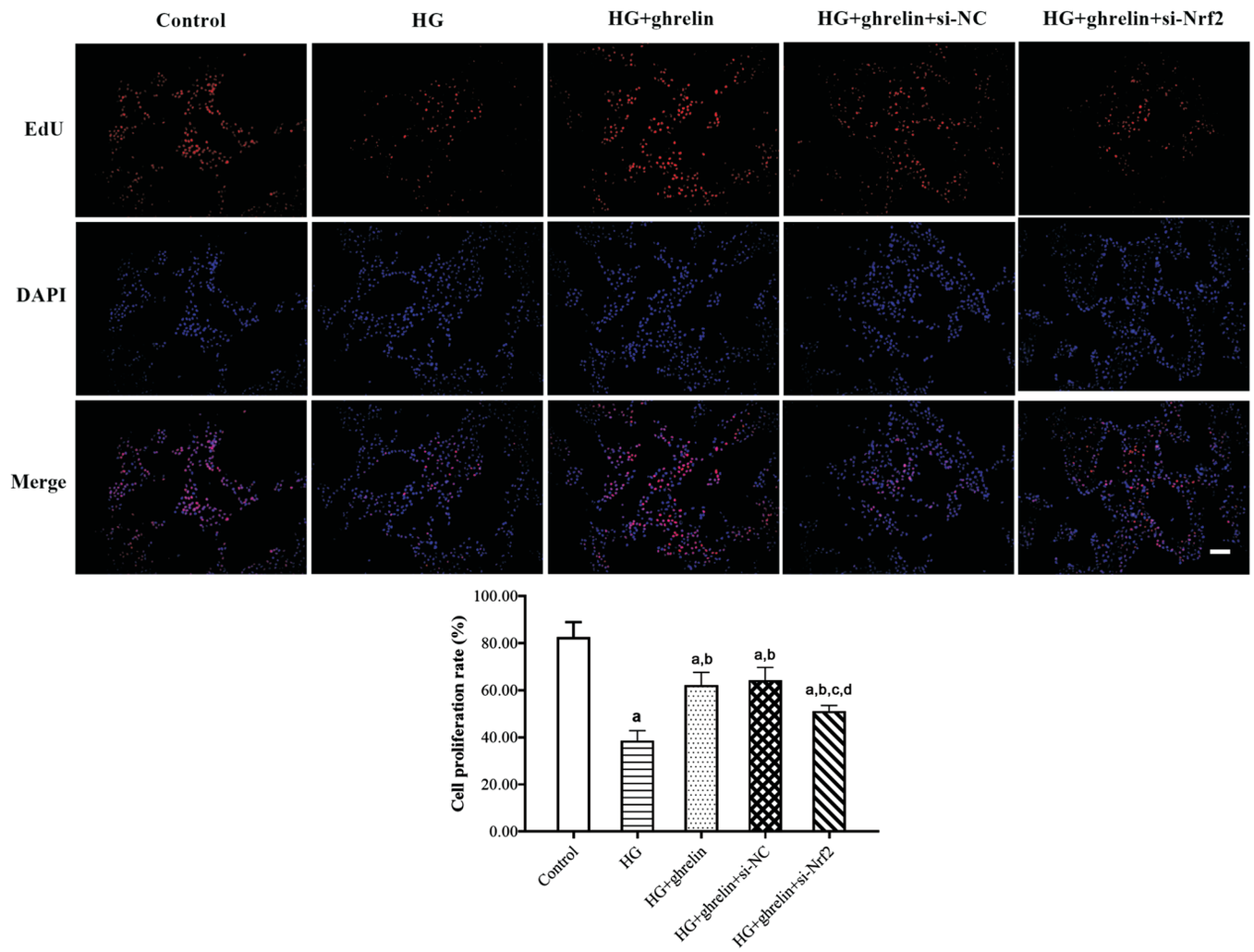

Figure 2 At the 24-hour mark, the EdU assay was used to assess the cellular proliferation of human retinal microvascular endothelial cells (HRMECs) in various groups Bar=50 μm. n=3, [a]P<0.05 *vs* control group; [b]P<0.05 *vs* HG group; [c]P<0.05 *vs* HG+ghrelin group; [d]P<0.05 *vs* HG+ghrelin+si-NC group. HG: High glucose; NC: Negative control; Nrf2: Nuclear factor erythroid 2-related factor 2; DAPI: 4',6-diamidino-2-phenylindole.

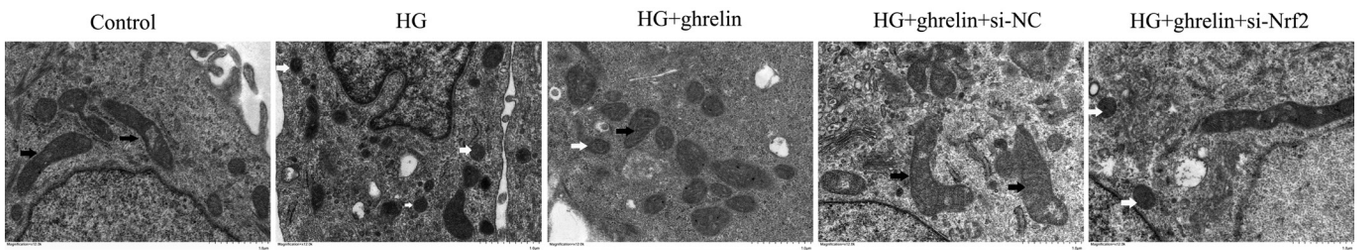

Figure 3 Representative TEM images of mitochondria showing ferroptosis induction in human retinal microvascular endothelial cells (HRMECs) in different groups Bar=1 μm. Black arrow: Normal mitochondria; White arrow: Mitochondria during ferroptosis. HG: High glucose; NC: Negative control; Nrf2: Nuclear factor erythroid 2-related factor 2.

human trabecular meshwork cells[26]. In addition, ghrelin was found to attenuate HG-induced loss of cell viability, reduced oxidative damage, and cell apoptosis in human lens epithelial cells, and effectively maintain the transparency of lens[27]. Oxidative stress, one of the major effects of hyperglycemia, is a pathological state in which elevated production of reactive oxygen species damages cells and tissues. The retina is relatively prone to oxidative stress due to its high metabolic activity[3]. In view of this, attention has been paid to the role of ghrelin in DR and its association with oxidative stress recently. In diabetic rats, ghrelin was discovered to diminish the production of ROS, inhibit retinal cell apoptosis, and safeguard the retina from HG-induced dysfunction[28]. Our previous studies demonstrate that ghrelin can inhibit the endoplasmic reticulum stress induced by HG and play a protective role in retinal microvascular endothelial cells[18]. The current research also indicates that ghrelin has the potential to alleviate the oxidative stress caused by high glucose, as

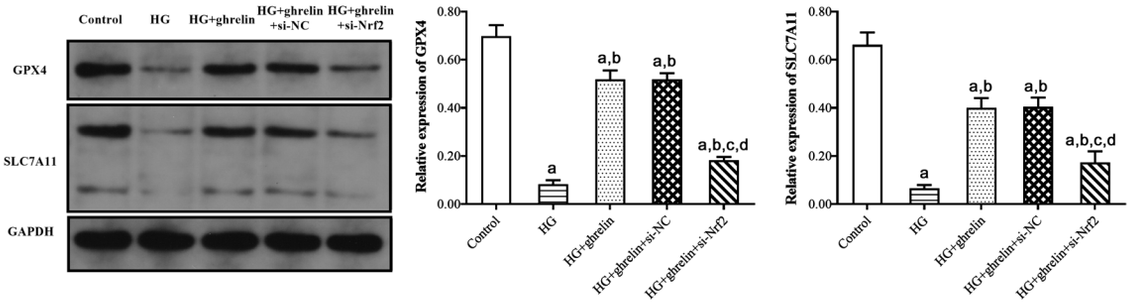

Figure 4 Comparison of expression of glutathione peroxidase 4 (GPX4) and solute carrier family 7 member 11 (SLC7A11) proteins by Western blotting in different groups n=3, [a]P<0.05 *vs* control group; [b]P<0.05 *vs* HG group; [c]P<0.05 *vs* HG+ghrelin group; [d]P<0.05 *vs* HG+ghrelin+si-NC group. HG: High glucose; NC: Negative control; Nrf2: Nuclear factor erythroid 2-related factor 2.

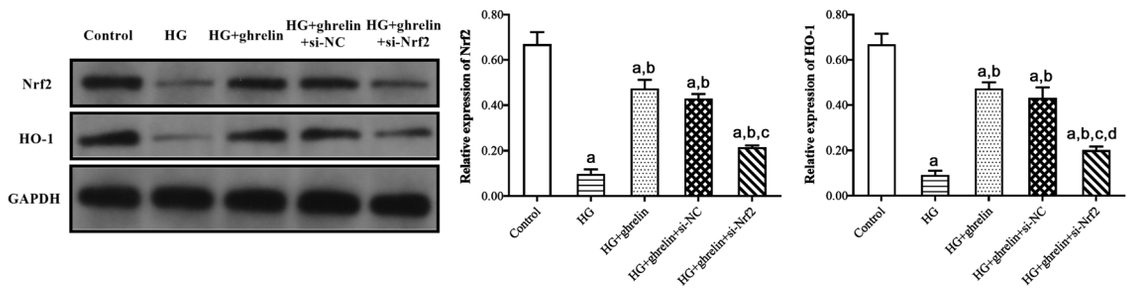

Figure 5 Comparison of expression of nuclear factor erythroid 2-related factor 2 (Nrf2) and haemoxygenase-1 (HO-1) proteins by Western blotting in different groups n=3, [a]P<0.05 *vs* control group; [b]P<0.05 *vs* HG group; [c]P<0.05 *vs* HG+ghrelin group; [d]P<0.05 *vs* HG+ghrelin+si-NC group. HG: High glucose; NC: Negative control.

evidenced by reduced levels of ROS and MDA, and elevated levels of the antioxidant markers GSH and SOD. Moreover, ghrelin can improve the vitality of retinal vascular endothelial cells under HG conditions. Simultaneously, a newly conducted clinical trial revealed a notable decrease in ghrelin levels in both non-proliferative DR (NPDR) and proliferative DR (PDR), with levels in the PDR stage being even lower than those in NPDR. This suggests that ghrelin plays a protective function in DR. These results may provide a certain reference for the use of ghrelin as a pharmacological target for the prevention and treatment of DR.

Cell death is the leading cause of neurovascular injury in DR. Oxidative stress leads to neurovascular cell death through various types of programmed cell death (RCD) pathways, such as pyroptosis, apoptosis, autophagy, necroptosis, and ferroptosis, and the resulting neurodegeneration leads to neurovascular and retinal tissue damage[29]. Ferroptosis, first reported by Dixon *et al*[19] in 2012, is a unique form of RCD. The essence of this complex process is to induce oxidative stress reaction under the catalysis of high concentration of iron, resulting in excessive peroxidation of peroxidation of polyunsaturated fatty acids (PUFAs)-containing phospholipids in cell membrane and triggering cell death. Morphologically, cells undergoing ferroptosis have dysmorphic and small mitochondria with decreased crista, a condensed membrane, and a ruptured outer membrane[19]. In this study, we also observed typical mitochondrial changes by TEM in HG group and ghrelin treatment significantly improved these malformations of mitochondria. As an essential trace element of life, iron is involved in a variety of biological processes. Iron is a redox-active metal, which is essential for free radical formation and propagation of lipid peroxidation, thus, excess iron can increase the vulnerability to ferroptosis *via* the accumulation of phospholipid hydroperoxides in the cell membrane[30]. Here, we observed elevated levels of iron in HRMECs in HG group and this alteration was almost turned to normal by ghrelin. A major protective mechanism against peroxidation damage of membranes is achieved through the activity of glutathione peroxidase 4 (GPX4), which plays a master role in blocking ferroptosis[31]. GPX4 can be inactivated through a variety of mechanisms such as depletion of intracellular GSH, which is an essential cofactor of GPX4[32]. The import of cystine, a necessary component for GSH synthesis, is hindered by the light chain SLC7A11 in system Xc-. This exchange occurs between intracellular glutamate and extracellular oxidized form of cysteine[33]. Consequently, inhibition of SLC7A11 leads to depletion of GSH and GPX4 being deactivated, thus causing lethal lipid peroxides and ferroptosis[34]. This study suggests that GSH levels decreased under HG conditions, accompanied by a reduction in GPX4 and SLC7A11 protein levels. Ghrelin can restore the GSH level and SLC7A11/GPX4 activity in HRMECs to some extent. Taken together, all these results suggest that HG can induce ferroptosis in HRMECs, and combined treatment with ghrelin can significantly attenuate ferroptosis of the cells.

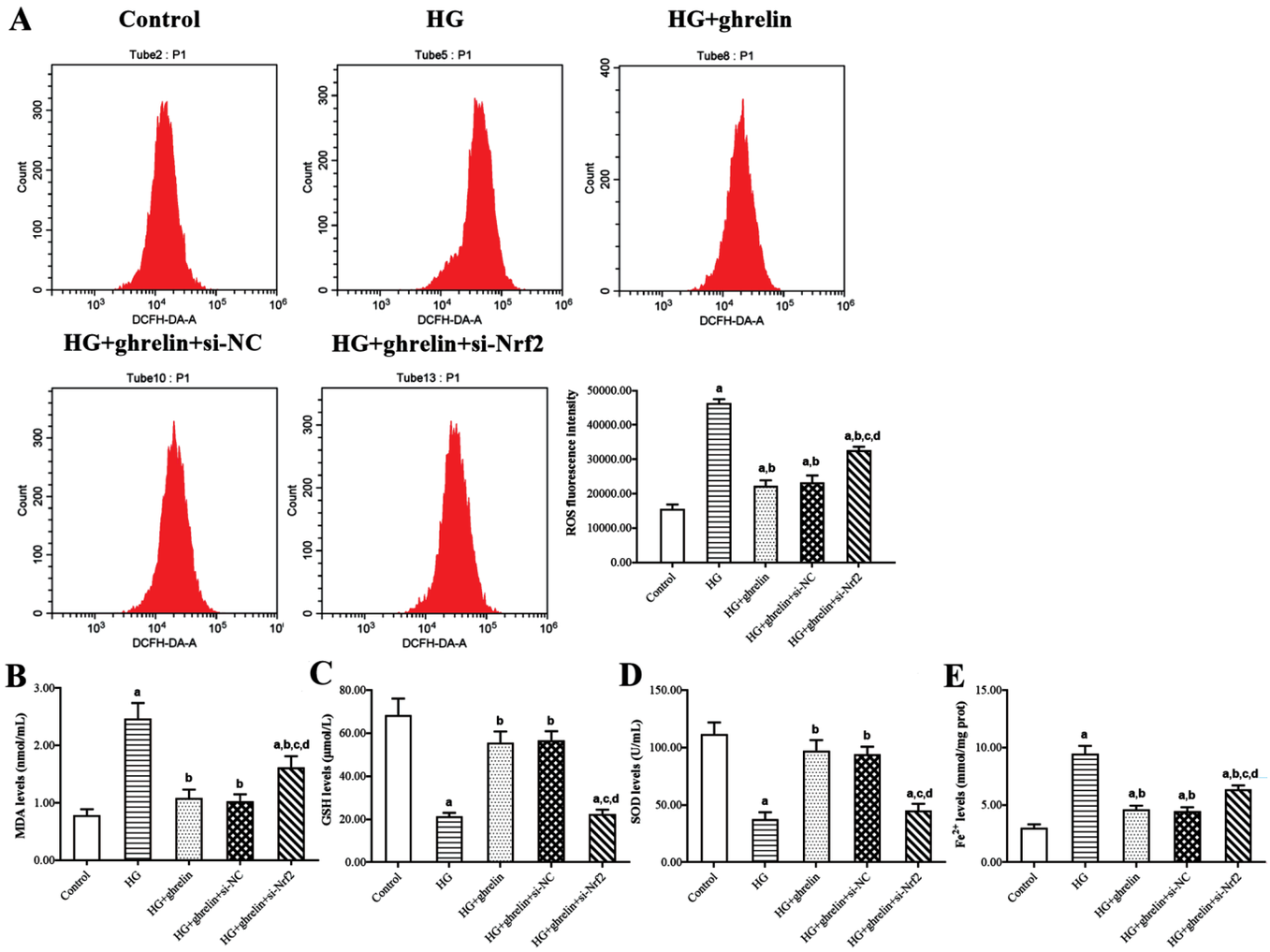

Figure 6 Comparison of intracellular ROS levels (A) measured by flow cytometry, and MDA (B), GSH (C), SOD (D) and Fe^{2+} (E) levels detected by biochemical assay in different groups n=3, [a]P<0.05 *vs* control group; [b]P<0.05 *vs* HG group; [c]P<0.05 *vs* HG+ghrelin group; [d]P<0.05 *vs* HG+ghrelin+si-NC group. ROS: Reactive oxygen species; MDA: Malondialdehyde; GSH: Glutathione; SOD: Superoxide dismutase; Fe^{2+}: Ferrous iron; HG: High glucose; NC: Negative control.

The transcription factor Nrf2 plays a crucial role in regulating antioxidant activity, protecting against lipid peroxidation and ferroptosis[35] through the upregulation of various cytoprotective enzymes, including HO-1[36]. Therefore, the sensitivity of cells to ferroptosis can be increased *via* inactivation of Nrf2/HO-1 signalling[37] and silencing of Nrf2-related genes[38]. In this study, decreased expressions of Nrf2 and HO-1 proteins in HG group suggests the vulnerability to ferroptosis of HRMECs. When ghrelin was added, the Nrf2/HO-1 axis was up-regulated to inhibit HG induced ferroptosis, which was proved through knockdown Nrf2 in HRMECs by siRNA-mediated interference. Besides, while si-Nrf2 was introduced, the anti-oxidative stress and cell proliferation promoting ability of ghrelin was diminished. These results directly demonstrate that ghrelin exerts protective effects in HRMECs against HG-induced ferroptosis through activating Nrf2/HO-1 pathway. However, since multiple factors are involved in the pathogenesis of DR besides hyperglycemia, whether ghrelin can play a protective role in its development by inhibiting ferroptosis needs to be confirmed. Additionally, due to the constraints of *in vitro* studies, it is necessary to conduct additional *in vivo* investigations using animal models or clinical samples in order to elucidate the connection between ghrelin and ferroptosis in DR.

To summarize, this research offers the initial proof linking ghrelin's anti-ferroptosis effects to the stimulation of the Nrf2/HO-1 pathway in human retinal microvascular endothelial cells exposed to high levels of glucose.

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Conflicts of Interest: Wang FF, None; **Li R,** None; **Liao D,** None; **Liu CQ,** None; **Yang XL,** None.

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