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Special Topic for 973 Program

Effects of moxibustion pretreatment on extracellular signal-regulated kinase signaling transduction pathway in the gastric tissues of rats with gastric mucosal damage

艾灸预处理对胃黏膜损伤大鼠胃组织细胞外调节蛋白激酶信号转导通路的影响

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Abstract

Objective: To observe the effects of moxibustion pretreatment on the protein expressions of epidermal growth factor receptor (EGFR), phosphorylation extracellular signal-regulated kinase 1/2 (p-ERK1/2) and activated protein-1 (AP-1), the key factors of extracellular signal-regulated kinase signaling transduction pathway in gastric tissue of rats with stress-induced gastric mucosal damage, and to discuss the mechanisms of moxibustion therapy in promoting the restoration of damaged gastric mucosa.

Methods: Thirty Sprague-Dawley (SD) rats were randomly divided into a normal group, a model group, and a moxibustion group using the random digits table, 10 in each group. Except the rats in the normal group, rats in the other two groups were used to make stress-induced gastric mucosal damage model using restraint and cold stress. Before modeling, rats in the moxibustion group were alternately treated with moxibustion at Zusanli (ST 36) and Zhongwan (CV 12), or Pishu (BL 20) and Weishu (BL 21), once a day, for a total of 8 d. Histological changes of gastric mucosa were observed under the light microscopy, the expression of gastric tissue p-ERK1/2 was detected by immunohistochemistry assay, and the protein levels of EGFR and AP-1 were measured by Western blots.

Results: Compared with rats in the normal group, gastric mucosal damage was more serious, and protein expressions of gastric tissue EGFR, p-ERK1/2 and AP-1 increased in the model group ($P<0.01$, $P<0.05$, $P<0.05$). Compared with rats in the model group, gastric mucosal damage was milder, and protein expressions of gastric tissue EGFR, p-ERK1/2 and AP-1 increased in the moxibustion group (all $P<0.01$).

Conclusion: Moxibustion at Zusanli (ST 36), Zhongwan (CV 12), Pishu (BL 20) and Weishu (BL 21) could increase EGFR, p-ERK1/2 and AP-1 expression levels in gastric tissue of stress-induced gastric mucosal damage rats, maintain the information transfer function of ERK signaling transduction pathway, and promote restoration of gastric mucosal damage.

Keywords: Moxibustion Therapy; Moxa Stick Moxibustion; Gastric Mucosal Damage; Receptor, Epidermal Growth Factor; Extracellular Signal-regulated Kinases; Rats

【摘要】目的：观察艾灸预处理对应激性胃黏膜损伤大鼠胃组织细胞外调节蛋白激酶(extracellular signal-regulated kinase, ERK)信号转导通路中关键因子——表皮生长因子受体(epidermal growth factor receptor, EGFR)、磷酸化细胞外信号调节激酶(phosphorylation extracellular signal-regulated kinase 1/2, p-ERK1/2)与激活蛋白 1 (activated protein-1, AP-1) 蛋白表达的影响，探讨艾灸促进胃黏膜损伤修复的作用机制。**方法：**将 30 只 Sprague-Dawley (SD)大鼠按照随机数字表随机分为正常组、模型组、艾灸组，每组 10 只。除正常组外，其余 2 组大鼠采用束缚冷应激法制作应激性胃黏膜损伤模型。在造模之前，艾灸组大鼠交替灸足三里、中脘或脾俞、胃俞，每日 1 次，共 8 d。光镜下观察胃黏膜组织形态学变化，免疫组织化学法检测胃组织 p-ERK1/2 的表达，蛋白质印迹法检测 EGFR 与 AP-1 蛋白

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的水平。**结果：**与正常组比较，光镜下模型组大鼠胃黏膜损伤严重，且胃组织 EGFR、p-ERK1/2 与 AP-1 蛋白表达升高($P<0.01$, $P<0.05$, $P<0.05$)；与模型组比较，光镜下艾灸组大鼠胃黏膜损伤较轻，且胃组织 EGFR、p-ERK1/2 与 AP-1 蛋白表达升高(均 $P<0.01$)。**结论：**艾灸足三里、中脘、脾俞和胃俞能提高应激性胃黏膜损伤大鼠胃组织 EGFR、p-ERK1/2、AP-1 的表达水平，维持 ERK 信号转导通路的信息传递功能，促进胃黏膜损伤修复。

【关键词】灸法；艾条灸；胃黏膜损伤；受体，表皮生长因子；细胞外信号调节激酶；大鼠
【中图分类号】R2-03　　**【文献标志码】**A

According to the known repair mechanism of gastric epithelial damage[1], trefoil factor (TFF) can cause the release of transforming growth factor-α (TGF-α) and bind to its specific receptor (epidermal growth factor receptor, EGFR) by acting on the 'intermediary' epithelial cell membrane receptor, the protease activated receptors, which then induce the cell migration signaling pathways related with extracellular signal-regulated kinase (ERK). ERK signaling pathway plays an important role in regulation of the body's stress, inflammation and cell proliferation and apoptosis[2]. ERK is usually located in the cytoplasm, once activated for phosphorylation, ERK will pass the nuclear membrane rapidly, and then activate the transcription factors such as activated protein-1 (AP-1), nuclear factor-κB (NF-κB), and regulate growth-related gene transcription. Our previous studies have shown that moxibustion at Zusanli (ST 36), Zhongwan (CV 12) and other acupoints could stimulate the synthesis and secretion of endogenous protective factors in vivo, such as epidermal growth factor (EGF), TGF-α, after binding to the specific receptor EGFR in gastric mucosal membrane, which will initiate the mucosal repair mechanism, induce the synergistic action of TFF1, EGF and TGF-α, and provide the material conditions to promote the proliferation and repair of gastric mucosal cells[3-5]. Based on previous studies, in this experiment, we investigated the effects of moxibustion on the protein expressions of EGFR, p-ERK1/2 and AP-1, the key factors of ERK signaling transduction pathway of the gastric tissues in stress-induced gastric mucosal damage rats, and explored the mechanisms of moxibustion therapy in promoting the restoration of damaged gastric mucosa, and further clarified the mechanisms of promoting gastric mucosal damage repair by the warming effects of moxibustion.

1 Materials and Methods

1.1 Animals and grouping

SPF grade healthy Sprague-Dawley (SD) rats, 3-4 month old and weighing (220 ± 20) g, half male and half female, were purchased from Hunan Slack King of Laboratary Animals Co., Ltd.[animal license number: SCXK (Xiang) 2009-0004]. The rats were randomly divided into three groups: a normal group, a model group and a moxibustion group, with 10 rats in each group. Experimental procedures were approved by Animal Ethics Committee of Experimental Animal Center in Hunan University of Chinese Medicine.

1.2 Main reagents and instruments

Pure moxa (model: mg-454, specification: 18 mm × 200 mm; Nanyang Wolong Hanyi Moxa Factory, China); self-made simple bracket for moxibustion, thermal insulation film; ready-to-use SABC immunohisto-chemistry (IHC) staining kit, brown DAB chromogenic agent (Wuhan Boster Biological Engineering Co., Ltd., China); rabbit anti-rat EGFR, p-ERK1/2, AP-1 polyclonal antibodies, BCA protein quantification kit (Changsha Virgin Biotechnology Ltd., China); TGL16M mode desktop high-speed refrigerated centrifuge (Changsha Kewei Industrial Co., Ltd., China); AEU-210 electronic analytical balance (Changsha Xiangyi Balance Instrument Factory, China); Motic B1 microscope (Fujian Motic China Group Co., Ltd., China); OLYMPUS Bx-70 microscope imaging system (OLYMPUS Corporation, Japan); image-Pro Plus image analysis software (Media Cybernetics, Inc., USA); 820 AO paraffin microtome (Applied Optoelectronics, Inc., USA).

1.3 Modeling method

Rat models of stress-induced gastric mucosal damage were made using restraint and cold stress[6], on the 8th day after the intervention, in addition to the normal group, rats in the other two groups were fasted for 24 h with normal water, and followed by keeping in 20 ℃ water after being bound to the mouse board, at a water level to the sternum xiphoid, and the rats were unloaded and released 10 h later. The modeling was successful if we could see the following phenomenon in the HE stained sections by light microscope, significant damage and ablation in the gastric mucosa, significant dilatation and congestion of submucosal blood vessel, and large number of cytoplasmic red staining in the dilatated blood vessels, visible inflammatory cell (predominant lymphocytes) infiltration in submucosa.

1.4 Treatment method for each group

1.4.1 Normal group

Rats in the normal group were restricted using the same method and time as rats in the other 2 groups, once a day, for a total of 8 d, without modeling.

1.4.2 Model group

Rats in the model group and moxibustion group were restricted using the same method for 8 d, and modeling started on the 9th day.

1.4.3 Moxibustion group

Rats in the moxibustion group were immobilized to the mouse board for moxibustion for 8 d, and modeling started on the 9th day, the specific interventions were as follows.

Points: Zusanli (ST 36) on both sides and Zhongwan (CV 12) were a group, bilateral Pishu (BL 20) and Weishu (BL 21) were a group.

Method: Points were positioned according to *Experimental Acupuncture Science*[7]. Moxa sticks were fixed on the self-made small moxibustion bracket. Pointed to the moxibustion sites (local unhair) and lit moxibustion from about 2 cm over the skin. Selected a group of acupoints every day, and performed moxibustion for 30 min. Two groups of acupoints were alternately selected for 8 d.

1.5 Observed indicators and detection methods

On the second day after the end of modeling, rats were anesthetized with 20% urethane at 10 mL/(kg·bw) by intraperitoneal injection. Ligated the pylorus and the cardia of the stomach with hemostatic forceps and cut the esophagus and duodenum at the ends of ligature, to remove the whole stomach. Opened the stomach along the greater curvature of the stomach and washed the residue in stomach with iced saline. Then took a small piece of stomach tissue (approximately 5 mm × 10 mm), placed it in 4% paraformaldehyde and fixed it at 4 ℃ for 24 h, and embedded it in paraffin for HE staining and p-ERK1/2 IHC staining; took another small piece of stomach tissue (approximately 5 mm × 10 mm), and quickly put it into the cryopreservation vials and transferred it into the liquid nitrogen tank for detection of EGFR and AP-1 protein expression by Western blot.

HE staining: Routinely embedded in paraffin, dehydrated with graded ethanol, and cleared with xylene. Sliced serial sections (4 μm of thickness), toasted, and then routinely dewaxed, followed by hematoxylin-eosin (HE) staining. Finally mounted with neutral gum, and observed histological changes of gastric mucosa under the light microscope.

Immunohistochemical detection of p-ERK1/2: Poly-L-Lysin handle slides, paraffin blocks serial sections (4 μm in thickness), patch, grilled slices, xylene hydration, graded alcohol dewaxing. And placed in 3% H_2O_2 in block endogenous antigen for 15min, washed with distilled water for 2 min × 3 times; slices into pH 6.0 citrate buffer, microwave high heat until boiling off after, repeated 10 min later for twice, 0.1 mol/L PBS wash, 3 min × 3 times; dropping normal goat serum blocking solution, closed at room temperature 20 min (wet box), shook off the excess liquid, no need to wash; dropping 1:125 dilution of rabbit anti-p-ERK1/2 in rat polyclonal antibodies in a humid box at 37 ℃ for 2 h, 0.1 mol/L PBS wash, 3 min × 3 times; added biotinylated goat anti-rabbit IgG, and kept in a humid chamber at 37 ℃ for 30 min. Washed with 0.1 mol/L PBS for 3 min × 3 times; added horseradish peroxidase labeled streptavidin working solution, kept in a humid chamber at 37 ℃ for 30 min., washed with 0.1 mol/L PBS for 3 min × 3 times; colored with DAB at room temperature, controlled the coloration time under microscope, washed with distilled water; slightly stained with hematoxylin. PBS was used instead of primary antibody as a negative control. The positive standard was brown fine granules in the cytoplasm. Two sections were randomly selected from each rat gastric mucosa specimen, and observed by 400 magnification using microscope imaging system. Four visual fields were randomly selected in the gastric mucosa layer for photograph from each slice. Analyzed by image-Pro Plus image analysis software. Set a pane with a length of 10 cm, and a width of 5 cm, and measured the optical density (OD) values of the positive results in the pane of each photo, average value of eight photos of each specimen was calculated and used as the representative value of this specimen.

Detection of AP-1 and EGFR by Western Blot: Added lysate (radio immunoprecipitation assay, RIPA) and protease inhibitors (phenylmethanesulfonyl fluoride, PMSF) into liquid nitrogen grinded tissues, with a ratio of PMSF and RIPA at 1: 100. Set at 4 ℃ for 1 h for clearage. Centrifuged at low temperature for 7 min, 800 r/min. The supernatant was taken out. The standard curve was made using the BCA protein quantification method. Equal volume of 5 × sample buffer was added into the protein for each swimming lane, then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) after boiling for the separation, gel, film and filter paper were put into the transmembrane apparatus for transmembrane, proteins were transferred to the PVDF (polyvinylidene fluoride, PVDF) membrane, and blocked in 1% bovine serum albumin (bovine serum albumin, BSA) at room temperature for 1 h. EGFR and AP-1 polyclonal antibody (1: 100) were added respectively and kept at 4℃ overnight for antigen-antibody binding. Washed the membrane for three times with wash membrane buffer (tris buffered saline with tween, TBST), 15 min per time. Added horseradish peroxidase (horseradish peroxidase, HRP) labeled secondary antibody (1: 400), incubated at room temperature for 1 h, washed the membrane for three times with TBST, 15 min each time. Color reagent was added and developed using X-ray film. Each film contained one target protein and the corresponding internal reference protein. Scanned the image and saved as computer files,

and digitized the gray value of each specific band on the image using Image J analysis software. The gray value of target protein was divided by gray value of β-actin internal reference to correct the errors; the ratio (relative gray value) was used as the result.

1.6 Statistical processing

Statistical analyses were performed using SPSS 16.0 version statistical software. All data were subjected to normal distribution test and described as mean ± standard deviation ($\bar{x} \pm s$) when the measurement data fit the normal distribution; multiple sets of measurement data were compared using ANOVA. Least significant difference (LSD) and the Student-Newman-Keuls (SNK) method were used for homogeneity of variance, Tamhane's T2 or Dunnett's T3 method was used for heterogeneity of variance. A P value <0.05 indicated a statistical significance.

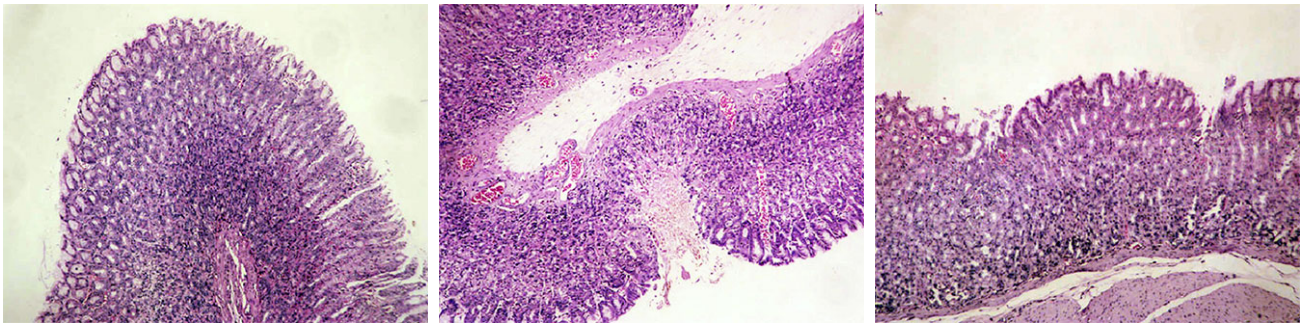
2 Results

2.1 Effects of moxibustion on histomorphologies of gastric mucosa tissue in rats with gastric mucosal damage

Under light microscope, rats in the normal group showed complete gastric mucosal epithelium, lamina propria, part muscularis mucosa, and intact gastric mucosa superficial cells, without obvious damage; compared with the normal group, rat's gastric mucosa in the model group showed significant breakage and ablation, and gastric mucosal damage; compared with the model group, rat's gastric mucosa in the moxibustion group showed partial surface ablation, a marked improvement of the breakage, and more intact gastric mucosa superficial cells (Figure 1).

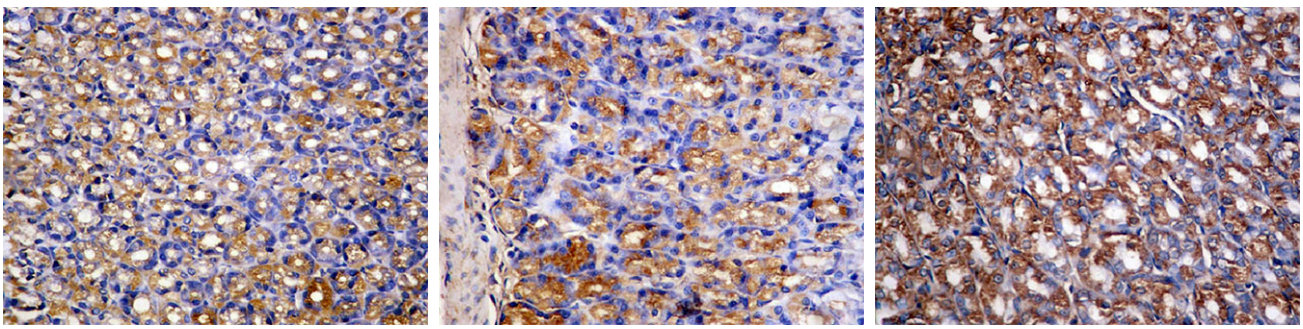
2.2 Effects of moxibustion on p-ERK1/2 protein expressions of gastric tissues in rats with gastric mucosal damage

Compared with the normal group, rats in the model group showed positive p-ERK1/2 expression, light brown in clolor in the cytoplasm of gastric mucosal cells, and the phosphorylated ERK1/2 protein expression significantly increased ($P<0.05$), which indicated that the activation levels of ERK1/2 in the tissues were gradually increased during the autotherapy process of damaged gastric mucosal tissues after restraint and water immersion stress; compared with the model group, rats in the moxibustion group showed a large number of brown positive p-ERK1/2 expression in the cytoplasm of gastric mucosal cells, and the phosphorylated ERK1/2 protein expression further enhanced ($P<0.01$), (Figure 2 and Table 1).



Normal group　　Model group　　Moxibustion group

Figure 1. Rat's gastric mucosal histomorphology of each group (HE staining, ×100)



Normal group　　Model group　　Moxibustion group

Figure 2. p-ERK1/2 protein expression of rat's gastric tissue in each group (S-P, ×400)

2.3 Effects of moxibustion on EGFR and AP-1 protein expressions of gastric tissues in rats with gastric mucosal damage

Compared with the normal group, protein expressions of EGFR and AP-1 in rat's gastric tissue were significantly increased in the model group ($P<0.01$), which indicated that the expressions of EGFR and AP-1 in rat's gastric tissue were increased during the autotherapy process of damaged gastric mucosal tissues after restraint and water immersion stress; compared with the model group, protein expressions of EGFR and AP-1 in rat's gastric tissue were further enhanced in the moxibustion group ($P<0.01$), (Figure 3 and Table 2).

Table 1. Comparison of p-ERK1/2 protein expression of rat gastric mucosa among groups ($\bar{x}\pm s$)

Group	*n*	p-ERK1/2 (OD value)
Normal	6	0.088±0.027
Model	6	0.131±0.009[1)]
Moxibustion	6	0.296±0.009[2)3)]

Note: Compared with normal group, 1) $P<0.05$, 2) $P<0.01$; compared with model group, 3) $P<0.01$

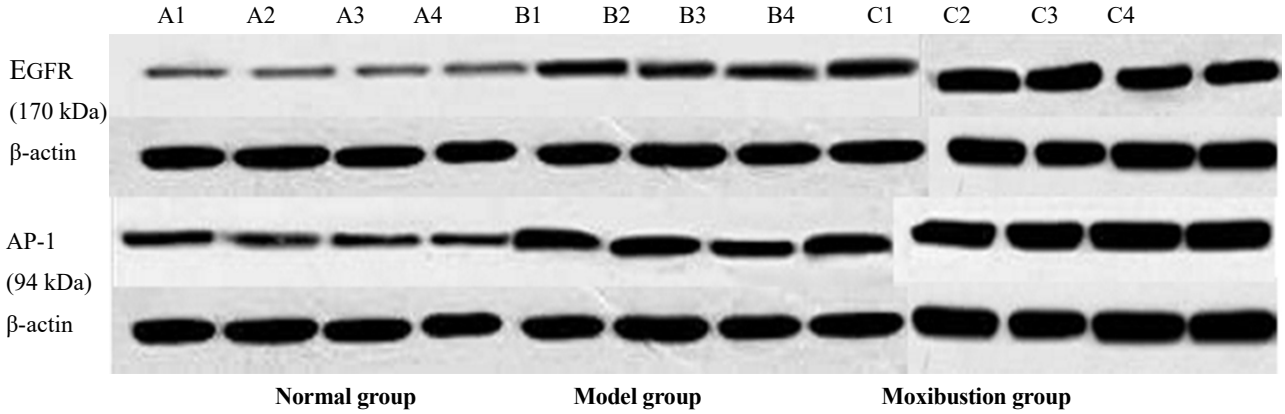


Figure 3. EGFR and AP-1 protein expressions of rat gastric tissue in each group

Table 2. Comparison of EGFR and AP-1 protein expressions of rat's gastric mucosa ($\bar{x}\pm s$)

Group	*n*	Average gray value	
		EGFR	AP-1
Normal	4	0.585±0.017	0.670±0.032
Mode	4	0.788±0.013[1)]	0.837±0.126[1)]
Moxibustion	4	0.920±0.024[1)2)]	0.975±0.013[1)2)]

Note: Compared with normal group, 1) $P<0.01$; compared with model group, 2) $P<0.01$

3 Discussion

ERK, one of the important members of mitogen-activated protein kinase (MAPK) family, including ERK1 and ERK2 two subtypes, is the key for the transduction of signals from the surface receptors to the nucleus. They are usually activated by the mitogen, such as endogenous growth factor (EGF, TGF-α, etc.) and participate in signal transduction. Phosphorylated ERK is its active form, which enters into the nucleus to act on transcription factors to promote the expression of genes involved in cell growth, development, division and function synchronization among cells, and so on, a variety of physiological processes, and has an important regulatory role in cell cycle running and the gene expression[8]. The basic steps of ERK signaling pathway have been known to the three enzymatic cascades of MAPKs. In the ERK pathway, Ras is an upstream activated protein, and Raf is mitogen-activated protein kinase kinase kinase (MAPKKK), MAPK/ERK kinase (MEK) is the mitogen-activated protein kinase kinase (MAPKK), ERK is mitogen-activated protein kinase (MAPK), namely the Ras-Raf-MEK-ERK pathway. ERK1/2 is an important signaling molecule in Ras pathway, locates at downstream of Ras, mainly activated by the growth factor receptors (such as EGFR), which needs the involvement of Ras, PKC and Raf proteins[9]. Only phosphorylated ERK1/2 is active. Normally ERK locates within the cytoplasm, and once activated, ERK will quickly pass through the nuclear membrane, then activates transcription factors (AP-1, NF-κB, etc.), and regulates growth-related gene transcription. Thus, in this experiment, we investigated the effects of moxibustion on the protein expressions of EGFR, p-ERK1/2 and AP-1, the key factors of ERK signaling transduction pathway in the gastric tissues of stress-

induced gastric mucosal damage rats, and explored the mechanisms of moxibustion therapy in promoting the restoration of damaged gastric mucosa, as a starting point to further discover the warming reinforcing action mechanisms of moxibustion in promoting the repair of gastric mucosal damage.

Currently there are more studies about the effect of moxibustion in regulating the growth factor; few studies are associated with transduction pathway mechanisms after the binding of growth factor to its receptor. Zhang H, *et al* found that treatment of spleen deficiency gastric ulcer by moxibustion may be through activation of EGFR-mediated ERK phosphorylation, thereby activating the EGFR/ERK signaling pathway[10]. In a recent study, we found that moxibustion at Zusanli (ST 36) and other acupoints showed a better regulatory effect on ERK key enzymes after the binding of growth factor to its receptor. But the mechanism of interfering nuclear transcription factors after activation of ERK signaling pathway needs further study[11].

The results showed that in the stress-induced gastric mucosal damage rats, the ERK1/2 phosphorylation gradually increased, EGFR and AP-1 protein expressions in gastric tissue increased, indicating the initiation of cell proliferation activity during the ulcer autotherapy process; moxibustion at Zusanli (ST 36), Zhongwan (CV 12) and other acupoints further enhanced the expression of the above key factors in ERK signal transduction pathway, so as to promote the repair of gastric mucosal damage.

Warming reinforcing effect of moxibustion is a combination of physical, pharmacological and acupoint characteristics, not only improving anxiety, depression, and other emotional changes caused by chronic visceral pain[12-14], but also promoting the repair of gastric mucosal damage related diseases. Gastric mucosal damage related diseases, mainly including acute gastric mucosal diseases, chronic gastritis, peptic ulcer, etc., which can be attributed to the category of 'epigastria pain' of traditional Chinese medicine (TCM). Combination of Zusanli (ST 36) and Zhongwan (CV 12) belongs to matching acupoints of the Lower He-Sea acupoint and the Front-Mu acupoint, which specializes in treatment of gastric disease; Pishu (BL 20) and Weishu (BL 21) respectively are the Back-Shu points of spleen and stomach, which are used to treat cold syndrome and deficiency syndrome. For example, epigastric pain due to deficient cold of spleen and stomach is usually treated with moxibustion at Pishu (BL 20) and Weishu (BL 21); conjunction of Weishu (BL 21) and Zhongwan (CV 12) belongs to matching acupoints of the Back-Shu acupoint and the Front-Mu acupoints, which has significant therapeutic effects on the Zang-fu organs' disease due to the coexistence of deficiency and excess, and cold-heat complex; alternate moxibustion at the above four acupoint can regulate spleen and stomach, invigorate spleen-stomach and replenish qi, with significant warming reinforcing effect[15-17].

The results in this study showed that moxibustion pretreatment could promote the proliferation and repair of gastric mucosal cells in gastric mucosal damaged rats, and enhance the protective function of gastric mucosa. This warming reinforcing effect of moxibustion at Zusanli (ST 36), Zhongwan (CV 12) and other acupoints could increase EGF, TGF-α and other growth factors in serum and the gastric tissue. After binding to specific receptor EGFR on the gastric mucosal cell membrane, EGF and TGF-α[3] also could induce the expression of TFF1, proliferating cell nuclear antigen, heat shock protein 70 (HSP70), metallothionein (MT) and other proliferation and repair-related factors[4,18], increase ERK and nuclear transcription factor AP-1 expression in gastric mucosal cells through ERK pathway, thereby promoting the proliferation of gastric mucosal cells. This further indicated that the influence of moxibustion pretreatment on signal transduction for repair of gastric mucosal damage may be achieved through ERK signaling pathway.

Basically, the key pathways of moxibustion pretreatment in promoting proliferation and repair of gastric mucosal damage could be described as: moxibustion at acupoints → release of periphery EGF, TGF-α and endogenous protective proteins expression (HSP70, MT) → expression of EGFR in gastric mucosal cells → phosphorylation of key enzyme of ERK (p-ERK1/2) → expression of nuclear transcription factor AP-1 → strengthening of gastric mucosal cell proliferation → repair of gastric mucosal damage. While, whether the influence of moxibustion pretreatment on signal transduction for repair of gastric mucosal damage is achieved through ERK signaling pathway still needs further study. In this study, we only discussed the mechanisms of moxibustion in promoting the repair of gastric mucosa by ERK signaling pathway related proteins, but if this mechanism is associated with other signal transduction pathways needs further investigation. In addition, we will block ERK signaling pathway in following experiments to explore the specificity of ERK signal transduction pathway in the action of moxibustion in promoting gastric mucosal repair.

Conflict of Interest

The authors declared that there was no potential conflict of interest in this article.

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Statement of Human and Animal Rights

The treatment of animals conformed to the ethical criteria in this experiment.

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