Effect of moxa-burning heat stimulating Liangmen (ST 21) and Zusanli (ST 36) on proliferation and apoptosis signaling proteins in rats with stress-induced gastric ulcer

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Abstract

OBJECTIVE: To observe the effect of moxa-burning heat stimulating acupoints of Liangmen (ST 21) and Zusanli (ST 36) on the proliferation and apoptosis signaling proteins in rats with stress-induced gastric ulcer.

METHODS: Forty rats were randomly divided into four groups: negative control (NC), ulcer control (UC), acupoints of stomach meridian (ASM), and acupoints control (AC). The acute gastric ulcer model was established by bound and water immersion. Rats in NC and UC groups didn't receive any moxa-burning heat stimulating treatment, while rats in ASM and AC groups were treated with burningmoxa heat stimulating the acupoints of Liangmen (ST 21) and Zusanli (ST 36) and their controlled points, respectively. Rats in all groups were sacrificed after 12 consecutive days treatment. The ulcer index was evaluated by using Guth's method. The expression of tumor necrosis factor-alpha (TNF-α), apoptotic protease activating factor-1 (Apaf-1), Caspase-3, p21 activated kinase 1 (PAK1), extracellular regulated protein kinases 2 (ERK2), phosphorylated ERK2 (pERK2), phosphoinositide 3-kinase (PI3K) and RAC-alpha serine/threonine-protein kinase (Akt) in gastric mucosa was detected by enzyme linked immunosorbent assay (ELISA).

RESULTS: Compared with UC group, the ulcer index of ASM and AC groups decreased, and the injured gastric mucosa was improved, the expression of TNF-α, Apaf-1 and Caspase-3 in gastric mucosa was significantly reduced ($P < 0.05$), while the ex-
pression of PAK1, ERK2, pERK2, PI3K and Akt in gastric mucosa was significantly increased (P < 0.05). And ASM showed better effect than AC group (P < 0.05).

CONCLUSION: Moxa-burning Heat stimulating of Liangmen (ST 21) and Zusanli (ST 36) could promote the recovery of gastric mucosal lesion probably by inhibiting cell apoptosis and promoting cell proliferation in stress-induced gastric ulcer.

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Key words: Moxibustion; Point ST 21 (Liangmen); Point ST 36 (Zusanli); Stomach ulcer; Apoptosis; Cell proliferation

INTRODUCTION

Peptic ulcer is one of the common and frequent occurring gastrointestinal diseases, which can be induced by many factors including cold stimulation, ethanol intake, emotional stress, high-dose use of non-steroidal anti-inflammatory drugs, etc.\(^1\) Erosion, ulceration and hemorrhage following gastric mucosal protection broken are the major pathological foundation of stress-induced gastric ulcer.\(^2\) Now, the treatment strategy of this disease include two approaches, one is to inhibit the secretion of gastric acid, the other one it to strength the gastric mucosal protection.\(^3\) However, the present medications can not cure the ulcers, and have some side effects.\(^4\) Considering its lethal complication and the defects of present drug treatment, it is necessary to seek a new and more effective therapeutic mean for stress-induced gastric ulcer.

At present, the detailed mechanism of stress-induced gastric ulcer is not clear. It is reported that gastric mucosal cell apoptosis, mucosal ischemia, mucus-mucosal barrier damage and inflammatory cytokines over-expression are believed to be involved in its mechanism.\(^2\) Moxa-burning heat stimulating acupoints of Zusanli (ST 36) and Zhongwan (CV 12) could alleviate gastric mucosal lesion by over-expressing HSP60 and HSP70, down-regulating Smac and inhibiting mucosal cell apoptosis, the selected two acupoints located on stomach meridian.\(^5\) Our preliminary results showed that moxa-burning heat stimulating acupoints of stomach meridian-Liangmen (ST 21) and Zusanli (ST 36) could regulate phosphorylation level of lots of signaling proteins, promote the recovery of injured gastric mucosa in rat model of stress gastric ulcers.\(^6\) But we still don’t know which signaling pathway can be targeted by moxa-burning heat stimulating treatment. This study focused on the effect of moxa-burning heat stimulating acupoints on cell apoptosis and proliferation during the repair of gastric mucosa.

MATERIALS AND METHODS

Animals

All experiments were carried out using specific pathogen free level healthy Sprague-Dawley rats (200-220 g, 10-11 weeks old, half male and half female) with free access to food and water and in a 12 h dark-light cycle at 20-22 °C and 65%-70% relative humidity. All these rats were provided by Xiamen University Laboratory Animal Center [Certificate number: SCXK (Min) 2008-0001] and housed in an air-conditioned atmosphere. The rats were handled in accordance with the regulations for the administration of affairs concerning experimental animals (Approved by People’s Republic of China State Council on October 31, 1988 and promulgated by Decree No. 2 of the State Science and Technology Commission on November 14, 1988). International rules were strictly followed when dealt with animals and the animal protocol has been previously approved by the ethics committee for administration of experimental animals in Medical College of Xiamen University.

Reagents and instruments

Microplate reader (type 352, LabSystems Multiskan MS) and plate washer (type AC8, LabSystems Multiskan MS) were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA); Homothermal incubator (type GNP-9080) was purchased from Shanghai Jing Hong Laboratory Instrument Co., Ltd. (Shanghai, China); Anti-rat ELISA kits, including tumor necrosis factor-alpha (TNF-α), apoptotic protease activating factor-1 (Apaf-1), Caspase-3, p21 activated kinase 1 (PAK1), extracellular regulated protein kinases 2 (ERK2), phosphorylated ERK2 (pERK2), phosphoinositide 3-kinase (PI3K), RAC-alpha serine/threonine-nitrogen-protein kinase (Akt) were bought from Wuhan Nolitai Biological Technology Co., Ltd. (Wuhan, China).

Modeling and grouping

Forty rats were divided randomly into 4 groups according to the random number table method: negative control (NC), ulcer control (UC), acupoints of stomach meridian (ASM) and acupoints control (AC) (10 rats for each group). Briefly, each group need at least 8 rats according to our preliminary results, here we use 10 rats in each group. Next, all 40 rats were labeled with 1-40 number and put these numbers in random number table. We chose the first 10 numbers as NC group, the next 10 numbers as UC group, the next 10 numbers as ASM group and last 10 numbers as AC group. Modeling method of stress-induced gastric ulcer as follow: the four limbs of all rats were bound on the boards for 20 min. Except the rats in negative control, the other rats were dipped into thermostat water tank (20 ± 1) °C vertically for 10 h (the level of the water reached the sternum xiphoid). Rats were taken out of water and the acute gastric ulcer model was established successfully by the pathological diagnosis.
Localization of acupoints and controlled points

Acupoints of Liangmen (ST 21) and Zusanli (ST 36) were pinpointed following the methods described in Experimental Acupuncture & Moxibustion, a textbook for National Higher Institutions of Traditional Chinese Medicine in the New Century. Their controlled points were 0.5 cm away from them.

Treatment

Rats in ASM and UC groups were treated with moxa-burning heat stimulating acupoints of Liangmen (ST 21) and Zusanli (ST 36) and their controlled points, respectively after the gastric ulcer model was established. Rats in the other two groups received no such treatment. Rats in ASM and AC groups were fixed on the boards. Hair was clipped around treated acupoints and the controlled points. Moxa-burning sticks were fixed with hand to make sure that the lit ends were 2 cm away from the skin. Each acupoint was treated for 20 min per day for 12 consecutive days. Every treatment use unilateral acupoints for one time and the other lateral acupoints for next time.

Specimen collection

Fasting for 24 h after the treatment, rats were anesthetized intraperitoneally with 10% chloral hydrate, and then were sacrificed and their stomachs were dissected. Each stomach was opened along the greater curvature and washed with ice-cold saline. Injured gastric tissue with the size of 1 cm x 0.5 cm was cut out and homogenized in saline. The homogenate was put in refrigerator with the temperature of –20°C. Cell membrane was damaged by repeated freezing and thawing twice and the homogenate was centrifuged for 3 min (5000 rpm/min, 2-8 °C), and the supernatant fluid was used for protein test. In addition, representative stomach specimens were taken from each group for histopathological examination.

Ulcer index of gastric mucosa

The ulcer index was recorded and calculated according to the method of Guth’s. Ulcer length ≤ 1 mm (including erosion foci) was scored 1; 1 mm < ulcer length ≤ 2 mm scored 2; 2 mm < ulcer length ≤ 3 mm scored 3; 3 mm < ulcer length ≤ 4 mm scored 4; ulcer length > 4 mm scored 5; ulcer width > 2 mm, score was doubled.

Pathological morphological analysis

The ulcer area of 0.5 cm x 0.5 cm was collected from gastric mucosa and immobilized for 24 h in 10% neutralized formaldehyde solution. After paraffin was embedded, the specimens were ready for cut. Paraffin sections (5 μm) were stained with hematoxylin-eosin (HE) and observed under the microscope magnified 100 times or 400 times respectively.

Enzyme linked immunosorbent assay (ELISA)

Signaling molecules of cell apoptosis and proliferation in stress-induced gastric ulcer were assessed by measuring the levels of TNF-alpha, Caspase-3, Apaf-1 and PAK1, ERK2, pERK2, PI3K, Akt in the gastric tissue of different groups respectively. The steps were carried out according to the manufacturer’s instructions of anti-rat ELISA kits. Briefly, Pipette 50 μL of standards, controls, and diluted samples to the appropriate microtiter wells. Cover wells with plate cover and incubate for 30 min at 37 °C. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. Pipette 50 μL Biotin-conjugated detection antibody was put into each well except the chromogen blank. Cover wells with plate cover and incubate for 30 min at 37 °C. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. Pipette 50 μL streptavidin-HRP solution was added in each well except the chromogen blank. Cover wells with plate cover and incubate for 30 min at 37 °C. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. Pipette 50 μL of stabilized chromogen to each well. The liquid in the wells will begin to turn blue. Incubate for 15 min at 37 °C and in the dark. Pipette 50 μL of stop solution was added in each well. The solution in the well should change from blue to yellow. Read the absorbance of each well at 450 nm (OD values) within 2 h after adding the stop solution.

Statistical analysis

Parameter data were expressed as mean ± standard deviation ( ± s). Analysis of variance were performed to test the differences between the groups) with SPSS for Windows (SPSS Inc., Version 11.5, Chicago, IL, USA). P < 0.05 was considered to be statistically significant.

RESULTS

Stress-induced gastric lesions

The ulcerated area in gastric mucosa has been measured and the ulcer index was used to evaluate injury in all groups (Figure 1). Ulcer index for rats in negative controls, and diluted samples to the appropriate microtiter wells. Cover wells with plate cover and incubate for 30 min at 37 °C. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. Pipette 50 μL Biotin-conjugated detection antibody was put into each well except the chromogen blank. Cover wells with plate cover and incubate for 30 min at 37 °C. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. Pipette 50 μL streptavidin-HRP solution was added in each well except the chromogen blank. Cover wells with plate cover and incubate for 30 min at 37 °C. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. Pipette 50 μL of stabilized chromogen to each well. The liquid in the wells will begin to turn blue. Incubate for 15 min at 37 °C and in the dark. Pipette 50 μL of stop solution was added in each well. The solution in the well should change from blue to yellow. Read the absorbance of each well at 450 nm (OD values) within 2 h after adding the stop solution.

Figure 1 Effect of moxa-burning heat stimulating acupoints on ulcer index in ulcerated rats.

The figure show ulcer index of four groups. Rats in NC and UC groups didn’t receive any treatment. Acupoints of stomach meridian-Liangmen (ST 21) and Zusanli (ST 36) in ASM group were treated with moxa-burning heat stimulating. Their control points in AC group were treated with moxa-burning heat stimulating. NC: negative control; UC: ulcer control; ASM: acupoints of stomach meridian; AC: acupoints control. Data are expressed as mean ± standard deviation (n = 10). P < 0.05, compared with NC group; P < 0.05, compared with UC group; P < 0.05, compared with AC group.
control group was very low. Rats received stress injury got a serious gastric mucosal injury and their ulcer index increased about 59-fold compared with negative control. Moxa-burning heat stimulating acupoints decreased the ulcer index, especially selecting the acupoints of stomach meridian, which suggested that moxibustion of acupoints of stomach meridian could promote gastric mucosa recovery.

**Gastric mucosal pathology**

Figure 2 showed the histopathological alterations in stomach specimens of different groups. Gastric mucosa from the negative control showed that epithelium structure was intact and cell had no hyperaemia and edema, submucosa and muscularis contained no inflammatory cells. The stress-induced gastric ulcer from ulcer control showed that the structure of gastric mucosa had been damaged totally, gastric gland cell necrosis, cell contained hyperaemia and edema, lots of inflammatory cells infiltrated in mucosa, submucosa as well as muscularis. The stomach specimen of moxa-burning heat stimulating acupoints in stomach meridian showed slight injury, epithelium structure was intact and no inflammatory cells infiltrating in it. While moxa-burning heat stimulating the control acupoints showed unintact epithelium structure, there was no blood capillary proliferation and hyperaemia, only little inflammatory infiltration in gastric wall. This result suggested that moxa-burning heat stimulating the acupoints of stomach meridian could reduce gastric mucosa injury induced by stress.

**Cell apoptosis-related factors**

Signaling molecules, such as TNF-α, Apaf-1 and Caspase-3, are the markers of cell apoptosis. The normal level of TNF-α, Apaf-1 and Caspase-3 in gastric mucosa were 168.13, 295.75 and 5.93 ng/mL respectively. Stress-induced gastric ulcer could increase to 1.24, 1.36-fold in gastric mucosa. Moxa-burning heat stimulating the acupoints significantly attenuated the stress-induced increase in these signaling molecules by about 15%, 12% and 20% respectively, which were better effective than moxa-burning heat stimulating on the controlled points (Figure 3). These findings suggested that moxa-burning heat stimulating the acupoints could reduce cell apoptosis in stress-induced gastric ulcer.

**Cell proliferation-related signaling proteins**

The effect of moxa-burning heat stimulating acupoints on the intracellular signaling pathway was showed in Figure 4, including PAK1/ERK2 pathway and PI3K/Akt pathway. These targets were significantly increased in stress-induced gastric ulcer compared to negative control. Moxa-burning heat stimulating the acupoints or the control points could increase their expression significantly in gastric mucosa compared to ulcer control. When compared to AC group, moxa-burning heat stimulating the acupoints was more effective in increasing their expression. These results implied that moxa-burning heat stimulating the acupoints in stomach meridian activated PAK1/ERK2 and PI3K/Akt signaling pathways and following proliferation signal transduction.

**DISCUSSION**

Peptic ulcer is a common digestive disease. Modern medical treatment, including the eradication of H. pylori, inhibition of gastric acid secretion and protection of gastric mucosa, do not cure this disease, and accompanied with many side effects. Previous studies have shown that moxa-burning heat stimulating the acupoints of stomach meridian can reduce the gastric mucosal injury, increase the gastric protection mechanism, and so can be used as an effective treatment of gastrointestinal diseases. Bound and water dipping induced severe gastric mucosal injury, which was confirmed by

![Figure 2](image-url)
the histopathological examination, including cell hyperaemia, edema and necrosis, inflammatory cells infiltration, and the damage of stomach mucosa structure. The ulcer recording showed a significant high ulcer index in ulcer control group (about 59-fold compared with negative control). In contrast, the ulcer index was significantly decreased by moxa-burning heat stimulating the acupoints, especially better effect when chose the acupoints of stomach meridian than the control points. Moxa-burning heat stimulating acupoints also...
absolutely attenuated stress-induced acute stomach mucosa injury by protecting stomach mucosal structure and decreasing inflammatory cells infiltration in submucosa and muscularis. Based on the above results, moxa-burning heat stimulating the acupoints of stomach meridian showed a significant protective role in stress-induced gastric ulcer.

A lot of evidence showed that the induction of apoptosis was involved in the mechanism of acute gastric ulcer in both human and rats, the signaling molecules including TNF-α and caspase-3 were positive related to gastric mucosal damage. The role of TNF-α is to activate NF-κB and MAPK signaling pathway and start caspase-3-mediated apoptotic program, and Apaf-1 play an important role in Akt1-mediated cell apoptosis. In this study, TNF-α, Apaf-1 and caspase-3 were significantly increased in ulcer control group. Moxa-burning heat stimulating the acupoints decreased their content in ulcerated stomach and the better effect of acupoints of stomach meridian had been observed. This suggested that moxa-burning heat stimulating the acupoints ameliorated stress-induced gastric ulcer via anti-apoptotic activity.

Previous studies have showed that the balance between cell apoptosis and cell proliferation play an important role in maintaining gastric mucosa integrity. Both apoptosis and cell proliferation were augmented in chronic ulcers induced by acetic acid, which suggested a complex mechanism in the restore homeostatic state of gastric mucosa. Our previous study have found that moxa-burning heat stimulating the acupoints of stomach meridian had regulated lots of protein phosphorylation by using genomics technology, including down-regulating TRAF2, Stat1, p53, Mkp-3 and Nf-κb, up-regulating MEK, Ras, P38MAPK, P13K, PAK, B-Raf and ERK2, suggesting a balance regulating role of moxa-burning heat stimulating the acupoints on apoptosis and cell proliferation. The activation of PAK1/ERK2 pathway and P13K/Akt pathway can induce cell proliferation and plays a pivotal role in gastric mucosal healing. The present study showed that both PAK1/ERK2 pathway and P13K/Akt pathway had been up-regulated by stress induction. Moxa-burning heat stimulating the acupoints of stomach meridian had further up-regulated these two pathways, suggesting a healing effect for ulcerated mucosa recovery via cell proliferation.

In conclusion, the moxa-burning heat stimulating Liangmen (ST 21) and Zusanli (ST 36) had a significant gastroprotective effect on bound and water immersion-induced gastric ulcer, and the underlying mechanism might involve inhibiting cell apoptosis and promoting cell proliferation.

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