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The antioxidative effects of empagliflozin on high glucose-induced epithelial-mesenchymal transition in peritoneal mesothelial cells via the Nrf2/HO-1 signaling

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ABSTRACT

High glucose (HG)-induced epithelial-mesenchymal transition (EMT) and oxidative stress play an important role in peritoneal fibrosis, which could be regulated by the nuclear factor erythroid-2-related factor 2 (Nrf2)/heme oxygenase-1 (HO-1) signaling pathway. This study aimed to investigate whether empagliflozin could inhibit HG-induced EMT and oxidative stress *via* activating the Nrf2/HO-1 signaling pathway. We used HG-based peritoneal dialysis (PD) solution in rats and HG in human peritoneal mesothelial cells (HPMCs) to induce EMT *in vivo* and *in vitro* respectively. The peritoneal structure and function were evaluated by hematoxylin and eosin, Masson's trichrome staining, and the peritoneal equilibrium test. Oxidative stress was measured by assay kits. EMT was analyzed using immunohistochemistry and western blot. The PD rats showed decreased ultrafiltration capacity and increased levels of oxidative stress. Histopathological analysis revealed markedly peritoneal thickening, excessive collagen deposition, increased expression of α-SMA, Collagen-I, and Fibronectin, and decreased expression of E-cadherin. Empagliflozin significantly ameliorated the aforementioned changes. The protein expression levels of nuclear Nrf2 (N-Nrf2) and HO-1 increased in PD rats, which were further promoted by treatment with empagliflozin. In *in vitro* experiments, the EMT of HPMCs was induced with 60 mM glucose for 24 h and inhibited by empagliflozin. Empagliflozin suppressed oxidative stress and promoted the protein expression of N-Nrf2 and HO-1 in HG-stimulated HPMCs, which was reversed by the Nrf2 inhibitor. In conclusion, empagliflozin exerted a protective effect against HG-induced EMT and suppressed oxidative stress in PMCs by activating the Nrf2/HO-1 signaling pathway.

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High glucose; epithelial-mesenchymal transition; peritoneal mesothelial cell; empagliflozin; oxidative stress; Nrf2/HO-1

Introduction

Peritoneal dialysis (PD) is an ideal renal replacement therapy for patients with end-stage renal disease (ESRD), which has the advantages of being simple, economical, and hemodynamically stable. Moreover, it better preserves residual renal function [1,2]. However, long-term PD patients are prone to peritoneal fibrosis and even ultrafiltration failure, which forces patients to withdraw from PD [3,4]. Therefore, it is crucial to protect the structural and functional integrity of the peritoneum for the treatment of peritoneal fibrosis and the maintenance of PD treatment [5].

Accumulating evidence indicates that the epithelial-mesenchymal transition (EMT) is a pivotal process in peritoneal fibrosis [6,7]. High glucose (HG) could induce oxidative stress in peritoneal mesothelial cells (PMCs) and stimulate cells to produce a large number of reactive oxygen species (ROS), which activates the downstream inflammatory signaling pathways, upregulates fibrogenic factors, and enhances the EMT process, thus inducing and accelerating the occurrence of peritoneal fibrosis [8,9]. Therefore, inhibiting oxidative stress is an effective method to prevent the progression of peritoneal fibrosis [10,11]. Nuclear factor erythroid-2-related factor 2 (Nrf2) is an important transcription factor, which could regulate the induction of downstream antioxidant proteins heme oxygenase-1 (HO-1) to suppress the generation of oxidative stress [12,13]. The activation of the Nrf2/HO-1 signaling pathway in the protection against EMT and peritoneal fibrosis has been reported in many studies [14,15]. Hence, the Nrf2/HO-1 signaling pathway may become a promising target for the therapy of peritoneal fibrosis.

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Empagliflozin (Emp), a selective sodium-glucose cotransporter 2 (SGLT2) inhibitor, is a novel oral antidiabetic agent [16]. It can reduce hyperglycemia in an insulin-independent manner *via* enhancing renal glucose excretion or glycosuria [17]. In our previous study, SGLT2 has been confirmed to be positively expressed in PMCs and SGLT2 inhibition exerts a glucose-lowering effect in the peritoneum exposed to peritoneal dialysis solution (PDS) [18]. And it has been found that empagliflozin can inhibit transforming growth factor-β1 (TGF-β1)-induced EMT of PMCs and attenuate peritoneal fibrosis by downregulating the TGF-β/Smad signaling pathway [19]. Nonetheless, there are few reports on the prevention of HG-induced EMT in PMCs by empagliflozin, and whether empagliflozin can alleviate peritoneal fibrosis through other signaling pathways remains unclear. Increasing studies have found that empagliflozin can exhibit antioxidative effects to alleviate fibrosis in the heart, lung and other organs by activating the Nrf2/HO-1 signaling pathway [20,21]. The link between oxidative stress and empagliflozin has not been verified in peritoneal fibrosis yet. In this study, we not only investigated the role of oxidative stress as a mediator of the phenotype transition of PMCs but also examined the effects of empagliflozin on EMT and oxidative stress with an exploration of potential mechanisms of peritoneal protection.

Materials and methods

Main reagents

Empagliflozin (BI10773, MedChemExpress, China), ML385 (S8790, Selleck Chemicals, USA), 4.25% dextrose PDS (Baxter Healthcare Ltd.), dulbecco's modified eagle's medium/f12 (DMEM/F12, Gibco, USA), fetal bovine serum (FBS, Gibco, USA), penicillin-streptomycin solution (Beyotime, China), phosphate buffer saline (PBS, Hyclone, China), 4% paraformaldehyde (Biosharp, China), triton X-100 (Biofroxx, Germany), bovine serum albumin (BSA, Biofroxx, Germany), DAPI staining solution (Beyotime, China), a nuclear protein extraction kit (Sigma-A Aldrich; Merck KGaA), ROS assay kit(Jiancheng Biotech, China), glutathione peroxidase (GSH-Px) assay kit (Jiancheng Biotech, China), malondialdehyde (MDA) assay kit (Jiancheng Biotech, China), total superoxide dismutase (SOD) assay kit(Jiancheng Biotech, China), total antioxidant capacity assay kit with a rapid ABTS method(Beyotime, China), primary antibodies against Cytokeratin-18 (CK-18, ab133263, Abcam, UK), Vimentin (ab92547, Abcam, UK), fibroblast specific protein-1 (FSP-1, ab124805, Abcam, UK), von willebrand factor (VWF, ab6994, Abcam, UK), E-cadherin (ab76055, Abcam, UK), α-smooth muscle actin (α-SMA, ab5694, Abcam, UK), Collagen-I (ab260043, Abcam, UK), Fibronectin (ab268020, Abcam, UK), N-cadherin (ab76011, Abcam, UK), HO- 1 (ab13243, Abcam, UK), Nrf- 2 (ab92946, Abcam, UK), β-actin (ab8226, Abcam, UK), GAPDH (ab8245, Abcam, UK) and Lamin B (cat.no.13435, Celling Signaling Technology, Inc), horseradish peroxidase (HRP)-conjugated goat anti-rabbit (CW0103S, CWbio, China) and HRP-conjugated goat anti-mouse antibodies (CW0102S, CWbio, China).

Animal model

24 male Sprague-Dawley (SD) rats (200-240 g, 7-8weeks) were obtained from the Experimental Animal Center of Soochow University (Jiangsu, China). Rats were housed in controlled conditions, at a temperature of 20- 25 °C with 40-70% humidity under a 12:12-h light/dark cycle, with access to the drink/feed boxes. All animal procedures conformed to the China Animal Welfare Legislation and were reviewed and approved by the Soochow University Committee on Ethics in the Care and Use of Laboratory Animals.

Rats were randomly grouped into four groups with 6 rats in each group: the negative control (NC) group, which was treated daily *via* intraperitoneal administration of 100 mL/kg of 0.9% saline; the Emp group, the NC group administered orally by gavage at a daily dose of 3 mg/kg empagliflozin; the PD group, which received daily intraperitoneal administration of 100 mL/kg of 4.25% dextrose PDS with 0.6 mg/kg LPS on day 1, 3, 5, and 7; the PD + Emp group, the PD group administered orally by gavage at a daily dose of 3 mg/kg empagliflozin. The dose of empagliflozin was determined by our previous study [18]. The rats were sacrificed on day 28 and peritoneal tissues were obtained from the contralateral side of the injection for subsequent analyses.

Peritoneal function

A 4-h peritoneal equilibrium test (PET) was performed at the end of the experiments. 100 mL/kg of 4.25% dextrose PDS was intraperitoneally administered to the rats, and 4 h later rats were anesthetized with pentobarbital sodium (0.8 mL/100 g). The abdominal cavity was opened along the linea alba and all the fluid contents were immediately collected to determine the volume (mL). Blood samples were obtained by cardiac puncture. The net ultrafiltration volume (UV) was determined by subtracting the drained volume from infused volume. The peritoneal permeabilities of glucose and blood creatinine were expressed as the peritoneal

absorption of glucose (D4/D0) from the dialysate and the dialysate-to-plasma (D/P) ratio of blood creatinine.

Histology and immunohistochemistry

Histology and immunohistochemical staining of 4-μm-thick tissue sections were performed as described previously [22]. The thickness of the submesothelial tissue was measured in tissue sections stained with hematoxylin and eosin (H&E) and Masson's trichrome under light microscopy (original magnification, ×100). Semiquantitative analysis of peritoneal thickness and collagen deposition was performed with ImageJ version 1.8.0 software and was expressed as the mean of five independent measurements for each section.

For immunohistochemical staining, the sections were incubated with primary antibodies against E-cadherin, α-SMA, Collagen-I, and Fibronectin. The results were observed under light microscopy (original magnification, ×100). Image-Pro Plus version 6.0 software was used to calculate the number of cells positive in each field. According to the proportion of positive cells, the following standard was used: 0, negative; 1, 1-25%; 2, 26-50%; 3, 51-75%; 4, 76-100%.

Cell culture and treatment

HPMCs were collected from PDE of patients with ESRD who underwent PD catheterization within 2 weeks by centrifugation and cultured in DMEM/F12 supplemented with 10% FBS and antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin)[18]. All cells were incubated at 37 °C in a humidified culture chamber supplied with 5% CO_2. The culture medium was changed every three days when cells reached 80% confluence. HPMCs were identified by morphological observation under the inverted phase contrast microscope and immunofluorescence staining. All experiments were performed using cells between the second and fourth passages.

Subconfluent HPMCs grown in a culture dish were incubated with 0.5% FBS medium for 24 h to arrest and synchronize the cell growth. The EMT model of cells was induced with HG. Cells were stimulated with different concentrations of glucose (17.5, 30, 60, 120 mM) for 48 h to find the suitable glucose concentration. Then cells were treated with the suitable glucose concentration for 12, 24, and 48 h to determine the appropriate stimulation time. Cells were allocated into four groups: the negative control (NC) group, cells were treated with normal glucose (NG) (17.5 mM); the Emp group, cells were cotreated with NG and empagliflozin (1 μM); the HG group, cells were treated with HG; the HG + Emp group, cells were cotreated with HG and empagliflozin (1 μM). The dose of empagliflozin was chosen according to previous reports [18,19]. In the part of the mechanism study, the cells were pretreated with ML385 at the concentration of 10 μM for 24 h [15].

All patients included signed informed consents. The study was approved by the Research Ethics Committee of The Second Affiliated Hospital of Soochow University, Jiangsu, China.

Measurement of oxidative stress

Oxidative stress was evaluated by detecting MDA levels, SOD and GSH-Px activities, total antioxidant capacity, and ROS generation in peritoneal tissue and HPMCs according to the detection kit instructions.

Immunofluorescence staining

Cells that grew on a confocal dish were fixed with 4% paraformaldehyde for 20 min. After washing with PBS, 0.5% Triton X-100 was added and cells were incubated for 10 min. Cells were again washed with PBS three times and blocking buffer (1% BSA) was added for incubation for 30 min. Primary antibodies, including anti-CK-18 (1:50 dilution), anti-Vimentin (1:250 dilution), anti-FSP-1 (1:250 dilution), or anti-VWF (1:250 dilution) antibody was added and incubated overnight at 4 °C in the dark. Cells were washed three times with PBS and then incubated with fluorescent secondary antibody (1:1000 dilution) in a wet box at 37 °C for 1 h without light. Cells were then extensively washed with PBS. DAPI staining for 2 min was performed. Images were observed with the confocal microscope.

Western blot analysis

The nuclear and cytoplasmic proteins of peritoneal tissues and HPMCs were extracted using a nuclear extract kit according to the manufacturer's protocol. Total proteins were homogenized in RIPA lysis buffer with protease and phosphatase inhibitors. Protein (30 μg) determined by BCA assay was separated *via* 10% SDS-PAGE and transferred to PVDF membranes, which were blocked with 5% nonfat milk in Tris-Buffered saline containing 0.1% Tween-20 for 1 h at room temperature, and then incubated with specific primary antibodies against E-cadherin (1:1000 dilution), α-SMA (1:1000 dilution), Collagen-I (1:1000 dilution), Fibronectin (1:1000 dilution), N-cadherin (1:5000 dilution), Nrf2 (1:1000 dilution), HO-1(1:1000 dilution), GAPDH (1:1000 dilution),

β-actin (1:5000 dilution), and Lamin B (1:1000 dilution) at 4 °C overnight. After incubation, membranes were washed three times with TBST for 10 min and subsequently incubated with HRP-conjugated anti-mouse IgG or anti-rabbit IgG for 1 h at room temperature. Proteins were visualized by an ECL system and a Bio-Rad electrophoresis image analyzer. Densitometric analysis of the western blots was performed using ImageJ version 1.8.0 software.

Statistical analysis

All experiments were performed at least three times. Data were presented as mean ± standard error of the mean (SEM) and analyzed using GraphPad Prism 8.0 statistical software. Comparisons between multiple groups were made using ANOVA with Tukey's *post hoc* test. $p < 0.05$ was considered to indicate a statistically significant difference.

Results

Empagliflozin attenuated PDF-induced peritoneal fibrosis in the rat model

To evaluate the effects of empagliflozin on the peritoneal structure and function, we established a rat model of peritoneal fibrosis by intraperitoneal injection of 4.25% glucose PDS. Sections of the rat peritoneum were evaluated by H&E and Masson's trichrome staining. The PD group significantly developed overt typical characteristics of peritoneal fibrosis, such as significantly peritoneal thickening, greater loss of mesothelial monolayer cells, and higher fibroblastic proliferation. The area of collagen accumulation was also larger in PD rats. Cotreatment with empagliflozin significantly reversed the changes described above (Figure 1(A,D)).

Likewise, the peritoneal function was significantly affected by HG-based PDS. Rats in the PD group exhibited significantly reduced UV values and an increased permeability for blood creatine compared with the NC and Emp groups. In contrast, changes were significantly attenuated in the PD + Emp group (Figure 1(E,F)). In addition, glucose absorption was significantly increased in PD rats and improved in the PD + Emp group (Figure 1(G)).

To evaluate whether empagliflozin inhibits EMT- and fibrosis-related markers levels *in vivo*, we performed immunohistochemical staining and western blot. Results revealed significantly increased expression of α-SMA, Collagen-I, and Fibronectin as well as decreased expression of E-cadherin in the PD group. Cotreatment with empagliflozin reversed the expression levels of EMT-and fibrosis-associated proteins (Figure 2).

Empagliflozin attenuated HG-induced oxidative stress in peritoneal tissue

Excessive oxidative stress is an inducer of EMT in response to HG. To determine the effects of empagliflozin on oxidative stress in PD rats, we measured the levels of ROS, GSH-Px, SOD, and MDA in peritoneal tissue. Oxidative stress parameters are shown in Figure 3. ROS and MDA levels were significantly higher in PD rats than in the control and Emp groups, whereas the activities of SOD and GSH-Px were significantly lower. Compared with the PD group, these changes could be inhibited by empagliflozin treatment.

The Nrf2 antioxidant pathway was activated by empagliflozin in PD rats

The Nrf2 antioxidant pathway is an active participant in regulating ROS generation and EMT. Accordingly, the effects of empagliflozin on the activity of the Nrf2 antioxidant pathway in peritoneal tissue were demonstrated by the protein expression levels of nuclear Nrf2 (N-Nrf2) and its downstream targets HO-1. It was found that the protein expression of N-Nrf2 in the PD + Emp group increased dramatically compared with that in the PD group. Consequently, the protein level of HO-1 was elevated in the PD + Emp group (Figure 4).

Identification of cells isolated from PDE as HPMCs

To confirm cultured cells isolated from PDE were HPMCs, we performed the morphological identification and immunofluorescence staining. On the one hand, the growth condition of HPMCs was observed under an inverted phase-contrast microscope. As shown in Figure 5, suspension cells obtained from PDE would adhere to the culture flask within 3 days of seeding. Then the adherent cells showed a fusiform shape and often projected extensive filopodia to contact with neighboring cells upon adherence to the flask on day 5. When reaching confluency, cells formed a confluent monolayer and exhibited a typical cobble-stone appearance on day 7. On the other hand, the phenotype of HPMCs was confirmed by immunofluorescence staining. Results showed that cells were positively expressed of Vimentin and CK-18 but negatively expressed of FSP-1 and VWF.

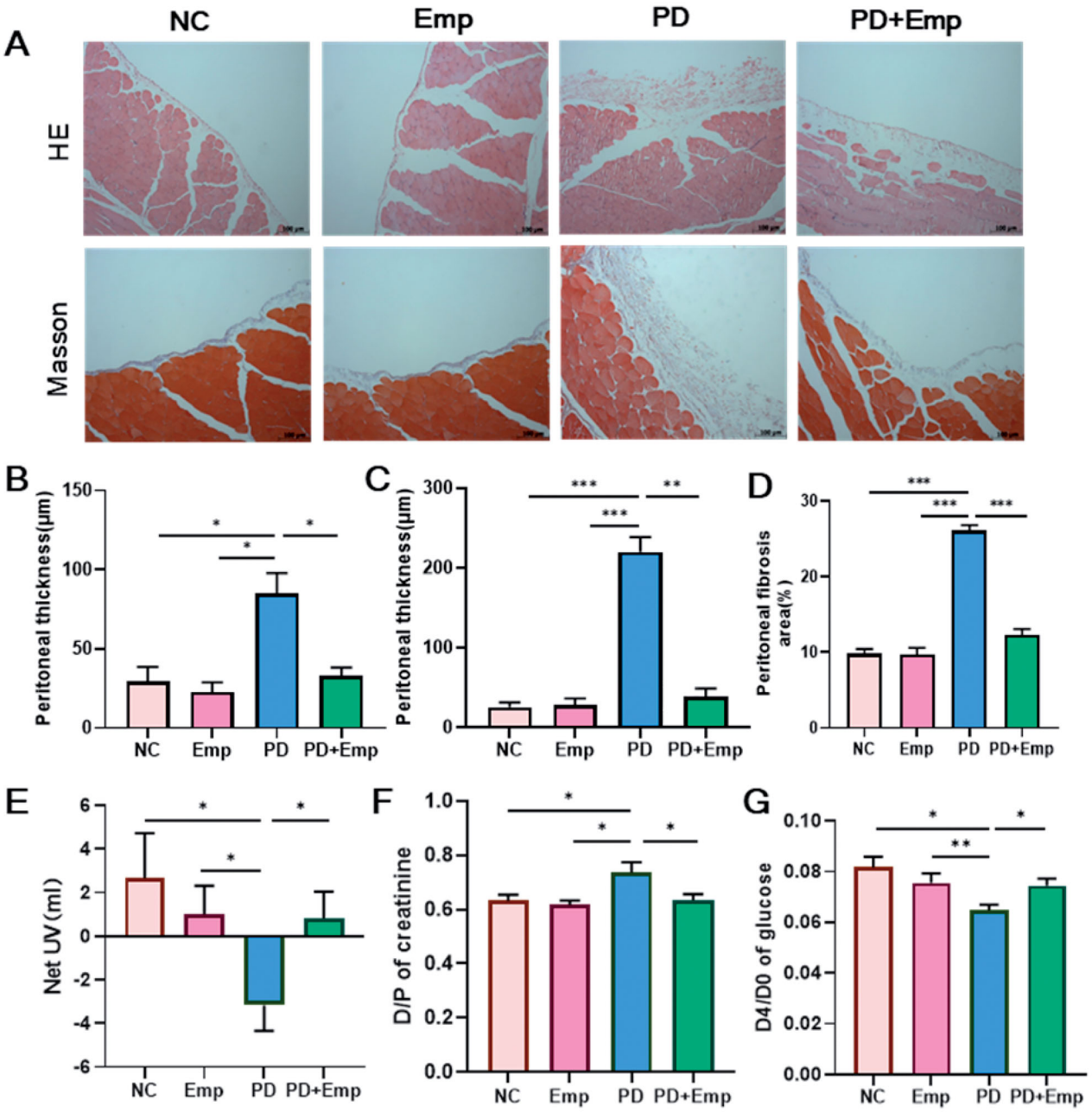

Figure 1. Effects of empagliflozin on peritoneal histopathological and functional damage in a rat model of peritoneal fibrosis. (A) Representative images of H&E and Masson's trichrome staining of rats from each group. (Scale Bar = 100μm, magnification, x100). (B) and (C) Mean peritoneal membrane thickness of rats from each group demonstrated by H&E and Masson's trichrome staining. 'Peritoneal thickness' was defined as the thickness of the submesothelial zone. (D) Peritoneal fibrosis score. (E) Net UV, (F) D/P of creatinine, and (G) D4/D0 of glucose were used to indicate the peritoneal function in each group. Data were expressed as the mean ± SEM. $n = 3$. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. NC: negative control; PD: peritoneal dialysis; Emp: empagliflozin.

Empagliflozin inhibited HG-induced EMT in HPMCs

To determine the effects of HG on the occurrence of EMT in HPMCs, we explored the most suitable glucose concentration and time for HPMCs stimulation. Cells were first treated with four different concentrations of glucose (control and 30, 60, and 120 mM) for 48 h to determine the suitable glucose concentration respectively. The results suggested that exposure of HPMCs to HG (60 and 120 mM) for 48 h decreased protein expression of epithelial cell marker E-cadherin, and increased the protein expression of fibrotic markers: α-SMA, Collagen-I, and Fibronectin. However, there was no statistical difference between the 60 mM and 120 mM groups (Figure 6(A,B)), suggesting that

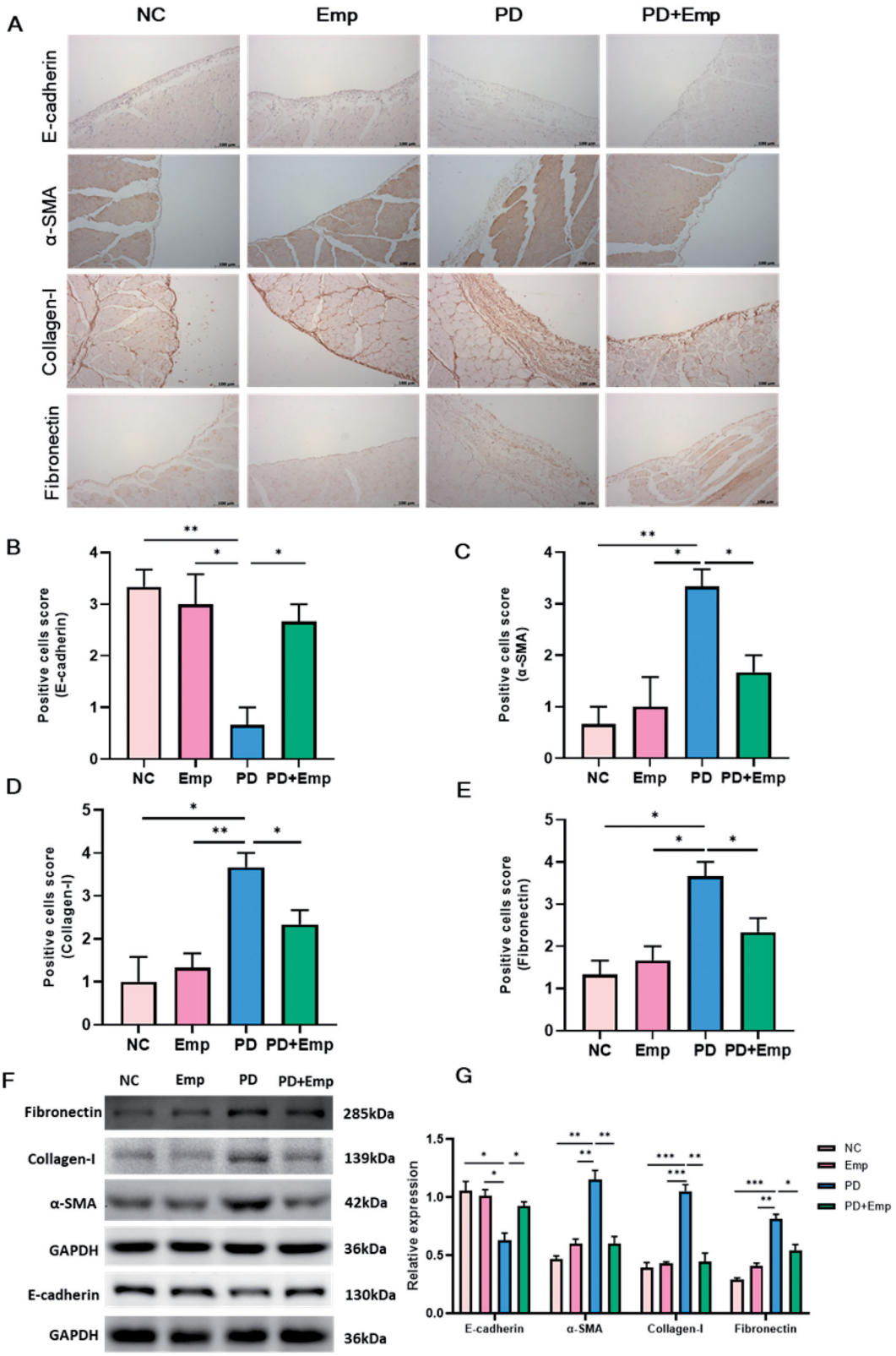

Figure 2. Effects of empagliflozin on the expression levels of EMT- and fibrosis-associated markers in the peritoneum of PD rats. (A-E) Representative immunocytochemistry of E-cadherin, α-SMA, Collagen-I, and Fibronectin in the peritoneum of rats from each group (original magnification × 100). (F) and (G) Representative western blots with quantitation bars of EMT- and fibrosis-associated proteins in the peritoneum of rats from each group. GAPDH was used as a loading control. Data were expressed as the mean ± SEM. $n = 3$. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. NC: negative control; PD: peritoneal dialysis; Emp: empagliflozin.

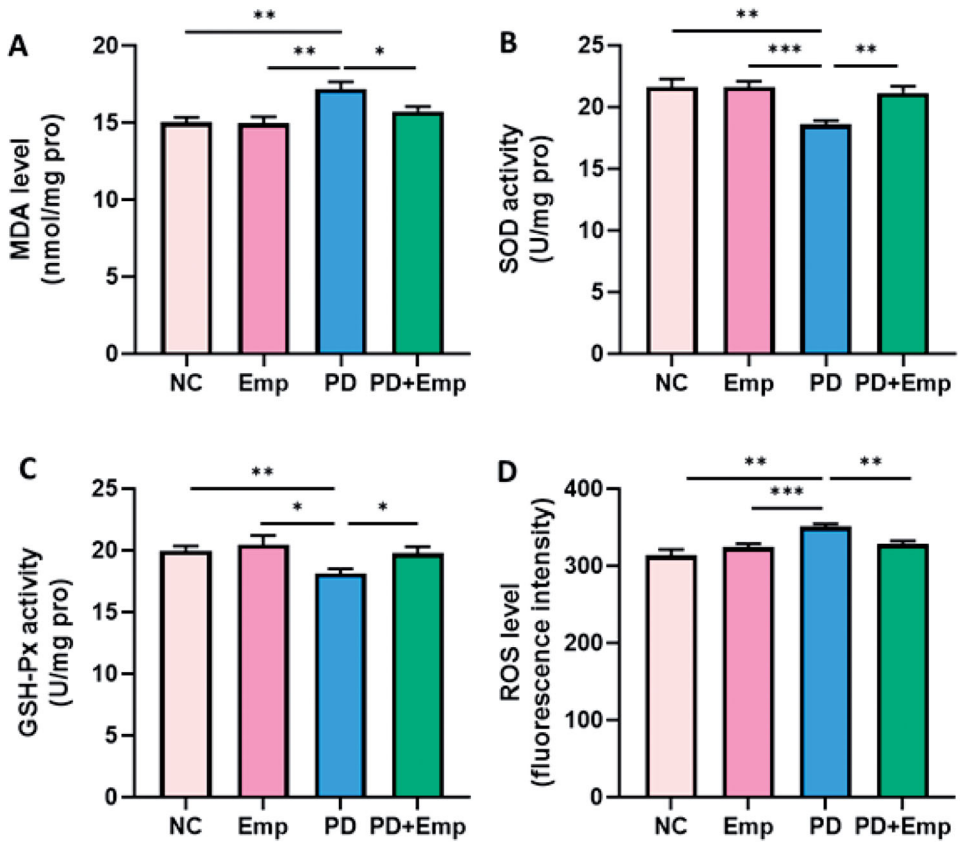

Figure 3. Effects of empagliflozin on oxidative stress levels in peritoneal tissue. (A) MDA levels, (B) SOD activity, (C) GSH-Px activity, and (D) ROS generation. Data were expressed as the mean ± SEM. $n = 3$. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. NC, negative control; PD: peritoneal dialysis; Emp: empagliflozin.

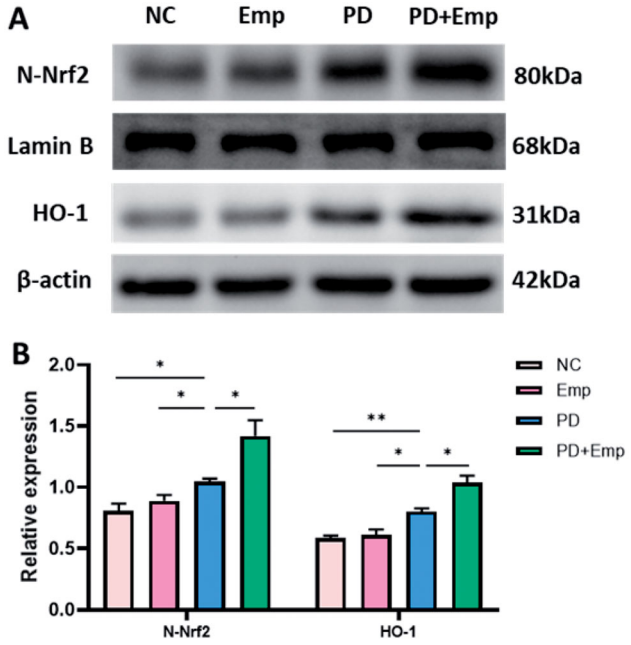

Figure 4. Effects of empagliflozin on the expression of N-Nrf2 and HO-1 in peritoneal tissue. (A) Representative western blots and (B) densitometric analysis for N-Nrf2 and HO-1. LaminB and β-actin were used as the loading control for Nrf2 and HO-1 respectively. Data were expressed as the mean ± SEM. $n = 3$. $^{*}p < 0.05$; $^{**}p < 0.01$. NC: negative control; PD: peritoneal dialysis; Emp: empagliflozin.

60 mM was the most suitable glucose concentration. Then cells were treated with 60 mM glucose for varying duration (12, 24, and 48 h) to find the appropriate stimulation time respectively. Stimulating HPMCs with 60 mM glucose for 24 or 48 h could significantly induce the EMT in HPMCs. There was also no statistical difference between the 24 h and 48 h groups (Figure 6(C,D)), suggesting that 24 h was the most appropriate stimulation time. In summary, these results indicated that EMT of PMCs could be successfully induced after stimulation with 60 mM glucose for 24 h. For subsequent analyses, we treated cells with 60 mM glucose for 24 h to produce significant EMT of HPMCs.

Next, we performed western blot analysis to demonstrate the effects of empagliflozin on HG-induced EMT. Consistent with *in vivo* findings, in the HG group, E- cadherin expression levels decreased, whereas the expression levels of α-SMA, Collagen-I, and Fibronectin were significantly upregulated compared with the NC and Emp groups. Cotreatment with empagliflozin resulted in a significant attenuation of HG-induced EMT- and fibrosis-associated alterations (Figure 6(E,F)).

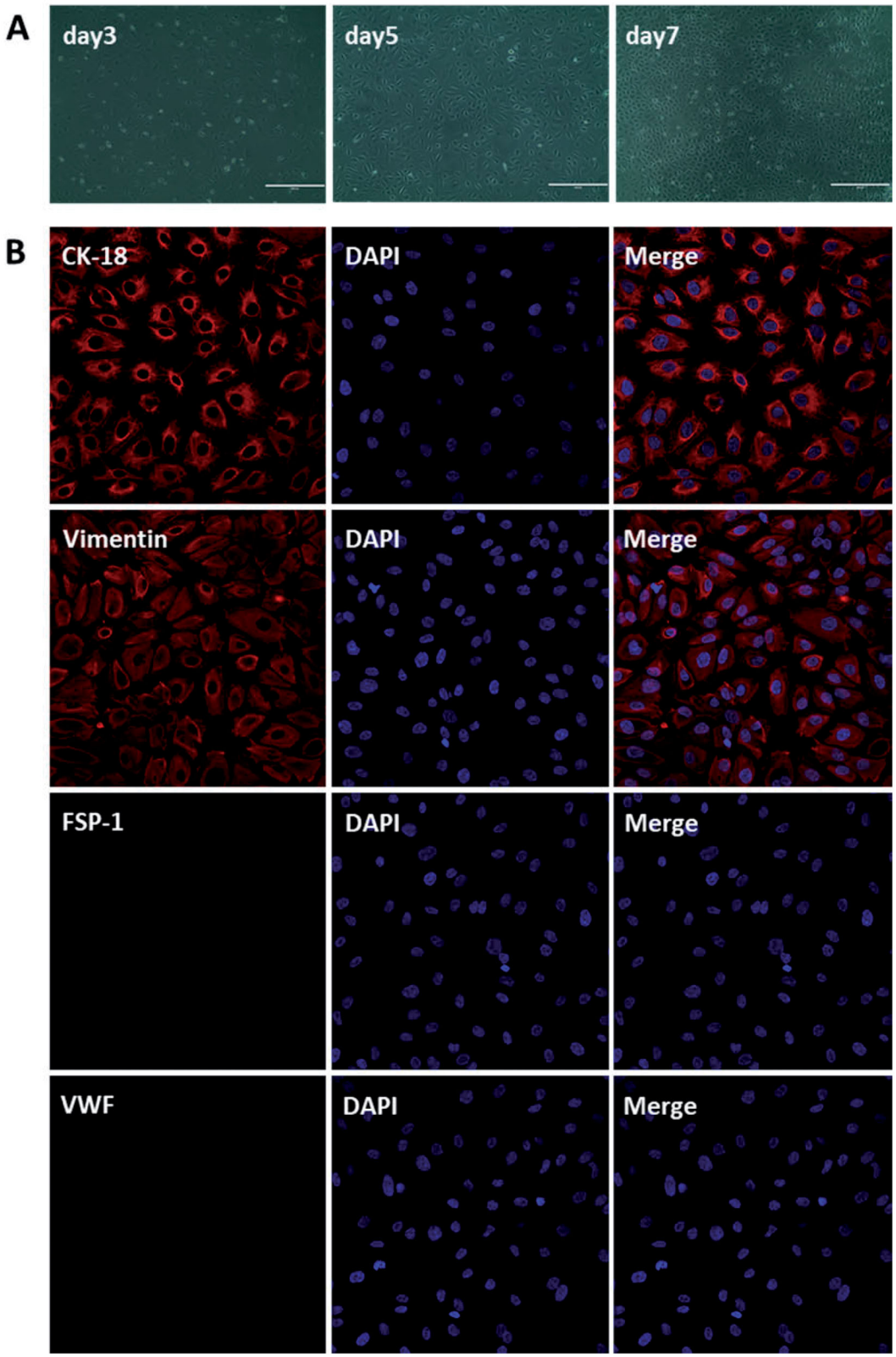

Figure 5. Identification of HPMC isolated from PDE. (A) Representative morphology of HPMC under the inverted phase-contrast microscope (scale bar = 100 μm, original magnification × 100). (B) Representative immunofluorescence staining showed that cultured HPMCs were positive for CK-18 and Vimentin but negative for FSP-1 and VWF. Red color indicates positive staining with anti-CK-18 or Vimentin antibody. Blue color indicates positive staining with DAPI staining (original magnification × 200).

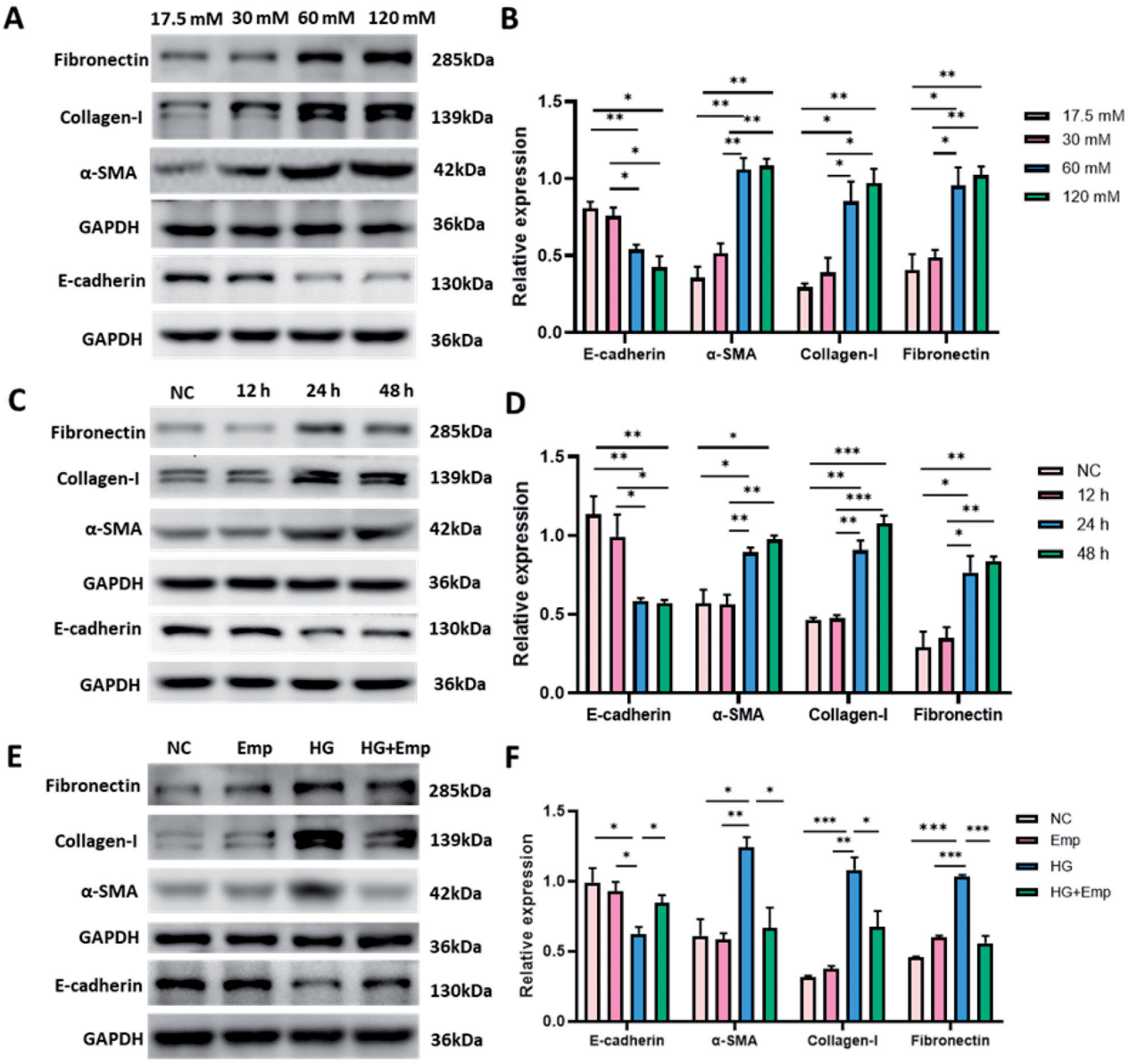

Figure 6. Effects of empagliflozin on the HG-induced EMT in HPMCs. Representative western blot analysis. (A) and (B) HPMCs were stimulated with different concentrations of glucose (17.5, 30, 60, and 120 mM) for 24 h. (C) and (D) HPMCs were stimulated with 60 mM glucose for 12, 24, and 48 h. (E) and (F) HPMCs were cotreatment with 60 mM glucose and empagliflozin for 24 h. GAPDH was used as a loading control. Data were expressed as the mean ± SEM. $n = 3$. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. NC, negative control; HG: high glucose; Emp: empagliflozin.

Empagliflozin attenuated HG-induced oxidative stress in HPMCs

To evaluate the effects of empagliflozin on the status of oxidative stress in HPMCs under HG, we detected the levels of ROS, SOD, MDA, and total antioxidant capacity levels. The HG group has a sharp accumulation of intracellular ROS and MDA, while empagliflozin significantly inhibited the HG-stimulated increase in ROS and MDA levels. The activity of SOD and total antioxidant capacity were remarkably raised in the HG + Emp group. These results indicated oxidative stress induced by HG in HPMCs was mitigated by empagliflozin (Figure 7).

Empagliflozin activated the Nrf2 antioxidant pathway induced by HG in HPMCs

To further understand the mechanisms responsible for the HG-induced EMT in the HPMCs, we investigated the activation of the Nrf2 signaling pathway. The expression levels of N-Nrf2 and HO-1 were upregulated

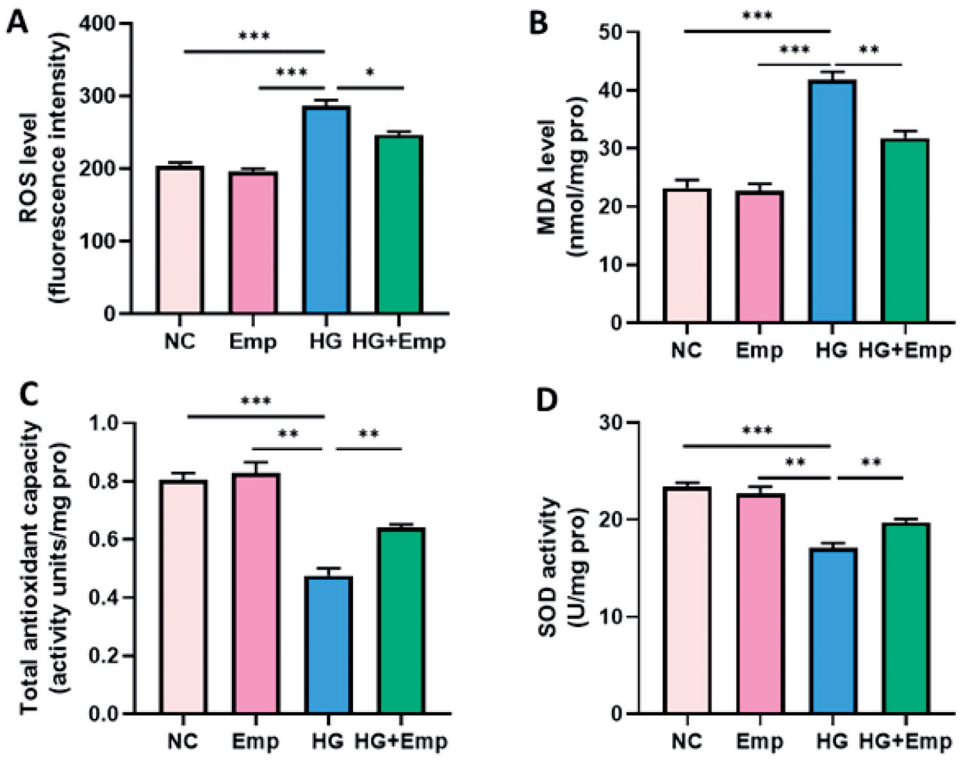

Figure 7. Effects of empagliflozin on the levels of oxidative stress in HPMCs under HG. (A) ROS level. (B) MDA level. (C) Total antioxidant capacity. (D) SOD activity. $n = 3$. Data were expressed as the mean ± SEM. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. NC: negative control; HG: high glucose; Emp: empagliflozin.

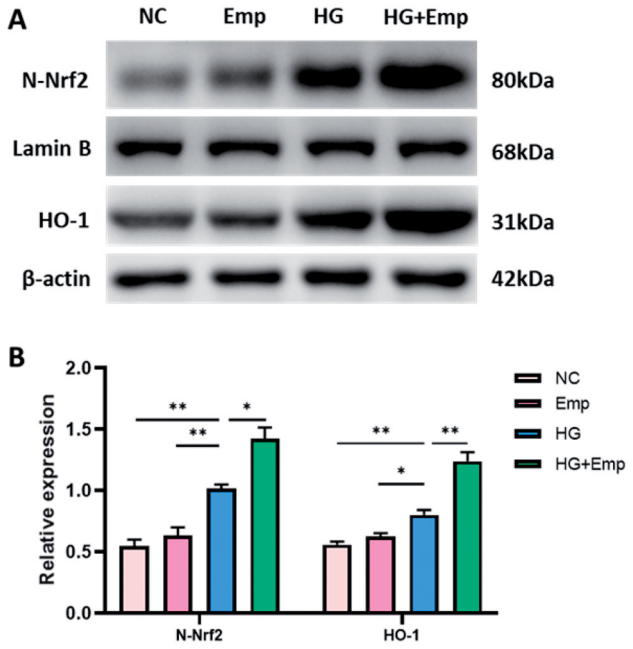

Figure 8. Effects of empagliflozin on the activity of the Nrf2 antioxidant pathway in HPMCs stimulated with HG. (A) Representative western blot and (B) densitometric analysis for N-Nrf2 and HO-1. LaminB and β-actin were used as the loading control for Nrf2 and HO-1 respectively. Data were expressed as the mean ± SEM. $n = 3$. $^{*}p < 0.05$; $^{**}p < 0.01$. NC: negative control; HG: high glucose; Emp: empagliflozin.

in HG-induced HPMCs compared with the NC group. Cotreatment with empagliflozin significantly promoted the expression of N-Nrf2 and HO-1 compared with the HG group. The group treated with empagliflozin alone presented no significant changes in expression compared with the NC group. These results were consistent with *in vivo* experiments (Figure 8).

Nrf2 activation played a key role in the inhibitory effects of empagliflozin on HG-induced oxidative stress and EMT

To verify the role of Nrf2 signaling activation mediated by empagliflozin *in vitro*, we measured oxidative stress levels and protein expression levels of EMT-associated markers after treatment with the specific Nrf2 inhibitor-ML385. It was verified that the inhibition of Nrf2 reversed the inhibitory effects of empagliflozin on oxidative stress and EMT (Figure 9). These results suggested that Nrf2 activation played a pivotal role for empagliflozin to exert inhibitory effects on HG-induced oxidative stress and EMT.

Discussion

Peritoneal fibrosis is a common complication of long-term PD and the main cause of ultrafiltration failure [4].

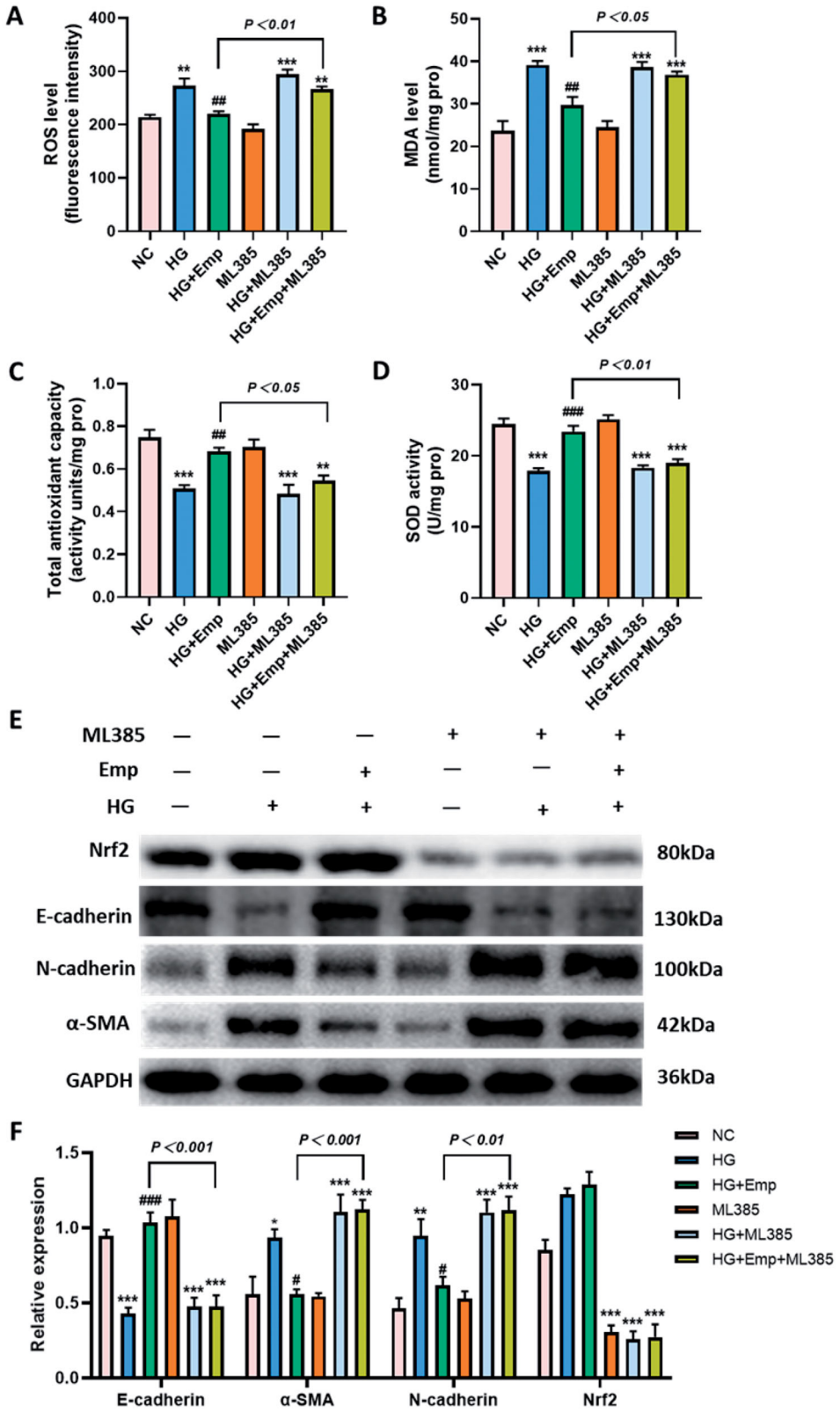

Figure 9. The role of Nrf2 activation in the inhibitory effects of empagliflozin on HG-induced oxidative stress and EMT in HPMCs. (A) ROS level. (B) MDA level. (C) Total antioxidant capacity. (D) SOD activity. $n = 3$. (E) Representative western blot and (F) densitometric analysis for EMT-related proteins and Nrf2 expression. GAPDH was used as the loading control. Data were expressed as the mean ± SEM. $n = 4$. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. NC: negative control; HG: high glucose; Emp: empagliflozin.

Scientists have devoted themselves to exploring the pathogenesis and treatment of peritoneal fibrosis. Regrettably, effective therapeutic methods are still limited. Therefore, it is urgent to find valuable treatments for peritoneal fibrosis. To the best of our knowledge, this is the first article to confirm that empagliflozin could inhibit HG-induced EMT in PMCs by suppressing oxidative stress. Additionally, we explored the potential mechanism and found that the protective effects of empagliflozin were associated with the activation of the Nrf2/HO-1 signaling pathway.

To evaluate the effects of empagliflozin *in vivo*, a rat model of peritoneal fibrosis was established by daily intraperitoneal injection of the 4.25% glucose PDS. Compared with the control and Emp groups, the PD group exhibited significant characteristics of peritoneal fibrosis. The current study demonstrated that empagliflozin could notably improve peritoneal function, relieve the thickness of the peritoneum and ameliorate collagen accumulation. Moreover, empagliflozin was able to inhibit the EMT by promoting the expression of epithelial marker E-cadherin and inhibiting the expression of fibrotic markers, including α-SMA, Collagen-I, and Fibronectin. Our previous study investigated the effects of varying empagliflozin concentrations (1, 3, and 6 mg/kg) on peritoneal glucose uptake in rats and found that rats with empagliflozin concentrations of 3 mg/kg reached the highest levels of both peritoneal glucose uptake ratios at D4/D0 and ultrafiltration ratios. Accordingly, the dose of empagliflozin used was 3 mg/kg in our study[18].

HG in PDS can improve the osmotic pressure on both sides of the peritoneum to improve the ultrafiltration volume, which plays an important role in ensuring the success of PD treatment [23]. However, HG is a potent inducer of EMT in PMCs [24]. EMT is a transdifferentiation process that converts epithelial cells into mesenchymal cells with migration and invasion capacities [25,26]. which has been identified as a crucial process in peritoneal fibrosis [5,27]. The EMT of HPMCs was induced by HG to explore the pathology and underlying mechanisms of peritoneal fibrosis in the present study. Based on our results and previous studies [28,29], the significant changes in expression levels of EMT- and fibrosis-associated biomarkers verified the successful establishment of EMT. However, treatment with empagliflozin suppressed the changes in the expression levels of representative markers. This is the first study to demonstrate that empagliflozin could inhibit EMT induced by HG in HPMCs. Our previous study has investigated the effects of different empagliflozin concentrations on HPMC viability and glycometabolism. Results demonstrated that 1 μM of empagliflozin was not toxic to the HPMCs and could enable HPMCs treated with HG to reach the lowest levels in both glucose uptake and consumption [18]. Consequently, the concentration of empagliflozin used was 1 μM in our study [19].

It was confirmed that empagliflozin could exert inhibitory effects on HG-induced EMT in our study, however, the potential mechanism is still unclear. Besides its excellent glycemic control effects, empagliflozin is a pleiotropic agent possessing anti-oxidative stress properties that make it a potential protective drug for fibrotic diseases [30,31]. Continuous exposure to HG could increase the generation of ROS which suppresses the activity of the antioxidant enzymes and in turn disrupts the balance between oxidants and antioxidants in PMCs, resulting in the occurrence and development of peritoneal fibrosis [7,32]. In particular, oxidative stress is regarded as a major mechanism of EMT in PMCs, thus aggravating the structural and functional damage of the peritoneum [11,33]. To investigate the antioxidant effects of empagliflozin in peritoneal fibrosis, we detected oxidative stress levels by assay kits. In the present study, HPMCs treated with HG were under substantial oxidative stress, indicated by enhanced accumulation of ROS and MDA as well as lower expression levels of SOD and total antioxidant capacity. However, empagliflozin was proven to decrease the level of oxidative stress. These results were also confirmed in the peritoneal tissue of PD rats. This was in agreement with the speculation that empagliflozin might attenuate the EMT of PMCs by inhibiting oxidative stress.

Emerging studies have confirmed that empagliflozin can act as an antioxidant to inhibit oxidative stress by activating the Nrf2/HO-1 signaling pathway, thus attenuating fibrotic diseases. It was found that empagliflozin ameliorated bleomycin-induced pulmonary fibrosis by enhancing the expression of Nrf2/HO-1 [20]. Empagliflozin not only improved diabetic myocardial structure and function but also decreased myocardial oxidative stress, thus ameliorating myocardial fibrosis, which was further confirmed to be correlated with the activation of Nrf2/HO-1 signaling [21]. It is worth noting that empagliflozin has been confirmed to alleviate peritoneal fibrosis by downregulating the TGF-β/Smad signaling pathway [19]. However, the potential mechanism underlying empagliflozin exerted antioxidative effects on HG-induced EMT in PMCs has not been reported. Thus, we speculated that empagliflozin could inhibit HG-induced EMT of PMCs by suppressing oxidative stress through the Nrf2/HO-1 signaling activation.

Nrf2 is a transcription factor with a basic-leucine zipper domain that can regulate the expression of some antioxidant enzymes through antioxidant response elements (AREs)[34]. The Nrf2 antioxidant pathway has been confirmed to play a vital role in the anti-fibrogenic process by regulating oxidative stress [35,36]. In addition, HO-1 is a downstream target gene of Nrf2. The expression of HO-1 is initiated and promoted by Nrf2, thus responding to oxidative stress and exerting antioxidant properties [37]. Nrf2/HO-1 signaling has been demonstrated to be activated in an HG-induced EMT of PMCs [14]. ML385, an inhibitor of Nrf2, can exacerbate HG-induced ROS production and EMT in PMCs, demonstrating that the protective role of Nrf2 signaling activation in EMT inhibition [15]. Herein, it was found that HG increased the expression of N-Nrf2 and HO-1. Notably, empagliflozin further promoted the expression of N-Nrf2 and HO-1. Combined with the results above, it might be concluded that HG could induce EMT of PMCs by activating the Nrf2/HO-1 signaling pathway. Empagliflozin may promote Nrf2 translocation to the nucleus and activate the Nrf2/HO-1 signaling to inhibit EMT and oxidative stress in PMCs. Given the close relationship between Nrf2/HO-1 expression and oxidative stress levels in HG-induced EMT of PMCs, empagliflozin may be considered a promising agent for reducing oxidative stress in the peritoneal fibrosis by targeting the Nrf2/HO-1 signaling pathway.

Furthermore, a key role of Nrf2 in the antioxidative effects of empagliflozin-mediated suppression of peritoneal fibrosis was verified by the Nrf2 inhibitor-ML385. ML385 can bind to the Neh1 binding region of Nrf2 and inhibiting binding of the Nrf2-MAFG complex to the promoter AREs sequence, thus reducing the transcriptional activity of Nrf2. In our study, pretreatment with ML385 reversed the inhibitory effects of empagliflozin on the HG-stimulated oxidative stress and EMT in HPMCs. These data confirmed that Nrf2 is a crucial target for empagliflozin to inhibit EMT of PMCs, followed by the alleviation of peritoneal fibrosis.

Nevertheless, the mechanism by which empagliflozin induces Nrf2 and HO-1 expression in HPMCs remains unclear. The cytoplasmic protein inhibitor Kelch-like ECH-associated protein 1 (Keap1) is the master regulator of Nrf2 activation, and the formation of an Keap1/Nrf2 signaling pathway represents the major modulator of the cellular antioxidation system [38]. Under stress conditions, Nrf2 dissociates from the Keap1 protein and translocates into the nucleus to increase the expression of the AREs-bearing target genes, which encode electrophile detoxifying enzymes such as NAD(P)H quinone dehydrogenase 1, antioxidant proteins such as HO-1, and GSH biosynthesis enzymes such as glutamate-cysteine ligase catalytic subunit [39]. The antioxidant axis Keap1/Nrf2 has been reported to relieve organ fibrosis, such as liver, lung and heart [38,40,41]. It has also been confirmed that the modulation of the Keap1/Nrf2 pathway could effectively attenuate oxidative stress and renal fibrosis [42,43]. Empagliflozin has been reported to protect the liver from oxidative stress and inflammation by activating the Nrf2/HO-1 signaling *via* the modulation of Keap1 [44]. Interestingly, empagliflozin has also been reported to play a similar role as above in the kidney [45]. Additional studies will be required to further decipher whether the regulatory effect of empagliflozin on Nrf2/HO-1 signaling in HG-stimulated EMT of PMCs is associated with Keap1.

In conclusion, the Nrf2/HO-1 signaling activation played a vital role in the HG-induced EMT of PMCs. Empagliflozin effectively attenuated HG-induced EMT *via* amelioration of oxidative stress, and potential mechanisms of the antioxidant effect provided by empagliflozin were associated with Nrf2/HO-1 signaling activation both *in vivo* and *in vitro*. These results indicated that empagliflozin could be a novel therapeutic option to protect the peritoneum from the development of EMT and peritoneal fibrosis.

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Ethical approval

The study protocol was consistent with the ethical principles of the Helsinki Declaration and was approved by the Ethics Committee of the Second Affiliated Hospital of Soochow University (approval number: JD-LK-2021-003-01; approval date: February 2, 2021). All participants were asked for permission to use their PDE for a noncommercial study and written informed consent was obtained.

Author contributions

YL performed study concept and design; PS and ZBZ performed development of methodology and writing; PRY and HYS performed the review and revision of the paper; XJY and SF provided acquisition, analysis and interpretation of data, and statistical analysis; KS provided technical and material support. All authors read and approved the final paper.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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