Xuanfudaizhe Decoction Alleviate Reflux Esophagitis through Inhibition of NLRP3/Caspase-1 Pathway

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Abstract

**Background:** Reflux esophagitis (RE) is a clinically common digestive disease, and the main pathological manifestation of RE is esophageal mucosa inflammatory damage. Xuanfu Daize (XFDZ) decoction is a traditional Chinese herbal compound that is famous for RE treatment, but the pharmacological and molecular mechanism of XFDZ remains largely unknown.

**Methods:** The active ingredients of XFDZ were detected using the ultra-performance liquid chromatography/quadrupole time-of-flight tandem mass spectrometry (UPLC/Q-TOF-MS). The rat model of RE was established with pylorus clamp and 2/3 fundus of stomach ligated. XFDZ (8.55 g/kg) and Omeprazole + Mosapride (1.35 g/kg) were orally administered for 14 days. Pathology of esophageal mucosal inflammation was evaluated under microscopy by hematoxylin-eosin (HE) staining. In vitro, by inducing cellular inflammatory response with glycocholic and taurocholic acid mixture (PH 4.7 acid medium added 500 μmol/L concentration of mixed bile acids) in human esophageal epithelial cells (HEEC). The mitochondrial membrane potential was detected using JC-1 fluorescence mitochondrial imaging. The mtDNA copy number was determined via quantitative real-time polymerase chain reaction (qRT-PCR). Fluorescent probe DCFH-DA was used to detect ROS. The relative fluorescence expression of NLRP3 inflammasome was determined by high-intensity cell imaging and quantitative fluorescence techniques. ELISA was used to detect and quantify inflammatory cytokines related to the NLRP3 inflammasome. Western blot analysis was performed to investigate proteins that are associated with the NLRP3 inflammasome.

**Results:** Twenty chemical components such as alkaloids and flavonoids were identified in the analysis of XFDZ, which may be the material basis for XFDZ to exert its effect. XFDZ can significantly reduce the contents of Caspase-1, IL-1β and IL-18 in serum of rats, and down-regulate the protein expression levels of NLRP3, Caspase-1, IL-1β and IL-18 in esophageal tissue. The simulated reflux could decrease the membrane potential, increase the ROS production, decrease the relative expression of mtDNA and activate the NLRP3/Caspase-1 signaling pathway in vitro. XFDZ had no obvious protective effect on the membrane potential and mtDNA, but could inhibit ROS production and the activation of NLRP3/Caspase-1 signaling pathway.

**Conclusion:** We concluded that XFDZ could reduce the inflammatory damage in RE by inhibiting NLRP3/Caspase-1 signaling pathway both in vitro and in vivo, indicating the capability of XFDZ as a promising drug for the treatment of RE.

Introduction

Reflux esophagitis (RE) is one of the most common manifestations of gastroesophageal reflux disease (GERD). It is characterized by esophageal inflammation and endoscopically visible breaks of the distal esophageal mucosa [1, 2]. RE is mainly caused by abnormal reflux with prolonged exposure of gastric contents and associated with the development of Barrett's esophagus, a condition that is the precursor of esophageal adenocarcinoma [3]. Thus, to prevent the development of Barrett's metaplasia and esophageal adenocarcinoma, the pathogenesis of RE has to be understood.

The RE pathogenesis could be attributed to esophageal mucosal inflammation, in this process, the presence of cytokines and inflammatory mediators is indisputable [4]. Recent studies have demonstrated that interleukin-1β (IL-1β), interleukin-18 (IL-18) participate in the development of RE, particularly, significant increases in IL-18 were seen in GERD colonised by *Campylobacter*[5]. Inflammasomes are involved in pathophysiology of diseases by coupling detection of pathogens and cellular stress to active Caspase-1, and consequent maturation of proinflammatory cytokines such as IL-1β and IL-18, which could lead to mitochondrial damage and cell death [6, 7]. Yin et al. found that CaSR, NOD-like receptor protein 3 (NLRP3) inflammasome, Caspase-1, and IL-1β were significantly up-regulated in the oesophagus of modified RE rats [8]. Specifically, aberrant overactivation of the NLRP3 inflammasome has been identified in Barrett's epithelial cells [9]. Despite these findings, the role of NLRP3/Caspase-1 inflammasome signalling pathway participated in RE is unclear.

Xuanfu Daize (XFDZ) decoction is a traditional Chinese medicine that is composed of seven medicinal herbs: *Inulae Flos(xuanfuhua), Haematitum(daizheshi), Pinelliae Rhizoma Praeparatum(qingbanxia), Ginseng Radix Et Rhizoma(renshen), Roast Radix Glycyrrhizae(zhigancao), Jujubae Fructus (dazao) and Zingiberis Rhizoma Recens (shengjiang)* [10]. XFDZ have been recorded in the 2020 edition of Chinese Pharmacopoeia (Part I) [11]. In China, this famous formula has been widely applied for the treatment of digestive system diseases, such as chronic gastritis, stomach neurosis and reflux esophagitis [10, 12-14]. However, there is no sufficient experimental evidence for the effects of XFDZ on RE.

In the present study, we aim to investigate the inflammatory antagonism of XFDZ in RE. The effects of XFDZ on esophageal mucosal tissue morphology, NLRP3 inflammasome and inflammatory factors in RE model rats were observed through experiments in vivo. In vitro, by inducing cellular inflammatory response in human esophageal epithelial cells (HEEC), the effect of XFDZ and its dissolution on the
expression of cytokines related to the NLRP3/Caspase-1 signaling pathway in HEEC injury model was observed. This study would provide new insights into the pathogenesis and treatment of RE.

**Materials And Methods**

**Drug and reagents**

XFDZ includes seven traditional Chinese herbs: Inulae Flos (15 g, Jiangsu, 200515), Haematitum (5 g, Sichuan, 200728), Pinelliae Rhizoma Praeparatum (15 g, Hubei, 200314), Zingiberis Rhizoma Recens (25 g, Shandong, Buy from the supermarket), Ginseng Radix Et Rhizoma (10 g, Jilin, 200321), Roast Radix Glycyrrhizae (15 g, Neimenggu, 200716), Jujubae Fructus (10 g, Shandong, 200706) all of which are provided by the Pharmacy Department of Kunshan Hospital of Traditional Chinese Medicine. The herbs were mixed with an 8-fold volume of distilled water evenly. After the herbs were moistened thoroughly, the mixture was boiled at 100˚C for 40 min and filtered through a gauze to obtain the filtrate. The decoctions were stored at 4˚C. Omeprazole magnesium enteric-coated capsules were purchased from AstraZeneca Pharmaceutical Co., Ltd. (Batch No. J20130093). Mosapride citrate tablets were produced by Lunanbet Pharmaceutical Co., Ltd. (Batch No. H19990317). The antibodies: NLRP3, Caspase-1, IL-1β, IL-18 were purchased from Abcam (UK). All the other chemicals and reagents were of standard commercially available biochemical quality.

**Detection of active ingredients in XFDZ by UPLC/Q-TOF-MS**

The active ingredients of XFDZ were detected using the ultra-performance liquid chromatography/quadrupole time-of-flight tandem mass spectrometry (UPLC/Q-TOF-MS). UPLC/Q-TOF-MS method was adapted from previously established method [15].

**Animals**

Sixty healthy SPF male Wistar rats (220±20 g, 12 weeks) for the experiments were provided by Beijing Speightford Biotechnology Co., Ltd. [Animal license number of the rats was SCXK (Beijing) 2016-0006]. The rats were raised in Tianjin Nankai Hospital Laboratory Animal Research Center. The study protocol was approved by the Animal Ethics Committee of Tianjin Nankai Hospital Laboratory, China (Approval No. NYKK-DWL-2019-083).

**Experiment grouping and establishment of the RE rat model**

Sixty normal SPF Wistar male rats were randomly divided according to their body weight into the following four groups: (a) Sham group; (b) Model group; (c) XFDZ group; (d) Omeprazole + mosapride group. In addition to the sham group, the remaining 45 rats underwent pyloric clip and section ligation for modeling [16]. All the 45 rats were anesthetized using 0.3% sodium pentobarbital (30 mg/kg, intraperitoneally) and were fasted for 24 h preoperatively. The junction of pylorus and duodenum was covered with pylorus clamp with the internal diameter of 4.2 mm avoiding the blood vessels and mesenteric tissues. Then, the closure was clamped tightly with hemostatic forceps to avoid scratching of blood vessels on both sides of pylorus clamp. Then, 2/3 fundus of stomach was ligated to enhance reflux of gastric contents into the esophagus. Fasting and not watering for 24 h postoperatively. Within 3 days after surgery, each rat was injected with levofloxacin hydrochloride injection at 1.5 mg/kg per day in the abdominal cavity, and was given normal saline for injection, then the rats returned to normal diet.

**Drug adminstration**

The rats of model group and sham group were gavaged with normal saline in the ratio of 1 ml/100 g body weight on the 8th day after surgery. The rats of treatment groups were gavaged with the XFDZ (8.55 g/kg) and omeprazole enteric-coated tablets + mosapride (1.35 g/kg) on the 8th day after the operation twice a day. All rats were sacrificed after 14 days to collect specimens for index detection.

**Haematoxylin and eosin (HE) staining and pathological evaluation**

HE staining was performed on paraffin-embedded oesophageal section (0.4 μm). The embedded section was deparaaffinized, hydrated and stained with HE. The stained sections were subsequently observed under an optical microscope.

**Cell culture**

HEEC purchased from Beijing Beinachuanglian Biotechnology Research Institute were cultured in DMEM complete medium containing 10% fetal bovine serum (Gibco, USA). The cell were cultured in a humidified incubator containing 5% CO₂, 37 °C.

**Glycocholic and taurocholic acid exposure**
HEEC were exposed to glycocholic and taurocholic acid mixture to induce cell inflammatory damage[17]. The HEEC were exposed to different concentrations (PH 3, 4, 5, 6) of acid medium and different concentrations (1000, 750, 500, 375 μmol/L) of alkaline medium (A mixture of gallinocholic acid and taurocholic acid in a ratio of 3:1) for 1 h to induce cell damage. A CCK-8 assay was performed to determine the viability of HEEC. Cells were seeded into 96-well plates at 1×10^5 cells/well with complete medium. After exposure to various stimuli, 10 µl CCK8 reagent (Dojindo, Japan) and 100 µl DMEM was then added. The plates were incubated at 37 °C for 2 hour. The absorbance was measured using a microplate reader (ThermoFisher, USA) at 450 nm. Based on the effect on HEEC cell viability, PH 4.7 acidic medium was finally selected with a concentration of 500 μmol/L mixed bile acid as gastroesophageal reflux mimic to construct a reflux injury model of HEEC. When the cells reached 80% confluence, they were treated with the mixture for 1 hour.

### Drug and experiment grouping of the HEEC

In order to further analyze the key active drugs in the XFDZ, the whole prescription can be divided into three groups of drugs according to the taste and function of TCM. Inulae Flos and Haematitum (IH) group are tasting bitter and have the descending effect. Pinelliae Rhizoma Praeparatum and Zingiberis Rhizoma Recenslt (PZ group) have the pungent taste and the dispersing effect. Ginseng Radix Et Rhizoma, Roast Radix Glycyrrhizae and Jujubae Fructus (GRJ group) are tasting sweet and have the role of nourishment. All the herbs'weight, brand, origin, batch as above. XFDZ and its decomposed recipes were used to treat HEEC for 24 h. Then, the cells were exposed to glycocholic and taurocholic acid mixture for 1 h.

### JC-1 fluorescence measurement of the mitochondrial membrane potential

The mitochondrial membrane potential was detected using JC-1 fluorescence mitochondrial imaging. The HEEC were incubated with JC-1 solution for 20 minutes at 37 °C. The cells were then washed twice with JC-1 buffer and medium was added to each well. Images were taken using a microplate reader (ThermoFisher, USA). The ratio of red to green fluorescence represented the mitochondrial membrane potential.

### Relative quantification of mtDNA copy number using real-time PCR

The total genomic DNA was extracted using a DNA extraction kit (DP304, Tiangen, China) from HEEC according to the manufacturer's instructions. The mtDNA copy number was determined via quantitative real-time polymerase chain reaction (qRT-PCR). For qRT-PCR, each genomic DNA sample was mixed with primers (Sangon Biotech, Shanghai, China) and SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). The expression of β-actin gene in the nucleus was used as a control, and the mt-Nd1 gene was used as a target mitochondrial gene. The sequences of the primers used for amplification of the target genes were as follows: 5’-CTCCATCTGGCTCGCTGT-3’ and 5’-GCTGCTACCTTCAACGTCCC-3’ for β-actin; 5’-CCTTCCACCTTATCAAC-3’ and 5’-CATATTAGCCAAGGTAT-3’ for Nd1. The target genes of the mixture were amplified using a CFX96™ Real-Time system (Bio-Rad, Hercules, CA, USA) with the following protocol: 95 °C for 30 s, followed by 42 cycles of 95 °C for 5 s, and 58 °C for 30 s. The relative mtDNA copy number was determined by the 2^-ΔΔCt method [18].

### Detection of intracellular reactive oxygen species (ROS) levels

After stimulation, the cells were placed in test tubes and washed three times with cell culture medium (without serum) after undergoing 2,7-dichlorodi-hydrofluorescein diacetate (DCFH-DA, Beyotime, China) staining. The other cells were treated with Rosup as the positive control. Adherent cells were rinsed with pancreatic enzymes and incubated with DCFH-DA at 37 °C for 20 min. All the cells were observed and analyzed under a fluorescence microscope (Leica, USA). The reaction between the ROS and DCFH-DA resulted in dichlorouorescein (DCF), a compound that emits green fluorescence [19]. The relative fluorescence value, which indicated the relative level of ROS, was determined with Microplate fluorescence wavelength detection (Tecan, Mannedorf, Switzerland).

### NLRP3 expression was detected by high-intensity cell analysis

After treatment with the XFDZ and its decomposed recipes. HEEC exposed to glycocholic and taurocholic acid mixture for 1 h. The cells were washed 3 times with PBS and fixed with 4% paraformaldehyde for 15 min. 0.5% Triton X-100 was permeable at room temperature for 20 min and washed with PBS for 3 times. NLRP3 (1:1000, Santa, sc-518122) was added as primary antibody and incubated overnight at 4°C in a wet box. On the second day, the cells were washed with PBST for 3 times, followed by fluorescent secondary antibody, incubated in a wet box for 2 h at room temperature, soaked with PBST, and stained with DAPI. The fluorescence intensity was observed and calculated under a high-content cell imaging analyzer (IN Cell Analyzer 2500 HS, GE, USA). HEEC were double-stained with NLRP3 and DAPI fluorescence. The effect of drug on NLRP3 expression was investigated by high-connotation cell imaging and fluorescence quantitative techniques.
**Enzyme-Linked Immunosorbent Assay (ELISA)**

XFDZ and its decomposed recipes were used to treat HEEC for 24 h. MCC950 as a specific small-molecule NLRP3 inhibitor, given with a low level (MCC950L group, 10 µmol/L) and the high level (MCC950H group, 50 µmol/L) for 2 h before administration of the simulated reflux. Then, the cells were exposed to glycocholic and taurocholic acid mixture for 1 h. Serum of RE rats and the supernatants from HEEC cell cultures were collected and centrifuged to remove cell debris. The concentration of Caspase-1, IL-1β and IL-18 in serum and cells were detected by ELISA kit (Multi sciences Biotech.CO., China) according to the manufacturer's instructions. All assays were performed in triplicate in three independent experiments.

**Western blot**

Oesophageal strips or cultured cells were collected to extract the protein with RIPA lysis buffer (Beyotime Biotech. CO., China). After complete lysis, the samples were centrifuged at 12,000 r/min for 10 minutes at 4 °C to precipitate the tissue debris. The supernatants were used to measure the protein concentration by a BCA Protein Assay Kit (Biosharp, China), according to the manufacturer's instructions. The proteins were electrophoresed in 5% SDS-PAGE gels and then transferred to PVDF membranes. After blocking with 5% skim milk for 1 hour at room temperature, the membranes were incubated with the following primary antibodies: NLRP3 (1:1,000, ab263899, Abcam), caspase-1 (1:1,000, ab179515, Abcam), IL-1β (1:1,000, ab216995, Abcam), IL-18 (1:200, ab207324, Abcam), β-Tubulin (1:5,000, ab68193, Abcam) and GAPDH (1:1,000, ab181603, Abcam) at 4 °C overnight. The membrane was washed with TBST and incubated with secondary antibodies (1: 5,000, CW0103S, CWBIO) for 2 hours at 25 °C. β-Tubulin and GAPDH were used as loading control. Protein bands were visualized on the membrane with a Gel Imaging System (BIO-RAD, ChemiDocTM XRS+, USA), and the protein bands were quantified with Image Lab software. A high-content analysis system was used to analyze NLRP3 protein expression.

**Statistical analysis**

Each experiment was conducted independently and at least three times for statistical analysis. Student's t-tests were performed to analyze the differences between two groups. The data are shown as the mean ± standard deviation (SD), which were analyzed by one-way analysis of variance (ANOVA), followed by the least significant difference (LSD) test, using SPSS 22.0 software. P < 0.05 was considered statistically significant.

**Results**

**UPLC/Q-TOF-MS analysis of chemical constituents of XFDZ**

By using UPLC/Q-TOF-MS analysis, XFDZ could be analyzed within 21 mintues and exhibited some major peaks in the total ion chromatography. According to the software, the chemical constituents were identified qualitatively. As a result, a total of 17 chemical constituents of XFDZ were identified in positive ion mode and 3 chemical constituents in negative ion mode in UPLC/Q-TOF MS analysis, as shown in Fig. 1. Most of these chemical constituents were glycosides, esters, organic acids and saccharide. Detailed information on the identified chemical constituents is listed in Table 1.
Table 1: UPLC/Q-TOF-MS data of 20 characterized compounds in XFDZ.

<table>
<thead>
<tr>
<th>NO.</th>
<th>RT(min)</th>
<th>M/Z (theoretical value)</th>
<th>M/Z (measured value)</th>
<th>Mode</th>
<th>Secondary ionic fragments</th>
<th>Formula</th>
<th>Error(ppm)</th>
<th>Compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.782</td>
<td>203.0532</td>
<td>203.0532</td>
<td>[M+Na]</td>
<td>145,175</td>
<td>C₆H₁₂O₆</td>
<td>5.9</td>
<td>Fructose</td>
</tr>
<tr>
<td>2</td>
<td>0.789</td>
<td>365.106</td>
<td>365.106</td>
<td>[M+Na]</td>
<td>70,127,296,325</td>
<td>C₁₂H₂₂O₁₁</td>
<td>-1.9</td>
<td>Sucrose</td>
</tr>
<tr>
<td>3</td>
<td>0.818</td>
<td>138.0555</td>
<td>138.0555</td>
<td>[M+H]</td>
<td>70,84,116</td>
<td>C₇H₁₄NO₂</td>
<td>-5.1</td>
<td>Trigonelline</td>
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<tr>
<td>4</td>
<td>0.888</td>
<td>136.0623</td>
<td>136.0623</td>
<td>[M+H]</td>
<td>70,116</td>
<td>C₅H₈N₅</td>
<td>9.6</td>
<td>Adenine</td>
</tr>
<tr>
<td>5</td>
<td>1.03</td>
<td>317.0662</td>
<td>317.0621</td>
<td>[M+HCOO]</td>
<td>85,97,191</td>
<td>C₁₃H₁₂O₅</td>
<td>0.3</td>
<td>Naringenin</td>
</tr>
<tr>
<td>6</td>
<td>1.209</td>
<td>268.1046</td>
<td>268.1046</td>
<td>[M+H]</td>
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<td>C₁₀H₁₃N₅O₄</td>
<td>2.6</td>
<td>L-adenosine</td>
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<td>7</td>
<td>1.437</td>
<td>132.1025</td>
<td>132.1025</td>
<td>[M+H]</td>
<td>81,84,127</td>
<td>C₆H₁₃NO₂</td>
<td>6.8</td>
<td>L-leucine</td>
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<tr>
<td>8</td>
<td>3.566</td>
<td>127.0395</td>
<td>127.0395</td>
<td>[M+H]</td>
<td>70,84,118</td>
<td>C₆H₈O₃</td>
<td>4.7</td>
<td>5-hydroxymethyl furfural</td>
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<td>9</td>
<td>4.862</td>
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<td>355.1029</td>
<td>[M+H]</td>
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<td>Chlorogenic acid</td>
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<td>10</td>
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<td>565.1557</td>
<td>[M+H]</td>
<td>85,270,481</td>
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<td>Isoschaftoside</td>
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<tr>
<td>11</td>
<td>8.428</td>
<td>419.1342</td>
<td>419.1342</td>
<td>[M+H]</td>
<td>85,147</td>
<td>C₂₁H₂₂O₉</td>
<td>2.6</td>
<td>Liquiritin</td>
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<tr>
<td>12</td>
<td>12.308</td>
<td>267.1596</td>
<td>267.1596</td>
<td>[M+H]</td>
<td>70,127,206</td>
<td>C₁₅H₂₂O₄</td>
<td>4.5</td>
<td>4q,6q-Dihydroxyeudesman-8β,12-olide</td>
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<tr>
<td>13</td>
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<td>263.1283</td>
<td>263.1283</td>
<td>[M+H]</td>
<td>59,133,143,183</td>
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<td>-1.9</td>
<td>Helenalin</td>
</tr>
<tr>
<td>14</td>
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<td>431.1342</td>
<td>431.1342</td>
<td>[M+H]</td>
<td>114,269</td>
<td>C₂₂H₂₂O₉</td>
<td>2.3</td>
<td>Ononin</td>
</tr>
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<td>15</td>
<td>12.81</td>
<td>839.4225</td>
<td>839.4283</td>
<td>[M-H]</td>
<td>79,1837</td>
<td>C₄₂H₆₅NO₁₆</td>
<td>-2.5</td>
<td>Mon ammonium glycyrrhizinate</td>
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<td>16</td>
<td>16.088</td>
<td>249.1491</td>
<td>249.1491</td>
<td>[M+H]</td>
<td>88,145,185</td>
<td>C₁₅H₂₀O₃</td>
<td>-0.4</td>
<td>4-epi-isoinuviscolide</td>
</tr>
<tr>
<td>17</td>
<td>16.095</td>
<td>249.1491</td>
<td>249.1491</td>
<td>[M+H]</td>
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<td>C₁₅H₂₀O₃</td>
<td>-0.4</td>
<td>Parthenolide</td>
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<tr>
<td>19</td>
<td>17.184</td>
<td>277.1804</td>
<td>277.1804</td>
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<td>117,137,145,177</td>
<td>C₁₇H₂₄O₃</td>
<td>4.7</td>
<td>6-shogaol</td>
</tr>
<tr>
<td>20</td>
<td>36.78</td>
<td>821.396</td>
<td>822.4055</td>
<td>[M-H]</td>
<td>153,303,351,795</td>
<td>C₄₂H₆₂O₁₆</td>
<td>2.1</td>
<td>Glycyrrhizic acid</td>
</tr>
</tbody>
</table>

**Weight change in the rat**

Eight of 45 model group rats died after surgery (survival rates: 62%), and sham group rats were all survival until acquirement of the esophageal tissue. After surgery, the weight loss of the rats in the model group was rapid and lower than before the experiment, and the weight loss was obvious compared with that of the sham group of rats. Although the weight of the rats in the XFDZ group and the omeprazole+mosapride group was still lower than the preoperative weight, they were both higher than those in the model group. After treatment with XFDZ and omeprazole + mosapride, there was no significant difference in the weight change of rats, as shown in Table 2.
Table 2 The weight change before and after treatment.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>W2-W1 (X±s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>15</td>
<td>32.0±4.5</td>
</tr>
<tr>
<td>Model</td>
<td>12</td>
<td>-40.7±5.0▼</td>
</tr>
<tr>
<td>XFDZ</td>
<td>13</td>
<td>-17.2±5.1△</td>
</tr>
<tr>
<td>Omeprazole+mosapride</td>
<td>12</td>
<td>17.7±1.0△</td>
</tr>
</tbody>
</table>

Note: ▼Compared with normal group, P<0.05. △Compared with model group, P<0.01. W1: the weight of rats before treatment. W2: the weight of rats after treatment.

Evaluation of Esophagus Lesions

The esophagus lesions of rats were observed under the light microscopy. The surface of mucosal layer in sham group was presented as non-keratinized multilayer squamous epithelium. There were a few melanoblasts and endocrine cells in the basal cell layer. The basal layer cell proliferation was seen in the model group. The papillae of the lamina propria of the squamous epithelium become longer and edematous, and there were neutrophil infiltrations. In addition, the mucosal erosion and ulceration could be seen. These changes were gradually lighter at the lower end of the esophagus (Fig. 2). In XFDZ group, there was no obvious change in the squamous epithelium and the mucous membrane was relatively intact. Moreover, the erosion and ulceration were rare. A few lymphocytes and monocytes were infiltrated in the mucosal interstitium (Fig. 2). In omeprazole + mosapride group, the squamous epithelial cells were slightly edematous, and the mucous membrane was relatively complete. Lesions such as erosion ulcers were observed. The chronic inflammatory cell infiltration was seen in the propria membrane (Fig. 2).

Modified RE rats exhibited up-regulation of the NLRP3/Caspase-1 inflammasome signalling pathway, and XFDZ effectively blocked NLRP3 inflammasome activation

To assess whether the NLRP3/Caspase-1 inflammasome signalling pathway is up-regulated in the impaired oesophagus, we used western blot techniques to test the expression of pathway-related proteins. We found that compared with the sham group, NLRP3, Caspase-1, IL-18 and IL-1β were significantly up-regulated in the oesophageal tissues of the treatment groups. Moreover, the contents of NLRP3, Caspase-1, IL-18 and IL-1β proteins in the esophageal tissues of the treatment groups were reduced with a statistically significant difference, and there was no significant difference between the XFDZ group and the omeprazole + mosapride group (Fig. 3).

By detecting the serum Caspase-1, IL-1β and IL-18 contents of the four groups, it was found that in serum Caspase-1, IL-1β and IL-18 levels of the rats in the model group were higher than those in the sham group. The serum Caspase-1, IL-1β and IL-18 levels were lower than those in the model group after drug intervention in XFDZ group and omeprazole + mosapride group, and there was no statistically significant difference between XFDZ group and omeprazole + mosapride group (Fig. 4).

Glycocholic and taurocholic acid treatment reduced the mitochondrial membrane potential and mtDNA copy number in HEEC, while XFDZ failed to improve mitochondrial membrane potential and mtDNA

After stimulation with glycocholic and taurocholic acid, the mitochondrial membrane potential decreased in HEEC. However, XFDZ and its decomposed recipes failed to increase the mitochondrial membrane potential of HEEC (Fig. 5a). After treatment, the relative expression of mtDNA in HEEC decreased, but XFDZ failed to increase mtDNA expression (Fig. 5b). It was suggested that XFDZ has no significant effect on the mitochondrial membrane potential and mtDNA copy number in glycocholic and taurocholic acid-stimulated HEEC.

Exposure of HEEC to glycocholic and taurocholic acid improves the ROS levels, and XFDZ declines the ROS induced by glycocholic and taurocholic acid in HEEC

The intracellular fluorescence intensity, which indicated the relative level of ROS, presented increased after stimulation of HEEC with glycocholic and taurocholic acid mixture. The fluorescence intensity in XFDZ group, IH group and GRJ group all showed decreased when compared with the model group, which suggested that XFDZ and its decomposed recipes could antagonize the oxidative stress induced by reflux according to inhibiting ROS level (Fig. 6).

Exposure of HEEC to glycocholic and taurocholic acid accelerates NLRP3 inflammasome activation, and XFDZ downregulates the NLRP3/Caspase-1 inflammasome signalling pathway induced by glycocholic and taurocholic acid in HEEC
To further confirm that NLRP3/Caspase-1 inflammasome signalling pathway is up-regulated in the oesophageal epithelium, we detected the expression of NLRP3, Caspase-1, IL-1β and IL-18 in HEEC stimulated by glycocholic and taurocholic acid mixture. The results of high-content analysis and western blot showed that exposure to glycocholic and taurocholic acid mixture significantly increased the expression of NLRP3 (Fig. 7).

Western blot and ELISA indicated that exposure to glycocholic and taurocholic acid mixture increased the expression of Caspase-1, IL-1β and IL-18, as shown in Fig. 8 and Fig. 9 respectively. XFDZ and MCC950 treatment effectively reduced the glycocholic and taurocholic acid-induced up-regulation of NLRP3, Caspase-1, IL-1β and IL-18 expression.

**Discussion**

The NLRP3 inflammasome is linked to sterile and pathogen-dependent inflammation, and its dysregulation underlies many chronic diseases. Recent studies have shown that inflammasome is closely related to the occurrence and development of a variety of digestive diseases. Most of the research has focused on inflammatory bowel disease, there are few studies on NLRP3 inflammatory bodies in reflux esophagitis. Li et al. gave the mouse model of ulcerative colitis (UC) buzhongyiqi decoction after 2 weeks of modeling, and found the intestinal NLRP3 inflammasome component of mice was downregulated after 3-4 weeks, which suggested that tonic decoction ameliorate UC by regulating NLRP3 inflammasomes [20]. Qin et al. showed that the mechanism of action in the treatment of UC might be related to the inhibition of the mRNA expression of NLRP3 inflammasome [21]. Zhang et al. found that Samling Baishusan could reduce intestinal inflammation and relieve intestinal mucosal damage by regulating NLRP3 inflammatory factors, and play a therapeutic role in 3% dextran sodium sulfate (DSS)-induced UC mice[22]. Liu et al. showed that peony glycosides could ameliorate the pathological symptoms of UC mice by inhibiting NLRP3 inflammasome [23].

For clarity the correlation between NLRP3/caspase-1 signaling pathway and esophageal inflammation and research the mechanism of XFDZ in the treatment of RE we observed the effects of XFDZ on the composition of inflammatory bodies and the expression of related inflammatory factors in rats with RE. The results suggest that NLRP3 inflammasories are activated in the model of RE, and NLRP3 recruits caspase-1 to form NLRP3 inflammasories, which increase the expression of IL-1β and IL-18 inflammatory factors downstream of the signaling pathway. However, XFDZ can effectively reduce the expression of NLRP3, Caspase-1, IL-1β and IL-18 cytokines in RE rats, suggesting that XFDZ can effectively block the assembly and activation of NLRP3 inflammasome.

**Conclusions**

NLRP3 inflammasome is a multimolecular complex that plays an important role in the body's immunity. NLRP3 inflammasome could be activated by a variety of internal and external factors. Mitochondria have been implicated as regulators of the NLRP3 inflammasome through several mechanisms including generation of mitochondrial ROS[24]. To further study the main components and effects of XFDZ on NLRP3 inhibition, the effect of XFDZ and its dissolution on the expression of cytokines related to the NLRP3/Caspase-1 signaling pathway in HEEC injury model was observed in vitro. We found that XFDZ could effectively ameliorate the cell damage induced by simulated reflux, and the inhibitory effect of XFDZ on NLRP3 inflammasome may be achieved by clearing ROS. The clearance rate of ROS in IH group was even better than that in XFDZ group, which was considered as the key drug of XFDZ. ROS are mainly clusters of highly reactive oxygen species produced by the synthesis of ATP in mitochondria, which is mainly produced when the electron transport chain of mitochondria changes from state I to state IV. Whether the ROS clearance effect of XFDZ is realized by affecting the mitochondrial respiratory chain and the down-regulation effect of IH group remains to be further studied.

NLRP3 inflammasome is an important part of the body's natural immunity. Inflammatory responses are response to resist damage caused by various dangerous stimuli, which play an important role in the normal physiological activities of the body. In general, inflammatory response is one of trigger factors leading to a variety of diseases. Therefore, antagonistic inflammation is closely related to the treatment of digestive system diseases in modern medicine. Rsophageal inflammatory response could be reduced by regulating ROS, interfering with NLRP3/Casapsae-1 signaling pathway, and inhibiting NLRP3 inflammasome activation. Our study may provide new insights into the clinical treatment of RE.

**Conclusion**

XFDZ can reduce the inflammatory response in RE rats, and promote the recovery of esophageal mucosal injury by regulating NLRP3/Caspase-1 signaling pathway. In addition, the ROS eliminates and anti-inflammatory effects of XFDZ were confirmed on glycocholic and taurocholic acid mixture induced HEEC, and at the same time down-regulates the activation level of NLRP3/Caspase-1 signaling pathway. Therefore, XFDZ has the possibility of being a good choice for RE treatment.
Declarations

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Abbreviations

RE: Refux esophagitis; GERD: Gastroesophageal reflux disease; NLRP3: NOD-like receptor protein 3; IL-1β: Interleukin-1β; IL-18: Interleukin-18; CaSR: Calcium sensitive receptor; HEEC: Human esophageal epithelial cells; ROS: Reactive oxygen species; DMEM: Dulbecco’s modified eagle medium; TBST: Tris Buffered Saline Tween; UC: Ulcerative colitis

Availability of data and materials

The datasets used in this study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

The study protocol was approved by the Animal Care and Use Committee of Tianjin Nankai Hospital (Approval No. NKYY-DWLL-2019-082, date of approval, May 7, 2019).

Competing interests

The authors declare that they have no competing interests

Consent for publication

Not applicable.

Author’s contributions

LJ, WY and DL are the co-first author, they performed the experiments, data analysis, partial molecular biology experiments and manuscript writing, made the same contribution in this study. YHX supervised the study; MJM, LYT, LY and QJT finished the animal experiment; GMM provided the quality control of Chinese traditional medicine; All the authors read and approved the final version of the manuscript.

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References


Figures

**Figure 1**

UPLC/Q-TOF-MS was used to detect the composition of XFDZ. **a** Positive ion detection mode of XFDZ extract total ion current; **b** Negative ion detection mode of XFDZ extract total ion current. Structures of these compounds are listed in Table 1.
Figure 2

XFDZ improves the esophageal lesions of rats with RE. **a** HE staining of the oesophagus in each group, scale bar = 200 μm. **b** Pathological scores of esophageal mucosa in each group. Bar graphs represent the means ± SD. *P <0.05, compared with the sham group; #P <0.05 and +P <0.01, compared with the model group.
Figure 3

XFDZ reduces the contents of NLRP3, Caspase-1, IL-1β and IL-18 proteins in RE rats. a Western blot for the protein expression of NLRP3, caspase-1, IL-1β and IL-18 in the oesophagus. b-e Quantitative analysis of the expression levels of NLRP3, caspase-1, IL-1β and IL-18. Bar graphs represent the means ± SD. *P < 0.05 and #P < 0.01, compared with the sham group; †P < 0.05 and ‡P < 0.01, compared with the model group.
Figure 4

XFDZ decreases the serum Caspase-1, IL-1β and IL-18 contents of RE rats. a-c ELISA for the expression of caspase-1, IL-1β and IL-18 in each group. Bar graphs represent the means ± SD. *P < 0.05 and ■P < 0.01, compared with the sham group; #P < 0.05 and +P < 0.01, compared with the model group.
Figure 5

XFDZ not really effective to rise mitochondrial membrane potential and mtDNA in HEEC. **a**, **c** JC-1 accumulates in the matrix of mitochondria and forms polymer when the mitochondrial membrane potential is high (red), JC-1 is a monomer when the mitochondrial membrane potential is low (green), scale bar = 100 μm. **b** Real-time PCR was used to relative quantification of mtDNA copy number. Bar graphs represent the means ± SD. *P < 0.05 and **P < 0.01, compared with the sham group.
Figure 6

XFDZ could inhibit ROS production in HEEC. 

a The fluorescence probe DCFH-DA was used to detect ROS (green), scale bar = 50 μm.

b ROS fluorescence value. Bar graphs represent the means ± SD. ■P < 0.01, compared with the sham group; +P < 0.05 and +P < 0.01, compared with the model group.
Figure 7

XFDZ effectively reduced the NLRP3 expression in HEEC. **a,b** Western blot for the expression of NLRP3. **c,d** High-intensity cell assay for the expression of NLRP3, fluorescently labeled NLRP3 inflammasome (red) and DAPI reagent labeled total cell volume (blue), scale bar = 100 μm. Bar graphs represent the means ± SD. ■P < 0.01, compared with the sham group; ◊P < 0.05 and ♦P < 0.01, compared with the model group.
XFDZ reduces the contents of Caspase-1, IL-1β and IL-18 proteins in HEEC. **Figure 8**

*a* Western blot for the protein expression of caspase-1, IL-1β and IL-18 in the HEEC.

**Figure 9**

Quantitative analysis of the expression levels of caspase-1, IL-1β and IL-18. Bar graphs represent the means ± SD. *P < 0.05 and ■P < 0.01, compared with the sham group; ♠P < 0.05, compared with the model group.
XFDZ effectively decreases the Caspase-1, IL-1β and IL-18 contents of HEEC. a-c ELISA for the expression of caspase-1, IL-1β and IL-18 in each group. Bar graphs represent the means ± SD. ▼P < 0.01, compared with the sham group; ▲P < 0.01, compared with the model group.