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Electro-Acupuncture treatment Promotes Regeneration and Recovery of Spinal Cord Injured Nerve by Regulating HIF-1 α /VEGF Signal Pathway

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Abstract

Objective: The present study aimed to explore the effect of Electro-acupuncture (EA) on the mechanism of nerve regeneration and recovery in spinal cord injury rats by interfering with the expressions of factors associated with HIF-1α/VEGF signaling pathway, covering HIF-1α, VEGF, PKA, Synapsin I, CaMKII as well as BDNF.

Methods: 120 SD female rats after achieving the successful modeling of spinal cord injury were randomly split into Jia ji EA group (EA-1 group), Yang ming EA group (EA-2 group), as well as Sham group(n=48 per groups); subsequently, the rats were split into 5 subgroups (1-5week group, n=8 per subgroups). EA treatment was performed on the 3rd day after the mice achieved the modeling, and specimens were taken at different time points after the treatment. BBB score was employed to assess the variations of lower limb function in mice after they had spinal cord injury. Histological variations of mice after they had spinal cord injury were observed under an optical microscope after undergoing HE staining. The variation of positive cell count was characterized under an optical microscope. RT-PCR and WB techniques were adopted to identify the variations of mRNA and protein expressions of factors associated with HIF-1α/VEGF signaling pathway.

Results: Over time, the lower limb function of mice having spinal cord injury was to a certain extent recovered, and EA-1 and EA-2 groups were more obviously recovered than the control group. According to the results of HE staining, as compared with Sham group, the nerve cell structure of EA-1 and EA-2 groups could be noticeably repaired, and the number of neurons was remarkably up-regulated. As suggested by immunohistochemical results, the number of positive cells in the injured site of Sham group was evidently risen in EA-1 and EA-2 groups as compared with that of Sham Group. PCR and WB results indicated that EA-treatment could up-regulate the expressions of HIF-1α, VEGF, PKA, Synapsin I, BDNF genes, as well as the corresponding proteins, while suppressing the expressions of CaMKII genes and their corresponding proteins. The expression of EA-2 group was partially better than that of EA-1 group.

Conclusion: EA treatment can stimulate the expression of HIF-1α, VEGF, PKA, synapsin I, CaMKII, BDNF mRNA and their corresponding proteins associated with the activation pathway of HIF-1α/VEGF signal transduction pathway. Moreover, this treatment is capable of regulating blood oxygen microenvironment (e.g., local energy consumption, oxygen consumption and blood circulation) after rats get injured, while being critical to the recovery of spinal cord injury.

Keywords EA treatment; Rats with spinal cord injury; HIF-1α/VEGF signaling pathway; Blood oxygen microenvironment; Mechanism of nerve regeneration and recovery

Introduction

Spinal Cord Injury (SCI) refers to the primary cause of paraplegia; it is often accompanied by significant complications (e.g., urinary tract infection and bedsore). Thus, nerve regeneration and recovery of spinal cord injury has been a research hotspot in the medical field [1, 2]. After SCI, ischemia and hypoxia, regeneration and perfusion disorders in local tissues can lead to nerve cell necrosis and axon myelin sheath variation, thereby leading to the formation of glial scar, and seriously hindering axon regeneration and myelination; these are the major obstacles affecting nerve regeneration [3, 4]. Tissue ischemia and hypoxia are also a critical link in glial scar formation. The critical factors determining nerve regeneration are the amelioration of tissue ischemia and hypoxia after SCI and the building of a good blood oxygen microenvironment [5]. Several studies reported that HIF-1α/VEGF signaling pathway was critical to sense and regulate the balance of blood oxygen microenvironment [6]. Hypoxia-inducible factor-1α (HIF-1), regulating gene expression after variations at intracellular oxygen concentration, refers to one of the critical transcription factors regulating oxygen metabolism [5, 7]. Vascular endothelial growth factor (VEGF) is capable of stimulating the proliferation and migration of vascular endothelial cells and indirectly protecting nerves from ischemia and hypoxia injury by the promotion of angiogenesis [8]. Ca2+/calmodulin-dependent kinase II (CaMKII) and camp-dependent protein kinase a (PKA) are capable of regulating Ga2+ signaling; they act as vital factors in hypoxia-oxidation regulatory response after the

subjects have spinal cord injury [9]. Synapsin I first gets combined with synaptic vesicles in dephosphorylated state, then gets dephosphorylated by PKA protein and CaMK II protein, subsequently dissociated from vesicles, and finally allows exocytosis, which is crucial for synaptic regeneration and extension formation of nerve cells [10, 11]. Brain-derived neurotrophic factor (BDNF) has neurotrophic effect; it can accelerate neurite growth and promote the recovery of injured nerves [12]. The interaction of the six factors is significantly beneficial to rebuild the blood oxygen microenvironment.

SCI is termed as "flaccidity disease" in Traditional Chinese medicine. Chinese medicine has a long history in treating SCI. Since the beginning of Neijing, the theory, "treating flaccidity only by taking Yang ming", has been proposed and has been adopted as the treatment criterion followed by subsequent generations in treating flaccidity disease. Foot Yang ming Stomach Meridian is the sea of five zang-organs and six fu-organs, the source of qi and blood metaplasia. It nourishes the bones and muscles, the joints. Thus, it is said that weak qi and blood is the root cause of erectile dysfunction, and supplementing qi and blood is the basic treatment principle of erectile dysfunction. However, acupuncture therapy emphasizes balancing qi and blood, harmonizing yin and yang; it follows the principle of tonifying deficiency and purging excess. For this reason, it is capable of becoming the major method to treat flaccidity. It is noteworthy that the theory of treating flaccidity disease from qi and blood in traditional Chinese medicine exhibits remarkable similarity to SCI nerve recovery since the enhancement of tissue oxygen microenvironment acts as the vital link. Acupuncture has effectively treated various nerve injuries in long-term practice, and it is one of the most frequently applied methods for clinical rehabilitation of this disease [13]. Accordingly, our research group has proposed a scientific hypothesis that "EA treatment is capable of improving spinal cord oxygen microenvironment to promote nerve regeneration by interfering with HIF-1α/VEGF signal pathway conduction". In the present study, the SCI rat model was built to observe the effect of EA treatment on the expressions of factors associated with HIF-1α/VEGF signaling pathway in SCI rats, and to delve into the mechanism of EA treatment on nerve regeneration and recovery of spinal cord injury.

Material and Methods

Animal and groups

120 SPF female Sprague Dawley (SD) rats weighing 180-220 g provided by Animal Experimental Center of Guangxi Medical University were randomly split into 3 groups (EA-1, EA-2, Sham). The rearing environment was set as 40%-47% humidity, 20-25

Experimental materials

primary reagents included SDS-PAGE protein loading buffer, biyun biotechnology co., ltd; PMSF, Bi Yun Tian Biotechnology Co., Ltd.; RIPE protein lysate, Soledad Technology Biology Co., Ltd.; Trizol, Solebo Technology Biology Co., Ltd.; SYBR Green mix, solable technology biology co., ltd. Reverse transcription kit: AffIInity Company; HiF-1α, VEGF, PKA, Synapsin I, CaMKII, BDNF antibody: AffIInity Company; Main equipment: Tanon-4200 automatic chemiluminescence image analysis system; Electrophoresis Instrument, Beijing Liuyi Biotechnology Co., Ltd.; Semi-dry film transfer apparatus, ATTO Company of Japan; ABI7500 fluorescence quantitative PCR instrument.

Model building of experimental rats

Mice underwent intraperitoneal injection of 10% chloral hydrate (3.5ml/kg); after sufficient anesthesia, prone position was performed, and the approach of T9-T11 level on the back of rats was touched, while T10 spinous processes were in horizontal direction, to locate T10 spinal cord segment and disinfect with T10 as the center.

After iodophor disinfection was performed, the skin was cut, and the fascia and interspinous muscle were removed layer by layer to progressively expose the T9-T11 lamina, rigorously and slowly bite the T9-T11 lamina, and completely bite off the pedicle of vertebral arch on each side of T10 to clearly expose the spinal cord wrapped by dura mater.

Clamping the spinal cord with micro blood vessels and keeping the clamped state for 20 sec for the building of a spinal cord clamping injury model. After the model was built, visible congestion marks on the clamping injury could be observed, suggesting that the model was successfully built.

After modeling was successfully achieved, muscle and skin were sutured layer by layer. Penicillin was injected to mice intraperitoneally for 3 consecutive days to prevent mice from infection and reduce their mortality.

Mortality rate of experimental rats and treatment methods

After sufficient pre-experimental preparation before the formal experiment of the subjects, the mortality rate of rat modeling was significantly less than 1/4 of the mortality rate required by the subject. If rat death took place during modeling and treatment, the modeling and treatment of rats should be supplemented in line with the group of dead rats.

EA treatment of rats after modeling

In sham group, no treatment measure was taken on the mice after modeling, and EA treatment once a day was started on the 3rd day after operation in the spine clamping mice of EA and EA-2 groups. Point selection of spine clamping EA group: it was located between spinous processes of vertebral bodies of two spinal cord segments, namely T8- T9 and T11- T12 (EX-B2, T9-T11), at the complete transection of spinal cord. For EA-2 group, bilateral Futu (LI 18) and Zusanli (ST36) were selected. A filiform needle was inserted into respective point, the depth of insertion was nearly 2mm after skin penetration. Besides, the treatment intensity was 12-15mv, the treatment frequency was 2Hz, the waveform was taken as density wave, the needle retention time was 30min, and the time was 5 times per week 5.

Detection method

BBB Score: Baso Beattie Bresnahan Loco Motor Rating Scale (BBB Scale) has been extensively as the scoring standard in animal experiments to study spinal cord injury, which is critical to the study of neurological function of severe spinal cord injury [14]. BBB score of lower limb function of rats: The tests were performed by the examiners blinded to the animals' treatments.

HE staining: Rats were anesthetized at 1,2,3,4,5 weeks after the treatment, and subsequently injured spinal cord tissue specimens were taken out. Routine H&E staining was performed to observe the histological variations at various time points after the subjects had

spinal cord injury under an optical microscope after embedding, sectioning, dewaxing, hydration, staining and other steps.

Immunohistochemical staining: The expressions and distribution of HIF-1α, VEGF, PKA, Synapsin I, etc. in the injured spinal cord of rats were observed by microscopic examination after the processes of embedding, sectioning, dewaxing, baking, ethanol soaking, PBS immersion, dehydration as well as sealing. The cytoplasm and/or nucleus were observed under an optical microscope and then stained in brown-yellow as positive cells. The staining results complied with the semi-quantitative immunohistochemical scoring standard [15]: at high magnification (× 400), the number of positive cells in each field was calculated by randomly intercepting 3 fields in respective picture, and the expression intensity of positive cells was measured.

Real-time fluorescence quantitative PCR (RT-PCR): RT-PCR was performed to detect the expression of each factor.

- The obtained spinal cord tissue was ground in a mortar. The spinal cord tissue was placed in an EP tube without RNA enzyme. 1ml of trizol RNA lysate was added for soaking. The samples were uniformly mixed. The mixture was placed in a-20 deg. refrigerator for freezing. The extracted tissue lysate was displaced into a centrifuge tube. The extracted tissue lysate was repeatedly blew with a 1 ml gun head until no obvious precipitate was found in the lysate, and then it was centrifugated at 4 deg. c and 4 ℃12000 rpm for 15 min. 1.5ml of supernatant was sucked. The supernatant was placed into a new EP tube. An equal volume of isopropanol precooled at-20 deg c was added and then mixed uniformly. The mixture was placed in a refrigerator at-20 deg c for precipitation for 10 min. 20 ul of RNase-free water was added until it was completely dissolved. Subsequently, ultraviolet analysis was conducted to determine the concentration of extracted RNA.
- Table 1 lists PCR primer sequence and the PCR products length.

Gene	Primer sequence(5'-->3')	Products
β-actinSense	GCTATGTTGCCCTAGACTTCGA	173bp
β-actinAntisense	GATGCCACAGGATTCCATACC	
HIF-1α: Sense	AGAGGAAGCGAAAAATGGAACA	102bp
HIF-1α: Antisense	AAAGCGACATAGTAGGGGCACG	
VEGF: Sense	CGTCCAACTTCTGGGCTCTT	150bp
VEGF: Antisense	CCCCTCTCCTCTTCCTTCTC	
PKA: Sense	GGCTTCCAACTCCAACGAT	99bp
PKA: Antisense	TCCAACTGGGCTGTATTCT	
synapsin I: Sense	GATGGTTCGACTACACAA	116bp
synapsin I: Antisense	TCTTCACAACTACAGGGT	
CaMKII: Sense	GTATATCTTGCTGGTTGGG	97bp
CaMKII: Antisense	TGGTGATGGGAAATCGTAG	
BDNF: Sense	GCAGGTTCGAGAGGTCTGAC	110bp
BDNF: Antisense	TTATGGTTTTCTTCGTTGGG	

Table 1: Primers for RT-PCR.

Western blot: The total protein of spinal cord was extracted, and then the expression of each factor protein was detected by Western blot assay.

- The mixed part of the spinal cord tissue was ground with liquid nitrogen. 1ml of rip lysate was added and then centrifugated at 4℃ 12000r / min for 10min. The supernatant was transferred to a new centrifugation tube, sub pack 100ul / tube. The above samples were placed in the protein buffer, boiled at 100℃ for 10min, and then centrifugated at 12000rpm for 1min for standby.
- Different concentrations of glue were prepared according to the molecular weight of different proteins, and the prepared samples were placed on the sample. Electrophoresis was performed under 80 V at constant pressure and under 120 V at constant pressure after 30 min, 1 h.
- 1 h at ambient temperature with blocking solution, one antibody was diluted with newly configured blocking solution, covering 1:400 dilution ratio of HIF-1α, PKA, synapsin I, CaMKII protein antibody, 1:1000 dilution ratio of VEGF and β-actin protein, 1:500 dilution ratio of BDNF protein antibody; one antibody was incubated overnight at 4℃, the membrane was cleaned three times with tbst, 5 min each time, dilute it with blocking solution. The membrane was incubated at ambient temperature for 1h, the membrane was washed three times with tbst, each time for 5 min, drained, put into the chemiluminescence imager, exposed, and recorded in photos.

Statistical Analysis: All data are expressed as means ± SEM. Significant differences were assessed by two-tailed Student's t test or one-way ANOVA followed by the Student – Newman – Keuls test. Differences were considered to be statistically significant at $p<0.05$.

Results

BBB Locomotor Rating Scale

Table 2 lists the comparison of nerve function scores of rats in each group after operation. As compared with sham group, the scores of 1-5 week neurological function in rats of EA-1 and EA-2 groups increased over time, and the differences were of statistical significance ($P<0.05$). To be specific, the scores of 1-3 week neurological function in rats of EA-2 and EA-1 groups were only higher than those of EA-1 group, and the diversifications were of statistical significance ($P <0.05$). No statistical difference existed in other time points.

Group	0 D	1W	2W	3W	4W	5W
Sham	21.00 ± 0.00	2.50 ± 0.58	2.50 ± 0.58	1.50 ± 0.58	6.00 ± 0.82	8.00 ± 0.82
EA-1	21.00 ± 0.00	4.00 ± 0.82a	4.75 ± 0.96a	7.00 ± 0.82a	10.50 ± 1.29a	12.75 ± 0.96a
EA-2	21.00 ± 0.00	5.25 ± 0.50ab	7.50 ± 0.58ab	9.75 ± 0.96ab	12.00 ± 0.82a	14.00 ± 1.83a

NotesAs compared with Sham group, a P < 0.001As compared with EA-1 group, b P < 0.05

Table 2: BBB scores of all groups.

Pathological Examination

HE staining results: Figure 1 suggests that in sham group, the structure of the spinal cord was extremely disordered, neurons disappeared, and some myelin sheath was lost in 1week; in 2week, the structure of the spinal cord was extremely disordered, neurons disappeared, and some myelin sheath was lost; in 3week, the structure of the spinal cord was moderately disordered, some neurons appeared, no obvious myelin sheath was lost, no obvious hemorrhage took place; in 4week, the structure of the spinal cord was slightly disordered, and the number of neurons was obviously up-regulated, no obvious demyelination or hemorrhage took place, slight disorder of spinal cord structure occurred, neurons increased obviously, and no demyelination or obvious hemorrhage took place.

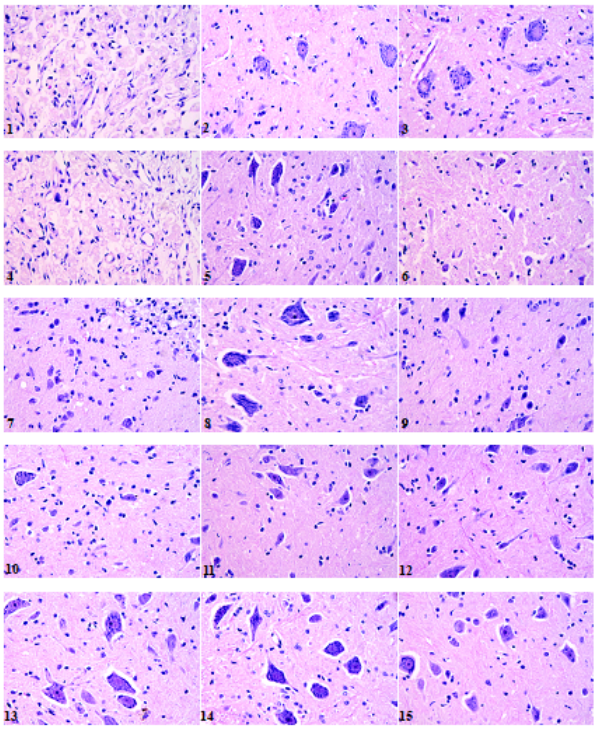

Figure 1: Morphological picture of spinal cord stained with HE in different groups 400 × Notes: 1. Sham group-1week; 2.EA-1 group-1week; 3. EA-2 group-1week; 4. Sham group-2week; 5.EA-1 group-2week; 6. EA-2 group-2week; 7. Sham group-week; 8.EA-1 group-3week; 9. EA-2 group-3week; 10.Sham group-4week; 11.EA-1 group-week; 12. EA-2 group-4week; 13.Sham group-5week; 14.EA-1 group-5week; 15.EA-2 group-5week

The EA-1 and EA-2 groups had the up-regulated number of spinal capillaries with slight hemorrhage. As compared with that in 1week, the number of 2week capillaries was reduced, no hemorrhage took place, and the structure remained disordered. As compared with 2week, no hemorrhage took place in 3week, and the structure was slightly disordered. As compared with 3week, no hemorrhage took place in 4week, the structure was close to the normal level. In 5week, no hemorrhage was observed, and the structure basically returned to the normal level.

Neuron count results: The number of sham group 1-5-week neurons first decreased and then increased, respectively (10.00 ± 1.00; 7.33 ± 0.58; 5.00 ± 1.00; 9.00 ± 1.00; 11.00 ± 1.00). As compared with sham group, 1-5week neuron number of EA-1 group was noticeably higher, marking the significant difference (P < 0.05); 4week neuron number was the highest, up to 24.33 ± 2.52; 1-5week neuron number of EA-2 group was remarkably higher than that of sham group, the difference was significant (P < 0.05); 5week neuron number was up to 29.67. As compared with EA-1 group, 1-3week neuron number of EA-2 group increased evidently (P < 0.05), and there was no statistical difference at other time points, as listed in Table 3.

Group	1W	2W	3W	4W	5W
Sham	10.00 ± 1.00	7.33 ± 0.58	5.00 ± 1.00	9.00 ± 1.00	11.00 ± 1.00
EA-1	14.00 ± 1.00a	16.00 ± 1.00a	19.33 ± 0.58a	24.33 ± 2.52a	24.00 ± 4.36a
EA-2	21.00 ± 2.00ab	21.67 ± 1.53ab	24.67 ± 0.58ab	27.00 ± 1.00a	29.67 ± 2.52a
Notes: As compared with Sham group, a P<0.001As compared with EA-1 group, b P<0.05					

Table 3: Number of neurons at different time points of spinal cord injury.

Immunohistochemistry: The positive expression of HIF-1α protein was located in the nucleus and cytoplasm. The first week sham group had slightly higher positive expression than those of EA-1 and EA-2 groups. The difference was not significant, and there was of no statistical significance. The positive expression of the first week sham group tended to decrease from 2week to 5week. The positive expression of EA-2 group was obviously higher than that of sham group at 2 to 4 weeks (P < 0.05), whereas the difference was not statistically significant at 5 weeks. The overall expression of 2 to 5 weeks EA-2 group was slightly higher than that of EA-1 group, whereas the difference was not statistically significant, as presented in Figure 2.

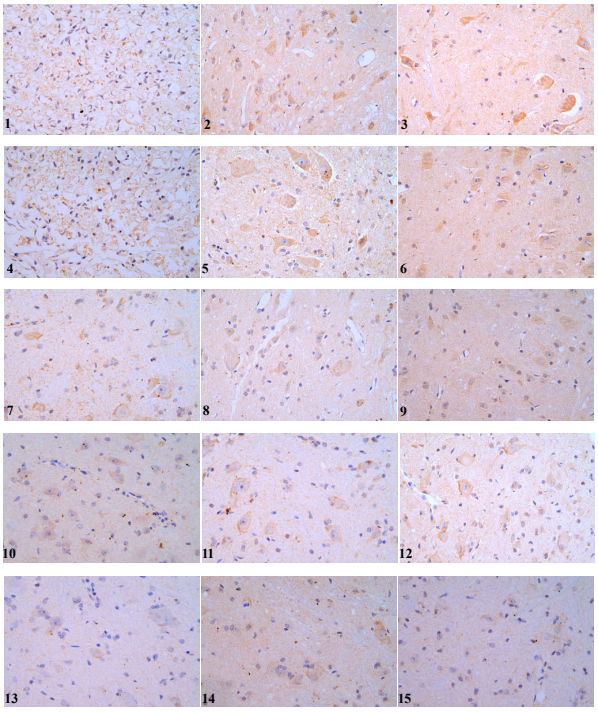

Figure 2: Expression of HIF-1α protein in each group 400 × Notes: 1.Sham group-1week ; 2. EA-1 group-1week ; 3. EA-2 group-1week; 4. Sham group-2week; 5. EA-1 group-2week; 6. EA-2 group-2week ; 7. Sham group-3week ; 8. EA-1 group-3week; 9. EA-2 group-3week; 10. Sham group-4week; 11. EA-1 group-4week ; 12. EA-2 group-4week ; 13. Sham group-5week; 14. EA-1 group-5week; 15. EA-2 group-5week.

As observed from Figure 3, the positive expression of VEGF protein was located in the cytoplasm, and the positive expression of sham group tended to be strengthened from the first week; the second week reached the peak and then gradually decreased. As compared with sham group, the expression of 1-4week EA-2 Group was noticeably higher, marking the statistically significant difference ($P < 0.05$). Moreover, the expression of the fifth week remained higher than that of sham group, whereas the difference was not statistically significant. As compared with sham group, 1-2week of EA-1 group was noticeably higher, marking the statistical difference ($P < 0.05$). 3-5week remained higher than that of sham group, whereas the difference was not statistically significant. See Figure 3 for details.

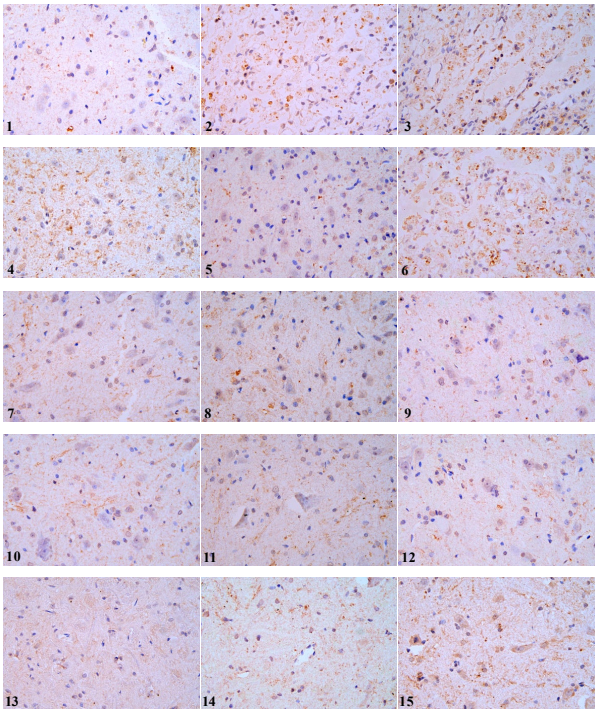

Figure 3: Expression of VEGF protein in each group 400 × Notes1. Sham group-1week2. EA-1 group-1week3. EA-2 group-1week4. Sham group-2week5. EA-1 group-2week6. EA-2 group-2week7. Sham group-3week8. EA-1 group-3week9. EA-2 group-3week10. Sham group-4week11. EA-1 group-4week12. EA-2 group-4week13. Sham group-5week14. EA-1 group-5week15. EA-2 group-5week.

As observed from Figure 4, PKA protein positive expression was located in the cytoplasm and cell membrane, and sham group protein positive expression was the strongest at 1week; from 2week, the positive expression was progressively down-regulated; since 5week, the positive expression was very low; there was a statistically significant difference between each week ($P < 0.05$). The expressions of EA-1 and EA-2 groups were remarkably higher than that of sham group ($P < 0.05$).

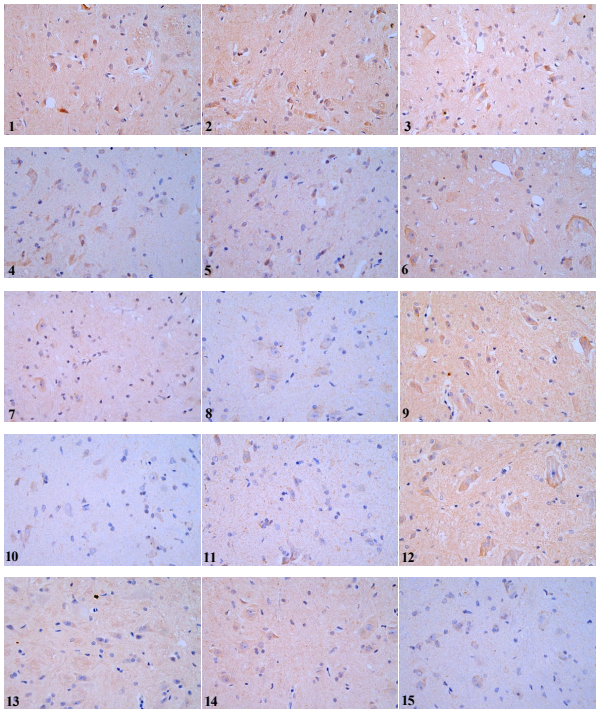

Figure 4: Expression of PKA protein in each group 400 × Notes：1. Sham group-1week ； 2. EA-1 group-1week ； 3. EA-2 group-1week；4. Sham group-2week；5. EA-1 group-2week；6. EA-2 group-2week ； 7. Sham group-3week ； 8. EA-1 group-3week；9. EA-2 group-3week；10. Sham group-4week；11. EA-1 group-4week ； 12. EA-2 group-4week ； 13. Sham group-5week；14. EA-1 group-5week；15. EA-2 group-5week

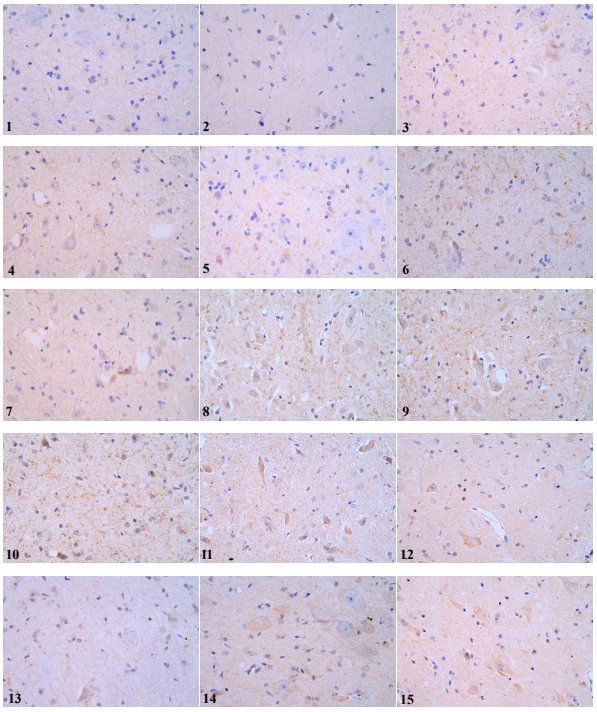

Figure 5: Expression of Synapsin I protein in each group 400 × Notes：1. Sham group-1week；2. EA-1 group-1week；3. EA-2 group-1week；4. Sham group-2week；5. EA-1 group-2week；6. EA-2 group-2week ； 7. Sham group-3week ； 8. EA-1 group-3week；9. EA-2 group-3week；10. Sham group-4week；11. EA-1 group-4week ； 12. EA-2 group-4week ； 13. Sham group-5week；14. EA-1 group-5week；15. EA-2 group-5week.

As observed from Figure 5, the positive expression of synapsin I protein was located in the cell membrane and cytoplasm. From the first week, the positive expression was gradually up-regulated; in the fourth week, the positive expression reached the peak; in the fifth week, the positive expression was progressively down-regulated; there was statistical significance in the expression difference between each week ($P < 0.05$). The expressions of EA-1 and EA-2 groups were evidently higher than that of sham group ($P < 0.05$). As compared with EA-1 group, the expression of synapsin I in EA-2 group was slightly higher than that in EA-1 group, marking no noticeable difference.

As observed from Figure 5, the positive expression of synapsin I protein was located in the cell membrane and cytoplasm. From the first week, the positive expression was gradually increased; in the fourth week, the positive expression reached the peak; in the fifth week, the positive expression was progressively down-regulated; there existed statistical significance in the expression difference between each week ($P < 0.05$). The expressions of EA-1 and EA-2 groups were obviously higher than that of sham group ($P < 0.05$). As compared with EA-1 group, the expression of synapsin I in EA-2 group was slightly higher than that in EA-1 group, marking no significant difference.

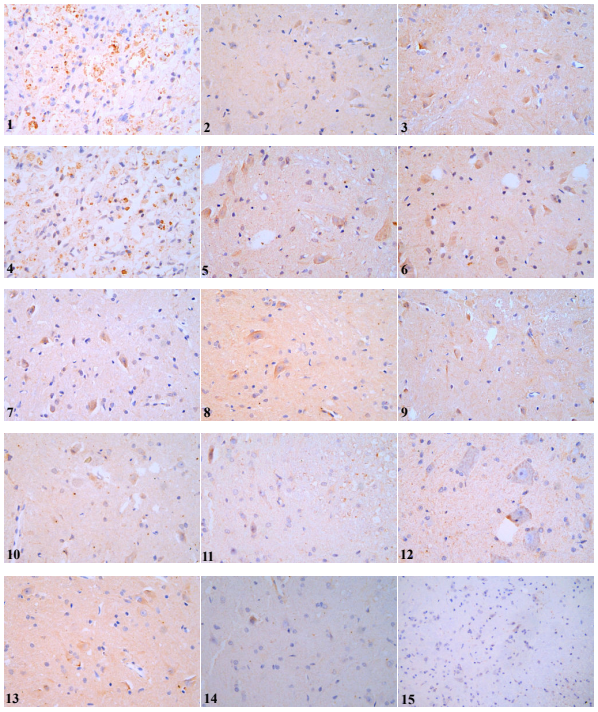

Figure 6: Expression of CaMKII protein in each group 400 × Notes： 1. Sham group-1week; 2. EA-1 group-1week; 3. EA-2 group-1week; 4. Sham group-2week; 5. EA-1 group-2week; 6. EA-2 group-2week; 7. Sham group-3week; 8. EA-1 group-3week; 9. EA-2 group-3week; 10. Sham group-4week; 11. EA-1 group-4week; 12. EA-2 group-4week; 13. Sham group-5week; 14. EA-1 group-5week; 15. EA-2 group-5week.

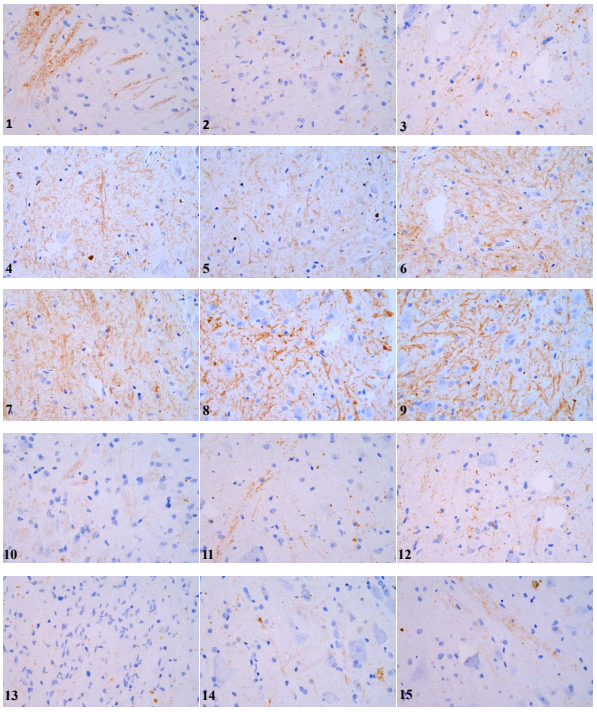

Figure 7: Expression of BDNF protein in each group 400× Notes： 1. Sham group-1week; 2. EA-1 group-1week; 3. EA-2 group-1week; 4. Sham group-2week; 5. EA-1 group-2week ; 6. EA-2 group-2week; 7. Sham group-3week; 8. EA-1 group-3week; 9. EA-2 group-3week; 10. Sham group-4week; 11. EA-1 group-4week; 12. EA-2 group-4week; 13. Sham group-5week; 14. EA-1 group-5week; 15. EA-2 group-5week.

As observed from Figure 7, BDNF falls to the category of secretory protein, and the positive expression was located outside the cell. As spinal cord injury was prolonged, the positive expression of protein was up-regulated gradually; in the third week, the positive expression was up-regulated to the highest; since the fourth week, the positive expression has been decreased progressively; in the fifth week, the expression was down-regulated to the lowest; there existed a significant difference between each week ($P < 0.05$). The expressions of EA-1 and EA-2 groups were noticeably higher than that of sham group ($P < 0.05$).

RT-PCR Analysis

As spinal cord injury was prolonged, the expression of HIF-1α gene mRNA was progressively down-regulated; it decreased to the lowest level at 4 weeks and rose at 5 weeks. The expression of HIF-1α gene mRNA of 1-week EA-2 and EA-1 groups was lower than that of sham group, and after 2 weeks, such expression of EA-2 and EA-1 groups was higher than that of sham group. As spinal cord injury was prolonged, VEGF gene mRNA was higher than that of sham group. The expression of VEGF gene mRNA in EA-2 and EA-1 groups was higher than that in sham group, and EA-1 group could facilitate the expression of VEGF gene mRNA. The expressions of PKa, synapsin I and BDNF gene mRNA in sham group were up-regulated first and then down-regulated over time. The overall expression of Ming and EA-1 groups was higher than that of sham group, and EA treatment could up-regulate the expressions of PKA, synapsin I and BDNF gene mRNA. Moreover, the expression of CaMKII gene mRNA in sham group was up-regulated first and then down-regulated over time, the overall expression of EA-2 and EA-1 groups was lower than that of sham group, and EA treatment could suppress CaMKII gene mRNA from being expressed Figure 8.

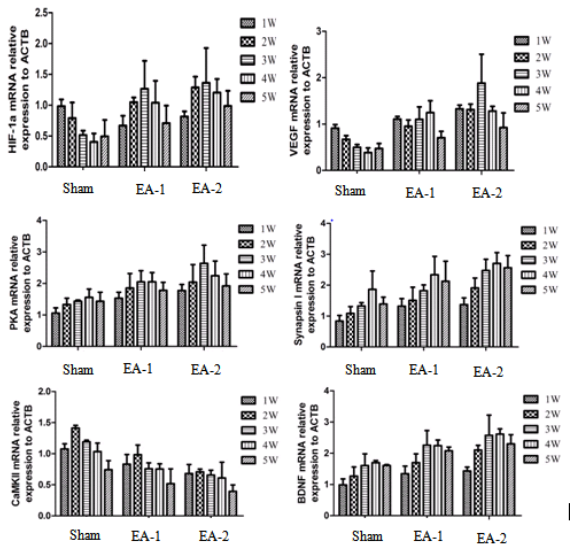

Figure 8: mRNA expression of each factor.

Western blot Analysis

EA treatment was capable of facilitating the expressions of HIF-1α, VEGF, PKA, synapsin I and BDNF regulatory proteins, as well as inhibiting CaMKII protein from being expressed. The overall expression was regulated by related mRNA, and its expression trend complied with that of related factors Figure 9.

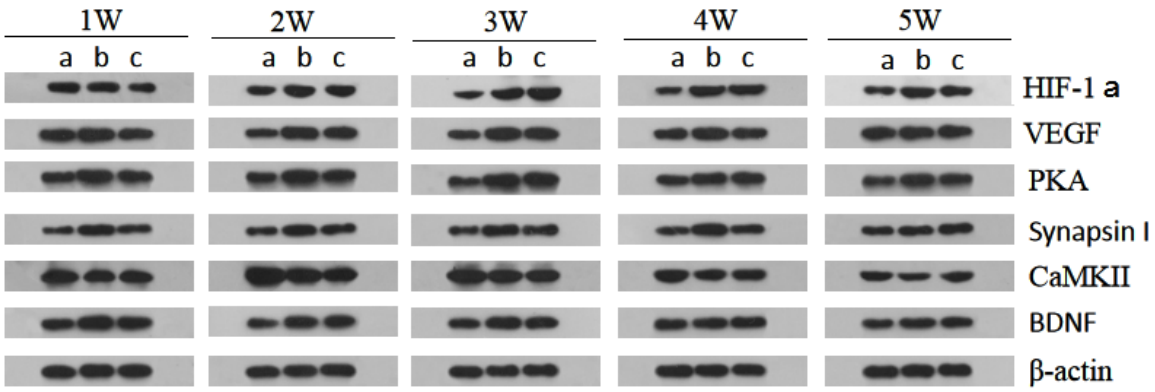

Figure 9: Protein expression of each factor, a represents sham group; b represents EA-1 group; C represents EA-2 Group.

Discussion

Spinal cord injury, as a medical hotspot worldwide, has been arousing huge attention from researchers and clinical scholars. With the discovery of endogenous neural stem cells, nerve regeneration can be achieved [16, 17]. Nevertheless, regeneration has sophisticated physiological and pathological mechanisms, leading to injury ischemia, hypoxia and edema reactions after trauma [18], and subsequently causing axonal demyelination, glial cell proliferation, neuron necrosis, etc. Eventually, cavities or scars are formed at the injured site [19], thereby forming microenvironment that adversely affects nerve regeneration. Accordingly, the reconstruction of regeneration microenvironment should be urgently solved for spinal cord nerve regeneration and recovery.

After spinal cord injury occurs, the nerve is under oxidative stress after ischemic injury, the body's antioxidant balance will be destroyed [20], ROS will be accumulated in large quantities, considerable ROS will be generated; however, the body's antioxidant mechanism cannot be balanced, and the generated ROS break cell membranes and mitochondria [21-23], Ca2+ homeostasis is unbalanced, numerous Ga2+ ions flow in to activate CaMK II[24]. The results of this study revealed that after the subjects had spinal cord injury, CaMKII rose first and then declined, while the decrease trend of CaMKII in EA treatment group was noticeably faster than that in Sham group. The overall expression of EA-2 and EA-1 groups was lower than that of Sham group. EA treatment was capable of suppressing the expression of CaMKII. The expression of CaMKII was down-regulated; subsequently, Ga2+ influx in cells was reduced, ATP consumption was lowered, and cell oxygen consumption was reduced, and neuronal ischemia was improved [25, 26]. In the meantime, the results of this study suggested that the overall mRNA and protein expressions of EA-2 and EA-1 groups PKA, Synapsin I, BDNF were higher than Sham

group. EA treatment could promote the mRNA and protein expressions of PKA, Synapsin I, BDNF. Research suggested that PKA is a ubiquitous protein kinase participating in phosphorylation of HIF-lα under intermittent hypoxia conditions [27]; it can stimulate the combination of co-activator p300 and HIF-1αand enhanced the transcription activity of HIF-lα [28, 29]. HIF-1α enhanced the oxygen carrying capacity of red blood cells, promoted erythropoiesis, alleviated tissue hypoxia, improved oxygen supply and blood circulation in ischemic regions, stimulated neovascularization, and lastly repaired damaged nerve tissue [30, 31]. As suggested by the results of this study, the expression of HIF-1α in EA treatment group was remarkably higher than that in Sham group. As a downstream gene of HIF-1α, the expression of VEGF was directly associated with HIF-1α; it acted as a critical factor for promoting angiogenesis. By promoting angiogenesis, the local blood oxygen microenvironment was improved to maintain the stable oxygen environment in vivo [32, 33]. Given the results of this study, it was reported that the expression of VEGF in EA-stimulated EA-1 group and foot Yangming Group was evidently higher than that in Sham group. Synapsin I widely exists in the presynaptic membrane and critically impacts the signal transmission between neurons; it is a vital marker of neurotransmitter release and synaptic strength between neurons [34], and also significantly impacts the regeneration and extension of synapses[35]. In the meantime, BDNF has the function of nourishing nerve. It accelerates the recovery of injured nerve by activating endogenous neural stem cells (ENSCs), reducing glial scar formation, as well as accelerating nerve axon growth [36, 37]. As revealed by the results of this study, EA treatment could promote the expression of mRNA and protein of Synapsin I and BDNF. Besides, pathological results suggested that EA treatment obviously increased the number of capillaries as compared with Sham group, the amount of hemorrhage was reduced, and neuron cell count results revealed that the number of neurons in the same period was noticeably higher than Sham group, which proved that EA treatment therapy could facilitate the recovery of cell neurons.

At the same time, the BBB scores of the mice were measured. By macroscopic observation of the lower limb function of the rats, it was reported that the lower limb function scores of the three groups of rats began to gradually recover over time. This study reported that the mentioned result might be part of the central nervous system self-recovery mechanism, and the 1 ~ 5 weeks BBB scores of the EA-1 and EA-2 groups rats increased noticeably faster than that of Sham group, marking statistically significant differences ($p<0.05$). In the meantime, EA-2 and EA-1 groups were reported to have higher neurological function scores than EA-1 group only in 1 ~ 3 weeks ($P<0.05$), and no statistical difference existed at other time points. EA treatment on stomach meridian of foot Yangming had relatively better therapeutic effect than EA-1 group, whereas its mechanism was not clear, which is also the subsequent research to be conducted by our research group. The key of this study is to use BBB score to prove that whether EA treatment can promote the recovery of lower limb function in rats after SCI, and to verify macroscopically that whether EA treatment can promote the regeneration and recovery of spinal cord nerve after the subjects had spinal cord injury.

Conclusion

In conclusion, EA treatment is capable of reducing the consumption of cellular ATP, improving the oxygen consumption in the injured area to promote angiogenesis and blood circulation, enhancing the oxygen microenvironment in the injured area to facilitate the recovery of spinal cord cells and neurons, and finally perform the function of regeneration and recovery of spinal cord nerves by regulating six factors associated with HIF-1α/VEGF signal pathway, namely, CaMKII, PKA, HIF-1α, VEGF, Synapsin I and BDNF.

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