Int J Clin Exp Med 2020;13(4):2879-2887
www.ijcem.com /ISSN:1940-5901/IJCEM0101130

Original Article

Electroacupuncture inhibits apoptosis in anal sphincters of rabbits with neuropathic fecal incontinence

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Received August 19, 2019; Accepted December 3, 2019; Epub April 15, 2020; Published April 30, 2020

Abstract: Objective: To determine whether electroacupuncture (EA) regulates NGF-TrkA signaling during neurogenic fecal incontinence (FI). Methods: A total of 110 rabbits, 10 of which were randomly selected as the control group, were used for this study. Radiofrequency ablation was used to create FI models, and the successfully modeled animals were randomly divided into the following groups: FI model, sacral nerve stimulation (SNS), posterior tibial nerve stimulation (PTNS), and EA (25 per group). Electrical stimulation therapy was started at 2 months, in which control and FI model groups were bundled for 20 min without treatment, while SNS, PTNS, and EA groups were treated (20 min) every other day for 3 months. At the end of treatment, anal sphincters and nerve tissues were collected, and NGF, TrkA, Bax, Bcl-2, p-AKT, and AKT levels were examined by real-time PCR or Western blot. Results: EA induced the expression of NGF, TrkA and Bcl-2, but it reduced the expression of Bax and p-AKT, in the fourth sacral nerves and anal sphincter tissues compared with the FI model group. In addition, EA dramatically decreased cell apoptosis. Compared with SNS or PTNS group, EA group showed significantly elevated expression of NGF, TrkA and Bcl-2, but decreased expression of Bax and p-AKT, which led to inhibition of apoptosis. Conclusion: EA may be beneficial to neurogenic FI by inducing NGF-TrkA expression.

Keywords: Electroacupuncture, NGF, TrkA, fecal incontinence

Introduction

Fecal incontinence (FI) is a pelvic condition and belongs to defecation disorders that are characterized by defects in anorectal control, in which feces and gas cannot be restrained and hence flow out of the anus involuntarily. FI is especially common in elderly people [1], as well as in patients with birth trauma or neuropathy [2]. The physical and mental pain associated with this condition poses serious interference with patients' daily life [3]. Currently, treatments for FI mainly include diet management, medication, nerve stimulation and surgery. However, the efficacy of these approaches remains low [4-6].

Nerve growth factor (NGF) was first identified as a neurotrophic factor [7] that affects nerve growth, maturation, and injury, as well as plasticity of the central nervous system [8]. In addition, NGF protects cells from oxidative stress and cytotoxin-mediated apoptosis [9]. When motor neurons are injured, NGF selectively binds to its specific high-affinity receptor TrkA, which initiates biological effects of NGF on the development, repair and regeneration of the central nervous system [10, 11]. Activation of TrkA receptors and intracellular kinase pathways, including PI3K/AKT and proliferating protein kinase pathways, is important in neuroprotection and neurotrophic factor protection [12].

Electroacupuncture (EA) is a therapy that has been efficiently applied in a variety of diseases, including asthma, rhinitis, inflammatory bowel syndrome, rheumatoid arthritis and chronic pain conditions [13]. EA is modified from traditional Chinese acupuncture. Sacral nerve stimulation (SNS) is mainly used and generally effective in improving continence and quality of life [14-16]. Posterior tibial nerve stimulation (PTNS) has a few important advantages, namely its less invasive approach and lacks the need

for surgical procedures, as well as its economical use. Given these advantages, PTNS has been successfully used as the least invasive form of neuromodulation for overactive bladder, yielding results that are satisfying for idiopathic FI [17-19]. Previous studies have shown that EA markedly improved bowel function and promoted spinal cord repair after injuries [20-22]. However, whether EA can treat neurogenic FI has not been investigated.

This current study aimed to investigate the effects of EA on the expression of NGF and TrkA using FI model in rabbits. In addition, the effects of EA on cell apoptosis in anal sphincters of rabbits with FI were explored. In summary, our results showed that NGF, TrkA, Bax/Bcl-2, and p-AKT/AKT levels were upregulated in the fourth sacral nerves and anal sphincter tissues after EA administration, suggesting potential therapeutic approaches towards FI.

Materials and methods

Animals

In total, 110 male New Zealand rabbits (8 months) were provided by China Academy of Chinese Medical Sciences Guang'anmen Hospital Experimental Animal Center.

FI model

Animals were slowly, intravenously anesthetized with 3% sodium pentobarbital at 1 ml/kg. After routine disinfection, Kimberly-Clark-dedicated puncture needles was used for puncture according to the anatomical site of the fourth sacral nerve puncture. Radiofrequency (RF) needle cores were replaced after placement, and current was increased until the experimental rabbit's anus had twitching, contraction, and we repeatedly adjusted the direction of the puncture needle and depth. RF energy transmission began when the observed current was between 0.6 mA and 1.0 mA (temperature setting: 85°C and time setting: 90 sec). After the RF needle temperature dropped below 40°C, the RF energy was transmitted again. Postoperatively, animals were intraperitoneally injected with 200,000 units of penicillin sodium every 12 h for three days. The anal pressure was measured 14 days after the procedure. If it was less than 15 mmHg, the model was established successfully.

Group

All animals were fed standard diets for 1 week and individually identified. There were 10 rabbits that were randomly selected as the control group. Radiofrequency ablation was used to create the FI model. Two weeks after modeling, the animals with successful FI were randomly divided into the following groups: model group, SNS group (electrical stimulation of the sacral nerves on the S3-S4 level), PTNS group (electrical stimulation of the posterior tibial nerve originates in the spinal segments L4 to S2), and EA group [EA stimulation of "Zhongliao" (BL33), "Sanyinjiao" (SP6) and "Zusanli" (ST36)] (25 per group). Bundle of electrical stimulation therapy was started at 2 months, in which control and FI model groups were only bundled for 20 min without treatment, whereas SNS, PTNS and EA treatment groups were treated every other day for 3 months, with each treatment lasting 20 min.

Anal sphincter and nerve tissue collection

The external anal sphincters, fourth sacral nerve root, and surrounding nerve tissue were dissected and collected. Tissues were washed with saline, placed in a cryovial, and then stored at -80°C for Weston blot analysis. H&E and lead uranium staining were performed using the fixed tissues to examine morphology and cell ultrastructure under optical microscope or projection electron microscope.

Real-time PCR analysis

RNA was isolated from the fourth sacral nerves and anal sphincters using Trizol reagent (Invitrogen). cDNA was synthesized using SYBR Green dye (Thermo) and the following primer sequences: Oryctolagus cuniculus nerve growth factor (NGF), mRNA (5'-CAAGGGCAATGAGGTGAAGG-3', 5'-CTGTGTCGATGCGGATGAAG-3'), Oryctolagus cuniculus neurotrophic receptor tyrosine kinase 1 (TrkA), mRNA (5'-CAACAGCACCTCTGGAGAC-3', 5'-GCCTTTGCCATCAGTAGGG-3'), Oryctolagus cuniculus glyceraldehyde-3-phosphate dehydrogenase (GAPDH), mRNA (5'-GTCGGAGTGAACGGATTTG-3', 5'-ATCTCGCTCCTGGAAGATG-3'). Samples were analyzed using 7000 Sequence Detection System hardware and software (Applied Biosystems).

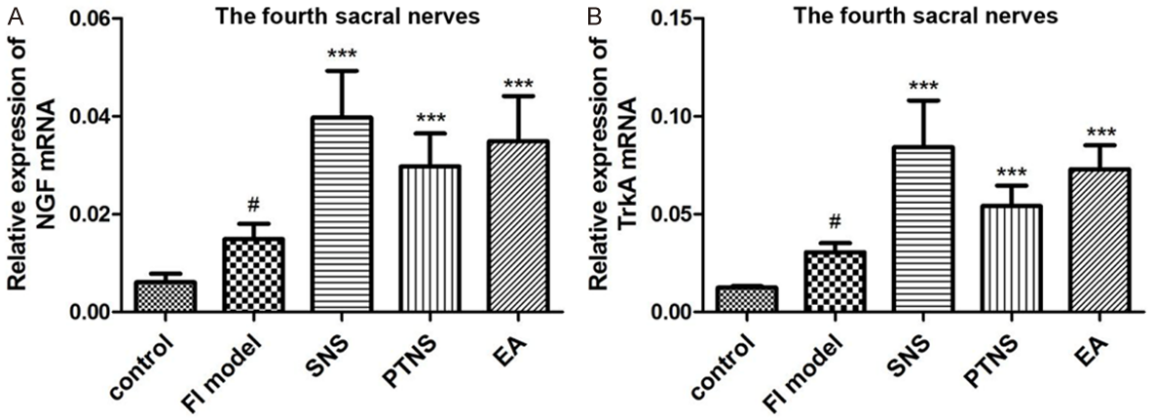

Figure 1. Real-Time PCR (RT-PCR) analyses of NGF and TrkA mRNAs in the fourth sacral nerves. A. NGF mRNA relative expression in each group (n=8 per group). B. TrkA mRNA relative expression in each group (n=8 per group). $^{\#}P<0.05$ compared with control group; $^{***}P<0.001$ compared with FI model group. FI, fecal incontinence. EA, electroacupuncture.

Weston blot analysis

Tissues were homogenized in sample buffer (20 mM Tris-acetate, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, pH 7.5) and centrifuged at 10,000×g for 25 min at 4°C. Supernatants were collected for Western blotting and ELISA. Total protein (30 μg) from each sample was dissolved with a loading buffer (0.1 M Tris-HCl buffer, pH 6.8, containing 0.2 M DTT, 4% SDS, 20% glycerol, and 0.1% bromophenol blue), separated by 12.5% SDS-PAGE, and transferred to polyvinylidene fluoride (PVDF) membranes overnight. Membranes were incubated overnight at 4°C with the following primary antibodies: anti-TrkA (1:1000, Abcam, Cambridge, MA, USA), anti-AKT (1:500, Bioss, Atlanta, GA, USA), anti-p-AKT (1:1000, Abcam), anti-NGF (1:1000, Abcam), anti-Bax (1:2000, Proteintech Group, Inc., Rosemont, IL, USA), anti-Bcl-2 (1:2000, proteintech), and anti-GAPDH (1:2000, Cell Signaling Technology, Danvers, MA, USA). Blots were developed with an enhanced chemiluminescent (ECL) detection kit (Amersham Bioscience UK limited, England). The optical density of GAPDH bands was used as an internal control for difference in sample loading.

Transferase dUTP nick end labeling (TUNEL) staining

Anal sphincter tissues cells were plated onto chamber slides at a density of 5×10^4 cells per chamber. TUNEL assay was performed using Apoptosis Detection Kit.

Statistical analysis

Data were expressed as mean ± SEM. Data between multiple groups were compared by GraphPad Prism software Version 6.0 (San Diego, CA, USA) with one-way ANOVA followed by post-hoc Tukey's test.

Results

NGF and TrkA mRNA was upregulated during FI and after EA administration

To determine the roles of NGF and TrkA in neuropathic FI, we analyzed the changes in NGF and TrkA mRNA expression in the fourth sacral nerves and anal sphincter tissues by real-time PCR. Interestingly, the mRNA expression of NGF in the FI model group was dramatically increased compared to the control group ($P<0.05$). In SNS, PTNS and EA groups, NGF mRNA expression was elevated compared with the FI model group ($P<0.001$, $P<0.001$, $P=0.001$, respectively) (**Figure 1A**). Consistent with this, TrkA mRNA expression in the FI model group was upregulated ($P<0.05$). Likewise, TrkA mRNA expression was increased in the SNS, PTNS and EA groups compared with the FI model group ($P=0.001$, $P<0.01$, $P<0.001$, respectively) (**Figure 1B**). Similar patterns were observed in anal sphincters. NGF displayed higher expression in the FI model group compared to the control group ($P<0.01$), while its expression increased in the SNS, PTNS and EA groups compared to the FI model group ($P=$

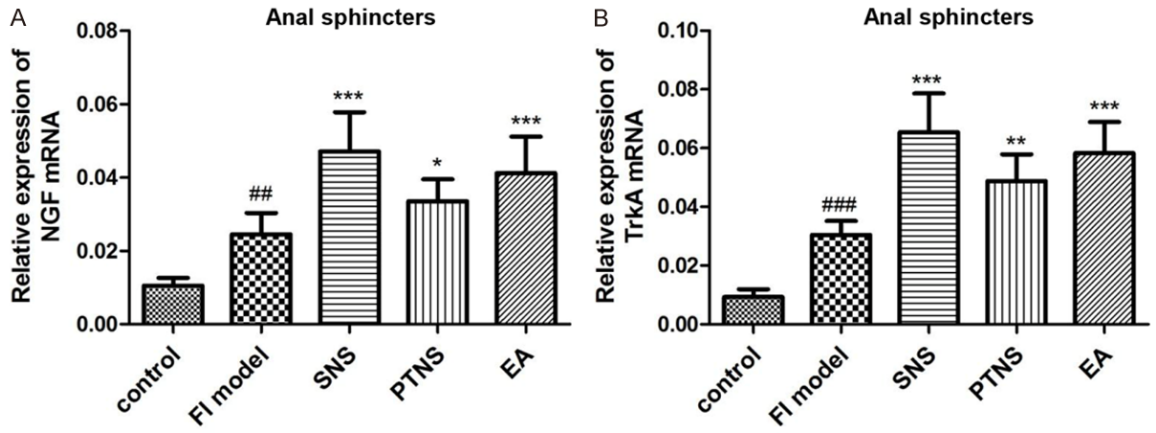

Figure 2. Real-Time PCR (RT-PCR) analyses of NGF mRNA and TrkA mRNAs in anal sphincters. A. NGF mRNA relative expression in each group (n=8 per group). B. TrkA mRNA relative expression in each group (n=8 per group). $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ compared with control group; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared with FI model group. FI, fecal incontinence. EA, electroacupuncture.

0.001, $P<0.05$, $P<0.001$, respectively) (**Figure 2A**). Analogously, TrkA mRNA expression in the FI model group was increased compared with the control group ($P<0.001$). Moreover, TrkA mRNA was upregulated in the SNS, PTNS and EA groups compared with the FI model group. ($P<0.001$, $P<0.01$, $P=0.001$, respectively) (**Figure 2B**). These results suggested that NGF and TrkA mRNAs were induced in the FI model.

NGF and TrkA were upregulated at the translational level during FI and after EA administration

We wondered whether the protein levels of NGF and TrkA displayed similar changes. NGF expression in the fourth sacral nerves was drastically increased in the FI model group compared with the control group ($P<0.05$) (**Figure 3A** and **3B**). Likewise, NGF expression was markedly increased in the SNS, PTNS and EA groups compared with the FI model group ($P<0.001$, $P<0.01$, $P<0.001$, respectively) (**Figure 3A** and **3B**). Consistently, TrkA relative expression in the FI model group was elevated compared with the control group ($P<0.001$), as well as in the SNS, PTNS and EA groups compared with the FI model group ($P<0.001$, all) (**Figure 3A** and **3C**). On the other hand, p-AKT/AKT and Bax/Bcl-2 ratios in SNS, PTNS and EA groups were reduced compared with the FI model group (**Figure 3A**, **3D**, **3E**). Together, these results suggested that apoptosis was inhibited in SNS, PTNS and EA groups. Similar patterns were observed in anal sphincters. NGF expression in the FI model group was dramatically induced compared with the control group ($P<0.01$), as well as in the SNS and EA groups compared with the FI model group ($P<0.01$, $P=0.001$, respectively). However, there was no significant difference in NGF expression between the FI model group and PTNS group (**Figure 4A** and **4B**). Consistent with this, TrkA relative expression was elevated in the FI model group compared with the control group ($P=0.01$), as well as and in the SNS and EA groups compared with the FI model group ($P=0.005$, $P=0.009$, respectively), but there was no significant difference between FI model group and PTNS group (**Figure 4A** and **4C**). Furthermore, p-AKT/AKT and Bax/Bcl-2 ratios in the SNS, PTNS and EA groups were decreased compared with the FI model group (**Figure 4A**, **4D**, **4E**). These results suggested that EA strongly induced NGF and TrkA expression, exhibiting the most dramatic increase in all groups.

Anal sphincter apoptosis

Since we observed changes in Bax/Bcl-2 ratio, we wondered whether apoptosis was affected during these administrations. To further examine the apoptotic changes at histological and cellular levels, we performed TUNEL staining in the anal sphincter tissues (**Figure 5A**). Consistent with results from Western blots, TUNEL staining showed increased apoptotic rates in the FI model group compared with the control group ($P=0.001$). On the other hand, SNS, PTNS

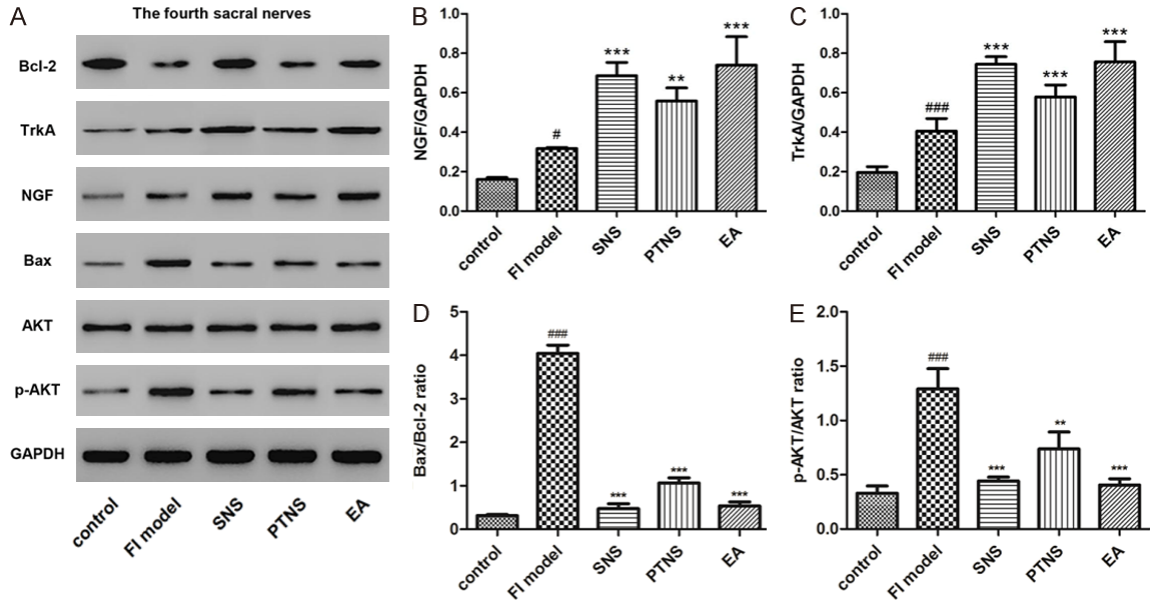

Figure 3. NGF and TrkA protein levels were determined by Western blot in the fourth sacral nerves. A. Western blot analyses of NGF and TrkA protein. B. NGF relative expression in each group (n=3 per group). C. TrkA relative expression in each group (n=3 per group). D. Bax/Bcl-2 expression ratio in each group (n=3 per group). E. p-AKT/AKT expression ratio in each group (n=3 per group). $^{\#}P<0.05$, $^{\#\#\#}P<0.001$ compared with control group, $^{**}P<0.01$, $^{***}P<0.001$ compared with FI model group. FI, fecal incontinence. EA, electroacupuncture.

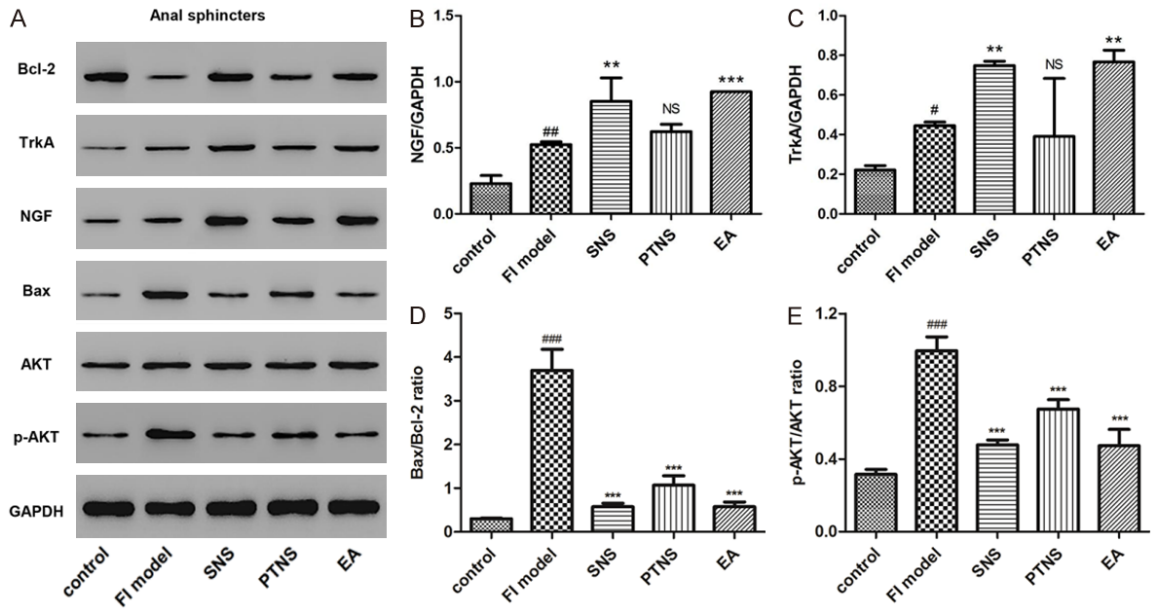

Figure 4. NGF and TrkA relative protein levels were determined by Western blot in anal sphincters. A. Western blot analyses detecting NGF and TrkA protein. B. NGF relative expression in each group (n=3 per group). C. TrkA relative expression in each group (n=3 per group). D. Bax/Bcl-2 expression ratio in each group (n=3 per group). E. p-AKT/AKT expression ratio in each group (n=3 per group). NS, non-significant; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ compared with control group; $^{**}P<0.01$, $^{***}P<0.001$ compared with FI model group. FI, fecal incontinence. EA, electroacupuncture.

and EA groups exhibited reduced apoptotic rates compared with the FI model group (P=0.016, P=0.044, P=0.007, respectively) (**Figure 5B**). The EA group displayed the least apop-

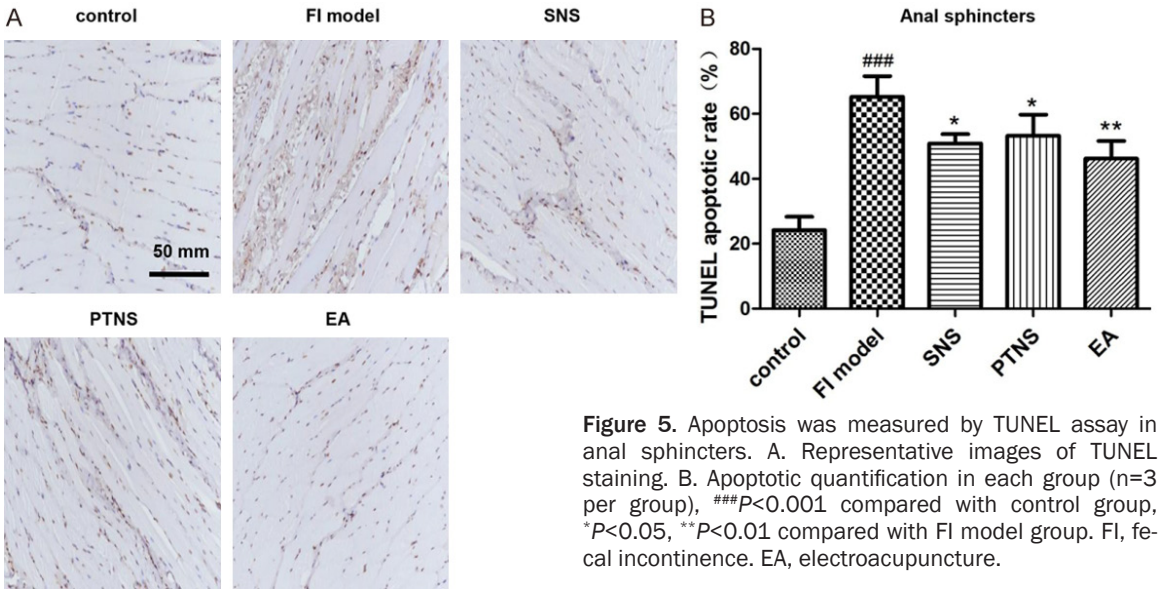

Figure 5. Apoptosis was measured by TUNEL assay in anal sphincters. A. Representative images of TUNEL staining. B. Apoptotic quantification in each group (n=3 per group), ###P<0.001 compared with control group, *P<0.05, **P<0.01 compared with FI model group. FI, fecal incontinence. EA, electroacupuncture.

tosis among these groups, suggesting efficiency of EA in preventing apoptosis *in vivo*.

Discussion

In the current study, we found that EA upregulated NGF and TrkA expression, which can potentially be applied in future clinical interventions. Neuropathic FI refers to a decrease in the ability to control bowel movements due to functional deterioration or lesions of the nervous system. The clinical pathological changes are mainly due to weakened autonomic control of the puborectalis and external sphincters, decrease in the ability of the descending colon, and relative increase of the anal internal sphincter tension. The rectal mucosa is unable to sense swelling, resulting in the inability to proceed with intention or defecation. This in turn leads to anal sphincter expansion and relaxation. Surgery to repair external sphincter and uborectalis is a major clinical treatment, and can improve the excitatory reflex of the anal canal and rectum, increase rectal capacity and alleviate incontinence symptoms [23]. Nonetheless, there is a pressing need for less invasive approaches.

Several strategies have been proposed towards FI [24], but the management algorithm and trends continue to change as new options for efficacy, safety, simplicity, and affordability emerge. SNS and PTNS serve as alternative treatments. SNS has been recognized as an effective treatment for FI in several single and multicenter studies including those with long-term follow-up [25, 26]. Indirect modulation of the pelvic nerves using PTNS has the advantages of being even less invasive, almost complication-free, and potentially more cost-effective [27-29]. EA uses the EA instrument to output pulse current, and acts on the human body “acupoint” and “meridian” through the needle to achieve the purpose of treating diseases. EA, in which a galvanic method of stimulation replaces manual manipulation, has been widely used in clinical and research fields. Previous studies have suggested that EA can markedly improve bowel function [21, 22, 30-32], but whether it can be used to treat FI is unexplored. We established the novel EA parameters, and the acupuncture points were composed of three points: “Zhongliao”, “Sanyinjiao” and “Zusanli”.

Kitagawa *et al*. previously reported that neurotrophic factors may play a protective role in brain ischemic injury [33]. NGF is a key member of the neurotrophic factor family and promotes nerve growth, maturation, and injury. When motor neurons are damaged, NGF selectively binds to tyrosine kinase receptor A (TrkA), a high-affinity receptor, to initiate its biological effects. Previous research [34] reported that daily usage of low-frequency EA for 11 days to RCS rats during a critical developmental stage of retinal cell degeneration caused an increase of retinal NGF and TrkA expression. In 2007, Li

and his colleagues reported that EA treatment could upregulate TrkA expression after focal cerebral ischemia in rats and EA therapy clearly decreased the high expression of TRPM7 induced by ischemia [35]. In our study, we found that the expression of NGF and TrkA was increased in the model group. Consistent with these observations, we found that NGF and TrkA were higher in the treatment group, and the EA group had the most significant difference compared with the model group.

Cell survival is regulated by a balance between death and survival. Previous studies have revealed that the AKT signaling pathway is involved in the regulation of cell growth, proliferation, death and survival [36]. In addition, AKT has also been found to be involved in NGF-induced inhibition of apoptosis [37]. Our TUNEL staining analysis of the pathological changes of the anal sphincters revealed increased cell apoptosis in anal sphincters in the FI model group. Consistently, the ratios of p-AKT/AKT and Bax/Bcl-2 expression were also increased in the fourth sacral nerves and anal sphincters in the FI model group. On the other hand, the three treatment groups (SNS, PTNS, and EA group) showed reduced apoptosis, as well as p-AKT/AKT and Bax/Bcl-2 levels. Among the three, EA was most effective. Taken together, these results suggest potentially beneficial effects of EA treatment, and that the mechanism by which EA might exert its action on likely targets through regulation of NGF and its TrkA receptors in the fourth sacral nerves and anal sphincters. To our knowledge, this was the first time that NGF and TrkA were examined thoroughly in neuropathic FI. Our findings, hence, may provide potential therapeutic target for future clinical strategies.

Conclusions

In summary, our current study reveals that EA can stimulate the expression of NGF and TrkA, and inhibit apoptosis in anal sphincter tissues. The clinical efficacy of EA is better than SNS and PTNS *in vivo*. We provide compelling data that EA can be utilized to treat FI in clinics.

Acknowledgements

This work was sponsored by Beijing Natural Science Foundation (7162166) and National Natural Science Foundation of China (General Program; 81673980).

Disclosure of conflict of interest

None.

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