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Anwulignan Ameliorates the Intestinal Ischemia/Reperfusion

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ABSTRACT

Anwulignan is one of the monomer compounds in the lignans from *Schisandra sphenanthera*. In this study, we observed the effect of anwulignan on intestinal ischemia/reperfusion (II/R) injury in male Sprague-Dawley rats and explored the underlying mechanisms. The results showed that pretreatment with oral anwulignan could significantly increase the mesenteric blood microcirculatory flow velocity; relieve the congestion and pathologic injury of jejunum; enhance the autonomic tension of jejunum smooth muscle and its reactivity to acetylcholine; increase the activities of superoxide dismutase, catalase, glutathione *S*-transferase, and choline acetyltransferase; increase the contents of acetylcholine and glutathione in the serum or jejunal tissue; decrease the activities of myeloperoxidase, protein kinase C, and nicotinamide adenine dinucleotide phosphate oxidase; reduce the contents of malondialdehyde, 8-hydroxy-2-deoxyguanosine, nicotinamide adenine, reactive oxygen species, tumor necrosis factor-α, interleukin (IL)-6, and IL-1β; increase the expression levels of muscarinic receptor 3, PI3K, phosphorylation protein kinase B, p-GSK3β Ser9, Nrf2, p-Nrf2, heme oxygenase (decycling) 1, and b-cell lymphoma 2 in the jejunal tissue; and decrease the expression levels of p-GSK3β Tyr216, kelch-like ECH-associated protein 1, Bax, and cleaved caspase-3, suggesting that anwulignan can ameliorate II/R-induced jejunal tissue injury in rats and that the mechanism may be related to its activating the PI3K/protein kinase B pathway and then regulating the Nrf2/Anti-oxidative Response Element signaling pathway and the expression of apoptosis-related proteins to play antioxidant and antiapoptotic roles.

SIGNIFICANCE STATEMENT

Anwulignan can significantly reduce jejunal tissue injury and the production of inflammatory factors in rats with intestinal ischemia-reperfusion injury, improve the antioxidant capacity, and reduce the apoptosis of jejunal tissue, and it has the effect of significantly improving 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.

Introduction

Intestinal ischemia-reperfusion (II/R) injury is secondary to enteritis, volvulus, trauma, blood loss, and intestinal transplant rejection, with a morbidity of 13 of 100,000 and a mortality of 30%–80% in the 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 are generated in ischemia-reperfusion, exceeding 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 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 of II/R injury, but studies have shown that some antioxidants can ameliorate

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ABBREVIATIONS: ACh, acetylcholine; AKT, protein kinase B; Anwu, anwulignan; ARE, Anti-oxidative Response Element; Bax, bcl2-like protein 4; Bcl2, b-cell lymphoma 2; CAT, catalase; ChAT, choline acetyltransferase; GAPDH, Glyceraldehyde-3-phosphate Dehydrogenase; GSH, glutathione; GSK3β, glycogen synthase kinase-3β; GST, glutathione *S*-transferase; HO-1, heme oxygenase (decycling) 1; II/R, intestinal ischemia/reperfusion; IL, interleukin; Keap1, kelch-like ECH-associated protein 1; M3, muscarinic receptor 3; MDA, malondialdehyde; MPO, myeloperoxidase; NOX, reduced nicotinamide adenine dinucleotide phosphate oxidase; Nrf2, nuclear factor (erythroid-derived 2)-like 2; 8-OHdG, 8-hydroxy-2-deoxyguanosine; p-AKT, phosphorylation protein kinase B; p-GSK3β, phosphorylation glycogen synthase kinase-3β; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; p-Nrf2, phosphorylation nuclear factor (erythroid-derived 2)-like 2; ROS, reactive oxygen species; RT-PCR, Real Time–Polymerase Chain Reaction; SD, Sprague-Dawley; SOD, superoxide dismutase; TNF-α, tumor necrosis factor-α; Wnt, wingless integration-1.

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 (*S. sphenanthera*), first recorded in *Shennong's Herbal Classic* and included in *Chinese Pharmacopoeia* (2020), is a Chinese herbal medicine used in health foods issued by the Health Commission of the People's Republic of China, with the functions of nourishing and strengthening (Li et al., 2018a). *S. sphenanthera* is also commonly used as an ingredient in Chinese prescription medicines in Japan and in health foods in Korea and the United States (Zhu et al., 2019). Lignans are the main active components of *S. sphenanthera*, with significant antioxidant and hepatoprotective effects (Zhu et al., 2019). Anwulignan is one of the main monomeric active components in the lignans from *S. 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, until 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 to provide an experimental basis for the development of drugs for the prevention and treatment of II/R injury.

Materials and Methods

Materials

Animals. SD rats (male, aged 8–10 weeks) weighing 220–250 g 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 of Beihua University.

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); H&E, SOD, MDA, CAT, glutathione peroxidase, 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); Bicinchoninic Acid kit, HCl-Tris, ammonium persulfate, 30% acrylamide, glycine, N,N,N′,N′-Tetramethylethylenediamine, and 5× SDS loading buffer (Beijing Dingguo Changsheng Biotechnology Co., Ltd, Beijing, China); skimmed milk powder (Becton, Dickinson and Company, NJ); M3, Keap1, p-Nrf2, Nrf2, HO-1, PI3K, p-AKT, AKT, p-GSK3β Tyr216, p-GSK3β Ser9, GSK3β, Bcl2, Bax, cleaved caspase-3, Glyceraldehyde-3-phosphate Dehydrogenase, horseradish peroxidase goat anti-rabbit IgG (H+L), and horseradish peroxidase goat anti-mouse IgG (H+L) secondary antibodies (ABclonal); electrochemiluminescence chromogenic solution, One-Step PT-PCR Kit, and nucleic acid dye (Nanjing Vazyme Biotech Co., Ltd, Nanjing China); and polymerase chain reaction primers (Shenyang Dingguo Biotechnology Co., Ltd., Shenyang, China) were obtained.

Methods

Animal Grouping and Administration. In total, 44 male SD rats were randomly and evenly divided into four groups: 1) sham operation group (sham group), only laparotomy and no ischemic treatment; 2) intestinal ischemia/reperfusion group (II/R group), ischemic treatment of 45 minutes and then reperfusion for 60 minutes; 3) sham operation + anwulignan group (sham + Anwu group), only laparotomy and no ischemia/reperfusion, and gavage of anwulignan; and 4) sham operation + anwulignan group (II/R + Anwu group), ischemic treatment of 45 minutes and then the reperfusion for 60 minutes and gavage of anwulignan. Rats in the 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 the 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 the sham group and the sham + Anwu group was only isolated (without clamping), whereas that in the II/R group and II/R + Anwu group was isolated and clamped with a noninvasive vascular clamp for 45 minutes, and then the clamp was removed and the artery was reperfused for 60 minutes for the establishment of an II/R model (Camara-Lemarroy et al., 2009). After the II/R, the blood was collected from the abdominal aorta, left standing at room temperature for 30 minutes, and centrifuged at 4°C for 10 minutes. The serum was taken and stored at −80°C. After the collection of blood samples, rats were euthanized with 200 mg/kg pentobarbital, and then a 5- to 6-cm segment of the jejunal tissue was taken about 5 cm from the ileum and quickly rinsed with ice-cold normal saline. The contents in the jejunal tissue segment were washed away, and the water on its surface was blotted dry for further experiments. The experimental protocol is shown in Fig. 1.

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.

Measurement of Blood Flow Velocity of the Mesenteric Microcirculatory Capillary. After the II/R model of rats was established, as described in the *Animal Grouping and Administration* section, 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.

Histopathological Observation of the Jejunal Tissue. The jejunal tissue was obtained as described in the *Animal Grouping and Administration* section, 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 H&E. The histologic changes of the jejunal tissue were observed under an optical microscope, and the staining images were observed by 200× and 400× 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.

Measurement of the Jejunal Tension. The jejunal tissue was obtained as described in the *Animal Grouping and Administration* section. A 0.5-cm ring of the jejunum was cut and placed in a bath containing Tyrode's solution (Subramanya et al., 2015) (130 mM NaCl, 4.5 mM KCl, 2.2 mM $CaCl_2$, 0.6 mM $MgCl_2$, 24.2 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 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 1 g was loaded on the jejunal ring, and the solution was changed every 10 minutes and left stable for 60 minutes. The contraction and relaxation of the jejunum ring were recorded by a biologic signal recording system, and the number and height of contraction waves within 1 minute 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

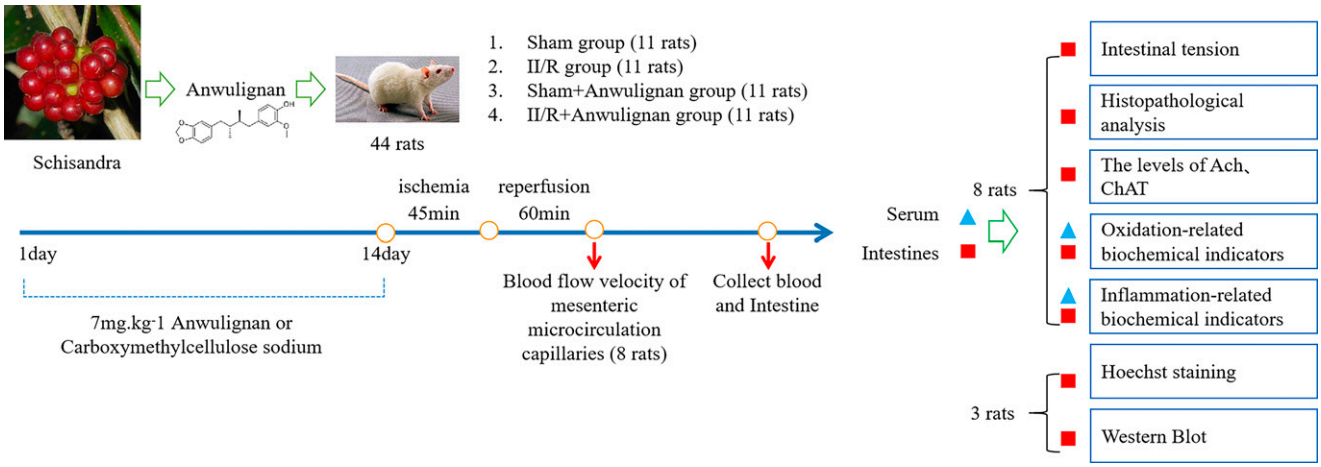

Fig. 1. Experimental protocol.

et al., 2016), and the contraction response curve and the maximum contraction tension were observed.

Detection of Biochemical Indexes. The serum and jejunal tissue were obtained as described in the *Animal Grouping and Administration* section. The activities of SOD, CAT, GST, NOX, MPO, and ChAT and the contents of GSH, MDA, 8-OHdG, PKC, ROS (reactive oxygen species), TNF-α, interleukin (IL)-6, IL-1β, and ACh in the serum and/or the jejunal tissue homogenate were detected according to the methods provided by the kit manufacturers.

Observation on the Apoptosis of Jejunal Tissue Cells by Hoechst Staining. The tissue sections were dewaxed and washed with PBS for 2 × 3 minutes on a shaking table. Each section was added with 0.5 ml Hoechst 33258 staining solution and dyed on the shaking table for 5 minutes and then washed with PBS for 2 × 3 minutes to remove the staining solution, and a drop of antiquenching 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:

$$Apoptosisrate = apoptoticcells/totalcells \times 100\%.$$

Detection of Keap1, Nrf2, HO-1, p53, Bcl2, Bax, and Caspase-3 Expressions in the Jejunal Tissue by RT-PCR. As in the *Animal Grouping and Administration* section, the jejunal tissues of three rats in each group were randomly taken. The RNA in the jejunal tissues were extracted according to the instructions of the RNA extraction kit, and the cDNA was synthesized according to the instructions of the reverse transcription kit. The primers were designed with Primer software 6 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: predenaturation at 94°C for 3 minutes, denaturation at 94°C for 30 seconds, annealing for 3 minutes and 30 seconds (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 caspase-3, and 55.6°C for β-actin), and extension at 72°C for 30 seconds, which was repeated 30 times, and then extension at 72°C for 7 minutes. Finally, the samples were stored at 4°C for the gel electrophoresis.

Detection of the Expressions of M3, Nrf2/ARE, PI3K/AKT, and Apoptosis-Related Proteins in the Jejunal Tissue by Western Blot. As described in the *Animal Grouping and Administration* section, jejunal tissues from three rats in each group were randomly taken, and the total protein was extracted using the total protein extraction kit. Bicinchoninic Acid 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 caspase-3 (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 hour. Then, the membranes were washed, and an electrochemiluminescence developer was used for the color development of the membranes.

Statistical Analysis

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

Results

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 (Fig. 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 (Fig. 2B) showed that, compared with the sham group, the blood flow velocity of mesenteric microcirculatory capillary in the II/R group was significantly decreased ($P < 0.01$); however, compared with the II/R group, the blood flow velocity in the II/R + Anwu group was significantly increased ($P < 0.01$).

The histopathological observation on the jejunal tissue (Fig. 2C) showed that, compared with the sham group, the jejunum congestion was more severe in the II/R group, and compared

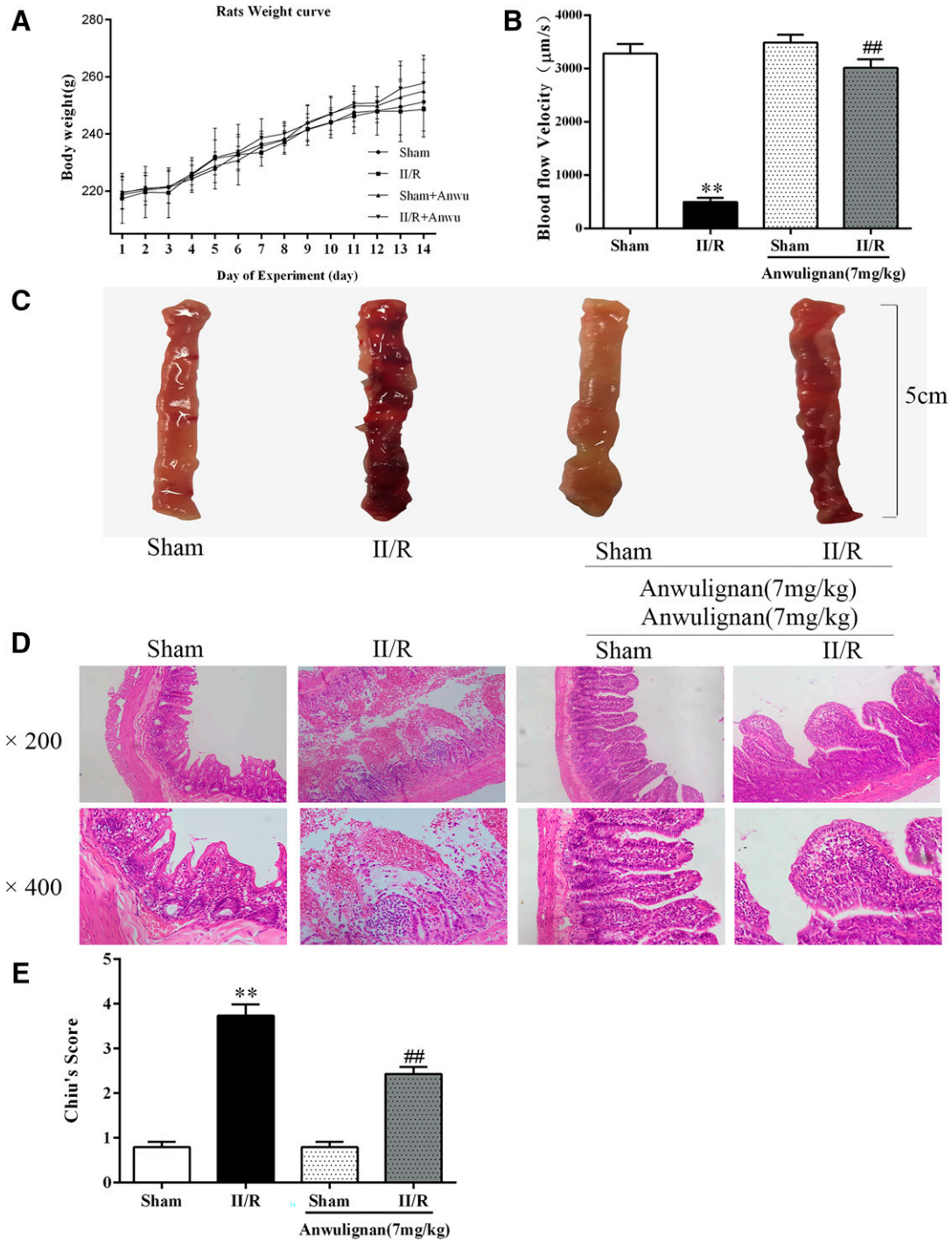

Fig. 2. Effects of anwulignan on the body weight curve, mesenteric microcirculatory capillary blood flow velocity, and histopathological changes of the jejunum (mean ± S.D.). (A) Body weight curve; $n = 11$. (B) Mesenteric microcirculatory capillary blood flow velocity; $n = 8$. (C) Gross pathologic 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, $^{\#\#}P < 0.01$.

with the II/R group, the jejunum congestion was significantly alleviated in the II/R + Anwu group. The observation on the effects of anwulignan on the jejunal tissue by H&E staining (Fig. 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 the 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 the II/R group + Anwu group, the necrosis of upper mucosa cells was mitigated, the muscle layer thickness was reduced, and the infiltration of inflammatory cells and bleeding were

alleviated. The results of intestinal histologic injury scores (Fig. 2E) showed that, compared with the sham group, the Chiu's score increased significantly after II/R injury ($P < 0.01$); compared with the II/R group, the Chiu's score decreased significantly in the 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 minutes and reperfusing it for 60 minutes, and the jejunal tissue injury was significantly alleviated after the pretreatment with anwulignan.

Effects of Anwulignan on the Jejunal Tension In Vitro. Intestinal autonomic contraction tension is a direct index to evaluate intestinal function (Ovsiannikov and Berezina, 2013). In this study, the autonomic contraction of jejunum rings was observed after the jejunum rings in the isolated tissue perfusion system were stable. As shown in Fig. 3, A–C, the autonomic contraction of jejunum rings in the sham group and the sham + Anwu group was slow and rhythmic; compared with the sham group, the autonomic contraction in the II/R group almost disappeared, and the frequency and the amplitude of autonomic contraction were significantly reduced ($P < 0.01$); compared with the II/R group, the frequency and amplitude in the 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 Fig. 3, D–F, the maximum contractile tension and amplitude induced by ACh in the II/R group were significantly smaller than those in the sham group ($P < 0.01$), and the maximum contractile tension and amplitude induced by ACh in the II/R + Anwu group were significantly larger than those in the 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.

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 Fig. 4, in II/R rats, serum SOD, CAT, GST, and GSH levels were 63.3%, 49.5%, 72.0%, and 52.7% of those in the sham group, respectively, whereas 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 levels were 3.9, 1.5, 1.9, and 1.7 times those in the sham group, respectively. Jejunal MDA, 8-OHDG, and ROS levels were 3.3, 1.9, and 1.7 times those in the 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 it 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.

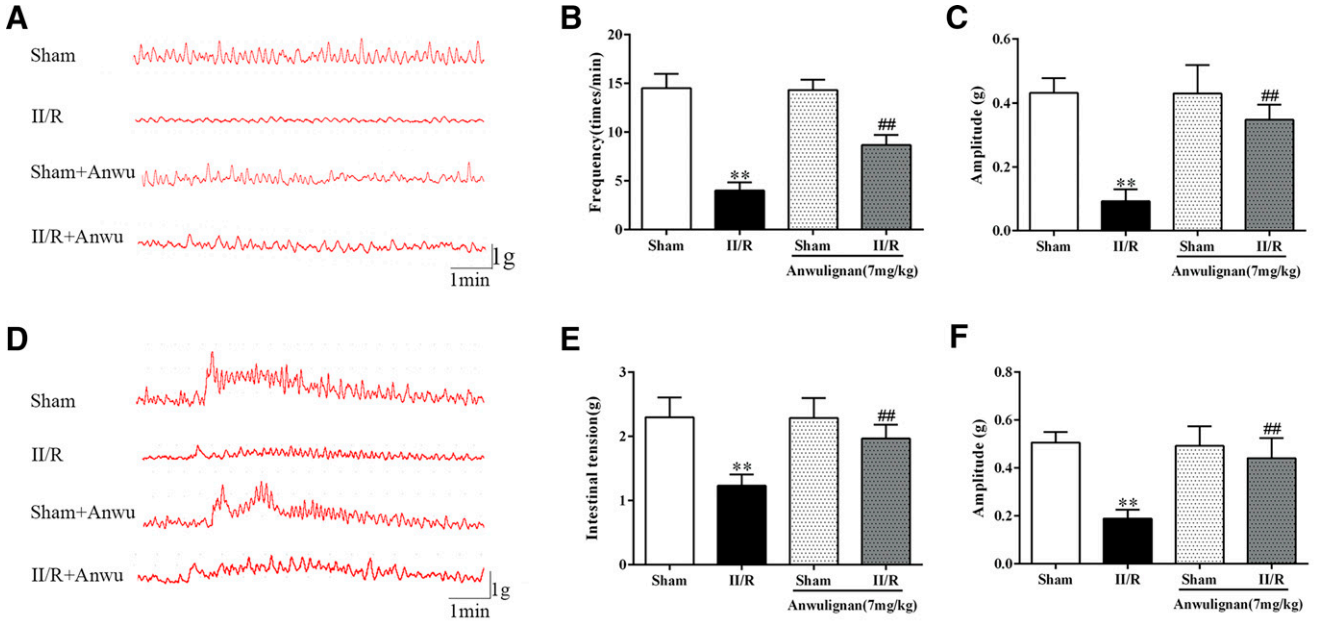

Fig. 3. Effects of anwulignan on the tension of isolated jejunum in rats (mean ± S.D.; $n = 8$). (A) Autonomic contraction curves of jejunal rings. (B) Frequency of autonomic contraction of jejunal rings. (C) Amplitude of autonomic contraction of jejunal rings. (D) Responsiveness of jejunal rings to 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, $^{\#\#}P < 0.01$.

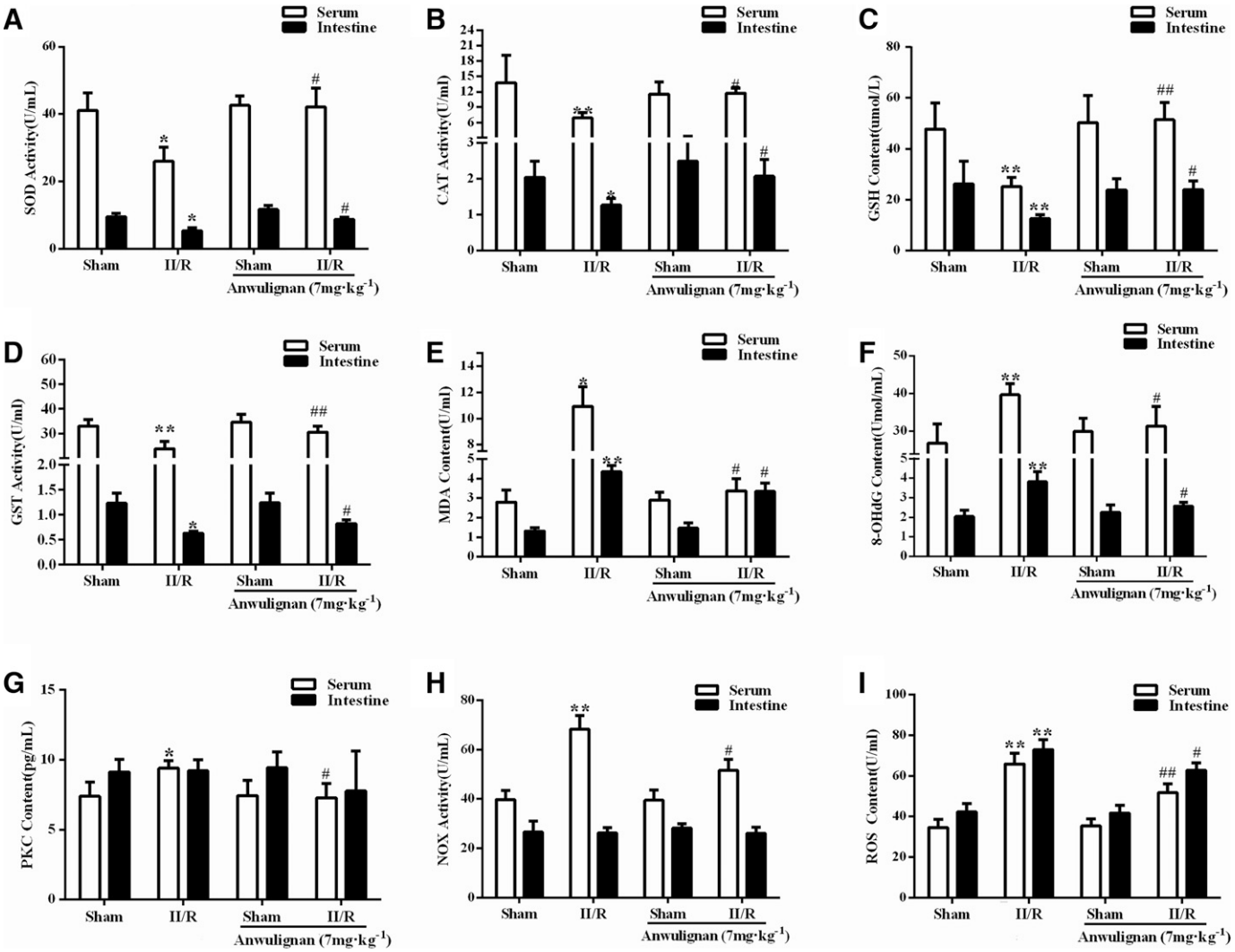

Fig. 4. Effects of anwulignan on oxidation-related indexes in the serum and jejunal tissue of rats (mean ± S.D.; $n = 8$). (A) SOD; (B) CAT; (C) GSH; (D) GST, (E) MDA; (F) 8-OHdG; (G) PKC; (H) NOX; (I) ROS. Compared with sham group, $^{*}P < 0.05$, $^{**}P < 0.01$; compared with II/R group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

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

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 M3 to mediate the contraction of gastrointestinal smooth muscle. ChAT 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 (Fig. 6) showed that, compared with the 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 the II/R group ($P < 0.01$); but all of them were significantly increased in the II/R + Anwu group ($P < 0.01$) compared with those in the II/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 II/R rats.

Effects of Anwulignan on the Expression of Nrf2/ARE Pathway–Related Genes in the Jejunal Tissue. The Nrf2/ARE pathway is the key pathway playing an antioxidant 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 Fig. 7, compared with the sham group, the expression level of Keap1 was significantly increased, and the

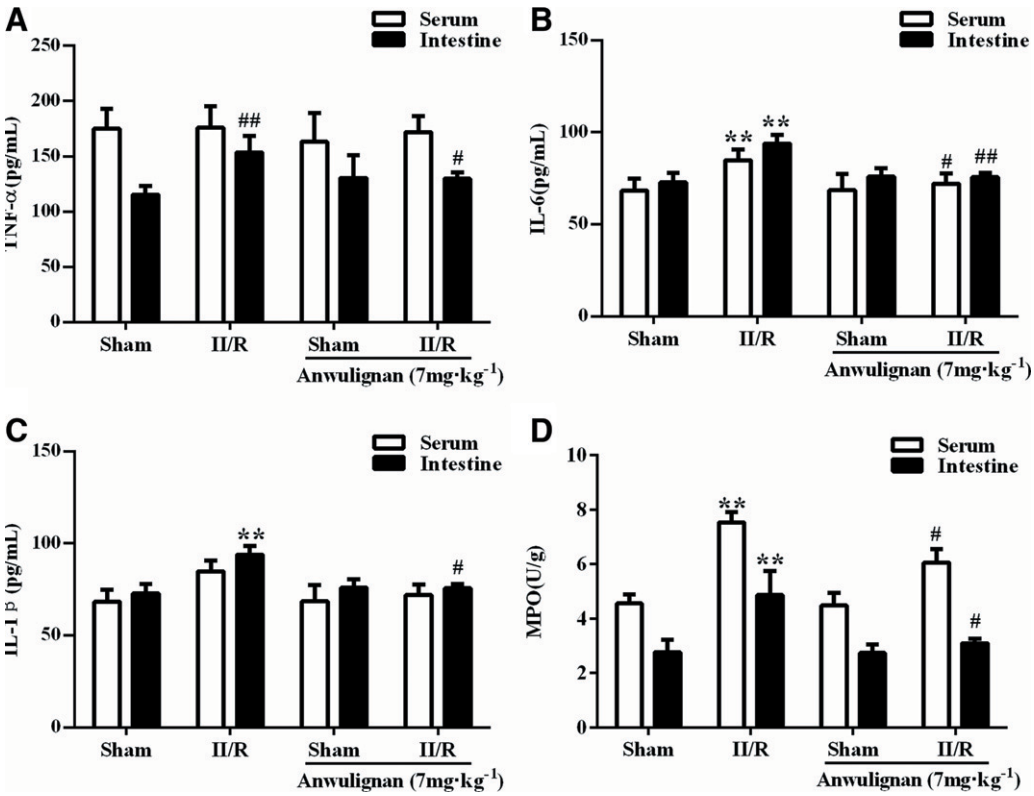

Fig. 5. Effects of Anwulignan on TNF-α, IL-6, and IL-1β contents and MPO activity in the serum and jejunal tissue (mean ± S.D.; $n = 8$). (A) TNF-α; (B) IL6; (C) IL-1β; (D) MPO. Compared with sham group, $^{*}P < 0.05$, $^{**}P < 0.01$; compared with II/R group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

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 the II/R group ($P < 0.05$, $P < 0.01$); however, compared with the II/R group, the expression level of Keap1 was significantly decreased, and the expression of Nrf2, the ratio of p-Nrf2 to Nrf2, and the expression level of HO-1 were significantly increased in the jejunal tissue of rats in the II/R + Anwu group ($P < 0.05$, $P < 0.01$), suggesting that anwulignan could alleviate the II/R injury by activating the Nrf2/ARE signaling pathway and then exerting an antioxidant effect in rats.

Effects of Anwulignan on PI3K/AKT Pathway–Related Protein Expression in the Jejunal Tissue. The PI3K/AKT signaling pathway is one of the important pathways involved in the regulation of Nrf2/ARE signaling pathway, and the activated PI3K can regulate 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 Fig. 8, compared with the 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 the II/R group. However, compared with the II/R group, the expression levels of PI3K, p-AKT, and p-GSK3β Ser9 were significantly increased ($P < 0.05$, $P < 0.01$), whereas the expression level of p-GSK3 Tyr216 was significantly decreased ($P < 0.05$) in the jejunal tissue of rats in the 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., 2019). Studies have shown that activation of the Wnt/β-catenin pathway significantly improves intestinal ischemia/reperfusion injury (El-Sayyad et al., 2017). As shown in Fig. 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 the Wnt/β-catenin signaling pathway, which may be another important mechanism of anwulignan in improving intestinal ischemia-reperfusion injury in rats.

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. As shown in Fig. 9A, the jejunal tissue cells of rats in the sham group and the 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 the II/R group increased, with an increased fragmented bright-blue chromatin, whereas the apoptosis of jejunal tissue cells of rats in the II/R group was significantly alleviated in the anwulignan-pretreated groups; the apoptosis rate of rats in the II/R group was significantly higher than that in the sham group ($P < 0.01$), and the apoptosis rate of rats in the II/

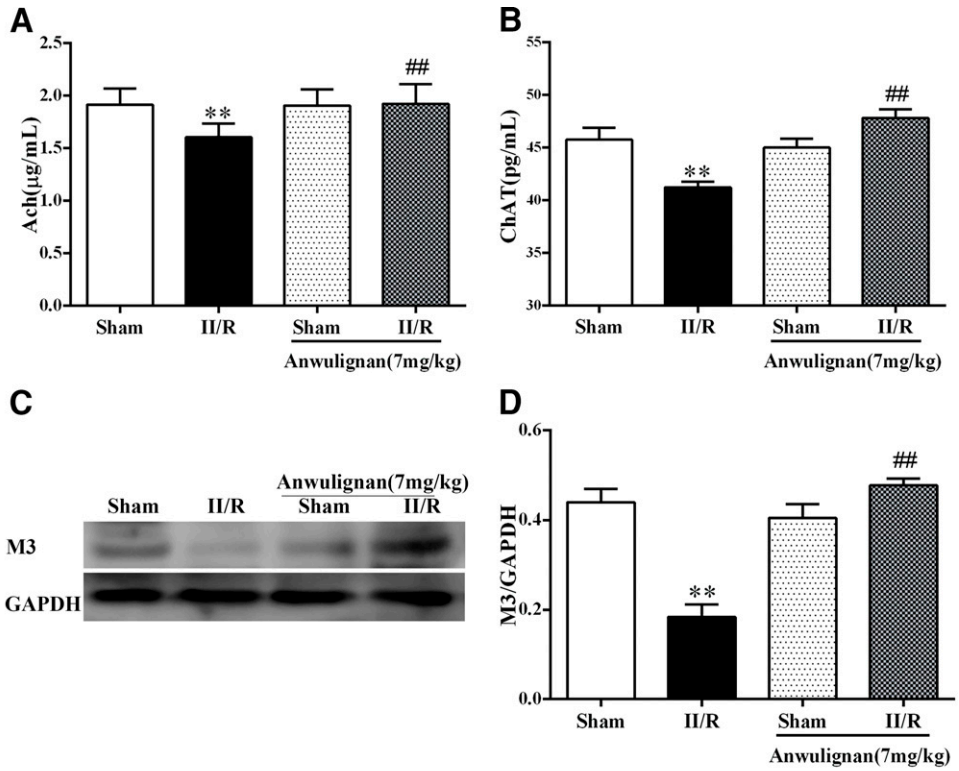

Fig. 6. Effects of anwulignan on the content of ACh and the activity of ChAT in the jejunal tissue (mean ± *SD*). (A) ACh contents, $n = 8$; (B) ChAT activities, $n = 8$; (C) M3 and GAPDH protein electrophoresis images (Western blot); (D) M3/GAPDH column charts, $n = 3$. Compared with sham group, $^{**}P < 0.01$; compared with II/R group, $^{\#\#}P < 0.01$.

R + Anwu group was significantly lower than that in the II/R group ($P < 0.01$).

Bax is a proapoptotic protein, and Bcl2 can bind to Bax to inhibit the occurrence of apoptosis (Tan et al., 2005). Caspase-3 is the key executor of apoptosis, and its expression represents the degree of apoptosis (Weng et al., 2019), and P53 can induce apoptosis by regulating the expression of Bax, Bcl2, and caspase-3 (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 Fig. 9, the expression level of Bcl2 in the jejunal tissue of rats in the II/R group was significantly lower than that in the sham group, and the expression levels of p53, Bax, caspase-3, and cleaved caspase-3 in the jejunal tissue of rats in the II/R group were significantly higher than in the sham group ($P < 0.01$), whereas the expression level of Bcl2 in the II/R + Anwu group was significantly higher than that in II/R group, and the expression levels of p53, Bax, caspase-3, and cleaved caspase-3 in the II/R + Anwu group were significantly lower than those in the 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 II/R injury in rats.

Discussion

Ischemia-reperfusion injury refers to the ischemia injury that occurs to tissue first, and this injury is not improved by the subsequent blood supply recovery, but is instead aggravated and even irreversible (Tőkés et al., 2015). Studies have shown that epithelial cells located at the tip of intestinal villi are very vulnerable to ischemia-reperfusion injury. Intestinal ischemia-reperfusion is more common in 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 classic method (Camara-Lemarroy et al., 2009), 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 (Camara-Lemarroy et al., 2009; Gonzalez et al., 2015), including histopathological changes, physiologic dysfunctions, biochemical abnormity, and excessive oxidative stress, etc. The present study showed that there was 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. (2005) reported that anwulignan could inhibit platelet aggregation, 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 (Ovsiannikov and Berezina 2013; Chen et al., 2018). It has been reported that after II/R injury, the content of ChAT (a key enzyme in the synthesis of ACh), ACh, and the expression level of M3 protein decreased (Paulino et al., 2011; Palombit et al., 2013),

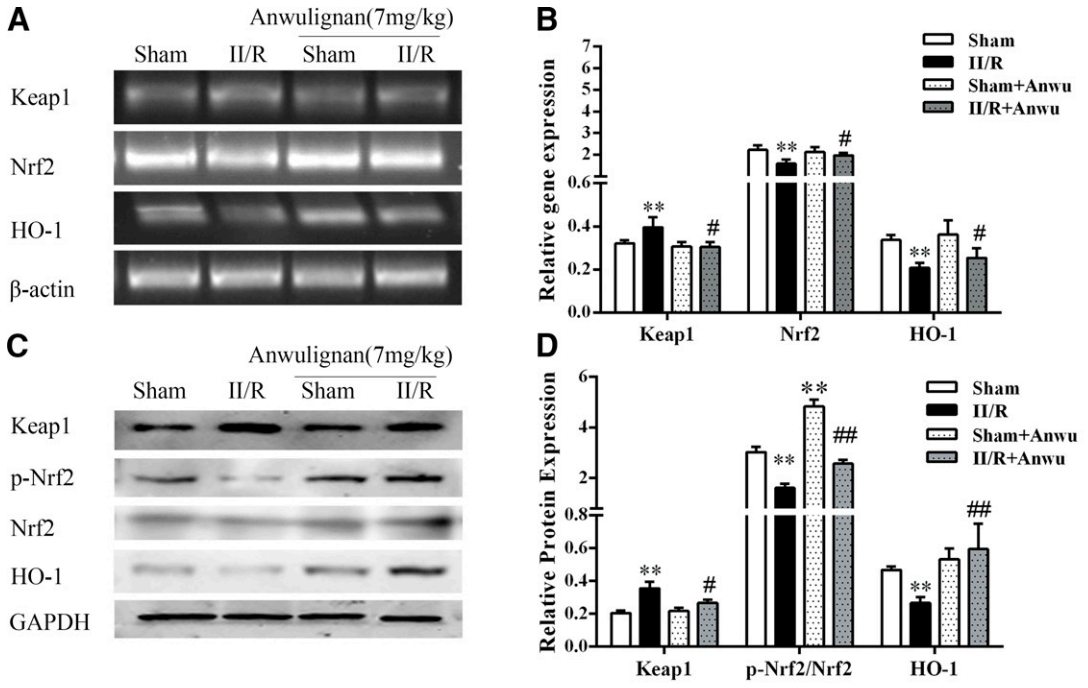

Fig. 7. Effects of anwulignan on Nrf2/ARE pathway–related gene expressions in the jejunal tissue (mean ± S.D.; $n = 3$). (A) Keap1, Nrf2, HO-1, and β-actin mRNA electrophoresis images (RT-PCR); (B) Keap1/GAPDH, Nrf2/GAPDH, and HO-1/GAPDH column charts; (C) Keap1, p-Nrf2, Nrf2, HO-1, and GAPDH protein 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, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

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 upregulate M3 receptor expression in II/R rats, indicating anwulignan can improve the physiologic 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

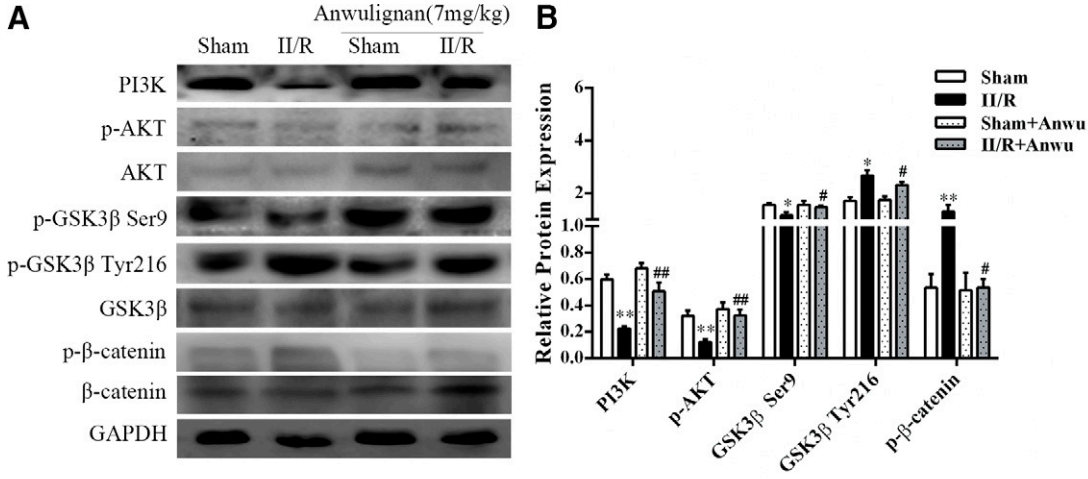

Fig. 8. Effects of anwulignan on expression of PI3K/AKT pathway–related proteins in the jejunal tissue (mean ± S.D.; $n = 3$). (A) PI3K, p-AKT, AKT, p-GSK3β Ser9, p-GSK3β Tyr216, and GAPDH protein electrophoresis images (Western blot); (B) PI3K/GAPDH, p-AKT/AKT, p-GSK3β Ser9/GAPDH, and p-GSK3β Tyr216/GAPDH column charts. Compared with sham group, $^{*}P < 0.05$, $^{**}P < 0.01$; compared with II/R group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

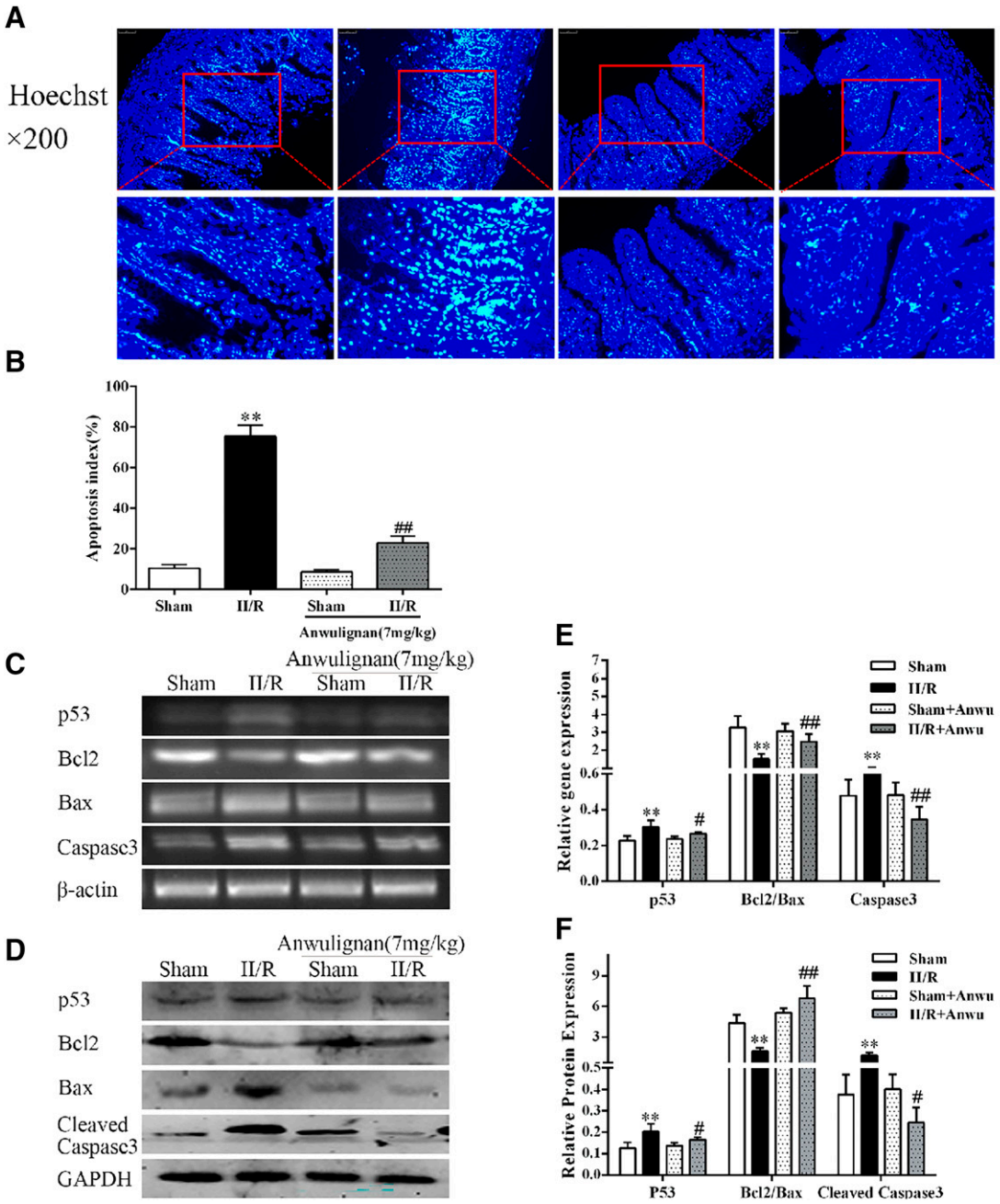

Fig. 9. Effects of anwulignan on the apoptosis in the jejunal tissue (mean ± S.D.; n = 3). (A) Apoptosis of jejunal tissue cells by Hoechst staining. (B) Apoptosis rates of jejunal tissue cells. (C) p53, Bcl2, Bax, caspase-3, and β-actin mRNA electrophoresis images (RT-PCR). (D) p53/β-actin, Bcl2/Bax, and caspase-3/β-actin column charts. (E) p53, Bcl2, Bax, cleaved caspase-3, and GAPDH electrophoresis images (Western blot). (F) p53/GAPDH, Bcl2/Bax, and cleaved caspase-3/GAPDH column charts. Compared with sham group, $^{**}P < 0.01$; compared with II/R group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

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 and 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.

The 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., 2018b). In a physiologic 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 upregulate the expression of p-Nrf2 s40 and HO-1 and downregulate 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 Fig. 10, 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 Tyr216 can lead to the activation of GSK3β, whereas the phosphorylation of 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, and 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, and 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 pathways and exert its antioxidant effects to protect rat II/R. The Wnt/β-catenin signaling pathway is also regulated by AKT (Fukumoto et al., 2001). Studies have shown that the Wnt/β-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 the 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 disfunction (Wang et al., 2017). The apoptosis is mainly determined by the ratio of anti-apoptotic protein Bcl2 and proapoptotic protein Bax. The death of apoptotic cells is completed by the caspase family, and caspase-3 is the most important executor of the apoptosis (Zhao et al., 2017). Although the degree of apoptosis can be directly reflected by the expression of cleaved caspase-3 (Zhang et al., 2017), p53, another important regulator of apoptosis, activated by oxidative stress, can upregulate the Bax gene and downregulate the Bcl2 gene to promote apoptosis and also promote the activation of caspase-3, directly leading to apoptosis (Zhao et al., 2017). Interestingly, p53 can also be regulated by the 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, whereas 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 antiapoptotic effect in the jejunal tissue of II/R rats. Based on the changes of the 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.

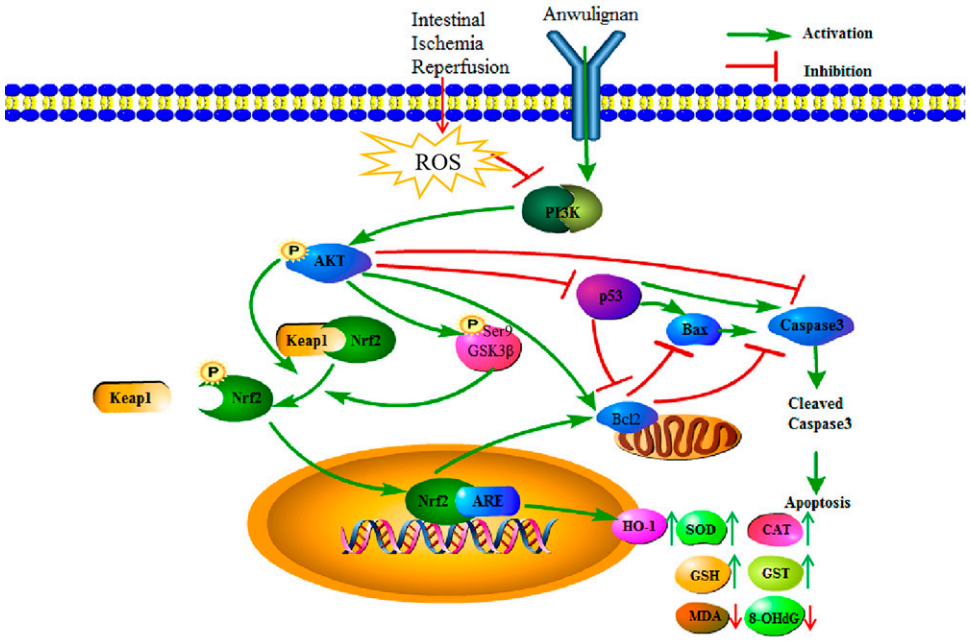

Fig. 10. Alleviation of anwulignan on the II/R injury through PI3K/AKT signaling pathway in rats.

In this study, experiments were performed by pretreating rats with anwulignan before performing intestinal ischemia/reperfusion treatment. The 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 controlled conditions, 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 antiapoptotic effects thorough its activation of the PI3K/Akt and Nrf2/ARE signaling pathways.

Authorship Contributions

Participated in research design: Li, Jing

Conducted experiments: Lin, Zhang, D. Wang, Jiaw. Liu, Yuan, Jial. Liu.

Performed data analysis: D. Wang, C. Wang, Sun, Chen.

Wrote or contributed to the writing of the manuscript: Lin, Li, Jing.

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