Effects of moxibustion and acupuncture at Zusanli (ST 36) and Zhongwan (CV 12) on chronic atrophic gastritis in rats

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Abstract

OBJECTIVE: To evaluate the effects of moxibustion and acupuncture of Zusanli (ST 36) and Zhongwan (CV 12) acupoints on chronic atrophic gastritis (CAG) in rats, and to study the mechanisms behind their actions.

METHODS: Forty-four male Sprague-Dawley rats were induced with CAG by intragastric administration of 40% ethanol combined with free drinking of N-methyl-N′-nitro-N-nitrosoguanidine and irregular feeding for 12 weeks, followed by daily treatment with moxibustion or acupuncture for 2 weeks. Histopathologic examination, Western blotting of cytokines [epidermal growth factor (EGF), EGF receptor (EGFR), extracellular signal-regulated kinase (ERK), phosphorylated ERK (p-ERK)], and 1H NMR-based metabolic profiling of gastric tissues were used to measure changes related to CAG modeling and treatment.

RESULTS: Moxibustion and acupuncture at Zusanli (ST 36) and Zhongwan (CV 12) each relieved CAG-induced abnormalities in histopathology and cytokine expression of ERK and p-ERK. Only moxibustion treatment regulated the expression of EGF and EGFR. Metabolites that were increased in gastric tissue by CAG induction (alanine, nicotinamide adenine dinucleotide phosphate, uracil DNA glycosylase, lactate, glycerol and adenosine) were restored to normal levels after moxibustion treatment; acupuncture treatment only normalized the levels of adenosine monophosphate and glycerol.

CONCLUSION: Our findings suggest that moxibustion or acupuncture at Zusanli (ST 36) and Zhongwan (CV 12) can significantly improve the condition of CAG in rats. These treatments exert their effects on CAG through different mechanisms.

Keywords: Gastritis, atrophic; Moxibustion; Acu-
puncture; Metabolomics; Proton magnetic resonance spectroscopy

INTRODUCTION

Chronic atrophic gastritis (CAG) is a common digestive disease characterized by upper abdominal pain, fullness, belching and lack of appetite. CAG is widely regarded as the precancerous stage of gastric cancer, and its pathologic features generally show gastric mucosal atrophy, intestinal metaplasia and gastric epithelial dysplasia. The modern medical treatment of CAG mainly comprises molecules with anti-inflammatory, acid suppressive and gastric mucosal protective properties, such as vitamin B12, proton pump inhibitors and antibiotics. Such treatments can effectively inhibit the deterioration of CAG, but may involve long treatment cycles, high cost and the potential for drug resistance. Thus, optimized complementary and alternative interventions are urgently needed. Currently, Traditional Chinese Medicine (TCM) is becoming increasingly popular because of its significant benefits, few side effects and comparatively low cost.

Moxibustion and acupuncture have been widely used in China for centuries. Increasingly, acupuncture and moxibustion are practiced as a form of ‘green’ therapy because of their perceived safety and few side effects. Acupuncture stimulates acupoints through mechanical irritation with needles, while moxibustion applies the heat from burning moxa to stimulate acupoints. These two treatments can be used alone or in combination across a range of different adaptations. The therapeutic effectiveness of moxibustion and acupuncture on CAG has been demonstrated in both humans and animals.

Metabolomics is a systems biology approach that can simultaneously monitor and access changes in metabolic profiles caused by disease and other types of stimulation in a holistic context, which is concordant with the holistic, dynamic approaches of moxibustion and acupuncture. Recently, 1H nuclear magnetic resonance (NMR)-based metabolomics has been widely used to elucidate the therapeutic mechanisms of moxibustion and acupuncture because of its non-destructive sample preparation and nonselective analysis. In previous studies, we showed that both acupuncture and moxibustion were effective in treating CAG via stimulation at stomach meridian acupoints. According to TCM theory, the gastric Front-Mu point known as Zhongwan (CV 12) can regulate gastric functions, and it was reported that stimulation of Zhongwan (CV 12) and Zusanli (ST 36) can improve gastrointestinal motor abnormalities. In this study, we used 1D 1H-NMR spectra to investigate the effects of moxibustion and acupuncture of Zusanli (ST 36) and Zhongwan (CV 12) on CAG in rats, and to examine the mechanisms behind the actions.

METHODS

Materials

Moxa sticks (1.8-cm diameter) were from Lishizhen Qi-ai Mugwort Group (Hubei, China) and 0.2 mm × 0.25 mm stainless steel acupuncture needles were from Suzhou Acupuncture Goods Co., Ltd., Suzhou, China. Chemical suppliers were as follows: N-methyl-N-nitro-N-nitrosoguanidine (MNNG); 75F71-TF, Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan); sodium 3-trimethylsilyl-(2, 2, 3, 3-d4)-1-propionate (TSP-d4, Cambridge Isotope Laboratories, Inc., Tewksbury, MA, USA); D,O (633178), Tris, ammonium persulfate, sodium dodecyl sulfate (SDS), tetramethylthelylenediamine, Tween-20, acrylamide, glycine, bisacrylamide, and Ponceau stain (Sigma, St. Louis, MO, USA); anhydrous ethanol, 4% polyoxymethylene and 10% chloral hydrate (Changsha Guge Bio-Technology Co., Ltd., Changsha, China); carbinol and chloroform (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China). Hyperpure water was prepared using a Milli-Q system (Millipore, Billerica, MA, USA). Horseradish peroxidase (HRP) goat anti-rabbit IgG, HRP goat anti-mouse IgG, epidermal growth factor receptor (EGFR)-specific rabbit polyclonal antibody (18986-1-AP), and β-actin mouse monoclonal antibody (60008-1-lg) were from Proteintech Group Inc., (Rosemont, IL, USA). Anti-extracellular signal-regulated kinase (ERK) 1 (pT202/pY204) + ERK2 (pT185/pY187) antibody (ab50011), anti-EGF antibody (ab77851), and anti-ERK1/2 antibody (ab184699) were from Abcam (Cambridge, MA, USA).

Animals

Forty-four healthy male specific pathogen free (SPF) degree Sprague-Dawley rats, weighing (200 ± 20) g, aged 5-6 weeks, were obtained from the Experimental Center of Hunan University of Chinese Medicine (Hunan, China). Rats were housed in the experimental animal room, which was controlled at a temperature of 20-25 °C and 50%-70% relative humidity, with a 12-h light-dark cycle. The entire experimental study protocol was approved by the Animal Care and Use Committee of Hunan Province [Permit No. SYXK (Xiang) 2013-0005]. All animals used in the study were treated in accordance with the Guide for the Care and Use of Laboratory Animals from the US National Institutes of Health.

Modeling

After 1 week of adjustment, according to the random number table, the rats were divided into 4 groups: control (n = 10), CAG (n = 10), moxibustion (n = 10) and acupuncture (n = 10). The control group rats were fed normally. Other three groups were established CAG by free drinking of MNNG (150 μg/mL) coupled with 40% ethanol gavage (1 mL/100 g bodyweight, twice/week) and irregularity of feeding (altering 1 d of feeding and 1 of day fasting) for 12 weeks.
Moxibustion and acupuncture treatment

The Zusanli (ST 36) and Zhongwan (CV 12) acupoints in rats were located according to the Veterinary Acupuncture of China and the Government Channel19 and Points Standard GB12346-90 of China.19

Once a day for 2 weeks, rats in the moxibustion and acupuncture groups were fixed on a rat frame in supine position, the selected acupoints were disinfect with 75% alcohol, and moxibustion or acupuncture treatment was given. For moxibustion treatment, the lit moxa sticks were placed 10-15 cm above the acupoints for 15 min. To ensure that the skin temperature reached 45-55 °C, we fabricated a body surface temperature sensor and placed it at the moxibustion region. During the treatment, heat-insulation material fitted with a 5-mm diameter hole was placed on the surface of the acupoints to ensure uniformity across the moxibustion range.

For acupuncture treatment, stainless steel needles were inserted into the acupoints at a depth of 5 mm for 15 min.

For the control and CAG groups, rats were only fixed on a rat frame in supine position for 15 min once a day for 2 weeks.

Sample collection

After the 2-week treatment completed, all rats were anesthetized by intraperitoneal injection with 10% chloral hydrate. Gastric tissue were removed and a part of gastric tissue was immersion in a microwave oven for 10 min for antigen retrieval. Then, the samples were dehydrated with an ascending series of ethanol. The samples were then embedded in paraffin and stained with hematoxylin and eosin (HE). Histopathology

Gastric tissue for biopsy was dehydrated with an ascending series of ethanol. The samples were then embbeded in paraffin by paraffin embedding station, sectioned at 5 μm by rotary microtome (Leica Biosystems , Nussloch GmbH, Nuffloch, Germany), and stained with hematoxylin and eosin (HE).

Immunohistochemistry (IHC)

Gastric tissue were selected, embedded in paraffin and cut into ultra-thin slices using a semiautomatic microtome. The slices were submerged in dimethylbenzene for 10 min, dewaxed using a graded alcohol series, dipped into 0.01 M citrate buffer (pH 6.0) and heated in a microwave oven for 25 min for antigen retrieval. Next, the samples were reacted with 3% hydrogen peroxide for 10 min to inactivate endogenous enzymes. The appropriate diluent primary antibody was dropped onto the slices and incubated at 4 °C overnight; after washing with phosphate-buffered saline, the secondary antibody was dropped onto the slices for 30 min at 37 °C followed by successive staining with 3, 3N-diaminobenzidine tetrahydrochloride (DAB) and hematoxylin, then dehydrated with a graded alcohol series. Finally, the samples were sealed with neutral gum for observation under a microscope. The mean IHC integrated optical density (IOD) of images was measured by Image-Pro Plus 7.0 (Media Cybernetics, MD, USA).

Western blotting

Total protein was extracted from gastric tissue samples by homogenization with RIPA lysis buffer and phenylmethylsulfonyl fluoride. The supernatants were collected and the protein concentration was measured using the bicinchoninic acid method. The total protein was separated by electrophoresis (Bio-Rad Laboratories, Inc., Hercules, CA, USA) in a 10% SDS-polyacrylamide gel and transferred to a polyvinylidene fluoride membrane. After successfully blocking the target proteins with primary and secondary antibodies, the bands containing target protein were detected using the enhanced chemiluminescence method; the grey level of each protein band was calculated by Quantity One (Bio-Rad Laboratories).

1H NMR experiments

Gastric tissue weighing about 200 mg were homogenized in a mixture of 300 mL H2O and 600 mL CH3OH, then vortexed for 1 min. Supernatants were collected after centrifugation (12000 × g, 15 min, 4 °C). The upper supernatant from each sample was lyophilized and mixed with 600 μL D2O containing 1 mM TSP. A 500-μL aliquot of this mixture was collected into a 5-mm NMR tube for analysis.

NMR spectra were collected by Bruker Avance III NMR spectrometer (850 MHz) (Bruker, Karlsruhe, Germany); 1D 1H spectra were conducted with Nuclear Overhauser Effect Spectroscopy (NOESY, RD-901-t1-90°-tm-90° -acquire) pulse sequence. Experimental parameters were as follows: experimental temperature, 298 K; number of sampling points (TD), 65 536; mixing time (τm), 120 ms; spectral width (SWH),16 ppm; relaxation time delay (D1), 4 s; mixing time (D8), 0.01 s; sampling time (AQ), 3.27 s; accumulation frequency (NS), 128; air sweep times (DS), 4. Resonance signals of metabolites were identified by Chenomx NMR Suit software (Chenomx Inc., Edmonton, Canada), published literature, and chemical shift databases of compounds including BMRB (http://www.bmrbr.wisc.edu/metabolomics/) and HMDB (http://www.hmdb.ca/).

The 1D 1H-NMR spectra were processed by MestReNova v 9.0.1 software (Mestrelab Research, Santiago de Compostella, Spain). After referencing to a single peak from TSP at 0.0 ppm, all spectra were manual phased and baseline corrected. To avoid the influence of water, all spectra were cut to the chemical shift range of 4.65-5.00 ppm. After binning from 0.5 to 9.5 ppm using a 0.01-ppm width for each integral region, the spectral data were normalized to a sum of all integrals in a spectrum and then imported into SIMCA-P+14.1 (Umetrics, Malmö, Sweden) for multivariate analysis. Pareto scaling was used to reduce the effect of noise in
the model, and principal component analysis was performed for each group by visual observation to evaluate the natural separation. The supervised orthogonal projection to latent structures discriminant analysis (OPLS-DA) was then applied to distinguish different groups. The $P$ value of cross-validated analysis of variance was calculated to evaluate the statistical significance of the OPLS-DA model, and the parameters for model fitness ($R^2$) and the predictive ability ($Q^2$) were used to assess quality of the OPLS-DA model. To find the potential variables for differentiation, the corresponding S-plot of OPLS-DA was also conducted, and the variable importance in the project (VIP ≥ 1.00) and an independent-sample $t$ test ($P < 0.05$) were used to screen potential biomarkers.

RESULTS

**Histopathology**

Histopathology results (Figure 1) shown that, in the control group, the glandular cells were clear and orderly, with the same shape and size. The epithelial cells formed a single columnar layer with transparent cytoplasm and membrane integrity; there was no hyperemia or edema in the glandular and mucosal tissues. Conversely, the histologic morphology of rats in the CAG group showed a thin gastric mucosa, broadening of the muscularis mucosa, exfoliated epithelial cells, lymphocyte infiltration, and irregular and reduced glands, which all indicate successful CAG modeling in the rat. Interestingly, in the moxibustion group, the glands were relatively intact and a small amount of lymphocytic infiltrate and sporadic punctate hemorrhage were observed in the mucous and submucosa. In the acupuncture group, inflammatory cell infiltration, sporadic punctate hemorrhage, complete gland structure and reduced vacuole-like changes in chief cells could be observed.

**Hc image analysis**

The positive expression of EGF, EGFR and ERK in gastric tissue appears yellow or brown on IHC images (Figure 2A-L). Image analysis revealed that the expression of EGF, EGFR and ERK were all increased in the CAG group compared with the control group, whereas treatment with moxibustion or acupuncture decreased the levels of all three factors to varying degrees.

**IOD analysis**

The results of IOD of IHC images (Figure 2M-O) showed that, compared with the control group, the expression of EGF, EGFR and ERK in the CAG group was increased ($P < 0.01; P < 0.05; P < 0.01$). Compared with the CAG group, the expression of EGF, EGFR and ERK were downregulated in the moxibustion group ($P < 0.01; P < 0.05; P < 0.05$), and the expression of EGF in moxibustion group was lower than acupuncture ($P < 0.01$). In acupuncture group, only ERK expression was downregulated ($P < 0.01$).

**Western blot assays of EGFR and phosphorylated ERK (p-ERK)**

The results of Western blot assays are shown in Figure 3. Compared with the control group, the expression of EGFR and phosphorylated (p)-ERK in gastric tissues of CAG rats was markedly increased. In the moxibustion group, both factors were significantly downregulated ($P < 0.01$), whereas only ERK expression showed an obvious decrease ($P < 0.01$) in the acupuncture group.

**$^1$H NMR experiments**

Typical $^1$H NMR spectra of gastric tissues of rats are shown in Figure 4. The different metabolites were confirmed based on pertinent literature, an NMR database that we developed, and the Human Metabolome Database (http://www.hmdb.ca/) and 43 endogenous metabolites were identified.

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**Figure 1** Pathologic section of gastric tissues in each group (HE staining)

A-D: the pathologic section ($\times$ 100) of hematoxylin-eosin staining; E-H: the pathologic section ($\times$ 400) of hematoxylin-eosin staining; A: control group (fed normally); B: CAG group (MNNG 150 µg/mL, 40% ethanol 1 mL/100 g twice/week and irregularity of feeding); C: G: moxibustion group (moxibustion treatment, once a day for 2 weeks); D: H: acupuncture group (acupuncture treatment, once a day for 2 weeks). HE: hematoxylin-eosin; CAG: Chronic atrophic gastritis; MNNG: N-methyl-N’-nitro-N-nitrosoguanidine.
Visual inspection of the complex 1H NMR spectra did not reveal obvious differences between the groups. To find possible variation in factors contributing to changes in rats undergoing CAG modeling and acupuncture or moxibustion treatment, we applied OPLS-DA to the data (Figure 5A). Through the analysis of S-plots...
and t tests, we found that the levels of inosinic acid, 3-hydroxybutyric acid, adenosine monophosphate, adenosine, nicotinamide adenine dinucleotide phosphate (NADP+) and uracil DNA glycosylase (UDG) were increased, whereas the levels of glycine, glycerol, lactate, glycerophosphocholine, alanine and leucine were decreased in CAG rats compared with controls (Figure 5B).

We also used OPLS-DA to show that there was a clear separation between the rats in the CAG group and those in the acupuncture and moxibustion groups (Figure 5C, 5E). Furthermore, according to the S-plot and t-test analysis the levels of lactate, alanine and glycerol were increased in rats in the moxibustion group, while the levels of adenosine and NADP+ were decreased, compared with the CAG rats (Figure 5D). In the acupuncture group, there was a decreased level of adenosine monophosphate and an increased level of glycerol in the gastric tissues compared with the CAG group (Figure 5F).

**DISCUSSION**

In this study, pathologic examination of gastric mucosa was used to evaluate stomach damage in CAG rats. The pathologic injury observed in our rats suggests that the CAG modeling protocol was successful and that both moxibustion and acupuncture of Zusanli (ST 36) and Zhongwan (CV 12) points led to different degrees of improvement of CAG.

Epidermal growth factor (EGF) is an important cytokine in the EGF family. EGF and its receptor EGFR participate in the pathogenesis of gastric cancer and play important roles in regulating cell proliferation, apoptosis and differentiation.\(^{11}\) In this study, increased expression of the growth factor in the CAG rats suggested that their CAG was in the process of transforming to gastric cancer. Decreased expression of EGF and EGFR in the gastric mucosa of CAG rats after moxibustion of Zusanli (ST 36) and Zhongwan (CV 12) effectively alleviated canceration of this tissue. By contrast, there was no obvious downregulation of EGF and EGFR in the acupuncture group, indicating that acupuncture at Zusanli (ST 36) and Zhongwan (CV 12) may impact CAG in other ways.

Upon activation by internal and external stimulators, extracellular signal-related kinase (ERK) is an important signaling pathway in the mitogen-activated protein kinase system, transmitting stimulus signals from the cell membrane to the cytoplasm and promoting the transcription of relevant genes to participate in various biologic reactions.\(^{12}\) As a key regulator of cellular proliferation, ERK participates in the pathological growth, proliferation and metastasis of cells in gastric cancer as well as CAG.\(^{13}\) In this study, the high expression of ERK in rats induced with CAG was reversed to normal levels by treatment with moxibustion or acupuncture, indicating that stimulation of Zusanli (ST 36) and Zhongwan (CV 12) with either acupuncture or moxibustion could be useful to treat CAG. To better understand the function of ERK in CAG, we also examined the expression of p-ERK and found results concordant with those of ERK, providing more evidence that either of these treatments can alleviate CAG via regulation of the ERK signaling pathway.

The stomach is considered a target organ in the CAG process. Metabolomics, as a systems biology approach, can provide a global overview of the integrated response to stress by an organism or its biofluids, which aligns with the holistic principles of TCM theory.\(^{14}\) In this study, \(^1\)H NMR-based metabolomics revealed changes in metabolites that were induced by CAG modeling and reversed by moxibustion or acu-
L-lactate is constantly produced from pyruvate via the glycolytic pathway. Glycerol and fatty acids are released and the glycerol component can be converted into fat. Glycine is involved in the release of energy by converting into glucose by the liver, providing energy for cellular metabolism. Glycine is involved in the release of energy by regulating NADP+, which is an important intermediate during glycolysis and the tricarboxylic acid cycle. L-lactate is constantly produced from pyruvate via the enzyme lactate dehydrogenase in a process of fermentation that occurs during normal metabolism and exercise. The organic compound 3-hydroxybutyric acid is involved in synthesis and degradation of ketone bodies when the body is in a state of hunger. Alanine and leucine, branched-chain amino acids, are critical to human life, and are especially important in stress, energy and muscle metabolism. The levels of all of the metabolites mentioned above were altered by CAG modeling, indicating that CAG may involve disorders in energy metabolism. Moxibustion at Zusanli (ST 36) and Zhongwan (CV 12) reversed these changes in adenosine, lactate, glycerol, alanine and NADP+; while acupuncture treatment at these acupoints normalized the levels of adenosine monophosphate and glycerol. These results suggested that both treatments exert beneficial effects on CAG in rats by regulating energy metabolism, but act through different pathways. In summary, the induction of CAG caused gastric

Figure 5 Plots of orthogonal projection to latent structures discriminant analysis scores and corresponding S-plots. Comparisons of control and CAG groups [A and B; R2X = 0.649, R2Y = 0.997, Q2 (cum) = 0.909; P value of cross-validated analysis of variance (CV-ANOVA) = 0.0001]; CAG and moxibustion groups [C and D; R2X = 0.495, R2Y = 0.96, Q2 (cum) = 0.515; P value of CV-ANOVA = 0.0019]; and CAG and acupuncture groups [E and F; R2X = 0.559, R2Y = 0.948, Q2 (cum) = 0.712; P value of CV-ANOVA = 0.0055]. CAG: Chronic atrophic gastritis.
gland atrophy and inflammatory infiltration in the stomach of rats, and these symptoms were improved with either acupuncture or moxibustion treatment. Moxibustion treatment normalized the levels of EGF, EGFR and ERK in gastric tissue of CAG rats, while acupuncture only affected the level of ERK. Thus, moxibustion may treat CAG by affecting the EGF and ERK signaling pathways, while acupuncture may be relevant to the ERK signaling pathway alone; these results provide the foundation for further investigations of their specific molecular mechanisms. Additionally, both acupuncture and moxibustion at Zusanli (ST 36) and Zhongwan (CV 12) had significant effects on CAG in rats that were exerted by regulating energy metabolism. The increased levels of metabolites adenosine, lactate, glycerol, alanine and NADP + were reversed by moxibustion, while acupuncture treatment normalized the levels of adenosine monophosphate and glycerol.

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