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Studies on Mechanism of Preventive Effect of Fermentation Product of *Calvatia lilacina* on Water-Immersion Stress Induced Gastric Ulcers in Mice

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Authors' Contribution
DH conceived the study, designed the experiments, supervised the research, analyzed the data and revised the manuscript. HW and JW completed the experiments and analyzed the data. DH wrote the manuscript. TYZ, XYL and SMW did the experiments.

Key words
***Calvatia lilacina*, Fermentation product, Gastric ulcer, Inflammation, Water-immersion, Restraint stress**

ABSTRACT

To explore the potential mechanism of the fermentation product (FP) of *Calvatia lilacina* on gastric ulcer, fifty mice were randomly divided into 5 groups (10 mice per group), namely control (normal saline), model (normal saline), ranitidine group (0.03 g/kg) and FP groups (1 and 2 g/kg). Stress gastric ulcer was induced by water-immersion and restraint stress (WIRS) after 10 days of drug administration. To observe the histopathological changes of gastric mucosa. the activities of pepsin, superoxide dismutase (SOD), malondialdehyde (MDA), nitric oxide (NO) in gastric tissue and the levels of tumor necrosis factor (TNF–α) and interleukin (IL-6) in serum were measured through ELISA, and the contents of NLRP3, ASC, caspase-1 and IL-1β in gastric tissue were detected by Western blot. The results showed that FP of *C. lilacina* could effectively prevent the stress gastric ulcer induced by WIRS in mice by reducing the expression level of NLRP3/ASC/caspase-1.

INTRODUCTION

Gastric ulcer is a common disease, the incidence of which is increasing year by year (Hung *et al*., 2019; Zakaria *et al*., 2011). There are many causes of gastric ulcer, including irregular diet, long-term drinking, the use of non steroidal anti-inflammatory drugs (NSAIDs), helicobacter pylori infection, work pressure, mental anxiety, etc., (Hunt *et al*., 2015). In recent years, with the acceleration of the pace of human life and the adjustment of dietary structure, negative emotions and life pressure have gradually become the main causes of gastric ulcers (Lee *et al*., 2017).

The occurrence of stress gastric ulcer is mainly acute gastric mucosal lesion characterized by inflammatory erosion, superficial ulcers and bleeding under stress condition, including various trauma, critical illness, shock, etc. (Cheng *et al*., 2017). In the stress state, the protective barrier of gastric mucosa was destroyed, and the increase of endogenous and exogenous attack factors accelerated the apoptosis of gastric mucosa cells, resulting in the disorder of gastric mucosa cell metabolism, the disorder of blood circulation of gastric mucosa, and the destruction of mucus mucosa barrier (Kubben *et al*., 2007). At present, the mechanism of stress gastric ulcer is not clear. Studies have shown that the main causes of stress gastric ulcer may be the decrease of pH value of gastric juice, mucosal ischemia, oxidative stress and over expression of inflammatory factors (Jo *et al*., 2013; Ye *et al*., 2013; Sindhu and Kuttan, 2012).

Puffballs are large fungus, mainly grown on the grassland in the wild. Edible when young, fruiting body can be used medicinally after maturity and have a variety of pharmacological effects, including bacteriostasis, anti-inflammatory, antitussive, antitumor and cell proliferation inhibition (Lee *et al*., 2018; Eroğlu *et al*., 2016; Tsay *et al*., 2009).

Therefore, the liquid fermentation products of *C. lilacina* was selected as a material in this experiment. Through this study, a stable, reliable, fast and high-yield fermentation technology of *C. lilacina* was determined, and a large number of fermentation products were obtained and the effect of fermentation produced by *C. lilacina* was further investigated.

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MATERIALS AND METHODS

Materials and reagents

Calvatia lilacina (Mont. et Berk.) Lloyd was provided by the Mycological Medicine Research Laboratory of Jilin Bioengineering Technology Center. The ELISA assay kits for IL (interleukin)-6, TNF-α (tumor necrosis factor α), Pepsin, SOD (superoxide dismutase), MDA (malondialdehyde), and NO (nitric oxide) were purchased from Meimian Biotechnology (Yancheng, Jiangsu, China). Mouse anti-NLRP3, ASC, caspase-1, IL-1β and GAPDH were obtained from Cell Signaling Technology Inc. (Beverly, MA, USA). All other reagents used in the experiment are analytically pure. All other reagents used in the experiment were analytically pure. PMSF and RIPA were obtained from Beyotime Biotechnology (Shanghai, China).

Preparation of fermentation products of C. lilacina

The fermentation was cultured in a liquid shake flask, 120 mL of liquid medium (maltose 4%, yeast powder 3%, KH_2PO_4 0.1%, $MgSO_4$ 0.05%) was put into a 500 mL conical flask, inoculated according to 10% volume ratio (V/V), the shake flask speed was 150r/min, at 25°C for 7 days. When the biomass reached 1.25g/100 mL, the fermentation broth was collected, concentrated, dried and pulverized as experimental drugs.

Animals and experimental groups

Total 50 healthy male BALB/c mice (an albino and laboratory-bred strain, weighing 19-21g) were provided by Liaoning Changsheng Biotechnology Co., Ltd., (Liaoning, China; Certificate No.: SCXK Liao 2015-0001). Before the experiment, the mice were allowed to adapt to the standard laboratory conditions for a week, with free diet and drinking water, and were randomly divided into 5 groups (n=10): the control group (physiological saline, healthy mice), model group (physiological saline, model mice), ranitidine group (0.03g/kg), and fermentation product (FP) groups (1 and 2g/kg, respectively). Each group was given corresponding drugs by intragastric administration, respectively, for ten consecutive days.

Water-immersion stress induced gastric ulcers in mice

Before modelling, all mice were fasted for 24 h with sufficient drinking water supplied. In addition to control group, mice in other groups were fixed on the self-made mouse board, immersed in water at 20±1°C for 20 h, and the water surface was kept at the same level as the xiphoid process of mice (Rahmadi, 2021). Subsequently, for all groups of mice, blood was collected from eyeballs and sacrificed by cervical dislocations. The stomachs of three mice in each group were ligated at the pylorus, injected with an appropriate amount of 4% paraformaldehyde solution, then ligated at the cardia, and finally the free gastric tissues were immersed in 4% paraformaldehyde solution for fixation. The stomachs of the other mice were opened along the great curvature of the stomach and the stomach contents were removed with cold normal saline and the stomach tissue was frozen immediately.

Histopathological examination

A part of stomach tissue fixed in 4% paraformaldehyde solution and was embedded in paraffin. Tissue slices were cut at 5μm thickness and stained with hematoxylin and eosin. The histopathological changes of the stomach were observed under the light microscope.

Determination of related biochemical indices in gastric tissues

The stomach tissues were weighed, and cold saline (1:9) was added according to the mass ratio of organ mass and fully grinded to get 10% tissue homogenate. The tissue homogenates were centrifuged at 3500 rpm at 4°C for 10 min. Supernatant was used for estimation of the activities of pepsin, SOD, MDA and the content of NO.

Determination of inflammatory factors in serum

The blood samples were centrifuged at 3500 r/min, at 4°C for 10 min. The serum was used to determine the levels of TNF-α and IL-6 according to the instructions of ELISA kit. The standard curve was used to calculate the level of the analytes.

Western blotting analysis of gastric tissue

Briefly, 100 mg gastric tissue samples were homogenized in 500 μL precooled protein lysate (containing PMSF) and incubated on ice for 30 min. The homogenate was then centrifuged at 3500 rpm, 4°C for 10 min.

The supernatant was used for determination of the protein concentration with BCA kit. Equal amounts of total protein were subjected to SDS-PAGE followed by electrophoretic transfer to nitrocellulose membranes. The nonspecific binding was prevented by incubation with 5% skimmed milk powder for 2 h at room temperature. Blots were incubated with primary antibody (anti-NLRP3, anti-Caspase-1, anti-ASC, anti-IL-1β, and anti-GAPDH) overnight at 4°C, and then, incubated the second antibody with fluorescent label for 2 h at room temperature. The bands were detected using the enhanced chemiluminescence detection system and band intensities were measured by densitometric analysis using Image J software. The ratio of NLRP3, Caspase-1, IL-1β or ASC

to the corresponding control GAPDH was calculated for each sample.

Statistical analysis

The data were processed by Graph Pad Prism (Version 5.0) and the difference between groups was analyzed by one-way ANOVA. The experimental data were expressed as mean ± SEM, and $P<0.05$ suggested that the difference was statistically significant.

RESULTS

FP of C. lilacina *could prevent gastric ulcer in mice induced by WIRS*

The stress gastric ulcer was established in the restrained water soaked mice (Fig. 1). The results of histopathology of gastric mucosa showed that the gastric mucosa of the control group showed normal flesh color, mucosal folds were smooth, and no bleeding spots were seen. In the model group, diffuse bleeding spots were seen, gastric mucosa showed congestion, edema, bleeding and erosion spots, and inflammatory cell infiltration was obvious; The surface of the stomach tissue in the tidine group, high-dose enzyme group and low-dose enzyme group was smoother, with mild congestion and edema in part of the mucosa, a small amount of erosion, no obvious ulcers, and a small amount of inflammatory cell infiltration (Fig. 1).

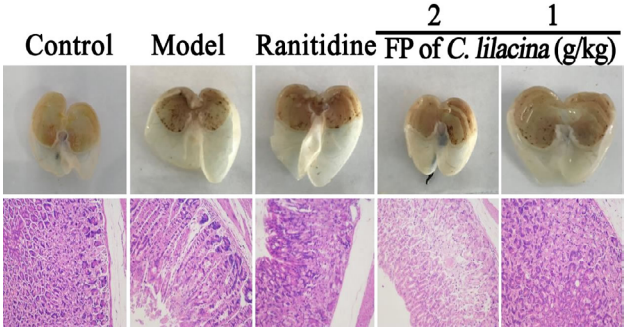

Fig. 1. Effect of FP of *C. lilacina* pretreatment on histological sections in the mice stomach. Gastric mucosa histopathological changes are shown following H and E staining (magnification, 200×).

Pepsin is a kind of proteolytic enzyme, which can decompose proteins and is one of the attack factors of gastric mucosa damage (Samuels and Johnston, 2020). Therefore, reducing pepsin activity is an important aspect of anti-gastric mucosal injury. Compared with the model group, the pepsin activity of ranitidine group and high-dose group of FP decreased significantly ($P<0.05$ or $P<0.01$) (Fig. 2).

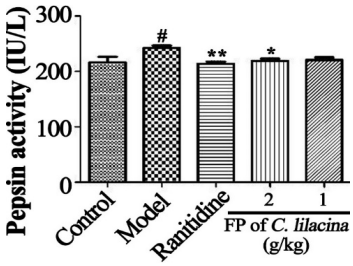

Fig. 2. Effect of FP of *C. lilacina* on the activity of pepsin in gastric tissue. Data are expressed means± SD (n= 6). $^{\#}P<0.05$ versus the injury group. $^{**}P<0.01$, $^{*}P<0.05$ versus the previous group.

FP of C. lilacina *could affect the related indices of oxidative stress in gastric tissue of model mice*

As shown in Figure 3, compared with the FP groups, the stage of MDA and NO in the gastric homogenate of the model group was elevated drastically ($P<0.05$ or $P<0.01$), and the degree of SOD in the gastric homogenate of the model group diminished extensively ($P<0.01$). Compared with the model group, the degree of MDA and NO in gastric homogenate of mice in ranitidine group, high-dose and low-dose of FP group decreased. The MDA of high dose and low dose of FP groups lowered substantially ($P<0.05$ or $P<0.01$). However, the degree of NO reduced barring vast difference. The degree of SOD in gastric homogenate of mice in ranitidine group and FP groups were first increased, though not significantly after that.

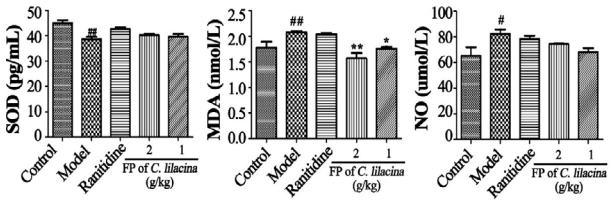

Fig. 3. Effect of FP of *C. lilacina* on the related indices of oxidative stress in gastric tissue. Data were expressed means± SD (n= 6). $^{\#\#}P<0.01$, $^{\#}P<0.05$ versus the injury group; $^{**}P<0.01$, $^{*}P<0.05$ versus the previous group.

FP of C. lilacina *could affect the anti-inflammatory indices in the serum of model mice*

The degrees of TNF-α and IL-6 in the model group were drastically decreased ($P<0.05$ or $P<0.01$) compared with the FP groups, as shown in Figure 4. Compared with the model group, the ranges of TNF-alpha and IL-6 in serum of ranitidine group, the high-dose and low-dose group of FP were decreased, and the degrees of TNF-α and IL-6 in serum of high dose and low dose of FP groups were decreased appreciably ($P<0.05$ or $P<0.01$).

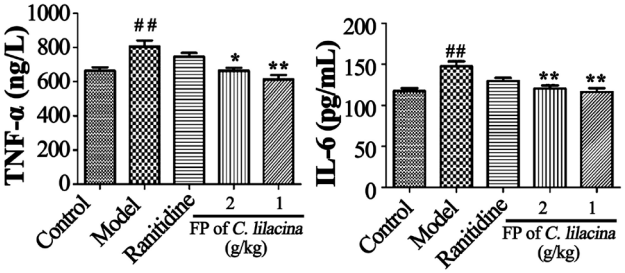

Fig. 4. Effect of FP of *C. lilacina* on the anti-inflammatory indices in the serum. Data are expressed means± SD (n=6). $^{\#\#}P$ <0.01 versus the injury group; $^{**}P$ <0.01, $^{*}P$ <0.05 versus the previous group.

FP of C. lilacina *can inhibit the activation of NLRP3/ASC/caspase-1 pathway in gastric tissue*

Figure 5 shows that compared with the control group, the expression levels of NLRP3, caspase-1, ASC and IL-1β protein in the gastric tissues of the model group were significantly increased (P <0.01). The expression levels of NLRP3, caspase-1, ASC and IL-1β protein in the gastric tissues of the ranitidine group, the high-dose group of FP and the low-dose group of FP were significantly decreased (P <0.05 or P <0.01), compared with the model group.

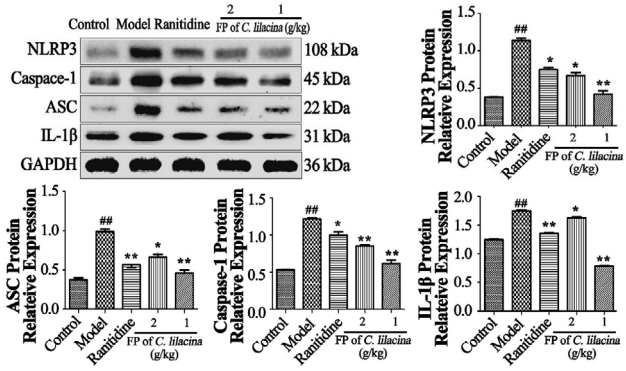

Fig. 5. Effect of FP of *C. lilacina* on the activation of NLRP3 / ASC / caspase-1 pathway in gastric tissue. Data were expressed means ± SD (n=3). $^{\#\#}P$ <0.01 versus the injury group; $^{**}P$ <0.01, $^{*}P$ <0.05 versus the previous group.

DISCUSSION

Acute gastric ulcers are superficial diffuse gastric mucosal lesions, which are usually caused by excessive smoking and drinking, heavy use of non-steroidal anti-inflammatory drugs and severe stress injuries, such as shock, burn and surgical injury (Jeon *et al*., 2020; Rahman *et al*., 2020). Acute gastric ulcer can lead to serious upper gastrointestinal bleeds with high incidence and mortality rates (Li and Song, 2020). The mechanisms un-derlying acute gastric ulcers remain largely unclear, but acute inflammation can appear as the main factor that affects and regulates the progression of the disease.

Although there is evidence that the *C. lilacina* exerts a wide range of health-promoting effects (Zhu *et al*., 2010), its role in water-immersion stress induced acute gastric ulcers remains largely unknown. Therefore, this study was aimed to determine the potential effects of oral administration of the FP of *C. lilacina* on ethyl alcohol-induced acute gastric ulcers. As mentioned above, acute gastric ulcers were triggered in mice via water-immersion stress method, and the FP of *C. lilacina* exhibited protective effects on Water-immersion stress induced acute gastric ulcers. water-immersion stress did cause significant increase in MDA and NO, but a remarkable reduction in SOD. After treatment with the FP of *C. lilacina*, the levels of MDA and NO were significantly reduced, while SOD activity was markedly increased.

NLRP3 inflammasome activation and IL-1β release are considered to be one of the important mechanisms of diseases such as acute gastric injury. The binding of NLRP3 and ASC under different stimuli can trigger the release of caspase-1, resulting in pro-IL-1β activation and IL- 1β maturation (Zhou *et al*., 2020). NLRP3/ASC/caspase-1 pathway plays an essential role in water-immersion stress induced gastric injuries Higashimori *et al*. (2021). In this study, the expression levels of ASC, caspase-1, NLRP3 and IL-1β were examined to elucidate the antiinflammatory mechanisms of the FP of C. lilacina.

CONCLUSION

In summary, this study reveals that the fermentation product of *Calvatia lilacina* can significantly down-regulate the expression of NLRP3 and ASC, inhibit the activation of Caspase-1, reduce the release of IL-1β, TNF-α and IL-6, and effectively reduce Water-immersion stress induced inflammation, enhance the anti-oxidation activity of gastric mucosa, and thus reduce the severity of gastric ulcer. It is suggested that the FP of *C. lilacina* may confer protective roles in Water-immersion stress induced acute gastric ulcer by inhibiting NLRP3 signaling pathway.

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Ethical compliance

All procedures and experimental methods were approved by the Animal Ethics Committee of Changchun University of Traditional Chinese Medicine (Certificate No.: 20180126).

Statement of conflicts of interest

The authors have declared no conflict of interest.

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