

## Effects and mechanisms of electroacupuncture at PC6 on frequency of transient lower esophageal sphincter relaxation in cats

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### Abstract

**AIM:** To investigate the effects of electroacupuncture (EA) at neiguan (PC6) on gastric distention-induced transient lower esophageal sphincter relaxations (TLESRs) and discuss the mechanisms of this treatment.

**METHODS:** Protocol I : Twelve healthy cats underwent gastric distention for 60 min on the first day. Electrical acupoint stimulation was applied at the neiguan or a sham point on the hip in randomized order before gastric distention, on the third day and fifth day. Those cats that underwent EA at neiguan on the fifth day were named "Neiguan Group" and the cats that underwent EA at a sham acupoint on the fifth day were named "Sham Group" (control group). During the experiment the frequency of TLESRs and lower esophageal sphincter (LES) pressure were observed by a perfused sleeve assembly. Plasma levels of gastrin (GAS) and motilin (MTL) were determined by radioimmunoassay. Nitrite/nitrate concentration in plasma and tissues were measured by Griess reagent. The nuclei in the brain stem were observed by immunohistochemistry method of c-Fos and NADPH-d dyeing. Protocol II : Thirty six healthy cats were divided into 6 groups randomly. We gave saline (2 mL iv. control group), phaclofen (5 mg/kg iv. GABA-B antagonist), cholecystokinin octapeptide (CCK-8) (1 µg/kg per hour iv.), L-Arginine (200 mg/kg iv.), naloxone (2.5 µmol/kg iv.) and tacrine (5.6 mg/kg ip. cholinesterase inhibitor) respectively before EA at Neiguan and gastric distention. And the frequencies of TLESRs in experimental groups were compared with the control group.

**RESULTS:** Protocol I : Not only the frequency of gastric distention-induced TLESR in 60 min but also the rate of common cavity during TLESRs were significantly

decreased by EA at neiguan compared to that of sham acupoint stimulation. C-Fos immunoreactivity and NOS reactivity in the solitarius (NTS) and dorsal motor nucleus of the vagus (DMV) were significantly decreased by EA at neiguan compared to that of the sham group. However, the positive nuclei of C-Fos and NOS in reticular formation of the medulla (RFM) were increased by EA at neiguan. Protocol II : The inhibited effect of EA at neiguan on TLESR's frequency was completely restored by pretreatment with CCK (23.5/h vs 4.5/h,  $P < 0.05$ ), L-arginine (17.5/h vs 4.5/h,  $P < 0.05$ ) and naloxone (12/h vs 4.5/h,  $P < 0.05$ ). On the contrary, phaclofen (6/h vs 4.5/h,  $P > 0.05$ ) and tacrine (9.5/h vs 4.5/h,  $P > 0.05$ ) did not influence it.

**CONCLUSION:** Electric acupoint stimulation at Neiguan significantly inhibits the frequency of TLESR and the rate of common cavity during TLESR in cats. This effect appears to act on the brain stem, and may be mediated through nitric oxide (NO), CCK-A receptor and mu-opioid receptors. But the GABAB receptor and acetylcholine may not be involved in it.

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**Key words:** Electroacupuncture; Transient lower esophageal sphincter relaxation; C-Fos; Nitric oxide; Cat

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### INTRODUCTION

Transient lower esophageal sphincter relaxations (TLESRs) is the most important mechanism of gastroesophageal reflux (GER) either in the patients of gastroesophageal reflux disease (GERD)<sup>[1]</sup> or in normal subjects<sup>[2]</sup>. Distention of the proximal stomach is a major stimulus for triggering TLESRs<sup>[3]</sup>. The stimulus passes through vago-vagal reflex and is integrated in the brain stem. There is ongoing interest in developing drugs that can decrease GER by interfering with TLESRs, including

GABA-B receptor agonist<sup>[4]</sup>, Cholecystokinin-A receptor antagonist<sup>[5]</sup>, nitric oxide synthase (NOS) inhibitor<sup>[5,6]</sup>, morphine<sup>[7]</sup> and atropine (act through central cholinergic blockade)<sup>[8-10]</sup>. The aim of our study is to explore new approaches (EA at Neiguan acupoint) to decrease the rate of TLESRs during gastric distention, and discuss the mechanisms of this treatment.

Acupuncture has been used to treat functional gastrointestinal disorders in eastern countries for centuries. It can modulate visceral sensation as well as function through stimulation at selected acupoints along the meridians (channels of acupoints)<sup>[11]</sup>. EA at zusanli (ST-36) can increase the basal LES pressure<sup>[12]</sup>. Transcutaneous electric nerve stimulation (TENS) at Hukou acupoint increased the degree of LES relaxation in volunteers<sup>[13]</sup> and reduced basal LES pressure in patients with achalasia<sup>[14,15]</sup>. Previous studies have suggested that TENS at neiguan may inhibit the rate of TLESRs triggered by gastric distention<sup>[16]</sup> and reduce the perception to gastric distention<sup>[17]</sup> in human beings. But the precise mechanisms for this phenomenon have not been extensively investigated and are not fully understood.

## MATERIALS AND METHODS

### Materials

Protocol I was performed on 12 adult cats weighing  $3.7 \pm 0.2$  kg (M/F: 8/4), and 36 adult cats weighing  $3.6 \pm 0.5$  kg (M/F: 25/11) were studied for protocol II. Cats were provided by the Animal Center of the First Hospital of Peking University. They were kept in individual cages in a controlled environment with a temperature of 22-26°C, 12/12-h light/dark cycles, and fed with standard cat diet. The animals were deprived of food 10 h before each experiment. All procedures were approved by the Committee for Animal Care and Usage for Research and Education of the Peking University. Anesthesia was initially induced with katamine (30 mg/kg im). Supplementary doses of katamine (15 mg/kg ip) were given whenever necessary to maintain an appropriate depth of anesthesia, as assessed they remained motionless yet still had cornea reflex. They were euthanized with pentobarbital sodium (0.5 mL/kg ip) at the end of the protocol.

Phaclofen, CCK octapeptide and tacrine were obtained from Sigma Chemical Co. (St. Louis, Missouri, USA). Naloxone was provided from Beijing Shuanghe Chemical Company. L-arginine was offered by Beijing Dingguo Chemical Company.

The GAS Radioimmunoassay kit was purchased from the China Institute of Atomic Energy, Beijing, China. The MTL Radioimmunoassay kit was purchased from the Neurobiological Technique Center of the Second Military Medical University, Shanghai, China. The Griess reagent for testing nitrite/nitrate concentration was purchased from Promega Corporation, USA. The reduced form nicotinamide adenine dinucleotide phosphate (NADPH) of and NBT (nitro blue tetrazolium) were obtained from Biomol Corporation, London, UK. The antibody of C-Fos was obtained from Santa Cruz Biotechnology, California, USA.

### Recording methods

The manometry catheter (outer diameter 0.5 cm) consisted of a multilumen silicone tube with five side holes located at 9, 6, 3, 0 and -6 cm from the upper margin of the 6 cm-long Dent sleeve sensor (Dentsleeve, Belair, Australia). The catheter was continuously perfused with distilled water by a low compliance pneumohydraulic capillary infusion system (UPS-2020, Holland) at a rate of 0.2 mL/min. The external pressures transducers were connected via an analog/digital converter to a personal computer system. The data were displayed continuously on a monitor and stored on the personal computer system (MMS B.V. the Netherlands).

After anesthesia the cat was set in a supine position. A manometry catheter was placed through the mouth into the esophagus and positioned so that the sleeve sensor straddled the LES to register LES pressure. The distal side hole was used as a reference point for intragastric pressure. And the upper LES side holes were used to measure esophageal body pressure.

A mylohyoid electromyography (MMS B.V. the Netherlands) was used to record swallowing<sup>[11,18]</sup>. The pinhead electrode was inserted in the mylohyoid muscle, and the reference electrode was fixed to the interscapular region of the back.

### Electroacupuncture

Two acupuncture needles of 0.22 mm in diameter (Suzhou global acupuncture instrument Co. Ltd, Suzhou, China) were inserted perpendicularly at the bilateral Neiguan acupoint (PC6, located 1.5-2.0 cm above the wrist between the ligaments of the flexor carpi radialis and the palmaris longus<sup>[19]</sup>) overlying the median nerve to a depth of 5 mm. An electrical stimulator (Model LH202H Hans, Beijing Huawei Medical Instrument Co. Ltd, Beijing, China) provided current to the needles. Wave patterns were sparse with dense pulse intervals ranging from 2 to 100 Hz (2/100 Hz), with constant amplitude and current flow (3-4 mA). The duration was 60 min. Correct positioning was confirmed by observing slight repetitive paw flexion during stimulation<sup>[20]</sup>.

Control stimulation on a sham acupoint was conducted at the hip, a point away from the traditional meridians and dermatomes.

### Gastric distention

Air was insufflated (at a rate of 15 mL/s) into the stomach through a 2.0-mm diameter tube intubated through mouth to stomach. Its depth equal to 5 cm plus the esophageal body length. 30 mL air into the stomach every 6 min amount to 300 mL were insufflated in the 1 h period of gastric distention.

### Study protocol I : Effects of EA on LES motor function

Twelve adult cats were divided into Neiguan group and Sham group randomly. Each cat was studied for three sessions, and an interval of each session is 2 d (Figure 1). In the first session, the basal rate of TLESRs triggered by gastric distention was observed for one hour. In session 2 and session 3, the influence of EA at neiguan or sham

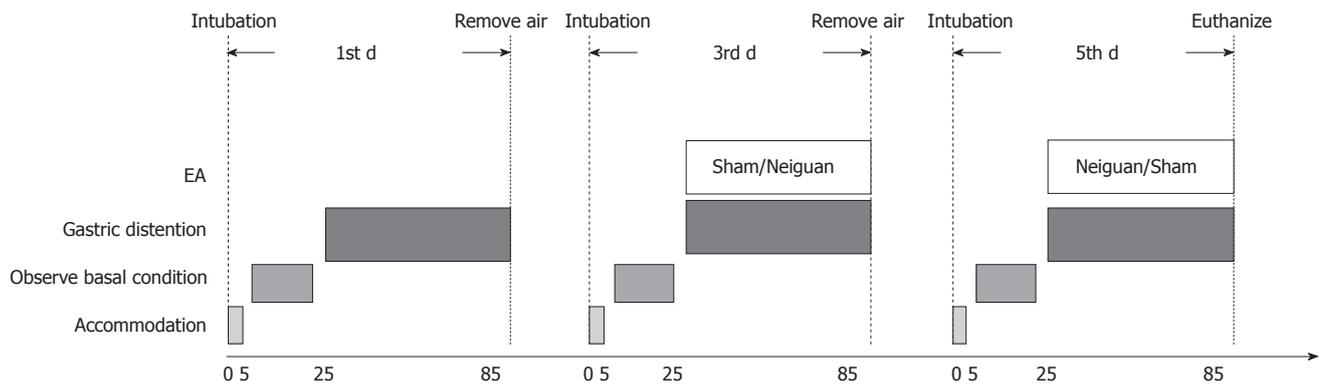


Figure 1 The flow chart of protocol I .

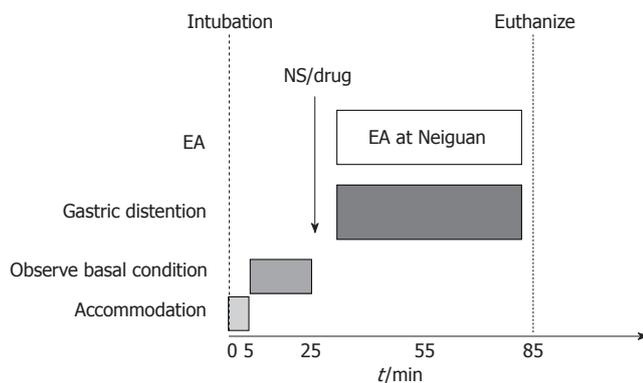


Figure 2 The process of protocol II .

acupoint to the rate of gastric distention induced TLESRs was observed in a random order.

After 5min of accommodation, the basal conditions were observed for 20 min (including basal rate of TLESRs and basal LES pressure). And then the gastric distention was applied in session 1. In session 2 and 3, gastric distention and electroacupuncture were applied at the same time. The remaining air was removed at the end of each session. At the end of session 3, the cats were euthanized and the blood, gastric fundus and brain stem were obtained for further research.

### Study protocol I : Effects of drugs (phaclofen, CCK octapeptide, L-arginine, naloxone and tacrine) on inhibition of TLESRs by electric acupoint stimulation

Thirty six healthy cats were divided into 6 groups randomly. We gave normal saline(NS) (2 mL iv.), GABA-B antagonist phaclofen<sup>[21]</sup> (5 mg/kg iv.), CCK octapeptide<sup>[5]</sup> (1 µg/kg per hour iv.), L-arginine<sup>[5]</sup> (200 mg/kg iv.), naloxone<sup>[22]</sup> (2.5 µmol/kg iv.) and tacrine<sup>[23]</sup> (5.6 mg/kg ip. cholinesterase inhibitor in central nerve system) respectively before EA at Neiguan and gastric distention, and observed the frequency of TLESR and LES pressure (Figure 2). At the end of this study, the cats were euthanized and the blood, gastric fundus and brain stem were obtained for further research.

### Data analysis

TLESRs were defined according to established methods<sup>[9]</sup>.

Basal LES pressure was measured at the end of expiration relative to gastric pressure. The LES pressure during gastric distention was measured for 1 min every 6 min, and an overall mean for each period of the study was calculated. Common cavities were defined as abrupt simultaneous and sustained rises of basal esophageal pressure to intragastric pressure in at least the two lower esophageal body manometry recording sites<sup>[24]</sup>. Common cavities are considered as markers of gas or liquid reflux from the stomach into the esophagus.

### Assay of gastrointestinal hormones and nitrite concentration

When the cats were euthanized, 15 mL venous blood was collected into a test tube containing 400 µL of 10% EDTA-Na<sub>2</sub> (an anticoagulant) and 200 µL of trasylol. The blood samples were centrifuged at 4°C at 3500 r/min for 20 min. The serum was separated and stored at -70°C before analysis. The tissue of gastric fundus is about 2 cm × 2 cm. After weighting, tissues were added to 1 mL of 1 mol/L acetic acid and mixed evenly in a homogenizer to obtain a homogenate and were refrigerated at 4°C for 100 min. The extracted homogenates were added to 1 mL of 1 mmol/l NaOH and centrifuged at 3500 r/min 4°C for 20 min. The supernatant fluid was collected and stored at -70°C.

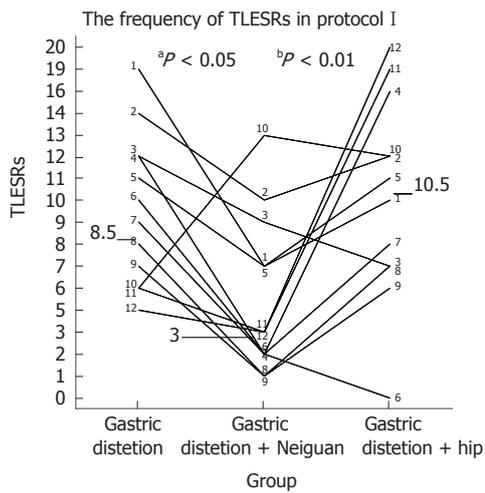
Gastrin (GAS) and motilin (MTL) levels were measured with commercial radioimmunoassay kits. Nitrite/nitrate levels were tested by Griess reagent.

### c-Fos immunohistochemistry and NADPH-d histochemistry

After 60 min of gastric distention on the fifth day, the animal was transcardially perfused with 9 g/L saline followed by 40 g/L paraformaldehyde in 0.1 mol/L phosphate buffer saline (PBS, pH 7.3). The brain stem was removed and postfixed in the same fixative overnight and cryoprotected by immersion in 200 g/L sucrose for 72 h.

Coronal sections (40 µm) of the brain were cut in a cryostat. Every fourth section was used to reveal c-Fos immunoreactivity and the second set of sections was used to reveal NADPH-diaphorase (NADPH-d) staining.

The first set of sections were placed into a 50 g/L goat serum for 30 min at room temperature (RT), and incubated overnight at RT in primary antibody c-Fos (1:200). After washing for 15 min with PBST, the sections were incubated in biotinylated anti-rabbit IgG



**Figure 3** EA at neiguan decreased the rate of TLESRs induced by gastric distention, compared with EA at sham acupoint (<sup>a</sup> $P < 0.01$ ) and baseline distention (<sup>b</sup> $P < 0.05$ ). Each number represents one cat.

(Zymed, South San Francisco, Canada) diluted 1:300 in PBST at RT for 2 h, and then incubated in peroxidase-conjugated streptavidin (1:300 dilution, Zymed) for 2 h at RT. The immunoreactivity was visualized by incubating with 0.05 mol/L Tris-HCl buffer containing 0.1 g/L 3, 3'-diaminobenzidine, and 0.3 mL/L H<sub>2</sub>O<sub>2</sub> for 10-20 min at RT. The stained sections were mounted on APES-coated glass slides, dehydrated and coverslipped.

The second set of sections were incubated at 37°C for 2 h in a solution containing 1 mmol/L NADPH, 0.5 mmol/L NBT, Tris-HCl 50 mmol/L, and Triton X-100 2 g/L. After a rinse in PBST, sections were mounted on APES-coated glass slides, dehydrated and coverslipped.

The distribution of c-Fos and NADPH-d positive cells was detected under a microscope (Olympus, Tokyo, Japan), and the cells were counted on LEICA Q550CW system (Leica Microsystems Imaging Solutions Ltd, Wetzlar, Germany), 10 sections for NTS/DMV, 8 sections for RFM. The average number of c-Fos or NADPH-d positive neurons per section for each cat was calculated, respectively.

**Statistical analysis**

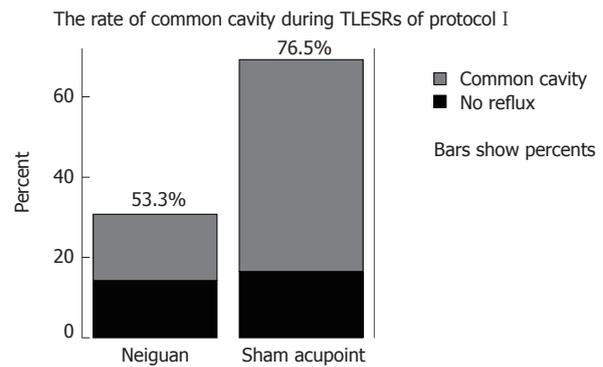
The number of TLESRs was compared using Wilcoxon signedrank test and expressed as median (interquartile range). Basal LES pressure and intragastric pressure were presented as means ± SD and were compared using repeated-measures. The rate of common cavity is compared using paired sample  $\chi^2$ . Nitrate concentrations, plasma hormone levels and the average number of c-Fos or NADPH-d positive nucleus of respective brain areas per section were expressed as mean ± SD and was compared using independent *t*.

SPSS 11.0 was used for statistical analysis, and  $P < 0.05$  was considered statistically significant.

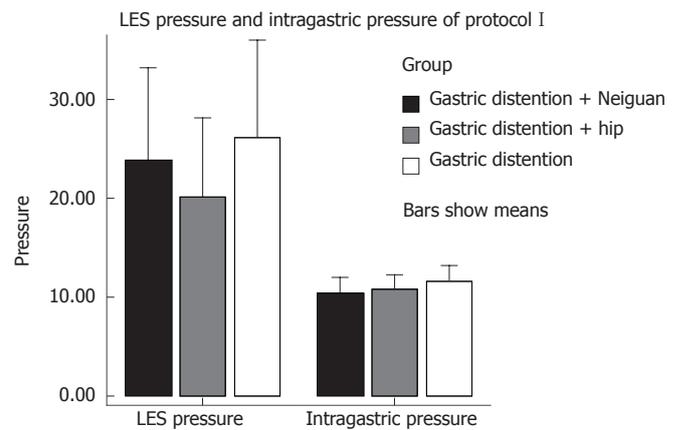
**RESULTS**

**Study protocol I : Effects of EA on LES motor function**

**Transient LES relaxations:** The frequency of TLESRs



**Figure 4** EA at Neiguan decreased the rate of common cavity during TLESRs compared with EA at sham acupoint ( $P < 0.05$ ).



**Figure 5** Electric stimulation at Neiguan didn't influence the LES pressure and intragastric pressure.

during acupoint stimulation at neiguan [3 per hour (range, 1-13)] was significantly lower than that during both the baseline period without any stimulation [8.5 per hour (range, 5-19),  $P < 0.05$ ] and the period of sham stimulation at the hip [10.5 per hour (range, 0-20),  $P < 0.01$ ] (Figure 3).

**Common cavities during TLESRs:** During EA at neiguan, a total of 60 TLESRs induced by gastric distention were observed in one hour, from it 32 were associated with common cavity, and its rate was 53.3%. During EA at sham acupoint, there are 136 gastric distention induced TLESRs in all, and 104 were associated with common cavity. The rate was 76.5%. Between-group comparisons showed that the rate of common cavity during EA at Neiguan was significantly lower than that occurring during EA at Sham acupoint ( $P < 0.05$ ) (Figure 4).

**LES pressure:** EA at neiguan (PC6) had no effect on LES pressure. Overall mean LES pressure during electrical acupoint stimulation at neiguan ( $34.33 \pm 18.16$  mmHg) was similar to that during stimulation at the sham acupoint ( $30.97 \pm 15.72$  mmHg,  $P > 0.05$ ) and during the baseline period without any acupoint stimulation ( $37.74 \pm 18.69$  mmHg,  $P > 0.05$ ) (Figure 5).

**Intragastric pressure:** EA at neiguan (PC6) had no effect on gastric pressure during gastric distention. Overall

**Table 1** Nitrite concentration in plasma and gastric fundus of protocol I (mean  $\pm$  SD)

Group	Serum (mmol/mL)	Gastric fundus (mmol/mg)
Neiguan	0.85 $\pm$ 0.62	3.50 $\pm$ 0.87
Sham	0.66 $\pm$ 0.59	4.18 $\pm$ 1.09

No significant difference was found between the two groups.

**Table 2** Plasma GAS and MTL in each group of protocol I (mean  $\pm$  SD)

Group	GAS (pg/mL)	MTL (pg/mL)
Neiguan group	79.43 $\pm$ 28.84	82.23 $\pm$ 43.79
Sham group	156.30 $\pm$ 72.53	103.6 $\pm$ 68.16

GAS: gastrin; MTL: motilin. No significant difference was found between the two groups.

**Table 3** c-Fos and NADPH-d staining in CNS

group	C-Fos		NOS	
	NTS and DMV	RFM	NTS and DMV	RFM
Neiguan group	21.9 $\pm$ 6.9 <sup>a</sup>	96.6 $\pm$ 16.5 <sup>a</sup>	23.6 $\pm$ 4.6 <sup>a</sup>	75.6 $\pm$ 17.0 <sup>a</sup>
Sham group	45.3 $\pm$ 10.1	61.0 $\pm$ 13.8	43.9 $\pm$ 11.0	30.6 $\pm$ 8.5

NADPH: nicotinamide adenine dinucleotide phosphate; CNS: central nerve system; NOS: nitric oxide synthase; NTS: nucleus tractus solitarius; DMV: dorsal motor nucleus of vagus; RFM: reticular formation of medulla. <sup>a</sup>*P* < 0.05 vs Sham group.

mean gastric pressure during EA at neiguan (10.37  $\pm$  3.17 mmHg) was similar to that during stimulation at the sham acupoint (10.81  $\pm$  2.89 mmHg, *P* > 0.05) and during the baseline period without any acupoint stimulation (11.58  $\pm$  3.16 mmHg, *P* > 0.05) (Figure 5).

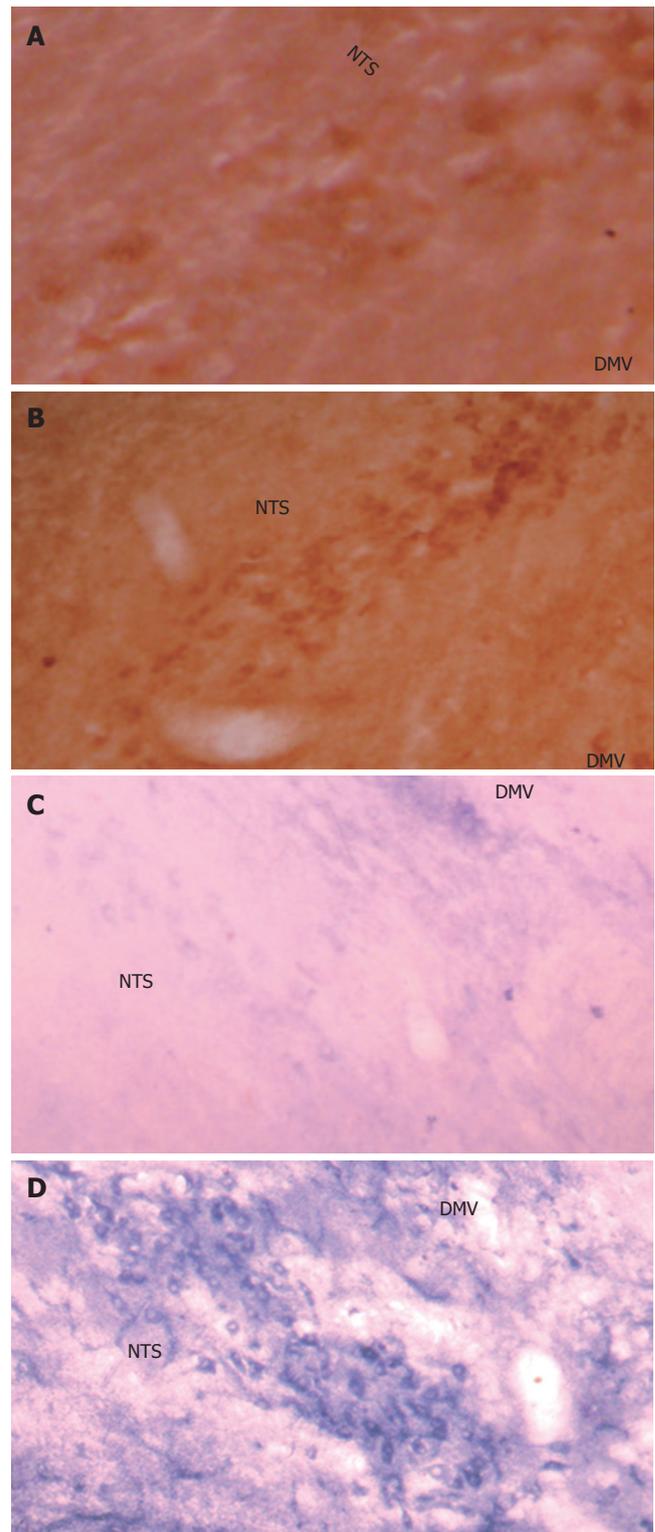
**Nitrite concentration:** EA at neiguan (PC6) did not influence the nitrite levels in plasma and gastric fundus tissues compared with Sham group (*P* > 0.05) (Table 1).

**Gastrin (GAS) and motilin (MTL) levels:** EA at neiguan (PC6) did not influence the GAS and MTL levels in plasma (Table 2).

### C-Fos and NADPH-d staining in the central nerve system (CNS)

The c-Fos positive cell nuclei of activated cells showed the characteristic dark brown staining of oxidized DAB. Compared with the Sham group, electroacupuncture at neiguan significantly inhibited the number of C-Fos-labeled neurons in nucleus tractus solitarius/dorsal motor nucleus of vagus (NTS/DMV) (Figure 6A and B). However, it stimulated a significantly greater number of C-Fos positive nuclei in areas of reticular formation of the medulla (RFM) (Figure 7A and B).

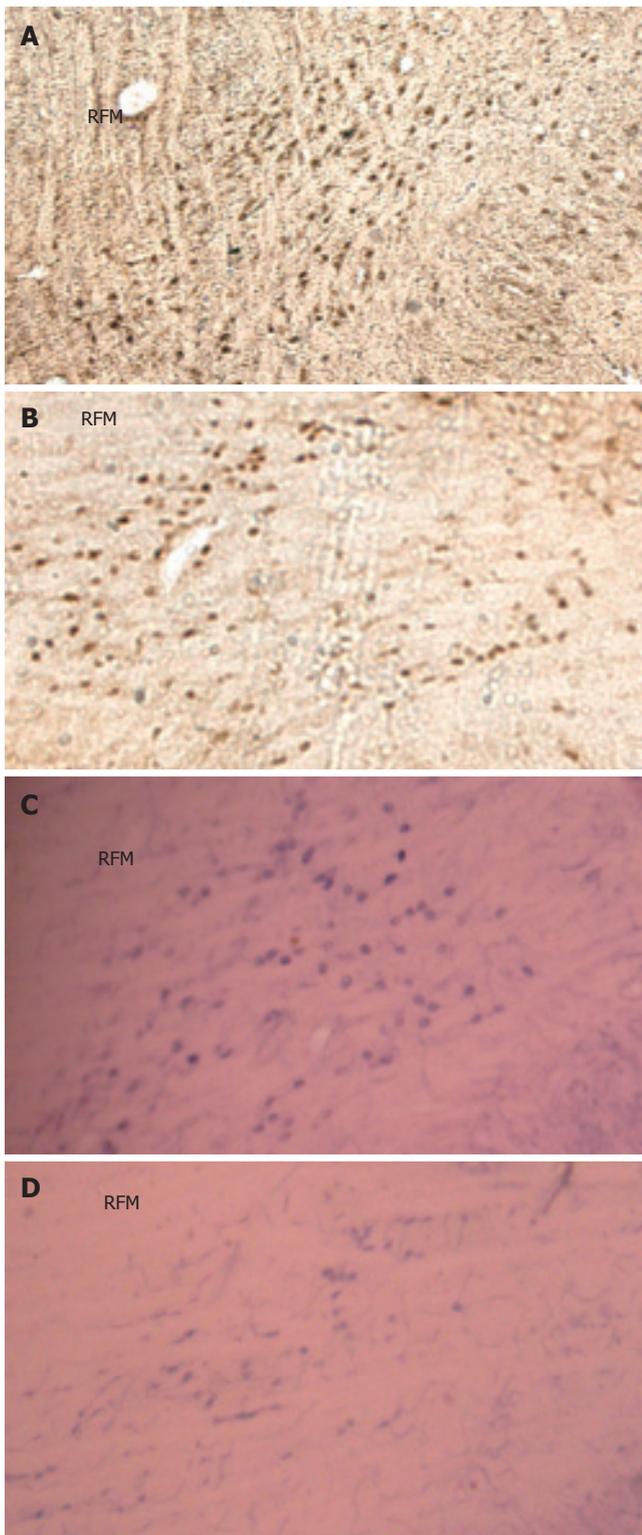
NADPH-d activity was visualized as a vibrant blue color within perikarya, dendrites and axons. Electroacupuncture at neiguan significantly decreased the number of



**Figure 6** Photomicrographs showing C-Fos and NOS positive neurons in nucleus tractus solitarius/dorsal motor nucleus of vagus (NTS/DMV). **A:** C-Fos immunohistochemistry of neiguan group; **B:** C-Fos immunohistochemistry of sham acupoint group; **C:** NADPH-d histochemistry of neiguan group; **D:** NADPH-d histochemistry of sham acupoint group ( $\times$  10).

NADPH-d stained cells in NTS/DMV (Figure 6C and D), but increased the number of positive cells in RFM (Figure 7C and D).

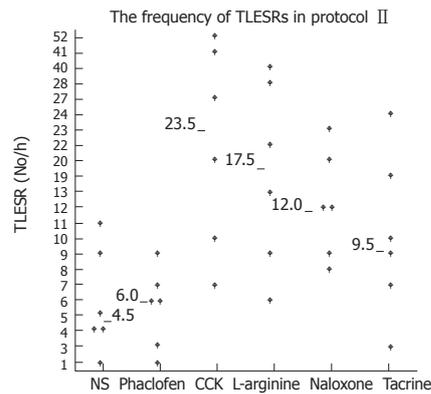
The number of positive nuclei in each group is listed in Table 3.



**Figure 7** Photomicrographs showing C-Fos and NOS positive neurons in reticular formation of medulla (RFM). **A:** C-Fos immunohistochemistry of neiguan group; **B:** C-Fos immunohistochemistry of sham acupoint group; **C:** NADPH-d histochemistry of neiguan group; **D:** NADPH-d histochemistry of sham acupoint group ( $\times 10$ ).

**Study Protocol II : Effects of Drugs (phaclofen, CCK octapeptide, L-arginine, naloxone and tacrine) on inhibition of TLESRs by Electric Acupoint Stimulation**

**Transient LES relaxations:** After saline infusion, the frequency of TLESRs during acupoint stimulation at neiguan [4.5 per hour (range, 1-11)] was still significantly



**Figure 8** CCK, L-arginine and naloxone significantly increase the frequency of TLESRs compared with control group ( $^eP < 0.05$ ). But phaclofen and tacrine did not change the frequency of TLESRs compared with control group ( $P > 0.05$ ). It was indicated that the inhibited effects of EA at neiguan were completely restored by pretreatment with CCK-A, L-arginine and naloxone. But it was not influenced by phaclofen and tacrine.

lower than that during sham stimulation at the hip in protocol I [10.5 per hour (range, 0-20),  $P < 0.05$ ].

Infusion of Phaclofen and tacrine did not influence the rate of TLESRs during acupoint stimulation at neiguan. They were [6 per hour (range 1-9) *vs* 4.5 per hours (range 1-11),  $P > 0.05$ ] and [9.5 per hour (range 3-24) *vs* 4.5 per hour (range, 1-11),  $P > 0.05$ ] respectively. However, during infusion of CCK octapeptide, L-arginine and naloxone, the inhibited effect of EA at neiguan had been completely restored. The rate of TLESRs were CCK [23.5 per hour (range 7-52) *vs* 4.5 per hours (range 1-11),  $P < 0.05$ ], L-arginine [17.5 per hour (range 6-40) *vs* 4.5 per hours (range 1-11),  $P < 0.05$ ] and naloxone [12 per hour (range 8-23) *vs* 4.5 per hours (range 1-11),  $P < 0.05$ ] respectively (Figure 8).

**DISCUSSION**

Acupuncture has been used to treat functional gastrointestinal disorders in the eastern countries for centuries. A large amount of clinical evidence supports the effectiveness of acupuncture for treating functional disorders of the gastrointestinal tract, and the most commonly used acupoints in treating gastrointestinal symptoms are Neiguan (PC6) and Zusanli (ST-36). In the present study, we found electric acupoint stimulation at the neiguan acupoint resulted in a significant reduction of the rate of TLESRs induced by gastric distention. This result was consistent with previous investigations on human beings [12]. Furthermore, the present work also demonstrated the action site and neurotransmitters of this effect.

**Speculation on the action site of electroacupuncture at neiguan**

We postulate that the site at which electric acupuncture stimulation acts to inhibit the occurrence of TLESRs may be as follows: First, it may increase the proximal gastric motility or increase the gastric fundus tone, so that to decrease the volume of gastric fundus, and inhibit

the stretch receptors localized in the gastric fundus (As we know, the stretch receptors were the major receptor in triggering TLESRs<sup>[25,26]</sup>) and then reduce the sensory input from gastric distention. Second, it may inhibit the integration of TLESRs in some area of the brain stem, such as NTS (nucleus tractus solitarius) and DMV (dorsomotor nucleus of the vagus nerve). Third, electric acupoint stimulation at Neiguan may exert its action primarily on the efferent motor pathway.

With present research, electric stimulation at Neiguan did not change the intragastric pressure and nitrite levels in the tissue of gastric fundus compared with EA at sham acupoint. Nitric oxide is well accepted as an inhibitory neurotransmitter in the gastrointestinal tract, and may exert a tonic inhibition on the proximal stomach<sup>[27]</sup>. So the result of our study suggested that EA at neiguan cannot change the tone of gastric fundus. And it is consistent with previous research<sup>[12,28]</sup>.

In the current research, electric stimulation at neiguan had no effect on the residual LES pressure, so it is unlikely that it exerts action primarily on the efferent motor pathway.

The dorsal vagal complex (DVC) comprising nucleus tractus solitarius (NTS) and dorsomotor nucleus of the vagus nerve (DMV) is the center of the integration of TLESRs<sup>[9]</sup>. The brain stem dyeing shows that when compared with sham group EA at neiguan significantly decreases the C-Fos and NOS positive nucleus in NTS/DMV. However, it stimulated a significantly greater number of C-Fos and NOS positive nucleus in areas of reticular formation of the medulla (RFM). And RFM may be one of the acupuncture action sites<sup>[29]</sup>.

Consequently, the action site of EA at neiguan may be localized within the brain stem. It may increase NOS in the nucleus of RFM, so that it inhibits NOS in NTS/DMV, and then decreases the frequency of TLESRs.

### **Speculation on the neurotransmitter of electroacupuncture at neiguan**

In the second part of this research, the inhibited effect of EA at neiguan on TLESR's rate was completely restored by pretreatment with CCK, L-arginine and naloxone. On the contrary, phaclofen and tacrine did not influence it. So this effect appeared to be mediated through nitric oxide (NO), CCK-A receptor and mu-opioid receptors. But the GABAB receptor and acetylcholine may not be involved in it.

In our study, electric stimulation at Neiguan also did not influence the gastrin and motilitin levels in plasma. It suggested that the inhibited effects of electric stimulation were not through these two neuropeptides.

### **The variance between previous research**

Endogenous opioid peptides (EOPs) are considered as major candidates for a role in acupuncture action because numerous investigations have clearly demonstrated that electroacupuncture effect is antagonized by the opioid receptor antagonist naloxone<sup>[30]</sup>. And in our research, the naloxone can reverse the inhibited effect of electroacupuncture on TLESRs. In contrast to our results in cats, Zou *et al.*<sup>[12]</sup> found the inhibited effect of

acupoint stimulation was not inhibited by naloxone. In that study, the frequency of electrical stimulation is 100 Hz. However, our wave patterns were sparse and dense pulse intervals ranging from 2 Hz to 100 Hz (2/100 Hz). Previous studies have demonstrated that low-frequency (2 Hz) electroacupuncture analgesia (EAA) is induced by the activation of  $\mu$ - and  $\delta$ -opioid receptors *via* the release of enkephalin,  $\beta$ -endorphin, and endomorphin; and high-frequency (100 Hz) EAA is caused by activation of  $\kappa$  opioid receptors *via* release of dynorphin<sup>[31]</sup>. So it may be the reason of the variance between our findings and Zou's research.

In conclusion, electric acupoint stimulation at the Neiguan result in a significant reduction of the rate of TLESRs induced by gastric distention. This effect appears to act at the brain stem, and may be mediated through NO, CCK-A receptor and mu-opioid receptor.

## **COMMENTS**

### **Background**

Gastroesophageal reflux disease (GERD) is a disorder characterized by an increased exposure of the esophagus to the intragastric contents. Recent studies have suggested that transient lower esophageal sphincter relaxation is the main mechanism underlying gastroesophageal reflux. It involves a prolonged relaxation of the lower esophageal sphincter, mediated by a vago-vagal neural pathway, synapsing in the brainstem. Acupuncture has been used to treat functional gastrointestinal disorders in the eastern countries for centuries. It can modulate visceral sensation as well as function through stimulation at selected acupoints along the meridians (channels of acupoints).

### **Research frontiers**

Transient lower esophageal sphincter relaxation (TLESR) is the most important mechanism of gastroesophageal reflux (GER) in the patients of GERD. So it had become an important target in dealing with gastroesophageal reflux disease.

### **Innovations and breakthroughs**

Although there have one article indicated that electroacupuncture at neiguan (PC6) may decrease the frequency of TLESRs, our research is the first paper to observe the mechanisms carefully.

### **Applications**

Our research observed the relationship between electroacupuncture at neiguan (PC6) and TLESRs. And there may be a significant clinical impact in the future.

### **Terminology**

TLESR: It is a spontaneous relaxation of LES without swallow induced. Its definition included: (1) absence of swallowing for 4 s before to 2 s after the onset of LES relaxation. (2) relaxation rate of  $>$  or  $=$  1 mmHg/s. (3) time from onset to complete relaxation of  $<$  or  $=$  10 s. (4) nadir pressure of  $<$  or  $=$  2 mmHg. Exception: a markedly prolonged LES relaxation  $>$  or  $=$  10 s, and nadir pressure  $<$  or  $=$  2 mmHg can also be classified as TLESR, irrespective of the relation with swallow; Common Cavity: It was defined as abrupt simultaneous and sustained rises of basal esophageal pressure to intragastric pressure in at least the two lower esophageal body manometry recording sites.

### **Peer review**

This manuscript it is well written and the experimental design is sound. In addition, there may be a significant clinical impact if these results will be confirmed in the future.

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