Int J Clin Exp Med 2019;12(3):2427-2434
www.ijcem.com /ISSN:1940-5901/IJCEM0088906

Original Article

Biological effects of electrical stimulation on pelvic floor muscle strength and neuropeptide Y expression

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Received November 25, 2018; Accepted January 8, 2019; Epub March 15, 2019; Published March 30, 2019

Abstract: Objective: This study aimed to evaluate neuropeptide Y (NPY) expression and pelvic floor muscle strength of stress urinary incontinence (SUI) and diabetes rats before and after ES (electrical stimulation), and to compare the neuromuscular morphology and pathological features. Materials and Methods: Animal models of SUI or diabetes were induced by vaginal balloon dilation, or high-fat high-energy diet plus STZ injection respectively. The strength of pubococcygeal muscles were measured utilizing a biomechanical system, NPY expression was detected by ELSIA assay and western blotting. The histology of pelvic floor muscles was analyzed by H&E staining. Results: Stationary contractility and forced contractility of pelvic floor muscles in rats of SUI or diabetes were significantly increased after ES treatment. The NPY concentration in plasm of SUI or diabetes groups were largely promoted, and increased expression of NPY amount was correlated with enhanced muscle contractility before and after ES treatment. The pubococcygeal muscle morphology of SUI or diabetes animal models has appeared similar myogenic neurogenic performance, which may be good simulated the etiology of pelvic floor dysfunction. Conclusions: The present study has shown ES increases the release of NPY as a neurotransmitter, which reflects the recovery of the damaged nerve to increase the muscle strength. ES may be an ideal physiotherapy for SUI patients in clinical.

Kewords: Stress urinary incontinence, SUI, electrical stimulation, ES, pelvic floor muscles, neuropeptide Y, NPY, muscle strength

Introduction

Pelvic floor dysfunction (PFD) is a position and function disorder of pelvic organs that has many different causes [1]. Patients who have PFD usually exhibit various clinical symptoms, such as pelvic organ prolapse (POP), chronic pelvic pain, and stress urinary incontinence (SUI) [2]. SUI is the complaint of involuntary urine loss on effort or exertion such as coughing or sneezing, and it is frequently diagnosed and recognized as the most common type of UI in women [3]. It has been reported that nearly 30 billion US dollars are spent each year on the clinical therapy of SUI, which is much more than total cost for breast, ovarian, and cervical cancer [4].

Although reasons for SUI are currently unknown, more and more research has been focused on the supporting system changes of the pelvic floor, especially dysfunction of pelvic muscles. Therefore, physiotherapies targeted to the recovery of pelvic muscles have become part of the clinical management of SUI and have achieved outstanding effects [5]. Specifically, electrical stimulation (ES), one of the most common interventions used by physiotherapists, manages to produce muscle hypertrophy, normalize the reflex activity of the lower urinary tract, and increase circulation to muscles or the capillary system [6].

Previously, an animal model of SUI was established by using vaginal balloon dilation, and dysregulation of transforming growth factor (TGF)/Smad signaling pathway was found to possibly play an important role in the pathogenesis of SUI. Expression of TGF-β receptor II (TβR-2) and Smad7 protein was significantly increased [7]. As for the current study, postpartum rats with SUI as well as rats with diabetes were used, because both of these groups have typical SUI symptoms. ES treatment was used

Table 1. Results of the sneezing experiment in the SUI animal model

Groups	Number	Sneezing Experiment	
		Positive (+)	Negative (-)
Control	5	0	5
SUI	10	10	0

to test whether it can increase pelvic muscle strength and resolve the dysfunction. To assess its effects, a neurotransmitter of neuropeptide Y (NPY) amount was detected by enzyme-linked immunosorbent assay (ELISA) and Western blotting and the histological characteristics of pelvic muscle tissue were analyzed.

Materials and methods

Establishment of SUI and diabetes in animal models

Eight-week-old healthy female Wistar rats were provided by the experimental animal center of Guangdong province, and randomized into three groups: the control group (n=5), the SUI group (n=10), and the diabetes group (n=10). Rats in the control group were non-pregnant and without any treatment, rats in the SUI group underwent normal parturition plus post-partum vaginal balloon dilation for 6 hours, and rats in the type 2 diabetes group were developed with high-fat high-energy diets plus Streptozocin (STZ, Sigma, USA) injections. Vaginal balloon dilation and STZ injections were performed as previously described [7]. Ethics were approved by the Institutional Animal Care and Ethics Committee of Guangzhou Medical University.

Evaluation of SUI and diabetes in animal models

The SUI and diabetes animal models were evaluated by sneezing experiment and glucose test, respectively. For the sneezing experiment, rats of the SUI group were intraperitoneally anesthetized with 2% pentobarbital sodium (0.2 mL/100 g), and the bladder was emptied by an epidural catheter. The maximum bladder capacity was measured by filling the bladder with methylene blue (Jili, Jiangsu, China), dissolved in sterile saline, until the first drop of urine leakage outside the external orifice of urethra was observed. Then, the bladder was emptied again and filled with a volume of methylene blue solution equaling half of the maximum bladder capacity. The sneezing reflex was induced by inserting a piece of severed rat's beard into the nostril to increase abdomen pressure. If any amount of methylene blue outflow was observed from the external meatus, the sneezing experiment was considered positive. For evaluating the diabetes animal model, the body weight and blood glucose were examined after 1 month on the high-fat, high-energy diet and injections. Only rats with typical symptoms of diabetes were considered to be induced with type 2 diabetes. Glucose standards of type 2 diabetes in the present study were set as fasting blood-glucose >11.1 mmol/L or postprandial blood sugar >16.7 mmol/L.

Electrical stimulation as the normal treatment

All rats in the control, SUI, and diabetes groups were given ES for the pubococcygeal muscles. ES was performed with the MyoBravo electro stimulation instrument (MTR+Vertiebs GmbH, Berlin). A vaginal probe was inserted, and a medium-frequency (25 Hz) alternating current was administered for stimulation with a duty cycle of 1, 3, 6, 12, and 30 times. The interval was 60 seconds, and total cycle time was 343 second. Each rat received a 30 minute session every 2 days, and the strength of pelvic floor muscles was assessed at baseline and after the completion of the experimental sessions (7 days).

Strength measurement of the pubococcygeal muscles

The pubococcygeal muscles of all rats were dissected on day 7 and attached to a tension transducer by using the non-plastic thread (10 cm). Muscle strength was measured by using a biomechanical test system (BL-420, Chengdu, China). Briefly, initial force was set as 1 gram and stabilized for 3 seconds, and then ES (25 Hz, 50 ms, 3.6 V) of the pubococcygeal muscles was performed for 3 seconds. Changes of muscle strength were recorded as results of stationary contractility. After 3 min of rest, the thread was extended to 10.5 cm, and ES of pubococcygeal muscles was performed immediately. Changes of muscle strength were recorded as results of forced contractility.

Table 2. Results of the glucose test in the diabetes animal model

Groups	Number	Before (mmol/L)	After (mmol/L)		
			1 month	2 months	3 months
Control	5	3.58±0.54	3.60±0.67	3.72±0.48	3.84±0.57
Diabetes	6	3.47±0.59	21.3±1.82	16.8±1.26	23.3±0.79

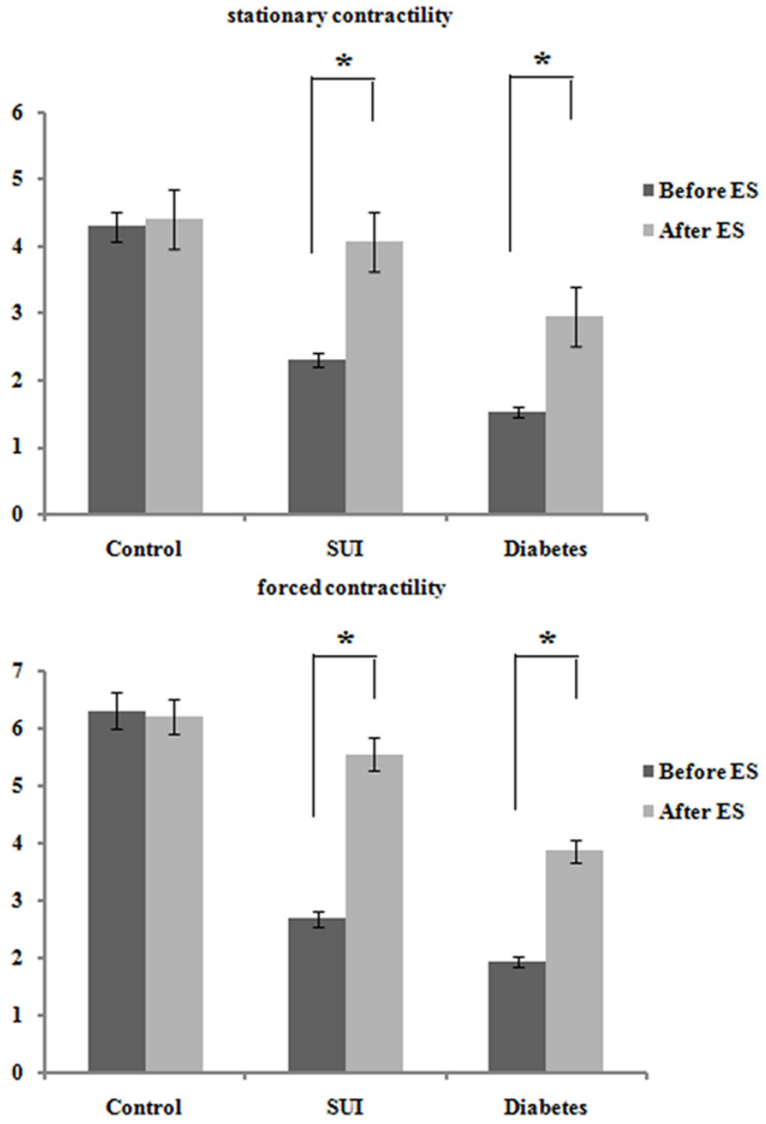

Figure 1. ES enhanced the strength of pelvic floor muscles in rats of the SUI and diabetes groups. Both stationary contractility and forced contractility of pelvic floor muscles in rats of the SUI and diabetes groups were significantly enhanced after ES treatment (*, $p<0.05$).

Enzyme-linked immunosorbent assay (ELISA)

Peripheral blood was collected from all rats through the abdominal aorta, stabilized for 2 hours, and then centrifuged for 15 minutes (3000 rpm) before and after ES treatment. Supernatant was obtained and used for NPY measurement. Ninety-six well plates were used, and NPY production was detected by using an enzyme-linked immunosorbent assay (ELISA) kits (CSB-E13431r, Wuhan, China) following the manufacturer's protocol.

Western blot analysis

The pubococcygeal muscle tissue was collected from rats in the diabetes group before and after ES treatment, homogenized, and lysed in radio immune-precipitation assay (RIPA, Bio-teke Corporation, China) buffer with 1 mM phenylmethanesulfonyl fluoride (PMSF, Bio-teke Corporation, China). The protein concentration was measured using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Forty micrograms of each protein was separated on 10% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) and then transferred to cellulose acetate membranes (Millipore, MA, USA). The membranes were blocked with 5% non-fat dry milk in Tween/Tris-buffered solution (TTBS) for 2 hours at room temperature, followed by incubation with rabbit anti-NPY (1:500, Abcam, USA) and rabbit anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:5000, Abcam, USA) antibodies overnight at 4°C. The membranes were washed with TBST three times and incubated with HRP-conjugated anti-rabbit IgG (1:5000, Jackson, USA) for 1 hour at room temperature. Proteins were detected by enhanced chemiluminescence (Thermo Scientific, MA, USA) and analyzed by Image J software.

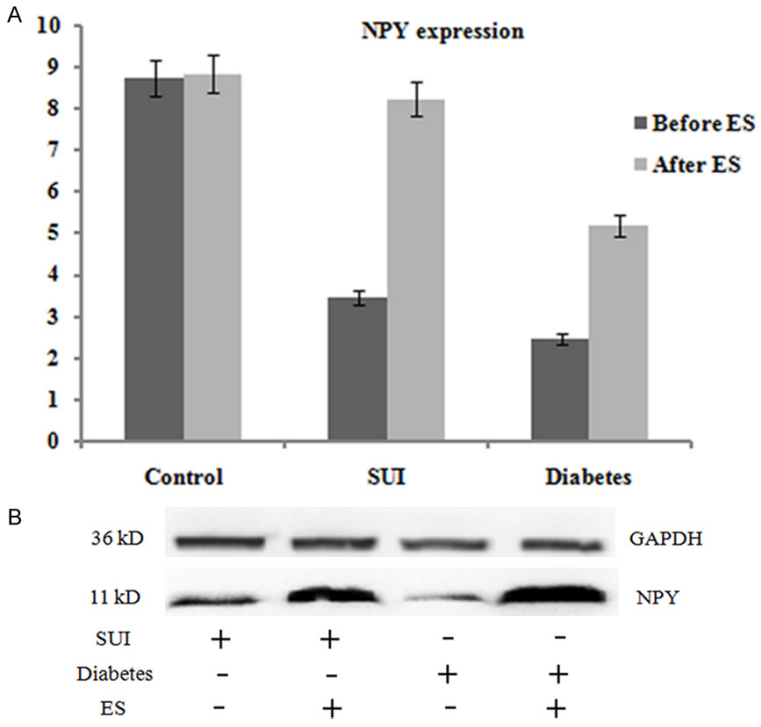

Figure 2. ES increased the amount of NPY expression in rats of SUI and diabetes groups. The NPY concentration in the supernatant of peripheral blood from rats in the SUI or diabetes groups was significantly increased after ES (A, *, $p<0.05$). Western blotting further confirmed the increased amount of NPY at the protein level (B).

Histological analysis of pubococcygeal muscles

Pelvic floor muscle tissue was harvested from rats in the diabetes group before and after ES treatment, fixed in 4% paraformaldehyde (PFA, Sigma, USA), embedded in paraffin wax, and cut into 5 μm thick sections. Serial sections were dewaxed twice in Xylene (10 minutes), and rehydrated with 100%, 90%, and 70% ethanol, respectively (5 minutes). After being rinsed with distilled water, sections were stained by Harris hematoxylin (5 minutes, Sigma, USA), differentiated with 1% HCl in 70% ethanol for one to two dips, and washed in running tap water for 15 minutes. Afterwards, sections were stained with alcoholic eosin (Sigma, USA) solution for 1 minute, dehydrated with 70%, 90%, and 100% ethanol respectively (5 minutes), and cleared twice with Xylene (10 minutes). Finally, all sections were mounted with mounting medium and microscope cover slips. Staining results were observed and assessed by two separate pathologists.

Statistical analysis

Data analysis was performed using SAS program (SAS version 8.1, USA). Student's t test was used to compare the two means. One-way analysis of variances (ANOVA) was used to compare more than two means. A p value of less than 0.05 was regarded as significant.

Results

Establishment of SUI and diabetes in animal models

According to the results of the sneezing experiment, all 10 rats in the SUI group were verified as having SUI (**Table 1**). As for the diabetes group, 6 of 10 rats were recognized as having type 2 diabetes based on the 3 months of fasting blood-glucose test, which were significantly more than that of control group (**Table 2**). Four other rats died due to increased blood glucose.

ES enhanced the strength of pelvic floor muscles in rats of both SUI and diabetes groups

Both stationary contractility and forced contractility of pelvic floor muscles in rats of the SUI and diabetes groups were significantly increased after ES ($p<0.05$, **Figure 1**). No significant differences of stationary or forced contractility were found in the control group before and after ES treatment ($p>0.05$, **Figure 1**).

ES increased the NPY expression amount in rats of SUI and diabetes groups

The NPY concentration in supernatant of peripheral blood from rats in both the SUI and diabetes groups was significantly enhanced after ES ($p<0.05$, **Figure 2A**). No significant differences of NPY expression amount were found in the control group before and after ES ($p>0.05$, **Figure 2A**). Results of Western blot an-

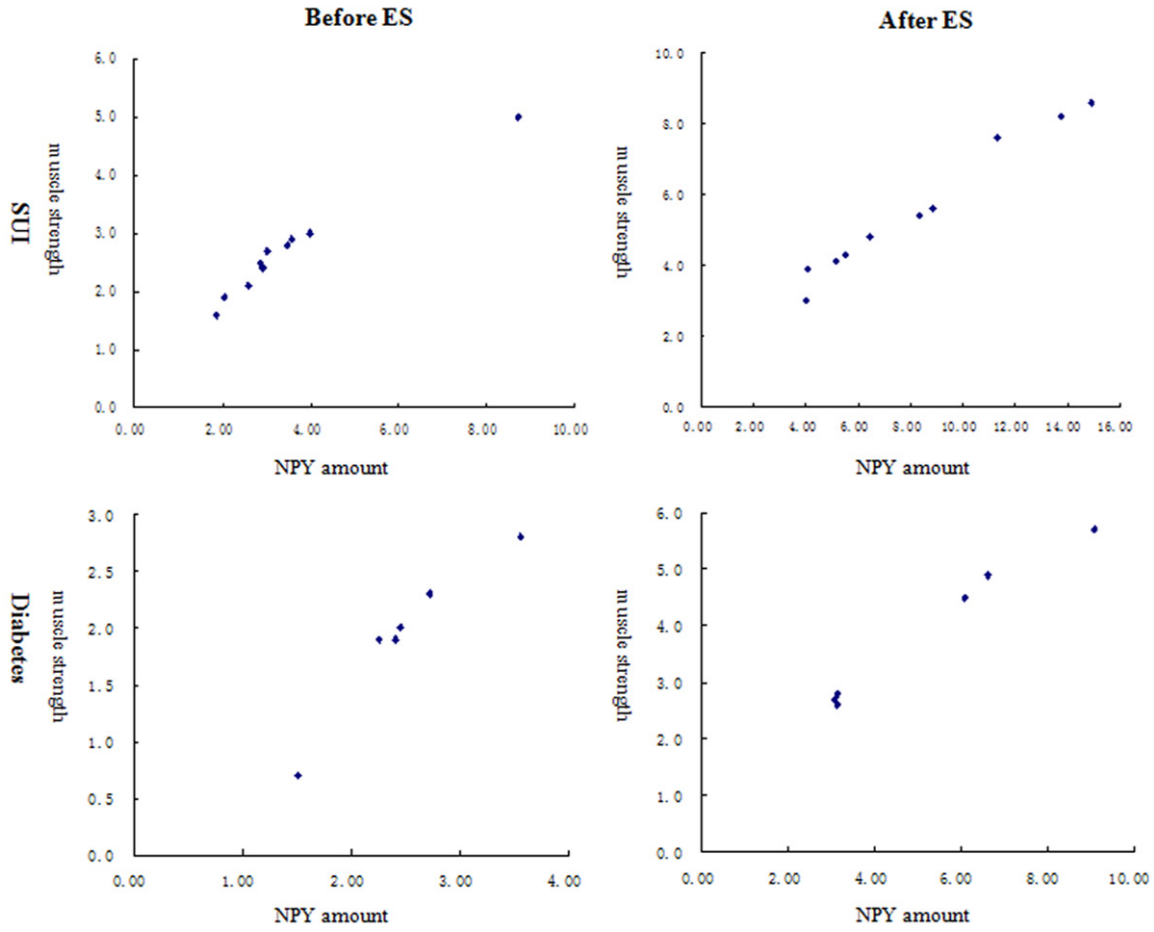

Figure 3. Increased NPY was correlated with enhanced strength of pelvic floor muscles before and after ES treatment (Pearson r=0.98, $p<0.05$).

alysis further confirmed the increased amount of NPY protein after ES treatment (**Figure 2B**). In both the SUI and diabetes groups, the expression amount of NPY correlated with muscle contractility before and after ES ($p<0.05$, **Figure 3**).

Histological characteristics of pelvic floor muscles before and after ES treatment

Histology of pelvic floor muscle tissue before and after ES treatment was assessed by hematoxylin and eosin (H&E) staining. The neurons and muscle fibers showed ischemic changes in the SUI group before ES. The target fibers displayed pathological changes. Muscle fibers were swollen, and the cytoplasm was lightly stained. The striations were not prominent. Most of muscle fibers broke down, with nuclei entering into sarcolemmas (**Figure 4**). A great number of inflammatory cells were also found to be infiltrated into the muscle fibers. Neural changes included the swelling neuronal cells, condensed nuclei with marked hyperchromatism, and the dissolution of Nissl bodies (**Figure 5**). After ES, pathological changes were not observed in the target fibers. Muscle bundles had integrated structures with a pink appearance, and were characterized by periodic striations of alternating dark and light bands of the myofibrils. Few neurons showed swelling, and there was partial dissolution of Nissl bodies.

The nucleus number under sarcolemma of most muscle fibers had increased significantly in the diabetes group before ES (**Figure 4**). Muscle fibers showed chain changes, which were similar with myogenic neurogenic changes. Neural changes included the congestion of blood vessels and the swelling of endothelial

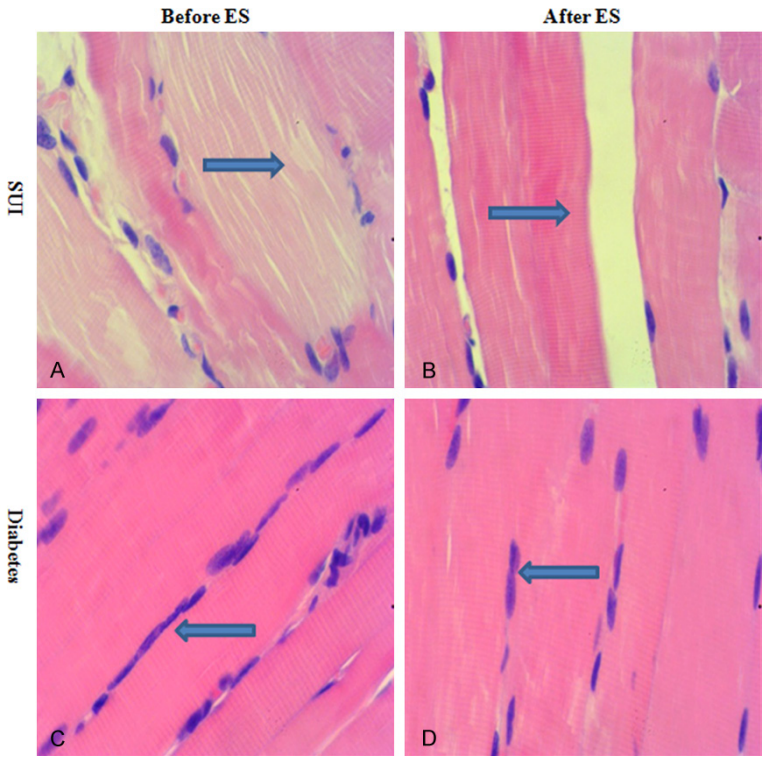

Figure 4. Histological characteristics of pelvic floor muscles before and after ES treatment. For SUI rats before ES, muscle fibers were swollen and the cytoplasm was lightly stained (arrow, A). The striations were not prominent. After ES, no pathological changes were observed in the target fibers. Muscle bundles had integrated structures with a pink appearance (arrow, B). For diabetes rats, muscle fibers showed chain changes, similar to myogenic neurogenic changes (arrow, C). After ES, the nucleus numbers under sarcolemma had significantly increased in partial muscle fibers (arrow, D).

cells (**Figure 5**). After ES, the nucleus numbers under sarcolemma had significantly increased in partial muscle fibers. Slightly swelling endothelial cells were found to be embedded in the endo-neural membrane, and the disarrangement of neuron fibers was observed less frequently.

Discussion

There is a growing need to understand how physiotherapies impact SUI symptoms, impairments, and functional limitations. The target of physiotherapies is the pelvic floor muscles. The pelvic floor consists of striated muscles arranged in a dome-shaped sheet, which is usually described as a sling [8]. These muscles are referred to as lying within either the deep or superficial pelvic floor. Although it is infrequently discussed, the pelvic floor has an important role in the function of core muscle stabilization. The core muscles are known as muscles of the trunk, such as the abdominals, quadrates lumborum, spinal muscles of multifidus, hip muscles, the diaphragm, and pubococcygeal muscles [9]. In essence, the pelvic floor muscles are the floor of the core. In the present study, contractility of pelvic floor muscles was found in rats of the SUI group and the diabetes group was significantly enhanced after ES treatment, which indicates its prominent effects. The physiological objectives of ES are to produce muscle hypertrophy and increase circulation to muscles. It may increase conscious awareness of muscles to produce improved ability to perform voluntary muscle contraction [10].

Interestingly, the enhanced contractility of pelvic floor muscles follows with the increased expression of NPY, which suggests its potential value to assess the effects of ES treatment. NPY, a 36-amino-acid neuropeptide, is involved in the regulation of blood flow and is commonly found among the nerve fibers [11, 12]. NPY is always synthesized in the neuron and then transported to the vaginal wall through the nerve axon; therefore, NPY is one of the most abundant neuropeptides observed underlying the vaginal epithelium [13]. For example, the staining of NPY protein was found in the anterior vaginal wall tissue of healthy women by Hu et al [14]. In menopausal patients with POP, as the symptoms intensify, NPY expression decreases progressively. Results of ELISA and Western blotting in the present study showed not only the NPY concentration but also its protein amount from rats of SUI and diabetes groups significantly increased after ES, and expression of NPY was correlated with muscle contractility. Based on these results, the increased amount of NPY in the pelvic floor muscles is proposed to be related to nerve recovery or regeneration, resulting in a change in blood flow, atrophy, and pelvic floor laxity [15].

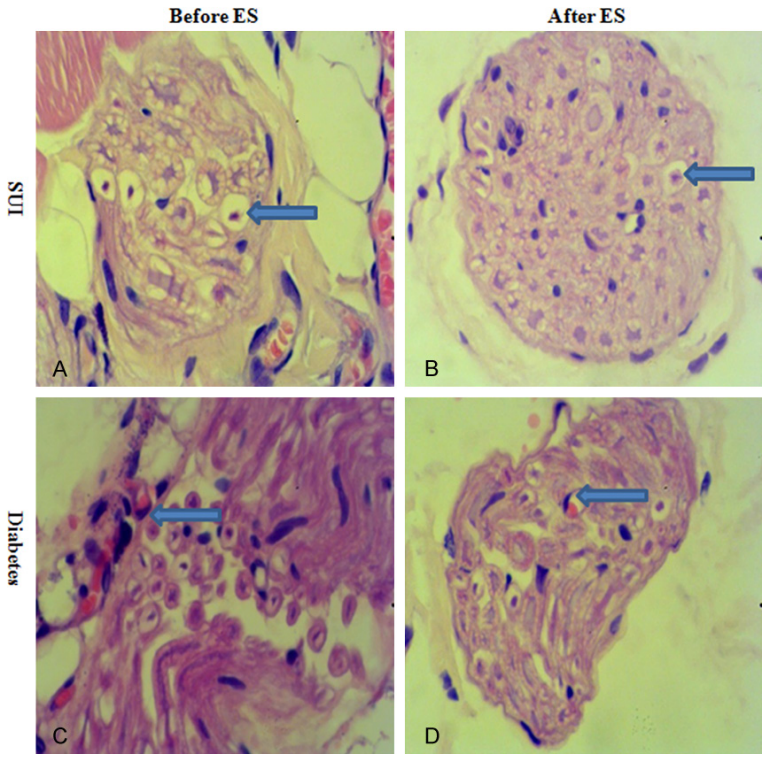

Figure 5. Neural changes of pelvic floor muscles before and after ES treatment. Swollen neuronal cells were observed in SUI rats before ES. Condensed nuclei with marked hyperchromatism and dissolution of Nissl bodies (arrow, A) were also observed. After ES, few neuron cells showed swelling and partial dissolution of Nissl bodies (arrow, B). For diabetes rats before ES, neural changes included the congestion of blood vessels and swelling of endothelial cells (arrow, C). After ES, slightly swollen endothelial cells were found to be embedded in the endoneural membrane, and disarrangement of neuron fibers was observed less frequently (arrow, D).

To further explore the histological characteristics of pubococcygeal muscles, H&E staining and histological analysis were performed. The neurons and muscle fibers showed ischemic changes in the SUI group before ES. Muscle fibers were swollen, and the cytoplasm was lightly stained. Neural changes included swelling neuronal cells, condensed nuclei with marked hyperchromatism, and the dissolution of Nissl bodies. The diabetes group showed typical pathological changes, muscle fibers displayed chain changes, the congestion of blood vessels, and swelling of endothelial cells. After ES, muscle bundles had integrated structures with a pink appearance in the SUI group, and few neurons showed swelling and partial dissolution of Nissl bodies. In the diabetes group, the nucleus numbers under sarcolemma had significantly increased in partial muscle fibers. Slightly swelling endothelial cells were embedded in the endoneural membrane. Results of the pubococcygeal muscle morphology in both SUI and diabetes rat models have shown similar myogenic changes. Neurogenic myopathology may provide the answer to the etiology of pelvic floor dysfunction, and clearly explain the pathological mechanisms of ES treatment on SUI symptoms.

Conclusion

In summary, the present study found that, the increased concentration of NPY in SUI and diabetes rats after ES treatment was related to the recovery of the pubococcygeal muscle strength, which may be related to NPY in promoting the generation of the pelvic floor muscles and blood vessels. ES treatment has shown considerable therapeutic effects, and would be an ideal physiotherapy to solve patients' SUI symptoms.

Acknowledgements

This study was supported by funds from National Natural Science Foundation of China (Grant No. 8167060599).

Disclosure of conflict of interest

None.

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