Donepezil ameliorates oxygen-glucose deprivation/reoxygenation-induced cardiac microvascular endothelial cell dysfunction through PARP1/NF-κB signaling

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Abstract. Ischemia/reperfusion (I/R) injury is a serious clinical condition characterized by high morbidity and mortality rates. Donepezil plays a neuroprotective role in I/R-associated diseases. The aim of the present study was to investigate the role and the potential mechanism of action of donepezil in I/R-induced myocardial microvascular endothelial cell dysfunction. An I/R model was simulated using oxygen-glucose deprivation/reoxygenation (OGD/R) injury in human cardiac microvascular endothelial cells (CMECs). Cell viability and lactate dehydrogenase release were examined following treatment with donepezil. Commercial kits were used to evaluate cell apoptosis, cell permeability and caspase-3 activity. The expression levels of apoptosis-associated proteins, as well as proteins found in tight junctions or involved in the poly(ADP-ribose) polymerase 1 (PARP1)/NF-κB pathway, were measured using western blotting. These parameters were also examined following PARP1 overexpression. The results demonstrated that donepezil increased cell viability and reduced toxicity in OGD/R-treated CMECs. The apoptotic rate, caspase-3 activity and protein expression levels of Bax and cleaved caspase-3 were significantly reduced following donepezil treatment, which was accompanied by Bcl-2 upregulation. Moreover, cell permeability was notably reduced, coupled with a marked increase in the expression of tight junction-associated proteins. The expression levels of proteins related to PARP1/NF-κB signaling were significantly downregulated in CMECs following donepezil treatment. However, the protective effects of donepezil on OGD/R-induced CMEC injury were reversed following PARP1 overexpression. In conclusion, donepezil suppressed OGD/R-induced CMEC dysfunction via PARP1/NF-κB signaling. This finding provided insight into the mechanism underlying myocardial I/R injury.

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Introduction

Ischemia/reperfusion (I/R) is a serious, life-threatening disease that can induce heart failure and other adverse cardiovascular outcomes following myocardial ischemia, cardiac surgery or circulatory arrest (1). The pathogenesis of I/R involves, at least in part, inflammation, myocardial necrosis, apoptosis, intracellular calcium overload and excess reactive oxygen species production (2-4). However, challenges remain in clinical practice.

Donepezil is a well-characterized reversible acetylcholinesterase inhibitor with a protective effect against neurodegenerative diseases, such as brain injury and Alzheimer's disease, following cardiac I/R injury (5). Previous studies have demonstrated that donepezil reduces myocardial I/R injury by balancing mitochondrial dynamics, mitochondrial phagocytosis and autophagy; in addition, it also markedly improved the long-term survival of rats with chronic heart failure after extensive myocardial infarction (6,7). Moreover, pretreatment with donepezil counteracts TNF-α-induced endothelial cell permeability (8). However, to the best of our knowledge, the role of donepezil in cardiac microvascular endothelial cells (CMECs) has not yet been reported.

Furthermore, the potential role of the poly(ADP-ribose) polymerase 1 (PARP1) signaling pathway in I/R injury has been demonstrated in several studies. For instance, inhibition of PARP1 activation and apoptosis-inducing factor (AIF) nuclear translocation attenuates caspase-independent cell death in a rat model of cerebral I/R (9). Another study has demonstrated the protective effect of modulating the PARP1/AIF signaling pathway in I/R-induced apoptosis (10). The oxygen-glucose deprivation/reoxygenation (OGD/R) cell model has been widely used to investigate the mechanisms of I/R injury (11,12). In addition, it has been suggested that regulation of the TLR4/PARP1/NF-κB pathway can ameliorate OGD/R injury (13). However, the roles of donepezil and the PARP1 signaling pathway remain unclear.

The aim of the present study was to investigate the role of donepezil in I/R by establishing a model of OGD/R injury using CMECs. In addition, the second objective was to determine whether the protective effects of donepezil are mediated through the PARP1/NF-κB signaling pathway.

Materials and methods

Blood samples. Blood samples were obtained intravenously from a total of 30 cases (age range, 16-34; male: female, 2:1) between April 2015 and January 2017 from the Cardiology Department of The Rizhao Central Hospital in Shandong Province (Rizhao, China), including 15 healthy controls (age range, 18-32; male: female, 2:1) and 15 patients (age range, 16-34; male: female, 2:1) with a confirmed diagnosis of coronary artery disease. The present study was approved (approval no. 2020-018) by The Medical Ethics Committee of Rizhao Central Hospital (Rizhao, China) and written informed consent was obtained from patients for all samples. The exclusion criteria was as follows: Patients with other comorbid syndromes; patients under 18 years of age; or patients who were unable to cooperate with the research. The samples were left to stand for 30 min, then centrifuged at 4˚C for 20 min at 1,000 x g to obtain serum.

Cell culture. The human CMECs (cat no. CP-H079) were purchased from Procell Life Science & Technology Co., Ltd. and cultured in DMEM supplemented with 10% FBS (both from Gibco; Thermo Fisher Scientific, Inc.) and 100 μg/ml penicillin/streptomycin (Beyotime Institute of Biotechnology) in a 5% CO_2 incubator at 37˚C. Then, cells were sub-cultured at a ratio of 1:2 when reaching 80-90% confluence and in passages two to three were used in cell experiments. All the experimental protocol was approved (approval no. 2020-018) by The Medical Ethics Committee of Rizhao Central Hospital.

OGD/R injury. CMECs were incubated for 4 h to simulate ischemia by deprivation of oxygen and glucose in serum/glucose-free DMEM in a 5% CO_2, 95% N_2 hypoxic chamber at 37˚C. After 4 h of incubation, cells were cultured in normal DMEM supplemented with 10% FBS under normoxic conditions for 12 h at 37˚C to recover. Different concentrations (25, 50 and 100 μM) of donepezil (cat no. D6821; Sigma-Aldrich; Merck KGaA) were used to treat the cells for 24 h before OGD/R. Control cells were incubated under normoxic conditions.

Cell Counting Kit-8 (CCK-8) assay. A CCK-8 (Shanghai Yusheng Biotechnology Co., Ltd.) was used to detect cell viability. Briefly, $1x10^4$ cells were plated into 96-well plates, then 10 μl CCK-8 reagent was added for 2 h at 37˚C. The absorbance at 450 nm was obtained using a spectrophotometer (Thermo Fisher Scientific, Inc.) as a measure of cell viability.

Lactate dehydrogenase (LDH) activity assay. Cytotoxicity was quantified using the LDH assay kit (cat. no. A020-2-2; Nanjing Jiancheng Bioengineering Institute). Briefly, the cells were centrifuged at 4˚C for 5 min at 600 x g to collect supernatant. The working solution from this kit was added to the 96-well plate in this order and the plate was incubated at 37˚C for 30 min according to the manufacturer's instructions. The absorbance values were measured at 450 nm.

Cell transfection. PARP1 overexpression vector (Ov-PARP1; 50 nM) and negative control vector (Ov-NC; 50 nM) were designed and amplified by Shanghai GenePharma Co., Ltd. CMECs were seeded into 6-well plates at a density of $1x10^6$ cells/well, then transfected with Ov-PARP1 or Ov-NC using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) for 24 h at 37˚C according to the manufacturer's protocols. Successful transfection was determined using reverse transcription-quantitative PCR (RT-qPCR). Subsequent experiments were completed within 48 h.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). RNA was then reverse transcribed into cDNA using the Revert Aid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. qPCR was performed with 2 μg cDNA using iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, Inc.) on an ABI PRISM 7500 Real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following thermocycling conditions were used for qPCR: Initial denaturation at 95˚C for 5 min; followed by 40 cycles at 95˚C for 10 sec, 55˚C for 20 sec and 72˚C for 20 sec, and final extension step at 72˚C for 2 min. The sequences of the PARP1 and GAPDH primers were as follows: PARP1 forward, 5'-GGCGATCTTGGACCGAGT AG-3' and reverse, 5'-AGCTTCCCGAGAGTCAGGAT-3'; and GAPDH forward, 5'-GGAGCGAGATCCCTCCAAAAT-3' and reverse, 5'-GGCTGTTGTCATACTTCTCATGG-3'. The mRNA levels of PARP1 were normalized to those of endogenous control GAPDH and were calculated using the $2^{-\Delta\Delta Cq}$ method (14).

Western blotting. Total protein was extracted from cells using RIPA lysis buffer (Beijing Solarbio Science & Technology Co., Ltd.) with 1% phenylmethanesulfonyl fluoride on ice, and the concentration was determined using a BCA kit (cat. no. P0012S; Beyotime Institute of Biotechnology). A mass of 30 μg of protein was loaded per lane and separated using 6, 10 or 15% SDS-PAGE, and then transferred to PVDF membranes. The membranes were then blocked with 5% skimmed milk for 2 h at room temperature. Subsequently, the membranes were incubated overnight at 4˚C with primary antibodies as follows: Anti-Bcl-2 (cat. no. 15071), anti-Bax (cat. no. 2774), anti-caspase-3 (cat. no. 14220), anti-cleaved caspase-3 (cat. no. 9661), anti-zona occludens-1 (ZO-1; cat. no. 13663), anti-occludin (cat. no. 91131), anti-vascular endothelial cadherin (VE-cadherin; cat. no. 2158), anti-claudin-5 (1:3,000; cat. no. ab131259; Abcam), anti-PARP1 (cat. no. ab191217; Abcam), NF-κB p65 (cat. no. 8242), phosphorylated (p)-NF-κB p65 (p-NF-κB p65; cat. no. 3033) and anti-GAPDH (cat. no. 5174). Following primary incubation, the membranes were incubated with HRP-conjugated goat anti-mouse IgG2c (cat. no. 56970) and goat anti-rabbit IgG2c (cat. no. 7074) secondary antibodies for 1-2 h at room temperature. All antibodies were purchased from Cell Signaling Technology, Inc. and used at 1 in 1,000 dilution

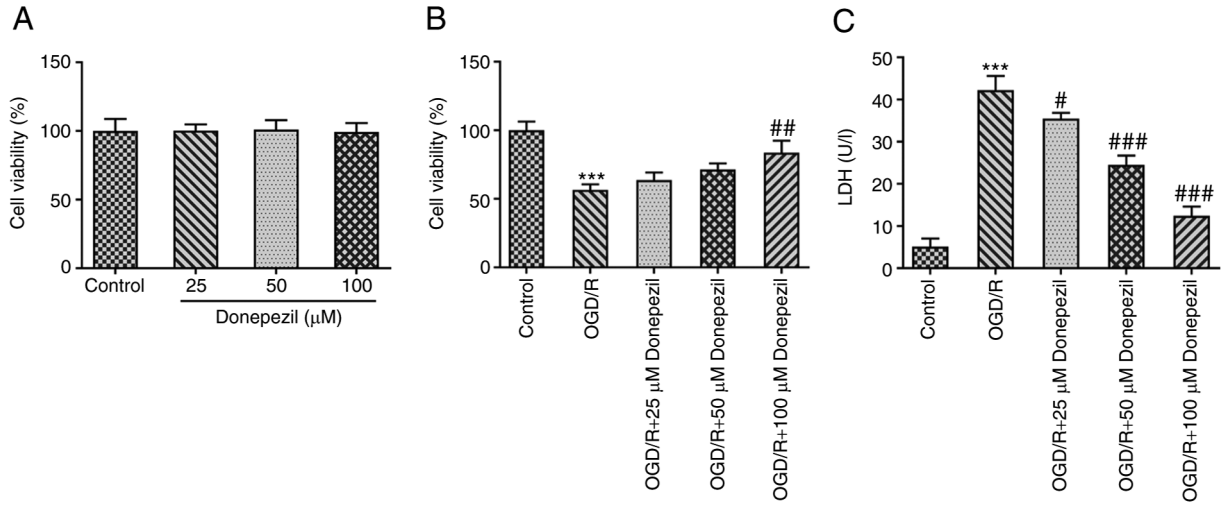

Figure 1. Donepezil increases cell viability and reduces toxicity in OGD/R-induced CMECs. (A) Cell viability following treatment with different concentrations of donepezil. (B) Cell viability in OGD/R-induced CMECs following treatment with different concentrations of donepezil. (C) LDH release in each group. ***P<0.001 vs. the control; #P<0.05, ##P<0.01 and ###P<0.001 vs. OGD/R. OGD/R, oxygen-glucose deprivation/reoxygenation; CMECs, cardiac microvascular endothelial cells; LDH, lactate dehydrogenase.

unless otherwise indicated. The membranes were visualized with enhanced chemiluminescence reagent (ECL System; MilliporeSigma) and ImageJ software (version 1.8.0; National Institutes of Health) was used to quantify the grayscale values. Protein levels were normalized to those of GAPDH.

TUNEL assay. Apoptosis of CMECs was detected by a One Step TUNEL assay kit (cat. no. C1088; Beyotime Institute of Biotechnology) according to the manufacturer's instructions. Briefly, following fixation with 4% paraformaldehyde at room temperature for 15 min, cells at the density of $1x10^6$ cells/well were washed twice with PBS in 24-well plates, then incubated with 50 μl TUNEL reaction buffer for 1 h at 37˚C in the dark. Subsequently, 1 μg/ml DAPI was used for counterstaining in the dark at room temperature for 15 min and cells were mounted using DAPI-containing mounting medium (Vector Laboratories, Inc.). Images were captured using a fluorescence microscope (magnification, x200; Olympus Corporation), and cells were counted in five randomly selected microscopic fields.

Caspase-3 activity assay. Caspase-3 activity was measured in CMECs using a caspase-3 activity assay kit (cat. no. C1116; Beyotime Institute of Biotechnology). The cells were lysed on ice for 15 min and centrifuged at 16,000 x g for 15 min at 4˚C, then added to a reaction buffer mixture containing the caspase-3-specific substrate Ac-DEVD-pNA in 96-well plates to incubate for 1-2 h at 37˚C. The absorbance value was measured at a wavelength of 405 nm using a spectrophotometer (Thermo Fisher Scientific, Inc.).

Human CMEC permeability assay. CMECs ($1x10^4$ cells/well) were seeded in the upper chamber of a Transwell (Corning, Inc.) with 8-μm pores, then incubated with 0.5 mg/ml fluorochrome-conjugated dextran (cat. no. D1830; Thermo Fischer Scientific, Inc.) for 30 min at 37˚C. 1 ml complete medium was added to the lower chamber. Fluorescence was measured in the lower chamber at 595 and 615 nm using a plate reader (Packard Bioscience Company).

Statistical analysis. Statistical analysis was performed using GraphPad Prism 8.0 software (GraphPad Software, Inc.). One-way ANOVA followed by Tukey's post hoc was used to examine the differences between multiple groups. P<0.05 was considered to indicate a statistically significant difference. The data are presented as the mean ± SD of at least three independent experiments.

Results

Donepezil inhibits OGD/R-induced CMEC injury. CMEC viability was detected using CCK-8 assays. A gradient concentration of donepezil (25, 50 and 100 μM) was used to pre-treat CMECs before OGD/R intervention. The results demonstrated that no significant difference was present in cell viability between each concentration alone (Fig. 1A). However, cell viability was significantly reduced following OGD/R treatment compared with the untreated group and a concentration-dependent increase was observed after donepezil treatment compared with the OGD/R group (Fig. 1B). Furthermore, as shown in Fig. 1C, LDH release (a measure of cytotoxicity) was significantly increased following OGD/R injury. In addition, treatment with different concentrations of donepezil gradually reduced the release of LDH. As demonstrated in Fig. 2A and B, the apoptotic rate and caspase-3 activity were both inhibited following donepezil treatment compared with OGD/R alone. Moreover, donepezil intervention significantly attenuated the expression of Bax and cleaved caspase-3/caspase-3 proteins, which was accompanied by upregulation of the Bcl-2 anti-apoptotic protein (Fig. 2C).

Donepezil ameliorates OGD/R-induced dysfunction in CMECs. Cell permeability and the expression of tight junction-associated proteins were analyzed to examine the function of CMECs. As presented in Fig. 3A, treatment with donepezil reduced the increase in permeability induced by OGD/R in a concentration-dependent manner, indicating restoration of endothelial cell barrier function. Similarly, the

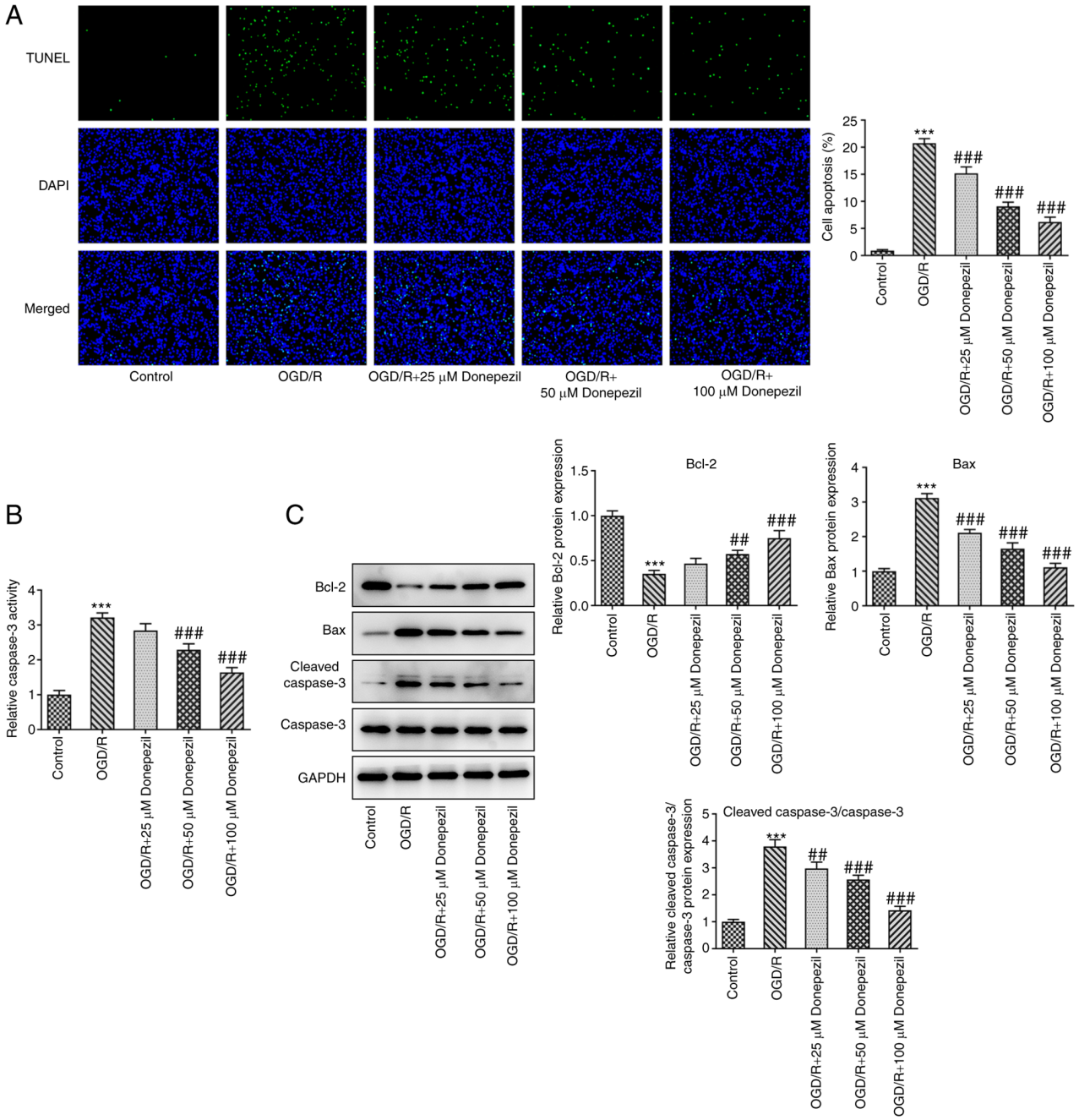

Figure 2. Donepezil attenuates OGD/R-induced apoptosis in CMECs. (A) Apoptosis was assessed using TUNEL staining. Magnification, x200. (B) Caspase-3 activity. (C) Protein expression levels of Bcl-2, Bax and cleaved caspase-3/caspase-3. ***P<0.001 vs. the control; ##P<0.01 and ###P<0.001 vs. OGD/R. OGD/R, oxygen-glucose deprivation/reoxygenation; CMECs, cardiac microvascular endothelial cells.

levels of tight junction-associated proteins, including ZO-1, occludin, VE-cadherin and claudin-5, were significantly reduced in OGD/R-treated cells compared with the control group. However, the levels of these aforementioned proteins were significantly increased following donepezil treatment (Fig. 3B). These results suggested that donepezil restored CMEC function.

Donepezil ameliorates OGD/R-induced CMEC dysfunction via the PARP1/NF-κB signaling pathway. PARP1 expression levels were significantly higher in serum from patients with myocardial infarction than in healthy individuals (Fig. 4A and B). Furthermore, the PARP1 expression levels and p-NF-κB p65/NF-κB p65 protein levels were significantly reduced in OGD/R-exposed CMECs by donepezil compared with the OGD/R group (Fig. 4C and D), suggesting a potential role for PARP1/NF-κB signaling. To further investigate the mechanism of action of donepezil, 100 μM donepezil was used for subsequent experiments. Ov-PARP1 was transfected into CMECs, which led to PARP1 upregulation compared with the Ov-NC group, as demonstrated by RT-qPCR and western blotting (Fig. 5). In addition, the protective effect of donepezil on OGD/R-induced cell viability was reversed following PARP1 overexpression (Fig. 6A). Treatment with donepezil in Ov-PARP1-transfected cells significantly increased LDH release in CMECs compared with the OGD/R + donepezil

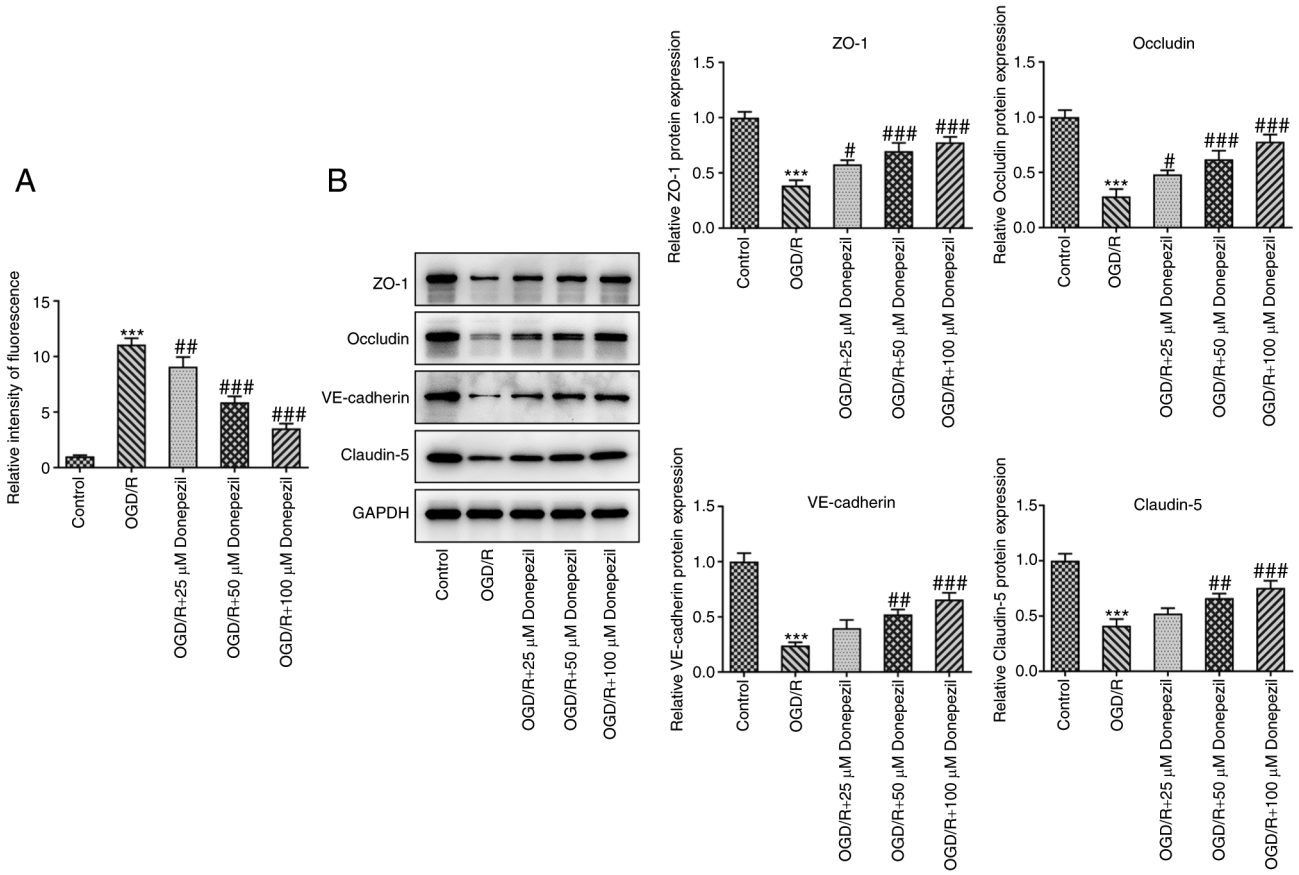

Figure 3. Donepezil enhances OGD/R-induced dysfunction of CMECs. (A) Cell permeability was measured using fluorochrome-conjugated dextran. (B) Protein expression levels of ZO-1, occludin, VE-cadherin and claudin-5. ***P<0.001 vs. the control; #P<0.05, ##P<0.01 and ###P<0.001 vs. OGD/R. OGD/R, oxygen-glucose deprivation/reoxygenation; CMECs, cardiac microvascular endothelial cells; ZO-1, zona occludens-1; VE-cadherin, vascular endothelial cadherin.

group (Fig. 6B). Similarly, apoptosis and caspase-3 activity were also increased (Fig. 6C and D). In addition, Bax and cleaved caspase-3 protein levels were upregulated, while those of Bcl-2 were reduced (Fig. 6E). The expression levels of tight junction-associated proteins were also significantly reduced after Ov-PARP1 transfection, which was accompanied by a notable increase in cell permeability. The barrier function of CMECs was disrupted following PARP1 overexpression (Fig. 7). This suggested that donepezil protects CMECs from OGD/R damage through the PARP1/NF-κB pathway.

Discussion

Myocardial I/R injury is a common public health concern worldwide and numerous studies have demonstrated that I/R-induced cardiac injury can be reduced following inhibition of apoptosis (15,16). Furthermore, OGD/R-treated cells have been studied *in vitro* to attenuate I/R-induced apoptosis (17). In the present study, the apoptotic rate and TUNEL-stained images were revealed to be significantly increased following OGD/R, which was accompanied by changes in the expression of apoptosis-associated proteins and caspase-3 activity.

Donepezil, an acetylcholinesterase inhibitor, enhances cholinergic neurotransmission by reversibly binding to acetylcholinesterase enzyme and blocking acetylcholine hydrolysis (18). Donepezil is approved for the treatment of Alzheimer's disease (19,20). However, a large body of studies have also reported a role for donepezil in ischemic or cardiovascular diseases. For instance, a previous clinical trial illustrated that treatment of acute ischemic stroke with donepezil enhanced recovery (21). Donepezil has also been shown to reduce I/R-induced brain damage through inhibition of Ca^{2+} overload and antioxidation (22), to inhibit apoptosis and protect I/R renal function in mice (23), and to improve long-term survival in rats with chronic heart failure after extensive myocardial infarction (6). Moreover, donepezil was demonstrated to protect rat primary cerebral cortical neurons against OGD/R-induced injury (24). In addition, it has been documented that donepezil plays a protective role against endothelial cell injury (8,25,26). Notably, it was revealed for the first time in the present study, to the best of our knowledge, that donepezil had a protective effect against OGD/R-induced apoptosis in a dose-dependent manner. Meanwhile, after donepezil pretreatment in the presence of OGD/R, the permeability of CMECs was markedly reduced and tight junction protein expression was increased, suggesting a protective effect of donepezil on the barrier function of CMECs.

PARP1 is upregulated following I/R injury (27). Furthermore, inhibition of the PARP1 signaling pathway has been reported to serve a protective role against I/R injury (28). A previous study showed that hypoxic preconditioning protects human brain endothelial cells from ischemic apoptosis (29).

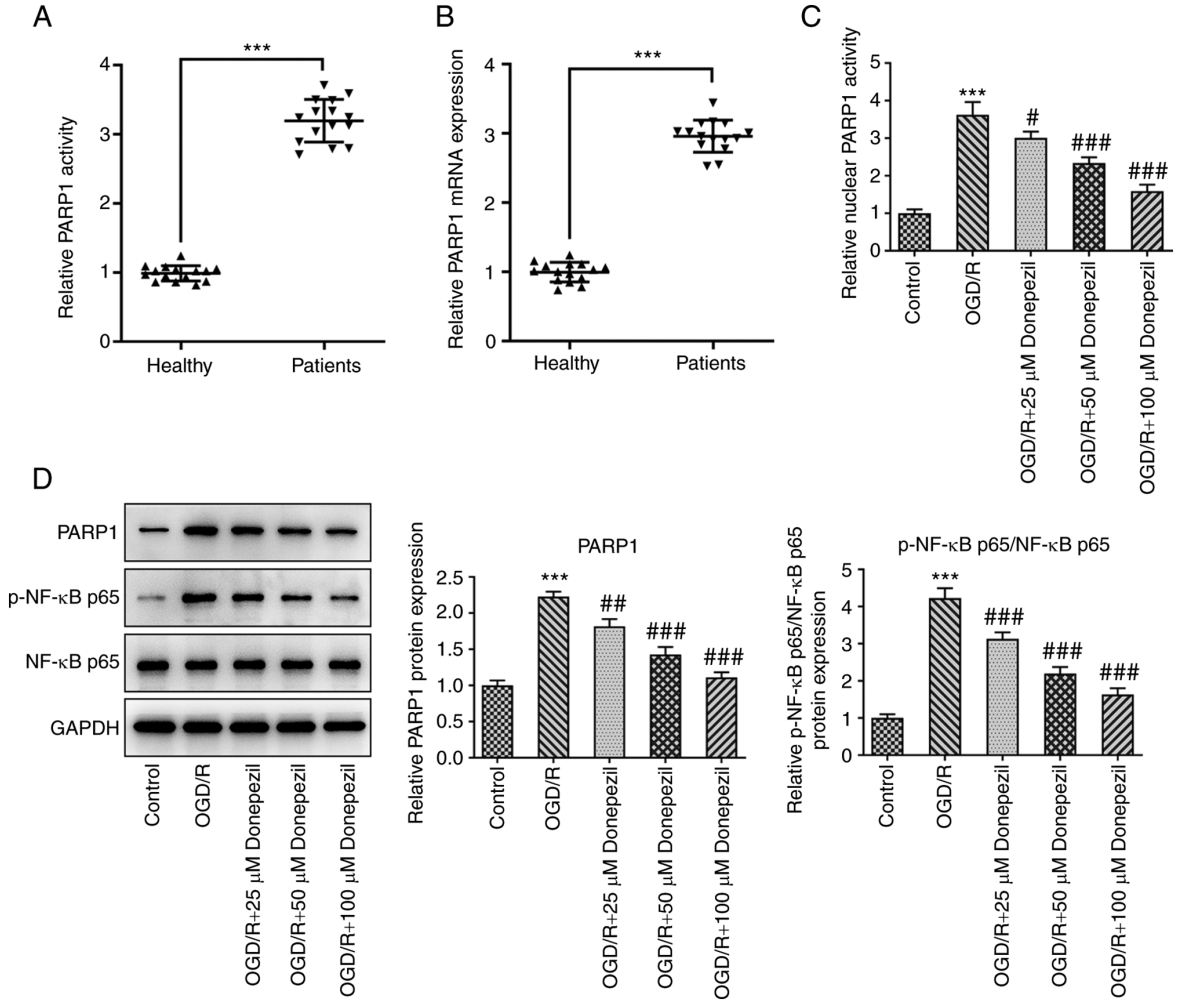

Figure 4. PARP1 pathway-associated proteins are upregulated in myocardial infarction patients and in OGD/R-treated cells. (A) PARP1 activity was measured using a PARP assay kit and (B) mRNA expression was measured using reverse transcription-quantitative PCR in serum samples from patients with myocardial infarction. (C) PARP1 activity in each group of CMECs. (D) Protein expression levels of PARP1 and p-NF-κB p65/NF-κB p65 (normalized to GAPDH). ***P<0.001 vs. the control; #P<0.05, ##P<0.01 and ###P<0.001 vs. OGD/R. PARP1, poly(ADP-ribose) polymerase 1; OGD/R, oxygen-glucose deprivation/reoxygenation; CMECs, cardiac microvascular endothelial cells; p-, phosphorylated.

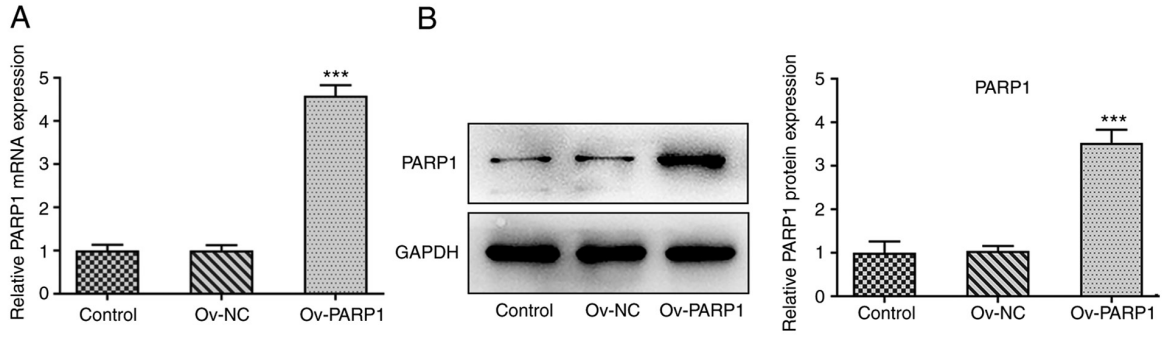

Figure 5. PARP1 is highly expressed in CMECs following transfection with Ov-PARP1. (A) mRNA and (B) protein expression levels of PARP1 following transfection with Ov-PARP1. ***P<0.001 vs. Ov-NC. PARP1, poly(ADP-ribose) polymerase 1; CMECs, cardiac microvascular endothelial cells; Ov-PARP1, PARP1 overexpression vector; Ov-NC, negative control vector.

Donepezil may work in OGD/R-induced CMEC by interfering with PARP1/NF-κB signaling. Consistent with the expected results, OGD/R-induced PARP1 and p-NF-κB p65 expression levels were found to be reduced following donepezil treatment. More importantly, overexpression of PARP1 significantly reversed the effects of donepezil on cell viability, apoptosis and cell barrier function. Thus, the present study provided the first evidence that donepezil affected cell function via the PARP1/NF-κB signaling pathway, which was the highlight of the study.

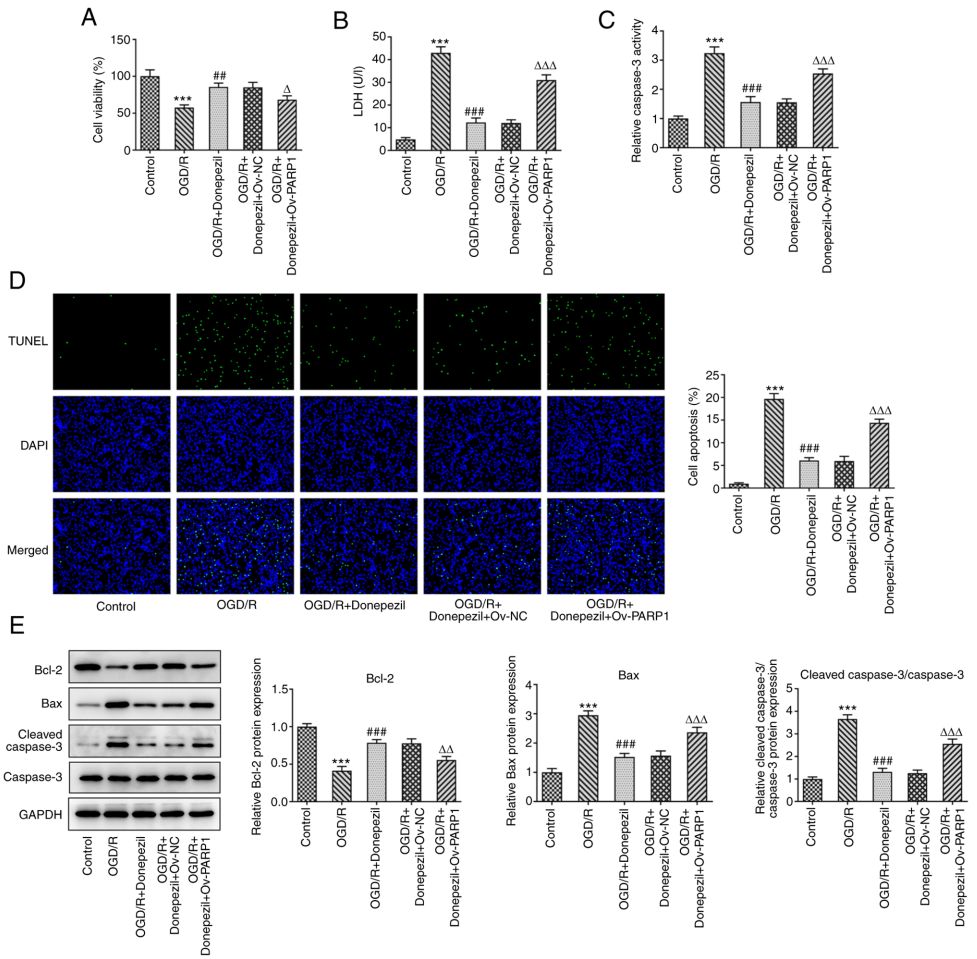

Figure 6. PARP1 overexpression reverses the protective effect of donepezil against OGD/R injury. (A) Viability, (B) LDH release and (C) caspase-3 activity in each group of CMECs. (D) Apoptosis was assessed using TUNEL staining. Magnification, x200. (E) Protein expression levels of Bcl-2, Bax and cleaved caspase-3 (normalized to GAPDH). ***P<0.001 vs. the control; $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. OGD/R; $^{\Delta}$P<0.05, $^{\Delta\Delta}$P<0.01 and $^{\Delta\Delta\Delta}$P<0.001 vs. OGD/R + Donepezil + Ov-NC. PARP1, poly(ADP-ribose) polymerase 1; OGD/R, oxygen-glucose deprivation/reoxygenation; LDH, lactate dehydrogenase; CMECs, cardiac microvascular endothelial cells; Ov-PARP1, PARP1 overexpression vector.

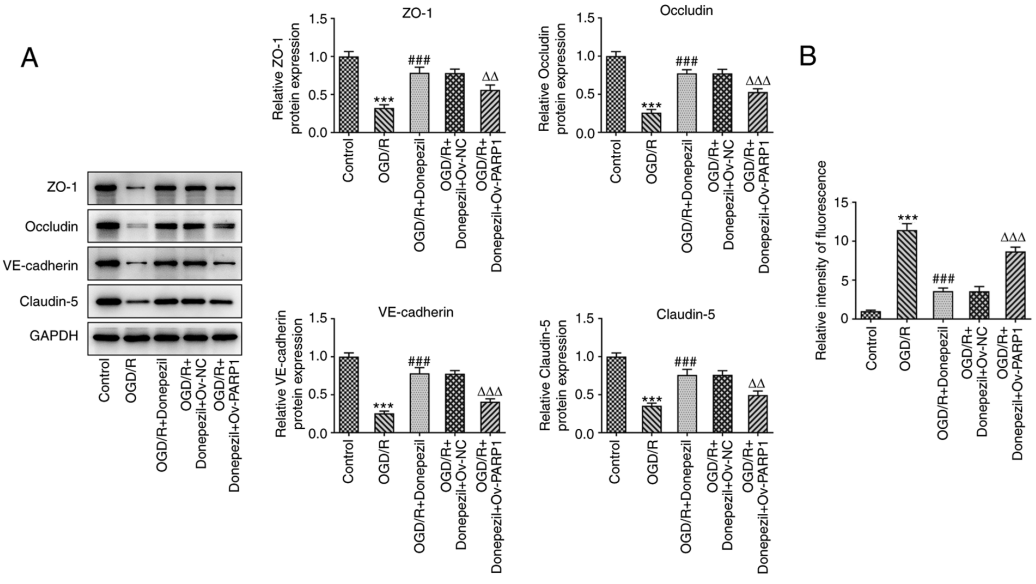

Figure 7. PARP1 overexpression reverses OGD/R-induced barrier dysfunction of CMECs following donepezil treatment. (A) Protein expression levels of ZO-1, occludin, VE-cadherin and claudin-5 (normalized to GAPDH). (B) Cell permeability was measured using fluorochrome-conjugated dextran. ***P<0.001 vs. control; $^{\#\#\#}$P<0.001 vs. OGD/R; $^{\Delta\Delta}$P<0.01 and $^{\Delta\Delta\Delta}$P<0.001 vs. OGD/R + Donepezil + Ov-NC. PARP1, poly(ADP-ribose) polymerase 1; OGD/R, oxygen-glucose deprivation/reoxygenation; CMECs, cardiac microvascular endothelial cells; ZO-1, zona occludens-1; VE-cadherin, vascular endothelial cadherin; Ov-NC, negative control vector; Ov-PARP1, PARP1 overexpression vector.

In summary, the present findings suggested that donepezil effectively protects against OGD/R injury by inhibiting apoptosis and maintaining cell function via PARP1/NF-κB signaling in CMECs. These results provided insight into the mechanisms underlying I/R-induced microvascular endothelial cell disorders. However, future studies are required to further demonstrate this mechanism in an *in vivo* animal model to exclude the existing limitations of *in vitro* studies, including the possible effects of donepezil on processes such as cellular senescence.

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Availability of data and materials

The datasets used and analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

YL and CY conceived and designed the study. YL, XS, JZ and CY performed the experiments. XS, JZ and JW collected the data and reviewed the manuscript. YL and XS drafted the manuscript. YL, XS, JW and CY were responsible for analyzing the data. All authors read and approved the final manuscript. YL, XS, JW and CY confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The present study was approved (approval no. 2020-018) by The Medical Ethics Committee of Rizhao Central Hospital (Rizhao, China) and written informed consent was obtained from patients for all samples.

Patient consent for publication

The patients consented to the publication of their data in the present study.

Competing interests

The authors declare that they have no competing interests.

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