Title page

**Protective effect of Anwulignan on gastric injury induced by indomethacin in mice**

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Running title page

**Running Title:** Effect of Anwulignan on gastric injury in mice.

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**Abbreviations**


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Abstract

AN (Anwulignan) is a monomer lignan from *Schisandra sphenanthera* Rehd. Et Wits (*Schisandra sphenanthera* fructus, *Schisandra sphenanthera*). The protective effect of AN against the indomethacin (IND)-induced gastric injury to mice and the related mechanism of action was investigated in this study. The effect of AN was mainly assessed by observing the gastric tissue morphology, GUI (gastric ulcer index), UIR (ulcer inhibition rate), GJV (gastric juice volume) and pH value. Chemical colorimetry, immunofluorescence, and ELISA, Western blot were used to detect related factors in the gastric tissue. The results showed that AN reduced the GUI, increased the UIR, inhibited the GIV, and increased the gastric pH value. AN significantly increased COX-1, COX-2, and PGE2 expression levels in the gastric tissue, activated Nrf2, increased the HO-1 expression, enhanced the activity of SOD and GSH-Px, and decreased the MDA content. AN reduced the phosphorylation of NF-κB p65 and its nuclear translocation, the key protein of NF-κB signaling pathway in the gastric tissue, and the content of the pathway downstream signaling molecules, including IL-6, IL-1β and TNF-α, to play an anti-inflammatory role. AN inhibited the downstream signals Bax and Cleaved Caspase-3 in gastric tissue, and activated Bcl-2, to play an anti-apoptotic role, which were further verified by Hoechst staining. Therefore, AN has a significant protection against the gastric injury induced by IND in mice, and the mechanism may be concerned in its activation of Nrf2, inhibition of NF-κB signaling pathway, and anti-apoptotic effect.

**Significance statement:** AN significantly reduced the IND-induced gastric injury in mice, and its anti-oxidation, anti-inflammation and anti-apoptosis were considered to be involve in the effect, suggesting that AN should be a potential drug or food supplement for gastric injury induced by indomethacin.
Introduction

Gastric ulcer, one of the most common diseases in the digestive tract, can be found in more than 4 million people every year in the world, and has become a major health problem in human beings (Yismaw et al., 2020). It is well known that the pathophysiology of gastric ulcer is associated with the imbalance between gastric mucosal protection factors and gastric mucosal invasion factors (Tamawski et al., 2014). With the continuous effect of some invasive factors, the gastric mucosal defense function will be weakened and the mucosa eventually forms ulcer (Zhang et al., 2020). The increased application of NSAIDs has caused more gastrointestinal adverse reactions, which has attracted an extensive attention of researchers. Although NSAIDs have good anti-inflammatory effects, its long time administration is prone to a series of adverse reactions such as gastroduodenal ulcer, gastric bleeding, and even gastric perforation (Schmassmann et al., 1998; Wallace et al., 2000). It has been found in some studies that NSAIDs, including indomethacin (IND), can inhibit the activity of cyclooxygenase, decrease the PGE2 synthesis, affect the mucosa regeneration, and finally induce the gastric mucosa inflammation and ulcer (Sostres et al., 2013; Fang et al., 2019). NSAIDs can also directly damage gastrointestinal mucosa (Kuczyńska et al., 2021), causing mitochondria to release a huge number of oxygen free radicals and evoke the oxidative stress of neutrophil infiltration, releasing inflammatory factors, and then producing inflammatory response. Meanwhile, these oxygen free radicals also lead apoptosis of gastric mucosal cells by lipid peroxidation, protein denaturation, and DNA damage. Therefore, the key ways to protect gastric mucosa are considered to reduce the oxidative stress, inflammation and apoptosis in the gastric tissue. Currently, H⁺-K⁺-ATPase inhibitors including omeprazole are considered as first-line drugs to treat NSAIDs-induced gastrointestinal damages. However, proton pump inhibitors themselves also have severe adverse effects (Melcarne et al., 2016), so to find some
new drugs for gastric protection should be an urgent issue.

Herbal medicine has a long treatment history for a variety of diseases, with remarkable therapeutic effects (Hatre et al., 2018). *Schisandra*, firstly recorded in Shennong Herbal Classic, is the dry and mature fruit of *Schisandra sphenanthera* fructus. It has been widely applied as medicine, health supplement, food and beverage (Panossian et al., 2008; Li et al., 2018) in China, South Korea, and Russia (Nowak et al., 2019). Lignan is the main active component in *Schisandra*, with a significant antioxidant, anti-inflammatory, and liver protective effect (Luo et al., 2018). AN is a lignan and representative monomer active component in *Schisandra sphenanthera* fructus. In our previous studies, we found that AN protected the hydrochloric acid/ethanol-induced gastric ulcer in mice, and its mechanism was connected to its antioxidation and anti-inflammation (Liu et al., 2021). Furthermore, AN also showed anti-oxidation, anti-inflammation, and anti-apoptosis effects in D-galactose-induced aging and overtired mouse models (Li et al., 2020; Zhang et al., 2019). However, up to now, there is no report available about the effect of AN on NSAIDs-induced gastric injury. Therefore, we exploited an IND-induced gastric injury model of mice (one of NSAIDs) to examine the protective effect of AN against it. We hope that this study will promote AN to be a new candidate for anti-ulcer drugs and health foods.
Materials and Methods

Animals. Forty-eight ICR mice (male, 6-8 weeks and 18-20g) were purchased from Changchun Yisi Experimental Animal Co., Ltd., Changchun, China [SCXK (Ji)-2020-0002]. All animal experiments were approved by IACUC of Beihua University and carried out according to the Regulations on the Administration of Experimental Animals issued by the State Council, PRC. The animals were raised in a pathogen-free laboratory at 22-25°C, and with a humidity of 40-50%, a 12-hour light/dark cycle and a free access to food and water.

Reagents. Anwulignan (AN, Sichuan Weikeqi Biological Technology Co., Ltd., Chengdu, China); Omeprazole (AstraZeneca Pharmaceutical Co., Ltd., UK); Indomethacin (IND, Shanghai Jinbuhuan Lankao Pharmaceutical Co., Ltd., Shanghai, China); SOD, GSH-Px and MDA test kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China); Hoechst cell apoptosis detection kit and periodic acid-Schiff (PAS) staining kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China); ROS, MPO and PGE2 test kits (Shanghai Enzyme-Linked Biotechnology Co., Ltd., Shanghai, China); TNF-α, IL-6 and IL-1β test kits (ABclonal Biotechnology Co., Ltd., USA); COX-1, COX-2, p-Nrf2 (S40), Nrf2, Keap1, HO-1, NF-κB p-65, p-NF-κB p-65, MMP-9, Bcl2, Bax, Cleaved Caspase-3 and GAPDH antibodies (ABclonal Biotechnology Co., Ltd., USA).

Methods

Protocol. Forty eight mice were randomly divided into 4 groups, ten in each group (Wang et al., 2020; Abd El-Ghffar et al., 2018): (1) Blank control group (CON): solvent + normal saline; (2) Indomethacin group (IND): solvent + 18 mg/kg IND; (3) AN + IND group (AN): 4 mg/kg AN + 18 mg/kg IND; (4) OME + IND group (OME): 20 mg/kg OME (Venzon L et al., 2018; Badr AM et al., 2019; Li Q et al., 2018) + 18 mg/kg IND). AN alone had no significant effect on healthy mice (Zhang et al., 2019; Lin et al, 2021) and the dose for this study was determined
based on the results reported by us previously (Liu et al., 2021; Gao et al., 2018; ). Mice in IND, AN and OME groups were intragastrically given the corresponding dose of the different agents as described above once daily for 14 days, while those in CON group were administered with an equal volume of the solvent (CMC-Na) in the same way. Before the experiment, mice in each group were forbidden to eat for 24 hours and to drink for 4 hours. On the first hour after the last treatment, mice in CON group were administered with an equal volume of normal saline by gavage, and those in the other groups with 18 mg/kg IND in the same way (dissolved in 5% NaHCO₃) for building a mouse model of acute gastric injury. Six hours after the animal modeling, enough sodium pentobarbital (50 mg/kg) was intraperitoneally given to the mice for the euthanasia of mice, and then the mice gastric tissues were taken and stored in a refrigerator at -20 °C. Fig. 1 shows the experimental protocol.

**Measurement of the body weight of mice**

The body weights of all mice were measured daily for 14 days.

**Gross observation of gastric tissue, calculation of gastric ulcer index and ulcer inhibition rate**

Gastric ulcer index (GUI) was evaluated according to gastric injury evaluation criteria provided by Guth and Nwafor et al (Guth et al., 1979; Nwafor et al., 2000). The scores were calculated according to the length of ulcer or erosion, i.e. no damage was scored as 0 point, minute hemorrhagic lesions as 1 point, lesions less than 2 mm as 2 points, those from 2 to 3 mm as 3 points, those from 3 to 4 mm as 4 points, and those more than 4 mm as 5 points. The score of erosion with a width greater than 1 mm was multiplied by 2, and the sum of the total gastric scores was GUI. The ulcer inhibition rate (UIR) was calculated according to GUI, and formula is as follows: 

\[
UIR (%) = \frac{(\text{control lesion area} - \text{sample lesion area})}{\text{control lesion area}} \times 100.
\]

UIR was
used to estimate the gastric mucosal injury.

**Measurement of pH value and gastric juice volume**

Gastric content was collected from the pylorus of the stomach. The gastric mucosa was washed with 2 mL distilled water, and the volume and pH value of gastric juice were measured immediately.

**Histopathological evaluation of gastric injury.**

The gastric tissue was firstly fixed in 10% formaldehyde solution. Two days later, the tissue was dehydrated in gradient alcohol and embedded with paraffin, then cut into slices of a thickness of 4 μm, and the slices were stained with H&E (hematoxylin and eosin), or PAS (periodic acid-Schiff) for evaluating the synthesis of gastric mucus glycoprotein. Histopathological changes in the gastric tissue were examined under an optical microscope.

**Examination of biochemical markers related to oxidative stress and inflammation.**

The gastric tissue pieces were mixed with precooled PBS in a ratio of 1:9 for preparing the gastric tissue homogenate. The homogenate supernatant was obtained by centrifuging the homogenate at 3500 rpm and 4 °C for 15 min, and then frozen at -80 °C. GSH-Px and SOD activities, and ROS, MDA, IL-1β, TNF-α, IL-6, and PGE2 contents in the supernatant were measured as procedures provided by the kit manufacturers.

**Western blot analysis**

Gastric tissues from 3 mice in each group were added to the lysis buffer, which was performed on ice for 1 h. The lysis buffer contains phosphatase inhibitor and protease inhibitor. Then, the supernatant of gastric tissue-lysis buffer solution was obtained by centrifuging it at 12000 rpm for 10 min. BCA method was used to determine the total protein in the supernatant. The sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on COX-1, COX-2, p-Nrf2,
Nrf2, Keap1, HO-1, NF-κB p65, p-NF-κB p65, MMP-9, Bcl2, Bax, Cleaved Caspase3, and GAPDH proteins was performed. The proteins were transferred onto PVDF membranes for 2 h. The membranes were washed with Tween/Tris-buffered salt solution (TBST) for 5 min, and then blocked with a blocking TBST buffer with 5% skimmed milk at room temperature for 2 h. The blocking buffer was discarded, then the membranes were incubated with the primary antibodies of COX-1, COX-2, Nrf2, p-Nrf2, Keap1, HO-1, MMP-9, Bcl2, NF-κB p65, p-NF-κB p65, Bax and Cleaved Caspase3 (1:1000) at 4 ℃ overnight. Next day, the membranes were washed with TBST 3 times, 10 min each, then incubated with the secondary antibody (1:5000) at room temperature for 1 h, and washed with TBST in the same way again. An enhanced chemiluminescence (ECL) developer was used for the development of the images.

**Examination of the nuclear translocation of NF-κB p65 protein by immunofluorescence assay**

Immunofluorescence analysis was used to confirm that AN could reverse the NF-κB p65 translocation from cytoplasm to nucleus induced by indomethacin. Firstly, paraffin slices of gastric tissue were routinely dewaxed and hydrated, and then placed in ethylenediamine tetraacetic acid (EDTA) for the recovery of antigen. The slices were washed with PBS 3 times, 3 min each, then blocked for 1 h at room temperature, and incubated with the primary antibody of NF-κB p65 (1:500) overnight in a humidity cabinet at 4 ℃. Next day, the sections were rinsed again with PBS 3 times, 3 min each, and incubated with the secondary antibody for 1 h at room temperature. Subsequently, 4, 6-diamino-2-phenylindole (DAPI) was used to stain the nuclei, and a fluorescence microscope was applied to photograph the images. Finally, Image Pro Plus 6.0 software was used to analyze the nuclear translocation of NF-κB p65.

**Observation of the apoptosis of mouse gastric tissue cells**

The pathological slices of mouse stomach were dewaxed and hydrated, and rinsed with PBS
twice on a shaking table for 3 min each time, a total of three times. The slices were stained with Hoechst 33258 staining solution for 5 min, then rinsed with PBS, and finally the glass slides were covered using an anti-quenching mounting medium. A fluorescence microscope was used for examining the sections. The cell membranes of normal cells were intact, with a uniform blue staining, and the nucleus of apoptotic cells was concentrated, with a bright blue staining. Five visual fields were randomly selected in each section, the normal cell number and the apoptotic cell number were counted, respectively, and the apoptosis rate was calculated as: apoptosis rate = apoptotic cell number/total cell number × 100%.

**Statistical analysis**

SPSS20.0 software was used to analyze experimental data, and the data were expressed as mean ± SD. Differences among multiple groups were compared by one-way analysis of variance. Differences between groups were compared by Tukey test. A value of $P < 0.05$ was taken as a significant difference in statistics.
Results

Effects of AN on gastric ulcer index, gastric ulcer inhibition rate, gastric juice pH and gastric juice volume

The weights of all mice were increasing, but without significant difference in statistics among the groups (Fig. 2A).

As shown in Fig. 2B (a-d), in CON group, the gastric mucosa of mice was smooth and flat, without ulcer, and the overall color was light pink; in IND group, a large number of ulcer spots and abnormal changes such as slight congestion and erosion were visible; however, in AN and OME groups, ulcer spot, congestion, and erosion, were significantly alleviated.

The calculation of GUI and UIR is a common method to estimate the gastric injury degree. As shown in Fig. 2C, the GUI in IND group was 6.875 ± 0.99; in comparison to that in IND group, the GUI decreased significantly in AN group (2.625 ± 0.52) and OME group (1.812 ± 0.53), respectively, and the UIR was also significantly reduced in AN group (60.58%) and OME (73.08%), respectively.

As shown in Fig. 2D, compared with CON group, the gastric juice volume was significantly increased in IND group (0.625 ± 0.07), while in comparison to IND group, the gastric juice volume in AN (0.438 ± 0.08) and OME (0.319 ± 0.07) groups were significantly decreased, respectively. Compared with that in CON group, the pH value of gastric juice was significantly decreased in IND group (2.22 ± 0.38), but was significantly increased in AN (2.625 ± 0.37) and OME (2.918 ± 0.26) groups, indicating that AN could alleviate the gastric ulcer induced by IND in mice by reducing the gastric juice secretion and acidity.

Effects of AN on histopathological changes of gastric injury

As shown in Fig. 3A (a-d), in CON group the gastric mucosa was intact, without obvious
bleeding and inflammatory cell infiltration; in IND group, a severe epithelial cell defect of the mucosa and an infiltration of inflammatory cells could be found; in contrast, in AN and OME groups, the above pathological changes were significantly alleviated, the gastric mucosa was relatively intact, the epithelial cell defect and inflammatory cell infiltration became less and tended to be normal, suggesting that AN could alleviate the acute gastric ulcer induced by IND.

The PAS staining results in Fig. 3B (e-h) showed that in CON group, a completely positive PAS staining could be found; in IND group, the mucosal epithelium of gastric tissue was damaged, the PAS positive substance disappeared, and the mucin injury was serious; while in AN and OME groups, the disappearance of PAS positive substances was lessened, and the mucin injury was alleviated.

**Effects of AN on COX-1, COX-2, and PGE2 expressions in gastric tissue.**

As shown in Fig. 4, in the model group, the contents of COX-1, COX-2, and PGE2 proteins in the gastric tissue of mice decreased ($P < 0.05$), but increased in AN and OME groups ($P < 0.01$).

**Effects of AN on oxidative stress-related factors in gastric tissue.**

The acute gastric injury induced by IND is associated with oxidative stress. Therefore, in this study, SOD and GSH-Px activities, and ROS and MDA contents in the gastric tissue of mice were measured. Compared with those in CON group, SOD and GSH-Px activities in the gastric tissue of mice were decreased ($P < 0.05$ or $P < 0.01$), and the ROS and MDA contents were increased ($P < 0.05$ or $P < 0.01$) in IND group; however, compared with those in IND group, SOD and GSH-Px activities in the gastric tissue of mice were increased ($P < 0.05$ or $P < 0.01$), and the ROS and MDA contents were decreased ($P < 0.05$ or $P < 0.01$) in AN group and OME group (Fig. 5), indicating that AN can alleviate the IND-induced oxidative damage to the gastric tissue by enhancing the
body’s antioxidant capacity.

**Effects of AN on inflammatory factors in gastric tissue of mice.**

In comparison to those in CON group, TNF-α, IL-6, and IL-1β levels and MPO activities in the gastric tissue of mice increased in IND group ($P < 0.05$ or $P < 0.01$), and those decreased ($P < 0.05$ or $P < 0.01$) in AN and OME groups (Fig. 6).

**Effects of AN on the expression of Nrf2/ARE pathway-related proteins in the gastric tissue of mice.**

Nrf2/ARE signaling pathway is involved in oxidative stress response, and the expression of Nrf2/ARE signal pathway-related proteins in the gastric tissue of mice was detected. Compared with that in CON group, the expression of Keap1 protein increased ($P < 0.05$), and the ratio of p-Nrf2/Nrf2 and the expression of HO-1 protein decreased ($P < 0.05$ or $P < 0.01$) in IND group; in AN and OME group, the ratio of p-Nrf2/Nrf2 and the expression of HO-1 protein increased ($P < 0.05$ or $P < 0.01$), and the expression of Keap1 protein decreased ($P < 0.05$) compared with that in IND group (Fig. 7), suggesting that AN may play an antioxidant role in mitigating the acute gastric injury induced by IND through activating the Nrf2/ARE signaling pathway in mice.

**Effects of AN on the expression levels of NF-κB signaling pathway-related proteins in the gastric tissue of mice.**

NF-κB signaling pathway is a typical inflammatory response regulation pathway in the body, and the changes in the NF-κB p65 nuclear translocation and its downstream signal molecules (MMP-9) can be used to evaluate an inflammatory response. In the present study, Western blot was used to analyze NF-κB p65, p-NF-κB p65, and MMP-9 protein expressions in the gastric tissue of mice. Compared with those in CON group, the p-NF-κB p65/NF-κB p65 ratio and the MMP-9 protein expression in the gastric tissue of mice was increased in IND group ($P < 0.05$) (Fig. 8 A and
B), while those in AN and OME groups decreased ($P < 0.05$). Furthermore, the effect of AN on the NF-κB p65 nuclear translocation in the gastric tissue (Fig. 8C) was also confirmed by immunofluorescence, showing that AN inhibited the NF-κB p65 nuclear translocation and the MMP-9 expression to play an anti-inflammatory role, which may be considered as a main factor to alleviate the gastric mucosal injury of mice.

**Effects of AN on the apoptosis of gastric tissue cells of mice**

One of the main characteristics of IND-induced gastric injury is apoptosis in the gastric tissue in mice. We used Hoechst staining for the observation of the apoptosis of mouse gastric tissue. It was found that in CON group, the gastric tissue cells were complete in morphology and evenly stained, and the chromatin in the nucleus showed a light blue fluorescence; but in IND group, the chromatin condensation in the nucleus increased, and the chromatin showed with a flaky bright blue fluorescence, and the apoptosis rate increased ($P < 0.01$); however, in AN and OME group, the bright blue chromatin in the gastric tissue of mice decreased significantly, and the apoptosis rate also decreased significantly ($P < 0.01$) (Fig. 9 A and B).

Caspase family is the mediator and executor of apoptosis, and Caspase3 is located at the downstream of the orderly cascade of apoptosis and the convergence point of a variety of apoptosis stimulation signals, representing the degree of apoptosis. Bcl2 can bind to the pro-apoptotic protein Bax to inhibit the apoptosis (El-Lekawy et al., 2019; Hegab et al., 2018). Western blot was used to detect the expression of the above proteins in gastric tissue in this study. Compared with that in CON group, the Bcl2/Bax ratio decreased ($P < 0.01$) and the expression of cleaved Caspase3 increased ($P < 0.01$) in IND group; however, in AN and OME group, the Bcl2/Bax ratio increased ($P < 0.01$) and the cleaved Caspase3 expression decreased ($P < 0.05$ or $P < 0.01$) (Fig. 9 C and D), suggesting that AN may mitigate the acute gastric injury induced by IND through inhibiting the apoptosis.
Discussion

Mice were administered with indomethacin (IND) by gavage for establishing a mouse gastric injury model. The preventive gavage of Anwulignan (AN) for 14 days could protect against gastric injury by reducing the ulcer index of gastric mucosa, increasing the ulcer inhibition rate, decreasing the gastric juice secretion, and lowering the gastric juice pH value. Moreover, above effects were also verified by the gross observation and histopathological examination of gastric tissue.

Studies have shown that drug gastrointestinal adverse reactions often happen in the therapy of NSAIDs, including IND, but less in selective COX-2 inhibitors, such as celecoxib. Although in many cases, COX-1 can play a protective role in the gastric tissue through the regulation of vasoconstriction, gastric mucosal blood flow, and gastric juice secretion, and COX-2 can be described as an inflammatory mediator (Chatterjee et al., 2012), it is undeniable that COX-1 and COX-2 are inhibited simultaneously after the administration of IND (Wallace et al., 2000), and this inhibition of cyclooxygenase activity and prostaglandin E2 (PGE2) synthesis in gastric tissue is believed to be one of the main reasons for the gastric injury induced by IND (Yadav et al., 2012; Suleyman et al., 2010). PGE2, a metabolite of arachidonic, participates in maintaining gastric mucosal defense and various gastrointestinal functions (Yildirim et al., 2015). After the administration of IND, the gastric tissue was damaged obviously, COX-1 and COX-2 activities, and PGE2 contents in the gastric tissue decreased significantly. However, AN and OME (omeprazole) could increase all of them, thus playing gastric protective effects.

In addition, NSAIDs can also cause direct damage to gastrointestinal mucosa. Studies have shown that when the body is stimulated by NSAIDs, a lot of reactive oxygen species (ROS) substances will be produced in the gastrointestinal tract, leading to the tissue oxidative damage to
affect the cell metabolism (Maziero et al., 2021). MDA is often taken as an indicator to reflect oxidative damage in tissues, since it is the final product of lipid peroxidation (Ibrahim et al., 2015). The body has a strong antioxidant system, such as GSH-Px and SOD, which can remove oxygen free radicals to keep free radicals at a low level (Qiu et al., 2020). In this study, IND could cause severe damages to gastric mucosa, increasing the content of ROS and MDA, and decreasing the activity of GSH-Px and SOD, consistent with previous results on the IND-induced gastric injury (Barboza et al., 2018; Ugan et al., 2020). However, AN could improve these parameters, suggesting that AN may have a strong antioxidant activity to alleviate the IND-induced gastric injury in mice. Nrf2, a main antioxidative stress transcription factor in cells, can bind to Keap1 in cytoplasm. In an oxidative stress injury, Keap1 is degraded to release Nrf2, which can be phosphorylated, then migrate into the nucleus, bind to ARE, induce the expression of HO-1, and finally enhance GSH-Px and SOD activities (Carrasco-Pozo et al., 2016; Arafa et al., 2017). HO-1, as a stress response protein, can reduce the sensitivity of gastrointestinal cells to oxidative damage (Allam et al., 2017). Therefore, we speculated that AN might protect the gastric mucosa through its regulation of Nrf2/ARE signaling pathway. To confirm this hypothesis, we detected the Nrf2/ARE signaling pathway-related protein expressions and found that AN could down-regulate the Keap1 expression and up-regulate the p-Nrf2 (S40), Nrf2, and HO-1 expressions in the gastric tissue. Moreover, in our previous study, we found that AN has similar effects on the ischemia-reperfused intestinal, liver, brain, and spleen tissues in D-galactose-treated mice, suggesting that the Nrf2/ARE signaling pathway activation may be the common antioxidant mechanism of AN.

Inflammation and oxidative stress are closely related in some pathological processes, and they both exist at the same time under many pathological conditions (Fagundes et al., 2021). A continuous inflammation can aggravate the gastric mucosa injury, so that to control the content of
proinflammatory factors can effectively inhibit and prevent some gastric lesions (Pineda-Peña et al., 2020). NF-κB, a key transcription factor linking oxidative stress and inflammatory response, normally exists in the cytoplasm in an inactive form (Ko et al., 2020) and can induce the expression of a variety of pro-inflammatory factors. When a large amount of ROS is produced in the gastrointestinal tract, NF-κB is activated, and then NF-κB p65 can be phosphorylated to enter the nucleus and interact with DNA, inducing the expression of TNF-α, IL-6 and IL-1β, and then inflammation (Akanda et al., 2018). TNF-α can increase the neutrophil migration to gastric mucosa and the oxygen free radical release to delay the healing of gastric ulcer. TNF-α at a high level can also facilitate the secretion of some cytokines IL-6 and IL-1β, which, in turn, further aggravate the inflammatory response (Wang et al., 2018). A lower MPO (a specific marker of severe tissue inflammation) level indicates a greater anti-inflammatory activity in gastric tissue. (Rozza et al., 2014). In this study, IND increased the ratio of p-NF-κB p65 to NF-κB p65, the nuclear translocation of NF-κB-p65, the TNF-α, IL-6 and IL-1β levels, and the MPO activity significantly in the gastric tissue of mice, while AN reversed the above effects of IND, suggesting that AN exerts this anti-inflammatory effect by activating the NF-κB signaling pathway. NF-κB signaling pathway also promotes the up-regulation of extracellular matrix regulators, such as matrix metalloproteinase-9 (MMP-9). NF-κB, as one of the main gene transcripts after activation, can enhance a series of inflammatory reactions in the body (Mahmoud et al., 2021), while MMP-9 is an important member in metalloproteinase family, also involved in the extracellular matrix remodeling imbalance in the gastric ulcer formation, and the imbalance of MMP-9 will lead to the insufficient angiogenesis and poor healing of gastroduodenal ulcer (Yadav et al., 2017). Therefore, the progression of gastric ulcer is usually related to the increased MMP-9 in the gastric tissue (Park et al., 2017). It was found in our study that AN could reduce the MMP-9 expression in the gastric tissue of mice with gastric ulcer, and then exert its anti-inflammatory and gastric protective
roles.

Besides inflammation and oxidative stress, apoptosis also participates in the occurrence and development of IND-induced gastric ulcer (Ahmed et al., 2021). In fact, ROS and TNF-α are related to the activation of apoptosis pathway. TNF-α binds to tumor necrosis factor receptor-1 (TNFR-1) to stimulate the exogenous apoptotic pathway (Chen et al., 2016), and ROS can activate the mitochondrial apoptosis pathway. Mitochondrial apoptosis pathway is activated by some pro-apoptotic proteins, Bax for example, and inhibited by some anti-apoptotic proteins, Bcl-2 for example. Bax can stimulate the release of cytochrome C and then activate caspase3, while Bcl-2 can bind and neutralize mitochondrial pro-apoptotic proteins to regulate the apoptosis pathway (Correia et al., 2015). Therefore, the ratio of pro-apoptotic proteins/anti-apoptotic proteins controls the fate of cells (Arab et al., 2015; Badr et al., 2019). The apoptosis of cells mainly comes from the imbalance of Bcl2/Bax in gastric ulcer animal models. This study showed that the ratio of Bcl2/Bax decreased significantly, and the expression of cleaved Caspase3 protein and apoptosis index increased significantly in the gastric tissue of mice with gastric ulcer induced by IND, while AN could increase the Bcl2/Bax ratio, decrease the expression of cleaved caspase 3 protein and the apoptosis index, confirming that AN can also improve the gastric injury induced by IND by its anti-apoptosis in mice.

The gastric protective effect of AN was considered mainly due to its regulating the Nrf2/ARE signaling pathway. In order to confirm this hypothesis, Nrf2, NF-κB signaling pathway and key proteins of apoptosis in stomach tissues were detected by Western Blot.

Conclusion

In conclusion, AN could alleviate the indomethacin-induced gastric mucosal injury by its antioxidant, anti-inflammatory, and anti-apoptosis in mice. Further mechanisms of actions could be related to its regulations of Nrf2/ARE pathway, NF-κB signaling pathway, and apoptosis-related
protein expressions (Fig. 10).

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Authorship Contributions.

Participated in research design: He Li, Shu Jing, Jia Wei Liu.

Conducted experiments: Jia Wei Liu, Jia Hui Fang, Jun Xiong Zhang, Zhi Hong Zhang.

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

Wrote or contributed to the writing of the manuscript: Jia Wei Liu, He Li, and Shu Jing.

All authors critically reviewed the manuscript and approved its final version.
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14.


Footnotes

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Conflicts of interest

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

Figure. 1 Experimental protocol

Figure. 2 Effects of AN on body weight, gross observation of stomach, gastric ulcer index, ulcer inhibition rate, pH value of gastric juice and gastric juice volume in mice (mean ± SD, n = 8). A. Body weight curve; B. Gross observation of stomach; a: CON group; b: IND group; c: AN group; d: OME group; C. Gastric ulcer index and ulcer inhibition rate; D. PH value and gastric juice volume. Compared with CON group, * P < 0.05, ** P < 0.01; Compared with IND group, # P < 0.05, ## P < 0.01.

Figure. 3 Effects of AN on histopathological changes in the gastric injury of mice (mean ± SD, n = 3). A. H&E staining (×200); a: CON group; b: IND group; c: AN group; d: OME group; B. PAS staining (×200); e: CON group; f: IND group; g: AN group; h: OME group.

Figure. 4 Effects of AN on the expression of COX-1, COX-2 and PGE2 proteins in the gastric tissue of mice (mean ± SD, n=3). A. COX-1 and COX-2 expressions; B. PGE2 contents. Compared with CON group, * P < 0.05, ** P < 0.01; Compared with IND group, # P < 0.05, ## P < 0.01.

Figure. 5 Effects of AN on oxidative stress-related factors in the gastric tissue of mice (mean ± SD, n = 8). A. ROS content; B. SOD activity; C. MDA content; D. GSH-Px activity; Compared with CON group, * P < 0.05, ** P < 0.01; Compared with IND group, # P < 0.05, ## P < 0.01.

Figure. 6 Effects of AN on inflammatory factors in the gastric tissue of mice (mean ± SD, n = 8). A. TNF-α content; B. IL-6 content; C. IL-1β content; D. MPO activity. Compared with CON group, * P < 0.05, ** P < 0.01; Compared with IND group, # P < 0.05, ## P < 0.01.

Figure. 7 Effects of AN on the expression of Nrf2/ARE pathway-related proteins in the
gastric tissue of mice (mean ± SD, n = 3). A. Electrophoretogram of nuclear factor-E2-related factor 2 (Nrf2), p-Nrf2(S40), Keap1, and HO-1 proteins; B. Relative expressions of p-Nrf2(S40), Nrf2, Keap1, and HO-1 proteins. Compared with CON group, * P < 0.05, ** P < 0.01; Compared with IND group, # P < 0.05, ## P < 0.01.

Figure. 8 Effects of AN on the expression levels of NF-κB signaling pathway-related proteins in the gastric tissue of mice (mean ± SD, n=3). A. Phosphorylated nuclear kappa-B (p-NF-κB) p65, nuclear kappa-B (NF-κB) p65 and matrix metalloproteinase-9 protein levels; B. Relative expressions of p-NF-kB p65/NF-kB p65 and MMP-9; C. Immunofluorescence analysis for the effect of AN on the nuclear translocation of NF-κB. Compared with CON group, * P < 0.05, ** P < 0.01; Compared with IND group, # P < 0.05, ## P < 0.01.

Figure. 9 Effects of AN on the apoptosis of gastric cells in mice (mean ± SD, n = 3). A. Apoptosis of gastric cells detected by Hoechst staining; B. Apoptosis rate of gastric tissue; C. Bcl2, Bax and Cleaved Caspase3 protein levels; D. Relative expressions of Bcl2/Bax and Cleaved Caspase3. Compared with CON group, * P < 0.05, ** P < 0.01; Compared with IND group, # P < 0.05, ## P < 0.01.

Figure. 10 Mechanism of protective effect of AN against the gastric ulcer in mice.
Figure 1
Figure 2

A

Body weight (g)

Experimental time (Day)

CON

AN

IND

OME

B

a

b

c

d

C

Ulcer index

Ulcer inhibitory

**

***

IND (18 mg/kg)  +  +  +  +  +  +  +  +
AN (4 mg/kg)    +  +  +  +  +  +  +  +
OME (20 mg/kg)  +  +  +  +  +  +  +  +

D

pH

Volume

**

***

IND (18 mg/kg)  +  +  +  +  +  +  +  +
AN (4 mg/kg)    +  +  +  +  +  +  +  +
OME (20 mg/kg)  +  +  +  +  +  +  +  +

Gastric juice volume (ml)
Figure 4

A

<table>
<thead>
<tr>
<th></th>
<th>COX-1</th>
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<th>GAPDH</th>
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<td>+</td>
</tr>
<tr>
<td>AN (4 mg/kg)</td>
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<td>–</td>
<td>+</td>
</tr>
<tr>
<td>OME (20 mg/kg)</td>
<td>–</td>
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B

![Graph showing PGE2 (pg/ml) levels for different treatments](chart.png)
**Figure 6**

**A**

```
<table>
<thead>
<tr>
<th>Treatment</th>
<th>TNF-α (pg/ml)</th>
</tr>
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<tbody>
<tr>
<td>IND (18 mg/kg)</td>
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<td>OME (20 mg/kg)</td>
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<table>
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<td>AN (4 mg/kg)</td>
<td>-</td>
</tr>
<tr>
<td>OME (20 mg/kg)</td>
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**B**

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**C**

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**D**

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<td>AN (4 mg/kg)</td>
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<td>OME (20 mg/kg)</td>
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Figure 7

A

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<th>HO-1</th>
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<tbody>
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<td>IND (18 mg/kg)</td>
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<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AN (4 mg/kg)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
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</tr>
<tr>
<td>OME (20 mg/kg)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

B

- P-Nrf2/Nrf2
- Keap-1/GAPDH
- HO-1/GAPDH

Relative protein expression

- -
- +
- +
- +

Legend (
- 
+ 
**
***
###
##

Downloaded from jpet.aspetjournals.org at ASPET Journals on September 4, 2022
Figure 9

A

Hoechst ×200

(a) (b) (c) (d)

B

Apoptosis index (%)

IND (18 mg/kg) = + + + +
AN (4 mg/kg) = + + + +
OME (20 mg/kg) = + + + +

C

Bcl2

IND (18 mg/kg) = + + + +
AN (4 mg/kg) = + + + +
OME (20 mg/kg) = + + + +

D

Relative protein expression

IND (18 mg/kg) = + + + +
AN (4 mg/kg) = + + + +
OME (20 mg/kg) = + + + +
Figure 10

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