

Protective effects of syringic acid on inflammation, apoptosis and intestinal barrier function in Caco-2 cells following oxygen-glucose deprivation/reoxygenation-induced injury

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Abstract. Syringic acid (SA) is an abundant phenolic acid compound that has been demonstrated to yield therapeutic benefits in myocardial and renal ischemia/reperfusion (I/R). However, the role of SA in intestinal I/R injury is unclear. Thus, the present study aimed to investigate the protective effect of SA against intestinal I/R injury. Caco-2 cells were incubated with different doses of SA before oxygen-glucose deprivation/reoxygenation (OGD/R) induction. The viability of Caco-2 cells, the activity of lactate dehydrogenase (LDH), the production of pro-inflammatory cytokines and the levels of reactive oxygen species, superoxide dismutase and malondialdehyde were measured. Apoptosis was evaluated using a TUNEL assay and western blotting. Transepithelial electrical resistance and western blotting were performed to evaluate intestinal barrier function in Caco-2 cells. The present study revealed that pretreatment with SA significantly increased cell viability and reduced LDH release in Caco-2 cells subjected to OGD/R treatment. In addition, SA suppressed OGD/R-induced inflammatory responses by reducing pro-inflammatory cytokine levels. Furthermore, the levels of oxidative stress and apoptosis were ameliorated by SA. SA also alleviated the intestinal barrier disruption exhibited by Caco-2 cells after OGD/R injury. Overall, the present study revealed that SA may potentially protect Caco-2 cells from OGD/R injury, and that this effect may be attributed to its anti-inflammatory and

anti-apoptotic activities, as well as its ability to protect the function of the intestinal barrier.

Introduction

Intestinal ischemia/reperfusion (I/R) is a clinical vascular emergency that is mainly caused by hemorrhagic or septic shock, cardiac arrest, occlusion of the mesenteric artery and surgical procedures (1). This pathophysiological process can cause systemic inflammatory response syndrome and multiple organ failure, which ultimately results in high morbidity and mortality rates (2,3). Intestinal I/R injury is a complex process involving oxidative stress, inflammation, apoptosis and intestinal barrier function disruption (4). Increased microvascular permeability and mucosal barrier damage may occur following ischemia of the intestine, while reperfusion leads to serious oxidative stress and inflammatory cell infiltration (5,6). At present, the strategies for treating intestinal I/R injury mostly concentrate on the reperfusion period, since intestinal ischemia is rarely preventable (7). Several common reagents have been used to treat intestinal I/R injury, such as glutamine, palmitoylethanolamide and inducible nitric oxide synthase (iNOS) inhibitors, but the clinical efficacy of these treatments have not been determined (8-10). Novel therapeutic approaches, including natural products for attenuating inflammation and apoptosis, and protecting the intestinal mucosal barrier from injury, are under development (11).

Syringic acid (SA), an abundant phenolic acid compound, is extracted from olives, spices, grapes, dates and other plants (12). It has been demonstrated that SA exerts biological effects in various diseases due to its anti-inflammatory, antioxidant and antitumor properties (13). Previous studies have demonstrated that SA alleviates injury from hypoxia/reoxygenation or I/R in neuronal, myocardial and kidney cells (14-16). In addition, SA was revealed to protect from dextran sulfate sodium-induced experimental colitis in BALB/c mice (17). However, the functional role of SA in intestinal I/R injury remains unclear. Therefore, the present study aimed to explore whether SA exhibits a protective role from intestinal I/R injury *in vitro*.

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Materials and methods

Cell culture and treatment. The human colon carcinoma immortalized cell line Caco-2 was purchased from the Cell Bank of Shanghai Institutes for Biological Science. Cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 1% penicillin and streptomycin, and maintained in a humidified atmosphere with 5% CO₂ at 37°C.

SA was purchased from Sigma-Aldrich (Merck KGaA) and dissolved in 0.1% DMSO (v/v). Caco-2 cells were cultured for 24 h at 37°C and fed with DMEM culture medium supplemented with different concentrations of SA (0.1, 1.0 and 10.0 μ M). After incubation for 24 h at 37°C, cells were collected by trypsin application.

Oxygen-glucose deprivation/reoxygenation (OGD/R) treatment. To simulate intestinal I/R injury *in vitro*, an OGD/R model was established using Caco-2 cells. Caco-2 cells were cultured in glucose-free DMEM and maintained in an incubator under conditions of 94% N₂, 1% O₂ and 5% CO₂ at 37°C for 8 h. The cells were subsequently placed in a microaerophilic system in the presence of 95% O₂ and 5% CO₂ at 37°C in normal DMEM for 20 h to induce reoxygenation.

Cell viability assay. Cell Counting Kit-8 (CCK-8) assay was performed to assess cell viability. Caco-2 cells were seeded into 96-well plates at a density of 5x10³ cells/well and pretreated with various concentrations of SA (0.1, 1 and 10 μ M) for 24 h. After OGD/R, 10 μ l CCK-8 reagent (Dojindo Molecular Technologies, Inc.) was added to each well, and the cells were incubated for 2 h at 37°C. The resulting absorbance was detected at 450 nm using a microplate reader (Bio-Rad Laboratories, Inc.).

Determination of lactate dehydrogenase (LDH) activity. Cell injury was detected by measuring the LDH activity using an LDH assay kit (cat. no. A020-2; Nanjing Jiancheng Bioengineering Institute). Caco-2 cells were seeded into 96-well plates (5x10³ cells/well) and treated with 0.1, 1.0 and 10.0 μ M of SA followed by OGD/R injury. The cell culture medium was then collected, and the LDH activity was measured at 530 nm using a microplate reader (Benchmark; Bio-Rad Laboratories, Inc.).

ELISA. Caco-2 cells were pretreated with 0.1, 1 and 10 μ M SA for 24 h and underwent OGD/R injury for 8/20 h. The concentration levels of TNF- α (cat. no. H052-1), IL-6 (cat. no. H007-1), IL-1 β (cat. no. H002) and monocyte chemoattractant protein-1 (MCP-1; cat. no. H115) in the cell culture medium of Caco-2 cells were determined using ELISA kits (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's instructions. Absorbance at 450 nm was measured using a microplate reader (BioTek Instruments, Inc.). A total of 6 parallel wells were set for ELISA.

Western blotting. Total protein was extracted from Caco-2 cells using RIPA lysis buffer (Beyotime Institute of Biotechnology), after which the protein concentration was measured using

a BCA protein assay kit (Beijing Dingguo Changsheng Biotechnology Co., Ltd.). A total of 30 μ g/lane protein samples were then separated by 10% SDS-PAGE and transferred onto PVDF membranes (MilliporeSigma). After blocking with 5% non-fat milk for 1 h at 25 °C, the membranes were incubated at 4°C overnight with the following primary antibodies obtained from Abcam: p65 (1:1,000; cat. no. ab16502), phosphorylated (p)-p65 (1:1,000; cat. no. ab86299), iNOS (1:1,000; cat. no. ab178945), cyclooxygenase 2 (COX2; 1:1,000; cat. no. ab179800), Bcl-2 (1:1,000; cat. no. ab32124), Bax (1:1,000; cat. no. ab32503), cleaved caspase 3 (1:500; cat. no. ab32042), cleaved peroxisome proliferator-activated receptor (PPAR; 1:1,000; cat. no. ab178860), Claudin-3 (1:1,000; cat. no. ab214487), Claudin-2 (1:1,000; cat. no. ab53032), zonula occludens 1 (ZO-1; 1:1,000; cat. no. ab216880) and GAPDH (1:3,000; cat. no. ab125247). The membranes were washed with 0.05% PBS-Tween-20 thrice and then incubated with horseradish peroxidase-labeled anti-mouse (cat. no. 7076; Cell Signaling Technology, Inc.; 1:1,000) and anti-rabbit IgG (cat. no. 7074; 1:1,000; Cell Signaling Technology, Inc.) for 1 h at 37°C. The bands were detected using an enhanced chemiluminescence detection system (Thermo Fisher Scientific, Inc.). Finally, the protein bands were analyzed using ImageJ software (Version 1.49; National Institutes of Health) with GAPDH as a loading control. All the experiments were performed ≥ 3 times, and representative data are presented in the corresponding figures.

Determination of reactive oxygen species (ROS), superoxide dismutase (SOD) and malondialdehyde (MDA) levels. Caco-2 cells were seeded in 6-well plates at a density of 4x10⁵ cells/well, and treated with 0.1, 1 and 10 μ M SA for 24 h. Following OGD/R induction, the expression levels of ROS and MDA, and the level of SOD activity in the culture medium were evaluated using corresponding commercial kits (cat. nos. E004-1-1, A003-1-2 and A001-3-2, respectively; Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's protocols.

TUNEL assay. TUNEL assay was performed to determine apoptosis. Upon treatment, cells were washed twice with PBS and fixed with 4% paraformaldehyde at room temperature in the dark for 30 min. Next, the cells were incubated with proteinase K for 15 min at 37°C and placed in 3% H₂O₂ for 15 min at room temperature. After washing several times with PBS, the cells were treated with TUNEL working solution at 37 °C for 90 min and co-labeled with DAPI working solution (1 μ g/ml) for 10 min at room temperature. Next, labeled Caco-2 cells were visualized using a fluorescence microscope (Leica Microsystems GmbH, x200) and at least 10 fields per section for each sample were examined.

Transepithelial electrical resistance (TEER) assay. To investigate the function of the intestinal barrier *in vitro*, a TEER assay was carried out to examine the integrity of the intestinal barrier. Caco-2 cells were treated with 0.1, 1 and 10 μ M SA for 24 h followed by OGD/R induction. The cells were subsequently harvested and the TEER assay was performed in 24-well Transwell plates (0.4- μ m pore size; Costar; Corning, Inc.) as previously described (18).

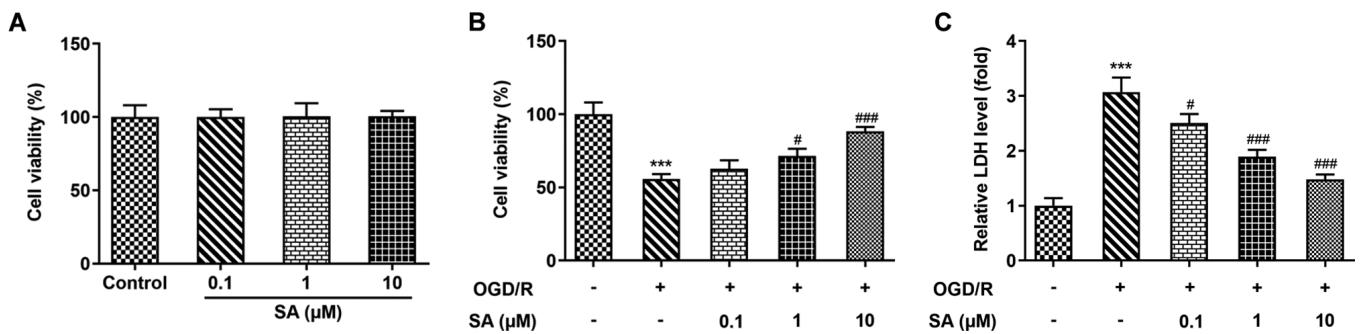


Figure 1. SA pretreatment protects against OGD/R-stimulated cell injury in Caco-2 cells. Caco-2 cells were pretreated with 0.1, 1 and 10 μ M of SA for 24 h before OGD/R injury induction for 8/20 h. (A) Cell viability was measured using a CCK-8 assay in untreated or SA-treated Caco-2 cells. (B) Cell viability was detected using CCK-8 assay in untreated or OGD/R-induced Caco-2 cells with or without SA. (C) LDH levels were evaluated in untreated or OGD/R-induced Caco-2 cells with or without SA. *** P <0.001 vs. the control; # P <0.05 and ### P <0.001 vs. the OGD/R group. SA, syringic acid; OGD/R, oxygen-glucose deprivation/reoxygenation; CCK-8, Cell Counting Kit-8; LDH, lactate dehydrogenase.

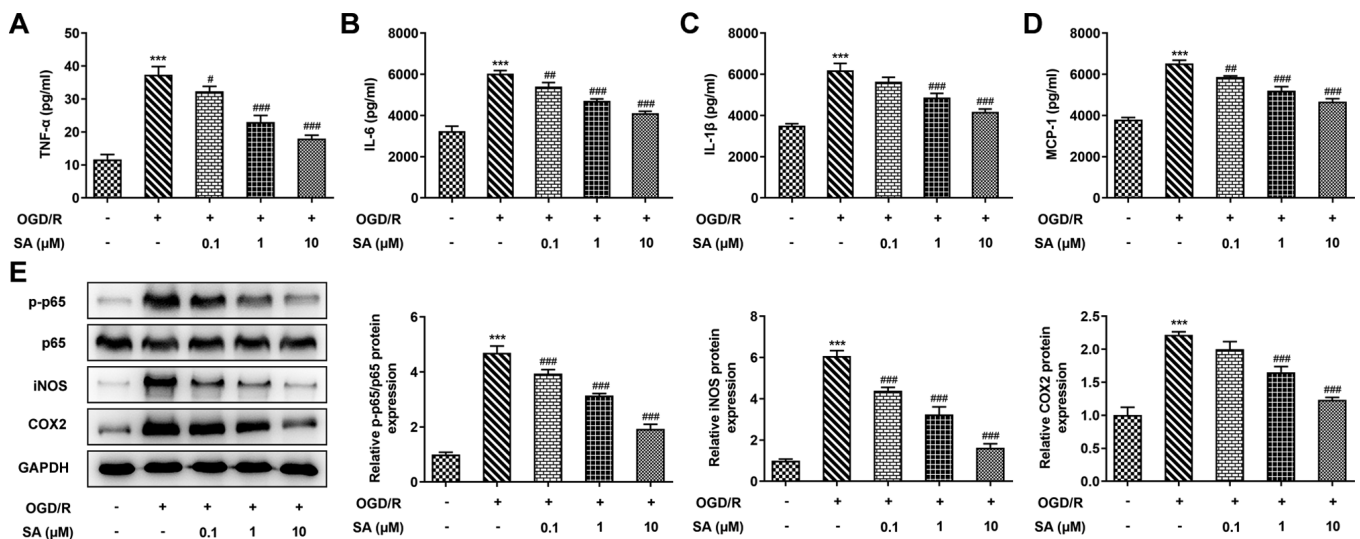


Figure 2. SA pretreatment suppresses the release of inflammatory cytokines in Caco-2 cells subjected to OGD/R. Caco-2 cells were pretreated with 0.1, 1 and 10 μ M of SA for 24 h and underwent treatment with OGD/R injury for 8/20 h. Levels of (A) TNF- α , (B) IL-6, (C) IL-1 β and (D) MCP-1 were detected using ELISA kits. (E) Western blotting was used to determine protein levels of p-p65, p65, iNOS and COX2. *** P <0.001 vs. the control; # P <0.05, ## P <0.01 and ### P <0.001 vs. the OGD/R group. SA, syringic acid; OGD/R, oxygen-glucose deprivation/reoxygenation; MCP-1, monocyte chemoattractant protein-1; p-, phosphorylated; iNOS, inducible nitric oxide synthase; COX2, cyclooxygenase 2.

Statistical analysis. Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, Inc.). All values are expressed as the mean \pm SD from at least three independent experiments and were analyzed using one-way ANOVA followed by Tukey's post hoc test. P <0.05 was considered to indicate a statistically significant difference.

Results

Effects of SA pretreatment on the viability and injury of OGD/R-stimulated Caco-2 cells. To investigate the role of SA in Caco-2 cell viability and injury following OGD/R induction, a CCK-8 assay and LDH activity measurements were carried out. As presented in Fig. 1A, pretreatment with 0.1-10 μ M SA had no significant effect on the viability of Caco-2 cells. In addition, compared with that of the control group, cell viability was significantly reduced by OGD/R injury; however, SA restored the viability of OGD/R-treated

cells in a dose-dependent manner (Fig. 1B). Consistently, OGD/R injury led to a significant increase in LDH release, while SA administration significantly reduced LDH release following OGD/R injury (Fig. 1C). These data suggest that SA decreased Caco-2 cell viability and cytotoxicity injury induced by OGD/R.

Effects of SA pretreatment on the release of inflammatory cytokines in Caco-2 cells subjected to OGD/R. To explore the protective effect of pretreatment with SA, the production of inflammatory cytokines was evaluated *in vitro*. As presented in Fig. 2A-D, OGD/R significantly enhanced the levels of TNF- α , IL-6, IL-1 β and MCP-1 in Caco-2 cells compared with the normoxia group. However, the concentrations of the four aforementioned inflammatory cytokines were significantly reduced following SA treatment in a dose-dependent manner. Western blot analysis revealed that OGD/R significantly increased the protein expression of p-p65, iNOS and

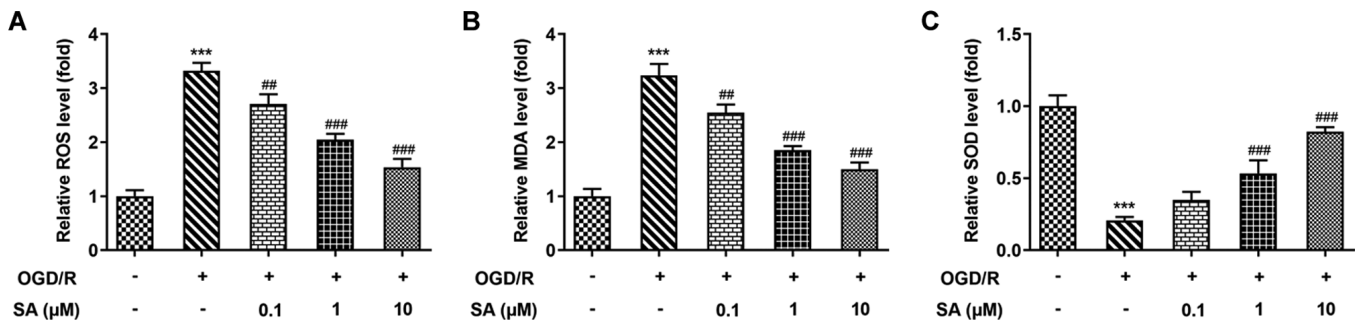


Figure 3. Pretreatment with SA attenuates oxidant stress in OGD/R-induced Caco-2 cells. Caco-2 cells were pretreated with 0.1, 1 and 10 μ M of SA for 24 h and underwent treatment with OGD/R injury for 8/20 h. Levels of (A) ROS, (B) MDA and (C) SOD were analyzed in the media. *** P <0.001 vs. the control; ** P <0.01 and *** P <0.001 vs. OGD/R group. SA, syringic acid; OGD/R, oxygen-glucose deprivation/reoxygenation; ROS, reactive oxygen species; MDA, malondialdehyde; SOD, superoxide dismutase.

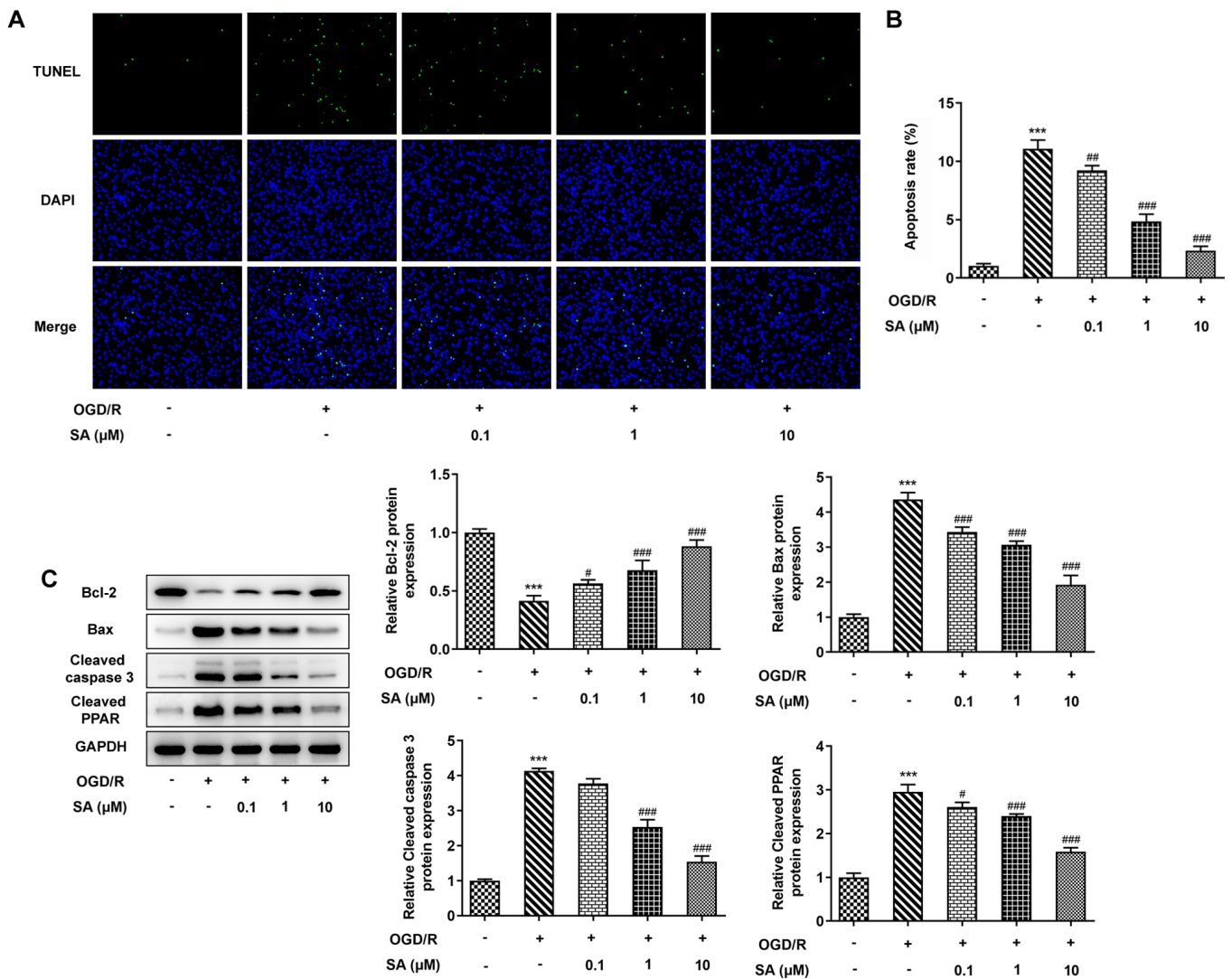


Figure 4. SA pretreatment ameliorates apoptosis in OGD/R-induced Caco-2 cells. Caco-2 cells were pretreated with 0.1, 1 and 10 μ M of SA for 24 h and underwent OGD/R injury for 8/20 h. (A) TUNEL staining was performed to assess apoptosis and was (B) quantified. Original magnification: 200x. (C) Protein expression levels of Bcl-2, Bax, cleaved caspase3 and cleaved PPAR were evaluated using western blotting. *** P <0.001 vs. control; # P <0.05, ** P <0.01 and *** P <0.001 vs. the OGD/R group. SA, syringic acid; OGD/R, oxygen-glucose deprivation/reoxygenation; PPAR, peroxisome proliferator-activated receptor.

COX2, whereas pretreatment with SA reversed this effect in a dose-dependent manner (Fig. 2E). The results indicate that SA inhibited inflammatory release in OGD/R-stimulated Caco-2 cells.

Effects of SA pretreatment on oxidative stress in OGD/R-treated Caco-2 cells. Oxidative stress plays a notable role in intestinal cell injury induced by OGD/R. Thus, the present study explored the protective effect of SA on OGD/R-induced oxidative stress

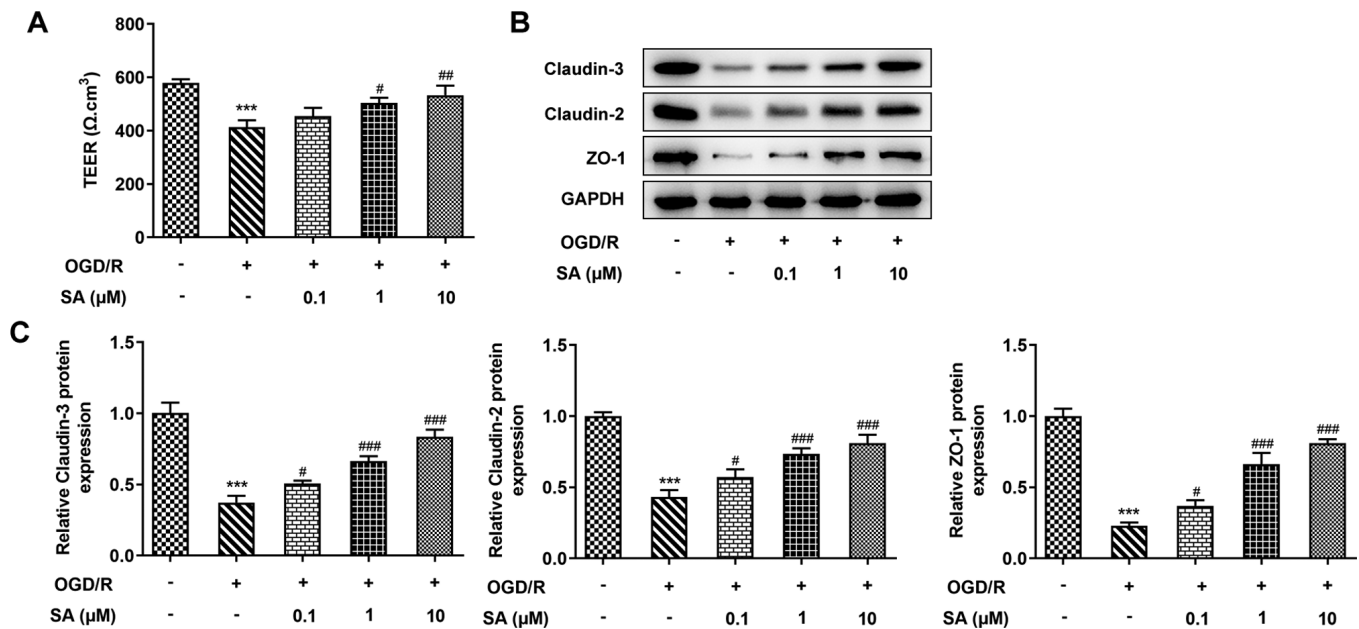


Figure 5. SA pretreatment inhibits intestinal barrier disruption in Caco-2 cells response to OGD/R. Caco-2 cells were pretreated with 0.1, 1 and 10 μ M of SA for 24 h and underwent treatment with OGD/R injury for 8/20 h. (A) A TEER assay was implemented to measure intestinal epithelial integrity. (B) Western blotting was performed to elucidate the (C) protein expression levels of Claudin-3, Claudin-2 and ZO-1. *** $P < 0.001$ vs. the control; # $P < 0.05$, ## $P < 0.01$ and ### $P < 0.001$ vs. the OGD/R group. SA, syringic acid; OGD/R, oxygen-glucose deprivation/reoxygenation; ZO-1, zonula occludens1.

in Caco-2 cells. As presented in Fig. 3, levels of ROS and MDA were significantly increased, while SOD activity was significantly decreased after OGD/R induction, compared with the normoxia group. Conversely, pretreatment with SA significantly suppressed the production of ROS and MDA, but elevated SOD activity levels in Caco-2 cells after OGD/R injury. Therefore, SA may protect intestinal tissue from I/R injury via its anti-inflammatory and antioxidant activities. These results suggested that SA suppressed oxidative stress in OGD/R-treated Caco-2 cells.

Effects of SA pretreatment on apoptosis in OGD/R-treated Caco-2 cells. To further analyze the role of SA in intestinal I/R injury *in vitro*, apoptosis was evaluated. As presented in Fig. 4A and B, the results of the TUNEL assay revealed a significant increase in the apoptosis rate of Caco-2 cells after OGD/R induction, which was significantly decreased following SA treatment. Similarly, western blotting revealed that, compared with the control cells, OGD/R injury significantly inhibited Bcl-2 protein expression, but increased the levels of Bax, cleaved caspase 3 and cleaved PPAR. However, SA reversed the OGD/R-induced alteration in the levels of these proteins in a dose-dependent manner (Fig. 4C). The results suggested that SA inhibited cell apoptosis in OGD/R-stimulated Caco-2 cells.

Effects of SA pretreatment on the intestinal barrier function of Caco-2 cells in response to OGD/R. The current study assessed whether SA administration ameliorated OGD/R-induced intestinal barrier disruption. A TEER assay was performed to examine intestinal epithelial integrity. The results revealed that the value of TEER was significantly reduced by OGD/R injury, whereas SA pretreatment significantly increased TEER in Caco-2 cells (Fig. 5A). Consistent with these results, OGD/R

injury significantly decreased the protein expression levels of Claudin-3, Claudin-2 and ZO-1, but these were increased after treatment with SA under OGD/R conditions in a dose-dependent manner (Fig. 5B and C). The findings indicated that SA protected Caco-2 cells from OGD/R-induced intestinal barrier function injury.

Discussion

SA is a type of monomer extracted from traditional Chinese medicines, including *Dendrobium nobile* Lindl. Previous studies have revealed that SA plays a role in disorders associated with I/R (16,19,20). However, to the best of our knowledge, the biological role of SA in intestinal I/R has not been reported thus far. The present study demonstrated that pretreatment with SA increased cell viability and abated cytotoxic injury in Caco-2 cells following OGD/R. Additionally, SA inhibited the OGD/R-induced release of inflammatory cytokines and oxidative stress, and decreased the rate of apoptosis in Caco-2 cells. SA also improved epithelial barrier function and protected intestinal epithelial integrity damaged by OGD/R.

It is widely accepted that I/R contributes to the inflammatory response that occurs during the occurrence and development of intestinal I/R (21). Inflammation can aggravate intestinal I/R injury (22,23). The present study established an *in vitro* intestinal I/R model using OGD/R injury. The data revealed that OGD/R significantly promoted the production of inflammatory cytokines, including TNF- α , IL-6, IL-1 β and MCP-1 in Caco-2 cells, as well as increasing the expression levels of p-p65, iNOS and COX2. SA pretreatment inhibited the OGD/R-induced increase in these inflammatory cytokines in a dose-dependent manner. In addition, evidence indicates that inflammation is associated with oxidative stress in intestinal

I/R injury (24,25). The antioxidant enzyme system is required to mitigate injuries induced by I/R (26). The present study revealed that OGD/R injury resulted in higher levels of oxidative stress by increasing the production of ROS and MDA, and by reducing SOD activity. Therefore, SA may protect intestinal tissue from I/R injury via its anti-inflammatory and antioxidant activities.

Reperfusion after ischemia in intestinal tissues leads to the activation of caspases and an imbalance of pro-/anti-apoptotic proteins (27). Apoptosis, as an active gene directed cell death process, is easily induced by external stimuli and is relevant to intestinal reperfusion injury (28). Bcl 2 is combined with Bax to form a heterodimer that prevents Bax homodimerization and the activation of caspase 3 (29,30). Caspase 3 and PPAR serve an important role in the apoptosis of intestinal epithelial cells (31,32). In the present study OGD/R specifically downregulated the expression of Bcl 2 and upregulated expression of Bax, cleaved caspase 3 and cleaved PPAR in Caco-2 cells. Nevertheless, pretreatment of SA reversed the effects of OGD/R on the expression levels of Bcl 2, Bax, cleaved caspase 3 and cleaved PPAR.

It has been reported that intestinal I/R injury causes dysfunction of the intestinal mucosal barrier, which is regarded as a complication that may lead to life-threatening bacterial translocation from the gut, multi-system organ failure and mortality (10,33). Thus, protecting the intestinal barrier from injury or enhancing the ability of the organism to repair the intestinal barrier is an important step for blocking pathophysiological processes from intestinal I/R (34). Tight junction proteins, such as Claudin-2, Claudin-3 and ZO-1, are associated with intestinal barrier function and intestinal permeability (35). In the present study, transepithelial electrical resistance and western blotting were detected to measure intestinal barrier function *in vitro*. The results revealed that OGD/R injury reduced the value of transepithelial electrical resistance and decreased the expression levels of Claudin-3, Claudin-2 and ZO-1 in Caco-2 cells. Treatment with SA for 24 h increased the transepithelial electrical resistance and enhanced the levels of these tight junction proteins, indicating the regulatory role of SA in epithelial barrier function. However, in the present study, the effects of SA on intestinal I/R were explored *in vitro*. The role of SA in animal experiments and clinical trials need to be performed in further study.

In summary, the present study demonstrated that SA increased cell viability and abrogated OGD/R cell injury in Caco-2 cells. Furthermore, the current study demonstrated that SA exerted a protective role in OGD/R-treated Caco-2 cells by inhibiting inflammation, oxidative stress, apoptosis and mucosal barrier injury. These results may provide a novel therapeutic drug for the treatment of disorders derived from intestinal I/R injury.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

SX and JX designed the study, performed the experiments, drafted and revised the manuscript. SX analyzed the data and examined the literature. SX and JX confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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