Electroacupuncture with high frequency at acupoint ST-36 induces regeneration of lost enteric neurons in diabetic rats via GDNF and PI3K/AKT signal pathway

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Du F, Liu S. Electroacupuncture with high frequency at acupoint ST-36 induces regeneration of lost enteric neurons in diabetic rats via GDNF and PI3K/AKT signal pathway. Am J Physiol Regul Integr Comp Physiol 309: R109–R118, 2015. First published May 13, 2015; doi:10.1152/ajpregu.00396.2014.—Background electroacupuncture (EA) at acupoint ST-36 (Zusanli) has been used to alleviate gastrointestinal symptoms and improve gastrointestinal motility, but the effects and mechanisms of EA on enteric nervous system (ENS) have scarcely been investigated. SD rats were randomly divided into eight groups: normal control group, diabetes mellitus group (DM), chronic high-frequency EA (C-HEA), chronic low-frequency EA (C-LEA), chronic sham stimulation group (C-SEA), acute high-frequency EA group (A-HEA), acute low-frequency EA group (A-LEA), and diabetic with acute sham stimulation group (A-SEA). The parameters of HEA included a frequency of 100 Hz and an amplitude of 1 mA, while the parameters for LEA were 10 Hz and 1 mA. The expressions of PGP9.5, neuronal nitric oxide synthase neurons, CHAT neurons, glia cell line-derived neurotrophic factor (GDNF) and p-Akt were measured by immunofluorescence or immunohistochemistry, real-time PCR, and Western blotting methods in colon tissues of each rat. The total neurons and the two types of enteric neurons (neuronal nitric oxide synthase and choline acetyl transferase neurons), together with GDNF and p-Akt in the mRNA and protein level were significantly decreased in DM group compared with the normal control group in colon (P < 0.01). Compared with DM or all other DM with EA groups, the chronic HEA could induce a more significant quantitative increase in the mRNA and protein level of the enteric neurons and GDNF and p-Akt in colon (P < 0.01). EA with high-frequency and long-term stimuli at acupoint ST-36 can induce regeneration of lost enteric neurons in diabetic rats, and GDNF and PI3K/AKT signal pathway may play an important role in EA-induced regeneration of impaired enteric neurons.

electroacupuncture; diabetes; enteric neuropathy; glial cell line-derived neurotrophic factor-PI3K/Akt; neuron regeneration

GASTROINTESTINAL MOTILITY dysfunction is characterized by a series of complex gastrointestinal (GI) symptoms in the absence of mechanical obstruction of the GI tract. As we know, GI dysfunction may not increase patients’ mortality; however, it can significantly reduce patients’ quality of life and increase our nation’s health care burden. The incidence rate of diabetes is about 10% (1, 2), as well as our previous studies (13a, 13b) showed that EA at acupoint ST-36 could be used to alleviate gastrointestinal symptoms and improve gastrointestinal motility in both animal models and clinical patients. However, the mechanisms of EA on ENS have scarcely been investigated.

So far, the therapy of diabetic GI dysfunction is largely symptomatic and clearly suboptimal. Also, most of the treatment methods are nonspecific and not uniformly effective. In recent years, studies indicated that electroacupuncture (EA) may come to a potential effective therapy method to treat diabetic GI dysfunction. EA, which is the combination of acupuncture and electrical current stimulation instead of the manual manipulation of needles, is more consistent and reproducible. Some studies (11, 21), as well as our previous studies (13a, 13b) showed that EA at acupoint ST-36 could be used to alleviate gastrointestinal symptoms and improve gastrointestinal motility in both animal models and clinical patients. However, the effects and mechanisms of EA on ENS have scarcely been investigated.

The purpose of this study was to investigate whether EA affects ENS and which type of EA may be effective and to illustrate the possible mechanisms of stimulatory effects of EA on ENS neurons. The current study takes diabetic rats as models, gives the rats short-term and long-term EA with different frequencies to investigate the effect on nNOS neurons, CHAT neurons, total neurons [protein gene product 9.5 (PGP9.5) stained], and further explores whether GDNF and p-Akt are involved in EA-induced change of enteric neurons.

MATERIALS AND METHODS

Animals

A total of 64 adult male Sprague-Dawley rats weighing ~250–350 g were used in this study. All of the rats were obtained from the...
Experiment Animal Center of Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China. They were housed in temperature- and light-controlled rooms (20–25°C, 12:12-h light-dark cycle) and were given water ad libitum. All received a week of adaptive feeding before the experiment began. Rats received humane care, and all experiments were approved by the Animal Care and Use Committee of Huazhong University.

Experimental Protocols

Randomization. All of the 64 SD rats were randomly divided into eight groups (n = 8): normal control group, diabetic control (DM), diabetic with chronic high-frequency EA (C-HEA), diabetic with chronic low-frequency EA (C-LEA), diabetic with chronic sham stimulation group (C-SEA), diabetic with acute high-frequency EA group (A-HEA), diabetic with acute low-frequency EA group (A-LEA), and diabetic with acute sham stimulation group (A-SEA).

Diabetic models. Diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ; Alexis Biochemicals, San Diego, CA) at a dose of 60 mg/kg, in 0.05 mol/l citrate buffer (pH 4.5; Sigma, St. Louis, MO), while the controls were injected with citrate buffer at the same dose as the diabetic groups. Blood glucose and body weight of each rat were measured before and 1 wk after injection of STZ, as well as prior to obtaining specimens. Diabetes was diagnosed with a random blood glucose higher than 16.7 mmol/l.

EA stimulation. As ST-36 (Zusanli point of the lower limb) is one of the most commonly used acupoints for GI diseases, it was chosen for the EA stimulation acupoint in our experiment. ST-36 is located at 5 mm lateral and lower from the anterior tubercle of the tibia in rats (9). Needles were inserted to a depth of 5 mm into the rats’ skin and underlying muscles at ST-36 bilaterally. For the HEA/LEA groups, needles were inserted into the acupoint and were stimulated by using an electrical stimulator (G6805-2A; Shanghai Huayi Medical Instrument, Shanghai, China). The parameters of HEA included a frequency of 100 Hz and an amplitude of 1 mA, while those of the LEA were 10 Hz and 1 mA; for the SEA group, needles were only inserted into the acupoint without current. The three long-term chronic EA groups were given high-frequency, low-frequency or sham EA 30 min/day for 6 wk after diabetes was diagnosed; the three short-term acute EA groups were given high- and low-frequency EA or sham EA 30 min/day since the 6th wk and EA treatment continued for only 1 wk after diabetes was diagnosed. Schematic representation of EA study protocol is shown in Fig. 1.

Preparation of specimen. When all of the experimentation on the rats finished, they were killed, and specimens of the colon were obtained from each rat. Each specimen collected from the rats was divided into three pieces of equal size, which were then either placed into 4% paraformaldehyde for immunohistochemistry/immunofluorescence analysis or immediately snap frozen in liquid nitrogen and stored at –80°C for both real-time PCR and Western blot analysis.

Materials

In our study, we used the following materials: primary antibodies (for immunohistochemistry and Western blot analysis) to GDNF (Santa Cruz Biotechnology, Santa Cruz, CA), nNOS (Cell Signaling, Boston, MA), CHAT (Abcam, Cambridge, UK), PGP9.5 (Abcam), p-Akt (R&D Systems, Minneapolis, MN), and rabbit anti-rat GAPDH (Boster Biotech, Wuhan, China). Secondary antibodies for immunohistochemistry include MaxVision HRP (horseradish peroxidase)-polymer anti-mouse/rabbit immunohistochemistry kit (Maixin Biotech, Fuzhou, China). Secondary antibodies for Western blot include HRP-linked goat anti-rabbit IgG and HRP-linked goat antimouse IgG (Boster Biotech, Wuhan, China). Secondary antibodies for immunohistochemistry include MaxVision HRP (horseradish peroxidase)-polymer anti-mouse/rabbit immunohistochemistry kit (Maixin Biotech, Fuzhou, China). Secondary antibodies for Western blot include HRP-linked goat anti-rabbit IgG and HRP-linked goat antinauseous IgG (Invitrogen, Carlsbad, CA). There were some other reagents as follows: STZ (Alexis Biochemicals), TRRzol Reagent (Invitrogen), Oligo(dT) (Promega, Madison, WI), RNase inhibitor (BioStar International, Toronto, Canada), dNTPmix (Tiangen Biotech, Beijing, China), MMLV (Invitrogen), SYBR Green PCR Master Mix (Invitrogen), NC membrane (Millipore, Bedford, MA), and enhanced chemiluminescence (ECL) (ThermoScientific Pierce, Rockford, IL).

Immunohistochemistry Analysis

The colon tissue specimens were immediately fixed by immersion in 4% paraformaldehyde for 24 h, and then processed for paraffin embedding in a vacuum and cut to a thickness of 5 μm. Sections were deparaffinized in xylene and hydrated in a graded solution of ethanol. After endogenous peroxidase activity was quenched with 3% hydrogen peroxide, the sections were immersed in 3% hydrogen peroxide for 20 min to block endogenous peroxidase activity, and then treated with 10% normal donkey serum for 1 h to block non-specific binding. After incubation with primary antibodies, the sections were then immersed in secondary antibodies for 1 h, and then incubated with HRP-labeled secondary antibody for 1 h. Finally, the sections were treated with DAB for visualization. Sample sections were processed in parallel with negative controls (no primary antibody) or treated with unrelated primary antibodies.

Fig. 1. Schematic representation of electroacupuncture (EA) study protocol. After streptozotocin injection, the diabetic rats were divided into six groups depending on the total EA span and frequency: chronic high-frequency EA (C-HEA), chronic low frequency EA (C-LEA), chronic sham stimulation group (C-SEA), acute high frequency EA group (A-HEA), acute low frequency EA group (A-LEA), and diabetic with acute sham stimulation group (A-SEA).
gen peroxide (H$_2$O$_2$) for 10 min and microwaved (750 W) for 5 min, then nonspecific binding was blocked by treatment with normal goat serum for 30 min at 37°C. The primary antibodies for GDNF (1:100), p-Akt (1:50), nNOS (1:100), CHAT (1:50), and PGP9.5 (1:250) were applied to the sections, and each specimen was incubated in a moist chamber overnight at 4°C. The slides were washed three times in 0.01 mol/l PBS (pH 7.2) and incubated with HRP-linked polymer anti-mouse/rabbit IgG for 30 min at 37°C. After washing in PBS three times, the localization of target protein was visualized by incubating the sections for 10 min in freshly prepared 3,3-diaminobenzidine solution. The slides were washed again, counterstained in hematoxylin, and then dehydrated. Specificity of the antibody was confirmed by negative control in the absence of primary antibody treatment. Two observers evaluated the slides using an Olympus FV500 optical microscope (Olympus, Tokyo, Japan). Positive immunostaining was evaluated at a magnification of ×400.

### mRNA Expression Analysis

mRNA expression levels were measured by real-time quantitative reverse-transcription PCR. Total RNA was extracted using the TRIZol reagent, and RNA samples were reversely transcribed into cDNA, according to the manufacturer’s instructions. The cDNA was subsequently diluted in nuclease-free water and stored at −20°C. The specific primers used in our study are listed in Table 1. PCR was performed as described by the manufacturer using the SYBR Green PCR Master Mix. The reaction was performed in triplicate in total volume of 20 μl containing 10 μl SYBR Green/enzyme reaction mix, 1.5 μl of each sense and antisense primer (initial concentration 10 pmol/μl), 1 μl diluted cDNA and 6 μl ddH$_2$O. The PCR conditions were 95°C for 10 min first, then followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Real-time detection was performed on an ABI-StepOne Detection System (Applied Biosystems, Carlsbad, CA). Fluorescence values of SYBR Green dye, representing the amount of product amplified at that point in the reaction, were recorded in real time at both the annealing step and the extension step of each cycle. The amplification was followed by a melting curve analysis, which was applied to all reactions to ensure homogeneity of the amplification product. Melting curve analysis was performed by increasing the temperature by 1°C increments from 60°C to 95°C and measuring the fluorescence at each temperature for a period of 10 s. Anti-rat GAPDH (1:500) served as the internal control. After that, the membranes were washed in TBST (with 0.1% Tween-20) for three times and incubated with HRP-linked secondary antibody (HRP-linked goat anti-rabbit or HRP-linked goat anti-mouse, 1:3,000) for 1 h at room temperature. Detection of protein was achieved by ECL reagents, and the blot was subjected to autoradiography. A semiquantitative measurement of the band intensity was performed by Quantity One (Bio-Rad Technical Service Department, version 4.6.2).

#### Statistical Analysis

Experiments were repeated in eight independent experiments, and the mean values were used for statistical analysis. The results were expressed as mean values ± SE. Differences between multiple groups were evaluated by applying a one-way ANOVA and a post hoc test (Bonferroni correction). *P* < 0.05 was considered to be a significant difference. All statistical analysis was performed by using SPSS 17.0 statistics package (SPSS, Chicago, IL).

### RESULTS

The mRNA expression of enteric neurons in colon tissue by real-time PCR In Fig. 2, the mRNA expression of total neurons for marker PGP9.5, together with nNOS and CHAT neurons, was assessed. Statistical analysis demonstrated that chronic HEA dramatically enhanced the expression of PGP9.5, nNOS, and CHAT neurons compared with the DM group, and both chronic LEA and SEA groups in the proximal and distal colon (*P* < 0.05). In the meantime, when giving low-frequency EA stimulation, we found the expression of CHAT and nNOS neurons increased in the distal colon (*P* = 0.032, *P* = 0.006) compared with the DM group. However, no significant differences were found between the acute EA stimulation groups and the DM group in the proximal and distal colon (*P* > 0.05).

#### Effects of Diabetes and EA Stimulation on Total Neurons- PGP9.5 Protein Expression in Colon Tissue

Figure 3 shows the immunohistochemical staining and Western blot results of PGP9.5. The brown immunoreactive

### Table 1. List of designed primer sets for real-time quantitative PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Size, bp</th>
<th>GeneBank Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>sense</td>
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<tr>
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<td>antisense</td>
<td>270–314 bp</td>
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</tr>
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<td>305–348 bp</td>
<td>NM_052799</td>
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<tr>
<td></td>
<td>antisense</td>
<td>1425–1465 bp</td>
<td>XM_224626</td>
</tr>
<tr>
<td>nNOS</td>
<td>sense</td>
<td>428–469 bp</td>
<td>NM_017237</td>
</tr>
<tr>
<td></td>
<td>antisense</td>
<td>1425–1465 bp</td>
<td>XM_224626</td>
</tr>
<tr>
<td>CHAT</td>
<td>sense</td>
<td>428–469 bp</td>
<td>NM_017237</td>
</tr>
<tr>
<td></td>
<td>antisense</td>
<td>1425–1465 bp</td>
<td>XM_224626</td>
</tr>
<tr>
<td>PGP9.5</td>
<td>sense</td>
<td>428–469 bp</td>
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</tr>
<tr>
<td></td>
<td>antisense</td>
<td>1425–1465 bp</td>
<td>XM_224626</td>
</tr>
</tbody>
</table>
PGP9.5 fibers and positive ganglial neuronal bodies were easily found in the colon specimen of the control group. After 6 wk of diabetes, the PGP9.5-positive cells were markedly decreased compared with the control group. When giving chronic stimulation, comparing with the DM, SEA, and LEA groups, the immunopositive area of PGP9.5 was increased markedly in the HEA group. But no obvious changes were found between the three acute stimulation groups and the DM group.

The protein expression of PGP9.5 was significantly decreased in the DM group compared with the control group in both the proximal (P = 0.004) and distal colon (P = 0.003) and was increased obviously in the chronic HEA group compared with the DM group in the proximal (P = 0.001) and distal colon (P = 0.001) and also had significant difference in the chronic HEA group compared with the chronic LEA (P = 0.006, P = 0.004) and sham stimulation groups (P = 0.004, P = 0.005). However, in the three acute stimulation groups, the protein expression of PGP9.5 had no difference compared with the DM group (all P > 0.05).

Effects of Diabetes and EA Stimulation on nNOS Neurons and CHAT Neurons Protein Expression

Figure 4 shows the immunofluorescence double staining and Western blot results of nNOS-staining neurons and CHAT-staining neurons. Neurons double-labeled with nNOS/PGP9.5 were used to reveal the expression of nNOS neurons in colon tissue. In Fig. 4A, a small number of nNOS/PGP9.5 double-labeled cells could be seen in the DM colon tissue, and much more could be seen in the chronic HEA group than in the DM group. The proportion of enteric cells colabeled with nNOS and PGP9.5 in Fig. 4A were 4.59 ± 0.02% (control group) and
Fig. 3. Effects of different EA stimulation on the protein expression of colon total neurons PGP9.5 (immunohistochemical staining and Western blot). In immunohistochemical staining pictures, tissue with brown granular deposits were positive reaction (as arrows indicated ×400). Statistical analysis indicated that the chronic HEA group increased greater than the diabetic group and other EA groups. \( n = 8 \) for each group *Significantly different, EA group compared with DM group, \( P < 0.05 \). **Significantly different, EA group compared with DM group as \( P < 0.01 \). #Significantly different, HEA group compared with other EA group, \( P < 0.05 \). ##Significantly different, HEA group compared with other EA group, as \( P < 0.01 \).
1.63 ± 0.008% (DM group) and 2.24 ± 0.009% (C-HEA group), respectively. Furthermore, extraction of colon tissue protein for Western blot analysis showed that the protein expression of nNOS was decreased in the DM group compared with the control group \((P = 0.006, P = 0.004)\). The expression of nNOS was increased dramatically in the chronic HEA group compared with the DM group in both proximal and distal colon \((P = 0.001, P = 0.001)\) and also had markedly significant difference compared with the chronic LEA and sham stimulation groups \((P = 0.001, P = 0.002; P = 0.026, P = 0.003)\). However, in the three acute groups, the protein expression of PGP9.5 had no difference compared with the DM group (all \(P > 0.05\)). Figure 4B shows the immunofluorescence double staining and Western blot results of CHAT neurons. The proportion of enteric cells colabeled with CHAT and PGP9.5 in Fig. 4B were 4.72 ± 0.075% (control group) vs. 0.97 ± 0.008% (DM group), and vs. 2.58 ± 0.012% (C-HEA group). The trend observed for increased protein expression of CHAT neurons was matched in nNOS neurons; that is, statistical analysis indicated that the protein levels of CHAT neurons in the chronic HEA group were markedly increased compared with the diabetic group, LEA group, and SEA group \((P = 0.001, P = 0.003, P = 0.001; P = 0.001, P = 0.046, P = 0.027)\). The protein expression of PGP9.5 of the acute
Groups had no difference compared with the DM group (all $P > 0.05$).

**Effects of Diabetes and EA Stimulation on GDNF and PI3K/Akt Pathway**

Figures 5 and 6 show the real-time PCR and Western blot results of GDNF and p-Akt in colon tissue. As could be seen in Fig. 5, both in mRNA and protein levels, the expression of GDNF in the chronic HEA group were significantly increased compared with the DM, LEA, and SEA groups ($P = 0.001$, $P = 0.003$, $P = 0.001$; $P = 0.001$, $P = 0.002$, $P = 0.001$). Also, in the three acute groups, the mRNA and protein expression of GDNF had no significant difference compared with the DM group (all $P > 0.05$). Meanwhile, in Fig. 6, statistical analysis suggested that the protein level of p-Akt has the same downward trend in expression as GDNF, in both proximal and distal colon.

**DISCUSSION**

EA at acupoint ST-36 has been used to alleviate gastrointestinal symptoms and improve gastrointestinal motility (6, 11, 13a, 21, 24), but the EA’s mechanism of action is still unclear.
In the current study, we focus on the effect and the possible mechanism of EA at acupoint ST-36 on the enteric neuronal changes in the colon.

Our results showed that the number of enteric neurons decreased in 6-wk diabetic rats, and furthermore, compared with the other EA stimulation groups or the DM group, the chronic HEA stimulation could induce a more quantitatively significant increase in the mRNA and protein levels of the total enteric neurons and two neuron subtypes, together with GDNF and p-Akt in both the proximal and distal colon.

Previous researchers have discussed the damage caused to the ENS in diabetes models (5). Our previous study also found a clear decrease of PGP9.5, nNOS, and CHAT neurons in the colon of diabetic rats (both in 8- and 12-wk rats) (8). The current study showed the same changes of enteric neurons in diabetic 6-wk models as our previous research. Loss of nNOS-containing and CHAT-containing neurons in diabetes can result in disordered gastric emptying and intestinal transit (20, 25), which could explain the possible mechanism of GI motility disorder in diabetes. The loss of the total neurons is consistent with the former research we did, as well as other research (5, 8).

Acupuncture, one of the essences of Chinese traditional medicine, has more than a 3,000-year history. Acupuncture has already been used for treating functional GI disorders, including functional dyspepsia (24), irritable bowel syndrome (9), chronic constipation (2), and diarrhea (3). As an alternative to traditional acupuncture, EA is more consistent and reproducible, and it has the potential to be an effective method to treat gastrointestinal functional disorders. Few studies have reported the effects of central and peripheral neural pathways in EA ameliorating intestinal motility in rats (11). Some other research on the central nervous system have proven that EA stimulation can promote neural plasticity and induce cell proliferation and differentiation (10, 17). A previous research of our group found that EA could promote colonic contraction, and further research has shown that the effect may be partly due to an improvement of the cholinergic nerve pathways.
As ENS plays an important role in the GI motility, we inferred that enteric neurons in the ENS are also probably affected or changed after the EA stimulation. Indeed, our current study showed that EA at ST-36 rescued the lost enteric neurons in diabetic rats, both the inhibitory nNOS neurons and excitatory CHAT neurons in the colon. This means that EA may take a balance of different subtypes of the enteric neurons to repair the impaired ENS and ameliorate GI motility.

Different EA parameters have different effects on the organism itself and its parts. A report by Iwa et al. (11) showed that low-frequency EA could accelerate the distal colon movement and had no obvious effect on the proximal colon motility. However, a previous study of our group demonstrated that EA with high frequency (100 Hz) at ST-36, not the low-frequency group (10 Hz), accelerated the distal colonic motility in diabetic rats (23). A possible explanation for the differences in colonic motility response to low-frequency EA in different studies may be that rats in the state of disease respond differently to EA, while one possible reason that colonic motility responded differently to low- and high-frequency EA in the study may be that the low-frequency EA is not enough to cause changes in motility. Other researchers have reported that the high-frequency EA can prevent the central neuronal degeneration and protect the damaged neurons, while the low-frequency EA has no such clear effect in the Parkinson’s animal models (12). The different outcomes induced by a different course of EA may imply that there are still some other mechanisms participating in the EA mechanisms, such as gastrointestinal hormone, vagal activity, myoelectrical changes, and so on. On the basis of our study, we conclude that only by giving earlier and chronic EA stimulation at ST-36 to diabetic individuals can we acquire a measure of functional and structural recovery of the GI tract.

GDNF is one of the important members of neurotrophic factor family, and high concentrations of GDNF could be detected in the adult rat colon (16). There is some evidence that shows that GDNF can promote intestinal neuron differentiation, migration, proliferation, and survival (1, 15, 20). PI3K/Akt, the downstream signal pathway of GDNF, is one of the important pathways for the survival of intestinal neurons. Our previous study found that PI3K/Akt plays an important role in enteric neuropathy in diabetes (8). There have been only a few reports about the EA’s effect on GDNF and its downstream signal pathways. The few existing reports mainly focus on research in the central nervous system. For example, one report shows that EA can increase the GDNF expression in dorsal root ganglion and spinal cord dorsal horn (7), while another study shows that EA can restore the damaged brain neurons and increase the expression of brain-derived neurotrophic factor (12). In the current study, we investigated the effect of EA.
on the expression of GDNF and the change of PI3K/Akt pathway in ENS for the first time. We showed that after different time and EA stimulation parameters, high-frequency EA at acupoint ST-36 may induce regeneration of lost enteric neurons in diabetic rats via GDNF and PI3k/Akt signal pathways.

Perspectives and Significance

In this study, EA at ST-36 partly restored the loss of enteric neurons in diabetes, requiring a long duration and higher frequency of stimulation. Furthermore, GDNF and its downstream signal pathway PI3K/Akt may play an important role in this therapeutic process. The present study results, together with our previous animal and human studies, have demonstrated that EA at ST-36 is capable of improving impaired GI dysfunction, accelerating gastric emptying and colonic motility, and restoring the loss of enteric neurons. These findings suggest that EA may have therapeutic potential for GI dysfunction and ENS regeneration. However, as we know, the development of new treatment options has often been hampered by a poor understanding of the underlying mechanisms, so a deeper investigation is still needed on the effect of EA on ENS.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: F.D. conception and design of research; F.D. performed experiments; F.D. analyzed data; F.D. interpreted results of experiments; F.D. prepared figures; F.D. drafted manuscript; S.L. edited and revised manuscript; S.L. approved final version of manuscript.

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