Anwulignan ameliorates the intestinal ischemia/reperfusion

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Running Title:
Anwulignan improves intestinal ischemia-reperfusion injury.

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Abstract: 246 words
Introduction: 372 words
Discussion: 1830 words

Abbreviations:

I/R: Intestinal ischemia/reperfusion, SOD: superoxide dismutase, CAT: catalase, GST: glutathione S-transferase,
factor (erythroid-derived 2)-like 2, **HO-1:** heme oxygenase (decycling) 1, **Bcl2:** b-cell lymphoma 2, **Bax:** bcl-2-like protein 4

Recommended Section: Gastrointestinal, Hepatic, Pulmonary, and Renal
Abstract. Anwulignan is one of monomer compounds in the lignans from Schisandra sphenanthera. In this study, we observed the effect of anwulignan on the intestinal ischemia/reperfusion (I/R) injury in male SD rats and explored the underlying mechanisms. The results showed that the pretreatment with oral anwulignan could significantly increase the mesenteric blood microcirculatory flow velocity; relieve the congestion and pathological injury of jejunum; enhance the autonomic tension of jejunum smooth muscle and its reactivity to acetylcholine; increase the activities of superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST) and choline acetyltransferase (ChAT); increase the contents of acetylcholine (ACh) and glutathione (GSH) in the serum or jejunal tissue; decrease the activities of myeloperoxidase (MPO), protein kinase C (PKC), and nicotinamide adenine dinucleotide phosphate oxidase (NOX); reduced the contents of malondialdehyde (MDA), 8-hydroxy-2-deoxyguanosine (8-OHdG), nicotinamide adenine, reactive oxygen species (ROS), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-1 β (IL-1 β); increased the expression levels of M3 receptor, PI3K, p-AKT, p-GSK3β Ser9, Nrf2, p-Nrf2, HO-1 and Bcl2 in the jejunal tissue decreased the expression levels of p-GSK3β Tyr216, Keap1, Bax and Cleaved Caspase-3, suggesting that anwulignan can ameliorate the I/R-induced jejunal tissue injury in rats, and the mechanism may be related to its activating the PI3K/AKT pathway and then the to regulate Nrf2/ARE signaling pathway and the expression of apoptosis-related proteins to play antioxidant and antiapoptotic roles.

Significance Statement:
Anwulignan can significantly reduce the jejunal tissue injury and the production of inflammatory factors in rats with intestinal ischemia-reperfusion injury, improve the antioxidant capacity, reduce the apoptosis of jejunal tissue, and has the effect of significantly improving the intestinal ischemia-reperfusion injury in rats, suggesting that anwulignan may be used as a potential drug for the prevention and treatment of intestinal ischemia-reperfusion
injury or a resource for the development of health food.

Key words: Anwulignan; Intestinal ischemia-reperfusion (I/R) injury; PI3K; Nrf2
INTRODUCTION:

Intestinal ischemia-reperfusion (II/R) injury is secondary to enteritis, volvulus, trauma, blood loss, and intestinal transplant rejection, with a morbidity of 13/100000 and a mortality of 30% - 80% in population (Gonzalez et al. 2015; Yang et al. 2019). The pathophysiological mechanism of intestinal tissue injury induced by II/R is complex, and currently it is believed to involve oxidative stress, inflammatory reaction and intestinal cell apoptosis (Zu et al. 2018). A large number of ROS is generated in ischemia-reperfusion, which exceeds the scavenging capacity of the body's antioxidant defense system, and the excessive ROS can attack the macromolecules in the intestinal tissue, such as lipid, protein and DNA, to cause an oxidative stress injury (Civantos et al. 2017). A large number of ROS can also break the homeostasis of intestinal epithelium to induce the apoptosis of intestinal epithelial cells, eventually leading to a severe intestinal tissue injury (Li et al. 2021). At present, there is no special clinical treatment for II/R injury, but studies have shown that some antioxidants can ameliorate II/R injury (Zu et al. 2018), so drugs with an antioxidant effect may be a new direction to improve II/R injury.

**Schisandra sphenanthera** Rehd (**Schisandra sphenanthera**), firstly recorded in “Shennong's herbal classic”, is one of Chinese herbal medicines and can be used in health foods issued by Health Commission of the people's Republic of China, and included in Chinese Pharmacopoeia (2020), with the functions of nourishing and strengthening (Li et al. 2018). **Schisandra sphenanthera** is also commonly used as an ingredient in Chinese prescription medicines in Japan, and in health foods in Korea and USA (Chen et al. 2019). Lignans are the main active components of **Schisandra sphenanthera**, with significant antioxidant and hepatoprotective effects (Zhu et al. 2019). Anwulignan is one of the main monomeric active components in the lignans from **Schisandra sphenanthera** (Zhang et al. 2019; Li et al. 2020). It was found in our previous study that anwulignan could play a significant antioxidant role in a D-galactose-induced aging and fatigue mouse model (Zhang et al. 2019; Li et al. 2020). However, till now there was no relevant report available on its anti-II/R effect. In this study, we established an II/R...
injury rat model by clamping the superior mesenteric artery of rats to observe the effect of anwulignan on the II/R injury and explore the underlying mechanism, so as to provide an experimental basis for the development of drugs for the prevention and treatment of II/R injury.

1. Materials and Methods

1.1 Materials

1.1.1 Animals

SD rats (male, aged 8-10 weeks), weighing 220-250g, were purchased from Changchun Yisi Experimental Animal Technology Co., Ltd. (Changchun China), and the production license number was SCXK (Ji)-2018-0007. The animal experiment was approved by the Institutional Animal Care and Use Committee (IACUC) of Beihua University.

1.1.2 Reagents

Anwulignan (Sichuan Victory Biotechnology Co., Ltd., Chengdu, Sichuan); NaCl, KCl, CaCl_2, MgCl_2, NaHCO_3, NaH_2PO_4, glucose (Tianjin HengXing Chemical Reagent Co., Ltd, Tianjin, China); Tween-20 (analytical purity, Tianjin Yongda Chemical Reagent Co., Ltd, Tianjin, China); hematoxylin-eosin (HE), SOD, MDA, CAT, GSH-Px, GST, NOX and MPO kits, and Hoechst cell apoptosis detection kits (Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China); ACh, ChAT, 8-OHdG, PKC, ROS, TNF-α, IL-6 and IL-1β ELISA kits (Shanghai Enzyme-Linked biotechnology Co., Ltd., Shanghai China); BCA kit, HCl-Tris, ammonium persulfate, 30% acrylamide, glycine, TEMED and 5×SDS loading buffer (Beijing Dingguo Changsheng Biotechnology Co., Ltd, Beijing, China); skimmed milk powder (Becton, Dickinson and Company, New Jersey, USA); M3, Keap1, p-Nrf2, Nrf2, HO-1, PI3K, p-AKT, AKT, p-GSK3β Tyr216, p-GSK3β Ser9, GSK3β, Bcl2, Bax, Cleaved-Caspase3, GAPDH, HRP Goat Anti-Rabbit IgG (H+L) and HRP Goat Anti-Mouse IgG (H+L) secondary antibodies (ABclonal, USA); ECL chromogenic solution, One-Step PT-PCR Kit and nucleic acid dye (Nanjing Vazyme
1.2 Methods

1.2.1 Animal grouping and administration

Forty four male SD rats were randomly and evenly divided into 4 groups: ① sham operation group (Sham group), only laparotomy and no ischemic treatment; ② intestinal ischemia/reperfusion group (II/R group), ischemic treatment for 45 min and then the reperfusion for 60 min; ③ sham operation+anwulignan group (Sham+Anwu group), only laparotomy and no ischemia/reperfusion, and gavage of anwulignan; ④ sham operation+anwulignan group (II/R+Anwu group), ischemic treatment for 45 min, then the reperfusion for 60 min, and gavage of anwulignan. Rats in Sham+Anwu group and II/R+Anwu group were given anwulignan (7 mg/kg) by gavage once a day for 14 days, and those in Sham group and II/R group were given an equal volume of sodium carboxymethyl cellulose in the same way. All rats were fasted 24 hours before operation, and 30 minutes after the last administration, they were weighed and anesthetized by the intraperitoneal injection of 5% pentobarbital (50 mg/kg) (Mohamed et al. 2020). Then, the rats were fixed on a rat plate in a supine position, and a 2-cm incision was made along the median line of the abdomen below the xiphoid process to expose the superior mesenteric artery. The superior mesenteric artery of rats in Sham group and Sham+Anwu group was only isolated, without clamping, while that in II/R group and II/R+Anwu group was isolated and clamped with a non-invasive vascular clamp for 45 min, and then the clamp was removed and the artery was reperfused for 60 min for the establishment of an II/R model (Camara-Lemarroy et al. 2009; Durmaz et al. 2020). After the II/R, the blood was collected from the abdominal aorta, left standing at room temperature for 30 min, and centrifuged at 4 °C for 10 min. The serum was taken and stored at -80 °C. After the collection of blood samples, rats were euthanized with 200 mg/kg pentobarbital (Pang and Chisholm 2016), and then a 5-6 cm segment of the jejunal tissue was taken about 5 cm from the ileum and quickly rinsed with ice normal saline. The contents in the jejunal tissue segment were washed...
away and the water on its surface was blotted dry for the further experiments. The experimental protocol is shown in Figure 1.

1.2.2 Measurement of the daily weight of rats

During the experiment, the body weight of rats in each group was weighed, and its change curve was drawn.

1.2.3 Measurement of blood flow velocity of the mesenteric microcirculatory capillary

After the I/R model of rats was established as described in Section 1.2.1, the small intestinal loop was pulled out through the abdominal incision to expose the mesentery and a segment of jejunum with abundant blood vessels was selected, and spread on a thermostatic tank at 37 °C. A microcirculation observation system and a BI-2000 medical image analysis system were used to observe and record the blood flow velocity of mesenteric microcirculatory capillary.

1.2.4 Histopathological observation of the jejunal tissue

The jejunal tissue was obtained as described in Section 1.2.1, immersed in formalin solution (10%) for 72 hours, and then dehydrated with gradient concentration ethanol, embedded in paraffin, sliced with a 5 μm thickness, and stained with HE. The histological changes of the jejunal tissue were observed under an optical microscope, and the staining images were observed by 200 and 400 times with the microscope. According to Chiu's score (Chiu et al. 1970; Li et al. 2017), the integrity of villi, epithelial layer and lamina propria were evaluated, and the severity of intestinal injury was scored from 0 to 5 as follows: (0) normal villi; (1) capillary congestion, slight uplift of epithelial layer; (2) the epithelial layer is moderately; (3) a large number of uplift of epithelial layer, villi tip ulceration; (4) villi abscission, telangiectasia; (5) disintegration, bleeding and ulcer of lamina propria.

1.2.5 Measurement of the jejunal tension

The jejunal tissue was obtained as described in Section 1.2.1. A 0.5 cm ring of the jejunum was cut and placed in a bath containing Tyrode solution (Subramanya et al. 2015) (130 mM NaCl, 4.5 mM KCl, 2.2 mM CaCl₂, 0.6
mM MgCl₂, 24.2 mM NaHCO₃, 1.2 mM NaH₂PO₄, and 11 mM glucose). One end of the jejunal ring was fixed in the bath with a stainless steel hook, and the other end was connected with a tension transducer. A resting tension of 1g was loaded on the jejunal ring, and the solution was changed every 10 min and left stable for 60 min. The contraction and relaxation of the jejunum ring were recorded by a biological signal recording system, and the number and height of contraction waves within 1 min were regarded as the contraction frequency (times/min) and amplitude, respectively (Wang et al. 2019). Then 10⁻⁴ M ACh was used to stimulate the jejunum ring (Montgomery et al. 2016), and the contraction response curve and the maximum contraction tension were observed.

1.2.6 Detection of biochemical indexes

The serum and jejunal tissue were obtained as described in Section 1.2.1. The activities of SOD (superoxide dismutase), CAT (catalase), GST (glutathione S-transferase), NOX (reduced nicotinamide adenine dinucleotide phosphate oxidase), MPO (myeloperoxidase) and ChAT (choline acetyltransferase), and the contents of GSH (glutathione), MDA (malondialdehyde), 8-OHdG (8-hydroxy-2 deoxyguanosine), PKC (protein kinase C), ROS (reactive oxygen species), TNF-α (tumor necrosis factor-α), IL-6 (interleukin 6), IL-1β (interleukin-1β) and ACh (acetylcholine) in the serum and/or the jejunal tissue homogenate were detected according to the methods provided by the kit manufacturers.

1.2.7 Observation on the apoptosis of jejunal tissue cells by Hoechst staining

The tissue sections were dewaxed, and washed with PBS for 2 × 3 min on a shaking table. Each section was added with 0.5 ml Hoechst 33258 staining solution and dyed on the shaking table for 5 min, then washed with PBS for 2 × 3 min to remove the staining solution, and a drop of anti-quenching sealing solution was dropped onto it for mounting. The sections were examined and photographed under a fluorescence microscope, in which five visual fields in each group were randomly selected for counting the number of normal cells (cells with an intact cell membrane stained with uniform blue) and apoptotic cells (cells with a nucleus stained with bright blue),
respectively, and the apoptosis rate was calculated according to the following formula.

\[
\text{Apoptosis rate} = \frac{\text{apoptotic cells}}{\text{total cells}} \times 100\%
\]

1.2.8 Detection of Keap1, Nrf2, HO-1, p53, Bcl2, Bax and Caspase3 expressions in the jejunal tissue by RT-PCR

As in Section 1.2.1, the jejunal tissues of 3 rats in each group were randomly taken. The RNA in the jejunal tissues were extracted according to the instructions of RNA extraction kit, and the cDNA was synthesized according to the instructions of reverse transcription kit. The primers were designed with a primer software and synthesized by Beijing Dingguo Changsheng Technology Co., Ltd, in which β-actin was used as the internal reference gene. The amplification conditions were as follows: pre-denaturation at 94 °C for 3 min, denaturation at 94 °C 30 sec, annealing for 3 min for 30 sec (annealing temperature: 59 °C for p53, 57.5 °C for Keap1 and HO-1, 55.4 °C for Nrf2, 57.8 °C for Bcl2, 54 °C for Bax, 55.8 °C for Caspase3, and 55.6 C for β-actin) and extension at 72 °C for 30 sec, which was repeated 30 times, and then extension at 72 °C for 7 min. Finally, the samples were stored at 4 °C for the gel electrophoresis.

1.2.9 Detection of the Expressions of M3, Nrf2/ARE, PI3K/AKT and apoptosis-related proteins in the jejunal tissue by Western blot

As described in Section 1.2.1, jejunal tissues from 3 rats in each group were randomly taken, and the total protein was extracted using the total protein extraction kit. BCA method was used to detect the protein concentration and SDS-PAGE gel electrophoresis was performed. The first antibodies of M3 (1:1000), Keap1 (1:1000), p-Nrf2 (1:1000), Nrf2 (1:1000), HO-1 (1:1000), p53 (1:1000), Bcl2 (1:1000), Bax (1:1000), Cleaved Caspase3 (1:1000), PI3K (1:1000), p-Akt (1:1000), Akt (1:1000), p-GSK3β Tyr216 (1:1000), p-GSK3β Ser9 (1:1000), GSK3β (1:1000) and GAPDH (1:1000) were added onto PVDF membranes after the transmembrane, and the membranes were incubated at 4 °C overnight. The second antibodies (1:2000) were added onto the
membranes after they were washed, and then the membranes were incubated at room temperature for 1 h. Then, the membranes were washed, and an ECL developer was used for the color development of the membranes.

1.3 Statistical analysis

SPSS 20.0 statistical software was used for the analysis of the data. The above data were expressed as mean ± SD. One-way analysis of variance was used for the comparison among multiple groups and Tukey test was used for the comparison between groups, in which a value of $P < 0.05$ was considered as a significant difference in statistics.

2 Results

2.1 Effects of anwulignan on the body weight curve, mesenteric microcirculatory capillary blood flow velocity and histopathological observation of the jejunal tissue

The results showed that the body weight of rats in each group increased during 14 days, but there was no significant difference among the groups (Figure 2A).

A microcirculation blood flow disturbance is often accompanied by an II/R, microcirculation is the basis of material exchange between blood and tissues, and the blood flow velocity of mesenteric circulation capillary is directly related to the intestinal tissue injury (Yeh et al. 2012). In this study, the blood flow velocity of mesenteric microcirculatory capillary was observed using a microcirculation observation system. The results (Figure 2B) showed that compared with that in Sham group, the blood flow velocity of mesenteric microcirculatory capillary in II/R group was significantly decreased ($P < 0.01$); however, compared with that in II/R group, the blood flow velocity in II/R+Anwu group was significantly increased ($P < 0.01$).

The histopathological observation on the jejunal tissue (Figure 2C) showed that compared with that in Sham group, the jejenum congestion was severer in II/R group, and compared with that in II/R group, the jejenum congestion was significantly alleviated in II/R+Anwu group. The observation on the effects of anwulignan on the
jejunal tissue by HE staining (Figure 2D) showed that in Sham and Sham+ Anwu groups, the mucosal epithelial cells were intact, the muscle layer was intact, and the goblet cells were clearly visible; in II/R group, the mucosal epithelial cells of jejunum were necrotic and shed, the villus height was reduced, the villus of jejunum was detached from the lamina propria, the villus integrity was damaged, and the lamina propria was necrotic, with bleeding, ulceration and inflammatory cell infiltration; in II/R group+ Anwu group, the necrosis of upper mucosa cells was mitigated, the muscle layer thickness was reduced, the infiltration of inflammatory cells and bleeding were alleviated. The results of intestinal histological injury scores (Figure 2E) showed that compared with that in Sham group, the Chiu's score increased significantly after the II/R injury \( (P < 0.01) \); compared with that in II/R group, the Chiu's score decreased significantly in II/R+Anwu group \( (P < 0.01) \). These results suggest that the jejunal tissue of rats was significantly damaged by clamping the superior mesenteric artery for 45 min and reperfusing it for 60 min, and the jejunal tissue injury was significantly alleviated after the pretreatment with anwulignan.

2.2 Effects of anwulignan on the jejunal tension in vitro

Intestinal autonomic contraction tension is a direct index to evaluate the intestinal function (Ovsiannikov & Berezina 2013). In this study, the autonomic contraction of jejunum rings was observed after the jejunum rings in the isolated tissue perfusion system was stable. As shown in Figure 3A-C, the autonomic contraction of jejunum rings in Sham group and Sham+Anwu group was slow and rhythmic; compared with that in Sham group, the autonomic contraction in II/R group almost disappeared, and the frequency and the amplitude of autonomic contraction were significantly reduced \( (P < 0.01) \); compared with those in II/R group, the frequency and amplitude in II/R+Anwu group were significantly increased \( (P < 0.01) \).

Detecting the responsiveness of a jejunum ring to the acetylcholine-induced contraction is another classic method to evaluate the contractile function of jejunum smooth muscle (Wei et al. 2013). In this study, \( 10^{-4} \) M
Acetylcholine was added into the in vitro organ experiment bath to induce the contraction of jejunum rings, and the maximum contractile tension and the contractile amplitude of in vitro jejunum were observed. As shown in Figure 3D-F, the maximum contractile tension and amplitude induced by ACh in II/R group were significantly smaller than those in Sham group ($P < 0.01$), and the maximum contractile tension and amplitude induced by ACh in II/R+Anwu group were significantly larger than those in II/R group ($P < 0.01$), indicating that anwulignan could play a regulatory role in the II/R-induced dysfunction of intestinal smooth muscle contraction and relaxation.

2.3 Detection of SOD, CAT, GSH, GST, MDA, 8-OHdG, PKC, NOX and ROS in the serum and jejunal tissue

SOD, CAT, GST and GSH are important endogenous antioxidants in the body, MDA and 8-OHdG are products of oxidative stress (Mesole et al. 2020), and PKC and NOX play a catalytic role in the generation of ROS (Pejenaute et al. 2020). In this study, the above oxidation-related indexes in the serum and jejunal tissue of rats were detected. As shown in Figure 4, in II/R rats, serum SOD, CAT, GST, and GSH were 63.3%, 49.5%, 72.0% and 52.7% of those in the sham group, respectively, while those in jejunum were 56.4%, 62.0%, 51.1% and 47.8% of those in the sham group, respectively ($P < 0.05, P < 0.01$). The serum MDA, 8-OHdG, ROS and NOX were 3.9, 1.5, 1.9 and 1.7 times of those in the sham group, respectively. Jejunal MDA, 8-OHdG and ROS were 3.3, 1.9 and 1.7 times of those in sham group ($P < 0.05, P < 0.01$). However, anwulignan elevated SOD, CAT, GST and GSH in II/R rats in the serum by 1.62, 1.71, 1.28 and 2.04 times, and in the jejunum by 1.63, 1.64, 1.31 and 1.91 times ($P < 0.05, P < 0.01$), but decreased the levels of MDA, 8-OHdG, ROS and NOX in the serum by 30.8%, 79.1%, 78.7% and 75.5, and the jejunal levels of MDA, 8-OHdG and ROS to 30.7%, 67.5% and 80.0% of those in II/R in jejunum ($P < 0.05, P < 0.01$). These results suggest that anwulignan could significantly improve the antioxidant capacity of II/R rats to reduce the oxidative stress injury of jejunum.

2.4 Effects of anwulignan on TNF-α, IL-6 and IL-1β contents and MPO activity in the serum and jejunal...
Ischemia/reperfusion injury can increase the level of TNF-α, IL-6, IL-1β and the activity of MPO (Arda-Pirincci and Bolkent 2014). As shown in Figure 5, in I/R rats, the levels of IL-6 and the activity of MPO in serum were 1.26 times and 1.65 times, while the levels of TNF-α, IL-6, IL-1β and the activity of MPO in jejunum were 1.40, 1.29, 1.40 and 1.76 times of those in sham group, respectively (P < 0.05, P < 0.01). However, anwulignan decreased the levels of TNF-α, IL-6, IL-1β and the activity of MPO in jejunum of I/R rats to 81.2%, 82.6%, 76.3% and 63.5% of those in I/R group, respectively (P < 0.05, P < 0.01), suggesting that anwulignan could play a protective role against the I/R injury by reducing the inflammatory response of jejunum in rats.

2.5 Effects of anwulignan on the content of ACh, the activity of ChAT and the expression of M3 in the jejunal tissue

ACh released by cholinergic nerves is the most important excitatory neurotransmitter in the gastrointestinal tract (Yeh et al. 2019). ACh binds to muscarinic receptor 3 (M3) to mediate the contraction of gastrointestinal smooth muscle. ChAT (choline acetyltransferase) is an important catalytic enzyme in the synthesis of ACh, and its activity is positively correlated with the level of ACh (Yeh et al. 2019). The experimental results (Figure 6) showed that compared with those in Sham group, the content of ACh, the activity of ChAT and the expression of M3 protein in the jejunal tissue of rats were significantly decreased in I/R group (P < 0.01); but all of them were significantly increased in I/R+Anwu group (P < 0.01) compared with those in I/R group, indicating that anwulignan could protect the contractile function of jejunal smooth muscle by increasing the ACh content, ChAT activity and M3 protein expression in the jejunal tissue of I/R rats.

2.6 Effects of anwulignan on the expression of Nrf2/ARE pathway-related genes in the jejunal tissue

Nrf2/ARE pathway is the key pathway to play an anti-oxidant role in the body (Zheng et al. 2019). The expression levels of Keap1, p-Nrf2, Nrf2 and HO-1 in the jejunal tissue of rats were detected at mRNA and protein
levels in this study, and the Western blot and RT-PCR results were consistent. As shown in Figure 7, compared with that in Sham group, the expression level of Keap1 was significantly increased, and the expression of Nrf2, the ratio of p-Nrf2/Nrf2 and the expression level of HO-1 were significantly decreased in the jejunal tissue of rats in II/R group ($P < 0.05$, $P < 0.01$); however, compared with that in II/R group, the expression level of Keap1 was significantly decreased, and the expression of Nrf2, the ratio of p-Nrf2/Nrf2 and the expression level of HO-1 were significantly increased in the jejunal tissue of rats in II/R+Anwu group ($P < 0.05$, $P < 0.01$), suggesting that anwulignan could alleviate the II/R injury by activating Nrf2/ARE signaling pathway and then exerting an antioxidant effect in rats.

### 2.7 Effects of anwulignan on PI3K/AKT pathway-related protein expressions in the jejunal tissue

PI3K/AKT signaling pathway is one of the important pathways involved in the regulation of Nrf2/ARE signaling pathway, and the activated PI3K can regulative downstream AKT and GSK3β, and then activate Nrf2 (Qiu et al. 2010; Zhang et al. 2020). In this experiment, the expression of PI3K/AKT-related proteins PI3K, p-AKT, p-GSK3β Ser9 and p-GSK3β Tyr216 was detected by Western blot. As shown in Figure 8, compared with that in Sham group, the expression levels of PI3K, p-AKT and p-GSK3β Ser9 were significantly decreased ($P < 0.05$, $P < 0.05$), and the expression level of p-GSK3 Tyr216 was significantly increased ($P < 0.05$) in the jejunal tissue of rats in II/R group. However, compared with that in II/R group, the expression level of PI3K, p-AKT and p-GSK3β Ser9 were significantly increased ($P < 0.05$, $P < 0.01$), while the expression level of p-GSK3 Tyr216 in was significantly decreased ($P < 0.05$) in the jejunal tissue of rats in II/R+Anwu group, indicating that anwulignan could alleviate the II/R injury by activating the PI3K/AKT signaling pathway and then activating the Nrf2/ARE pathway in the jejunal tissue of rats.

The Wnt/β-catenin signaling pathway is regulated by AKT (Fukumoto et al. 2001) and plays an important role in improving inflammation and reducing apoptosis (Liu et al. 2009; Mao et al. 2009). Studies have shown that
activation of the Wnt/beta-catenin pathway significantly improves intestinal ischemia/reperfusion injury (El-Sayyad et al. 2017). As shown in Figure 8, the results of this study showed that anwulignan significantly reduced P-β-catenin protein expression in jejunum of II/R rats, and significantly activated Wnt/β-catenin signaling pathway, which may be another important mechanism of anwulignan in improving intestinal ischemia-reperfusion injury in rats.

2.8 Effects of anwulignan on the apoptosis in the jejunal tissue

Apoptosis in intestinal tissue is one of the main characteristics of II/R (Arda-Pirincci and Bolkent, 2014). In this study, Hoechst staining was used to observe the apoptosis of jejunal tissue of rats. Results as shown in Figure 9A, the jejunal tissue cells of rats in Sham group and Sham+Anwu group were intact in shape and stained evenly, with a light blue fluorescence of the chromatin in the nucleus, and the chromatin concentration in the nucleus of jejunal tissue cells of rats in II/R group increased, with an increased fragmented bright blue chromatin, while the apoptosis of jejunal tissue cells of rats II/R group was significantly alleviated in the anwulignan-pretreated groups; the apoptosis rate of rats in II/R group was significantly higher than that in Sham group, ($P < 0.01$), and the apoptosis rate of rats in II/R+Anwu group was significantly lower than that in II/R group ($P < 0.01$).

Bax is a pro-apoptotic protein and Bcl2 can bind to Bax to inhibit the occurrence of apoptosis (Tan et al. 2005). Caspase3 is the key executor of apoptosis and its expression represents the degree of apoptosis (Weng et al. 2019), and P53 can induce an apoptosis by regulating the expression of Bax, Bcl2 and Caspase3 (Weng et al. 2019). The expression of the above genes in the jejunal tissue of rats was detected by RT-PCR and Western blot in this study, and the detection results obtained by the two methods were consistent. As shown in Figure 9, the expression level of Bcl2 in the jejunal tissue of rats in II/R group was significantly lower than that in Sham group, and the expression levels of p53, Bax, Caspase3 and Cleaved Caspase3 in the jejunal tissue of rats in II/R group were significantly higher than that in Sham group ($P < 0.01$), while the expression level of Bcl2 in II/R+Anwu group
was significantly higher than that in II/R group, and the expression levels of p53, Bax, Caspase3 and Cleaved Caspase3 in II/R+Anwu group were significantly lower than that in II/R group ($P < 0.05$, $P < 0.01$), suggesting that anwulignan can inhibit the apoptosis of jejunal tissue cells in II/R rats, which may be another important mechanism for anwulignan to improve the II/R injury in rats.

3. Discussion

Ischemia reperfusion injury refers to the ischemia injury that occurs to tissue first, but this injury is not improved by the subsequent blood supply recovery, but aggravated, even irreversible (Tokes et al. 2015). Studies have shown that epithelial cells located at the tip of intestinal villi are very vulnerable to the ischemia reperfusion injury (Guan et al. 2009). Intestinal ischemia-reperfusion is more common in the surgery with serious burns, blood loss, trauma and surgical procedures (Gonzalez et al. 2015). In this study, a rat II/R injury model was successfully established by a classical method (Camara-Lemarroy et al. 2009; Durmaz et al. 2020), and the protective effect of anwulignan on the jejunum was confirmed.

A majority of studies have revealed that II/R can cause a series of disorders (Gonzalez et al. 2015; Camara-Lemarroy et al. 2009; Durmaz et al. 2020), including histopathological changes, physiological dysfunctions, biochemical abnormity, and excessive oxidative stress etc. The present study showed that there were obvious congestion in the jejunal microcirculation with slow blood flow velocity, the infiltration of inflammatory cells in the jejunal tissue, high activity of serum and jejunal MPO. Anwulignan could improve the congestion and injury of jejunal tissue and tissue necrosis, and then the blood flow velocity, decrease the MPO content, showing a significant protection on II/R tissue. Jiang et al reported that anwulignan could inhibit platelet aggregation (Jiang et al. 2005), so we speculated that the effect of anwulignan on the blood flow velocity may be related to its inhibition on the platelet aggregation.

The tension of intestinal smooth muscle is an important index to evaluate the degree of intestinal injury
It has been reported that after II/R injury, the content of ChAT, a key enzyme in the synthesis of ACh, ACh as well as the expression level of M3 protein decreased (Paulino et al. 2011; Palombit et al. 2013), which may be one of the main reasons for the decrease of intestinal smooth muscle tension after II/R injury. Our results showed that anwulignan could significantly enhance the autonomic contractility of jejunal smooth muscle and the response to ACh in II/R rats, increase ChAT and ACh levels and up-regulate M3 receptor expression in II/R rats, indicating anwulignan can improve the physiological dysfunctions of II/R tissue.

Oxidative stress injury is the main mechanism of II/R injury (Gubernatorova et al. 2017). Compared with other organs, intestinal tissue is more likely to produce ROS during II/R (Sasaki and Joh 2007). An excessive ROS not only consumes endogenous antioxidants, such as SOD, GSH, GST and CAT, but also attacks lipids, proteins and other components in the body, leading to the lipid peroxidation and the generation of MDA to severe cell damage (Civantos et al. 2017). The accumulation of ROS can also damage DNA, producing 8-OHdG, a marker of oxidative stress (Mesole et al. 2020; Pejenaute et al. 2020). In addition, the levels of PKC and NOX are positively correlated with the level of ROS (Castaldo et al. 2019; Pejenaute et al. 2020). Our results showed that the activities of SOD, CAT and GST, as well as the content of GSH in the serum and jejunal tissue of II/R rats were significantly decreased, and the contents of MDA, 8-OHdG and ROS, and the activities of PKC, NOX were significantly increased, which were consistent with the results of other reports on the II/R induced intestinal injury (Zu et al. 2018). In the present study, anwulignan could increase the activities of SOD, CAT and GST, as well as the content of GSH in the serum and jejunal tissue of II/R rats, decrease the contents of MDA, 8-OHdG and ROS, and also decrease the activities of PKC and NOX, suggesting that anwulignan may play its roles through the antioxidation.

Nrf2/ARE pathway is a key regulatory pathway in the body against oxidative stress, and Nrf2 is a key regulator of antioxidation (Li et al. 2018). In a physiological state, Nrf2 binds to its inhibitor Keap1 to maintain the
homeostasis, and it is released from Keap1 after its phosphorylation (Zhang et al. 2020) to regulate the downstream factors including HO-1 and then activate a variety of antioxidant enzymes to improve the body’s antioxidant capacity and alleviate the oxidative stress injury (Zhang et al. 2020). Our results showed that anwulignan could up-regulate the expression of p-Nrf2 s40 and HO-1, and down-regulate the expression of Keap1 in the jejunal tissue of II/R rats, suggesting that anwulignan can activate the Nrf2/ARE pathway to play a strong antioxidant role against the II/R injury in rats.

As shown in Figure 11, PI3K/AKT is the upstream regulatory pathway of Nrf2 (Nakaso et al. 2003). The activated PI3K can promote the translocation of the protein onto the cell membrane to activate AKT, which then inhibits the downstream GSK3β and regulates the downstream target proteins to play corresponding roles (Nakaso et al. 2003). The phosphorylation of tyrosine 216 residue (Tyr216) can lead to the activation of GSK3β, while the phosphorylation of serine 9 residue (Ser9) reduces its activity (Rath et al. 2015). Studies have shown that the inhibition of PI3K, p-AKT or p-GSK3β Ser9 activity can weaken the activation of Nrf2 (Nakaso et al. 2003; Li et al. 2006). p-AKT and p-GSK3β can promote the separation of Nrf2 from Keap1, Nrf2 can promote the expression of downstream Nrf2-dependent antioxidant enzymes (Li et al. 2006; Zhang et al. 2020). The results of the present study showed that anwulignan increased the expression of PI3K, p-AKT, p-GSK3β Ser9, but decreased the expression of p-GSK3β Tyr216 in the jejunal tissue of II/R rats, indicating that anwulignan can activate the PI3K/Akt and Nrf2/ARE signaling pathway and exert its antioxidant effects to protect rat II/R. Wnt/beta-catenin signaling pathway is also regulated by AKT (Fukumoto et al. 2001). Studies have shown that the Wnt/beta-catenin signaling pathway plays a key role in intestinal ischemia-reperfusion and its activation reduces intestinal tissue damage (El-Sayyad et al. 2017). The present study showed that Anwulignan significantly increased the expression of p-β-catenin, which may be related to the activation of PI3K/AKT signaling pathway, and may be another important mechanism for the improvement of intestinal ischemia-reperfusion injury.
The apoptosis of intestinal epithelial cells is also involved in the mechanism of intestinal mucosal injury during II/R (Feng et al. 2017). A large amount of oxygen free radicals can damage the intestinal epithelial cells through lipid peroxidation, protein denaturation and DNA damage, and eventually induce their apoptosis and the intestinal dysfunction (Wang et al. 2017). The apoptosis is mainly determined by the ratio of anti-apoptotic protein Bcl2 and pro-apoptotic protein Bax. The death of apoptotic cells is completed by Caspase family, and Caspase3 is the most important executor of the apoptosis (Zhao et al. 2017). While the degree of apoptosis can directly reflected by the expression of Cleaved Caspase3 (Zhang et al. 2017). p53, another important regulator of apoptosis, activated by oxidative stress, can up-regulate Bax gene and down-regulate Bcl2 gene to promote an apoptosis, and also promote the activation of Caspase3, directly leading to an apoptosis (Zhao et al. 2017). Interestingly, p53 can be also regulated by PI3K/AKT signaling pathway (Zhang et al. 2016), and inhibited by p-AKT (Zeng et al. 2011). In II/R rats of the present study, the expression levels of p53, Bax and Cleaved Caspase 3 in the jejunal tissue were significantly increased, while the expression level of Bcl2 was significantly decreased, and the apoptosis index was significantly increased, indicating that II/R could induce the apoptosis in the jejunal tissue. Anwulignan, however, significantly increased the expression level of Bcl2, decreased the expression levels of p53, Bax and Cleaved Caspase 3, and decreased the apoptosis index in the jejunal tissue of II/R rats, suggesting that anwulignan has a significant anti-apoptotic effect in the jejunal tissue of II/R rats. Based on the changes of PI3K/AKT and Nrf2/ARE signaling pathway-related proteins, it is speculated that anwulignan may play roles in inhibiting the apoptosis in the jejunal tissue of II/R rats by activating the PI3K/AKT and Nrf2/ARE signaling pathways.

In the study, experiments were performed by pre-treating rats with Anwulignan before performing intestinal ischemia/reperfusion treatment. Current study may serve to show the importance of Anwulignan in modulating oxidative stress reactions, inflammatory immune responses and apoptosis during intestinal ischemia/reperfusion caused in a controlled condition such as transplants. Further basic and clinical studies need to be done to explore
its effects in an uncontrolled situation such as thrombosis, necrotizing enterocolitis and trauma.

In conclusion, anwulignan can significantly alleviate the jejunal tissue injury in rats with intestinal ischemia/reperfusion injury, and the mechanism may be related to its antioxidant and anti-apoptotic effects through its activation of PI3K/Akt and Nrf2/ARE signaling pathways.

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No author has an actual or perceived conflict of interest with the contents of this article.

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Performed data analysis: Dan Wang, Chunmei Wang, Jinghui Sun, Jianguang Chen.

Wrote or contributed to the writing of the manuscript: Huijiao Lin, He Li, and Shu Jing.

All authors critically reviewed the manuscript and approved its final version.

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Legends

Figure 1. Experimental protocol

Figure 2. Effects of anwulignan on the body weight curve, mesenteric microcirculatory capillary blood flow velocity and histopathological changes of the jejunum (Mean ± SD). (A) Body weight curve, n=11; (B) Mesenteric microcirculatory capillary blood flow velocity, n = 8; (C) Gross pathological appearances of the jejunum, n = 3; (D) Histopathological changes of the jejunum, n = 3 (E) Histopathological scores after II/R (Chiu’s scores), n = 3. Compared with Sham group, **P < 0.01; compared with II/R group, ##P < 0.01.

Figure 3. Effects of anwulignan on the tension of isolated jejunum in rats (Mean ± SD, n = 8). (A) Autonomic contraction curves of jejunal rings; (B) Frequency of autonomic contraction of jejunal rings; (C) Amplitude of autonomic contraction of jejunal rings; (D) Responsiveness of jejunal rings to the ACh-induced contraction; (E) Changes of intestinal tension after ACh stimulation; (F) Amplitude of autonomic contraction of jejunal rings after ACh-induced contraction. Compared with Sham group, **P < 0.01; compared with II/R group, ##P < 0.01.

Figure 4. Effects of anwulignan on oxidation-related indexes in the serum and jejunal tissue of rats (Mean±SD, n=8). (A) SOD; (B) CAT; (C) GSH; (D) GST; (E) MDA; (F) 8-OHdG; (G) PKC; (H) NOX; (I) ROS. Compared with Sham group, *P < 0.05, **P < 0.01; compared with II/R group, *P < 0.05, **P < 0.01.

Figure 5. Effects of Anwulignan on TNF-α, IL-6 and IL-1β contents and MPO activity in the serum and jejunal tissue (Mean ± SD, n = 8). A: TNF-α; B: IL6; C: IL-1β; D: MPO. Compared with Sham group, *P < 0.05, **P < 0.01; compared with II/R group, *P < 0.05, **P < 0.01.

Figure 6. Effects of anwulignan on the content of ACh and the activity of ChAT in the jejunal tissue (mean ± SD). A: ACh contents, n = 8; B: ChAT activities, n = 8; C: M3 and GAPDH protein electrophoresis images (Western blot); D: M3/GAPDH column charts, n = 3. Compared with Sham group, **P < 0.01; compared with II/R group, ##P < 0.01.
Figure 7. Effects of anwulignan on Nrf2/ARE pathway-related gene expressions in the jejunal tissue (Mean ± SD, n = 3). A: Keap1, Nrf2, HO-1 and β-actin mRNA electrophoresis images (RT-PCR); B: Keap1/GAPDH, Nrf2/GAPDH and HO-1/GAPDH column charts; C: Keap1, p-Nrf2, Nrf2, HO-1 and GAPDH protein electrophoresis images (Western Blot); D: Keap1/GAPDH, p-Nrf2/Nrf2 and HO-1/GAPDH column charts. Compared with Sham group, ** P < 0.01; compared with II/R group, # P < 0.05, ## P < 0.01.

Figure 8. Effects of anwulignan on expression of PI3K/AKT pathway-related proteins in the jejunal tissue (Mean ± SD, n = 3). A: PI3K, p-AKT, AKT, p-GSK3β Ser9, p-GSK3β Tyr216 and GAPDH protein electrophoresis images (Western Blot); B: PI3K/GAPDH, p-AKT/AKT, p-GSK3β Ser9/GAPDH and p-GSK3β Tyr216/GAPDH column charts. Compared with Sham group, * P < 0.05, ** P < 0.01; compared with II/R group, # P < 0.05, ## P < 0.01.

Figure 9. Effects of anwulignan on the apoptosis in the jejunal tissue (Mean ± SD, n = 3). A: Apoptosis of jejunal tissue cells by Hoechst staining; B: Apoptosis rates of jejunal tissue cells; C: p53, Bcl2, Bax, Caspase3 and β-actin mRNA electrophoresis images (RT-PCR); D: p53/β-actin, Bcl2/Bax and Caspase3/β-actin column charts; E: p53, Bcl2, Bax, Cleaved Caspase3 and GAPDH electrophoresis images (Western Blot); F: p53/GAPDH, Bcl2/Bax and Cleaved Caspase3/GAPDH column charts. Compared with Sham group, ** P < 0.01; compared with II/R group, # P < 0.05, ## P < 0.01.

Figure 10. Alleviation of Anwulignan on the II/R injury through PI3K/AKT signaling pathway in rats.
Figure 1

1. Sham group (11 rats)  
2. I/R group (11 rats)  
3. Sham+Anwulignan group (11 rats)  
4. I/R+Anwulignan group (11 rats)

44 rats

1 day  
14 day

ischemia  
45min  
reperfusion  
60min

Blood flow velocity of mesenteric microcirculation capillaries (8 rats)

Collect blood and Intestine

Serum

Intestines

8 rats

7mg.kg⁻¹Anwulignan or Carboxymethylcellulose sodium

Histopathological analysis  
The levels of Ach, ChAT  
Oxidation-related biochemical indicators  
Inflammation-related biochemical indicators  
Hoechst staining  
Western Blot
Figure 2

A. Rats Weight curve

Day of Experiment (day)

B. Blood flow Velocity (μm/s)

Sham | II/R | Sham + Anwuliganan (7mg/kg) | II/R + Anwuliganan (7mg/kg)

C. Images of tissue samples:

Sham | II/R

Sham | II/R

Anwuliganan (7mg/kg) | Anwuliganan (7mg/kg)

D. Magnification: ×200 and ×400

E. Chin's Score

Sham | II/R | Sham | II/R

Anwuliganan (7mg/kg)
Figure 3

A
Sham

II/R

Sham+Anwu

II/R+Anwu

D
Sham

II/R

Sham+Anwu

II/R+Anwu

B
Frequency (times/min)

Sham
II/R
Sham+Anwu
II/R+Anwu

C
Amplitude (g)

Sham II/R Sham+Anwu
II/R+Anwu

E
Intestinal tension (g)

Sham II/R Sham+Anwu
II/R+Anwu

F
Amplitude (g)

Sham II/R Sham+Anwu
II/R+Anwu
Figure 5

A

B

C

D

TNF-α (pg/mL)

IL-6 (pg/mL)

IL-1β (pg/mL)

MPO (U/g)

Serum

Intestine

Sham

II/R

Sham

II/R

Sham

II/R

Anwulignan (7mg·kg⁻¹)

Anwulignan (7mg·kg⁻¹)

Anwulignan (7mg·kg⁻¹)

Anwulignan (7mg·kg⁻¹)

# #

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Figure 6

A

B

C

D

Sham II/R Sham II/R Anwulignan(7mg/kg)

Sham II/R Sham II/R Anwulignan(7mg/kg)

Sham II/R Sham II/R Anwulignan(7mg/kg)

Sham II/R Sham II/R Anwulignan(7mg/kg)

M3

GAPDH

M3/GAPDH
Figure 8

A

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<tr>
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B

Relative Protein Expression

- Sham
- I/R
- Sham + Anwu
- I/R + Anwu

Legend:
- ∗ indicates significant difference from Sham group.
- ∗∗ indicates significant difference from I/R group.
- # indicates significant difference between groups.