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RESEARCH ARTICLE

"Warming *yang* and invigorating *qi*" acupuncture alters acetylcholine receptor expression in the neuromuscular junction of rats with experimental autoimmune myasthenia gravis

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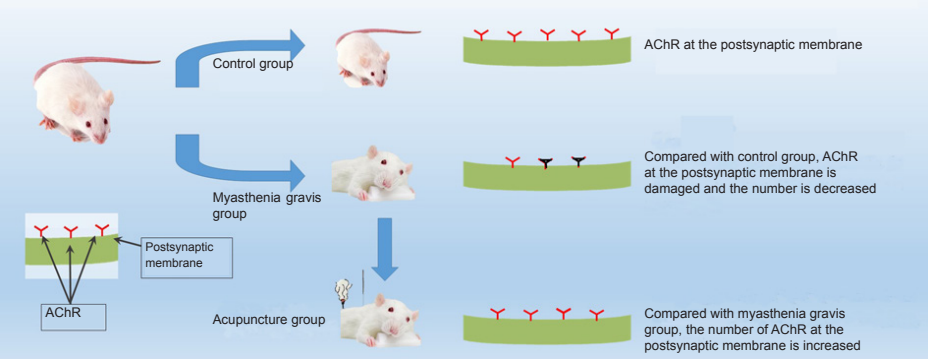
Graphical Abstract

Acetylcholine receptor (AChR) expression in postsynaptic membrane of neuromuscular junction of rats



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Abstract

Myasthenia gravis is an autoimmune disorder in which antibodies have been shown to form against the nicotinic acetylcholine nicotinic postsynaptic receptors located at the neuromuscular junction. "Warming *yang* and invigorating *qi*" acupuncture treatment has been shown to reduce serum inflammatory cytokine expression and increase transforming growth factor beta expression in rats with experimental autoimmune myasthenia gravis. However, few studies have addressed the effects of this type of acupuncture on the acetylcholine receptors at the neuromuscular junction. Here, we used confocal laser scanning microscopy to examine the area and density of immunoreactivity for an antibody to the nicotinic acetylcholine receptor at the neuromuscular junction in the phrenic nerve of rats with experimental autoimmune myasthenia gravis following "warming *yang* and invigorating *qi*" acupuncture therapy. Needles were inserted at acupressure points *Shousanli* (LI10), *Zusanli* (ST36), *Pishu* (BL20), and *Shenshu* (BL23) once daily for 7 consecutive days. The treatment was repeated after 1 day of rest. We found that area and the integrated optical density of the immunoreactivity for the acetylcholine receptor at the neuromuscular junction of the phrenic nerve was significantly increased following acupuncture treatment. This outcome of the acupuncture therapy was similar to that of the cholinesterase inhibitor pyridostigmine bromide. These findings suggest that "warming *yang* and invigorating *qi*" acupuncture treatment increases acetylcholine receptor expression at the neuromuscular junction in a rat model of autoimmune myasthenia gravis.

Key Words: *nerve regeneration; myasthenia gravis; acupuncture; "Warming yang and invigorating qi"; experimental autoimmune myasthenia gravis; neuromuscular junction; acetylcholine receptor; neural regeneration*

Introduction

Myasthenia gravis (MG), the most common disease affecting neuromuscular transmission, is an autoimmune disease mediated by a acetylcholine receptor antibody (AChR-Ab) that reacts with its complement to lead to a neurological muscle disorder (Peng, 2004). The incidence of MG has significantly recently increased from 1–15 persons per million to 3–175 persons per million, with the incidence increasing steadily in children (Aguiar Ade et al., 2010). Therefore, an improved cure rate for MG is particularly urgent.

Various clinical treatments for MG exist, including cholinesterase inhibitors, immunosuppressants, plasma exchange, and resection of thymoma in patients with combined thymoma. However, the adverse effects associated with these treatments are significant, such as diarrhea, nausea, vomiting, salivating, muscle twitching, and the treatments suffer from short effectiveness, difficult dosage control, strong dependence, and high cost (Rodnitzky and Goeken, 1982; Guo et al., 1999). Compared with western medicine, Chinese medicinal herb treatments and acupuncture for treating MG have marked advantages, including few adverse effects and notably successful outcomes. Compared with Chinese medicinal herb treatment, acupuncture is more convenient and has better outcomes (Yang and Cheng, 2003). "Warming *yang* and invigorating *qi*" acupuncture treatment has been shown to reduce serum inflammatory cytokine expression and increase transforming growth factor beta expression in rats with experimental autoimmune myasthenia gravis. However, few studies have reported on the effect of this treatment on the cholinergic system in MG.

Previous studies have demonstrated that serum tumor necrosis factor α, interleukin-12, and interleukin-18 expression decease, but transforming growth factor β expression increases in a rats with experimental autoimmune myasthenia gravis (EAMG) (Wang et al., 2013, 2014). Although the number of AChRs in the neuromuscular junction is reduced in patients with myasthenia gravis (Fambrough et al., 1973; Almon et al., 1974; Stanley and Drachman, 1978; Pestronk et al., 1985), it remains poorly understood whether "warming *yang* and invigorating *qi*" acupuncture therapy affects AChRs. Thus, this study tested the hypothesis that "warming *yang* and invigorating *qi*" acupuncture alters AChR levels toward a therapeutic effect on EAMG in rats.

Materials and Methods

Ethics statement

The animal studies were approved by the Medical Ethics Committee of Affiliated Hospital of Changchun University of Chinese Medicine and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Care was taken to minimize the suffering and number of animals used in each experiment.

Experimental animals

A total of 70 female (more susceptible to make model compared with male rats) specific-pathogen-free Lewis rats aged 7–8 weeks and weighing 160 ± 10 g were provided by Vital River in Beijing, China (SCXK (Jing) 2012-0001). The rats were housed in a standard medical laboratory after quarantine inspection.

Establishment of the EAMG model

The EAMG model was established in 60 randomly selected rats. A water-in-oil emulsion was made by complete emulsion of the AChRα1 129–145 peptide fragment (Jill Peptides Co., Ltd., Shanghai, China) and Freund's complete adjuvant (Sigma-Aldrich Trading Co., Ltd., Shanghai, China) (Wu et al., 2006). Rats were anesthetized by intraperitoneal injection of 4% chloral hydrate, and subcutaneously injected with 200 μL of the antigen emulsion (0.125 mg/mL) at six points along the back, two foot pads, and the tail close to the base. Four weeks later, a supplementary immunization was administered in the same manner as the first immunization. Four weeks after the second immunization, rats were administered a third immunization. This was followed by a supplementary immunization four weeks later (Yang and Cheng, 2003; Liu et al., 2007). Animals displaying a Lennon syndrome-classification score ≥ 1 and AChR-Ab-positive serum (positive/negative ≥ 2.1) (Christadoss et al., 2000) were considered successful models of EAMG. Thirty rats modeling EAMG were equally and randomly divided among the following three groups: MG, acupuncture, and drug. Ten experimentally naïve rats were used as controls.

Acupuncture and drug treatments

On day 2 after model establishment, rats in the acupuncture group were treated with "warming *yang* and invigorating *qi*" acupuncture. According to the principle of "Cure flaccidity only need to focus on *Yang Ming*" and "*Yin* disease cures *Yang*, *Yang* disease cures *Yin*", it selected the following acupoints. The acupoint *Shousanli* (LI10) is located bilaterally within intramuscular spaces in the first quarter of the radial side of dorsal forearm. The acupoint *Zusanli* (ST36) is located (bilaterally) 5 mm under the capitular fibula, on the posterolateral corner of the knee. The acupoint *Pishu* (BL20) is located bilaterally under the 12th dorsal vertebra at the interspace of the ribs. *Shenshu* (BL23) is located at both sides of the second lumbar vertebra (Guo and Fang, 2012). Experimental acupuncture study shows that the acupoints selected above for rats and humans are similar. A needle was perpendicularly inserted at *Shousanli* to a depth of 5 mm and at other acupoints to a depth of 6 mm. The reinforcing-attenuating method was conducted in accordance with a previous study (Guo and Fang, 2012). After needling at each acupoint, a moxa cone (Nanyang Hanyi Moxibustion Technology Development Co., Ltd., Nanyang, Henan Province, China) of about 1 cm was added by igniting it. The needle was maintained in place for 30 minutes. Two treatment courses were performed, with each course consisting of once daily treatments for 7 days. One day of rest was given between the two courses. Rats in the drug group were intragastrically administered the orally active cholinesterase inhibitor pyridostigmine bromide (18.5 mg/kg; Chinese

and Western Three-dimensional Pharmaceutical Co., Ltd., Shanghai, China) (Wei et al., 2010) once daily for 15 days. Rats in the control and MG groups were housed under the same conditions and removed from their cages and handled but were given no other intervention.

Sample preparation

On day 14 after acupuncture and drug treatments, the phrenic nerve was intraperitoneally injected with the nerve tracer Dil (Sigma-Aldrich Trading Co., Ltd., Shanghai, China). On day 2 after the DiI injection, rats were deeply anesthetized with 4% chloral hydrate (2 mL/200 g, intraperitoneal). The phrenic nerve was excised, embedded, frozen, and sliced into sections.

Fluorescence immunohistochemistry

The phrenic nerve sections were washed three times for 10 minutes each with 0.01 M phosphate-buffered saline (PBS). After the sections were blotted with filter paper, they were blocked with 5% normal donkey serum (Santa Cruz Biotechnology Co., Ltd., Shanghai, China) for 40 minutes, incubated in a CO_2 incubator at 37°C for 1 hour, and treated with a monoclonal anti-nicotinic acetylcholine receptor (a1, a3, a5 subunits) antibody (1:200 dilution; Abcam Trading Co., Ltd., Shanghai, China) at 4°C overnight. On the following day, the samples were washed three times with 0.01 M PBS, incubated with donkey anti-rabbit IgG-CFL488 (1:400 dilution; Santa Cruz Biotechnology [Shanghai] Co., Ltd.) in the dark at room temperature for 2 hours. Afterward, the sections were washed twice with 0.01 M PBS, stained with the nuclear dye Hoechst 33342 (Sanofi China Company, Shanghai, China) for 3 minutes at room temperature, washed with 0.01 M PBS, and maintained in place for 10 minutes. The sections were then mounted with glycerol and observed using a confocal laser scanning microscope (Olympus, Tokyo, Japan). Image-Pro Plus 6.0 image analysis software (Media Cybernetics Company, Shanghai, China) was used to determine the area and integrated optical density values of the immunofluorescence at the neuromuscular junction in the phrenic nerve.

Statistical analysis

Data were analyzed with SPSS 17.0 software (SPSS, Chicago, IL, USA) and are expressed as the mean ± SD. One-way analysis of variance and the *post hoc* test (Student-Newman-Keuls method) were used to compare the differences among the groups. A value of $P < 0.05$ was considered statistically significant.

Results

Compared with the control group, the averages of the immunofluorescence-positive area and the integrated optical density of the nicotinic AChR antibody immunoreactivity at neuromuscular junction in the phrenic nerve were lower in all three groups of rats with EAMG ($P < 0.01$). However, these values were higher in the acupuncture group than those in the MG group ($P < 0.01$), while they were similar between the acupuncture and drug groups ($P > 0.05$; **Figure 1**).

The *post hoc* test declared that no significant difference was detected between acupuncture group and drug group. Significant difference was observed between MG group and other three groups. Significant difference was found between blank group and other three groups (**Figure 1**).

Discussion

AChR antibodies, T lymphocytes, T lymphocyte subgroups, the thymus, cytokines, and other antibodies of the body and genetic factors play important roles in different aspects of the onset of MG (Le Panse et al., 2008). However, alteration of the AChRs in the neuromuscular junction is the most crucial point in the progression of MG. That is, no matter how the immune system and cytokines may change, the ultimate target in this disease is the AChR on postsynaptic membrane of the neuromuscular junction (Yang, 2004). Relatively few studies have examined the mechanisms of EAMG from the perspective of cell biology. The present study utilized immunofluorescence and confocal microscopy techniques to identify AChRs in a rat model of EAMG and observed the change in AChRs in the neuromuscular junction to demonstrate whether the acupuncture treatment of "warming *yang* and invigorating *qi*" is effective from the perspective of cell biology.

Our results indicated that the "warming *yang* and invigorating *qi*" acupuncture treatment increased AChR expression in the neuromuscular junction. Significant difference was observed in the integrated optical density of the AChR immunoreactivity between the acupuncture and MG groups, which proved curative effectiveness of acupuncture treatment. In modern clinical medicine, the most common drug for treating MG is cholinesterase inhibitor therapy. Our research found that no significant difference in the curative effect, that is, increased levels (density and area) of AChR immunoreactivity, was detected between the acupuncture and drug group. Thus, we believe that the pathological changes of AChRs in neuromuscular junction of the rats warrant greater emphasis, and different experimental techniques should be developed to enlarge the scope of research aimed at treating MG through the acetylcholine system.

In conclusion, acupuncture treatment shows promise for good therapeutic effects in MG. Thus, in the clinical treatment and research of MG, acupuncture may effectively improve MG and reduce the pain and burden of patients with this disease.

Author contributions: *HPH, HP and HFW conceived and designed the experiments. HPH and HP performed the experiments and analyzed the data. HPH wrote the paper. HFW reviewed the article. All authors approved the final version of the paper.*

Conflicts of interest: *None declared.*

Plagiarism check: *This paper was screened twice using Cross-Check to verify originality before publication.*

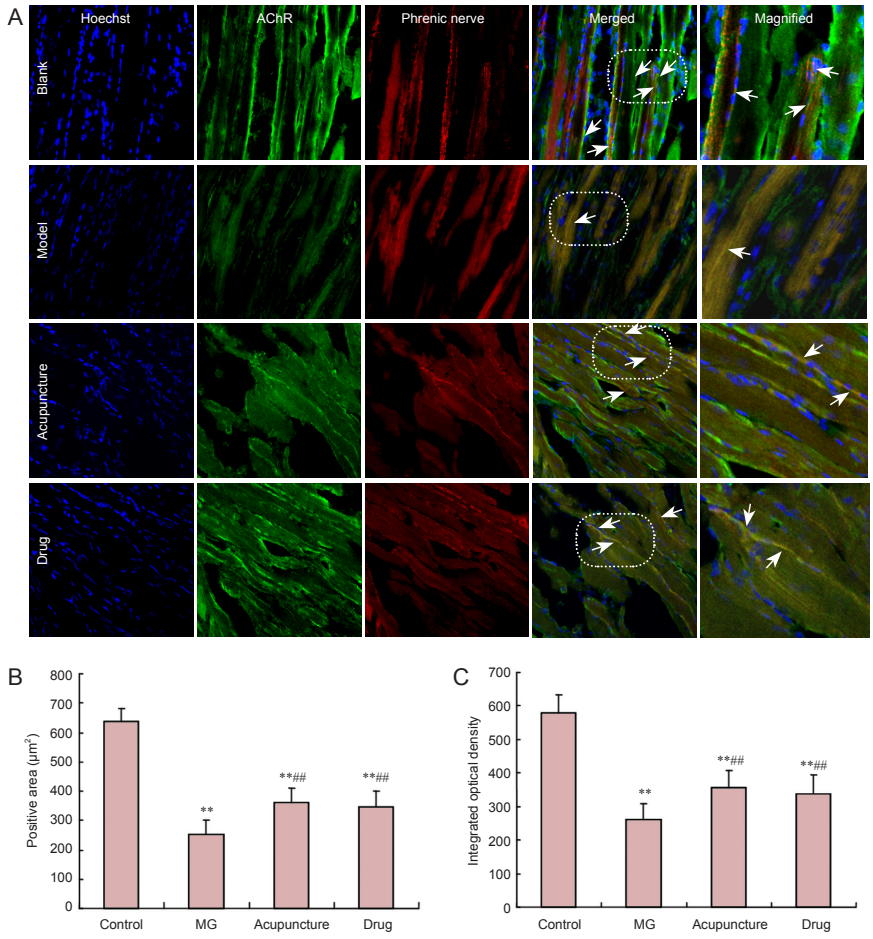

Figure 1 Effect of "warming *yang* and invigorating *qi*" acupuncture treatment on the expression of the nicotinic acetylcholine receptor (AChR) at the neuromuscular junction in the phrenic nerve of rats with experimental autoimmune myasthenia gravis (MG).

(A) Immunofluorescence photomicrographs show AChR immunoreactivity at the neuromuscular junction of rats (confocal laser scanning microscopy; all images captured at × 40 except the magnified images, which are at × 80). Blue (Hoechst 33342), nuclei; green, monoclonal anti-nicotinic AChR (a1, a3, a5 subunits) antibody immunoreactivity; red, the nerve tracer Dil in the phrenic nerve; arrows, AChR expression at the neuromuscular junction. Magnified views of the areas within the dotted-line rectangles represent magnifications of typical variations. (B, C) Averages of the area and integrated optical density of AChR immunoreactivity at the neuromuscular junction. Data are expressed as the mean ± SD (n = 10 per group; one-way analysis of variance, followed by the *post hoc* test (Student-Newman-Keuls method). $^{**}P < 0.01$, *vs.* control group; $^{\#\#}P < 0.01$, *vs.* MG group. Blank: experimentally naïve rats (control group); model: rats with experimental autoimmune myasthenia gravis.

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