

Terlipressin protects intestinal epithelial cells against oxygen-glucose deprivation/re-oxygenation injury via the phosphatidylinositol 3-kinase pathway

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Abstract. Intestinal ischemia/reperfusion (I/R) injury is associated with a high morbidity and mortality. Vasopressin is administered to critically ill patients with potential intestinal I/R. However, the impacts of vasopressin on intestinal epithelia under ischemic/anoxic conditions remain unclear. The aim of the present study was to evaluate the effects of terlipressin, a highly selective vasopressin V1 receptor agonist, on oxygen and glucose deprivation/re-oxygenation (OGD/R)-induced damage in intestinal epithelial cells (IEC-6). IEC-6 cells were subjected to OGD for 4 h, followed by 4 h re-oxygenation. Terlipressin was incubated with cells for 4 h following OGD. Following OGD/R, IEC-6 cell viability, proliferation and apoptosis, as well as cell cycle dynamics, were assessed and the levels of tumor necrosis factor (TNF)- α and 15-F2t-isoprostane in the culture medium were measured. In addition, wortmannin, a specific phosphatidylinositol 3-kinase (PI3K) inhibitor, was administered to investigate the mechanism of terlipressin action. The results demonstrated that IEC-6 cell viability and proliferation decreased, and cell apoptosis increased, following OGD/R. However, IEC-6 cell cycle dynamics did not significantly change 4 h after OGD. Incubation with 25 nM terlipressin significantly improved cell viability, proliferation and apoptosis. Furthermore, terlipressin inhibited the secretion of TNF- α and 15-F2t-isoprostane from IEC-6 cells following OGD/R. The aforementioned effects of terlipressin were completely abolished following the application of 2 μ M wortmannin. Therefore, the current study

demonstrated that terlipressin administration following OGD attenuates OGD/R-induced cell damage via the PI3K signaling pathway. These results may help physicians to better understand and more effectively use terlipressin in a clinical setting.

Introduction

Intestinal ischemia/reperfusion (I/R) injury usually occurs following acute mesenteric ischemia, severe trauma or burns, hemorrhagic or septic shock, and major surgical procedures (1). Intestinal I/R causes excessive inflammation, oxidative response and cell apoptosis, and subsequently leads to the disruption of the intestinal barrier and distant organ damage (1). The development of intestinal I/R is associated with multiple organ dysfunction syndrome and a high probability of mortality (2,3). Indeed, a recent multicenter study determined that the mortality rate from acute mesenteric ischemia in intensive care units (ICU) was as high as 58% (4). In clinical settings, vasopressors including norepinephrine and dobutamine are essential to maintain blood pressure and systemic perfusion in critically ill patients with intestinal I/R injury.

Vasopressin is generally used for cardiovascular support in the ICU (5) and is recommended for the treatment of severe sepsis and septic shock (6). However, it remains unclear whether vasopressin aggravates intestinal I/R injury. Previous studies have suggested that vasopressin decreases intestinal mucosal perfusion and diminishes the beneficial effect of norepinephrine due to its potent vasoconstriction (7-9). This suggests that the use of vasopressin may further deteriorate intestinal mucosal ischemia in critically ill patients with intestinal I/R. However, other studies have indicated that vasopressin does not compromise gut mucosal microcirculation and oxygen supply (10,11). To the best of our knowledge, there have been no studies investigating the effects of vasopressin on intestinal mucosal epithelial cells, the key component of the intestinal barrier, under I/R conditions. Thus, it is of clinical relevance to clarify the effect of vasopressin and its analogue on intestinal epithelial tissues in patients at risk of intestinal ischemia.

Performing oxygen and glucose deprivation and re-oxygenation (OGD/R) *in vitro* is a popular method to simulate organic I/R *in vivo*. Therefore, the present study was designed to assess

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the effects of terlipressin, a highly selective vasopressin V1 receptor agonist, on the production of inflammatory and oxidative cytokines in rat intestinal epithelial cells (IEC-6), and on cell viability and proliferation in the OGD/R model.

Materials and methods

IEC-6 cell culture. Rat IEC-6 intestinal epithelial cells (CRL-1592; passage 16-20; American Type Culture Collection, Manassas, VA, USA) were cultured as previously described (12). Briefly, IEC-6 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/l D-glucose, 10% v/v fetal bovine serum (all from Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and 1% penicillin/streptomycin (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and maintained under standard cell culture conditions of 37°C, 5% CO₂ and 21% O₂ for 24 h.

OGD/R. OGD intervention was conducted as previously described (12). Briefly, after the IEC-6 cells were grown under normal conditions up to 80% confluence, the DMEM was replenished with D-Hanks buffer (Sigma-Aldrich; Merck KGaA) and cells were incubated in a modular incubator chamber filled with a 95% N₂ and 5% CO₂ gas mixture for 4 h at 37°C. Following completion of OGD, medium was changed back to normoxic medium and the cells were incubated under normal conditions for 4 h (re-oxygenation).

Study groups and experimental protocol. IEC-6 cells were randomly assigned to receive different concentrations (1, 5, 25 or 125 nM) terlipressin (Hybio Pharmaceutical Co., Ltd., Shenzhen, China) following OGD, and then an appropriate concentration of terlipressin (25 nM) was selected according to the results of the cell viability test. Subsequently, cells were incubated with the chosen concentration of terlipressin and/or phosphatidylinositol 3-kinase (PI3K) inhibitor (Wortmannin; Beyotime Institute of Biotechnology, Haimen, China). Cells were randomly assigned to one of 5 groups (5 samples per group) as follows: i) Sham group: IEC-6 cells were incubated in normoxic medium for 8 h; ii) OGD/R group: IEC-6 cells were incubated for 4 h OGD followed by 4 h re-oxygenation; iii) terlipressin group: Following 4 h OGD, IEC-6 cells were incubated with 25 nM terlipressin for 4 h; iv) wortmannin group: Following 4 h OGD, IEC-6 cells were incubated with 2 µM wortmannin, a specific PI3K inhibitor, for 4 h; or v) T+W group: 2 µM wortmannin and 25 nM terlipressin were simultaneously administrated at the beginning of re-oxygenation. The detailed experimental protocol is presented in Fig. 1.

Cell viability assay. IEC-6 cells were seeded into 96-well plates (1x10⁵ cells/well) and incubated overnight at 37°C in 5% CO₂. Cells were then treated according to the aforementioned experimental protocol, with a sham cell group as a control. An MTT assay was used to determine cell viability as previously described (12). Briefly, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide; 5 mg/ml in phosphate-buffered saline) reagent (Sigma-Aldrich; Merck KGaA) was added to each well and incubated for 4 h at 37°C. Medium was then replaced with 150 µl dimethyl sulfoxide.

Optical density (OD) was recorded using a microplate reader at the wavelength of 490 nm. Cell viability was expressed as a percentage of the Sham.

Cell cycle analysis. Cell cycle distribution was measured by a flow cytometry assay with propidium iodide (PI) DNA staining. Briefly, IEC-6 cells were seeded into 96-well plates (1x10⁵ cells/well) and treated according to the aforementioned experimental protocol, with a sham cell group as a control. Cells were harvested and digested with 0.25% trypsin solution without EDTA (Sigma-Aldrich; Merck KGaA) for 15 min at 37°C, then isolated by centrifugation at 200 x g for 2 min at 4°C and washed twice with cold phosphate-buffered saline (PBS). Following 100 µm mesh sieve screening; the cell suspension was fixed with 75% ethanol at 4°C for 24 h. Cells were harvested by centrifugation at 200 x g for 10 min at 4°C and two washes in cold PBS, followed by centrifugation at 200 x g for 5 min at 4°C and cell collection by discarding of the supernatant. The cell suspension was then treated with 100 µl RNase (0.01 mol/l, Sigma-Aldrich; Merck KGaA) at 37°C for 30 min in a water bath, followed by treatment with PI staining solution (0.5 mg/l; Beyotime Institute of Biotechnology). Following 30 min of gentle mixing, cells were stored at 4°C in the dark for 30 min, then analyzed with a FACSCalibur™ Cell Analyzer (BD Biosciences, CA, USA). Red fluorescence at 488 nm was recorded using a microplate reader (Thermo Fisher Scientific, Inc.) and results were analyzed using Flowjo 7.6.3 software (Tree Star, Inc., OR, USA).

Cell proliferation assay. IEC-6 cell proliferation was measured using a cell counting kit-8 (CCK-8, Beyotime Institute of Biotechnology). At baseline (the start of experiment), and 24, 48 and 72 h after baseline, 5x10³ cells/well were propagated in a 5% CO₂ atmosphere at 37°C. Then, CCK-8 reagent was applied to the DMEM, incubated for 1 h and absorbance at 450 nm was measured using a multiwell spectrophotometer.

Apoptosis assay. IEC-6 cell apoptosis was measured by flow cytometry, following a previously described procedure (12). Cells were washed twice with cold PBS and stained with fluorescein isothiocyanate (FITC) Annexin V and PI using the Annexin V-FITC Apoptosis Detection kit I (BD Biosciences) for 15 min at room temperature in the dark. Stained cells were analyzed using flow cytometry within 1 h. The Annexin V⁺/PI⁻ and Annexin V⁺/PI⁺ cell populations were considered to represent apoptotic cells. The apoptosis index was calculated as: (Apoptotic cells/total cells) x100.

Tumor necrosis factor (TNF)-α and isoprostane assay. Following re-oxygenation, the culture medium was centrifuged at 600 x g for 5 min at 4°C, and the supernatant was collected. 15-F2t-isoprostane is a representative index of oxidative stress-induced lipid peroxidation (13). The concentrations of TNF-α and 15-F2t-isoprostane in the supernatant were determined using commercial kits (TNF-α: RTA00; R&D System, Inc., MN, USA and 15-F2t-isoprostane: 500431; Cayman Chemical Company, MI, USA), according to the manufacturer's procedure, as described previously (14,15).

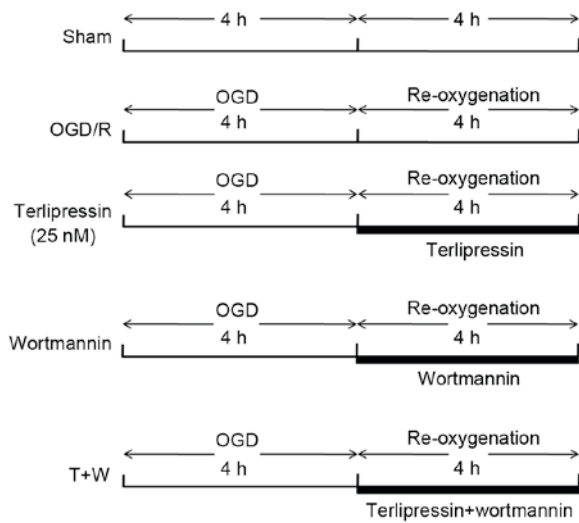


Figure 1. Experimental protocol. Sham, IEC-6 cells were incubated in normoxic medium for 8 h; OGD/R, IEC-6 cells were incubated for 4 h in OGD medium followed by 4 h re-oxygenation; Terlipressin, 25 nM terlipressin was administrated following OGD; Wortmannin, 2 μ M wortmannin was administrated following OGD; T+W, terlipressin and wortmannin were simultaneously administered following re-oxygenation. OGD/R, oxygen and glucose deprivation/re-oxygenation; T, terlipressin; W, wortmannin.

Statistical analysis. Results were analyzed using SPSS 15.0 software (SPSS Inc, Chicago, IL, USA). Data were expressed as mean \pm standard deviation. Data concerning cell proliferation were analyzed by two-way analysis of variance (ANOVA) with repeated measures. Other data were analyzed by one-way ANOVA with Tukey's post-test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Effect of terlipressin on IEC-6 cell viability following OGD/R. As presented in Fig. 2A, cell viability significantly decreased in the OGD/R group ($P < 0.01$ vs. Sham). Incubation with 25 nM and 125 nM terlipressin increased cell viability (both $P < 0.01$ vs. OGD/R), and cell viability in 125 nM group was similar to that in 25 nM group ($P = 0.756$). Therefore, 25 nM terlipressin was selected to use in subsequent experiments. As depicted in Fig. 2B, cell viability significantly decreased in the OGD/R, Wortmannin and T+W groups (all $P < 0.01$ vs. Sham). In addition, cell viability in the terlipressin group was significantly higher than that in the OGD/R, Wortmannin and T+W groups (all $P < 0.01$), and was significantly lower than that in the Sham group ($P < 0.01$).

Effect of terlipressin on IEC-6 cell cycle dynamics following OGD/R. As shown in Fig. 3, OGD/R intervention did not significantly alter cell cycle dynamics 4 h after re-oxygenation (all $P > 0.05$ vs. Sham). Furthermore, terlipressin did not significantly influence the percentage of IEC-6 cells in G1 phase and in S-phase (all $P > 0.05$ vs. OGD/R). Wortmannin and T+W did not significantly alter the percentage of IEC-6 cells in different phases of the cell cycle ($P > 0.05$; Fig. 3).

Effect of terlipressin on IEC-6 cell proliferation following OGD/R. As indicated in Fig. 4, 24 h following the beginning

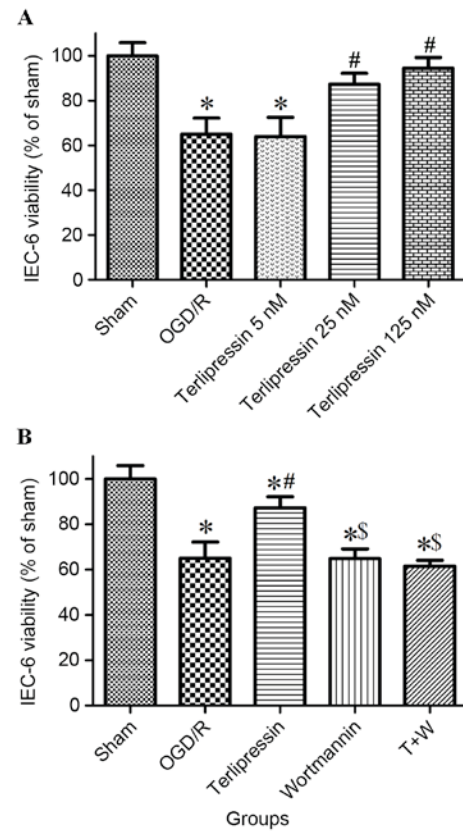


Figure 2. Changes in IEC-6 cell viability following OGD/R. (A) Effect of different concentrations of terlipressin on IEC-6 cell viability following OGD/R. (B) Impact of 2 μ M wortmannin on the improvement of cell viability conferred by 25 nM terlipressin. Data were expressed as mean \pm standard deviation. $n = 5$. Results were compared by one-way analysis of variance with a Tukey's post-test. * $P < 0.01$ vs. the Sham group; # $P < 0.01$ vs. the OGD/R group; § $P < 0.01$ vs. the Terlipressin group. OGD/R, oxygen and glucose deprivation/re-oxygenation; T, terlipressin; W, wortmannin.

of experiment (baseline), the OD value in the OGD/R group was similar to that in the Sham group ($P = 0.997$). OD values at 48 and 72 h were significantly lower in the OGD/R group compared with the control (both $P < 0.01$ vs. Sham). However, incubation with 25 nM terlipressin significantly increased the OD values at 48 and 72 h ($P = 0.034$ and $P = 0.035$ vs. OGD/R, respectively).

Effect of terlipressin on IEC-6 cell apoptosis following OGD/R. Apoptosis in IEC-6 cells was measured following OGD/R (Fig. 5). The flow cytometry gating strategy is depicted in Fig. 5A and representative histograms of each group are presented in Fig. 5B. The apoptosis index in the OGD/R group was significantly higher than that in the Sham group ($P < 0.01$; Fig. 5C). However, terlipressin attenuated OGD/R-induced cell apoptosis ($P < 0.01$ vs. OGD/R; Fig. 5C).

Effect of terlipressin on the production of TNF- α and isoprostane following OGD/R. TNF- α and 15-F2t-isoprostane levels increased following OGD/R (both $P < 0.01$ vs. Sham; Fig. 6). Furthermore, terlipressin significantly inhibited the production of TNF- α and 15-F2t-isoprostane from IEC-6 cells (both $P < 0.01$ vs. OGD/R; Fig. 6).

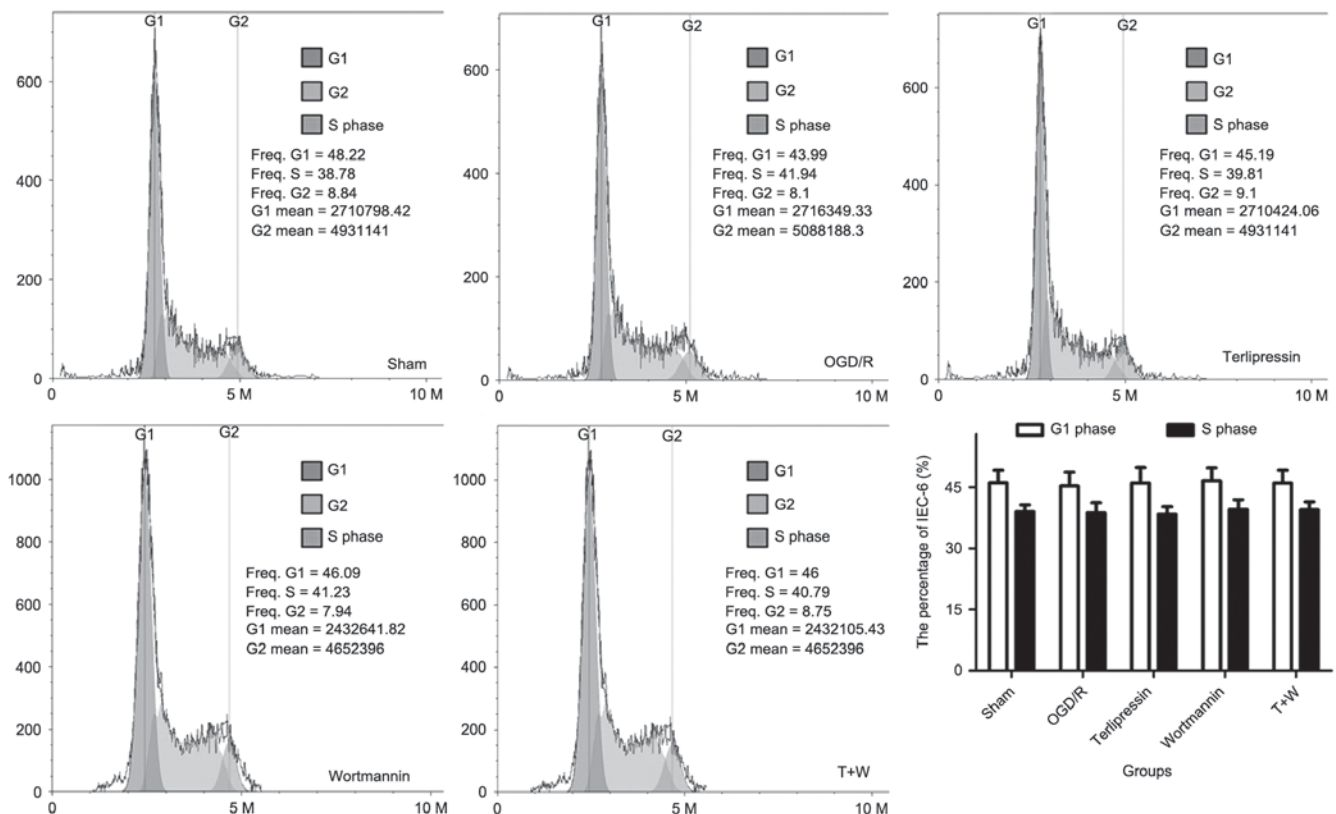


Figure 3. Changes in IEC-6 cell cycle dynamics following OGD/R. Representative flow cytometry cell cycle profiles in each group, and analysis of the percentage of IEC-6 cells in G1 and S phase were shown, respectively. Data are expressed as the mean \pm standard deviation, $n=5$. Results were compared by one-way analysis of variance with a Tukey's post-test. OGD/R, oxygen and glucose deprivation/re-oxygenation; T, terlipressin; W, wortmannin.

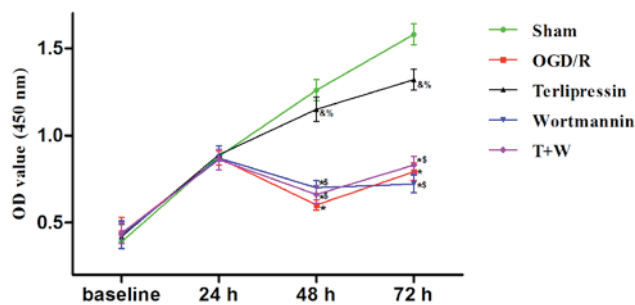


Figure 4. Changes in IEC-6 cell proliferation following OGD/R. IEC-6 cell proliferation (OD value) assessed by cell counting kit-8 assay in each group was shown. Data is expressed as the mean \pm standard deviation, $n=5$. Results were compared by two-way analysis of variance with repeated measures. * $P<0.01$ vs. the Sham group; $^{\#}P<0.05$ vs. the Sham group; $^{\circ}P<0.05$ vs. the OGD/R group; $^{\S}P<0.01$ vs. the Terlipressin group. OGD/R, oxygen and glucose deprivation/re-oxygenation; T: terlipressin, W, wortmannin; OD, optical density.

Effect of wortmannin on terlipressin-induced cell protection following OGD/R. Wortmannin alone (Wortmannin group) produced no significant impacts on the aforementioned variables compared with the OGD/R group (all $P>0.05$; Figs. 2-6). In the T+W group, wortmannin completely attenuated the effects of terlipressin on cell viability, proliferation and apoptosis (Figs. 2B, 4 and 5C). Moreover, the increased secretion of TNF- α and 15-F2t-isoprostane induced by terlipressin was also abolished by wortmannin (Fig. 6).

Discussion

In clinical settings, intestinal I/R injury is a serious condition and usually occurs prior to treatment with vasopressors. Therefore, in the present study, terlipressin was administered following OGD to assess its effects on intestinal epithelial cells. The results of the current study demonstrated that terlipressin decreased the production of TNF- α and isoprostane in IEC-6 cells and improved cellular viability and proliferation, and decreased rates of apoptosis during OGD/R. Furthermore, the aforementioned protective effects of terlipressin were abolished by the specific PI3K inhibitor wortmannin.

Previous studies have indicated that vasopressin reduces neuronal death and apoptosis via the vasopressin V1a receptor (16,17). Similarly, Higashiyama *et al* (18) determined that arginine vasopressin inhibits the serum deprivation-induced apoptosis of glomerular mesangial cells by activating the V1a receptor. It has been demonstrated that in animals, apoptosis is the predominant mechanism of mucosal epithelial cell death during destruction of the intestinal epithelial barrier induced by intestinal I/R injury (19). Furthermore, a recent study performed by the current authors indicated that OGD/R significantly enhanced IEC-6 apoptosis *in vitro* and that remifentanyl, a commonly used analgesic in general anesthesia, inhibited cell apoptosis and improved cell viability (12). To date however, no studies have investigated the effects of vasopressin on epithelial cell damage conferred by OGD/R. To the best of our knowledge, the current study is the first to demonstrate that terlipressin, an analogue of vasopressin, not only promoted IEC-6 viability but

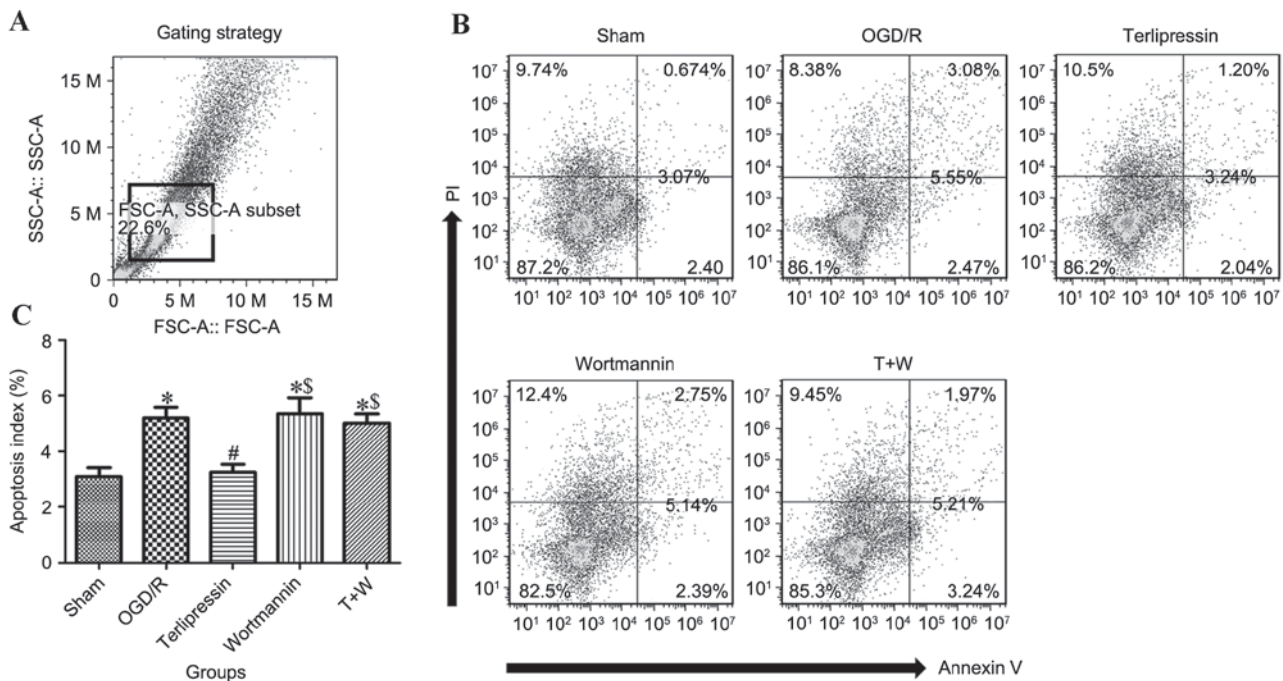


Figure 5. Changes in IEC-6 cell apoptosis following OGD/R. IEC-6 cell apoptosis in each group was assessed using flow cytometry. (A) The gating strategy for flow cytometry is presented. (B) Representative flow cytometric profiles are presented. The cell populations of annexin V⁺/PI⁺ and annexin V⁺/PI⁻ were considered to represent apoptotic cells. (C) Analysis of the apoptotic index of IEC-6 cells in each group is presented. Apoptotic index was calculated as (apoptotic cells/total cells) x100. Data were expressed as mean \pm standard deviation, n=5. Results were compared by one-way analysis of variance with Tukey's post-test. *P<0.01 vs. the Sham group; #P<0.01 vs. the OGD/R group; \$P<0.01 vs. the Terlipressin group. OGD/R, oxygen and glucose deprivation/re-oxygenation; PI, propidium iodide; T, terlipressin; W, wortmannin.

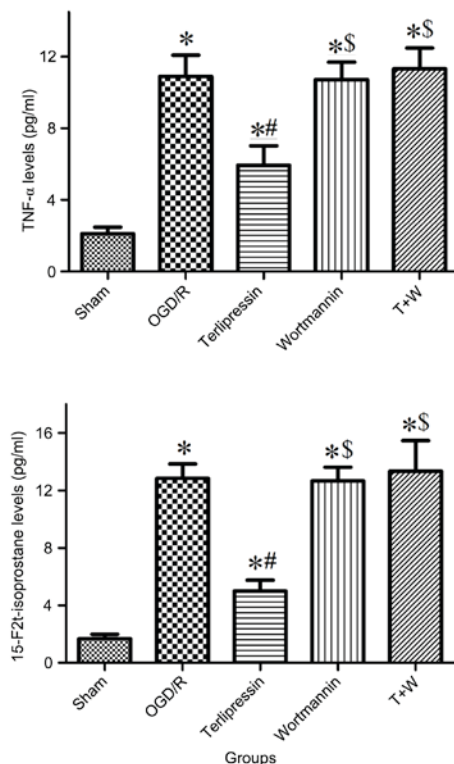


Figure 6. Changes in TNF- α and isoprostane production from IEC-6 cell following OGD/R. TNF- α and 15-F2t-isoprostane levels in the culture medium were assessed by immunoassay. Data are presented as mean \pm standard deviation, n=5. Results were compared by one-way analysis of variance with Tukey's post-test. *P<0.01 vs. the Sham group; #P<0.01 vs. the OGD/R group; \$P<0.01 vs. the Terlipressin group. OGD/R, oxygen and glucose deprivation/re-oxygenation; T, terlipressin; W, wortmannin; TNF- α , tumor necrosis factor α .

also decreased cellular apoptosis during OGD/R (Figs. 2 and 5), indicating that terlipressin management *in vivo* may provide direct protection for intestinal epithelial cells against I/R injury. Interestingly, incubation with terlipressin did not affect cell cycle and cell proliferation in the early period (<48 h) following re-oxygenation (Fig. 3) but significantly increased IEC-6 proliferation 48 and 72 h following OGD (Fig. 4). This delayed increase of cell proliferation induced by terlipressin may promote intestinal epithelial repair following intestinal I/R injury.

A previous study by the current authors indicated that overactivation of inflammatory response and oxidative stress was involved in the pathogenesis of intestinal I/R injury (15). Uncontrolled production of reactive oxygen species and inflammatory cytokines, including TNF- α and interleukin 1, is closely associated with the disruption of intestinal epithelia (20-21). A number of studies have determined that OGD/R increases the release of inflammatory cytokines or oxidative molecules in neural, renal tubular and vascular endothelial cells (22-25). The results of the present study demonstrated that OGD/R increased secretion of TNF- α and isoprostane from intestinal epithelial cells (Fig. 6). Ferrier *et al* (26) suggested that vasopressin increased TNF- α and myeloperoxidase levels through the V1b receptor in gut inflammatory disease. However, in septic animals, several studies have demonstrated that vasopressin may attenuate tissue inflammation and oxidative stress by activating the V1a receptor (27-29). Data from the current study indicate that terlipressin exerts anti-inflammatory and anti-oxidative effects during OGD/R attack (Fig. 6), suggesting that terlipressin may principally act on the V1a receptor of IEC-6 cells. Further

investigations are required to determine the definite impacts of terlipressin, *in vivo*, on intestinal inflammation and oxidation during intestinal I/R insult.

Previous studies have indicated that vasopressin V1 receptor agonists deliver their effects through the PI3K signaling pathway (16,30). V1 receptors couple to Gq protein and Gq subsequently activates phospholipase C and PI3K (31). Notably, the PI3K pathway is involved in inflammation, oxidation and cell apoptosis under ischemic conditions (32-34). Therefore, a PI3K specific inhibitor, wortmannin, was used to explore the potential signaling pathway of terlipressin in this study. The results indicated that the protective effects of terlipressin were completely abolished by wortmannin (Figs. 2B and 4-6), indicating that terlipressin reduced OGD-induced IEC-6 cell damage via the PI3K pathway *in vitro*.

The current study had several limitations. Firstly, the effects of terlipressin on IEC-6 cells were investigated using an *in vitro* OGD/R model. Further analyses should be conducted to clarify the effects and mechanisms of terlipressin on intestinal I/R injury in animals and in patients. Secondly, although terlipressin has a high binding affinity to V1 receptor agonist, a specific V1 receptor antagonist should be applied to verify that IEC-6 cell protection is mediated by V1 receptors. Finally, the downstream molecules of PI3K pathway were not measured in this study. An exact signal pathway may clearly interpret the protective effect of terlipressin following OGD injury.

In conclusion, the results of the current study demonstrate that, *in vitro*, OGD/R attack increases the production of TNF- α and isoprostane, as well as the apoptosis of IEC-6 cells, and decreases cell viability and proliferation. Moreover, the current results indicate for the first time that terlipressin reduces inflammation, oxidative stress and apoptosis, and directly protects IEC-6 cells against OGD/R-induced damage via the PI3K pathway. These important findings may help medical researchers and clinicians to better understand the pharmacological characteristics of terlipressin, and provide useful information for clinical trials and applications relevant to vasopressin.

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