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Basic Study

Effects of needling acupoints at different nerve segments on oxytocin neurons in rat's hypothalamic paraventricular nucleus and intragastric pressure

针刺不同神经节段腧穴对大鼠下丘脑室旁核催产素神经元及胃内压的影响

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Abstract

Objective: To compare and explore the effects of needling acupoints at different nerve segments on the oxytocin (OT) neurons in the paraventricular nucleus of hypothalamus (PVN) and the intragastric pressure, and discuss the possible mechanisms.

Methods: Thirty-two healthy adult Sprague-Dawley (SD) rats were numbered and divided into 4 groups according to the random number table, a Zusanli (ST 36) group, a Neiguan (PC 6) group, a Weishu (BL 21) group and a control group, with 8 rats in each group. Except the control group, rats in the other three groups received acupuncture at the corresponding acupoints. To observe the differences in double-labeled OT neurons and c-fos neurons of the hypothalamic PVN and the intragastric pressure after acupuncture among the three groups of needling acupoints at different nerve segments.

Results: Compared with the control group, the numbers of double-labeled cells in the PVN of the Zusanli (ST 36) group and the Neiguan (PC 6) group decreased significantly, while the intragastric pressure increased significantly (all $P<0.05$), and the inter-group differences were statistically significant ($P<0.05$). The intragastric pressure in the Weishu (BL 21) group decreased significantly, and the inter-group difference was statistically significant ($P<0.05$). Compared with the Weishu (BL 21) group, the numbers of OT/c-fos double-labeled cells in PVN of the Zusanli (ST 36) group and the Neiguan (PC 6) group decreased significantly, and the intragastric pressure increased significantly, the inter-group differences were statistically significant (all $P<0.01$).

Conclusion: Acupoints at different nerve segments have different regulation effects on intragastric pressure. The difference may be related to the different nerve conduction pathways by acupoints at different nerve segments in regulating the intragastric pressure. The PVN may be one common integration center for the regulation of gastric function in the three acupoints [Zusanli (ST 36), Neiguan (PC 6) and Weishu (BL 21)] at different nerve segments.

Keywords: Acupuncture Therapy; Point, Zusanli (ST 36); Point, Neiguan (PC 6); Point, Weishu (BL 21); Paraventricular Hypothalamic Nucleus; Neurons; Rats

【摘要】目的：比较和探讨不同神经节段腧穴对下丘脑室旁核(PVN)催产素(OT)神经元及胃内压的影响及其可能机制。**方法：**将32只健康成年Sprague-Dawley(SD)大鼠编号后按随机数字表法分为4组，即足三里组、内关组、胃俞组和对照组，每组8只。除对照组外，其余三组大鼠分别接受相应穴位针刺。观察三个不同神经节段腧穴组大鼠针刺后PVN内OT神经元细胞和c-fos神经元细胞的双标表达及胃内压的差异。**结果：**与对照组比较，足三里组和内关组的PVN内OT/c-fos的双标细胞数目减少，胃内压升高，组间差异均具有统计学意义(均$P<0.05$)；胃俞组的胃内压降低，组间差异有统计学意义($P<0.05$)。与胃俞组比较，足三里组和内关组PVN内OT/c-fos双标细胞数目减少，胃内压升高，组间差异均具有统计学意义(均$P<0.01$)。**结论：**不同神经节段腧穴对胃内压具有不同的调节作用，这种差异性可能与不同神经节段腧穴调节胃内压的神经传导途径不同有关。PVN可能是足三里、内关及胃俞三个不同神经节段腧穴调节胃功能的共同整合中枢之一。

【关键词】针刺疗法；穴，足三里；穴，内关；穴，胃俞；下丘脑室旁核；神经元；大鼠

【中图分类号】R2-03　　**【文献标志码】**A

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Correlation between meridians/acupoints and Zang-fu organs has always been a major frontier topic in acupuncture and meridian theory. As an external stimulus, acupuncture can regulate visceral function via a variety of ways such as nerve segment, neuroendocrine regulation, and central nerve regulation. Among them, the functions of nerves, blood vessels, muscle tissues, especially nerve tissues are particularly important for the local acupoints. The relative specificity of acupoints is related to the nerve segment where the acupoints locate[1].

Morphological studies have shown that Zusanli (ST 36), Neiguan (PC 6) and Weishu (BL 21) belong to three different nerve segments: the afferent impulses in the Zusanli (ST 36) area are mainly projected to the L_4-S_1 spinal nerve segment; afferent neurons of Neiguan (PC 6) mainly locate at C_6-T_1; the main afferent segment of Weishu (BL 21) locates at T_6-T_{10}, while the nerve segments that govern the stomach locate at T_5-T_9[2-3]. It can be seen that the afferent neurons of the stomach and Weishu (BL 21) overlap in the lower chest, which are not at the same nerve segment as the afferent neurons of Zusanli (ST 36) and Neiguan (PC 6).

Our previous research found that acupuncture at acupoints such as Zusanli (ST 36) and Neiguan (PC 6) regulated the gastric function through convergence and integration of the acupuncture information by the nucleus tractus solitarii (NTS), pointing out that the NTS is the primary integration center of regulating the stomach function by acupuncture[4-6]. These studies suggested that the mechanism of regulating the gastric function by the relevant acupoints was not limited to the peripheral nerve, but also related to the central nervous system. We further studied the high-level nervous system center and found that after acupuncture at Zusanli (ST 36) and Neiguan (PC 6), the relevant neuronal discharges could be recorded in the paraventricular nucleus of hypothalamus (PVN) of the gastric dilatation model rats, indicating that there were visceral convergent neurons in the PVN that responded to both gastric dilatation stimulation and acupuncture stimulation[7-9]. However, which neurons were recorded in the PVN still need further exploration.

Studies have shown that large cells and small cells of PVN synthesize a nonapeptide hormone: oxytocin (OT), which is also a neurotransmitter. OT receptors are widely expressed in human and rat digestive tract tissues, working to accelerate gastric emptying; OT receptor inhibitors delay gastric emptying, indicating that OT can directly act on digestive tract tissues[10-11]. Therefore, we concluded that OT neurons may be the main discharge neurons in the PVN during needling acupoints at different nerve segments associated with the stomach.

To further explore the effects of acupoints at different nerve segments on OT neurons in the PVN and intragastric pressure, and discuss the mechanism of acupoint regulating gastric function based on the spinal nerve segments, in this study, three acupoints at different nerve segments were acupunctured, and the differences in the activation of PVN OT neurons and intragastric pressure were observed by immunohistochemical double-labeling technique. By combination of the morphological expression of the high level center and the intragastric pressure of the subordinate effector, the differences in the conduction pathways of regulating the gastric function by acupoints at different nerve segments and the possible mechanisms were further explored.

1 Materials and Methods

1.1 Materials

1.1.1 Experimental animals

Thirty-two healthy male and female adult SD rats, weighing (300±20) g, were provided by the Animal Experimental Center of Hunan University of Chinese Medicine [animal certificate number: SCXK (Xiang) 2011-0003]. Rats were fasted for 12 h before the experiment and fed with 5% glucose saline. The animal disposal during the experiment all complied with the regulations of the related animal ethics standards from the 2009 Ethical Issues in Animal Experimentation[12].

1.1.2 Instruments and reagents

BL-420F biological information analysis experimental system (Chengdu Taimeng Technology Co., Ltd., China); JY3002 electronic balance (Shanghai Precision Scientific Instrument Co., Ltd., China); LEICA DM LB2 binocular microscope (LEICA Company, Germany); Haier medical microwave oven (Haier Group, China); MIAS medical image analysis system (Beihang Company, China); S2-93 automatic double pure water distiller (Shanghai Yarong Biochemical Instrument Factory, China); Shandon 325 paraffin slicer (Shandon, UK); DNP-9162 electric heating constant temperature incubator (Shanghai Jinghong Experimental Equipment Co., Ltd., China); Motic B5 micro camera system (Motic China Group Co., Ltd., China); c-fos primary antibody (item number: ab102699) and OT primary antibody (item number: ab78364) (Abcam Company, USA); secondary antibody kit (Cat. No. DS-0002, Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., China); G-6805 electroacupuncture treatment instrument (Shanghai Medical Electronic Instrument Factory, China); Hwato brand stainless steel sterile acupuncture needle (0.32 mm in diameter and 25 mm in length, Suzhou Medical Products Co., Ltd., China).

1.2 Methods

1.2.1 Animal groups

Animals were numbered and divided into 4 groups according to the random number table method, a Zusanli (ST 36) group, a Neiguan (PC 6) group, a Weishu (BL 21) group and a control group, with 8 rats in each group.

1.2.2 Gastric sacculus placing operation

At the beginning of the experiment, rats in each group were intraperitoneally injected with 20% urethane [50 mg/(kg·bw)] for anesthesia, then the rats were bound to a rat plate in a supine position to shave the hair on the upper abdomen. Under the xiphoid process, the abdomen was cut longitudinally along the median line to expose the gastric corpus. A sacculus with the original filling volume of 0.1 mL was pushed into the antrum through a small opening in the duodenum, about 0.5 cm from the pylorus. The basic pressure of each sacculus: after injecting 0.6 mL of distilled water, the water column of the water pressure gauge was raised to 46 cm H_2O. The sacculus was collected outside the body through a polyethylene hose and the wound was sutured. A polyethylene hose attached pressure transducer (through the tee with a 12 gauge needle) was connected to the BL-420F bioinformatics recording system.

1.2.3 Acupoint locations

Acupoints were located according to the *Experimental Acupuncture Science*[13].

Zusanli (ST 36): The posterolateral area of the knee joint and about 5 mm below the capitulum fibulae.

Neiguan (PC 6): The inner side of forelimb, between the ulna and the humerus, and about 3 mm away from the wrist joint.

Weishu (BL 21): Beneath the 12th thoracic spinous process and 5 mm away.

Attachment point: The attachment point was selected at 2 mm below each acupoint.

1.2.4 Acupuncture methods

Except the control group, all the rats in the other groups were subjected to acupuncture at the acupoints on the right limbs. Hwato brand stainless steel and sterile acupuncture needle was used for perpendicular needling to 3-5 mm, then connected to G-6805 electroacupuncture treatment instrument (the acupoint was connected to the positive electrode, and the attachment point was connected to the negative electrode). Stimulus parameters: sparse-dense wave (4 Hz for the sparse wave, 20 Hz for the dense wave, 0.5 ms for the pulse density, 2-4 V for the output voltage). The stimulation lasted for 20 min with the intensity causing slight vibration of limbs.

1.2.5 Intragastric pressure recording

After the gastric sacculus placement, except the control group, rats in each group received the corresponding electroacupuncture for 20 min, and then were injected with 2-4 mL warm normal saline via the syringe connected to one end of the T-tube. The experiment started after the gastric peristalsis waves were stabilized, and the intragastric pressure was continuously recorded for 30 min. If the basal peristaltic waves could not be recorded, the gastric sac size in the stomach was adjusted by a syringe through a three-way device. The intragastric pressure was input into the pressure transducer through the cannula of the three-way device, where the liquid pressure signal was converted into an electrical signal. After being amplified by the bio-amplifier, the electrical signal was input into the BL-420F biological information analysis experimental system. The rats in each group were recorded for 2 min before and after electroacupuncture, and the changes in the mean intragastric pressure amplitude per unit time were calculated.

1.2.6 Slice preparation

Two hours after acupuncture, the rats in each group were perfused with a cardiac cannula for fixing. The brain tissue was removed, and the cerebral cortex and other tissues were removed. The hippocampus and thalamus were preserved and fixed in 4% paraformaldehyde for 24 h. Serial sectioning (5 μm in thickness) was performed after dehydration and paraffin embedding.

1.2.7 Immunohistochemistry

Paraffin sections were dewaxed, rinsed with PBS for 2 min×3 times, and incubated with 3% H_2O_2 for 10 min at room temperature to eliminate endogenous peroxidase activity; rinsed with PBS for 2 min×3 times; microwave repaired with citrate buffer, then cooled naturally to room temperature; rinsed with PBS for 2 min×3 times; then added the primary antibody mixture [mouse monoclonal (4G11) to oxytocin, 1:100 dilution; rabbit polyclonal to c-fos, 1:100 dilution], and incubated for 2 h at 37 ℃. Rinsed with PBS for 2 min× 3 times; added the appropriately diluted secondary antibody [goat anti-mouse immunoglobulin G (IgG) polymer and goat anti-rabbit IgG polymer], incubated at 37 ℃ for 30 min; rinsed with PBS for 2 min×3 times; added reagent 3 (DAB, yellow); rinsed with PBS for 2 min×3 times. Added the mixture (red color) of reagent 4A (GBL-long red substrate solution), 4B (GBL-long red activator) and 4C (GBL-long red pigment substrate); fully rinsed with tap water, counterstained, dehydrated following the transparency and sealing.

1.3 Statistical analysis

The data were analyzed using the BL-420F biological

information analysis experimental system and MIAS medical image analysis system. The SPSS version 17 statistical software was used for statistical processing. Data were conducted descriptive statistics and expressed as mean ± standard deviation ($\bar{x}$ ±s). ANOVA was used for between-group comparisons of data with homogeneity of variance; the rank sum test and the q' test were used for the data with heterogeneity of variance. The significant level was bilateral α=0.05.

2 Results

2.1 C-fos expression of OT neurons in the PVN

Compared with the control group, the numbers of OT/c-fos double-labeled cells in the PVN of the Zusanli (ST 36) group and the Neiguan (PC 6) group decreased, and the inter-group differences were significant (both P<0.05); the number of OT/c-fos double-labeled cells in the PVN of the Weishu (BL 21) group increased, and the inter-group difference was significant (P<0.05). These results suggested that the activation of OT neurons in the PVN was different after acupuncture at acupoints at different nerve segments. Compared with the Weishu (BL 21) group, the c-fos expressions in the Zusanli (ST 36) group and the Neiguan (PC 6) group decreased, and the inter-group differences were significant (both P<0.01), indicating that the activation effect on OT neurons was more obvious when needling acupoints at the same nerve segment as the target organ than at the different nerve segments from the target organ (Figure 1).

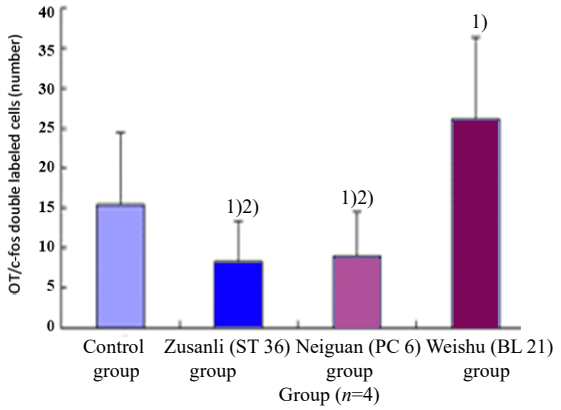


Figure 1. Effect of acupuncture at acupoints at different nerve segments on c-fos expression of the OT positive neurons in PVN

Note: Compared with the control group, 1) P<0.05; compared with the Weishu (BL 21) group, 2) P<0.01

2.2 Immunohistochemical observation

Three types of the positive cells were displayed on the double-labeled stained sections under the light microscope: the c-fos single-label positive cells were brown, mostly round or oval; the cytoplasm of OT single-label positive cells was red, and the nucleus was not colored. For the c-fos and OT double-labeled cells, the nuclei were brown and the cytoplasm was red. Double-labeled cells were bilaterally distributed in the PVN without significant difference, and the cells were mainly oval, but fusiform and multipolar ones could also be found (Figure 2 and Figure 3).

2.3 Changes in intragastric pressure

Compared with the control group, the intragastric pressure of the Zusanli (ST 36) group and the Neiguan (PC 6) group increased, and the inter-group differences were significant (both P<0.05); the intragastric pressure in the Weishu (BL 21) group decreased, and the inter-group difference was significant (P<0.05). Compared with the Weishu (BL 21) group, the gastric pressure in the Zusanli (ST 36) group and the Neiguan (PC 6) group increased, and the inter-group differences were significant (both P<0.01). These results suggested that acupuncture at acupoints at different nerve segments could regulate the intragastric pressure in rats; needling acupoints at the different nerve segments from the target organ increased intragastric pressure, while needling acupoints at the same nerve segment as the target organ had the inhibitory effect on intragastric pressure, that is, the effects on the intragastric pressure of different acupoints at different nerve segments were different (Figure 4).

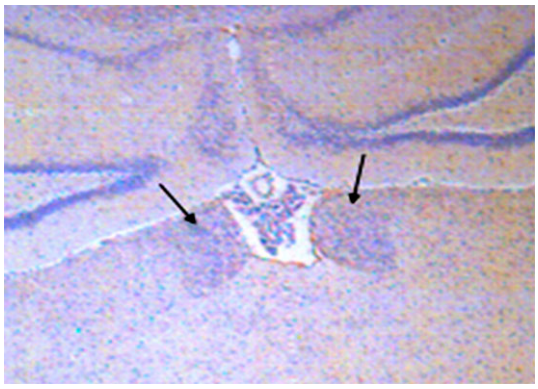
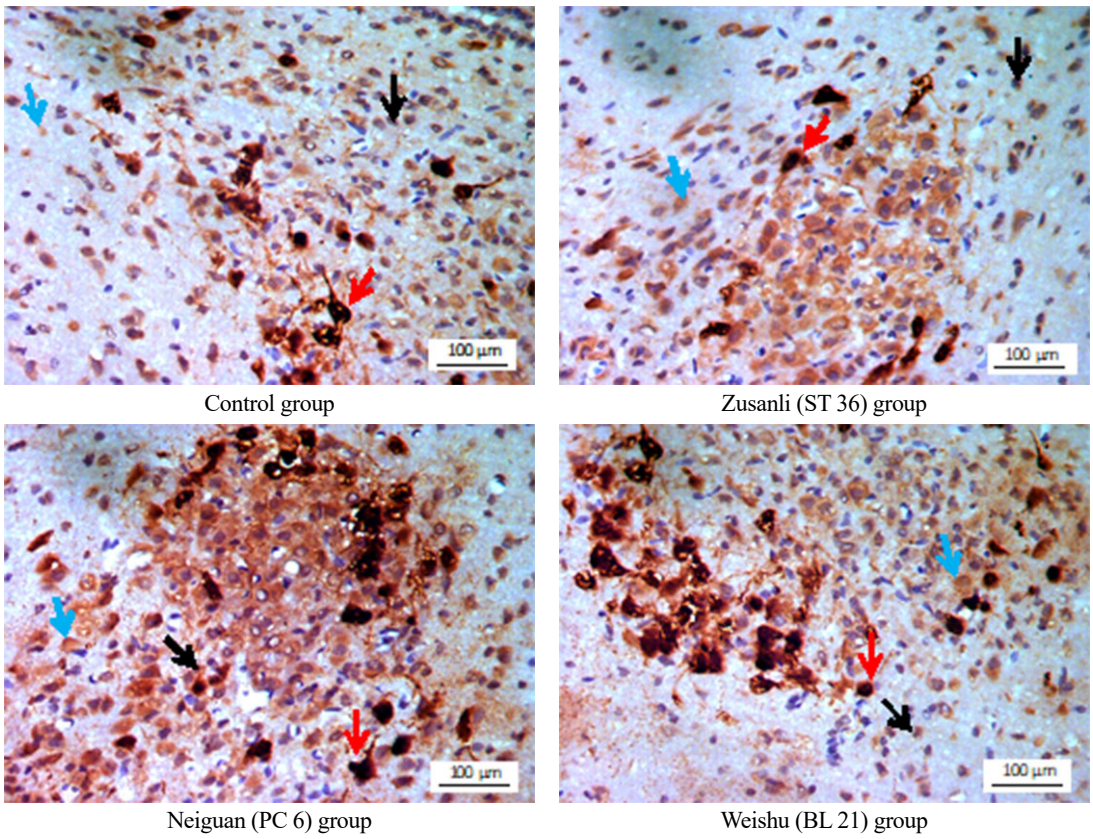


Figure 2. The position of PVN (immunohistochemistry double staining, ×40)



Control group | Zusanli (ST 36) group

Neiguan (PC 6) group | Weishu (BL 21) group

Figure 3. Immunohistochemical double staining of c-fos in OT neurons of the PVN (immunohistochemistry double staining, ×400)

Note: The red arrow indicates OT/c-fos double-labeled cells, the blue arrow indicates OT single-labeled cells, and the black arrow indicates c-fos single-labeled cells

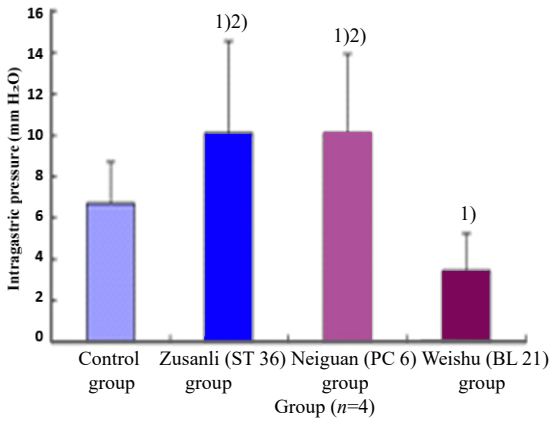


Figure 4. Effect of acupuncture at acupoints at different nerve segments on intragastric pressure

Note: Compared with the control group, 1) $P<0.05$; compared with the Weishu (BL 21) group, 2) $P<0.01$

3 Discussion

Japanese scholars Sato A, *et al*[2] studied the neurological mechanism of acupuncture-like stimulation on the visceral function reflex regulation, and demonstrated that acupuncture had a segmental and non-segmental regulation effect on the visceral reflex. The spinal cord is essential for the segmental reflexes, and the central nervous system is the key to non-segmental reflexes. It can be seen that the regulation on the stomach by Zusanli (ST 36) and Neiguan (PC 6), which are at different nerve segments from the stomach, is mainly mediated by non-segmental reflexes of the central nervous system; however, Weishu (BL 21), located at the same nerve segment, relies mainly on the segmental reflex of the spinal cord. At the same time, studies have shown that the effect of acupuncture at acupoints innervated by the same/near segments as the stomach to reduce the intragastric pressure may be achieved by the sympathetic nerve efferent fibers; the excitatory effect of acupuncture at acupoints far away from the stomach nerve segment to increase the intragastric pressure may be accomplished by the vagus nerve efferent fibers, and requires the involvement of the central nervous system[14]. According to neuroanatomy, the preganglionic fibers of the sympathetic nervous system that innervates the stomach originate from the C_8-L_2 cornu laterale medullae spinalis neurons, and the preganglionic fibers that govern the stomach vagus nerve originate from the dorsal vagal complex (DVC) of the brainstem. DVC consists of the dorsal motor nucleus

of vagus (DMV), nucleus tractus solitarii (NTS) and area postrema (AP), functioning as the primary center for regulating gastric function. Among them, NTS and AP mainly receive the afferent information from the stomach. After integrating the information, the fibers are sent to the DMV. The DMV and the nucleus ambiguous (NA) in the medulla send the parasympathetic nerve to innervate the stomach. Parasympathetic nerve endings are terminated in the gastric plexus (parasympathetic ganglia) and dominate the smooth muscle contraction of the stomach or secretion of gastric glandular cells[15-18]. On the other hand, DMV projects to the high level central nerve: PVN, and establishes a single synaptic connection with a large number of neurons in the PVN. This connection provides an anatomical basis for multiple peptide neurons in the PVN to affect the vagus nerve through DMV, thereby affecting the gastric motility function[19]. Peptide-energy neurons involved in this process have also been systematically studied in related literatures. It is found that the gastric motility inhibition after electrical stimulation of PVN is blocked by DMV or intracerebroventricular injection of OT receptor antagonists, indicating OT neurons in the PVN have important regulatory effects on gastric motility. Microinjection of OT into DMV or PVN can inhibit gastric motility. This inhibition is not sensitive to atropine, but can be blocked by the OT inhibitor injected into the PVN or disconnection of the vagus nerve[20]. The inhibitory effect of OT in the PVN on gastric function thus has been further clarified, and it is mainly by increasing the excitability of brainstem vagus neurons[21]. At the same time, the functional status of the stomach also plays an important role in the OT expression in the PVN. Gastric electrical stimulation with standard parameters to the distal gastric antrum also increases the OT expression in the PVN[22]. It can be seen that PVN and the stomach produce two-way connection through the action of OT. The synthesis and release of OT is important for PVN to regulate gastric function. As a marker of OT neuron activation, c-fos expression of OT neurons directly reflects the synthesis and release of OT.

In this study, double immunohistochemical technique was used to observe the effect of needling acupoints at different nerve segments on the c-fos double-labeled expression of OT neurons in the PVN, thus to study the effect of oxytocin neuron activation on intragastric pressure. After acupuncture at Zusanli (ST 36) or Neiguan (PC 6), the number of OT/c-fos double-labeled cells in the PVN and the intragastric pressure were decreased; the number of OT/c-fos double-labeled cells in PVN and the intragastric pressure of Weishu (BL 21) group were increased. Furthermore, the inhibitory effect of OT in the PVN on intragastric pressure was demonstrated, and the acupoints at the same nerve segment as the target organ had similar effects on the OT neuron activation in the PVN and intragastric pressure. However, the acupoints at the different nerve segments from the target organ had different effects on the activation of OT neurons in PVN and intragastric pressure. We speculated that this difference may be caused by the different conduction pathways of acupoints at different nerve segments in regulating gastric function.

For Weishu (BL 21), located at the same nerve segment as the stomach, our previous study found that after the high cervical spinal cord was transected, the acupuncture signaling pathway transmitted to the brain center was blocked, but its inhibitory effect on gastric pressure still existed, indicating the primary center of Weishu (BL 21) for regulating the intragastric pressure was in the spinal cord[23]. The neural pathway for acupuncture at Weishu (BL 21) to regulate the gastric function is shown in Figure 5. The acupuncture signal of Weishu (BL 21) may be uploaded to the T_5-T_9 cornu laterale medullae spinalis, then to the paravertebral ganglia and celiac ganglion directly through the anterior root of the spinal ganglia and white communicating branch for the neuron change. The post-ganglionic fibers are distributed along the nerve plexus to the smooth muscle of the stomach, thereby inhibiting the intragastric pressure. Because there is a two-way relationship between the PVN and the stomach, the inhibitory signal of gastric pressure can be uploaded to PVN through various levels of the central axis, activate OT neurons, and further reduce the intragastric pressure through the PVN-DVC vagus nerve pathway (Figure 5).

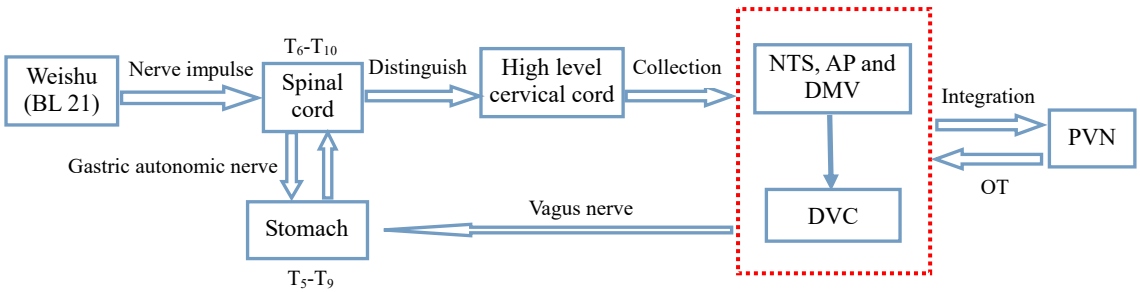


Figure 5. The nerve pathway of acupuncture at Weishu (BL 21) in regulating the gastric function

The nerve pathway for acupuncture at Zusanli (ST 36) and Neiguan (PC 6) to regulate gastric function is shown in Figure 6. After acupuncture at Zusanli (ST 36) and Neiguan (PC 6), the acupuncture signal goes up along the spinal cord, and may reach the brainstem NTS after preliminary discrimination in the high cervical spinal cord. After the initial integration by NTS[4], the signal is further projected upward to the PVN[7], thus to inhibit some OT neurons in the PVN, thereby reducing the OT projected from PVN to DMV[9]. The activated brainstem vagal motoneurons are also reduced, and the inhibitory nerve impulses reaching the gastrointestinal tract are weakened. Therefore, the intragastric pressure shows a stimulating effect (Figure 6).

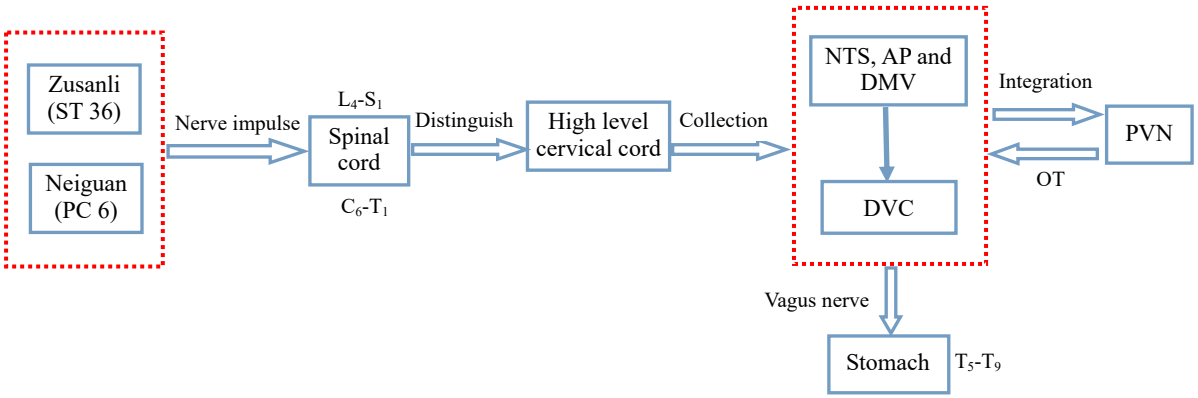


Figure 6. The nerve pathway of acupuncture at Zusanli (ST 36) and Neiguan (PC 6) in regulating the gastric function

In summary, by observing the double-labeled expression of OT neurons and c-fos neurons in the PVN of the hypothalamus in each group and the difference in intragastric pressure, this study found that acupoints at different nerve segments had different regulation effects on intragastric pressure, and the PVN may be one common integration center for regulating gastric function by the three different-nerve-segment acupoints of Zusanli (ST 36), Neiguan (PC 6) and Weishu (BL 21) in this study. Due to the current experimental level and technical conditions, this work only studied the effects of acupoints at different nerve segments on OT neurons in the hypothalamic PVN and intragastric pressure, and also their neural pathways. But, whether this neural pathway is complete, how to achieve the regulation of intragastric pressure, and whether there are other pathways need to be further studied.

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.

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