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Effects of sacral nerve stimulation with acupuncture on gut transit time and c-kit expression in colon of rats with slow transit constipation

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ABSTRACT. Sacral nerve stimulation (SNS) is an alternative surgical approach to alleviate fecal incontinence and constipation. This study aimed to explore the effects and underlying mechanisms of SNS with acupuncture on gut transit time and colon c-kit protein expression in rats with slow transit constipation (STC). Fifty Sprague-Dawley rats were randomly divided into five groups: blank control, SNS, Mosapride, sham SNS, and STC model control group. The STC model was established by subcutaneous injection of morphine. Each group

was treated over a 15-day period. Gut transit time was measured 1 day before the treatment started and after 5, 10, and 15 days of treatment. After the 15-day treatment, animals were sacrificed and colonic tissues were collected for analysis of c-kit protein expression, using western blot analysis. We found significant differences in gut transit time in the SNS group compared with the Mosapride group after 5 ($P = 0.001$) and 10 ($P = 0.004$) days of treatment. After 15 days of treatment, there were no differences in gut transit time among the SNS, Mosapride, and blank control groups. However, significant differences were observed when comparing the SNS and Mosapride groups with the STC model and sham SNS groups. A decreased c-kit protein expression was observed in the STC model control, sham SNS, and Mosapride groups, compared with the SNS group ($P = 0.001$). Our data indicate that SNS can decrease gut transit time and increase the expression of c-kit protein in rats with STC to improve colon transit function.

Key words: Sacral nerve stimulation; Acupuncture; Gut transit time; C-kit

INTRODUCTION

Constipation is a common complaint affecting between 2 and 27% of the population in western countries (Sonnenberg and Koch, 1989; Stewart et al., 1999; Pare et al., 2001). Every year, it is the cause of more than 2.5 million visits to physicians' offices, 92,000 hospitalizations, and laxative sales of several hundred million dollars in the United States (Sonnenberg and Koch, 1989). There is no single definition of constipation. Chronic constipation can be classified into three main categories: slow transit constipation (STC), disorders of defecation (or rectal evacuation), or a combination of both (Preston and Lennard-Jones, 1985, 1986; Thompson et al., 1999). Constipation is frequently multifactorial and can result from systemic or neurogenic disorders or medications. Mounting evidence has suggested that the interstitial cells of Cajal (ICCs) generate slow waves in the phasic gastrointestinal muscles, actively propagate slow waves, and mediate or transduce neural inputs from enteric motor neurons to smooth muscles (Huizinga et al., 1995; Ward and Sanders, 2001). Labeling of c-kit protein has provided an efficient means of identifying ICCs throughout the gastrointestinal tracts in humans and other species, using light microscopy (Ward et al., 1994; Huizinga et al., 1995; Ward et al., 1995). With this technique, several human motility disorders have been shown to be associated with a reduction in the number of ICCs or defects in their networks.

Conventional treatment for constipation involves dietary modification, pharmacologic agents, and behavioral therapy. However, conservative treatments fail in a significant proportion of patients (Chiotakakou-Faliakou et al., 1998). In these patients, surgical treatment may be considered. However, traditional operations have been found to be associated with substantial morbidity and a variable outcome (Lundin et al., 2002; Lees et al., 2004; Song et al., 2015).

An alternative surgical approach to both fecal incontinence and constipation is sacral nerve stimulation (SNS). Recently, a simultaneous-positive effect on coexisting chronic constipation was observed in some patients with urinary incontinence treated with SNS

(Caraballo et al., 2001). So far only a few studies have investigated SNS as a treatment of constipation, and early results are encouraging (Kenefick et al., 2002a; Malouf et al., 2002; Holzer et al., 2008; Kamm et al., 2010). Percutaneous tibial nerve stimulation as a new therapeutic option has been reported to increase stool frequency, decrease the use of laxatives, and improve the quality of life in patients with STC (Wong and Lubowski, 2007; Collins et al., 2012). During the past decades, electroacupuncture has been reported to accelerate gastrointestinal motility both in animals and humans (Iwa et al., 2006; Xu et al., 2006; Yin et al., 2010).

Loss of the important functions provided by ICCs would disturb the normal gastrointestinal motility patterns (Torihashi et al., 1999). Since c-kit has been recognized as a marker for the ICCs, c-kit protein expression can be used to indicate changes in ICCs.

In the present study, we used an acupuncture technique with SNS to treat a rat model with STC; sacral nerve posterior roots were electro-stimulated intermittently through the sacral foramina. The nerve roots and spinal segments controlling pelvic organs are mainly S1-S4 in Sprague-Dawley (SD) rat and S2-S4 in human. The sacral dorsal rami in SD rats are thicker than those in the human body, so S1-S2 were chosen to be stimulated and the stimulation parameters used were similar to human (Wang et al., 2003). Our aim was to evaluate the potential effects and underlying mechanisms of acupuncture with SNS in rats with STC.

MATERIAL AND METHODS

Animal model

We used 50 SD rats (including 25 males and 25 females) weighing 180-220 g that were provided by the Vital River Laboratory Animal Technology Co. Ltd. [certification No. SYXK (B) 2010-0009]. The rats were housed in plastic cages, kept at a 12-h controlled light cycle, and given free access to food and water.

After feeding with a standard rat diet for 1 week, the 50 SD rats were randomly divided into five groups: blank control, SNS, Mosapride, sham SNS, and STC model control group. The blank control group included normal SD rats, whereas the STC model control group included non-treated STC animals. Mosapride is a gastroprokinetic agent that can excite gastrointestinal cholinergic interneurons and the 5-hydroxytryptamine (5-HT) receptor in myenteric plexus. This acts to promote acetylcholine to release and, thus, enhance the movement of the gastrointestinal tract. The Mosapride group was used as positive control group.

Opioids, including morphine, inhibit gastric emptying and propulsive motor activity of the intestine. They can decrease the rate of intestinal transit and produce constipation. Based on the published literature (Xu et al., 2004), the rat models were subjected to subcutaneous morphine injections over a period of 45 days. The applied daily dosage was 2.5 mg/kg morphine. The blank control group was instead injected with the same amount of physiological saline. The weight of each rat was recorded every 5 days, and the dose of morphine/physiological saline was adjusted based on the weight of the animals. The methods of housing and handling of the animals were approved by the Ethics Committee of China Academy of Chinese Medical Sciences.

Experimental protocol

The posterior sacral foramina of SD rats can be located based on bony landmarks (Yu

et al., 2007). After the SD rat models had been established, the rats were anesthetized with an intraperitoneal injection of 2% pentobarbital sodium (30 mg/kg). The region of needling and electrodes were then carefully sterilized with iodophor. BL31-32 (S_{1-2} nerve roots) were acupunctured by stimulating electrodes bilaterally (the depth of needling was approximately 15 mm, based on the weight of the rat; the acupuncture needle was supplied by Suzhou Medical Appliance Co., Ltd., China), and stimulated by connecting to BL-420F Data Acquisition & Analysis System (Chengdu Technology & Market Co., Ltd., China). The stimulus mode used was continuous string pulse stimulation. The stimulus parameters used were waveform: sine wave; pulse amplitude: 2-6 mA; pulse width: 210 μs; stimulus frequency: 15 Hz; stimulus time: 30 min per day; treatment cycle: 15 days.

The method of sham SNS was to superficially acupuncture above the acupoints (not deep into the acupoints) using the same parameters of electric stimulation as the SNS group. The Mosapride group was treated with 2.5 mg/kg Mosapride, whereas the other groups did not receive any treatment. All animals were sacrificed on the 15th day of treatment. At this point, the colonic tissues were collected, washed out with physiological saline, and preserved in liquid nitrogen for further examined.

All animal experiments adhered to the institutional animal guidelines and were approved by the Ethics Committee of the Affiliated Hospital of Xuzhou Medical College.

Gut transit function test

The time when the first black particle of stool was discharged was documented, using a biological staining method with intragastric feeding of carbon black ink. The rats were fed 2 mL carbon black ink orally and the transit time was calculated from the beginning of intragastric feeding to discharge of feces. The measurement time points were the day before treatment and the 5, 10, and 15th day of treatment.

Western blot analysis

For the protein extraction, samples were homogenized in lysis buffer (50 mM Tris-HCl, 125 mM NaCl, 0.1% Nonidet P-40, 5 mM ethylenediaminetetraacetic acid, 50 mM NaF, 0.1% phenylmethylsulfonyl fluoride, and proteinase inhibitor), using a hand microhomogenizer, followed by centrifugation at 25,000 *g* for 5 min. The protein concentration of the supernatant was determined relative to a bovine serum albumin standard. Equal amounts of protein (50 μg) were loaded on each lane of 11% SDS polyacrylamide gels. After electrophoresis, the separated proteins were transferred to a nitrocellulose membrane (Millipore, Bedford, MA, USA) with a semidry apparatus for more than 30 min. The membrane was then incubated with 3% normal bovine serum overnight at 4°C, to block nonspecific binding sites. Anti-mouse CD117 (anti-c-kit, 1:500, BD Pharmingen, NewYork City, NY, USA) was applied for 1 h at room temperature on a rotating platform. After extensive washing with phosphate-buffered saline (PBS), the membrane was incubated with matching peroxidase-conjugated secondary antibody for approximately 45 min, followed by four more PBS washes. Specific protein bands were visualized by X-ray picture (Hyper lm ECL; Amersham, Arlington Heights, IL, USA) using the chemiluminescence detection kit (ECL; Amersham). The optical densities of the bands were analyzed by Quantity one (Bio-Rad, Hercules, CA, USA).

Statistical analysis

All data are reported as means ± SE. The Mauchly test of sphericity was used to judge whether there were relationships among the repeatedly measured data. If a relationship was found ($P \leq 0.05$), the significance of the among group differences on colon transit time was determined using repeated measures and multivariate analyses of variance (ANOVA) in SPSS 16.0. With the multivariate ANOVA, the repeatedly measured data in the different treatment groups at each measurement time was compared pairwise by the LSD *t*-test. The treatment effect was evaluated by estimating the between-subject variance. The repeated measurement effect and its interaction with treatment were evaluated by estimating the within-subject variance. The differences in colon c-kit protein expression among groups were examined by the Dunnett *t*-test. Comparisons among groups were evaluated by one-way ANOVA. $P < 0.05$ was considered statistically significant, in all analyses.

RESULTS

Effects of acupuncture with SNS on the colon transit time at each measurement time

With the multivariate ANOVA, the repeated measurement data at each time in different treatment groups were compared pairwise by the LSD *t*-test. As shown in Table 1, the gut transit time decreased both in the SNS and Mosapride groups, but the decrease was faster in the SNS group. After 5 days of treatment, we found that the gut transit time in the SNS group had decreased significantly (423.5 ± 18.6 min) compared with the STC model (523.5 ± 16.1 min), sham SNS (547.0 ± 11.2 min), and Mosapride (524.5 ± 25.4 min) groups, respectively. However, the gut transit time remained longer than in the blank control group (328.5 ± 18.6 min, $P = 0.001$). On the 10th day after treatment, the gut transit time in the SNS group (349.0 ± 17.7 min) was approaching that of the blank control group (325.0 ± 18.1 min, $P = 0.344$) and had reached the normal range of transit time. There was also a significant difference in gut transit time compared to the Mosapride group (426.0 ± 25.7 min, $P = 0.004$). On the 15th day after treatment, there was no statistical difference ($P = 0.847$) in gut transit time between the SNS group (328.5 ± 18.5 min) and that of the Mosapride group (344.5 ± 21.8 min). Likewise, there were no statistical differences in gut transit time in the blank control group (326.5 ± 17.4), compared to the SNS group ($P = 0.930$) or the Mosapride group ($P = 0.431$). In contrast, on the 15th day of treatment there were statistical differences in gut transit time in the SNS group (328.5 ± 18.5 min, $P = 0.001$) and Mosapride group (344.5 ± 21.8 min, $P = 0.001$), compared with the STC model (418.0 ± 9.2 min) and sham SNS (418.0 ± 8.9 min) groups.

C-kit expression in the colon of STC rats

The results of the western blot and densitometric c-kit analyses are shown in Figure 1. The presence of the 145-kDa c-kit protein in the colon was revealed by western blot analysis (Figure 1A). A different level of the 145-kDa transmembrane receptor protein was demonstrated in all groups. The ratio of c-kit was 0.87 ± 0.06 in the SNS group. A decreased c-kit expression was found in the STC model control, sham SNS, and Mosapride groups compared with the SNS group ($P = 0.001$). When compared with the blank control group (0.95 ± 0.27), the c-kit ratio in the colon was not significantly different from the SNS group ($P = 0.182$; Figure 1B).

Table 1. Comparison of transit time in different groups on the 1st, 5th, 10th, and 15th days of treatment.

Group	N	Transit time (min)			
		1st day	5th day	10th day	15th day
Blank	10	$326.5 \pm 15.4^{\triangle\triangle\#\#}$	$328.5 \pm 18.6^{\triangle\triangle\#\#}$	$325.0 \pm 18.1^{\#\#}$	326.5 ± 17.4
STC model	10	$615.0 \pm 18.2^{**}$	$523.5 \pm 16.1^{**\triangle\triangle}$	$438.5 \pm 11.8^{**\triangle\triangle}$	$418.0 \pm 9.2^{**\triangle\triangle\#\#}$
Sham SNS	10	$621.0 \pm 13.3^{**}$	$547.0 \pm 11.2^{**\triangle\triangle}$	$440.5 \pm 11.6^{**\triangle\triangle}$	$418.0 \pm 8.9^{**\triangle\triangle\#\#}$
SNS	10	$619.5 \pm 27.6^{**}$	$423.5 \pm 18.6^{**\#\#}$	$349.0 \pm 17.7^{\#}$	328.5 ± 18.5
Mosapride	10	$618.5 \pm 30.0^{**}$	$524.5 \pm 25.4^{**\triangle\triangle}$	$426.0 \pm 25.7^{**\triangle}$	344.5 ± 21.8

Values are reported as means ± SE; *P < 0.05; **P < 0.01: blank control group compared with other groups on the 1st day before treatment, 5th, 10th, and 15th days of treatment; $^{\triangle}$P < 0.05, $^{\triangle\triangle}$P < 0.01: SNS group compared with other groups on the 1st day before treatment, 5th, 10th, and 15th days of treatment; $^{\#}$P < 0.05, $^{\#\#}$P < 0.01: Mosapride group compared with other groups on the 1st day before treatment, 5th, 10th, and 15th days of treatment; using the LSD *t*-test.

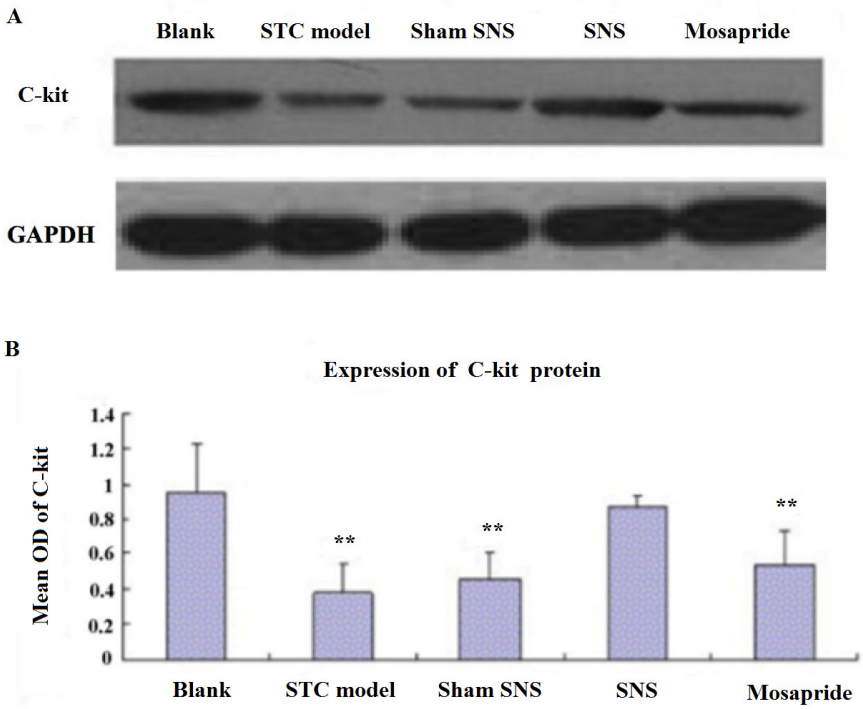

Figure 1. Effects of sacral nerve acupuncture stimulation on the expression of c-kit protein in the colon of rats with STC. **A.** Expression of c-kit protein in colonic tissues from each STC group evaluated by western blot analysis. The polyclonal antibody to c-kit is recognized as a single band at 145 kDa. GAPDH is a house keeping protein, as an internal control. **B.** Results of densitometric analysis of c-kit are shown as mean value of the optical density (OD). Each bar represents means ± SE (vertical line). **P < 0.01, compared with SNS by the Dunnett *t*-test.

DISCUSSION

In the present study, we measured the gut transit time and the expression of colonic c-kit protein in a rat model after electrical stimulation of the sacral nerve. To our knowledge, there have been few studies investigating the effect of sacral nerve stimulation as a means

to treat intractable constipation. STC is characterized by impaired colon motility. Although the pathophysiology has not yet been fully understood, alterations of ICCs (Wedel et al., 2002) could play a very important role in etiological mechanisms previously discussed in the literature (Yu et al., 2002a).

In the present study, we demonstrated that the SNS neuromodulation using acupuncture is an effective treatment in rats with STC. We found a significant improvement in gut transit time and a significant up-regulation in c-kit protein expression in the colonic tissues of STC rats. After 15 days of treatment, the gut transit time in the SNS and Mosapride groups had both decreased and the gut function had also improved. These results indicate that the SNS group had a better gut function and shorter gut transit time than the STC model and sham SNS groups. After 5 and 10 days of treatment, the gut transit time showed a significant improvement in the SNS group compared with the STC model, sham SNS, and Mosapride groups. The possibility of a placebo effect could be ruled out when comparing SNS group with the sham SNS group.

We found that the expression of c-kit protein in the SNS group was significantly increased compared to the Mosapride group. Mosapride also partially plays a role in the expression of c-kit protein, but it was not statistically different compared with the STC model control or sham SNS groups. Hence, we are not able to rule out the possibility that the ultimate motivational target of the 5-HT receptor agonist is also located in the ICCs. Further experimental evaluation is required to determine this. We speculate that the SNS mechanism may be different from that in the Mosapride group in regulating the c-kit protein expression to achieve the STC therapeutic effect.

The SNS effect on the pelvic floor or the lower intestine is still not yet fully understood. Some studies have suggested that the stimulation of afferent nerve fibers of the rectal wall and the pelvic floor could have a positive impact on pelvic floor dysfunction (Holzer et al., 2008; Kamm et al., 2010). The sacral nerve root itself is a mixed nerve containing voluntary somatic, afferent, sensory, and efferent autonomic motor nerves. These components may contribute to the clinical effect of SNS with acupuncture.

Acupuncture has an influence on neurons, nerve hormones, and various neurotransmitters together. At the same time, it has a certain regulatory effect on the sympathetic nervous system, immune function, and endocrine function of body systems (Maher et al., 2001; Wang, 2001; Yang et al., 2010). Previous studies have confirmed that acupuncture also has a regulatory effect on gene expression (Wu et al., 1998; Yu et al., 2002b; Li et al., 2010). Furthermore, evidence also suggests that the c-kit/SCF (stem cell factor) signal pathway plays a crucial role in ICC development and phenotypic maintenance (Hirota et al., 2000; Kitamura et al., 2001; Rich et al., 2003). To our knowledge, the latest research has suggested that acupuncture could repair the damaged c-kit/SCF signal pathway and increase the expression of c-kit protein in rat colon with STC (Sun et al., 2011). This may be one of the mechanisms by which acupuncture could treat rats with STC. A small, double-blind, crossover study has previously demonstrated that the beneficial effects of acupuncture with electric stimulation are unlikely to be related to a placebo effect (Kenefick et al., 2002b).

In this study, we used SNS with acupuncture to treat the STC rat models. Acupuncture is an important mainstay of traditional Chinese medicine. More than 2000 years of practice in China has shown that acupuncture is a simple, convenient, effective, and noninvasive therapy. Electroacupuncture is a form of acupuncture where a small electric current is passed between pairs of acupuncture needles to stimulate acupoint organization and treat diseases. There are some limitations in this experimental study; the sample size in each group was small, and

biases and heterogeneity between groups were inevitable. More animal models with STC should be investigated, and further experimental evaluation is required in the future.

To conclude, we were able to show that the mechanism of SNS with acupuncture in treating STC could be extrapolated to an experimental rat model. It provides evidence that treatment of STC may be realized through SNS with acupuncture.

Conflicts of interest

The authors declare no conflict of interest.

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