The effect and mechanism of electroacupuncture at LI11 and ST37 on constipation in a rat model

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ABSTRACT
Background Electroacupuncture (EA) is used clinically for the treatment of constipation. Serotonin (5-hydroxytryptamine, 5-HT) plays an important role in colonic motility; however it is unknown whether alterations in colonic 5-HT are associated with EA. In this study, the effect and mechanism of EA at acupuncture points LI11 and ST37 were examined using a cold saline-induced rat model of constipation.

Methods A rat constipation model was induced by cold saline gavage in 24 Sprague-Dawley rats. A further six rats were included as a Control group. The constipated rats were divided into four groups (n=6 each): a Constipation group that remained untreated; a Constipation+LI11 group that received EA at LI11; a Constipation +ST37 groups that received EA at ST37; and a Constipation+LI11+ST37 group that received EA at both LI11 and ST37. After EA treatment, faecal water content, defaecation frequency, and gastrointestinal (GI) transit were measured, as well as the expression of tryptophan hydroxylase (TPH) in colonic tissues (by Western blot analysis) and 5-HT in both faeces and colonic tissues (by ELISA).

Results All three EA-treated groups demonstrated significant improvements in faecal water content, defaecation frequency and GI transit (p<0.05). In addition, TPH and 5-HT expression were both increased by EA at LI11 and/or ST37 (p<0.05). There were no significant differences between the three EA groups for any outcomes.

Conclusions EA at LI11 and/or ST37 had a positive effect on objective markers of constipation in a rat model. In addition, EA increased 5-HT and TPH in the colonic tissues.

INTRODUCTION
Functional bowel disorders (FBD) are a set of gastrointestinal (GI) conditions with symptoms attributable to the mid or lower GI tract. FBD includes irritable bowel syndrome (IBS), functional abdominal bloating, functional constipation, functional diarrhoea, and unspecified FBD.1 Constipation is one of the most common symptoms in FBD. Diet, hormonal disorders such as hypothyroidism, side effects of medication and, rarely, heavy metal toxicity may induce constipation. In the general population the incidence of constipation ranges from 2–30%.2,3

Electroacupuncture (EA) stimulation of acupuncture points LI11 (Quchi) or ST37 (Shangjuxu) has been used classically for treatment of constipation in clinical practice. LI11 has been used historically to treat abdominal pain, vomiting, diarrhoea, and acute gastroenteritis. It is located at the lateral end of the transverse cubital crease when the elbow is flexed, midway between LU5 (Chize) and the lateral epicondyle of the humerus. ST37 is located 6 cun below ST35 (Dubi), one finger width lateral to the anterior crest of the tibia. It has been used historically for borborygmus, abdominal pain, diarrhoea, constipation, and intestinal abscess. Research has shown that sympathetic nerves inhibit GI peristalsis, while parasympathetic nerves promote it.4 Preclinical experiments have shown that EA stimulation of points on the abdomen can influence sympathetic nerves to the GI tract, while points in the four limbs can influence parasympathetic nerves (in view of their segmental relationships), and increased parasympathetic:sympathetic balance will theoretically promote GI movement.5–7

Serotonin (5-hydroxytryptamine, 5-HT) plays an important role in the regulation of GI motility, smooth muscle tone, mucosal secretion, and neuronal signalling. It has been estimated that 95%
of serotonin in the body is within the digestive tract, of which 90% is in the enterochromaffin cells distributed throughout the gut mucosa and 10% is in the neurons. The remaining 5% of the body's serotonin is in the central nervous system. Reports of plasma 5-HT concentrations in FBD suggest that circulating serotonin may play a role in determining the predominant pattern of bowel function. For example, 5-HT is increased in diarrhoea and coeliac disease, and decreased in constipation. Release of 5-HT activates intrinsic afferent neurons located on the terminals of myenteric neurons and GI smooth muscle cells, and the role of 5-HT in the gut is characterised by its interactions with various 5-HT receptors. Two important receptors for serotonin that are located in the neural circuitry of the intestines are the 5-HT3 and 5-HT4 receptors, which are the targets of drugs designed to treat GI disorders. 5-HT3 receptor antagonists are used during chemotherapy treatment to help alleviate symptoms, and 5-HT4 agonists are used as pro-motility agents to promote gastric emptying and to alleviate constipation. The rate-limiting enzyme of 5-HT synthesis is tryptophan hydroxylase (TPH).14–18

The aim of this study was to assess the effects of EA at LI11 and ST37 on faecal parameters and GI transit in a rat constipation model induced by cold saline. In addition, we aimed to measure TPH and 5-HT in the faeces and colonic tissue to examine for altered colonic cytokine production and to determine whether it is affected by EA at LI11 or ST37.

METHODS
Experimental animals
Thirty Male Sprague-Dawley rats (weighing approximately 250 g) were housed under environmentally controlled conditions (temperature 21±3°C under a 13/11 h light/dark cycle with lights on at 6:00) in a specific pathogen-free facility with food and water available ad libitum. All experiments were approved by the Laboratory Animal Ethics Committee at the Shaanxi University of Chinese Medicine (reference number #0228001/2011) and were conducted in accordance with local guidelines for animal welfare consistent with the National Research Council ‘Guide for the Care and Use of Laboratory Animals’.

Cold saline-induced model of constipation
An established rat model of constipation was used. Before experiments began, the rats underwent adaptation for 1 week. Six of 30 rats were assigned to the Control group, and thus did not undergo modelling. The remaining 24 rats received gavage with 10 mL/kg 0.9% saline (wt/vol) at 0°C and were immediately transferred into new individual cages. Gavage was repeated daily for a minimum of 5 consecutive days until the rats developed signs of constipation. The time between first administration of 0°C saline and the appearance of signs of constipation (excretion of dry and hard faeces) was assessed and compared between groups. Faecal water content and frequency of defaecation were taken as markers of model establishment.

EA treatment
After the animal model was successfully established, the 24 constipated rats were randomly subdivided into four groups (n=6 each): the Constipation group, which remained untreated; the Constipation+LI11 group, which received EA at LI11 only; the Constipation+ST37 group, which received EA at ST37 only; and the Constipation+LI11+ST37 group, which received EA at both LI11 and ST37. As described earlier, in the human LI11 is located at the lateral end of the transverse cubital crease when the elbow is flexed, midway between LU5 and the lateral epicondyle of the humerus. In the rats, LI11 was located at a depression anterior to the lateral aspect of the elbow, and was needled perpendicularly. In the human, ST37 is located 6 cun below ST35, one finger width lateral to the anterior crest of the tibia. In the rats, ST37 was located about 5 mm below ST36 on the hind leg, and was needled perpendicularly. Rats were fixed on the board lying on their right hand side, therefore the left-sided points were stimulated. After routine disinfection, stainless steel needles (diameter 0.32 mm, length 25 mm, GB2024-1994, Huatuo, Suzhou, China) were inserted at LI11 and/or ST37 to a depth of 4–5 mm, manipulated with the intention of generating needling sensation, and connected via their handles to a HANS (Han’s Acupoint Nerve Stimulator) electro-stimulator (LH202H, Huawei, Beijing, China). Electrical stimulation was applied in the form of disperse-dense waves at alternating 2 Hz and 15 Hz frequencies, respectively, and 1.5 mA intensity for 30 min. Two courses, each consisting of daily treatment for 6 days, were provided with a 2-day interval between the two. After treatment was completed, the general condition of the rats was assessed and functional indices were measured.

Measurement of faecal output and shape
Following gavage with 0°C saline, rat faeces were collected and the frequency of defaecation in 1 day was recorded. Collected faeces were weighed immediately (wet), and again after drying for 10 h at 80°C (dry). The faecal water content was calculated as follow:

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\text{Faecal water content (\%)} = \frac{(\text{wet faeces} - \text{dry faeces})}{\text{wet faeces}} \times 100
\]

The frequency of defection (per day) and faecal water content were used to confirm successful modelling and subsequently to evaluate the effect of EA.

Measurement of upper GI transit
An established model to measure transit through the upper GI tract of rats was used, because the measurement of colonic transit in rats is difficult due to the fluid reservoir function of the caecum during periods of intestinal hypersecretion. Following completion of EA treatment, the rats were fasted for 18–24 h in wire-bottom cages, to prevent the coprophagy that might otherwise have resulted from free access to faeces during a fast, but were allowed free access to water. The rats were then given an oral dose of 500 μL 10% activated carbon/10% gum arabic suspension in water. After 20 min the rats were euthanased by cervical dislocation. The abdomen was opened and the intestine from the stomach to the caecum was removed. GI transit (%) was expressed as the distance travelled by the charcoal relative to the total length of the small intestine, and the data were analysed using the equation $y = \sin^{-1}\sqrt{x}$, where $x=\text{travel distance of the charcoal in the GI tract}$.

Measurement of 5-HT by ELISA
Faeces were collected using a sterile tube, and samples were diluted in phosphate-buffered saline (PBS) (0.02 mol/L, pH 7.2) and shaken. After being allowed to stand for 10 min, samples were centrifuged for 20 min at 1000g. The supernatants were collected and stored at −80°C. Colonic tissue was rinsed thoroughly to remove any blood and weighed before being minced into small pieces and homogenised in PBS (10 mg tissue per 100 μL PBS). The resulting suspension was subjected to ultrasonication to further break down the cell membranes, following which homogenates were centrifuged for 15 min at 1500g. The supernatants were collected and stored at −80°C. The 5-HT concentration was measured separately in faeces and colonic tissues using an ELISA kit (MBS725497, MyBioSource, California, USA) as per the manufacturer’s instructions. Absorbance at 450 nm in each well was measured using a spectrophotometer.

Measurement of TPH by Western blot analysis
Colonic tissue samples from each group were homogenised in the same manner as samples prepared for determination of the expression of TPH. Concentration of the total protein in the solution of homogenised tissue was determined by measuring the absorption at 280 nm. Samples were diluted 1:1 (vol/vol) with 2×Laemmli sample buffer (62.5 mmol/L Tris-HCl (pH 6.8), 2% (wt/vol) sodium dodecyl sulfate (SDS), 50 mmol/L dithiothreitol, and 0.01% (wt/vol) bromophenol blue) and then subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) membranes. The total TPH protein level was detected using a specific anti-TPH antibody (ab46757, Abcam, UK), and alkaline phosphatase-conjugated secondary antibody (W3960, Promega, USA). The target band and the band for β-actin (as an internal control) were quantified by the Image J programme for optical density analysis.

Statistical analysis
All data are presented as mean±SE of the mean (SEM). Statistical analysis was conducted using SPSS V15.0 Software (SPSS Inc, Chicago, Illinois, USA). After testing for homogeneity of variance, the five groups were compared using one-way analysis of variance (ANOVA) and Student–Newman–Keuls (SNK) method post-hoc test. Differences were considered significant when p<0.05.

RESULTS
Effect of EA on defaecation
The establishment of the rat model of constipation was evaluated by the faecal water content and frequency of defaecation. After 3 h of the first gavage with 0°C saline, due to the acute stress of the rats, the faeces were swollen, moist or wet, and shapeless. After 4–24 h, the faeces become dry and hard. The frequency of defaecation (the number of faecal particles per day) gradually decreased. After the sixth gavage, compared to the normal condition (represented by the Control group), the faecal water content of the rats decreased from 40.42±2.41% to 29.17±1.72% (p<0.05) and the number of faecal particles per day decreased from 39.42±2.24 to 31.12±3.26 (p<0.05), demonstrating that cold saline effectively induced constipation in the rats (excretion of dry and hard faeces, at a reduced frequency).

The effect of EA on defaecation is shown in figure 1. The faecal water content in all three treated groups was significantly greater than the untreated constipated rats: 36.02±2.11%, 36.98±2.52%, and 39.78±2.09% in the Constipation+LI11, Constipation+ST37, and Constipation+LI11+ST37 groups, respectively, versus 29.17±1.72% in the Constipation group (p<0.05). Similarly, defaecation frequencies were higher, at 36.31±3.27, 36.62±3.18, and 38.52±3.25, respectively, compared with 31.12±3.26 (p<0.05). There were no statistically significant differences between the three groups receiving EA treatment (p>0.05).

Effects of EA on GI transit
The effects of saline gavage and EA stimulation on GI transit are shown in figure 2. All groups receiving EA stimulation displayed a statistically significant increase in the GI transit rate compared to the untreated constipated group (p<0.05); however, there were no statistically significant differences between rats receiving EA at LI11/ST37 only, or a combination of the two.

Effects of EA on 5-HT and TPH
The 5-HT content of the faeces and colonic tissue (as measured by ELISA) and the relative TPH protein...
levels in colonic tissue (measured by Western blot analysis) are shown in figures 3 and 4, respectively. 5-HT content in the faeces, and both 5-HT and TPH in colonic tissue, were significantly increased in all groups that received EA stimulation (p<0.05) compared to untreated rats with saline-induced constipation.

Figure 1 Faecal water content (A) and frequency defeacation (B) in six healthy rats (Control group) and 24 rats with cold saline-induced constipation that remained untreated (Constipation group, n=6) or received electroacupuncture at LI11 (Constipation+LI11 group, n=6), ST37 (Constipation+ST37 group, n=6) or both LI11 and ST37 (Constipation+LI11+ST37 group, n=6). Data are mean±SEM. #p<0.05 versus Control group; *p<0.05 versus Constipation group.

Figure 2 Gastrointestinal transit in six healthy rats (Control group) and 24 rats with cold saline-induced constipation that remained untreated (Constipation group, n=6) or received electroacupuncture at LI11 (Constipation+LI11 group, n=6), ST37 (Constipation+ST37 group, n=6) or both LI11 and ST37 (Constipation+LI11+ST37 group, n=6). Data are mean±SEM. #p<0.05 versus Control group; *p<0.05 versus Constipation group.
DISCUSSION

This study, by measurement of faecal output and GI transit, has demonstrated that EA stimulation at LI11, ST37 or a combination of the two acupuncture points, can have a positive effect on objective markers of constipation in a rat model. We also found that colonic TPH expression was decreased in rats following cold saline-induced constipation and that EA stimulation at either or both points attenuated this effect. Similar patterns were seen in the 5-HT content of the faeces and colonic tissue.

In this study, EA stimulation of LI11 and/or ST37 increased GI transit and improved defaecation frequency. Previous research has demonstrated that the sympathetic neurons that control the movement of the jejunum are derived from spinal levels T9–T10, and that the parasympathetic neurons originate in the dorsal nucleus of the vagus nerve in the medulla. The somatic afferent signals generated by stimulation at LI11 and ST37 project to C6–C7, and L4–L5, respectively. EA at both LI11 and ST37 appears to promote movement of the jejunum, which we suspect is mediated via the sacral parasympathetic nucleus, most likely involving a supraspinal pathway. Although LI11 and ST37 had equivalent effects and there were no significant differences between treatment at a single point or both points in the present study, previous research has indicated that the effects of point combinations are superior to that of a single point, suggesting that their actions are synergistic.

As a neurotransmitter, 5-HT plays an important role in GI motility and transit. It is well known that 5-HT stimulates intrinsic primary afferent neurons (IPANs) that synapse with ascending and descending interneurones in the myenteric plexus, thus inducing excitation and inhibition locally. Ascending interneurons activate excitatory motor neurons by releasing substance P and acetylcholine onto myocytes, resulting in circular muscle contraction. Descending cholinergic neurons stimulate inhibitory motor neurons releasing nitric oxide, vasoactive intestinal peptide, and adenosine triphosphate leading to circular muscle relaxation. The resulting peristaltic reflex is largely responsible for bolus movement from proximal to distal sites within...
the GI tract. Results from this study showed that the TPH expression and the content of 5-HT in colonic tissue was increased in all groups that received EA stimulation compared to untreated rats with cold saline-induced constipation. This suggests that EA upregulates 5-HT to promote bowel function.

In conclusion, EA stimulation at LI11, ST37, or LI11 and ST37 combined, increases defaecation frequency and GI transit in a cold saline-induced rat model of constipation, and increases faecal and colonic 5-HT content as well as colonic TPH expression. The positive effects of EA may be mediated by excitation of parasympathetic nerves supplying the GI tract, which may account for the higher levels of 5-HT in colonic tissue (although the exact mechanism of action is unknown). The results from the present study provide further evidence to support the potential role of EA in the treatment of constipation as well as preliminary data to suggest that 5-HT is involved in the beneficial effect of EA.

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Contributors XZ and ZL conceptualised and designed the research. HQ, LB and AZ drafted the English manuscript. WN analysed and interpreted the data from the Western blot analysis and 5-HT assay. LG analysed and interpreted the faecal output, faecal shape and GI transit data. YW provided the EA treatment.

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Competing interests None declared.

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