Acupuncture at heterotopic acupoints facilitates distal colonic motility via activating M₃ receptors and somatic afferent C-fibers in normal, constipated, or diarrhoeic rats

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Key Messages

• Acupuncture at heterotopic acupoints increases distal colonic motility regardless of normal or pathological conditions via predominately activating C-fibers of somatic afferent nerve and M₃ receptors.
• To investigate the effects of acupuncture on distal colonic motility, particularly in pathological conditions, and the precise mechanism employed in the regulation of acupuncture on colonic motility.
• A warm-water-filled manometric balloon inserted 5–6 cm into the rectum of anesthetized rats was used to assess distal colonic motility. Acupuncture was performed at heterotopic acupoints.
• Acupuncture at heterotopic acupoints increased distal colonic motility not only in normal rats but also in rats with constipation or diarrhea, and M₃ receptors play an important role in the facilitation of distal colonic motility. Activating afferent nerve C-fibers are essential for enhanced distal colonic motility by acupuncture.

Abstract

Background Previous studies have demonstrated the efficacy of somatic stimulation for patients with gastrointestinal motility disorders. However, little effort has been made to investigate the effects of acupuncture on colonic motility, particularly in pathological conditions. The precise mechanism employed in the regulation of acupuncture on colonic motility still remains unclear.

Methods We assessed the effect of acupuncture at heterotopic acupoints on distal colonic motility using a warm-water-filled manometric balloon inserted 5–6 cm into the rectum of anesthetized normal rats or rats with diarrhea or constipation. Choline chloride, 4-DAMP, cobra venom and capsaicin were separately applied to investigate the role of M₃ receptors in the regulation of distal colonic motility by acupuncture at heterotopic acupoints, and whether Aδ- and/or C-fibers are required for triggering distal colonic motility by acupuncture.

Key Results Acupuncture at heterotopic acupoints increased distal colonic motility not only in normal rats but also in rats with constipation or diarrhea. M₃ receptors play an important role in the facilitation of distal colonic motility triggered by acupuncture at heterotopic acupoints, and whether Aδ- and/or C-fibers are required for triggering distal colonic motility by acupuncture.

Conclusions & Inferences Our results reveal that acupuncture at heterotopic acupoints...
acupoints increases distal colonic motility regardless of normal or pathological conditions via predominately activating C-fibers of somatic afferent nerve and $M_3$ receptors.

**Keywords** acupuncture, C-fibers, colonic motility, heterotopic acupoints, $M_3$ receptors.

**INTRODUCTION**

Gastrointestinal motility disorders contribute to many diseases, such as impaired accommodation, constipation, and diarrhea, among others. A large number of studies have been conducted to explore the efficacy of somatic stimulation for the treatment of gastrointestinal motility disorders, and improvement in functional gastrointestinal disorders, including irritable bowel syndrome (IBS), functional dyspepsia, constipation and diarrhea has also been reported. However, previous investigations rarely addressed the effects of acupuncture on gastrointestinal motility in pathological conditions. Little effort has been made to investigate the effects of acupuncture on colonic motility. Furthermore, based on the innervation patterns and distribution of various receptors in the colon, the precise mechanism employed in the regulation of acupuncture on colonic motility still remains unclear. Addressing these questions can provide valuable clues for the development of effective therapeutics against gastrointestinal motility disorders.

It is believed that acupuncture at different points (acupoints) exerts different effects on internal organs to restore the homeostatic balance. Previous studies suggest site-specific inhibitory or stimulatory effects of acupuncture on gastric motility. Acupuncture could effectively produce dual effects on some visceral organs. However, the dual effects of acupuncture on the gastrointestinal tract and the heart are controversial.

Previous studies have demonstrated that gastrointestinal motility is influenced by somatic afferent stimulation. Pinching stimulation of the abdominal skin of rats inhibits gastric motility, whereas similar stimulation of a hind paw enhances gastric motility in rats. We have previously provided evidence that stimulation intensity above a threshold for activating A$\delta$- and/or C-fibers can effectively modulate gastric motility. Stimulation at homotopic acupoints, where afferent innervation is in the same segment from which the efferents innervate visceral organs, decreases intragastric pressure with or without spinalization.

Stimulation at heterotopic acupoints, which involve different segmental innervations of the spinal cord to visceral organs, induces gastrointestinal facilitation only in complete spinal rats. The effect of acupuncture on gastric motility is primarily associated with autonomic reflexes and the gut-brain axis. Parasympathetic nerves play a critical role in the excitatory regulation of gastrointestinal motility by somatic stimulation at heterotopic acupoints, such as ST36 (Zusanli), ST37 (Shangjuxu), and LI11 (Quchi). The majority of postganglionic C-fibers derived from parasympathetic nerves include a cholinergic component that releases acetylcholine (ACH) from the terminals when activated. Acetylcholine activates muscarinic receptors on the smooth muscle cell surface directly or indirectly, thus triggering various intracellular signaling pathways, which eventually lead to smooth muscle contraction.

In this study, we examined the effect of acupuncture at heterotopic acupoints on distal colonic motility in normal rats, constipated or diarrhoeic rats. We tested the hypothesis that the effects of acupuncture on distal colonic motility are associated with the distribution of cholinergic muscarinic receptors, and that activation of A$\delta$- and/or C-fibers may be required for increased colonic motility by acupuncture at heterotopic acupoints. Our data reveal that acupuncture at heterotopic acupoints enhances distal colonic motility via somatic afferent nerve C-fibers and $M_3$ receptor activation.

**MATERIALS AND METHODS**

**Animal preparation**

Animal experiments were performed in accordance with the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of China Academy of Chinese Medical Sciences (Permit Number: AE20110510-001). All surgery was performed under urethane anesthesia, and all efforts were made to minimize suffering. Totally 123 adult male Sprague-Dawley rats weighing 250–300 g were housed in 595 × 380 × 200 mm cages with ad libitum access to food and water at room temperature (23 ± 1 °C). The animals were housed in group of four with a 12:12 light-dark cycle (dark cycle 8:00 pm–8:00 am), and were allowed to acclimate to their housing for 7 days prior to the experiment. No adverse events were observed.

**Evaluation of constipation and diarrhea**

Constipation was induced via oral gavage of 0 °C normal saline, at a volume of 10 mL/kg/day for five consecutive days. Three hours after saline infusion on the first 2 days, defection increased and changed to soft and loose. Over the remaining part of day 1 and day 2, defection of hard stool was observed. Stool became harder with the increasing of infusion. The rats displayed fewer
and harder stools on day 3 to 5. The stool was collected 24 h after last infusion, weighed it (wet weight, WW) and then dried it for 6 h in the oven at 50 °C and weighed it again (dry weight, DW). The stool moisture (SM) = (WW - DW)/WW × 100%.

Acute diarrhea was induced in rats by administering a senna solution containing 300 mg/mL crude drug liquid via oral gavage, at a volume of 10 mL/kg/day for two consecutive days. The loose stool was determined based on whether there is wet mark in the filter paper and included four levels based on the diameter of the wet marks on the filter paper caused by stool: I, <1 cm; II, 1–2 cm; III, 2–3 cm; IV, >3 cm. The loose stool rate (LSR) = loose stool pellets (LSP)/total stool pellets (TSP) × 100%. The diarrhea index (DI) = LSR × the loose stool level (LSL).

Recording of colonic motility
Rats were fasted overnight prior to electrophysiological recordings. Animals were anesthetized with intraperitoneal injections of urethane (1.0–1.2 g/kg, i.p.) and supplementary anesthesia (0.1 g/kg i.p.) was administered if withdrawal of limbs or a significant fluctuation in heart rate was observed. Core body temperature was monitored and maintained at 37.0 ± 0.5 °C by a feedback-controlled electric blanket (ALC-HTP; Shanghai Alcott Biotech Co., Ltd, Shanghai, China). Heart rate was also monitored to maintain anesthetic depth and to avoid marked cardiac fluctuations caused by drug administration. A manometric balloon was inserted through anus and placed in 5–6 cm above anus. The balloon was filled with approximately 0.1 mL warm water and connected to a piece of polyethylene tube, providing a pressure of approximately 10 cmH2O. Pressure in the intestinal lumen was measured with a transducer through the polyethylene tube and was recorded using a Mac Lab system (AD Instruments, Bella Vista NSW, Australia). After recording stable basal colonic motility, acupuncture stimulation was conducted randomly on different acupoints. Localized spontaneous phasic pressure was recorded continuously for 30 min prior to and during acupuncture stimulation. An increase or decrease in more than 0.2 cmH2O from basal pressure was considered as a change in distal colonic motility.

Acupuncture stimulation
In this study, the unilateral heterotopic acupoints included LI11 (Quchi), ST37 (Shangjuxu), BL25 (Dachangshu) and ST25 (Tianshu). All above acupoints are in the left side of midline. Based on the heterotopic acupoints found in humans, analogous locations on rats are as follows: LI11 locates to the mid-point between the lateral end of the transversus cubicle crease and the lateral epicondyle of the humerus; ST37 is level with the navel, 2 mm lateral to anterior median line; ST37 is 5 mm lateral to the anterior tubercle of the tibia and 15 mm below the knee joint; BL25, at the waist, is under the fourth lumbar spine and 5 mm lateral to posterior midline. The acupuncture needles (0.25 × 25 mm, Suzhou Hwato Medical Instruments, Suzhou, China) were inserted vertically to a depth of approximately 5 mm in each acupoint and rotated right then left about 180° by an experimenter, at a frequency of 2 Hz for 60 s.

Afferent fiber inactivation
Ipsilateral sciatic A- or C-fibers of ST37 were inactivated. The left sciatic nerve A-fibers were demyelinated using cobra venom (Shanghai Pinzhen Biotechnology Co. Ltd, Shanghai, China) which was dissolved in distilled water to 0.3%. As described previously, the sciatic nerve trunk was isolated 1 cm over the fibular capitulum in anesthetized rats. A Hamilton syringe was used to aspirate 1 mL normal saline, 1 mL air and 1 mL 0.3% cobra venom solution, in sequence. The needle tip was then inserted into the sciatic nerve membrane, and the above solution was injected into the myelin sheath while avoiding drug leakage into the surrounding tissue. Thirty minutes after cobra venom injection and incision suture, acupuncture stimulation was performed and colonic motility was recorded.

Drug administration
Choline chloride [10 mg/kg, Sigma-Aldrich], a M3 cholinergic receptor agonist, and 4-DAMP [1 mg/kg, Sigma-Aldrich], a selective M3 receptor antagonist, and methoctramine [0.5 mg/kg, Sigma-Aldrich], a selective M3 cholinergic receptor antagonist, were used separately in this study. Choline chloride was diluted to 0.05% with normal saline, and 4-DAMP was dissolved to 10% in DMSO and then further diluted in distilled water to 0.02%; methoctramine was diluted to 0.05% with normal saline. The diluted agonist or antagonist was injected through carotid vein with a Hamilton syringe at a velocity of 0.1 mL/min. The drug injection was completed within 1–2 min to avoid marked fluctuation in heart rate. Generally, choline chloride induced the maximum stimulatory effect on distal colonic motility 1 min after injection and last for 4 min or so. The inhibitory effect of 4-DAMP on distal colonic motility stabilized after injection and at least continued 20 min. When the above drugs exerted maximum effects on distal colonic motility and did not cause fluctuation in heart rate, acupuncture stimulation commenced.

Statistical analysis
All data were analyzed online and offline using the Mac Lab system. For this study, both frequency and amplitude of distal colonic motility were analyzed. Statistical analysis was performed using SPSS17.0 software (IBM, Armonk, NY, USA). All data are displayed as the mean ± SE. To evaluate statistical significance, data sets with normal distributions were analyzed using paired or unpaired t-tests as appropriate. p < 0.05 was considered statistically significant.

RESULTS
Acupuncture at heterotopic acupoints increases distal colonic motility in normal rats
The manometric balloon was placed in the distal colon of normal rats and was filled with warm water to 10 cmH2O, which induced contractions. The intracolonic pressure stabilized at 4–8 cmH2O after 20 min.
The amplitude of the localized phasic pressure was stable 30 min after filling the balloon with water. The duration of the localized pressure wave was 2.5–4 s.

To determine whether acupuncture at heterotopic acupoints affects distal colon motility in normal rats, we designed an experiment in which manual acupuncture (MA) was applied at LI11, ST37, ST25, and BL25 separately. As shown in Fig. 1A–D, MA at LI11 increased distal colonic pressure to 135.84 ± 13.28% of baseline, a marked increase \( (p < 0.05) \), but did not significantly change motile frequency \( (102.68 \pm 11.68\% \text{ of baseline}, \ p < 0.01) \). MA at ST37 induced a significant increase not only in intracolonic pressure \( (136.19 \pm 11.04\% \text{ of baseline}, \ p < 0.01) \) but also in motile frequency \( (137.68 \pm 12.61\% \text{ of baseline}, \ p < 0.01) \). Manual acupuncture at ST25 significantly increased intracolonic pressure to 132.03 ± 9.83% of baseline \( (p < 0.05) \) and motile frequency to 125.92 ± 11.80% of baseline \( (p < 0.05) \). Manual acupuncture at BL25 enhanced intracolonic pressure to 122.03 ± 10.96% of baseline \( (p < 0.05) \), and motile frequency to 134.00% ± 13.13% of baseline \( (p < 0.05) \). These data suggest that MA at the heterotopic acupoints increases distal colonic motility.

**Acupuncture at heterotopic acupoints augments distal colonic motility in rats with constipation**

Previous studies reported dual effects of acupuncture at heterotopic acupoints on gastric motility.\(^{19,34–36}\) The inhibitory or stimulatory effects of acupuncture are determined by preexisting pathological conditions. For example, MA at ST36 induces dual effects in conscious rats: stimulation of gastric contractions in rats with hypomotility and inhibition of gastric contractions in rats with hypermotility.\(^{19}\) Now a question is raised: Does MA at heterotopic acupoints stimulate or inhibit distal colonic activity in rats with different pathological conditions? To answer this question, we generated constipated rats and diarrhoeic rats to identify the

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**Figure 1** Effects of acupuncture at different heterotopic acupoints on distal colonic motility in normal rats. [A] A representative trace of distal colonic motility in a normal rat; [B] Representative traces of distal colonic motility regulated by acupuncture at LI11, ST37, ST25 and BL25 separately; [C] Acupuncture at LI11, ST37 or ST25 increased intracolonic pressure significantly in normal rats \( (* p < 0.05, ** p < 0.01 \text{ vs baseline}, \text{paired } t\text{-test}, \ n = 17) \). Distal intracolonic pressure was normalized by baseline before acupuncture, the dashed line denotes basal distal intracolonic pressure before acupuncture; [D] Acupuncture at ST37, ST25 or BL25 increased frequency of distal colonic motility \( (* p < 0.05, ** p < 0.01 \text{ vs baseline}, \text{paired } t\text{-test}, \ n = 17) \). Frequency was normalized by baseline before acupuncture. The dashed line denotes basal frequency of distal colonic motility baseline before acupuncture. MA, manual acupuncture.
stimulatory or inhibitory effects of MA at the hetero-
topic acupoints on distal colonic motility in these
pathological conditions.

The irritable bowel model of constipation in rats was
generated by intragastrically infusing 0 °C normal
saline [10 mL/kg] serially over the course of 5 days.
The stool moisture reduced from 40.23 ± 2.19% to
29.37 ± 4.65% (p < 0.05, n = 17) following 5 days of
ice-saline infusion, indicating the model had been
developed successfully.

Manual acupuncture was performed at LI11, ST37,
ST25, and BL25 separately. As shown in Fig. 2B–D, MA
at LI11 significantly increased the intracolonic
pressure (149.91 ± 15.18% of baseline, p < 0.01), but
did not significantly alter frequency (110.43 ± 8.6% of
baseline). Manual acupuncture at ST37 caused signif-
icant changes in either intracolonic pressure (128.84 ±
11.13% of baseline, p < 0.05) or frequency (114.18 ±
5.98% of baseline, p < 0.05). Manual acupuncture at
ST25 increased both intracolonic pressure and motile
frequency significantly (intracolonic pressure: 142.40
± 12.93% of baseline, p < 0.01; frequency: 112.78 ±
7.58 of baseline, p < 0.05). Manual acupuncture at
BL25 induced significant increases not only in intra-
colonic pressure (151.55 ± 12.05% of baseline, p < 0.01) but also in frequency (112.66 ± 6.91% of
baseline, p < 0.05). These results suggest that in con-
stipated rats, MA at heterotopic acupoints increases
colonic motility.

Acupuncture at heterotopic acupoints augments
distal colonic motility in rats with diarrhea

Having identified the effects of MA at LI11, ST37,
ST25, or BL25 on distal colonic motility in rats with
constipation, we went on to identify the effects of MA
at these acupoints on distal colonic motility in diar-
hoeic rats. The rat model of diarrhea was gener-
ated by intragastric infusion of folium sennae deco-
cion [10 mL/kg] for 2 days. The decoction was prepared

![Figure 2](image_url)

**Figure 2** Effects of acupuncture at different heterotopic acupoints on distal colonic motility in constipated rats. (A) Representative traces of distal colonic motility in a normal rat (37 °C normal saline infusion) and a constipated rat. (B) Representative traces of distal colonic motility stimulated by acupuncture at LI11, ST37, ST25, and BL25 separately in constipated rats; (C) Intracolonic pressure was enhanced significantly by acupuncture at LI11, ST37, ST25, and BL25 separately in constipated rats (*p < 0.05, **p < 0.01 vs baseline, paired t-test, n = 17). Distal intracolonic pressure was
normalized by baseline before acupuncture, the dashed line denotes basal distal intracolonic pressure before acupuncture; (D) Acupuncture at ST25,
ST37, and BL25 separately facilitated frequency of distal colonic motility in constipated rats (*p < 0.05 vs baseline, paired t-test, n = 17). Frequency
was normalized by baseline before acupuncture. The dashed line denotes basal frequency of distal colonic motility baseline before acupuncture. MA,
manual acupuncture.
as 10 mL decoctum containing 0.3 g crude drug. Following 2 days of oral gavage, the rats appeared fatigued, showed a decrease in food consumption and movement, and feces were excessive and loose with an unpleasant odor. The LSR was approximately 49.5% of all gavaged rats, the LSL was 2.68, and the DI was 1.33 (49.5% × 2.68), indicating that the model had developed appropriately. In rats with diarrhea, the duration of baseline colonic contract wave was 2.5–4 s, and colonic motile frequency increased (Fig. 3A), consistent with the previous reports.37

Fig. 3B–D showed that in rats with diarrhea, MA at LI11 increased intracolonic pressure to 119.41 ± 9.50% of baseline, a significant difference (p < 0.05), but did not significantly change frequency [110.35 ± 9.13% of baseline]. Manual acupuncture at ST37 increased intracolonic pressure to 117.91 ± 7.94% of baseline (p < 0.05) and also increased contract frequency to 119.13 ± 5.93% of baseline (p < 0.05). Manual acupuncture at ST25 did not significantly enhance intracolonic pressure (112.22 ± 9.75% of baseline, p > 0.05), whereas frequency significantly increased 118.18 ± 7.08% of baseline, p < 0.05). After MA at BL25, we observed significant facilitation in either intracolonic pressure (137.63 ± 12.96 of baseline, p < 0.01) or frequency (119.73 ± 8.76% of baseline, p < 0.05). These data suggest that in rats with diarrhea, MA at heterotopic acupoints still increases distal colonic motility.

M₃ receptors play a role in mediating the excitatory effect of acupuncture at heterotopic acupoints on distal colonic motility

Previous evidence indicates that the dominant cholinergic muscarinic receptors in the jejunum are M₂ subtype, and the M₃ subtype is dominant in the colon.38,39 Therefore, we aimed to illustrate whether M₃ receptors play a specific role in mediating the effect of acupuncture at heterotopic acupoints on distal

![Figure 3](image-url)

**Figure 3** Effects of acupuncture at different heterotopic acupoints on distal colonic motility in diarrhoeic rats. (A) Representative traces of distal colonic motility in a normal rat (37 °C normal saline infusion) and a diarrhoeic rat; (B) Representative traces of distal colonic motility stimulated by acupuncture at LI11, ST37, ST25, and BL25 separately in diarrhoeic rats; (C) Intracolonic pressure was enhanced significantly by acupuncture at LI11, ST37, ST25, and BL25 separately in diarrhoeic rats (*p < 0.05, **p < 0.01 vs baseline, paired t-test, n = 17). Distal intracolonic pressure was normalized by baseline before acupuncture, the dashed line denotes basal distal intracolonic pressure before acupuncture; (D) Acupuncture at ST25, ST37, and BL25 separately facilitated frequency of distal colonic motility in diarrhoeic rats (*p < 0.05 vs baseline, paired t-test, n = 17). Frequency was normalized by baseline before acupuncture. The dashed line denotes basal frequency of distal colonic motility baseline before acupuncture. MA, manual acupuncture.
colonic motility. Firstly, we treated rats with 4-diphenyl-acetoxy-N-methyl-piperidine (4-DAMP, 1 mg/kg, iv), a M3 receptor antagonist, to identify the role of M3 receptors in augmentation of distal colonic motility induced by acupuncture at heterotopic acupoints. Fig. S1A–C showed that DMSO did not change the intracolonic pressure and frequency significantly. As shown in Fig. 4A, C and D, after 4-DAMP application, the intracolonic pressure reduced from 8.80 ± 0.58 cmH2O to 4.67 ± 0.41 cmH2O (p < 0.01), and motile frequency decreased from 12.26 ± 0.40 times/min to 6.32 ± 0.32 times/min (p < 0.01), consistent with previous reports.40,41 In the presence of 4-DAMP, MA at LI11, ST37, ST25, and BL25 separately failed to facilitate distal colonic motility. As shown in Fig. 4B, E and F, in LI11 group, intracolonic pressure was 70.26 ± 6.82% of baseline (p < 0.01), and frequency 59.40 ± 5.34% of baseline (p < 0.01); in ST37 group, intracolonic pressure was 66.04 ± 5.80% of baseline (p < 0.01) and motile frequency 74.78 ± 5.11% of baseline (p < 0.01); in ST25 group, intracolonic pressure was 69.52 ± 5.04% of baseline (p < 0.01), and frequency 77.40 ± 9.85% of baseline (p < 0.01); in BL25 group, intracolonic

Figure 4 Effect of 4-DAMP on distal colonic motility facilitated by acupuncture at different heterotopic acupoints. [A] Representative traces of distal colonic motility without and with 4-DAMP; [B] Representative traces of distal colonic motility changed by acupuncture at LI11, ST37, ST25, and BL25 separately following the administration of 4-DAMP; [C] 4-DAMP inhibited intracolonic pressure significantly (**p < 0.01 vs baseline, unpaired t-test, n = 16); [D] 4-DAMP decreased frequency of distal colonic motility significantly (**p < 0.01 vs baseline, unpaired t-test, n = 16); [E] 4-DAMP abolished the facilitation of distal intracolonic pressure evoked by acupuncture at LI11, ST37, ST25, and BL25, respectively (**p < 0.01 vs baseline, paired t-test, n = 16). Distal intracolonic pressure was normalized by baseline without any treatment, the dashed line denotes basal distal intracolonic pressure without 4-DAMP administration; [F] 4-DAMP prevent the effects of acupuncture at LI11, ST37, ST25, or BL25 significantly on frequency of distal colonic motility (**p < 0.01 vs baseline, shown as the dashed line, paired t-test, n = 16). Frequency was normalized by baseline without any treatment. The dashed line denotes basal frequency of distal colonic motility without 4-DAMP administration. MA, manual acupuncture.

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pressure is 73.25 ± 6.77% of baseline \((p < 0.01)\), and frequency 65.31 ± 4.32% of baseline \((p < 0.01)\).

To further characterize the role of M₃ receptors in mediating the facilitation of colonic motility induced by MA at these acupoints, we treated rats with choline chloride (10 mg/kg, i.v.), an agonist of M₃ receptors. As shown in Fig. S2A–C, the intracolonic pressure and frequency were not significantly changed by normal saline injection. Fig. 5A, C and D showed that choline chloride treatment caused a significant enhancement in intracolonic pressure [from 12.71 ± 1.56 cmH₂O to 22.96 ± 2.24 cmH₂O, \(p < 0.01\)], but by contrast, there was little change in motile frequency [from 11.79 ± 0.36 times/min to 12.08 ± 0.51 times/min, \(p > 0.05\)]. After choline chloride application, MA was conducted at LI11, ST37, ST25 and BL25 separately. As shown in Fig. 5B, E and F, MA at LI11 did not further enhance intracolonic pressure and motile frequency significantly [intracolonic pressure: 204.13 ± 24.11% of baseline for pre-MA vs 226.23 ± 30.87% of baseline for MA, \(p > 0.05\); frequency: 109.73 ± 6.0% of baseline for pre-MA vs 111.07 ± 4.9% for MA, \(p > 0.05\)]. Manual acupuncture at ST37 did not produce any significant changes in intracolonic pressure or frequency [intracolonic pressure: 212.65 ± 21.62% of baseline for pre-MA vs 201.94 ± 20.48% of baseline for MA, \(p > 0.05\); frequency: 109.72 ± 11.14% of baseline for pre-MA vs 115.43 ± 8.43% of baseline for MA]. Similarly, we did not observe marked effects of MA at ST25 or BL25 on intracolonic pressure after choline application [MA at ST25: 205.32 ± 22.86% of baseline intracolonic pressure and 105.79 ± 9.53% of baseline frequency for pre-MA vs 172.67 ± 20.63% of baseline intracolonic pressure and 116.30 ± 8.12% of baseline frequency for MA, \(p > 0.05\); MA at BL25: 202.30 ± 19.22% of baseline intracolonic pressure and 99.27 ± 11.63% of baseline frequency for pre-MA vs 212.84 ± 20.00% of baseline intracolonic pressure and 109.26 ± 9.93% of baseline frequency, \(p > 0.05\)]. Therefore, MA at the above acupoints could not further enhance intracolonic pressure after M₃ receptors were saturated and activated completely by choline. Taken together, these data suggested that M₃ receptors play an important role in mediating the facilitation of distal colonic motility induced by MA at heterotopic acupoints.

Our previous study demonstrated that M₂ receptors play a role in mediating the excitatory effect of acupuncture at heterotopic acupoints on jejunal motility. Here, we applied methoctramine (0.5 mg/kg, i.v.), a selective M₂ receptor antagonist, to detect whether M₂ receptors also play a role in the enhancement of distal colonic motility induced by acupuncture at heterotopic acupoints. As shown in Fig. S3A–C, intracolonic pressure and frequency were not changed by methoctramine significantly. As shown in Fig. S3D and E, after methoctramine application in normal rats, MA at LI11, ST37, ST25, or BL25 significantly increased intracolonic pressure and frequency separately [LI11: 128.61 ± 14.68% of basal intracolonic pressure, \(p < 0.05\); 98.56 ± 9.38% of basal frequency. ST37: 140.39 ± 16.74% of basal intracolonic pressure, \(p < 0.01\); 130.70 ± 11.24% of basal frequency, \(p < 0.05\). ST25: 135.86 ± 10.47% of basal intracolonic pressure, \(p < 0.05\); 124.39 ± 8.68% of basal frequency. BL25: 129.75 ± 8.94% of basal intracolonic pressure, \(p < 0.05\); 130.62 ± 10.07% of basal frequency, \(p < 0.05\)]. These data suggest that M₂ receptors did not play any role in mediating the excitatory effect of acupuncture at heterotopic acupoints on distal colonic motility.

**C-fiber activation is required for the regulation of distal colonic motility by acupuncture**

It has been well documented that the somatosensory inputs from the skin and/or muscle are involved in the control of various autonomic functions. Acupuncture modulates visceral organ function by inducing activation of the somato-visceral reflexes and changing the tone of the autonomic nervous system. Our previous studies demonstrated that modulation of gastric motility induced by acupuncture stimulation only involved the activation of fine-diameter afferent fibers including Aδ-fibers and C-fibers. Here, we set out to address which type of nerve fiber specifically mediates the afferent encoding of the heterotopic acupoint-colonic reflexes. In this study, we utilized cobra venom, an A-selective demyelination agent, and capsaicin, a C-fiber blocker. Cobra venom was applied for 30 min to demyelinate A-fibers of the sciatic nerve, then acupuncture at ipsilateral ST37 was performed. Fig. S4A–C showed that Cobra venom did not change distal intracolonic pressure and frequency significantly. As shown in Fig. 6A–C, without demyelination of Aδ-fibers of the sciatic nerve, acupuncture at ST37 significantly increased intracolonic pressure to 149.38 ± 4.76% of baseline \((p < 0.01)\) and motile frequency to 154.35 ± 2.10% of baseline \((p < 0.01)\). Following the demyelination of Aδ-fibers, MA at ST37 augmented intracolonic pressure to 132.44 ± 4.11% of baseline \((p < 0.05)\), and frequency to 131.35 ± 2.61% of baseline \((p < 0.05)\). There was a significant effect of Aδ-fiber demyelination on intracolonic pressure and frequency in MA-exposed animals \((p < 0.05)\). This suggests that Aδ-fibers may partially mediate afferent
encoding of MA at heterotopic acupoints, which enhances colonic motility.

To identify the role of C-fibers in mediating the regulation of colonic motility by MA at ST37, we administrated capsaicin to diminish activity of C-fibers of the sciatic nerve. Fig. S5A–C showed that capsaicin did not change distal intracolonic pressure and frequency. As shown in Fig. 7A–C, MA at ST37 increased intracolonic pressure to 130.65 ± 2.35% of baseline (p < 0.05), and frequency to 137.5 ± 10.3% of baseline (p < 0.05) in intact rats. Following a 24-h treatment with capsaicin [2%, 25 µL], the increases in intracolonic pressure and frequency induced by MA at ipsilateral ST37 were abolished completely [intracolonic pressure: 93.18 ± 3.98% of baseline, p < 0.05 vs baseline, frequency: 115.73 ± 6.10% of baseline, p > 0.05 vs baseline]. There was a highly significant effect of blocking C-fibers on intracolonic pressure and frequency in MA-exposed rats (p < 0.01). These data provide solid evidence that C-fiber activation is
essential for the enhancement of colonic motility induced by MA at heterotopic acupoints.

DISCUSSION

Disrupted colonic motility has been associated with various functional diseases, such as constipation, diarrhea, and IBS. In this study, we discovered for the first time that acupuncture at LI11, ST37, ST25, and BL25 accelerated colonic motility in all rats, regardless of gastrointestinal health state. We also noted that M₃ receptors play an important role in mediating the facilitation of colonic motility induced by MA at the above acupoints. The activation of C-fibers is required for the regulation of colonic motility by MA, while Aδ-fibers mediate this effect in part. Therefore, our study revealed that acupuncture at heterotopic acupoints facilitates colonic motility through activating afferent C-fibers. M₃ receptors play an important role in this process.

Dual directional regulation was considered as one key characteristic of acupuncture in Chinese medicine. Previous studies reported that acupuncture could effectively produce dual effects on some visceral organs, inhibiting overexcited activities and stimulating deficient activities.₁⁷,₁⁸ For example, acupuncture-like stimulation to perineal area could increase activity of the over-filling bladder, and decrease the activity of half-filling bladder.⁴³,⁴⁴ However, in this study, MA at heterotopic acupoints only exerted stimulatory effect on distal colonic motility not only in normal rats but also in rats with constipation or diarrhea, and did not exhibit dual effects on distal colonic motility, consistent with the previous reports.⁴⁵

Previous studies identified the site-specific inhibitory or stimulatory effects of acupuncture on gastric motility.₁⁵,₁⁶,₂⁰,₂⁶ Acupuncture at the hindlimb increases gastric motility, whereas acupuncture at the abdomen inhibits gastric motility in anesthetized rats.₁⁶,₄₆ In anesthetized rats, the cutaneogastric reflexes mediate the inhibition and the stimulation of gastric motility via sympathetic and parasympathetic efferents, respectively.₁⁵,₄⁷,₄₈ The cutaneo-sensory stimulation induced by pinching abdominal skin of rats inhibits gastric motility by increasing sympathetic activity. On the other hand, cutaneo-sensory stimulation induced by pinching the hindlimb enhances gastric motility via increased vagal activation.₁⁵ Acupuncture at ST36 could facilitate distal colonic motility,¹⁹,₄⁵,₄⁹ which could be diminished by atropine or transection of pelvic nerve.⁴⁵,⁵₀ This suggests that sacral parasympathetic nerve fibers, components of the pelvic nerve, play a critical role in the acceleration of distal colonic motility induced by acupuncture at ST36. Sacral parasympathetic efferents innervate the distal colon and the parasympathetic innervations of the proximal colon originate from the vagus nerve.⁴⁹ Our previous studies demonstrated that regardless of any acupoints,
MA at the acupoints of widespread territory (except homosegmental innervations with gastric innervations) induced a facilitative response on gastric motility. Acupuncture at the acupoints of local territory of homosegmental innervations with gastric innervations induces an inhibitory response on gastric motility.20 In this study, the afferent nerve fibers of LI11 area arrive at C5 spinal dorsal horn, the afferents of ST37 terminate at L5 spinal segment, ST25 enters T10 spinal segment and BL25 enters L3 spinal segment. It is well-established that the sacral parasympathetic efferent which innervates the distal colon mainly originates from S1 spinal segment in rats.51 Therefore, LI11, ST37, ST25, and BL25, as the acupoints of heterosegmental innervations with distal colonic innervations, facilitate distal colonic motility through activation of sacral parasympathetic nerve regardless of normal, constipated, or diarrhoeic state. However, MA at LI11 did not increase motile frequency in either normal or model rats, and MA at ST25 did not facilitate intracolonic pressure in diarrhoeic rats. These interesting points need to be clarified in the future.

It has been demonstrated that parasympathetic terminals release the contractile neurotransmitter ACH. Acetylcholine exerts excitatory effects on smooth muscle via muscarinic receptor binding.52 Although muscarinic ACH receptors comprise five distinct subtypes (M1-5) and are widely distributed in smooth muscle throughout the body including the gastrointestinal tract,53-55 in gastrointestinal smooth muscles, M3 and M4 muscarinic receptor subtypes are preferentially expressed.56 In rats, M3 is the primary subtype expressed in the colon.57 The recent use of mutant mice lacking specific muscarinic receptor subtypes has revealed that both M3 and M2 receptors may play a direct role in inducing contraction in gastric and ileal smooth muscles.56,58-61 Both M2 and M3 receptors mediate contractions induced by stimulation of cholinergic nerves.62 M2 receptors are less active than M3 receptors in mediating cholinergic contractions in wild-type tissues; cholinergic contractions in vitro are mediated predominantly by M3 receptors. However, a recent study verified that small but significant contractions remained in the homozygous muscles (bladder, 5%; ileum, 23–28%) and that M2 receptors mediated the residual contraction of M3 homozygous muscles directly.63 In this study, we found that the M3 receptor antagonist 4-DAMP abolished the stimulatory effect of MA at LI11, ST37, ST25, or BL25 on colonic motility, and acupuncture at these acupoints did not exhibit any stimulatory effect on distal colonic motility following a complete activation of M3 receptors by choline chloride. This indicates that M3 receptors play a critical role in mediating the facilitation of colonic motility triggered by acupuncture at heterotopic acupoints.

Somatic afferents from the skin and muscle are involved in the control of gastrointestinal motility.21-25

Figure 7 Effect of capsaicin on the regulation of distal colonic motility by acupuncture at ST37. (A) Representative traces of distal colonic motility without and with capsaicin; (B) Capsaicin completely abolished the increase in distal intracolonic pressure triggered by acupuncture at ST37 (*p < 0.05 vs baseline, paired t-test; ##p < 0.01 vs acupuncture without capsaicin treatment, unpaired t-test, n = 20). Intracolonic pressure was normalized by baseline, shown as the dashed line; (C) Capsaicin showed significant inhibitory effect on the increase in frequency of distal colonic motility attributed to acupuncture at ST37 (*p < 0.05 vs baseline, paired t-test; ##p < 0.01 vs acupuncture without capsaicin treatment, unpaired t-test, n = 20. Frequency was normalized by baseline, shown as the dashed line). MA, manual acupuncture.
Cutaneous stimulations such as acupuncture may stimulate the somatic afferent nerves of the skin and muscles that are important for evoking autonomic reflexes. Somato-visceral reflexes responsible for regulation of visceral organs are strongly associated with the effects of acupuncture. Previous work from our lab demonstrated that only stimulations greater than the threshold for activation of A\(\delta\) and/or C-fibers could profoundly modulate gastric motility.\(^\text{26}\) Stimulations below threshold for A\(\delta\)-fiber activation do not effectively trigger regulatory effects on gastric motility, regardless of the locations of the acupoints. Koizumi et al. conducted a systematical analysis of the relationship between the magnitude of cutaneo-intestinal reflex response and the groups of afferent fibers stimulated. They noted that stimulation of the sural afferent nerves of the hindlimb elicited facilitative jejunal reflexes when the stimulus intensity activated group III fibers, and maximal facilitation was achieved when the stimulus intensity activated group IV afferent fibers. Stimulating groups III-IV (particularly group IV) the stimulus intensity activated group IV afferent fibers are required for this enhancing effect of acupuncture on distal colonic motility. The findings in this study provide valuable clues for the development of effective therapeutics against intestinal motility disorders.

ACKNOWLEDGMENTS

We thank Kristin Schaeper for assistance with word polishing and grammar corrections.

FUNDING

This study was supported by grants from National Natural Science Foundation of China (no. 81173345, 81473778 to XG; 81273831 to HQ; 81130063 to BZ) and was also supported by the National Basic Research Program of China (no. 2011CB505201 to BZ) and Intramural Program of Institute of Acupuncture and Moxibustion (no. ZZ21006 to HQ).

DISCLOSURE

The authors have no competing interests.

AUTHOR CONTRIBUTION

XG and BZ Conceived and designed the experiments; QQ and KL performed the recordings; LL performed the acupuncture; XG, XY, HQ, and BZ wrote the paper in addition to providing financial support for this research.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher’s web site:

- **Figure S1** DMSO did not change basal distal colonic motility.
- **Figure S2** Normal saline did not exert effect on basal distal colonic motility.
- **Figure S3** Methoctramine did not block the facilitation of distal colonic motility induced by acupuncture at different heterotopic acupoints.
- **Figure S4** Cobra venom did not change basal distal colonic motility.
- **Figure S5** Capsaicin did not change basal distal colonic motility.