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Protective effects of Xiaoshi Lipi formula on gastric precancerous lesions via multi-target regulation of inflammation, oxidative stress, ferroptosis and apoptosis

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Abstract: **Background**: Xiaoshi Lipi formula, a traditional Chinese medicine, may alleviate Gastric precancerous lesion (GPL) progression through multi-target mechanisms. **Objectives**: To evaluate the protective effects of Xiaoshi Lipi formula on GPL and its underlying mechanisms. **Methods**: GPL was induced in rats by N-methyl-N'-nitro-N-nitrosoguanidine and sodium deoxycholate. Animals were assigned to normal, model, positive control and low-, medium-, or high-dose Xiaoshi Lipi formula groups. Gastric pathology was assessed by HE and AB-PAS staining. Serum inflammatory cytokines (TNF-α, IL-6, IL-1β), gastric function markers (G-17, PG I, PG II, PGE2), oxidative stress indices (ROS, MDA, SOD, GSH), ferroptosis- and antioxidant-related proteins and apoptosis (TUNEL) were analyzed. **Results**: Xiaoshi Lipi formula markedly alleviated gastric mucosal atrophy and intestinal metaplasia and improved gastric function. In the medium-dose group, G-17, PG I and PGE2 increased by ~90%, 70% and 62%, respectively, while PG Ⅱ decreased by ~40%. TNF-α, IL-6 and IL-1β were reduced by ~45%. ROS and MDA declined by ~48% and 50%, while SOD and GSH rose by ~38% and 83%. GPX4 and SLC7A11 were restored, Nrf2/HO-1 pathway activated and TUNEL-positive cells reduced by ~68%. Medium-dose effects were comparable to the positive control. **Conclusion**: Xiaoshi Lipi formula ameliorates GPL by targeting inflammation, oxidative stress and ferroptosis, supporting its potential in gastric cancer prevention.

Keywords: Ferroptosis; Gastric precancerous lesions; Inflammatory response; Oxidative stress; Xiaoshi Lipi formula

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INTRODUCTION

In clinical medicine, gastric cancer is still one of the most common cancers in the world in terms of incidence and mortality and early detection and treatment are still quite difficult (Conti *et al*., 2023; Thrift & El-Serag, 2020). The progression of gastric cancer generally entails a lengthy and gradual process, evolving from minor gastric mucosal damage to advanced malignancy. Gastric precancerous lesions (GPL), such as gastric mucosal atrophy, intestinal metaplasia and dysplasia, are recognized as essential phases in the development of gastric cancer (Drnovsek, Homan, Zidar, & Smid, 2024; Lai, 2019; Song, Kim, Jin, Lim, & Yang, 2016) and serve as key focuses for its prevention and management (M. Zhang *et al*., 2024). Consequently, discovering effective approaches to prevent the transition of GPL to gastric cancer has emerged as a central emphasis in research and preventive efforts related to gastric cancer.

The development of GPL is closely associated with persistent inflammatory responses, oxidative stress and cellular damage (Diaz, Valenzuela Valderrama, Bravo, & Quest, 2018). One of the main pathogenic causes of GPL is thought to be persistent inflammation of the gastric mucosa (Hoft, Noto, & DiPaolo, 2021). Research has indicated that inflammatory cytokines, including TNF-α, IL-6 and IL-1β, are consistently increased in GPL tissues. These molecules trigger signaling pathways like NF-κB, driving cell growth, suppressing programmed cell death and contributing to the establishment of a tumor-supportive microenvironment, ultimately hastening the development of gastric cancer (Tang *et al*., 2021). Another important element in the development of GPL is thought to be the excessive buildup of oxidative stress (Liu *et al*., 2023). Reactive oxygen species (ROS) overproduction exacerbates pathological damage to the gastric mucosa by causing lipid peroxidation, DNA damage and other negative consequences (Li, Pan, Pan, Li, & Tang, 2023). Ferroptosis, a new type of controlled cell death marked by lipid peroxidation of cell membranes brought on by oxidative stress, compromises cellular integrity and has been shown to be a major contributor to the development of gastric cancer (Gu *et al*., 2022; Jenke *et al*., 2024).

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Traditional Chinese Medicine (TCM) possesses a rich history and vast expertise in managing and preventing gastric cancer (L. Chen *et al*., 2023; Y. Liu *et al*., 2024; Yang *et al*., 2023; T. Zhang *et al*., 2022). Xiaoshi Lipi formula is an optimized and improved Chinese medicinal formula developed by the renowned TCM expert Professor Dong Mingguo. Based on years of clinical experience and the GPL theory of "food toxin," Xiaoshi Lipi formula was meticulously formulated by adding fried barley sprout and fried wheat germ, two digestion-promoting herbs, to the hospital-prepared Jianwei Xiaozhang Tablets. Xiaoshi Lipi formula follows the TCM theory of spleen and stomach, exhibiting a comprehensive therapeutic effect of regulating spleen and stomach functions, promoting qi and blood flow, resolving dampness and alleviating food stagnation (Dong & Lu, 2010).

Modern pharmacological research has demonstrated that Xiaoshi Lipi formula has significant anti-inflammatory, antioxidant, gastric mucosal repair-promoting and immunomodulatory effects. It is frequently used in the clinical treatment of gastrointestinal disorders because it successfully corrects pathological alterations in the gastrointestinal mucosa and restores gastric functional balance (Fang Zhike, Ma Xinlei, He Jing'er, & Dong Mingguo, 2017; Mo Qijun, Lu Hanqi, Chen Fanxiang, & Fang Xiaomei, 2023; Xiaomin, Mingguo, Yongbin, Zheng, & Hong Zhongyuan, 2020). However, the therapeutic effects of Xiaoshi Lipi formula in GPL and its underlying mechanisms have not been fully explored.

We propose that the Xiaoshi Lipi formula may enhance gastric mucosal healing and inhibit gastric cancer progression by modulating inflammatory responses, oxidative stress, ferroptosis and other associated pathways. This study seeks to explore the therapeutic effects and underlying mechanisms of the Xiaoshi Lipi formula in the context of gastric precancerous lesions (GPL), using a rat model induced by N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) and deoxycholic acid (DCA). The investigation will particularly focus on its potential to alleviate inflammation, oxidative stress, ferroptosis and apoptosis.

MATERIALS AND METHODS

Main reagents and equipment

The primary reagents used in this study included deoxycholic acid (DCA, 20 mol/L, Sigma-Aldrich, USA), N-methyl-N′-nitro-N-nitrosoguanidine (MNNG, 100 μg/mL, Sigma-Aldrich, USA), sodium salicylate (20 mol/L, Sigma-Aldrich, USA) and Xiaoshi Lipi formula (provided by the pharmacy of Dongguan Traditional Chinese Medicine Hospital, manufactured by Sinopharm Feng Liao Xing (Foshan) Chinese Herbal Medicine Co., Ltd.). Furthermore, ELISA kits (used to detect TNF-α, IL-6, IL-1β, G-17, PGI, PGII and PGE2, BD Biosciences, USA), HE staining reagents, AB-PAS staining reagents (Solarbio, Beijing) and a BCA protein assay kit (Thermo Fisher Scientific, USA) were employed. Additional chemicals included a TUNEL test kit (Roche, Switzerland), DAPI stain (Beyotime Biotechnology, Shanghai) and ROS, MDA, SOD and glutathione (GSH) detection kits (all from Beyotime Biotechnology, Shanghai).

The main equipment used in the experiment comprised: -80°C ultra-low freezer (Thermo Scientific ULT Freezer, Thermo Fisher Scientific, USA), Microplate reader (BioTek ELx800, BioTek, USA), Fluorescence microscope (Leica DMi8, Leica Microsystems, Germany), Electrophoresis apparatus (Mini-PROTEAN Tetra Cell, Bio-Rad, USA), Chemiluminescence imaging system (ChemiDoc MP, Bio-Rad, USA), Real-time quantitative PCR system (CFX96 Touch, Bio-Rad, USA), Vortex mixer (Vortex-Genie 2, Scientific Industries, USA), Centrifuge (Heraeus Labofuge 400, Thermo Fisher Scientific, USA).

Research subjects

Specific-pathogen-free (SPF) Sprague-Dawley (SD) rats, aged 6-8 weeks and weighing (280 ± 5) g, were selected as the research subjects. The rats were supplied by Guangzhou Riegle Biological Technology Co., Ltd., with the certification number SCXK (Yue) 2021-0059.

Preparation of the Xiaoshi Lipi formula

The formula of Xiaoshi Lipi formula includes: roasted malt (30 g), roasted rice sprout (30 g), gizzard lining (15 g), bitter orange (10 g), areca peel (10 g), agarwood (5 g), Chinese yam (15 g), eclipta (15 g), dendrobium (10 g), zedoary (10 g), perilla stem (15 g) and licorice (5 g). The herbs were accurately weighed according to their proportions in the formula, mixed thoroughly and soaked in six times their volume of water for 30 minutes. The mixture was subsequently simmered for 30 minutes, after which the liquid was filtered. The remaining solid was then re-extracted using three times its volume of water, simmered for an additional 30 minutes and filtered again. After being mixed together, the two filtrates were concentrated to yield a solution with a crude drug concentration of 2 g/mL. The preparation was sterilized and kept at 4°C in the refrigerator until use and it was gently warmed before administration. This formula is an optimized modification of the Jianwei Xiaozhang tablet and was provided by the pharmacy of Dongguan Traditional Chinese Medicine Hospital, manufactured by Sinopharm Feng Liao Xing (Foshan) Chinese Herbal Medicine Co., Ltd.

Animal model and group interventions

A total of 85 experimental rats were acclimatized for one week and then randomly divided into a normal group (n = 10) and a model group (n = 75) using a random number table method. Rats in the normal group were provided with regular drinking water and a standard diet, along with intragastric administration of physiological saline at 10

mL/kg daily for 16 weeks. Rats in the model group received 2 mL of 20 mol/L deoxycholic acid or 2 mL of sodium salicylate alternately via intragastric administration once daily and were given 100 μg/mL N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) as drinking water for 16 consecutive weeks to establish a rat model of chronic atrophic gastritis.

Model establishment was confirmed via hematoxylin and eosin (HE) staining, which showed structural changes in gastric mucosal parietal cells, loose and disordered cellular arrangement, nuclear atypia, goblet cell hyperplasia and signs of intestinal metaplasia, indicating successful induction of gastric precancerous lesions (GPL). Following successful modeling, the rats in the model group were split into five subgroups at random, each consisting of 15 rats: the GPL model group, the low-, medium- and high-dose Xiaoshi Lipi formula groups and a positive control group (Weifuchun). From the fifth week of modeling, the Weifuchun group received Weifuchun decoction at 1.0g/(kg·d), whereas the Xiaoshi Lipi formula low, medium and high dose groups received Xiaoshi Lipi formula at 0.5g/(kg·d), 1.0g/(kg·d) and 2.0g/(kg·d), respectively. Gavages of the same volume of normal saline were administered once day for 10 weeks to both the normal group and the GPL model group.

Sample collection

Following the final treatment, all experimental rats were fasted and deprived of water for 12 hours before specimen collection. Anesthesia was administered via intraperitoneal injection of 10% chloral hydrate and blood samples were drawn from the abdominal aorta into non-anticoagulant tubes. After mixing, the samples were centrifuged at 3000 rpm for 10 minutes at 4°C to isolate serum. The stomach was meticulously excised, opened along its greater curvature and emptied of its contents. Then, the stomach was washed thoroughly with physiological saline and then sectioned longitudinally on clean filter paper (including the gastric fundus, body and antrum). Part of the gastric tissue was preserved at -80°C for further analysis, while the remaining tissue was fixed in 10% neutral buffered formalin.

HE and AB-PAS staining for observing pathological changes in rat gastric tissue

HE Staining: Gastric tissues from rats were fixed in 10% neutral buffered formalin for 24 to 48 hours, then subjected to standard processes of dehydration, embedding and sectioning. Hematoxylin-eosin (HE) staining was performed and the tissue morphology was examined under a light microscope to identify pathological features, including mucosal hyperplasia, atrophy, intestinal metaplasia and dysplastic changes.

AB-PAS Staining: Paraffin-embedded sections underwent AB-PAS staining to evaluate mucus content in gastric epithelial cells. After staining, glandular metaplasia and alterations in mucus secretion by gastric glandular cells were observed using an optical microscope.

In addition to representative HE and AB-PAS staining, gastric mucosal atrophy and intestinal metaplasia were evaluated using a semi-quantitative grading system adapted from the updated Sydney system. For mucosal atrophy, grading was defined as: mild (≤30% gland loss), moderate (31–60% gland loss) and severe (>60% gland loss). For intestinal metaplasia, grading was defined as: focal (≤10% of glands), multifocal (11–30%) and extensive (>30%). For each animal, five high-power fields (×200) were randomly selected for assessment， and then the slides were scored. The number of animals falling into each grade is summarized in table 1 (atrophy) and table 2 (intestinal metaplasia).

Enzyme-linked immunosorbent assay (ELISA) detection

Serum and gastric tissue homogenates from rats were collected for analysis and commercially available ELISA kits were utilized to quantify inflammatory cytokines (TNF-α, IL-6, IL-1β) and indicators of gastric function (G-17, PGI, PGII, PGE2). Following the instructions provided with the kits, the samples underwent incubation and color development. A microplate reader was used to measure absorbance at 450 nm and the cytokine concentrations in the tissue and serum homogenates were computed and expressed in pg/mL.

Western blot (WB) analysis

The expression levels of proteins linked to ferroptosis as well as important proteins in oxidative stress and inflammatory signaling pathways were measured using WB. First, RIPA lysis buffer was used to extract total protein from rat stomach tissues. Equal amounts of protein were loaded onto SDS-PAGE gels for electrophoretic separation after protein quantification using the BCA technique. Non-specific binding sites were then inhibited when the proteins were moved onto PVDF membranes. Primary antibodies against ferroptosis-related proteins (GPX4, SLC7A11), oxidative stress signaling pathway proteins (Nrf2, HO-1) and inflammatory signaling pathway proteins (NF-κB p65, IκBα) were incubated on the membranes for an entire night. HRP-conjugated secondary antibodies were then used to incubate the membranes. An internal control protein called GAPDH (Santa Cruz) was employed. Target protein expression was examined and detection was accomplished using chemiluminescent reagents. Software like ImageJ was employed to quantify protein bands in order to compare the relative amounts of protein expression in each group.

Reverse transcription-polymerase chain reaction (RT-PCR)

The mRNA expression levels of pertinent proteins (GPX4, SLC7A11, Nrf2, HO-1, NF-κB p65 and IκBα) in stomach

Table 1: Semi-quantitative scoring of gastric mucosal atrophy in different groups based on HE staining.

Group	Number	Mild (≤30%)	Moderate (31-60%)	Severe (>60%)	Total
Normal	10	10	0	0	10
Model	15	2	5	8	15
Positive control	15	9	5	1	15
Low dose	15	7	6	2	15
Medium dose	15	11	4	0	15
High dose	15	10	5	0	15

Table 2: Semi-quantitative scoring of intestinal metaplasia in different groups based on AB-PAS staining.

Group	Number	Focal (≤10%)	Multifocal (11-30%)	Extensive (>30%)	Total
Normal	10	10	0	0	10
Model	15	3	6	6	15
Positive control	15	11	4	0	15
Low dose	15	6	7	2	15
Medium dose	15	12	3	0	15
High dose	15	9	6	0	15

Table 3: Primers sequence of related genes.

Gene	Primer direction	Sequence (5'-3')
GPX4	Forward	5'-TGTGGTGGTGTATGCCTTCC-3'
	Reverse	5'-CACCAGGTTTGAGAGCACTG-3'
SLC7A11	Forward	5'-GGGATTTGAACTTGGAGGCG-3'
	Reverse	5'-TGTAGGCACATTTGGTGGTG-3'
Nrf2	Forward	5'-TGAGGTTTGGGAGATGGAAGG-3'
	Reverse	5'-AAGTCGAAGGAACCTGAGCG-3'
HO-1	Forward	5'-CAGCCCCACCAAGTTCAAAC-3'
	Reverse	5'-TGGGGCAGAATCTTGCACTT-3'
NF-κB p65	Forward	5'-TGGGTGCGTCTGTGTTTATTG-3'
	Reverse	5'-AGTCCCATTCCGAGGAATTCC-3'
IκBα	Forward	5'-GCCAGGGAGGAGAGGAGATG-3'
	Reverse	5'-GCTGAGCGGTTTGGGGTGAG-3'
GAPDH	Forward	5'-TGCACCACCAACTGCTTAGC-3'
	Reverse	5'-GGCATGGACTGTGGTCATGAG-3'

tissues were determined by RT-qPCR. As directed by the kit, Trizol reagent was used to extract total RNA from gastric tissues. Nanodrop was used to measure the RNA concentration and purity (with an OD260/280 ratio between 1.8 and 2.0). A reverse transcription kit (like Takara's PrimeScript RT Reagent Kit) was then used to convert the isolated RNA into cDNA under the suggested time and temperature parameters. The SYBR Green qPCR kit was then used for amplification in real-time quantitative PCR. The SYBR Green Master Mix, primers and cDNA template were all part of the reaction system. The reaction conditions were as follows: initial denaturation at 95°C for 30 seconds, followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 30 seconds. The Ct values for each gene were obtained by continuously monitoring the fluorescence signals. Using GAPDH as the internal reference gene, the $2^{\wedge(-\Delta\Delta Ct)}$ technique was used to determine the relative expression levels of each gene. Table 3 lists the primer sequences for every protein.

Oxidative stress and antioxidant capacity detection

A ROS detection kit was used to measure the amounts of reactive oxygen species (ROS) in the stomach tissues. A fluorescence microplate reader was used to measure the fluorescence signals and a standard curve was used to determine the concentrations. An MDA detection kit was used to test the amount of malondialdehyde (MDA) and a microplate reader was used to assess absorbance at 532 nm. Using a colorimetric SOD detection kit, the activity of superoxide dismutase (SOD) was evaluated by measuring absorbance at 550 nm. Lastly, a GSH detection kit was used to test the amount of glutathione (GSH) and either colorimetric or fluorescence techniques were used to quantify the absorbance or fluorescence signals. All results were subjected to inter-group comparisons.

TUNEL evaluation of gastric tissue apoptosis

The proportion of apoptotic cells in gastric tissues was detected using a TUNEL assay kit. Tissue sections were dewaxed and rehydrated in water, followed by baking at

65°C for 1 hour. A sufficient amount of citrate buffer was used to immerse the slides and antigen retrieval was performed at around 95°C for 20 minutes. After cooling, the slides were washed with PBS. The TUNEL staining was performed according to the instructions of the *InSitu* Cell Death Kit Fluorescein. The slides were incubated at 37°C for 1 hour, followed by three 5-minute washes with PBS. DAPI was used for nuclear staining, incubated at 37°C for 3 minutes in the dark and washed with PBS three times for 3 minutes each. Fluorescence microscopy was used to capture images in the dark and the percentage of TUNEL-positive cells relative to total cells was calculated to reflect the apoptosis rate.

Statistical analysis

SPSS 25.0 statistical software was used to examine the experiment's data. The mean ± standard deviation ($\bar{x} \pm s$) is the format used to display quantitative data. One-way analysis of variance (ANOVA) was used for inter-group comparisons and the LSD-t test was used for additional post-hoc comparisons if statistical significance was discovered. A P-value of less than 0.05 was regarded as statistically significant and all statistical tests were two-sided. GraphPad Prism 8.0 was used to create the graphs.

RESULTS

Effect of Xiaoshi Lipi formula on gastric mucosal atrophy and intestinal metaplasia in rats

The pathological changes in gastric tissues from different groups were observed through HE and AB-PAS staining. According to the results of HE staining (Fig. 1A), the model group's stomach mucosal structure was markedly disturbed compared with the normal group, exhibiting characteristic features of atrophic gastritis, including mucosal gland atrophy, inflammatory cell infiltration and intestinal metaplasia. In contrast, the high- and medium-dose intervention groups of Xiaoshi Lipi formula showed significant improvement in the degree of gastric mucosal atrophy and glandular reduction, with pathological changes notably less severe than those in the model group ($P < 0.05$) and comparable to the effects of the positive control group. Semi-quantitative scoring further confirmed these findings (Table 2): the model group was dominated by moderate-to-severe atrophy, whereas treatment with Xiaoshi Lipi formula significantly shifted the distribution toward mild lesions, particularly in the medium-dose group. Furthermore, AB-PAS staining results (Fig. 1B) demonstrated that in the model group, gastric gland mucus secretion was reduced and intestinal metaplasia was prominent, while in the Xiaoshi Lipi formula treatment groups, mucus secretion and the extent of intestinal metaplasia were greatly improved ($P < 0.05$), with the medium-dose group showing slightly better results than the positive control group. This was supported by semi-quantitative grading (Table 3), which showed that the model group mainly exhibited multifocal or extensive intestinal metaplasia, whereas the majority of animals in the treatment groups, especially in the medium-dose group, were classified as focal only.

Our results indicate that Xiaoshi Lipi formula intervention can greatly reduce intestinal metaplasia and gastric mucosal atrophy in rats.

Effect of Xiaoshi Lipi formula on gastric function-related factors in rats

Rats in various groups were given serum and gastric tissue homogenates and gastric function markers were measured using ELISA. The model group's serum and gastric tissue had considerably lower levels of G-17, PG I and PGE2 ($P < 0.01$) than the normal group, but PG II was raised, indicating considerable gastric dysfunction, according to the results of gastric function indicators (Fig. 2). In contrast, the treatment groups with Xiaoshi Lipi Formula, at all doses, showed a significant increase in G-17, PG I and PGE2 levels, while PG II decreased to near normal levels ($P < 0.05$). Among these, the medium-dose group showed the most significant improvement ($P < 0.01$), followed by the high-dose group and the low-dose group showed a smaller improvement, demonstrating a dose-dependent effect. The recovery effect of positive control group was similar to that of medium dose, which further indicated the potential advantages of Xiaoshi Lipi formula in improving stomach function.

Effect of Xiaoshi Lipi formula on inflammatory response in gastric tissue of rats

The inflammatory factors were also evaluated using the ELISA method (Fig. 3 A-B). There was a strong inflammatory response in the GPL, as seen by the considerably higher blood and gastric tissue levels of TNF-α, IL-6 and IL-1β in the model group ($P < 0.05$) compared to the normal group. On the other hand, the positive control group and the Xiaoshi Lipi formula treatment groups (all doses) presented significantly reduced levels of inflammatory cytokines, particularly in the medium-dose group, where the levels were comparable to those in the normal group ($P < 0.01$). This suggests that the Xiaoshi Lipi compound successfully reduces the inflammatory reaction in the stomach tissue.

Additionally, the model group's stomach tissue exhibited markedly higher levels of NF-κB p65 protein and mRNA, but IκBα expression was substantially lower ($P < 0.05$), according to Western Blot (Fig. 3C-D) and RT-qPCR data (Fig. 3E). In contrast, there was no significant difference between the medium and high-dose groups of Xiaoshi Lipi formula and the positive control group ($P > 0.05$), although they did considerably block the expression of NF-κB p65 and enhance the expression of IκBα ($P < 0.01$). These findings suggest that the Xiaoshi Lipi formula considerably reduces the level of inflammation in the gastric tissue.

Effect of Xiaoshi Lipi formula on ferroptosis, oxidative stress and antioxidant capacity in gastric tissue of rats

According to the results of the Western Blot (Fig. 4 A-B) and RT-PCR (Fig. 4 C), Ferroptosis-related proteins (GPX4 and SLC7A11), antioxidant pathway proteins (Nrf2 and HO-1) and their associated mRNA levels were dramatically down-regulated ($P < 0.05$) in the gastric tissues of model group rats. This suggests that ferroptosis and oxidative stress are markedly increased during the progression of GPL. After treatment with Xiaoshi Lipi formula, the expression of GPX4 and SLC7A11 and their mRNA levels were upregulated in all dosage groups in comparison to the model group. The medium-dose group showed a restoration to levels that were comparable to those of the normal group ($P < 0.01$), with little distinction from the positive control group ($P > 0.05$). Meanwhile, the medium and high-dose groups showed substantially higher levels of the antioxidant pathway proteins Nrf2 and HO-1, as well as the mRNA levels associated with them ($P < 0.01$). This suggests that the Xiaoshi Lipi formula may reduce oxidative stress damage by activating the Nrf2/HO-1 antioxidant pathway and controlling ferroptosis-related proteins.

In addition, the oxidative stress indicators showed (Fig. 4D) that the model group rats' stomach tissue revealed markedly higher levels of ROS and MDA alongside markedly lower levels of SOD and GSH. Following the Xiaoshi Lipi formula therapy, the medium-dose group showed the most pronounced benefit, with ROS and MDA levels dramatically decreasing ($P < 0.01$) and SOD and GSH levels greatly increasing. These findings imply that Xiaoshi Lipi formula may significantly enhance the antioxidant capacity of gastric tissue by reducing ferroptosis and oxidative stress, thereby inhibiting the further progression of GPL.

Effect of Xiaoshi Lipi formula on cell apoptosis in gastric tissue of rats

TUNEL assay results (Fig. 5) showed the proportion of TUNEL-positive cells in the model group's stomach tissue was considerably higher ($P < 0.05$) versus the normal group, indicating that cell apoptosis contributes to the progression of GPL. However, the proportion of TUNEL-positive cells was far less in all Xiaoshi Lipi Formula dose groups when contrasted with the model group. The medium-dose group displayed a level of cell apoptosis that was comparable to that of the normal group ($P < 0.01$) and the outcome was similar to that of the positive control group ($P > 0.05$). According to these findings, Xiaoshi Lipi formula may have a major protective impact on the gastric mucosa by lowering cell apoptosis in gastric tissue through mechanisms such the prevention of oxidative stress and inflammation.

DISCUSSION

A number of pathological alterations, such as intestinal metaplasia, inflammation and atrophy of the stomach mucosa, are indicative of GPL, a crucial step in the development of gastric cancer (Drnovsek *et al*., 2024; Lai, 2019; M. Zhang *et al*., 2024). According to recent studies, ferroptosis, oxidative stress and chronic inflammation are important factors in the development and course of GPL. The breakdown of the gastric mucosal barrier and the malignant alteration of cells are the final results of these interconnected processes (S. Wang *et al*., 2024; S. Zhang *et al*., 2023). Based on this background, the current study investigated the possible mechanisms underlying the protective effects of Xiaoshi Lipi compound on GPL in rats. The findings provide scientific support for the possible development of Xiaoshi Lipi formula as a therapeutic approach for the prevention and treatment of gastric cancer by showing that it may dramatically improve GPL through the coordinated control of several pathways and targets.

Pro-inflammatory cytokines such TNF-α, IL-6 and IL-1β are prominent drivers of chronic inflammation, which is thought to be one of the most significant pathogenic processes in GPL (Bockerstett & DiPaolo, 2017; Liu *et al*., 2023). By triggering signaling pathways consisting of NF-κB, these cytokines encourage the prolonged production of inflammatory mediators, which damages the stomach mucosa, causes cell death and results in the formation of precancerous lesions (Bockerstett & DiPaolo, 2017; Liu *et al*., 2023). This investigation revealed that the Xiaoshi Lipi formula intervention considerably decreased the expression levels of TNF-α, IL-6, IL-1β, NF-κB and other inflammatory markers in comparison to the GPL group, enhancing the gastric tissue's function and inflammatory condition. These results are in line with those of other research (Gong *et al*., 2023; Lin *et al*., 2020), suggesting that Xiaoshi Lipi formula may suppress gastric mucosal damage and reduce the release of inflammatory mediators to preserve the gastric mucosa. In line with recent research on the potential of traditional Chinese medicine formulations for multi-target anti-gastritis activity (J. Liu *et al*., 2024), the results of this study further confirm the unique advantages of Xiaoshi Lipi formula in regulating inflammatory responses. Additionally, research has demonstrated that the anti-inflammatory properties of traditional Chinese medicine not only lessen damage to the stomach mucosa but also inhibit the production of pro-inflammatory cytokines by altering the mRNA and protein levels of markers of the NF-κB-related signaling pathway. This procedure has the potential to halt crucial phases in the development of stomach cancer from chronic gastritis (Yan-Rui *et al.,* 2024), which offers strong theoretical backing for the Xiaoshi Lipi formula's clinical development.

Significant increases in reactive oxygen species (ROS) and lipid peroxides (MDA) are the main indicators of oxidative stress, which is thought to be a major contributing factor in the development of GPL (Butcher, den Hartog, Ernst, & Crowe, 2017).

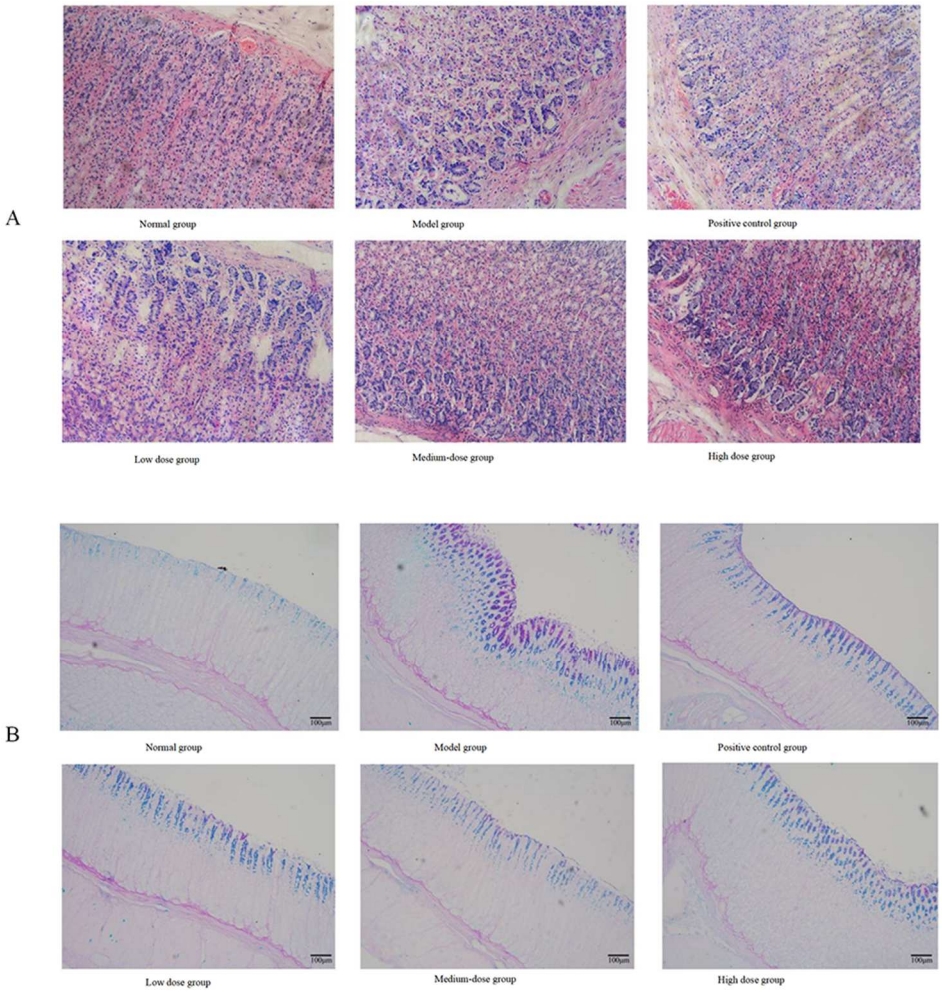

Fig. 1: HE and AB-PAS staining observed the effects of Xiaoshi Lipi formula on gastric mucosa atrophy and intestinal metaplasia in rats. Fig. 1A and B show the staining results of HE and AB-PAS, respectively.

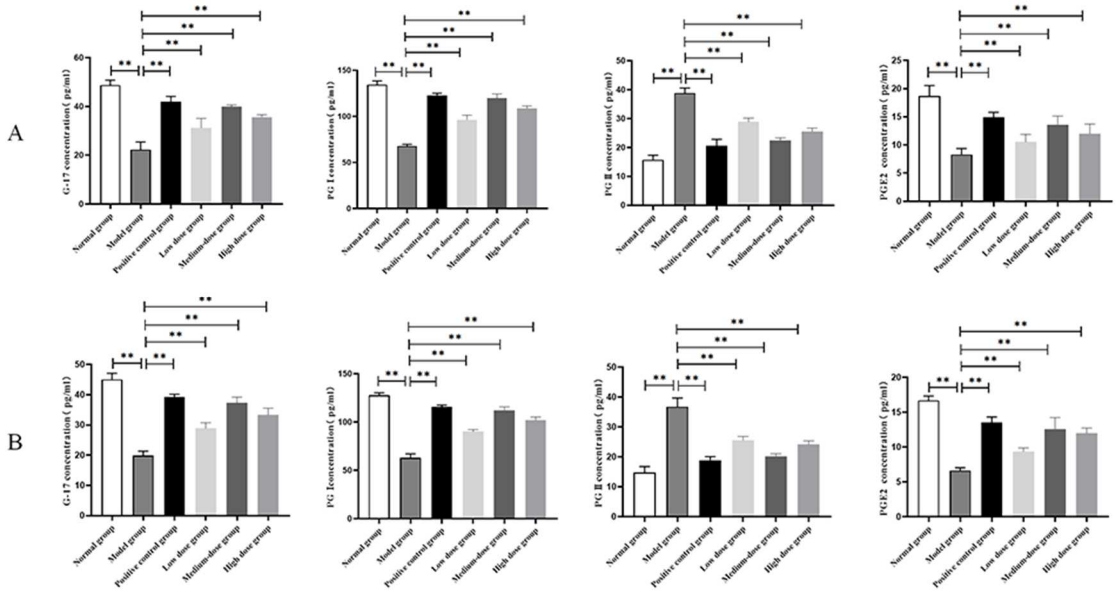

Fig. 2: Determination of the levels of gastric function indicators in rat serum and gastric tissue homogenates by ELISA. Fig. 2 A shows the levels of gastric function indicators in rat serum and Fig. 2B shows the levels of gastric function indicators in rat gastric tissue.

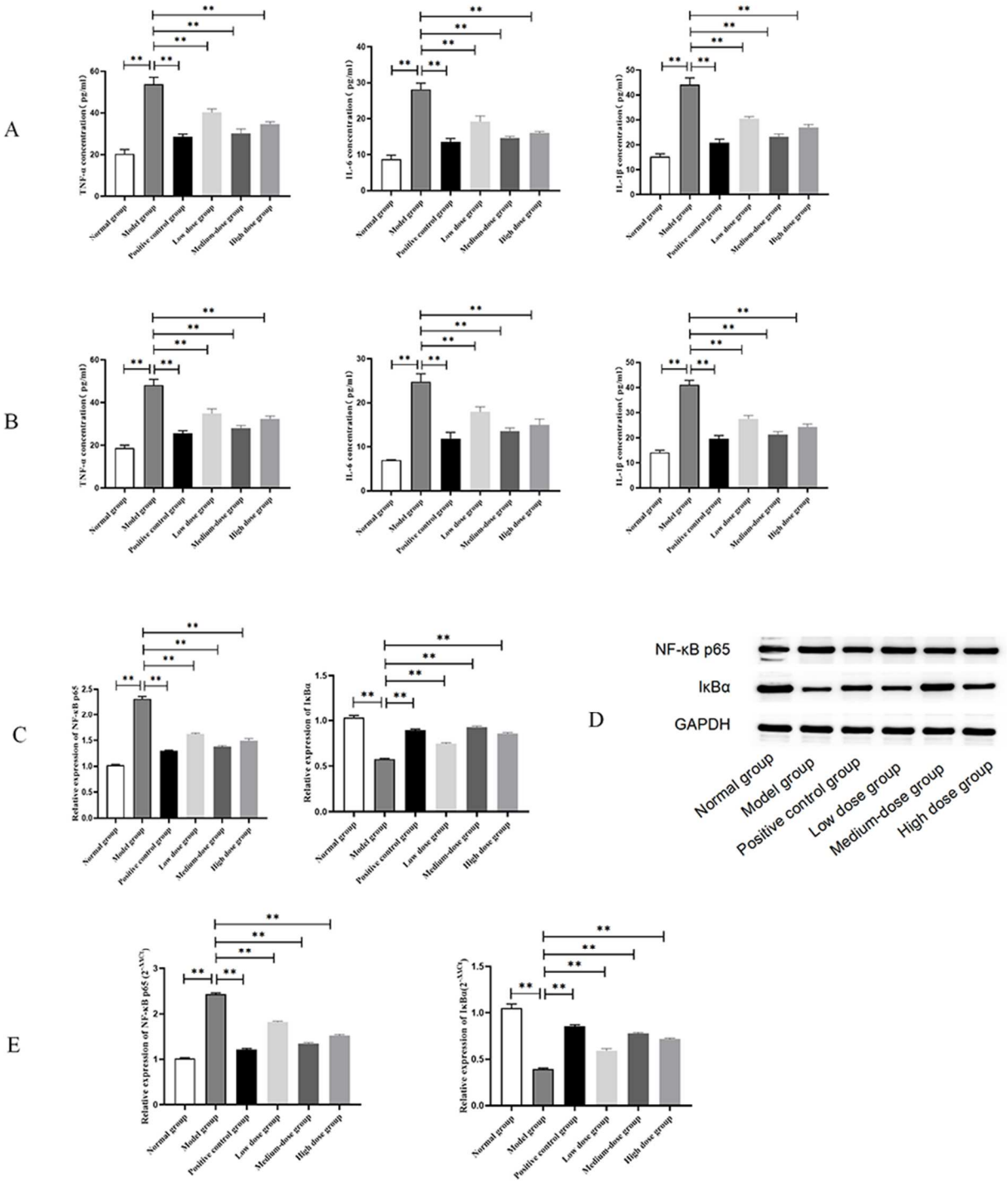

Fig. 3: Effect of Xiaoshi Lipi formula on the inflammatory response of gastric tissue in rats.
Fig. 3 (A and B) shows the levels of inflammatory factors in serum and gastric tissue of rats detected by ELISA. Fig. 3C, D and E show the protein and mRNA levels of NF-κB p65 and IκBα detected by Western Blot and RT-qPCR, respectively.

These oxidative products exacerbate the severity of the lesions by inducing DNA damage, cell death and amplifying inflammatory responses (Butcher *et al*., 2017). The Nrf2/HO-1 antioxidant pathway is a key defense mechanism the body uses to fend off oxidative stress, according to a number of studies (Kyung, Lim, & Kim, 2019; Paik *et al*., 2019; R. Wang *et al*., 2024). Its activation helps control the onset and progression of GPL by promoting the synthesis of antioxidant enzymes like SOD and GSH, efficiently scavenging ROS and shielding cells from oxidative damage. Based on this study, oxidative stress is markedly activated in GPL, as evidenced by the significantly higher levels of ROS and MDA in the gastric tissue of the GPL group.

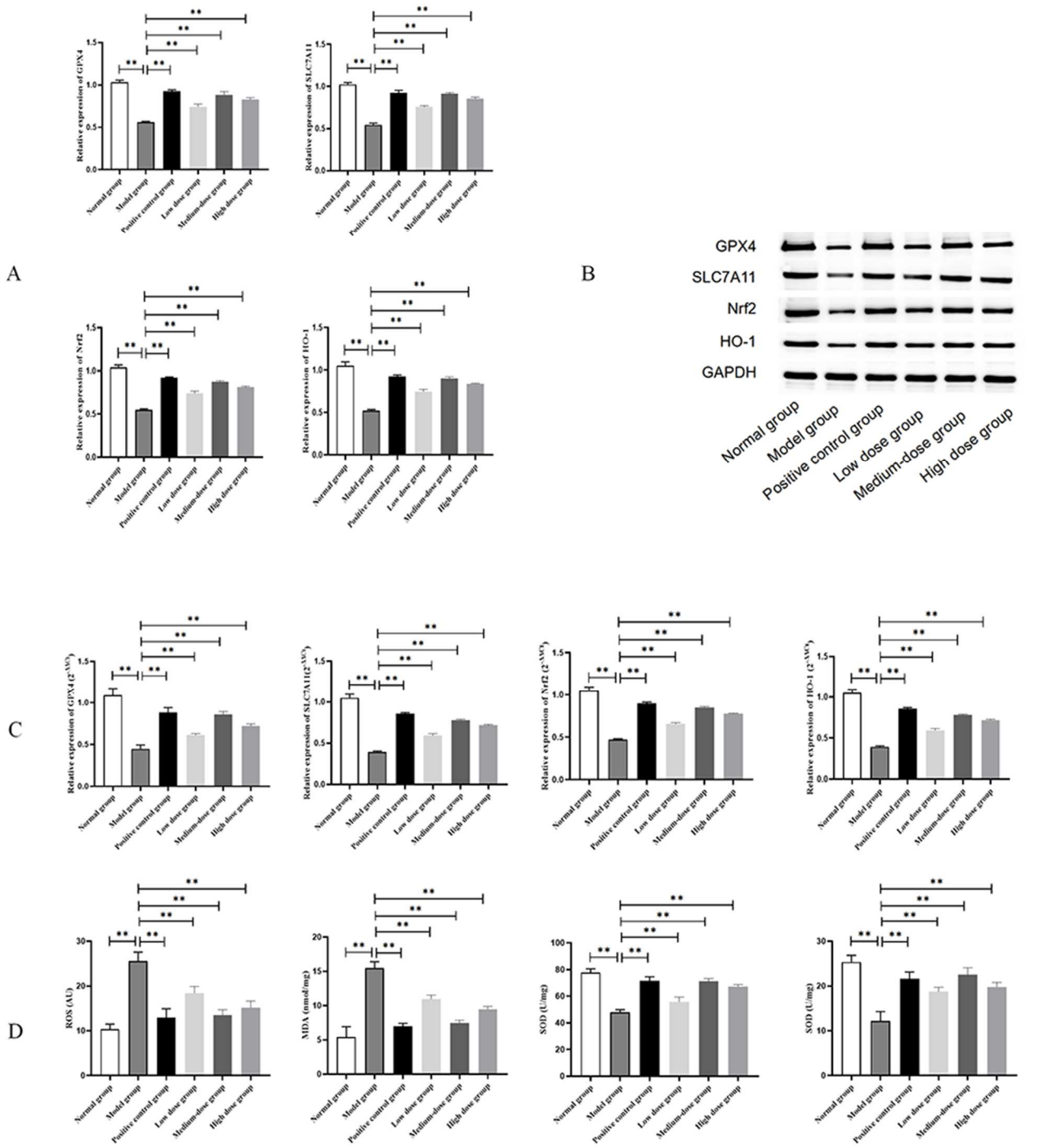

Fig. 4: Effect of Xiaoshi Lipi formula on ferroptosis, oxidative stress and antioxidant capacity of gastric tissue in rats. Fig. 4 A-B and C show the protein expression and mRNA levels of ferroptosis and oxidative stress detected by Western Blot and RT-PCR, respectively. Fig. 4D shows the oxidative stress and antioxidant capacity of rat gastric tissue detected by the kit.

At the same time, Nrf2 and HO-1 expression is significantly downregulated, indicating that the antioxidant defense mechanism is suppressed.

After intervention with Xiaoshi Lipi formula, the levels of ROS and MDA were significantly reduced, while the content of SOD and GSH significantly increased. Additionally, the expression of key proteins in the Nrf2/HO-1 pathway was significantly enhanced. This outcome is in line with findings from earlier research, which indicate that the Nrf2/HO-1 pathway is activated in traditional Chinese medicine to produce antioxidant benefits (Alomair *et al*., 2022; L. Chen *et al*., 2023; R. Wang *et al*., 2024).

Ferroptosis is a form of iron-dependent programmed cell death that has drawn more interest recently due to its connection to GPL (Kuang *et al*., 2023). GPX4 and SLC7A11 are key molecules involved in regulating ferroptosis. GPX4 is responsible for the elimination of lipid peroxides, while SLC7A11 maintains cellular antioxidant capacity by regulating glutamine metabolism (Yuan, Zhai, Chen, Xu, & Wang, 2021). This study discovered that the GPL group's stomach tissues had considerably lower levels of GPX4 and SLC7A11 expression. The expression of these proteins was considerably restored following the Xiaoshi Lipi formula intervention, particularly in the intermediate and high dose groups and was now close to normal levels. This finding suggests that by controlling the expression of proteins linked to ferroptosis, the Xiaoshi Lipi compound may reduce lipid peroxidation and cell damage in GPL. These results further support the significance of ferroptosis as a possible target for GPL treatment and are in line with recent research on the intervention of traditional Chinese medicine in ferroptosis (Guo, Du, & Wang, 2024). Moreover, there is significant crosstalk between ferroptosis, inflammation and oxidative stress (Chen, Fang, Yi, Wei, & Jiang, 2023) and the multifaceted effects of Xiaoshi Lipi formula suggest that it may improve gastric mucosal lesions through synergistic mechanisms across multiple pathways, which provides greater potential for its clinical application.

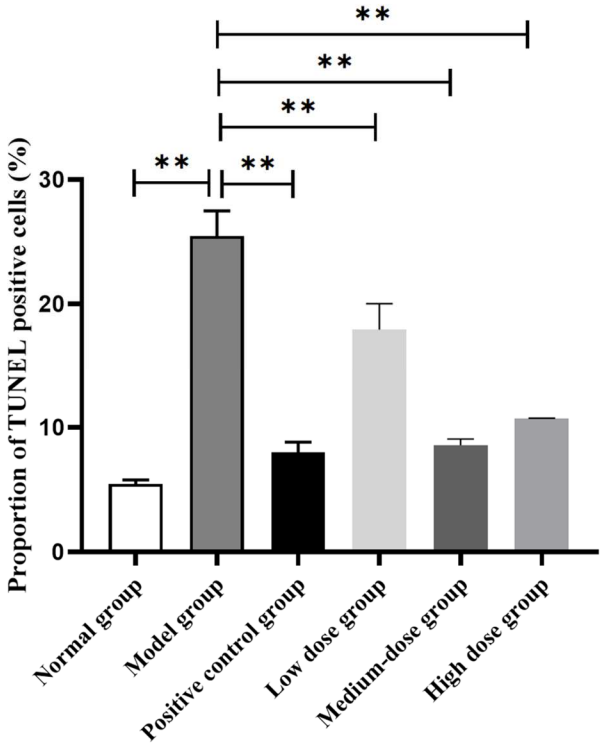

Fig. 5: The effect of Xiaoshi Lipi formula on apoptosis of gastric tissue in rats was detected by TUNEL.

Furthermore, apoptosis is another key pathological change in GPL. According to our findings, the GPL group's stomach tissues had a considerably higher level of apoptosis, which may indicate that apoptosis plays a role in the lesions. The percentage of TUNEL-positive cells in the gastric tissue was considerably lower following Xiaoshi Lipi formula intervention, suggesting that the formula may alleviate the lesions by preventing apoptosis. In addition, this study also found that Xiaoshi Lipi formula may regulate apoptosis through synergistic effects involving multiple mechanisms (including inhibiting inflammation, oxidative stress and ferroptosis). The reduction of apoptosis observed in Xiaoshi Lipi formula–treated groups was consistent with improvements in oxidative stress markers, as decreased ROS and MDA levels and increased SOD and GSH activity were accompanied by lower TUNEL-positive cells. This concordance suggests that attenuation of oxidative stress may contribute, at least in part, to the reduced apoptosis, further supporting the mechanistic link. This reflects a multi-pathway synergistic effect. This conclusion aligns with the perspective in previous studies (Xu *et al*., 2018; Zheng, Jiang, Huang, Ma, & Wang, 2023), which suggest that traditional Chinese medicine formulas can protect cells from pathological apoptotic damage through multiple mechanisms, further consolidating the theoretical and practical value of Xiaoshi Lipi formula.

In addition, we observed that the medium-dose group consistently exhibited the most pronounced therapeutic effects across gastric function markers, oxidative stress indices and apoptosis assays, whereas the high-dose group did not show further enhancement. This pattern suggests the presence of a therapeutic plateau at the medium dose rather than attenuation of efficacy at higher doses. Similar dose–response relationships have been reported in traditional Chinese medicine formulations, where an optimal dose achieves maximal benefit and higher doses do not necessarily confer additional effects, possibly due to saturation of pharmacological targets or homeostatic regulation.

CONCLUSION

In summary, Xiaoshi Lipi formula significantly ameliorated gastric precancerous lesions by alleviating inflammation, oxidative stress, ferroptosis and apoptosis and by improving gastric function. Among the treatment groups, the medium-dose Xiaoshi Lipi formula consistently demonstrated the most pronounced therapeutic effects, in several parameters showing outcomes comparable to or even superior to those of the positive control (Weifuchun). These findings indicate that Xiaoshi Lipi formula exerts dose-dependent protective effects, with a therapeutic plateau reached at the medium dose. Importantly, the results highlight the potential of Xiaoshi Lipi formula as a multi-target preventive strategy against the progression of gastric precancerous lesions,

thereby offering translational value for clinical gastric cancer prevention and intervention.

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Author's contributions
XML is responsible for the guarantor of integrity of the entire study, study concepts & study design, definition of intellectual content, literature research, clinical studies, manuscript preparation & editing & review; MGD is responsible for the study concepts & study design; ZYH is responsible for the manuscript editing; WSG is responsible for the literature research, data acquisition, statistical analysis, manuscript preparation; YXW is responsible for the experimental studies, data acquisition & analysis, statistical analysis; RXY is responsible for the guarantor of integrity of the entire study, clinical studies, manuscript review. All authors read and approved the final manuscript.

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Data availability statement
All data generated or analysed during this study are included in this. Further enquiries can be directed to the corresponding author.

Ethical approval
The animal experiment protocol was reviewed and approved by the Animal Experiment Ethics Committee of Guangzhou University of Chinese Medicine (Approval No. 20220415002).

Conflict of interest
There are no potential conflicts of interest to disclose.

Supplementary data

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