Original Research

Proteomic analysis of serum proteins in acute ischemic stroke patients treated with acupuncture

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Abstract

In the present study, we have investigated the effects of acupuncture on (1) serum protein expression that might have a beneficial effect on stroke patients and (2) the strength of limb muscles in stroke patients. A total of 35 acute ischemic stroke (IS) patients were divided into two groups, one receiving drug treatment alone and the other receiving electroacupuncture (EA) and drug treatment. EA treatment was performed on eight acupuncture points once a day for 10 consecutive days. Serum proteins were detected using a proteomics method based on two-dimensional gel electrophoresis, and the specificity of proteins was confirmed by Western blotting. Changes of limb muscle strength were measured using a modified Medical Research Council grading scale. After EA, SerpinG1 protein expression in serum was down-regulated while the expressions of gelsolin, complement component I, C3, C4B and beta-2-glycoprotein I proteins were up-regulated in patients. The changes of serum protein expression were further confirmed by Western blotting in a majority of the cases. The muscle strength of limbs was increased after EA in 18 patients. EA appears to be effective in regulating differential expression of multiple serum proteins involved in stroke, and also in enhancement of muscle strength recovery in acute IS patients despite an individual variation.

Keywords: electroacupuncture, two-dimensional electrophoresis, serum, stroke, muscle strength

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Introduction

Stroke is the second most common cause of death and a leading cause of adult disability worldwide.[1] In East Asia, many patients choose acupuncture as the alternative treatment for stroke recovery mainly because it is cost-effective and rarely causes side-effects. In China, acupuncture has been used for more than 2000 years and is widely regarded as a useful therapeutic method for treating strokes. However, while many studies have reported a positive effect of acupuncture on motor rehabilitation in stroke patients,[2,3] results from some other studies did not support the recovery effect of acupuncture.[4,5] The potential therapeutic effectiveness of acupuncture remains controversial, partly due to a lack of scientific evidence.

In acute stroke patients, it has been well documented that serum proteins including matrix metalloproteinase 9, vasoactive peptide hormone, D-dimer and S100b levels, are significantly higher than those in normal people.[6–9] These serum proteins have been suggested to be useful biomarkers for the diagnosis of strokes, and may also serve as surrogate markers in future neuroprotection trials. In view of the availability of high-throughput proteomics that offer the global profiling of proteins from tissues or organs, the present experiments were designed to investigate the effect of electroacupuncture (EA) on the expression of the various serum proteins that are beneficial for neuroprotection in acute stroke patients, using two-dimensional (2-D) electrophresis and matrix-assisted laser desorption/ionization two-stage time of flight (MALDI TOF/TOF) tandem mass spectrometry. In addition, a modified Medical Research Council (MRC, UK) scale was used for testing the muscle strength.[10,11]

Materials and methods

Study procedures

This study was approved by the institutional review board committee of the Medical School of Jinan University. All cases were anonymous, and all procedures followed were in accordance with the institutional guidelines of the Medical School of Jinan University.

Serum collection

According to the stroke subgroup classification using the Trial of ORG 10172 (danaparoid sodium) in Acute Stroke Treatment (TOAST) criteria,[12] acute ischemic stroke (IS) patients with small artery occlusion (lacune) were involved in this study. A total of 35 hospitalized voluntary patients (for detailed data, please see supplementary Tables 1–3) in the Department of Neurology, Nanhai Chinese Medicine Hospital, Foshan, Guangdong, from October 2007 to October 2008, were divided into two groups. One group of 24 patients received EA with the traditional Chinese herb *Erigeron breviscapus* (EA group) treatment (15 men and 9 women, with an average age of 68.79 ± 11.59), and the other group of 11 patients received the traditional Chinese herb *E. breviscapus* (drug group) treatment only (6 men and 5 women, with an average age of 78.82 ± 6.60). Twelve healthy elderly individuals with no treatment were enrolled as the normal control group (8 men and 4 women, with an average age of 74.25 ± 3.57). One or two days after treatment with piracetam and brain protein hydrolyzate, patients underwent EA treatment from the second day or the third day after hospitalization. Blood samples were taken from normal individuals, acute IS patients 10 d before and after treatment. After the blood clotted at room temperature for 30 min, serum was collected after centrifuging at 1000*g* for 10 min at 4°C and was stored at −80°C until analysis. Acupuncture was performed by a qualified acupuncturist without knowing the grouping of the patients. According to the Agilent protocol (www.chem.agilent.com/Library/usermanuals/Public/5188-5930.pdf), Agilent High Capacity (HC) Multiple Affinity Removal System Spin Cartridge Human-6HC (Agilent Technologies, Santa Clara, CA, USA) was used to remove six of high abundance proteins in the serum including albumin, IgG, IgA, transferrin, haptoglobin and antitrypsin. ReadyPrep™ 2-D cleanup kit (Bio-Rad, Hercules, CA, USA) was used to remove the salt in serum according to the manufacturer's instructions (cshprotocols.cshlp.org/cgi/reprint/2006/17/pdb.kit19.pdf), and then the serum was stored at −80°C.

EA treatment

EA apparatus (Model G6805-2A, Shanghai Huayi Medical Electronic Apparatus Company, China) was used in this study. Based on the Xingnao Kaiqiao acupuncture therapy principle, the acupoints selection principle of main yang meridians and subsidiary yin meridians for strokes was adopted.[13] The acupoints chosen included the scalp acupuncture motor areas (MS 6), the neck acupoints Tianzhu (BL 10) and Fengchi (GB 20), the upper limb acupoints Hegu (LI 4) and Neiguan (PC 6), the lower limb acupoints Weizhong (B 40), Sanyinjiao (SP 6) and Zusanli (ST 36) of the affected side. No. 32 1.5–3 inch acupuncture needles were used under 6 V voltage and 10 Hz frequency throughout this study. Needles were sterilized before use, and were kept at the acupoints for 30 min once a day, and a treatment course of 10 d was adopted.

2-D gel electrophoresis analysis

The protein concentration was determined with the Bradford assay using a bovine serum albumin standard. A sample size of 185 μL serum (120 μg protein) taken from three separate patients was used for 2-D polyacrylamide gel electrophoresis (PAGE) analysis. For the first-dimensional separation, each pH 5–8 range immobilized pH gradient (IPG) dry strip (11 cm) was rehydrated with serum for three hours at room temperature. Isoelectric focusing (IEF) was performed using the following parameters: initially 0–250 V for 30 min, 250–7000 V for three hours and finally 7000 V for seven hours. After the first-dimensional separation IEF, IPG gel strips were placed in an equilibration buffer (6 mmol/L urea, 50 mmol/L Tris-HCL, 2% sodium dodecyl sulfate [SDS], 20% glycerol, 2% dithiothreitol) for 15 min with gentle shaking, followed by another equilibration buffer (6 mmol/L urea, 50 mmol/L Tris-HCL, 2% SDS, 20% glycerol, 2.5% iodoacetamide) for 15 min.

The equilibrated IPG gel strips were placed on top of 12.5% SDS-PAGE gels. Gels were run at 120 V for 30 min, 150 V for 30 min and 200 V until Bromophenol blue dye reached the bottom of the gels. Then, the gels were fixed with fluid containing 10% methanol and 7% acetic acid for 30 min. Finally, the gels were stained with SYPRO RUBY protein stains (Bio-Rad) at room temperature for 12–16 h.

Image analysis

SYPRO RUBY-stained gel images were collected using a Typhoon-9400 (Amersham Biosciences, Uppsala, Sweden) scanner with 532 nm laser as excitation source. The PDQuestTM software (Version 7.4, Bio-Rad) was used to calculate average abundance changes and analyze the differential expression of the proteins statistically across all gels. The protein spots, whose expressions were beyond 2.5 ± 0.5 folds, were selected for in-gel digestion.

In-gel digestion and MALDI-TOF/TOF-MS

Each protein spot showing differential expression on 2-D gels was cut, and then placed in an Eppendorf tube with 100 μL double distilled water. The gels were cut into pieces and digested with 20 ng/mL trypsin (Roche Applied Science, Mannheim, Germany; sequencing grade) overnight at 37°C. The digested peptides were extracted with 50% acetonitrile and 0.1% trifluoroacetic acid in 50 mmol/L ammonium bicarbonate. A volume of 0.4 μL of peptide sample and 0.4 μL of matrix solution (10 mg/mL solution of α-cyano-4-hydroxy-cinnamic acid in 60% [v/v] acetonitrile with 0.1% trifluoroacetic acid) were eluted onto a MALDI sample plate for the analysis. The peptides were then analyzed by mass spectrometry using a 4800 MALDI-TOF/TOF (Applied Biosystems/MDS Sciex, Toronto, Ontario, Canada). Full scan mass spectra from 800 to 4000 m/*z* were collected in the positive mode. External mass calibration was performed using angiotensin II, angiotensin I, substance P, aombesin, adrenocorticotropic hormone clip 1–17, adrenocorticotropic hormone clip 18–39 and somatostatin 28 (Monoisotopic [MH^+] *m*/*z*1046.5420, 1296.6853, 1347.7361, 1619.8230, 2093.0868, 2465.1990 and 3147.4714, respectively). Trypsin-fragment peaks were used as internal standards for mass calibration. Each spectrum was generated by averaging 800 laser shots. The five

most intense ions per spot (S/N ratio > 50) were selected for subsequent tandem mass spectrometry (MS/MS) analysis. Each MS/MS spectrum was averaged over 1200 laser shots. Protein identification was performed by searching the MS/MS spectra against the international protein index (IPI) database (creation date: July 17, 2008), using a local MASCOT search engine (v2.1, Matrix, London, UK) on a global proteome server (v3.6, Applied Biosystems, Foster City, CA, USA). The following parameters were used for the data search: trypsin with a maximum of one missed cleavage was selected; precursor mass tolerance was 50 ppm; MS/MS mass tolerance was 0.3 Da; carboxymethyl modification was set as the fixed modification; oxidation modification was set as the variable modification; human were selected as the search species. Only peptides identified with confidence interval (CI) values of at least 95% were used for protein quantification and analysis.

Western blot

Serum samples were taken from healthy individuals and all patients. The protein concentration of the serum was determined with the Bradford Assay. The serum was heated in the $4 \times$ SDS sample buffer at 95°C for five minutes. A volume of 30 μg of sample was loaded into a 12.5% polyacrylamide gel and run. Proteins on the gel were then transferred to a polyvinylidene fluoride membrane. The membrane was incubated with Serpin G1/C1 inhibitor monoclonal antibody (1:500, R&D Systems, Minneapolis, MN, USA) or gelsolin monoclonal antibody (1:3000, Epitomics, Hayward, CA, USA) or C3 monoclonal antibody (1:500, Abgent, San Diego, CA, USA) overnight at 4°C, and rinsed with TBST buffer, and then incubated with anti-mouse IgG or anti-rabbit IgG (1:2000, Dako, Produktionsvej, Glostrup, Denmark) for two hours at room temperature. The immune complexes were detected by ECL (Cell Signaling Technologies, Danvers, MD, USA) and exposed to X-ray film.

Muscle strength tests

In this study, manual muscle testing was used to examine muscle strength. The score of the muscle strength was determined according to a modified MRC grading scale, and divided into 0, 1, 2, 3 −, 3, 3 +, 4 −, 4, 4 +, 5− and 5 (11). Muscles of the upper limb including deltoid muscle, biceps brachii, triceps brachii, pronator teres, wrist extensors and flexors, and muscles of the lower limb including gluteus maximus, quadriceps femoris, hamstring muscles, ankle dorsiflexors and plantar felxors, were tested. The muscle strength testing was examined three times for each patient before and after EA treatment. If the results from the three testings were different, the intermediate result was used to be analyzed. If there were two same results during the testing, the results were used to be analyzed.

Calculations and statistics

All data were presented as the means ± SEM. One-way analysis of variance (ANOVA) was used to analyze the A value in each group of serum samples with SPSS 13.0 software. Wilcoxon signed-ranks test was used to analyze the muscle strength before and after treatment. Mann–Whitney test was used to analyze the muscle strength before acupuncture or drug treatment. The significant difference was analyzed with $\alpha = 0.05$ as the significance level.

Results

2-D gel electrophoresis of serum protein of acute IS patients before and after EA

2-D gel electrophoresis of serum protein of acute IS patients before and after EA is shown in Figures 1a and b. After

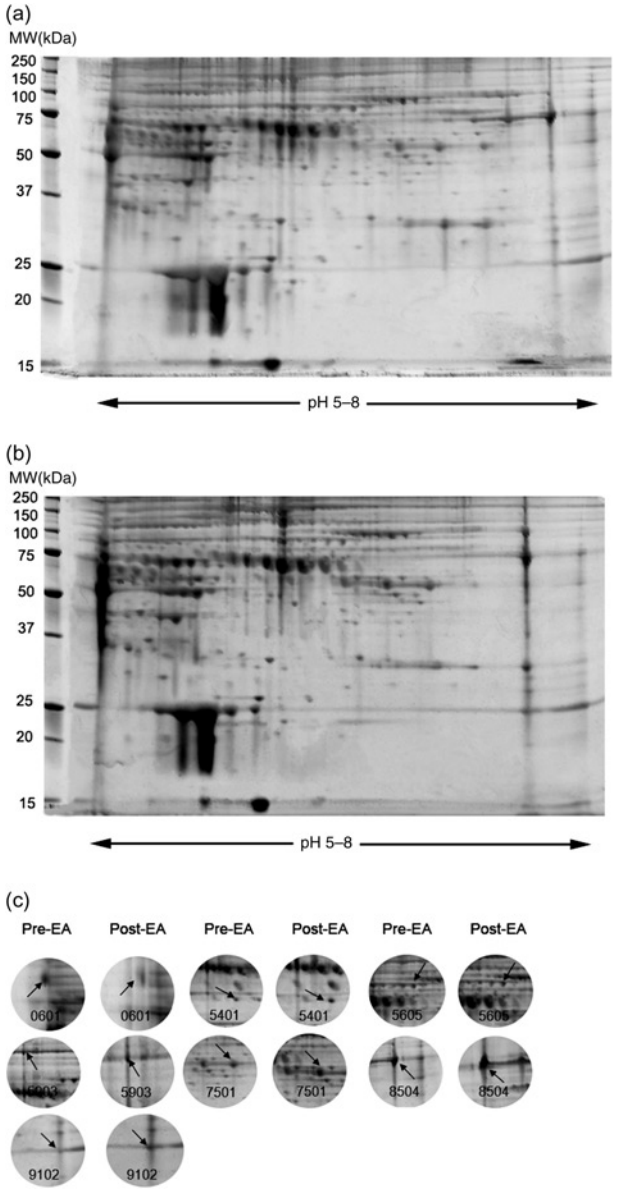

Figure 1 Images of 2-D gel electrophoresis of serum protein in acute IS patients. (a) Before EA. (b) After EA. (c) Significantly differentially expressed protein points of acute IS patients before and after EA were cut and used to be identified with mass spectrometry. Note: Differential protein points were indicated with arrows. The numbers were sample spot protein (SSP) numbers given by PDQuest software. EA, electroacupuncture; IS, ischemic stroke

analysis with PDQuest 7.4 software, it was found that there were 308 ± 13 protein points before EA, and 348 ± 23 protein points after EA. Over 2.5 ± 0.5-fold differential expression was found in seven protein points between the two groups. Among them, six points were up-regulated, and one point was down-regulated after EA (Figure 1c).

Identification of differentially expressed proteins in serum

According to the fingerprinting map and peptide sequence of peptide fragments of seven protein points and retrieval parameters selected in the Mascot Protein Data Bank, six proteins were identified with mass spectrum, which are shown in Table 1. According to their functions, six proteins were divided into following five groups: the first group was regulatory protein-gelsolin; the second group was enzyme inhibitor-SerpinG1; the third group was stress protein-complement component I, C3 and C4B; the fourth group was glycoprotein-beta-2-glycoprotein I. Two points were identified as beta-2-glycoprotein I. Among these differential proteins, SerpinG1 was down-regulated, while gelsolin, complement factor I, C3 and C4B were up-regulated after EA. Figure 2 shows the peptide mass fingerprinting and peptide sequence spectra of gelsolin protein.

Western blotting

According to the result of 2-D gel electrophoresis, SerpinG1, gelsolin and C3 were selected for further analysis. Western blotting was used to further confirm the changes of these proteins. No significant changes were observed either in the serum level of SerpinG1 or C3 between the normal group and pre-EA and predrug groups ($P > 0.05$) (Figure 3). However, gelsolin levels in patients of pre-EP and predrug were lower than those in the normal ($^{*}P < 0.05$). Serpin G1 content in the serum of acute IS patients was decreased after EA as compared with those before EA, with significant difference ($^{**}P < 0.01$) (Figure 4). Gelsolin and C3 contents in the serum of the patients were increased after EA as compared with those before EA, with significant difference ($^{**}P < 0.01$, $^{*}P < 0.05$). In contrast, no significant difference in all three proteins was observed before and after drug treatment with no EA ($P > 0.05$).

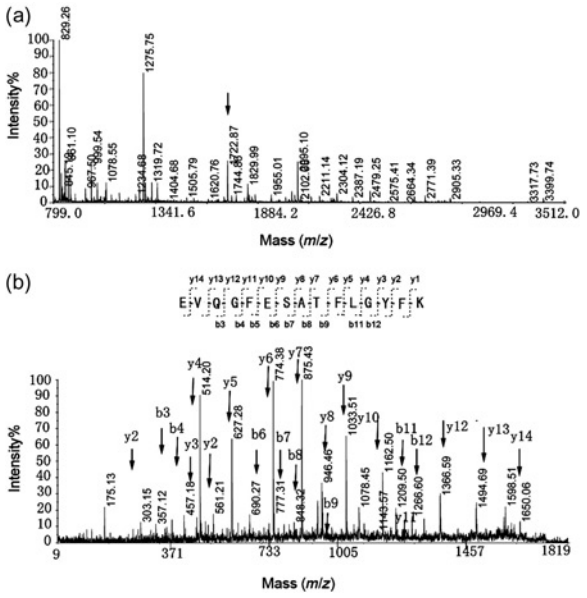

Figure 2 Showing mass spectra of gelsolin protein identified with mass spectrometry. (a) Peptide mass fingerprinting spectrum of gelsolin. The arrow indicates the peptide detected at m/z 1722.87. (b) Peptide sequence spectrum of gelsolin at m/z 1722.87. The amino acid sequence is EVQGFESATFLGYFK

Changes of muscle strength in acute IS patients

According to the MRC grading method, muscle strength of upper limbs and lower limbs of acute IS patients were graded before and after drug/EA treatment (Tables 2 and 3). Wilcoxon signed-ranks test showed that the muscle strength of upper limbs and lower limbs of the patients after EA was significantly increased as compared with that before EA ($P < 0.05$). The muscle strength of upper limbs and lower limbs of the patients was not significantly different before and after drug treatment ($P < 0.05$). Mann–Whitney test analysis showed that the muscle strength of CI patients was not significantly different between before EA and before drug treatment ($P > 0.05$).

Discussion

Results from this study indicated that the recovery of limb muscle strength was accompanied by differential effects of EA on the expression of a number of serum proteins. 2-D gel electrophoresis showed that there were six proteins

Table 1 Differentially expressed serum proteins between pre-EA and post-EA patients

SSP number	IPI number	Name of protein	Molecular weight (Da)/PI	Protein score CI%/total ion score CI%	Sequence coverage%	Ratio
1(0601)	IPI00556459	SerpinG1	37264.4/7.9	100/100	19%	$\downarrow 2.2 \pm 0.1$
2(5401)	IPI00291867	Complement factor I	42433.6/8.49	100/100	11%	$\uparrow 4.1 \pm 0.2$
3(5605)	IPI00026314	Gelsolin	85644.2/5.9	100/100	32%	$\uparrow 2.4 \pm 0.4$
4(5903)	IPI00298828	Beta-2-glycoprotein I	38272.7/8.34	100/100	21%	$\uparrow 2.9 \pm 0.5$
5(7501)	IPI00298828	Beta-2-glycoprotein I	38272.7/8.34	100/100	13%	$\uparrow 4.0 \pm 0.2$
6(8504)	IPI00783987	Complement C3	187029.9/6.02	100/100	27%	$\uparrow 4.4 \pm 0.3$
7(9102)	IPI00654875	Complement C4-B	192672.5/6.73	100/100	13%	$\uparrow 3.2 \pm 0.1$

CI, confidence interval; EA, electroacupuncture; IPI, international protein index; SSP, sample spot protein

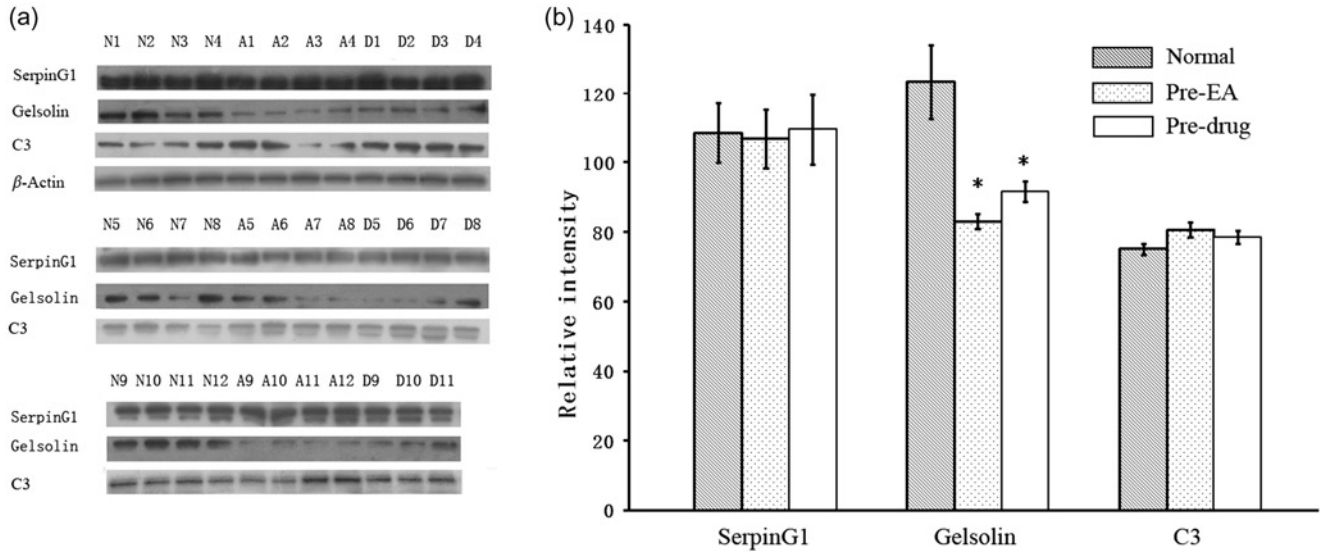

Figure 3 Serum SerpinG1, gelsolin and C3 levels between the healthy individuals and the acute IS patients before treatment with Western blotting. (a) *N* represents healthy individual serum in the normal group ($n = 12$); A represents patient serum before EA ($n = 12$); D represents patient serum before drug treatment ($n = 11$). (b) Histogram showing expressions of SerpinG1, gelsolin and C3 in the serum between the normal group and pre-EA and predrug groups with one-way ANOVA. Data are presented as mean $\pm$ SEM. $^{*}<0.05$ compared with the normal group. ANOVA, analysis of variance; EA, electroacupuncture; IS, ischemic stroke

with over 2.5 ± 0.5-fold expression before and after EA in patients' serum. Among them, SerpinG1 was down-regulated after EA, while gelsolin, complement component I, C3, C4B and beta-2-glycoprotein I were up-regulated after EA. The significance of these proteins is discussed in the following paragraph.

As far as SerpinG1 is concerned, down-regulation of SerpinG1 expression of serum was observed in three acute IS patients after EA using 2-D gel electrophoresis, and in 14 patients using Western blotting. On the other hand, up-regulation of SerpinG1 was observed in two patients while little changes (data not shown) were detected in eight patients with Western blotting. Moreover, there was no difference in the serum level of SerpinG1 between the normal group and pre-EA group and predrug group. These results together suggest that EA may regulate expression of serum SerpinG1, and different patients responded differently to EA, possibly due to the individual health history, age and physical condition.

More important, perhaps, is that the lack of change in SerpinG1 after drug treatment excludes the effect of Chinese patent medicine *E. breviscapus* on SerpinG1 expression, thus supporting a regulatory role of EA that was independent of *E. breviscapus*. The *E. breviscapus* belongs

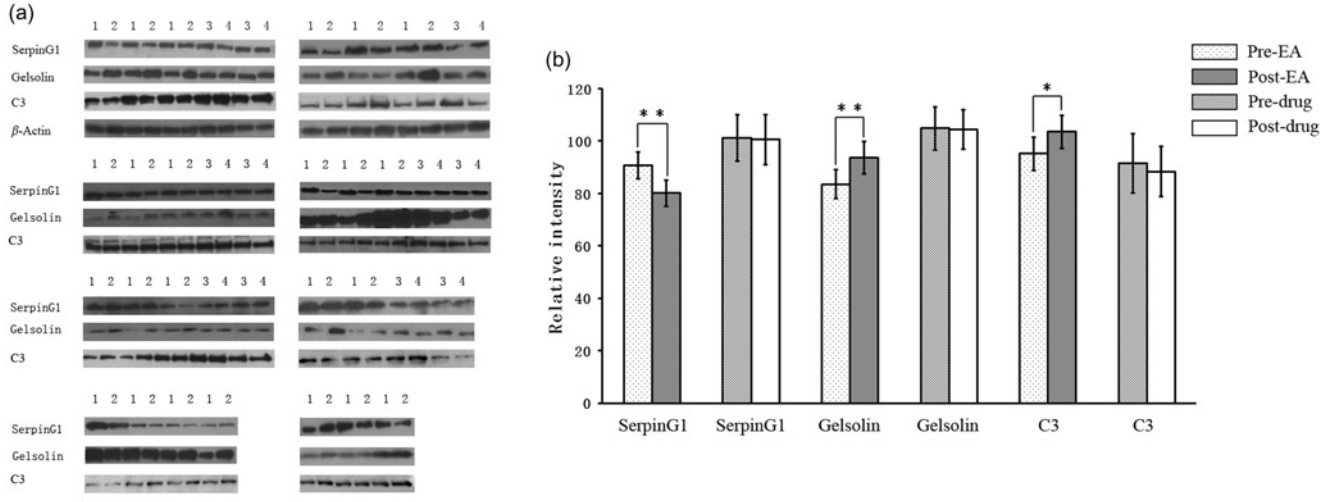

Figure 4 EA regulates expressions of SerpinG1, gelsolin and C3 proteins in the serum of the acute IS patients with Western blotting. (a) 1 represents patient serum before EA ($n = 24$); 2 represents patient serum after EA ($n = 24$). Adjacent 1 and 2 come from the same patient before and after EA; 3 represents patient serum before drug treatment ($n = 11$); 4 represents patient serum after drug treatment ($n = 11$). Adjacent 3 and 4 come from the same patient before and after drug treatment. (b) Histogram showing expressions of serpinG1, gelsolin and C3 proteins in the serum of 35 acute IS patients before and after treatment with one-way ANOVA. Data are presented as mean $\pm$ SEM. $^{*}<0.05$ compared with the pre-EA group. $^{**}<0.01$ compared with the pre-EA group. ANOVA, analysis of variance; EA, EA, electroacupuncture; IS, ischemic stroke

Table 2 Changes of muscle strength of EA-treated acute IS patients detected by a MRC grading scale

Patients	Muscle strength (pretreatment)	Muscle strength (post-treatment)	Patients	Muscle strength (pretreatment)	Muscle strength (post-treatment)
1	LUL G 3	No change	2	LUL G 3	LUL G 3 +
	LLL G 3	No change		LLL G 4	LLL G 5
	RUL G 4	No change		RUL G 5	RUL G 5
	RLL G 4	No change		RLL G 5	RLL G 5
3	LUL G 5	No change	4	LUL G 4	LUL G 5
	LLL G 5	No change		LLL G 5	LLL G 5
	RUL G 5	No change		RUL G 5	RUL G 5
	RLL G 4	No change		RLL G 5	RLL G 5
5	LUL G 5	LUL G 5	6	LUL G 4	LUL G 4 +
	LLL G 5	LLL G 5		LLL G 4	LLL G 4 +
	RUL G 4	RUL G 5		RUL G 5	RUL G 5
	RLL G 5	RLL G 5		RLL G 5	RLL G 5
7	LUL G 5	LUL G 5	8	LUL G 5	LUL G 5
	LLL G 5	LLL G 5		LLL G 5	LLL G 5
	RUL G 5	RUL G 5		RUL G 5	RUL G 5
	RLL G 5	RLL G 5		RLL G 4 +	RLL G 5
9	LUL G 4 +	LUL G 5	10	LUL G 4	LUL G 4 +
	LLL G 4 +	LLL G 5		LLL G 3 +	LLL G 4 +
	RUL G 5	RUL G 5		RUL G 5	RUL G 5
	RLL G 5	RLL G 5		RLL G 5	RLL G 5
11	LUL G 5	LUL G 5	12	LUL G 5	No change
	LLL G 5	LLL G 5		LLL G 5-	No change
	RUL G 3	RUL G 4		RUL G 5	No change
	RLL G 3	RLL G 4		RLL G 5	No change
13	LUL G 5	No change	14	LUL G 5	No change
	LLL G 5-	No change		LLL G 5	No change
	RUL G 5	No change		RUL G 4	No change
	RLL G 5	No change		RLL G 4	No change
15	LUL G 5	No change	16	LUL G 5	LUL G 5
	LLL G 5-	No change		LLL G 4	LLL G 4 +
	RUL G 5	No change		RUL G 5	RUL G 5
	RLL G 5	No change		RLL G 5	RLL G 5
17	LUL G 4-	LUL G 5	18	LUL G 3	LUL G 4
	LLL G 4-	LLL G 5		LLL G 3	LLL G 4
	RUL G 5	RUL G 5		RUL G 1	RUL G 2
	RLL G 5	RLL G 5		RLL G 1	RLL G 2
19	LUL G 5	LUL G 5	20	LUL G 2	LUL G 3
	LLL G 5	LLL G 5		LLL G 3	LLL G 4
	RUL G 2	RUL G 3-		RUL G 5	RUL G 5
	RLL G 2	RLLL G 4-		RLL G 5	RLL G 5
21	LUL G 4-	LUL G 4	22	LUL G 5	LUL G 5
	LLL G 4-	LLL G 4		LLL G 5	LLL G 5
	RUL G 4-	RUL G 4		RUL G 0	RUL G 4
	RLL G 4-	RLL G 4		RLL G 2	RLL G 4
23	LUL G 5	No change	24	LUL G 5-	No change
	LLL G 5-	No change		LLL G 5	No change
	RUL G 5	No change		RUL G 5	No change
	RLL G 5	No change		RLL G 5	No change

EA, electroacupuncture; G, Grade; IS, ischemic stroke; LUL, left upper limb; LLL, left lower limb; MRC, Medical Research Council; RUL, right upper limb; RLL, right lower limb

Based on negative ranks and Wilcoxon's signed-ranks test, the muscle strength was shown as follows: after EA versus before EA in LUL, $Z = 2.825$, $P = 0.005$; after EA versus before EA in LLL, $Z = 3{,}095$, $P = 0.002$; after EA versus before EA in RUL, $Z = 2.379$, $P = 0.17$; after EA versus before EA in RLL, $Z = 2.207$, $P = 0.027$

to the family of Compositae. Its active ingredients consist mainly of baicalein, naringenin, scopoletin, kaempferol, apigenin, scutellarin, luteolin, caffeic acid and protocatechuic acid.[14] It has a protective effect on cardiovascular and cerebrovascular ischemia,[15] probably through inhibiting platelet thromboxane B2 production which in turn reduces the permeability of the blood–brain barrier and/or up-regulates eNOS expression.[16,17]

SerpinG1, also known as complement component 1 inhibitor (C1 inhibitor), is a member of the serpin family of protease inhibitors. It can decrease the vascular permeability through inhibiting two proteases that are involved in the generation of bradykinin, factor XIIa and plasma allikrein.[18] It also plays an important anti-inflammatory role via inhibiting complement system proteases (C1r, C1s, MASP2) and the plasma kallikrein–kinin system proteases. In animal models of myocardial reperfusion injury and middle cerebral artery occlusion, administration of C1 inhibitor resulted in reduced myocardial injury and neuronal damage as well as suppression of ischemia-reperfusion-induced apoptosis.[19–22] Results from this study show a serum down-regulation of SerpinG1 after EA. Does it imply that EA not only has no neuroprotective effect but may also cause damage to neurons? The evidence

Table 3 Changes of muscle strength of drug-treated acute IS patients detected by a MRC grading scale

Patients	Muscle strength (pretreatment)	Muscle strength (post-treatment)	Patients	Muscle strength (pretreatment)	Muscle strength (post-treatment)
1	LUL G 4	LUL G 3	2	LUL G 3	LUL G 4
	LLL G 4	LLL G 3		LLL G 3	LLL G 4
	RUL G 5	RUL G 3		RUL G 4	RUL G 5
	RLL G 5	RLL G 3		RLL G 4	RLL G 5
3	LUL G 5	LUL G 5	4	LUL G 4	LUL G 5
	LLL G 5	LLL G 5		LLL G 4	LLL G 5
	RUL G 3 +	RUL G 5		RUL G 5	RUL G 5
	RLL G 3	RLL G 5		RLL G 5	RLL G 5
5	LUL G 0-1	LUL G 1	6	LUL G 5	LUL G 4
	RLL G 1	RLL G 1-2		LLL G 5	LLL G 4
	RUL G 5	RUL G 5		RUL G 0-1	RUL G 0-1
	RLL G 5	RLL G 5		RLL G 1-2	RLL G 1-2
7	LUL G 5	No change	8	LUL G 5	LUL G 5
	LLL G 5	No change		LLL G 5	LLL G 5
	RUL G 5-	No change		RUL G 3	RUL G 4
	RLL G 5-	No change		RLL G 3	RLL G 4
9	LUL G 4	No change	10	LUL G 4	LUL G 5
	LLL G 4	No change		LLL G 5	LLL G 5
	RUL G 5	No change		RUL G 5	RUL G 5
	RLL G 5	No change		RLL G 4 +	RLL G 4 +
11	LUL G 5	No change			
	LLL G 5	No change			
	RUL G 5	No change			
	RLL G 5	No change			

G, Grade; IS, ischemic stroke; LUL, left upper limb; LLL, left lower limb; MRC, Medical Research Council; RUL, right upper limb; RLL, right lower limb.
Based on negative ranks and Wilcoxon's signed-ranks test, the muscle strength was shown as follows: after drug treatment versus before drug treatment in LUL, $Z = 1.127$, $P = 0.26$; after drug treatment versus before drug treatment in LLL, $Z = 0{,}686$, $P = 0.493$; after drug treatment versus before drug treatment in RUL, $Z = 0.677$, $P = 0.498$; after drug treatment versus before drug treatment in RLL, $Z = 0.948$, $P = 0.343$. The comparison of muscle strength of LUL, LLL, RUL and RLL between before EA and before drug treatment with Mann–Whitney test analysis showed $Z = 1.55$, $P = 0.121$; $Z = 0.75$, $P = 0.453$; $Z = 1.015$, $P = 0.31$; and $Z = 1.179$, $P = 0.238$, respectively

that supports this notion remains fragmentary, because down-regulation of serum SerpinG1 can result in activation of C1r, C1s and MASP2 in the ischemic brain, the activation of the complement system could be conducive to the removal of the apoptotic cells in the presence of a large number of apoptotic cells in brain tissue for a long time,[23] and the activation of the complement 3 could be conducive to the regeneration of neurons.[24] This study also showed that the serum complement component I, C3, C4B were up-regulated, indicating that acupuncture could activate a variety of complements. It is worth noting that patients involved in this study received 10 d of treatment from the second or the third day after hospitalization, and it is likely that there was a large number of apoptotic cells in the ischemic brain of acute IS patients who were elderly and 69 years old in average. Therefore, the up-regulation of serum complement after EA may prove useful to enhance the clearance of the apoptotic cells.

Similarly, the results of Western blotting showed that C3 was up-regulated in 11 patients after EA treatment, but down-regulated in two patients and little changed (data not shown) in 11 patients, indicating that the therapeutic effect of EA also varied due to the physical conditions of individual patients. The lack of a significant change in serum C3 between the patients and healthy individuals may well reflect a decline in immune function associated with aging.[25,26]

Beta-2-glycoprotein I (APOH) expression was found to be up-regulated. As it is one of the non-complement molecules, APOH can bind to the apoptotic cells, and then activate complement, leading to the removal of the apoptotic cells.[27] Besides this, it also has antithrombotic effect.[28,29] Therefore, increase of serum APOH following EA would be beneficial to the deoppilation of obstructed vessels and the amelioration of acute IS symptoms.

Gelsolin level in patients of pre-EP and predrug was lower than that in normal individuals. Gelsolin was up-regulated after EA, but not after drug treatment. The finding of an increased expression of gelsolin is of interest and deserves some comments. Gelsolin is a secreted protein of phagocytic cells, platelets, fibroblasts, non-muscle cells, smooth and skeletal muscle cells. It was also found in cardiac muscle.[30] Its primary function is to control cytoskeletal rearrangement by binding, severing and caping actin filaments.[31] It is an actin-binding protein that can reduce the polymer size of F-actin at substoicheiometric amounts in a Ca^{2+}-dependent manner. The decrease in caldesmon-induced inhibition of actin-activated Mg^{2+}-ATPase activity of myosin by gelsolin may be relevant to the actin-linked regulation of smooth-muscle contraction.[32] D187N gelsolin is a mutant plasma gelsolin, and it has been reported recently that the loss of muscle strength was progressive in homozygous D187N gelsolin mice.[33] Therefore, it is suggested that gelsolin may well play a role in controlling muscle strength. This is supported further by the study by Unger and Hinssen that gelsolin appears to be important in maintaining the integrity of contractile structures and contributes to the efficiency of the contraction of invertebrate muscles.[34] Although the underlying mechanism is as yet not fully understood, results from the present experiments indicated clearly that EA could enhance gelsolin expression in the serum of acute IS patients

and will be one of the important mechanisms that underlie motor rehabilitation of EA in acute IS patients.

It is worth pointing out further that gelsolin is also involved in Fc receptor- and integrin- but not in complement-mediated phagocytosis.[35,36] Its overexpression can prevent caspase-3 activation to inhibit apoptosis.[37,38] This is in line with the previous report of a neuroprotective effect of gesolin in the ischemic brain.[39]

In this study, we also noted that serum gelsolin levels in the pre- and post-drug groups were higher than the values pre- and post-EA. This is probably due to the fact that the average age of patients in the drug group were older than those of patients in the EA group. The elevated level of gelsolin was also observed in senescent human diploid fibroblasts and in the liver, kidney, heart, spleen, stomach and brain of old rats.[40] Up-regulation of gelsolin in senescent cells would be partly responsible for age-related apoptosis resistance.[41]

In general, the limb muscle strength in the majority of acute IS patients after EA is significantly increased compared with that before EA. Considering that a significant muscle strength improvement was not observed only in three patients and that three patients had normal muscle strength before EA, our data indicate a 75% effective rate of EA in enhancing motor performance in acute IS patients. This observation, together with the finding showing a lack of an increased muscle strength in the group of patients that received drug treatment alone, suggest strongly a positive therapeutic effect of EA. Although the reason that a significant improvement of muscle strength was not observed in three patients is not clear, it is possible that increasing time of drug treatment may still be effective in recovery of muscle strength. Moreover, Mann–Whitney test indicated that the muscle strength of the patients was not significantly different before EA and drug treatment, thus excluding the effect of muscle strength before treatment on the therapeutic efficacy.

In summary, results from the present experiments have provided evidence that EA could differentially regulate expressions of a number of specific proteins in serum, which may play a role in rehabilitation of acute IS patients. The regulatory effect of EA in relation to neuroprotection could be mediated via mechanisms that may involve the activation of multiple complements, inhibition of cell apoptosis and antithrombotic effects, while the effect on promotion of recovery of limb muscle strength could be the correspondence of increase of gelsolin expression induced by EA. However, the effectiveness of EA may vary slightly from individual to individual, depending on the physical conditions and severity of stroke patients.

Author contributions: SP designed the experiments and wrote the manuscript. XZ, XS and LG conducted the experiments. LL analyzed the data. BS designed the experiments and supervised the project.

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