Weikangning therapy in functional dyspepsia and the protective role of Nrf2

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Abstract. Functional dyspepsia (FD) is a non-organic gastrointestinal disorder that has a marked negative impact on quality of life. Compared with conventional pharmacological therapies, the traditional Chinese medicine weikangning (WKN) is a safe and effective treatment for FD. The present study aimed to determine the molecular mechanisms underlying the efficacy of WKN. The effect of different concentrations of WKN on the proliferation of the human gastric mucosal epithelial cell line GES-1 was assessed. The optimal WKN concentration to promote cell proliferation was determined, and this concentration was used to examine the effect of WKN compared with a domperidone-treated positive control group on the antioxidant capacity of GES-1 cells. The effect of WKN treatment on the growth and antioxidant activity of GES-1 cells was also assessed following nuclear factor erythroid 2 like 2 (Nrf2) knockdown. The optimal WKN dose for promoting cell growth was determined to be 0.025 mg/ml; at this concentration the expression of the antioxidant proteins glutathione S-transferase P and superoxide dismutase 2 (SOD2) were significantly elevated ($P<0.0001$). Furthermore, the amount of reduced glutathione and activity of SOD2 were significantly increased ($P<0.0001$ and $P<0.01$, respectively), and malondialdehyde content was significantly decreased, compared with the controls ($P<0.001$). With WKN treatment, the transcription of Nrf2 and its downstream genes were significantly upregulated ($P<0.01$), and the level and nuclear distribution of Nrf2 protein was also markedly increased. Following Nrf2 silencing, the protective antioxidant effects of WKN treatment were impaired and GES-1 cell proliferation decreased. The results of the present study suggest that the efficacy of WKN in protecting gastric mucosal epithelial cells in FD is antioxidant-dependent and mediated by Nrf2 activation.

Introduction

Functional dyspepsia (FD) is a non-organic gastrointestinal (GI) disorder with persistent or recurrent sensations of early satiety, post-prandial fullness, nausea, and pain or discomfort in the upper abdomen (1,2). According to recent statistics, FD affects 5-11% of the population worldwide (3). Although FD is not a life-threatening disease, it necessitates numerous medical treatments and places a significant economic burden on patients (4). Furthermore, it has been reported that FD may develop into peptic ulceration (5).

Uncovering the pathogenesis of FD may contribute to the development of successful intervention strategies, which may in turn prevent symptomatic exacerbations of the disease and potentially even restore the digestive system to normal. There are a number of theories regarding the potential causes of FD, including genetic factors, infection with *Helicobacter pylori*, neurologic-hormonal disorders, autonomic disorders, stress and mental disorders, visceral hypersensitivity, and altered duodenal sensitivity to acids and lipids (1).

Several studies have explored the potential causes of FD, and efforts have been made to identify effective treatments; however, the optimal therapy for FD is yet to be determined (6). The majority of pharmacologic treatments that are currently available for FD have demonstrated limited efficacy (7). Several 5-hydroxytryptamine agonists, including tegaserod, cisapride, mosapride, pukabili, and renzapride, have demonstrated excellent efficacy in the treatment of FD via regulating GI dynamics; however, their safety has not been

adequately demonstrated (8). For example, evidence indicates that tegaserod may increase the risk of myocardial infarction, sudden cardiac death and other cardiovascular events (9,10). By contrast, cisapride may lead to QT interval prolongation, ventricular tachycardia and cardiac arrest (11).

The traditional Chinese medicine (TCM) weikangning (WKN) is a representative formula based on the classical TCM theory of 'Xi Kai Ku Jiang' (12). Several studies from the past decade have reported that WKN is a safe and effective treatment for FD (12,13). The composition of WKN includes the following nine Chinese herbs: *Radix Scutellariae*, *Rhizoma Zingiberis*, *Radix Codonopsis pilosulae*, *Rheum rhabarbarum*, *Radix Bupleuri*, *Radix Curcumae*, *Magnolia officinalis* bark, *Radix Paeoniae alba*, and *Rhizoma Corydalis* (12). Pharmacological studies have indicated that *Pinellia spp.*, *Radix Codonopsis pilosulae*, *Villosum spp.*, *Magnolia officinalis* and *Rheum rhabarbarum* may strengthen GI smooth muscle tension (14-19). Furthermore, *Scutellaria baicalensis* and *Coptis chinensis* have been reported to function as anti-inflammatory and antiallergic mediators, and serve a dual-directional role in the regulation of GI smooth muscle, which may provide a pharmacological basis for WKN as a treatment for FD (20-22).

To learn more about the detailed molecular mechanism underlying the efficacy of WKN in the treatment of FD, our group previously conducted a proteomic study to identify the differences in GI protein expression in an FD rat model with and without WKN treatment (12). The results revealed that the protein expression levels of glutathione-S-transferase P (GSTP1) and superoxide dismutase 2 (SOD2) were decreased in the disease model group compared with the normal control group. However, following the administration of WKN, the expression of GSTP1 and SOD2 was enhanced.

According to previous studies, GSTP1 and SOD2 serve an important role in combating oxidative stress (23,24). For example, Wang *et al* (23) demonstrated that upregulating the expression of GSTP1 and SOD2 significantly decreased oxidative stress by reducing reactive oxygen species (ROS) formation. In addition, a study by Dato *et al* (24) reported that genes belonging to pro-antioxidant signaling pathways, including GSTP1 and SOD2, were positively associated with longevity and functional status in very old age. Based on such observations and preliminary work by our group, it was hypothesized that oxidative stress may be an important component of the pathological mechanism of FD and that the efficacy of WKN may be due to reduced levels of oxidative stress. The results of these previous studies suggest that WKN may protect human GI cells by increasing antioxidant capacity, thereby maintaining the survival and growth of these cells and promoting recovery from FD. In addition, a previous study revealed that Nrf2 pathway played a critical role in antioxidation, anti-inflammation and protecting liver from oxidative damages in dyspepsia (25). Based on these results, it was investigated in the current study whether the antioxidant regulation of Nrf2, GSTP1 and SOD2 was involved underlying the WKN treatment for FD. Overall, the present study aimed to provide a theoretical basis for the use of TCMs in the treatment and prevention of FD, and to provide experimental data to support the hypothesis that oxidative injury is important in the development and exacerbation of FD.

Materials and methods

Cell culture. The human gastric mucosal epithelial cell line GES-1 was purchased from the cell bank of Culture Preservation Committee, Chinese Academy of Sciences (Beijing, China). GES-1 cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% heat inactivated fetal bovine serum (Zhejiang Tianhang Biological Technology Co., Ltd., Zhejiang, China), 100,000 U/l penicillin and 100 mg/l streptomycin in an atmosphere containing 5% CO_2 at 37˚C.

Establishment of the lentivirus-mediated nuclear factor erythroid 2 like 2 (Nrf2) gene-knockdown stable strain of GES-1 cells. Four pairs of short hairpin (sh)RNA species targeting Nrf2 mRNA were designed by Origene Technologies (Rockville, MD, USA; Table I) and cloned into Lenti-KD vectors (Pre-1102; Beijing Pregene Science and Technology Co., Ltd., Beijing, China) with blank vectors as controls. Using Lipofectamine® 2,000 Transfection reagent (Thermo Fisher Scientific, Inc.), these plasmids, along with the pMD2.0G and psPAX2 plasmids (both Addgene, Cambridge, MA, USA), were successively transfected into 293T cells (ATCC, Manassas, VA, USA) to form a virus package. After each type of transfected 293T cells were cultured in 5% CO_2 at 37˚C for 3 days, a 2 ml culture of supernatant-containing lentiviral particles was harvested by centrifugation at a speed of 300 x g at 4˚C for 5 min, and stored at -80˚C until required.

A total of $2x10^5$ GES-1 cells were seeded into a 6-well plate. Following overnight culture, the adherent cells were transfected at room temperature with the lentivirus-containing culture medium overnight, and cultured at 30˚C in a 5% CO_2 incubator. The following day, the medium was replaced. At 48-72 h following transfection, 3 μg/ml puromycin was added for selection and the cells were sub-cultured in a 10 cm^2 plate, with continued puromycin treatment. The Nrf2 expression of transduced cells was identified using western blotting as described below.

The gene knockdown mediated by the lentivirus with the shNrf2-3 sequence appeared to be the most effective (Fig. 1). The GES-1 cell strain transfected with this lentivirus was cryopreserved at -80˚C as the Nrf2 gene-knockdown stable strain, which is referred to as shNrf2 throughout the present study. At the same time, the GES-1 cells transfected with lentivirus containing shMock sequence (CCTAAGGTTAAGTCGCCC TCG) were cryopreserved and used as a control (shMock). After recovery, the culture conditions for the stable cell lines were the same as those described for the GES-1 cells above.

Drug preparation. WKN was prepared using the following ingredients: *Rhizome Pinelliae* Preparata, 9 g; *Rhizoma Coptidis*, 5 g; *Radix Scutellariae*, 9 g; *Fructus Amomi villosi*, 6 g; *Rhizoma Zingiberis*, 9 g; *Radix C. pilosulae*, 15 g; *Radix Curcumae*, 9 g; *M. officinalis* bark, 9 g; *Rhizoma Corydalis* vinegar, 10 g; *Radix Bupleuri*, 9 g; rhubarb, 6 g; fried almond, 9 g; inflammatory licorice, 9 g; *Radix Paeoniae* Alba, 15 g; and *Fructus Ziziphi jujubae*, 4 g. All components were supplied by the Department of Pharmacy of Wangjing Hospital of China Academy of Chinese Medical Sciences (Beijing, China). WKN was decocted according to traditional methods. The WKN was

Table I. Sequences of shRNAs targeting Nrf2 mRNA.

Oligo	Sequence
shNrf2-1	F:5'-GATCCGCACCTTATATCTCGAAGTTTCTTCCTGTCAGAAAACTTCGAGATATAAGGTGCTTTTTG-3'
	R:5'-AATTCAAAAAGCACCTTATATCTCGAAGTTTTCTGACAGGAAGAAACTTCGAGATATAAGGTGCG-3'
shNrf2-2	F:5'-GATCCCCGGCATTTCACTAAACACAACTTCCTGTCAGATTGTGTTTAGTGAAATGCCGGTTTTTG-3'
	R:5'-AATTCAAAAACCGGCATTTCACTAAACACAATCTGACAGGAAGTTGTGTTTAGTGAAATGCCGGG-3'
shNrf2-3	F:5'-GATCCCCCTGTTGATTTAGACGGTATCTTCCTGTCAGAATACCGTCTAAATCAACAGGGTTTTTG-3'
	R:5'-AATTCAAAAACCCTGTTGATTTAGACGGTATTCTGACAGGAAGATACCGTCTAAATCAACAGGGG-3'
shNrf2-4	F:5'-GATCCGCAGCAAACAAGAGATGGCAACTTCCTGTCAGATTGCCATCTCTTGTTTGCTGCTTTTTG-3'
	R:5'-AATTCAAAAAGCAGCAAACAAGAGATGGCAATCTGACAGGAAGTTGCCATCTCTTGTTTGCTGCG-3'

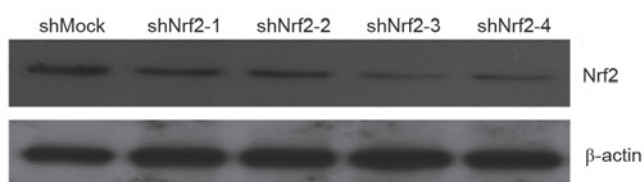shRNA, short hairpin RNA; Nrf2, nuclear factor erythroid 2 like 2; F, forward; R, reverse.

Figure 1. Expression of Nrf2 protein in GES-1 cell strains transfected with four types of shRNA or control shRNA. The expression of Nrf2 protein was assessed by western blotting. shRNA, short hairpin RNA; Nrf2, Nrf nuclear factor erythroid 2 like 2.

immersed in water for 30 min, then 500 ml water was added and decocted for 30 min; the extract of ~150 ml was poured out, and another 500 ml water was added followed by decocting again; finally the extract of the two steps were mixed, at a total volume of 300 ml. The solution was condensed to a volume of 400 ml. The 400 ml crude extract solution was vacuum lyophilized for at least 3 h to obtain a powder using a freeze dryer (ALPHA1-4, Martin Christ GmbH, Osterode, Germany). The powder was stored until use at -20°C, and was dissolved in PBS to make the stock solution (10 mg/ml). The stock solution was sterilized using ^{60}Co-γ radiation (26) and centrifuged at 15,000 x g, 37°C for 20 min to remove the insoluble ingredients. The stock solution was stored at -20°C and diluted to varying degrees depending on the concentration required for the different experimental procedures.

Domperidone (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was dissolved in dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA) to create a stock solution (0.1 mol/l) and a working solution (2.5 μmol/ml), and was used as a positive control. PBS was used as the negative control. The drug treatment groups varied depending on the different experimental procedures, as outlined below.

Proliferation activity assays
Cell Counting Kit-8 (CCK-8) assay. A total of 1x10^4 GES-1 cells/well were seeded into a 96-well plate. The cells were then cultured in an atmosphere containing 5% CO_2 at 37°C for 4-6 h. Drug treatments were administered following adherence. Cells were divided into 10 groups and WKN was added at different concentrations (0, 0.025, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 and 0.7 mg/ml). After 12, 24 and 48 h of incubation at 37°C,

20 μl CCK-8 solution (Beyotime Institute of Biotechnology, Haimen, China) was added to the wells. Cells were then cultured at 37°C for 1 h. Optical density was measured at 450 nm using a microplate reader (POLARstar® Omega; BMG Labtech GmbH, Ortenburg, Germany). Four replicates were performed at each time-point for each group.

MTS assay. A total of 5x10^4 shMock or shNrf2 cells/well were seeded into a 96-well plate. The cells were then cultured with 5% CO_2 at 37°C for 4-6 h. The drug treatment was performed following adherence. Cell culture media containing 0.025 mg/ml WKN or equivalent amounts of PBS (as a blank control) was added to the wells. After 4, 48 and 96 h of incubation, 20 μl MTS solution (Beijing Pregene Science and Technology Co., Ltd., Beijing, China) was added to the wells. Cells were then cultured at 37°C for 1 h. Optical density was measured at 490 nm using the microlate reader. Six replicates were performed at each time point for each group.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. A total of 1.5x10^6 cells/well were seeded into a 6-well plate. The cells were cultured with 5% at 37°C for 4-6 h. The drug treatments (see the corresponding figure legend for the specific group settings and processing times) were performed following adherence. After treatment, total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific, Inc.) and RT was performed using a Prime Script™ RT reagent kit (Takara Bio, Inc., Shiga, Japan) according to the manufacturer's protocol. PCR primers were synthesized by Shanghai Biotech Co., Ltd. (Shanghai, China) and are listed in Table II. The reaction was kept on ice in the dark until qPCR was performed and was comprised of the following components: 10 μl 2X SYBR Premix Ex Taq™ (Takara Bio, Inc.), 0.5 μl PCR forward primer (10 μM), 0.5 μl PCR reverse primer (10 μM), 2 μl cDNA template and 7 μl distilled H_2O. The thermocycling conditions were as follows: Initial denaturation at 95°C for 1 min; 40 cycles of 95°C for 15 sec, 60°C for 15 sec and 72°C for 45 sec; and a final extension at 72°C for 5 min. Relative mRNA expression was calculated using the 2$^{-\Delta\Delta Cq}$ method (27). β-actin was used as the internal reference gene.

Western blotting. A total of 1.5x10^6 cells/well were seeded into a 6-well plate and cultured with 5% CO_2 at 37°C for 4-6 h.

Drug treatments (see the corresponding figure legend for the specific group settings and processing times) were administered following adherence. Total protein was extracted when cells were collected. After ultrasonic homogenization in the RIPA lysis buffer (150 μl; Applygen Technologies, Inc., Beijing, China), cells were lysed at 4˚C for another 10 min, centrifuged (15,000 x g, 4˚C, 15 min), and the supernatant was transferred for analysis. Then protein was quantified using a BCA Protein Quantification kit (Beijing Biomed Biological Technology Co., Ltd., Beijing, China). Protein was denatured at 100˚C for 5 min, and 20-40 μg of protein samples were loaded Next, SDS-PAGE was performed using the following settings: 4% stacking gel, 80 V for 30 min; and a 12% resolving gel, 120 V for 1 h. Proteins were then electrotransferred onto PVDF membranes. The membranes were blocked with 5% nonfat milk in Tris-buffered saline (pH 7.5) with 0.1% Tween-20 for 1 h at room temperature. Primary antibodies directed against the following proteins were used: GSTP1 (P04906, Enzo Life Sciences, Farmingdale, NY, USA; 1:1,000), SOD2 (2299-1, Epitomics; Abcam, Cambridge, UK; 1:1,000), Nrf2 (ab62352, Abcam; 1:1,000), and β-actin (4970S, rabbit monoclonal antibody; Cell Signaling Technology, Danvers, MA, USA; 1:5,000). The membrane was incubated with the primary antibodies at 4˚C overnight. The secondary horseradish peroxidase-conjugated immunoglobulin G antibody (ZB-2301; 1:3,000) was purchased from Zhongshan Jinqiao Biotechnology Co., Ltd. (Beijing, China). The membrane was incubated with the secondary antibodies at room temperature for 1 h. Protein bands were visualized using an enhanced electrochemiluminescence western blot high-sensitivity detection kit (CW0049 M, Beijing Comwin Biotech Co., Ltd., Beijing, China) and images were captured using the Gel Doc™ XR Gel Imaging system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Semi-quantitative analysis was performed using ImageJ 1.47 software (National Institutes of Health, Bethesda, MA, USA). The results are presented as percentages relative to the control groups.

Immunofluorescent staining. GES-1 cells were seeded into a culture dish with pretreated coverslips at a density of 10^5 cells/500 μl. After adherence, 0.025 mg/ml WKN was added to the experimental groups, while an equivalent amount of PBS was added to the control group. After 24 h of treatment at 37˚C, the cells were harvested. The slides were soaked in PBS 3 times (3 min/soak) and fixed at room temperature for 15 min using 4% paraformaldehyde. Slides were subsequently soaked in PBS 3 times (3 min/soak) and penetrated with 0.5% Triton X-100 (in PBS) for 20 min at room temperature. Next, the slides were soaked in PBS and blotted dry. Subsequently, the slides were blocked with 6% normal goat serum (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) in PBS for 20 min at room temperature. The blocking solution was removed and the slides were treated with primary antibodies directed against Nrf2 (ab137550, Abcam; 1:40) overnight at 4˚C. The slides were then rewarmed at 37˚C for 45 min, soaked in PBS 3 times (3 min/soak) and blotted dry. A fluorescent secondary antibody, goat anti-rabbit IgG H&L Alexa Fluor® 647 (ab150079; Abcam; 1:800), was added and the slides were incubated for 30 min at 37˚C. The slides were soaked in PBS 3 times (3 min/soak) and blotted dry.

Table II. Primers used in reverse transcription-quantitative polymerase chain reaction.

Target gene	Sequence
GSTP1	F: 5'-GGCTCTATGGGAAGGACCAGC-3'
	R: 5'-CACATAGTCATCCTTGCCCGCC-3'
SOD2	F: 5'-TCAGCCCTAACGGTGGTGGA-3'
	R: 5'-AAGCCAACCCCAACCTGAGC-3'
Nrf2	F: 5'-AGCATGCTACGTGATGAAGATGG-3'
	R: 5'-AGCTCAGAAAAGGTCAAATCCTCC-3'
NQO1	F: 5'-CATCCCAACTGACAACCAGAT-3'
	R: 5'-CAGGGAAGCCTGGAAAGATAC-3'
HO-1	F: 5'-ATGACACCAAGGACCAGAGC-3'
	R: 5'-GCATAAAGCCCTACAGCAACT-3'
GCLM	F: 5'-CACGAGGTCAGGAGTTTGAGA-3'
	R: 5'-AGCTGAGATTACAGGCGAGTG-3'
GCLC	F: 5'-CCCTCCTCCAAACTCAGACAT-3'
	R: 5'-TGGTGAGCAGTACCACAAACA-3'
β-actin	F: 5'-GCTGCCCTGAGGCACTCTTC-3'
	R: 5'-ATCCTGTCGGCAATGCCAGG-3'

GSTP1, glutathione-S-transferase P; SOD2, superoxide dismutase 2; Nrf2, **nuclear factor erythroid 2 like 2; NQO1, NADPH dehydroge**nase quninone 1; HO, heme oxygenase; GCLM, glutamate cystteine ligase modifier subunit; GCLC, glutamate cysteine ligase catalytic subunit; F, forward; R, reverse.

The slides were treated with DAPI dye (Beyotime Institute of Biotechnology) at room temperature in the dark for 5 min to stain the cell nuclei. The slides were then washed with PBS 4 times (5 min/wash), sealed with mounting medium containing anti-fluorescence quenching reagent (Antifade Mounting Medium; Beyotime Institute of Biotechnology) and the edges were sealed with nail polish. The slides were observed under a fluorescence microscope and representative images were captured.

Detection of oxidative stress levels. Cells were lysed following drug treatment (see the corresponding figure legend for the specific group settings and processing times). The culture medium was removed and the cells were rinsed with pre-cooled PBS twice. Pre-cooled PBS (1 ml) was added and cells were swept with a cell lifter, then transferred into a 1.5 ml microcentrifuge tube. Centrifugation was performed at 4˚C and at 650 x g for 15 min. The sediment was harvested and cytopreserved at -80˚C. Different aspects of oxidative stress were detected using a reduced glutathione (GSH) content assay kit [intra-assay coefficient of variation (CV), 1.2%; inter-assay CV: 3.86%; sensitivity, 0.3-147.1 mg/l], a SOD enzyme activity assay kit (intra-assay CV, 5.05%; inter-assay CV, 3.32%; sensitivity, 0.5 U/ml), and a maleic dialdehyde (MDA) content assay kit (intra-assay CV, 3.5%; inter-assay CV, 4.11%; sensitivity, 0.5-113 nmol/ml). All kits were provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Cell lysis, the detection procedure and result calculations were all performed according to the manufacturer's protocol.

Statistical analysis. All experiments were repeated ≥3 times, and the results were analyzed using SPSS software (version 19.0; IBM Corp., Armonk, NY, USA). Data are represented as the mean ± standard deviation. Student's t-test was used to analyze differences between two groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Low concentrations of WKN promote GES-1 cell proliferation. A CCK-8 assay was used to investigate cell proliferation and it was demonstrated that WKN at lower concentrations had the optimal effect in promoting the proliferation of GES-1 cells at all time points (Fig. 2). The pro-proliferative effects gradually weakened with an increase in the concentration of WKN. The optimal effect was observed at 0.025 mg/ml.

Low concentrations of WKN increase the expression of GSTP1 and SOD2 in GES-1 cells. In our previous study of WKN treatment in FD model rats, proteomics were analyzed, and it was demonstrated that GSTP1 and SOD2 expression levels were decreased in the disease model group and upregulated following WKN treatment (12). It was therefore speculated that the growth-promoting effect of WKN treatment on GES-1 cells may be associated with the expression of GSTP1 and SOD2. Therefore, in the present study changes in GSTP1 and SOD2 expression following WKN treatment were investigated. Following treatment with WKN at different concentrations for 24 h, the expression level of GSTP1 mRNA in GES-1 cells was significantly elevated compared with the control group (P<0.05; Fig. 3A). However, no significant difference was observed between the domperidone treatment group and the control group (Fig. 3A). Although domperidone treatment significantly enhanced SOD2 mRNA levels (P<0.05; Fig. 3A), and GSTP1 and SOD2 protein levels (P<0.0001 and P<0.001, respectively; Fig. 3B), compared with the control group, 0.025 mg/ml WKN had a greater effect (P<0.0001; Fig. 3B). As the concentration of WKN increased, its effect on GSTP1 and SOD2 mRNA and protein levels decreased (Fig. 3). These results further confirmed that the efficacy of WKN is concentration-dependent. When the concentration of WKN was 0.025 mg/ml, the protective effect on GES-1 cells of promoting cell growth, and increasing GSTP1 and SOD2 expression levels, was the greatest. Therefore, this was chosen as the optimal concentration for subsequent experiments.

WKN decreases oxidative stress in GES-1 cells. GSTP1 and SOD2 serve important roles in combating oxidative stress. It was therefore hypothesized that the growth-promoting effect of WKN on gastric mucosal epithelial cells was associated with the regulation of oxidative stress. The GSH content, SOD activity and MDA content were measured as a proxy for the level of cellular oxidative stress (Fig. 4). When GES-1 cells were treated with 0.025 mg/ml WKN, GSH content and SOD activity increased significantly (P<0.0001 and P<0.01, respectively), while MDA content decreased significantly (P<0.001), compared with the control group. However, no significant changes in oxidative stress index levels were observed in the domperidone treatment group.
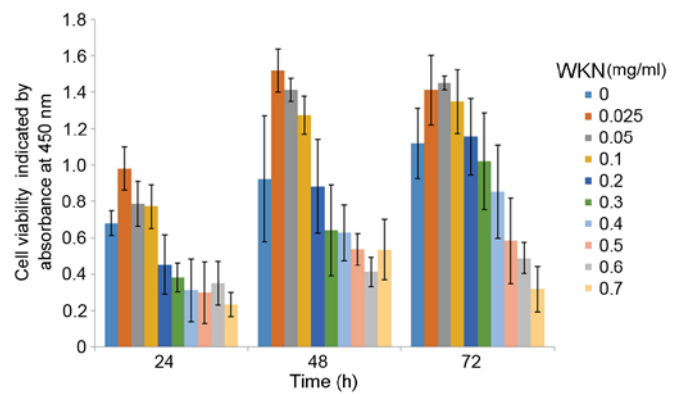

Figure 2. Proliferation GES-1 cells after 12, 24 or 48 h of WKN treatment at different concentrations (0, 0.025, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 and 0.7 mg/ml) detected using the Cell Counting Kit-8 assay. WKN, weikangning.

WKN increases the expression of Nrf2 and its target genes in GES-1 cells. A previous study identified *gstp1* and *sod2* as two of the putative Nrf2-dependent genes (27) and Nrf2 has been identified as an important transcription factor that regulates oxidative stress (28-32). To determine whether WKN treatment enhanced the antioxidant capacity of cells via the promotion of Nrf2, GES-1 cells were treated with 0.025 mg/ml WKN and the expression level of the Nrf2 gene was measured as the processing time increased. The transcription level of Nrf2 was significantly upregulated following 2 h of WKN treatment (P<0.001; Fig. 5A). This effect decreased with time. The transcription levels of four target genes of Nrf2 (NADPH dehydrogenase quinone 1, heme oxygenase 1, glutamate cysteine ligase modifier subunit and glutamate cysteine ligase catalytic subunit) were measured after 2 h (data not shown) and 4 h (Fig. 5B) of treatment with 0.025 mg/ml WKN. The results demonstrated that the transcription of the four genes increased significantly following 4 h of treatment (P<0.01; Fig. 5B).

WKN increases GES-1 cell Nrf2 content and nuclear translocation. Under normal conditions, Nrf2 is anchored in the cytoplasm by Kelch-like ECH-associated protein 1 (Keap1). However, when cells are exposed to inducing stimuli, Nrf2 is activated, released from Keap1 and transported into the nucleus (33). Nrf2 then combines with the antioxidant response element (ARE) in the promoter region of downstream target genes, activating the transcription of target genes and transducing antioxidant signals (34). To clarify the impact of WKN treatment on Nrf2 transcriptional activity, changes in Nrf2 content and nuclear localization after WKN treatment were assessed in the present study. Immunofluorescence staining revealed that the Nrf2 content of cells was markedly increased following WKN treatment and that Nrf2 protein was translocated into the nucleus (Fig. 5C).

Nrf2 silencing inhibits the protective effect of WKN on GES-1 cells. To further confirm that the growth-promoting effect of WKN on gastric mucosa epithelial cells was via the upregulation of Nrf2 and associated signaling pathways, a lentiviral-mediated Nrf2 gene knockdown GES-1 cell line was established. Western blotting revealed that Nrf2 protein expression decreased most markedly in shNrf22-3 silenced
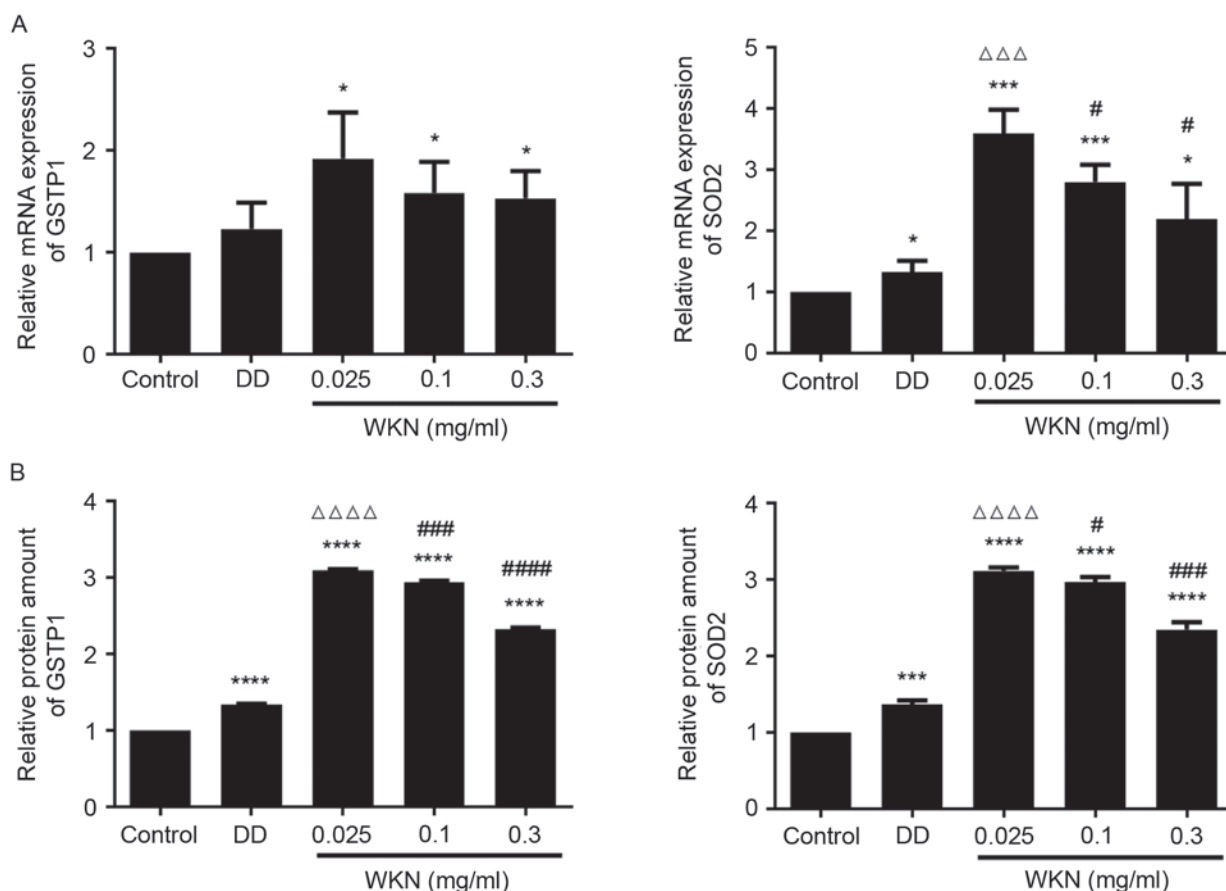
Figure 3. WKN treatment increases the expression of GSTP1 and SOD2 in GES-1 cells. GSTP1 and SOD2 (A) mRNA and (B) protein levels in GES-1 cells after 24 h treatment with 2.5 µmol/ml DD or WKN at. GES-1 cells treated with PBS were used as the control group and all results were normalized to the control. *P<0.05, ***P<0.001 and ****P<0.0001 vs. the control group. ΔΔΔP<0.001 and ΔΔΔΔP<0.0001 vs. the DD group. #P<0.05, ###P<0.001 and ####P<0.0001 vs. the 0.025 mg/ml WKN group. WKN, weikangning; GSTP1, glutathione-S-transferase P; SOD2, superoxide dismutase 2; DD, domperidone.
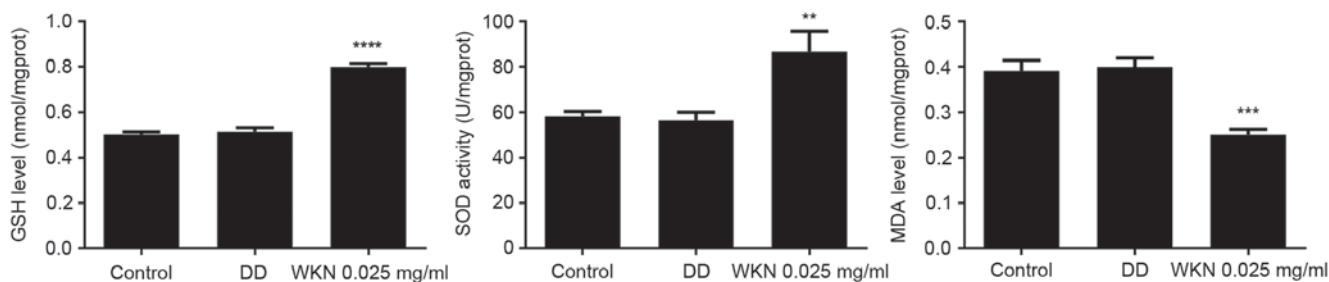

Figure 4. GSH content, SOD activity and MDA content of GES-1 cells following 24 h treatment with 2.5 µmol/ml DD or 0.025 mg/ml WKN. GES-1 cells treated with PBS solution were used as the control group. **P<0.01 and ****P<0.0001 vs. the control group. GSH, glutathione; SOD, superoxide dismutase; MDA, maleic dialdehyde; WKN, weikangning; DD, domperidone.

cells compared with the shMock cells (Fig. 1). Thus, these shNrf cells were taken forward for further experiments.

In the shMock cells, treatment with 0.025 mg/ml WKN significantly increased the mRNA and protein expression of GSTP1 and SOD2 compared with the control group (P<0.05; Fig. 6A and B). In shNrf2 cells, the mRNA and protein expression of GSTP1 and SOD2 were significantly lower compared with the shMock cells, before and after WKN treatment (P<0.01; Fig. 6A and B). Furthermore, when Nrf2 was knocked down, the GSH content and SOD activity of GES-1 cells decreased significantly, and MDA increased significantly, compared with the shMock group (P<0.001; Fig. 6C-E),

despite treatment with WKN. These results indicate that the protective effect of WKN against cellular oxidative stress was significantly impaired by Nrf2 silencing.

The proliferative abilities of shNrf2 and shMock cells were examined using the MTS assay following 4, 48 and 96 h of treatment with 0.025 mg/ml WKN (Fig. 6F). This demonstrated that 48 h of WKN treatment promoted the proliferation of shMock cells, but this effect decreased over time. Under Nrf2 silencing conditions, GES-1 cell growth was slower (P<0.0001; control + shNrf2 vs. control + shMock group). Furthermore, with Nrf2 knockdown, the effect of 48 h WKN treatment cell
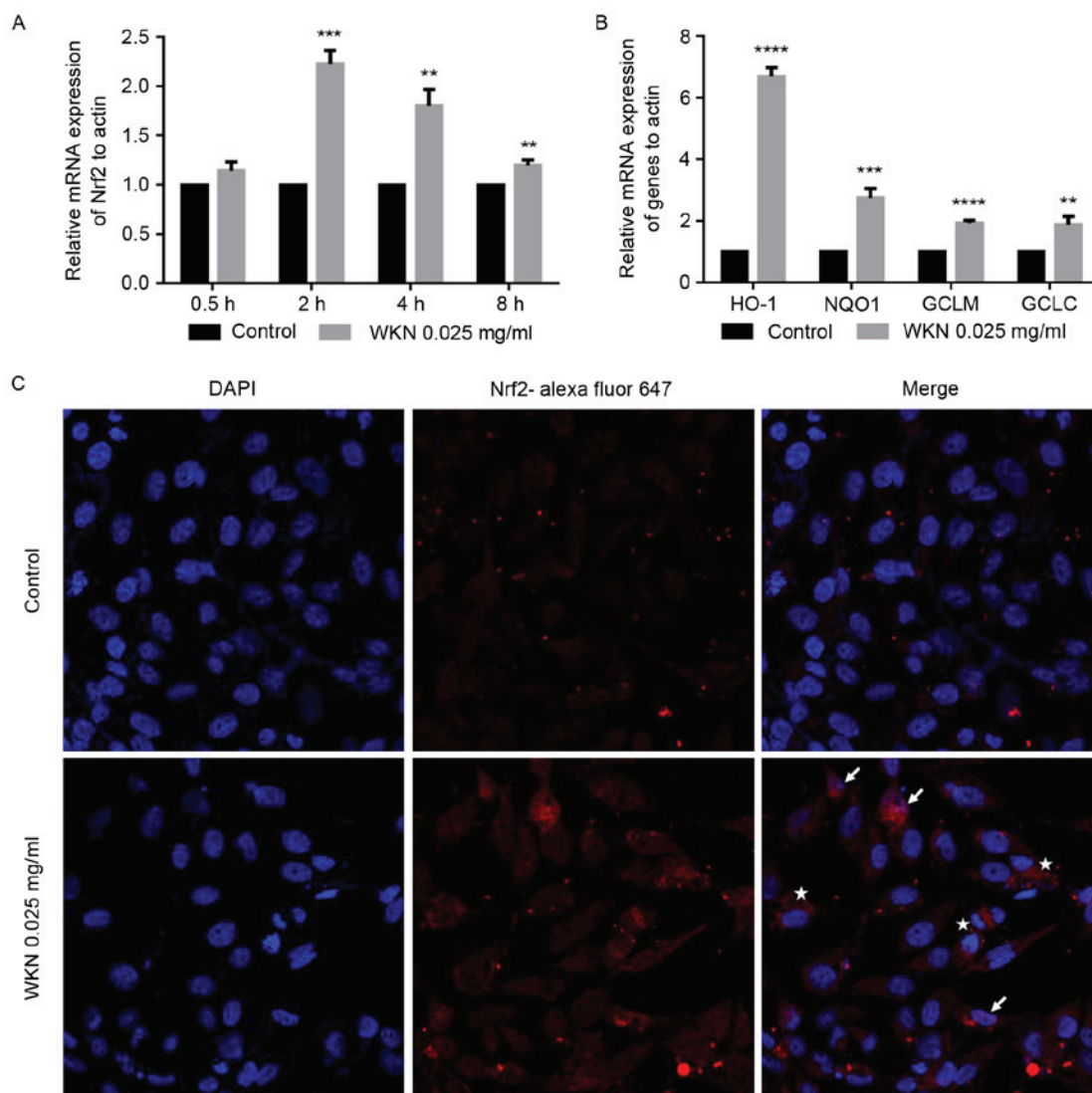
Figure 5. WKN treatment increases the transcription of Nrf2 in GES-1 cells. Transcription of (A) Nrf2 following 0.025 mg/ml WKN treatment for 0.5, 2, 4 and 8 h, and (B) Nrf2 downstream target genes, GCLC, HO-1, GCLM and NQO1 in GES-1 cells following 0.025 mg/ml WKN treatment for 4 h. The results were normalized to the PBS control. (C) Representative immunofluorescence images of Nrf2 staining in GES-1 cells after 24 h treatment with 0.025 mg/ml WKN or PBS solution. White stars indicate accumulated Nrf2 in the cytoplasm and white arrows indicate translocated Nrf2 in the nucleus. Magnification, x800. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ and $^{****}P<0.0001$ vs. the control group. WKN, weikangning; Nrf2, nuclear factor erythroid 2 like 2; GCLC, glutamate cysteine ligase catalytic subunit; HO-1, heme oxygenase 1; GCLM, glutamate cysteine ligase modifier subunit; NQO1, NADPH dehydrogenase quninone 1.

proliferation was significantly reduced (P<0.0001; WKN + shNrf2 vs. WKN + shMock group).

Discussion

In a previous study, our group investigated the pathogenesis of FD, in addition to the therapeutic effect of WKN and the underlying molecular mechanism of this effect, from the perspective of proteomic analysis in a rat model of FD (12). It was demonstrated that the expression levels of GSTP1 and SOD2 were significantly upregulated in the WKN treatment group compared with the disease model group.

GSH-dependent and SOD enzymes are important cellular antioxidants that serve vital roles in resisting the damaging effects of external toxin and ROS (35,36). GSTP1 is a member of the GSTP family, a subtype of GSTs. GSTP1 is capable of catalyzing the conjugate connections between GSH and charged complexes, so as to serve the function of detoxification. A previous study reported that different genotypes of the GSTP1 exon 5 are associated with intestinal metaplasia in the pre-gastric cancer stage, and positively interact with *Helicobacter pylori* infection in the intestinal metaplasia (37). SOD2 is primarily sub-cellularly located in the mitochondria and is an important component of the cellular antioxidant system (38-40). SOD2 maintains the balance between oxidation and antioxidation by reducing superoxides, including the superoxide anion (36). It has previously been reported that SOD2 knockout may be lethal in animals (41), whereas SOD2 overexpression significantly reduces lipid peroxidation, represses protein nitrosylation of protein tyrosine residues, inhibits cellular apoptosis and ameliorates ischemic tissue injury (42). In addition, SOD2 is able to inhibit the activation of the mitochondrial-dependent apoptotic signaling pathway by inhibiting the transfer of cytochrome *c* from mitochondria to the cytoplasm (43-45). There is also evidence that SOD2 serves a more important role than SOD1 in cellular resistance to stress and injury (46).
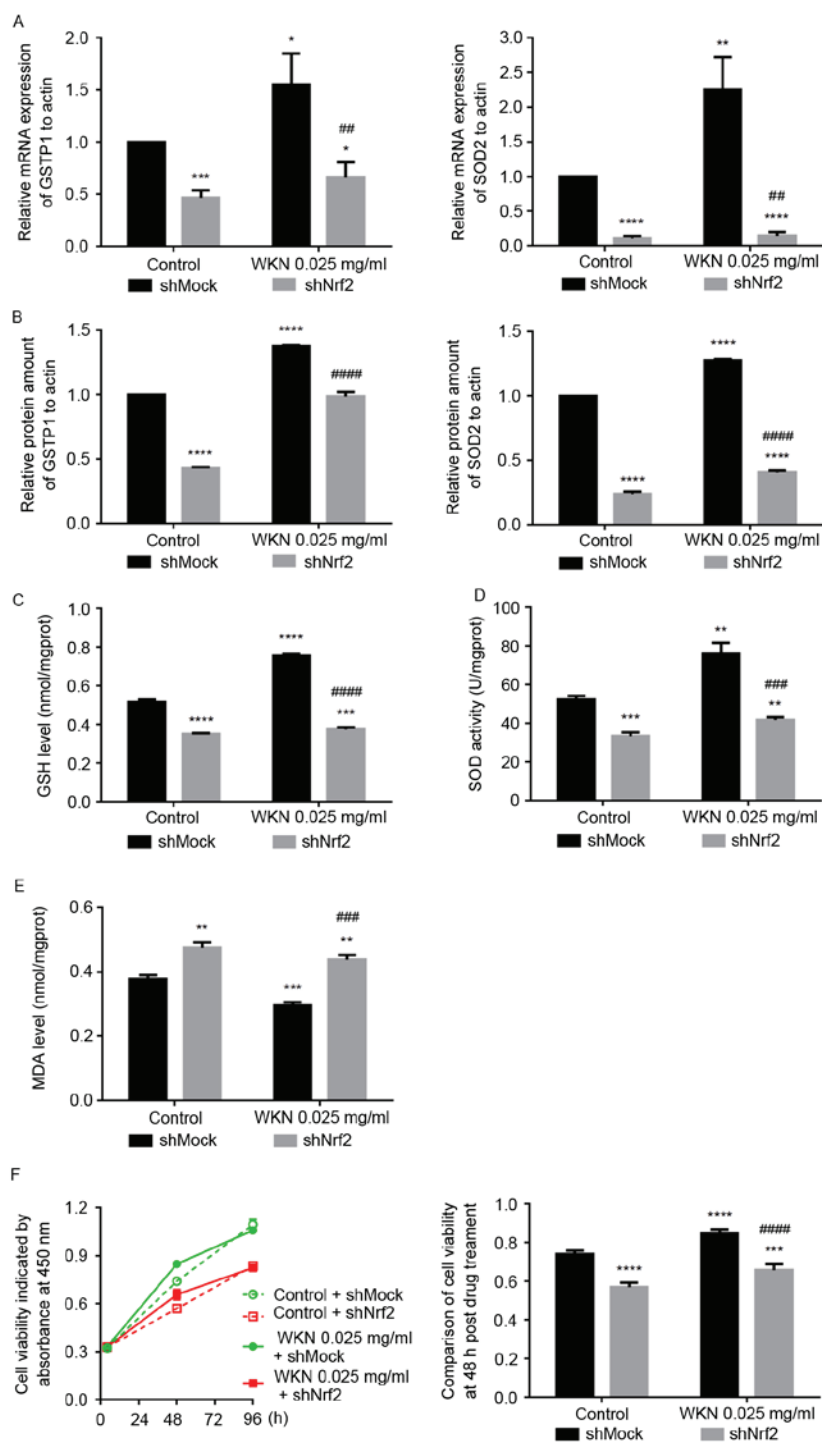
Figure 6. Nrf2 knockdown inhibits the protective effects of WKN on GES-1 cells. After 24 h treatment with 0.025 mg/ml WKN or PBS solution, the levels of expression of GSTP1 and SOD2 (A) mRNA and (B) protein, (C) GSH content, (D) SOD activity and (E) MDA content in shMock cells and shNrf2 cells were asessed. Results were normalized to the values of the control + shMock group. (F) Effect of 0.025 mg/ml WKN treatment on the growth of GES-1 cells prior to and after Nrf2 knockdown was assessed using the MTT assay. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ and $^{****}P<0.0001$ vs. the control + shMock group. $^{##}P<0.01$, $^{###}P<0.001$, $^{####}P<0.0001$ vs. the 0.025 mg/ml WKN + shMock group. Nrf2, **nuclear factor erythroid 2 like 2; WKN, weikangning; GSTP1, glutathione-S-trans**ferase P; SOD2, superoxide dismutase 2; GSH, glutathione; MDA, maleic dialdehyde; shRNA, short hairpin RNA.

Changes in the expression levels of GSTP1 and SOD2 in the FD rat model in our previous study suggested that oxidative stress may participate in the pathogenesis of FD (12). Previous studies have indicated that oxidative stress in GI tissues may serve an important role in the development of gastric damage and disorders, and the protective effects of certain pharmacological agents were reported to be associated with a reduction in oxidative stress (47-49). In the present study, SOD2 and GSTP1 were identified to be upregulated at the transcriptional and protein levels in human mucosal epithelial cells after WKN treatment. Furthermore, the GSH content, SOD activity and MDA content measured in the WKN-treated cells indicated that the WKN decreased oxidative stress in gastric mucosal epithelial cells.

The results of the present study suggest that WKN promotes the expression and thus effects of antioxidant enzymes via the Nrf2/ARE signaling pathway, which was supported by the results of lentiviral-mediated Nrf2 knockdown. Nrf2 is an important transcription factor that exerts a protective effect against oxidative and chemical stress, through regulating the expression of antioxidant proteins via ARE interaction (35). The Nrf2-ARE signaling pathway is the main pathway that induces cell resistance to damage by exogenous substances and oxidative stress. Nrf2 also serves key roles in maintaining homeostasis of the cell microenvironment, and promoting cell growth and reproduction (50,51). Nrf2 is able to protect the lungs, liver and digestive tract without cell or organ specificity (52,53). Consequently, Nrf2 has great therapeutic potential for multi-organ protection (53,54). The results of the present study demonstrated that Nrf2 may serve an essential role in GES-1 cells, as Nrf2 knockdown has an impact on growth and antioxidant activity. However, it remains to be elucidated whether the protective effect of WKN is entirely dependent on Nrf2 signaling.

In conclusion, combined with our previous research, the results of the present study suggest that the therapeutic efficacy of the TCM WKN as a treatment for FD relies on oxidative stress alleviation in GES-1 cells, which is mediated by Nrf2 signaling. The results of the present study also indicate indirectly that oxidative stress may be associated with the pathogenesis and development of GI disorders, something that has been proposed previously but is lacking in evidence (55,56). The beneficial effects of TCMs on inflammatory GI diseases and associated tumorigenesis have been extensively studied, with certain reports suggesting that these treatments act via modulating the Nrf2 signaling pathway (57,58). Future studies should test the results of the present study using an *in vivo* model of FD to increase the understanding of the pathology of functional GI disorders and potentially provide a clinical basis for WKN as a treatment for FD.

Acknowledgements

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