Protective Effect of Anwulignan on Gastric Injury Induced by Indomethacin in Mice

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ABSTRACT

Anwulignan (AN) 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, gastric ulcer index (GUI), ulcer inhibition rate (UIR), gastric juice volume (GJV) and pH value. Chemical colorimetry, immunofluorescence, ELISA, and Western blot were used to detect related factors in the gastric tissue. The results showed that AN reduced the GUI, increased the GJV, and increased the gastric pH value. AN significantly increased cyclooxygenase-1, cyclooxygenase-2, and prostaglandin E2 expression levels in the gastric tissue, activated B-cell lymphoma 2, to play an anti-inflammatory role. AN inhibited the downstream signals B-cell lymphoma 2-associated x protein and cleaved caspase-3 in gastric tissue, and activated B-cell lymphoma 2, to play an antiapoptotic 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 antiapoptotic effect.

SIGNIFICANCE STATEMENT

Anwulignan (AN) significantly reduced the indomethacin-induced gastric injury in mice, and its antioxidation, anti-inflammation, and antiapoptosis were considered to be involved 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 (Tarnawski et al., 2014). With the continuous effect of some invasive factors, the gastric mucosal defense function will be weakened, and the mucosa eventually forms an ulcer (Zhang et al., 2020). The increased application of nonsteroidal anti-inflammatory drugs (NSAIDs) has caused more gastrointestinal adverse reactions, which has attracted the extensive attention of researchers. Although NSAIDs have good anti-inflammatory effects, its long-term 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 prostaglandin E2 (PGE2) synthesis, affect the mucosa regeneration, and induce the gastric mucosal inflammation and ulcer (Sostres et al., 2013; Fang et al., 2019). NSAIDs can also directly damage gastrointestinal mucosa (Kuczyńska and Nieradko-Iwanicka, 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 an inflammatory response. Meanwhile, these oxygen free radicals also lead to apoptosis of gastric mucosal cells by lipid peroxidation, protein denaturation, and DNA damage. Therefore, the key ways to protect gastric mucosa are to reduce oxidative stress, inflammation, and apoptosis in the gastric tissue. Currently, H⁺-K⁺-ATPase inhibitors including omeprazole (OME) 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); therefore, finding 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 (Hatware et al., 2018). Schisandra, first recorded in Shennong Herbal Classic, is the dry and mature fruit of Schisandra sphenanthera fructus. It has been widely applied as medicine, a health supplement, food, and beverage (Panossian and Wikman, 2008; Li, He 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). Anwulignan (AN) is a lignan and representative monomer-active component in Schisandra sphenanthera fructus. In our previous studies, we found that AN protected hydrochloric acid/ethanol-induced gastric ulcer in mice, and its mechanism was connected to its antioxidant and anti-inflammation (Liu et al., 2021). AN also showed antioxidation, anti-inflammation, and antiapoptosis effects in D-galactose-induced aging and overtired mouse models (Li et al., 2020; Zhang et al., 2020). However, up to now, there is no report available about the effect of AN on NSAID-induced gastric injury. Therefore, we exploited an IND-induced gastric injury model of mice (of NSAIDs) to examine the protective effect of AN against it. We hope that this study will promote AN as a new candidate for antiulcer drugs and health foods.

Materials and Methods

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

Reagents. AN (Sichuan Weikeqi Biologic Technology Co., Ltd., Chengdu, China); OME (AstraZeneca Pharmaceutical Co., Ltd., UK); IND (Shanghai Jinhubuan Lankao Pharmaceutical Co., Ltd., Shanghai, China); superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and malondialdehyde (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); reactive oxygen species (ROS), myeloperoxidase (MPO), and PGE2 test kits (Shanghai Enzyme-Linked Biotechnology Co., Ltd., Shanghai, China); tumor necrosis factor-α (TNF-α), interleukin (IL)-6, and IL-1β test kits (ABclonal Biotechnology Co., Ltd., USA); cyclooxygenase (COX-1, COX-2), phosphorylation Nrf2 (p-Nrf2), S40, Nrf2, Kelch-like ECH-associated protein 1 (Keap1), heme oxygenase-1 (HO-1), nuclear factor-κ gene binding (NF-κB) p-65, p-NF-κB (phosphorylation nuclear factor-κ gene binding) p-65, matrix metalloproteinase-9 (MMP-9), B-cell lymphoma 2 (Bcl2), Bcl2-associated x protein (Bax), cleaved caspase-3, and glyceraldehyde-3-phosphate dehydrogenase antibodies (ABclonal Biotechnology Co., Ltd., USA).
NF-κB p65, p-NF-κB p65, MMP-9, Bcl2, Bax, cleaved caspase-3, and glyceraldehyde-3-phosphate dehydrogenase proteins was performed. The proteins were transferred onto polyvinylidene difluoride membranes for 2 hours. The membranes were washed with Tris-buffered saline/Tween 20 (TBST) for 5 minutes and then blocked with a blocking TBST buffer with 5% skimmed milk at room temperature for 2 hours. The blocking buffer was discarded, and then the membranes were incubated with the primary antibodies of COX-1, COX-2, Nrf2, p-NF-κB p65, p-NF-κB p65, Bax, and cleaved caspase-3 (1:1000) at 4°C overnight. The next day, the membranes were washed with TBST buffer for 5 minutes each time, and incubated with the secondary antibody (1:5000) at 4°C for 2 hours. The blocking buffer was discarded, and then the membranes were washed with Tris-buffered saline/Tween 20 (TBST) for 5 minutes each time, and incubated with the secondary antibody (1:5000) at room temperature for 1 hour and washed again with TBST in the same way. An enhanced chemiluminescence developer was used for the development of the images.

Statistical Analysis. The SPSS20.0 software was used to analyze the experimental data, and the data were expressed as mean ± S.D. Differences between multiple groups were compared by ANOVA. Differences between groups were compared by a Tukey test. A value of P < 0.05 was taken as a significant difference in statistics.

Results

Effects of AN on GUI, Gastric UIR, Gastric Juice pH, and Gastric Juice Volume. The weights of all mice increased, but without significant differences in statistics between the groups (Fig. 2A).

As shown in Fig. 2B, in CON group, the gastric mucosa of the 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 the AN and OME groups, ulcer spots, congestion, and erosion were significantly alleviated.

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

As shown in Fig. 2D, compared with the CON group, the gastric juice volume was significantly increased in the IND group (0.625 ± 0.07). In comparison with the IND group, the gastric juice volume in the AN (0.438 ± 0.08) and OME (0.319 ± 0.07) groups were significantly decreased. Compared with that in CON group, the pH value of gastric juice was significantly decreased in the 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 can 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, in the CON group, the gastric mucosa was intact, without obvious bleeding and inflammatory cell infiltration. In the IND group, a severe

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epithelial cell defect of the mucosa and an infiltration of inflammatory cells was found. In contrast, in the AN and OME groups, the previously described pathologic changes were significantly alleviated. The gastric mucosa was relatively intact, and the epithelial cell defect and inflammatory cell infiltration became less and tended to be normal, suggesting that AN can alleviate the acute gastric ulcer induced by IND.

PAS staining results in Fig. 3B showed that in the CON group, a completely positive PAS staining was found. In the IND group, the mucosal epithelium of gastric tissue was damaged, the PAS-positive substance disappeared, and the mucin injury was serious. Finally, in the 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, the contents of COX-1, COX-2, and PGE2 proteins in the gastric tissue of mice decreased ($P < 0.05$) in the model group but increased in the 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 the 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 the IND group. However, compared with those in the 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 the AN group and OME groups (Fig. 5), indicating that AN can alleviate 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 with those in the CON group, TNF-$\alpha$, IL-6, and IL-1$\beta$ levels and MPO activities in the gastric tissue of mice increased in the IND group ($P < 0.05$ or $P < 0.01$) and 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.** The Nrf2/ARE signaling pathway is involved in the 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 the 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 the IND group. In the AN and OME groups, 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 an acute gastric injury induced by IND in mice by activating the Nrf2/ARE signaling pathway.

**Effects of AN on the Expression Levels of NF-κB Signaling Pathway–Related Proteins in the Gastric Tissue of Mice.** The NF-κB signaling pathway is a typical inflammatory response regulation pathway in the body, and changes in the NF-κB p65 nuclear translocation and its downstream signal molecules (MMP-9) can be used to evaluate an inflammatory response. In this 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 the CON group, the p-NF-κB p65/NF-κB p65 ratio and MMP-9 protein expression in the gastric tissue of mice was increased in the IND group ($P < 0.05$) (Fig. 8, A and B), whereas those in the AN
and OME groups decreased ($P < 0.05$). Furthermore, the effect of AN on NF-κB p65 nuclear translocation in gastric tissue (Fig. 8C) was also confirmed by immunofluorescence, showing that AN inhibited NF-κB p65 nuclear translocation and 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 the CON group, gastric tissue cells were complete in morphology and evenly stained, and the chromatin in the nucleus showed a light blue fluorescence. However, in the IND group, chromatin condensation in the nucleus increased, and the chromatin showed a flaky bright blue fluorescence, and the apoptosis rate increased ($P < 0.01$). In contrast, in the AN and OME groups, the bright blue chromatin in the gastric tissue of mice decreased significantly, as did the apoptosis rate ($P < 0.01$) (Fig. 9, A and B).

The caspase family is the mediator and executor of apoptosis, and caspase-3 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 proapoptotic protein Bax to inhibit the apoptosis (Hegab et al., 2018; El-Lekawy et al., 2019). In this study, Western blot was used to detect the expression of the previously noted proteins in gastric tissue. Compared with that in the CON group, the Bcl2/Bax ratio decreased...
and the expression of cleaved caspase-3 increased ($P < 0.01$) in the IND group. However, in the AN and OME groups, the Bcl2/Bax ratio increased ($P < 0.01$) and cleaved caspase-3 expression decreased ($P < 0.05$ or $P < 0.01$) (Fig. 9, C and D), suggesting that AN may mitigate an acute gastric injury induced by IND by inhibiting apoptosis.

**Discussion**

Mice were administered IND by gavage to establish a mouse gastric injury model. The preventive gavage of AN for 14 days can protect against gastric injury by reducing the ulcer index of gastric mucosa, increasing the UIR, decreasing the gastric juice secretion, and lowering the gastric juice pH value. Moreover, these effects were 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). This inhibition of cyclooxygenase activity and

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*Fig. 8. Effects of AN on the expression levels of NF-κB signaling pathway-related proteins in the gastric tissue of mice (mean ± S.D., n = 3). (A) p-NF-κB p65, NF-κB p65, and MMP-9 protein levels; B. Relative expressions of p-NF-κB p65/NF-κB 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, #$P < 0.05$; ##$P < 0.01$. 

(P < 0.01), and the expression of cleaved caspase-3 increased ($P < 0.01$) in the IND group. However, in the AN and OME groups, the Bcl2/Bax ratio increased ($P < 0.01$) and cleaved caspase-3 expression decreased ($P < 0.05$ or $P < 0.01$) (Fig. 9, C and D), suggesting that AN may mitigate an acute gastric injury induced by IND by inhibiting apoptosis.
PGE2 synthesis in gastric tissue is believed to be one of the main reasons for gastric injury induced by IND (Suleyman et al., 2010; Yadav et al., 2012). 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 obviously damaged, and COX-1 and COX-2 activities and PGE2 contents in gastric tissue decreased significantly. However, AN and OME can increase all of them, thus playing gastric protective effects.

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

**Fig. 10.** Mechanism of protective effect of AN against the gastric ulcer in mice.
In addition, NSAIDs can cause direct damage to gastrointestinal mucosa. Studies have shown that when the body is stimulated by NSAIDs, a lot of ROS substances will be produced in the gastrointestinal tract, leading to tissue oxidative damage that affects cell metabolism (Maziero Alves et al., 2021). MDA is often taken as an indicator of 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 can cause severe damage to gastric mucosa, increasing the content of ROS and MDA and decreasing the activity of GSH-Px and SOD, consistent with previous results on IND-induced gastric injury (Barboza et al., 2018; Ugan and Un, 2020). However, AN can improve these parameters, suggesting that AN may have a strong antioxidant activity to alleviate IND-induced gastric injury in mice. Nrf2, a main antioxidant stress transcription factor, can bind to Keap1 in cytoplasm. In an oxidative stress injury, Keap1 is degraded to release Nrf2, which can be phosphorylated, and then migrates into the nucleus, binds to ARE, induces the expression of HO-1, and finally enhances GSH-Px and SOD activities (Carrasco-Pozo et al., 2016; Arafa Keshk et al., 2017). HO-1, as a stress response protein, can reduce the sensitivity of gastrointestinal cells to oxidative damage (Allam and El-Gohary, 2017). Therefore, we speculated that AN might protect the gastric mucosa through its regulation of the Nrf2/ARE signaling pathway. To confirm this hypothesis, we examined Nrf2/ARE signaling pathway–related protein expressions and found that AN can downregulate Keap1 expression and upregulate p-Nrf2 (S40), Nrf2, and HO-1 expressions in gastric tissue. Moreover, in our previous study, we found that AN has similar effects on the ischemia-reperfusion intestinal, liver, brain, and spleen tissues in D-galactose-treated mice, suggesting that Nrf2/ARE signaling pathway activation may be the common antioxidant mechanism of AN.

Inflammation and oxidative stress are closely related in some pathologic processes, and they both exist at the same time under many pathologic conditions (Fagundes et al., 2021). A continuous inflammation can aggravate the gastric mucosa injury; thus, controlling 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 thus increasing inflammation (Akanda et al., 2018). TNF-α can increase neutrophil migration to gastric mucosa and 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, such as 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 greater anti-inflammatory activity in gastric tissue (Rozza et al., 2014). In this study, IND significantly increased the ratio of p-NF-κB p65 to NF-κB p65; nuclear translocation of NF-κB-p65; TNF-α, IL-6, and IL-1β levels; and MPO activity in the gastric tissue of mice, whereas AN reversed the previously discussed effects of IND, suggesting that AN exerts this anti-inflammatory effect by activating the NF-κB signaling pathway. The NF-κB signaling pathway also promotes the upregulation of extracellular matrix regulators, such as 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), whereas MMP-9 is an important member in the metalloproteinase family and is also involved in the extracellular matrix remodeling imbalance in the gastric ulcer formation. The imbalance of MMP-9 can lead to 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). Our study finds that AN can reduce MMP-9 expression in the gastric tissue of mice with gastric ulcer and exert 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 the apoptosis pathway. TNF-α binds to tumor necrosis factor receptor-1 to stimulate the exogenous apoptotic pathway (Chen et al., 2016), and ROS can activate the mitochondrial apoptosis pathway. The mitochondrial apoptosis pathway is activated by some proapoptotic proteins (e.g., Bax) and inhibited by some antiapoptotic proteins (e.g., Bcl-2). Bax can stimulate the release of cytochrome C and activate caspase-3, whereas Bcl-2 can bind and neutralize mitochondrial proapoptotic proteins to regulate the apoptosis pathway (Correia et al., 2015). Therefore, the ratio of proapoptotic proteins/antiapoptotic 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 caspase-3 protein and the apoptosis index increased significantly in the gastric tissue of mice with gastric ulcer induced by IND whereas AN increased the Bcl2/Bax ratio and decreased the expression of cleaved caspase-3 protein and the apoptosis index, confirming that AN can improve the gastric injury induced by IND by its antiapoptosis in mice.

The gastric protective effect of AN was considered mainly due to its regulation of the Nrf2/ARE signaling pathway. To confirm this hypothesis, Nrf2, the NF-κB signaling, and AN participated in research design: Li, Jing, Liu. Conducted experiments: Liu, Fang, J.X. Zhang, Z.H. Zhang. Performed data analysis: D. Wang, Sun, Chen, C. Wang. Wrote or contributed to the writing of the manuscript: Liu, Li, Jing.
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