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Gastroprotective effects of Kangfuxin-against ethanol-induced gastric ulcer via attenuating oxidative stress and ER stress in mice

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ABSTRACT

Oxidative stress and ER stress play a role in the pathogenesis of gastric ulcer. Kangfuxin (KFX) has been used to treat gastric ulcer in patients. However, the underlying mechanisms of KFX action remain unclear. The current study was undertaken to evaluate the gastroprotective effects of KFX and to determine its potential mechanisms. Ethanol-induced gastric ulcer mouse model was employed. Ethanol pretreated mice were treated with low (0.02 g/kg) and high (0.05 g/kg) dose of KFX for 14 days. Cimetidine (0.8 g/kg) was used as positive control. Histological evaluation of the gastric mucosa revealed that mice treated with ethanol exhibited severe gastric mucosal damage. Ethanol treatment increased plasma and gastric MDA level, decreased plasma and gastric SOD activity, and reduced gastric HO-1 and GCL-c mRNA levels. ER stress markers (CHOP, GRP78, and caspase 12) were up-regulated upon ethanol administration. Moreover, increased cell apoptosis and pro-apoptotic protein Bax and caspase 3 were observed in ethanol treated mice, while the anti-apoptotic protein Bcl 2 was inhibited. Finally, KFX treatment reversed ethanol-induced phenotypes and ameliorated gastric ulcer. Our results demonstrated that the gastroprotective effects of KFX against ethanol-induced gastric ulcer could be attributed to its anti-oxidative stress, anti-ER stress and anti-apoptotic effects.

1. Introduction

Gastric ulcer is characterized by ulceration of the gastric mucosa and is one of the common diseases of the digestive system that affect many humans. Patients with gastric ulcer often suffer from abdominal discomfort, severe pain, nausea, and a burning sensation in the stomach. The aggressive factors such as hydrochloric acid, pepsin, and bile salts lead to gastric mucosal injury, but mucus bicarbonate layers serve to protect mucosa against aggressive factors [1]. These factors are maintained at a homeostatic state under normal physiological conditions. However, gastric ulcer could be occur when the balance of these factors is disrupted. The current treatments for gastric ulcer include proton pump inhibitors, M1-receptor blockers, and H2-receptor antagonists [2].

Consumption of alcohol has been implicated as contributing factors to gastric ulcer [3]. Previous study has demonstrated that alcohol promotes the development of gastroesophageal reflux disease [4]. Alcohol absorption into the bloodstream occurs throughout the gastrointestinal tract. Its direct contact with the mucosa could damage the mucosa, which could eventually result in various acute and chronic diseases such as gastrointestinal bleeding

Abbreviation: ER stress, endoplasmic reticulum (ER) stress; GRP78, glucose-regulated protein 78; CHOP, C/EBP homologous protein; LKFX and HKFX, low dose and high dose Kangfuxin; CMD, cimetidine; MDA, malondialdehyde; SOD, superoxide dismutase; HO-1, heme oxygenase-1; GCL-c, glutamate-cysteine ligase catalytic subunit; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; ROS, reactive oxygen species; GPx, glutathione peroxidase; CAT, catalase; Nrf2, NF-E2-related factor; NSAIDs, Non-steroidal anti-inflammatory drugs; CFDA, China Food and Drug Administration; bFGF, basic fibroblast growth factor.

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and ulcer [5]. In addition, alcohol-induced gastric ulcer model has been used for evaluate the protective effects of new potential therapeutic targets on the gastrointestinal tract [6].

Alcohol consumption has been demonstrated to lead to oxidative stress, which could result in overproduction of reactive oxygen species (ROS) that might play an important role in gastric ulcer [1,7]. Previous studies have shown that elevated lipid peroxidation and MDA, along with mucosal impairment, are associated with ethanol-induced gastric ulcer in rats [8,9]. By contrast, administration of antioxidants and/or increased antioxidant enzymes protect against ethanol-induced gastric ulcer via inhibiting oxidative stress-induced cell damage [10,11].

Oxidative stress induces endoplasmic reticulum (ER) stress, and ER stress has been shown to involve in the pathogenesis of stress-induced gastric ulcer [12,13]. For example, ER stress can be induced after non-steroidal anti-inflammatory drugs (NSAIDs) administration which could lead to gastric mucosal cells being resistant to NSAIDs-induced cell apoptosis *in vitro*, and amelioration of NSAID-induced gastric lesions *in vivo* [14,15]. On the contrary, hydrogen sulphide (H2S) has been shown to reduce gastric ulcer index, and improve stress-induced gastric ulcer through inhibiting glucose-regulated protein 78 (GRP78) and caspase 12, which are both markers of ER stress [16].

Kangfuxin (KFX) is a pure Chinese herbal medicine that is an extract of Peripleneta Americana, and has been approved by the China Food and Drug Administration (CFDA) (Z51021834). The chemical composition of KFX are mainly amino acids, small molecular peptides, and nucleotides. The therapeutic effects of KFX include promotion of tissue wound healing, especially in gastric and duodenal ulcer. Preclinical study has shown that administration of KFX promotes synthesis and secretion of extracellular matrix in dermal wound site, and facilitates wound healing [17]. In addition, KFX enhances pressure sore healing [18] and promotes diabetic foot ulcer in patients [19]. Clinical studies also reported that KFX exhibits therapeutic effects on ulcerative colitis [20,21] and gastrointestinal ulcer [22]. Recently, a multicenter prospective randomized clinical study demonstrated that KFX lowers incidence of oral pain and gastrointestinal mucositis induced by chemoradiotherapy [23].

As described above, accumulating clinical studies have demonstrated that KFX is a promising therapeutic drug for the treatment of wound healing, including gastric ulcer. However, to date, the underlying mechanisms of KFX in ameliorating gastric ulcer are still not well described. Based on previous reports from others, we hypothesize that KFX improves alcohol-induced gastric ulcer by inhibiting oxidative stress as well as ER stress. In this study, we utilized ethanol-induced gastric injury mice model to determine if ethanol treatment induces oxidative stress and ER stress, ultimately leading to cell apoptosis and gastric injury; and KFX improves ethanol-induced gastric ulcer via inhibition of oxidative stress, ER stress as well as cell apoptosis.

2. Materials and methods

2.1. Reagents and antibodies

Anti-GAPDH primary antibody, and appropriate secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-CHOP antibody was purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-active and procaspase 3, anti-caspase 12 and anti-GRP78 antibodies were obtained from Abcam (Abcam, Cambridge, MA). Cimetidine was ordered from Aladdin (Aladdin, Shanghai, China). SOD and MDA kit were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). TUNEL kit was obtained from Roche (Roche, Shanghai, China). Kangfuxin (KFX) was provided by Good Doctor Pharmaceutical Group (Z51021834, Good Doctor Pharmaceutical Group, Sichuan, China). All other reagents were obtained from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO).

2.2. Animal treatment

This study was reviewed and approved by the Ethics Committee for Experimental Animals of Wenzhou Medical University. 30 male C57BL/6 mice (age 4 months) were obtained from the Animal Center of the Chinese Academy of Sciences and maintained in a temperature-controlled room with a 12-h dark–light cycle and food and water were provided ad libitum. Gastric mucosal injury (GI) was induced using a single intragastric dose of absolute ethanol (10 ml/kg) by oral gavage [24]. The normal group received an equal volume of saline instead of ethanol. The mice were randomly assigned into five groups according to the intervention:(1) Normal group: non-injury mice were administered saline orally per day; (2) Control group: GI mice were administered saline orally per day; (3) Low dose KFX group (LKFX): GI mice were administered KFX (0.02 g/kg) orally per day; (4) High dose KFX group (HKFX): GI were administered KFX (0.05 g/kg) orally per day; (5) CMD group: GI mice were administered cimetidine (0.8 g/kg) orally per day. The animals were euthanized at the end of 14 days, and their stomachs were dissected. Macroscopic observation of the extent of damage was performed. Then, each stomach was dichotomized. One moiety was fixed for histopathological and immunohistochemical assessment while the other was stored at −80 °C for mRNA and protein analyses.

2.3. Haematoxylin–eosin (HE) staining and microscopic scoring of gastric damage

The specimens were processed using standard paraffin-embedding protocol and sections were cut at thickness of 5 μm. Sections were stained with haematoxylin and eosin for histological evaluation. Gastric microscopic damage was scored on a 0–9 scale with modified criteria as previously described [25,26]. Each section was examined for interstitial edema (score: 0–3), hemorrhagic damage (score: 0–3), and the presence of inflammatory cells (score: 0–3).

2.4. Mucosal glycoprotein detection (PAS staining)

Paraffin sections were deparaffinized, rehydrated, oxidized, and washed in distilled water. Periodic acid Schiff (PAS) staining were performed in each group as previously described [27]. Results were evaluated by light microscopy.

2.5. Immunohistochemistry

After deparaffinization and dehydration, paraffin sections were incubated in 3% H_2O_2 and 80% methanol for 30 min, and then in blocking solution for 1 h at room temperature. The sections were then incubated overnight at 4 °C with the following primary antibodies: caspase 3 (1:200) and caspase 12 (1:200). After washing with PBS, sections were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies for 2 h at 37 °C, followed by addition of 3, 3′-diaminobenzidine (DAB). The nuclear were stained with haematoxylin. Images were acquired at ×200 magnification on a Nikon digital camera. Optical density was measured automatically at eight randomly selected fields at lesion site per sample using the Image-Pro Plus 6.0.