ACUPUNCTURE RESEARCH

Effects of Electro-Acupuncture at Zusanli, Guanyuan for Sepsis Patients and Its Mechanism through Immune Regulation*

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ABSTRACT **Objective**: To evaluate the effect of electro-acupuncture on Zusanli (ST 36), Guanyuan (RN 4) in patients with sepsis, and explore its mechanism in term of immune regulation. **Methods**: In this prospective randomized controlled trial, 60 patients with sepsis were randomly assigned to the control group and the intervention group equally by block randomization. Patients in the control group received routine treatment and those in the intervention group received electro-acupuncture at bilateral Zusanli and Guanyuan in addition to routine treatment, respectively. The mortality at 28 days, Acute Physiology and Chronic Health Evaluation (APACHE)-Ⅱ score were compared to evaluate the effect, and the levels of T cell subsets (CD3+, CD4+, CD8+, CD4+/CD8+) and monocytes of human leukocyte antigen (HLA)-DR using flow cytometry were compared to explore the mechanism of this combined treatment. **Results**: Fifty-eight patients completed the trial with 29 in each group. There was no significant difference of mortality in the 28th day between the two groups, with 5 death of 29 patients in the intervention group (17.2%) and 9 of 29 in the control group (31.0%). After treatment, APACHE-Ⅱ score of both groups was significantly decreased, however, score of the intervention group was lower than the control group (13.28±7.07 *vs*. 17.10±5.83; *P*<0.01). The levels of CD3+, CD4+, CD8+ and CD4+/CD8+ ratio of the intervention group improved after treatment and were higher than the control group (59.71%±11.94% *vs*. 52.54%±11.86%; 36.46%±7.60% *vs*. 31.58%±10.23%; 18.40%±8.82% *vs*. 23.07%±7.30%; 2.38±1.14 *vs*. 1.54±0.80, respectively; all *P*<0.05). The expression of HLA-DR significantly increased after treatment in the intervention group than that in the control group (7.28%±9.26% *vs*. 1.27%±7.00%; *P*<0.01). **Conclusion**: Electro-acupuncture at Zusanli and Guanyuan could improve clinical curative effect in patients with sepsis, which might be achieved by regulation of the immune system.

KEYWORDS sepsis, electro-acupuncture, ST36, RN4, immunohematology, clinical trial

Sepsis is a systemic inflammatory response syndrome (SIRS) caused by infection, which is one of the most serious diseases in intensive care unit (ICU) and would develop into septic shock, multiple organ dysfunction syndrome (MODS) without early treatment. According to the recent statistics literature, mortality rate of sepsis has grown up to 2% per year in the United States.[1] Sepsis is the main cause of death in ICU. Despite the large number of clinical managements were used in the treatment of sepsis, the mortality rate remains high. In recent research, immune dysfunction was found to be an important pathological process of sepsis in addition to excessive inflammation, which led to the imbalance of pro-inflammatory and anti-inflammatory.[2] It is one of the important reasons for the development of severe sepsis and MODS. Acupuncture is one of the important treatments of Chinese medicine. A large number of experimental and clinical researches confirmed that electro-acupuncture treatment could bi-directionally regulate immune system.[3,4] However, few clinical trials explored whether electro-acupuncture can improve the immune dysfunction in sepsis patients. Therefore, this study was conducted to observe the effect of electro-acupuncture at Zusanli (ST 36), Guanyuan (RN 4) and its mechanism in term of immune regulation in sepsis patients.

METHODS

Diagnostic Criteria

Sepsis was diagnosed according to the new criteria

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established on the 2001 international meeting, which was defined as infection plus systemic inflammatory response syndrome.[5] Infection can be diagnosed based on confirmed presence of bacteria or highly suspicious infected lesions; and SIRS can be diagnosed when there are more than two signs following: (1) temperature> 38 ℃ or <36 ℃, (2) heart rate>90 beats/min, (3) respiratory rate>20 beats/min or $PaCO_2$<32 mm Hg, (4) white blood cell count>12×10^9/L or <4×10^9/L or immature granulocytes>10%, when excluding other reasons which could cause abnormal white blood cell count, such as chemotherapy, leukemia.

Inclusion Criteria

The male or female patients with definite sepsis according to above diagnostic criteria and with ICU duration longer than 72 h could be enrolled. All patients have signed informed consents before enrollment.

Exclusion Criteria

The patients meeting the following criteria were excluded: (1) patients with autoimmune diseases such as systemic lupus erythematosus, viral hepatitis and human immunodeficiency virus infection; (2) patients had used immunosuppressive drugs within 3 months; (3) pregnancy or lactating females; (4) those with history of psychiatric.

Ethical Review

The study protocol was approved by the institutional ethics committee of Guangdong Provincial Hospital of Traditional Chinese Medicine, and all subjects signed the informed consent before inclusion. Registration number: B2012-27-01.

Patients and Grouping

All subjects were enrolled from in-patient department and were observed in ICU of Guangdong Provincial Hospital of Traditional Chinese Medicine, Guangzhou Higher Education Mega Center Hospital from May 2013 to February 2014. The block randomization design and code distributed in equal ratio were adopted. The random number table was generated by a computer and saved in sealed envelope.

The sample size was estimated with the non-inferiority test. We determined the type Ⅰ error to be α=0.05 (single side) and the type Ⅱ error to be β=0.10. There were 30 patients in each group expected.

Treatment

Selection of acupuncture point: Zusanli is located one finger breadth lateral to inferior end of tibial tuberosity and three inches (four fingers) down of the knee eye. Guanyuan is located 3 inches below the belly button.

The control group was given conventional treatment (anti-infection, nutritional support, fluid management, mechanical ventilation and other therapeutic method) referenced to "sepsis treatment guidelines" which was formulated by Sepsis Salvation Movement (Surviving Sepsis Campaign, SSC) formulation initiated by American Association of Critical Care (SCCM), the European Association of Critical Care (ESICM) and the International Sepsis Forum (ISF) at 2008.[6] The therapeutic method excluded immune suppression or immune enhancers, including gamma globulin.

In addition to the conventional treatment as control group, electro-acupuncture was given to the intervention group, twice a day for a week. A qualified Acupuncturist carried out electro-acupuncture treatment for 30 min each time. Acupuncture needles of 0.35 mm size were used at Zusanli and 0.25 mm size were used at Guanyuan. The needles were inserted into the specific points on the surface of skin (10–30 mm depth) in the intervention group and a Deqi sensation was ensured in this group. The needles were attached to flexible wires connected by crocodile clips of electro-acupuncture instrument (Model: G6805-A, manufactured by Shanghai Huayi Medical Instrument Co., Ltd., China). The frequency was adjusted to continuous wave and the maximum intensity of current to the patients can be tolerated (local muscle twitching or patients can tolerate).

Measurements

The primary outcome included acute physiology and chronic health evaluation (APACHE)-Ⅱ scores and mortality at 28 days.[7] The secondary outcome include the level of T cell subsets (CD3+, CD4+, CD8+, CD4+/CD8+) and monocytes of human leukocyte antigen DR (HLA-DR) in patients at the 3rd day and 7th day.

Materials

CD45-fluorescein isothiocyanate (FITC)/CD4-phycoerythrin (PE)/CD3-phycoerythrin cyanin (PC)5/CD8-energy coupled dye (ECD) four-color fluorescence

standard flow type antibody were manufactured by Beckman company, USA. anti-human HLA-DR FITC was manufactured by eBioscience Inc, USA. IgG26-FITC/IgG1-PE/IgG1-PC5/IgG1-ECD isotype control antibody were manufactured by BECKMAN company, USA. IgG1-FITC isotype control antibody was manufactured by eBioscience Inc, USA. Optilyse C hemolysin was manufactured by BECKMAN company, USA. Phosphate buffered solution (PBS) was manufactured by HYclone company, USA.

Measurements of T lymphocyte Subsets and HLA-DR Expression

At the end of the treatment, peripheral venous blood was collected from patients. The expression rate of lymphocyte T or HLA-DR were determined using 50 μL of anti-coagulated blood sample according to the protocol. In brief, either 10 μg/mL antibody to CD45-FITC/CD4-PE/CD3-PC5/CD8-ECD were incubated with the blood samples for 15 min, or antibody to IgG1-FITC/IgG1-PE/IgG1-PC5/IgG1-ECD as isotype control at the same requested condition. Analogously, the level of HLA-DR was determined by immune-blotting the parallel sample with anti-human HLA-DR FITC in tris-buffered saline and Tween-20 (TBS-T) buffer at 4 ℃ for 15 min. In contrast, anti-IgG1-FITC isotype control antibody was incubated with the isotype control sample. After severe concussion, all blood samples were hemolysis for an additional 2 min with optilyse C hemolysin. After the rupture of red blood cells and clarification, PBS were blended with the samples. The positive expression rates of CD3+, CD4+, CD8+ in T lymphocytes as well as HLA-DR were detected using a flow cytometry (Model: Cytomics FC500, manufactured by Beckham Coulter company, USA). Correspondingly, the percentage of HLA-DR was determined by the lymphocyte count via FS/SS (forward scattered/side scattered light) chart.

Statistical Analysis

The measurement data were expressed as mean ± standard deviation ($\bar{x} \pm s$). Statistical analysis was performed with SPSS 18.0. The measurement data were tested by using paired *t* test, or using Mann-Whitney U test if variance was heterogeneous. If the measurement data were obtained from both groups at different points in time (≥3), the test used repeated measures analysis of variance or multivariate analysis of variance. Two-side variability test was used, with $P<0.05$ being regarded as statistically significant.

RESULTS

Subjects Enrollment

Sixty patients were enrolled in this study, with 30 patients in each group, and 58 patients completed this clinical trial. The analysis was performed with 29 cases in each group (Figure 1).

General Information

There were no significant difference in gender and age between the two groups ($P>0.05$). There were no significant differences in infected organs, source of infection and preexisting conditions between the two groups ($P>0.05$; Table 1).

APACHE-Ⅱ Scores

The difference of APACHE-Ⅱ scores of the two groups before treatment was not statistically significant

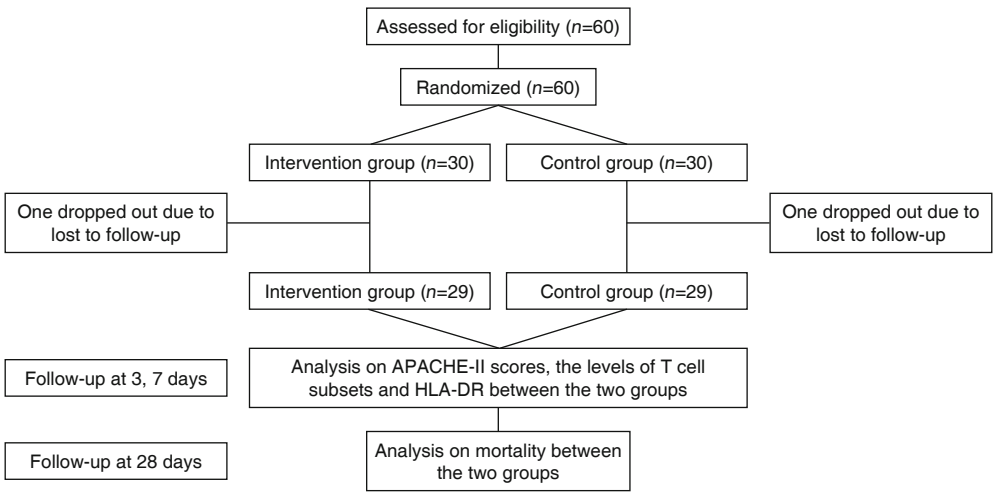

Figure 1. Flow Diagram of the Study in Sepsis Patients

Table 1. Comparison of Clinical Characteristics between the Two Groups (Case)

Item	Intervention (29 cases)	Control (29 cases)	*P* value
Male/Female	20/9	17/12	0.41
Age (Year, $\bar{x} \pm s$)	72.55 ± 12.84	71.17 ± 12.25	0.68
Number of infected organs	1.59 ± 0.68	1.62 ± 0.49	0.83
Lung	25	22	0.32
Circulation	7	11	0.26
Blood	4	8	0.20
Liver	2	2	1
Kidney	7	3	0.26
Source of infection			
Lung	27	22	0.07
Blood	7	7	1
Abdomen	0	2	0.15
Urinary tract	5	3	0.45
Skin	0	1	0.31
Other	0	0	1
Preexisting conditions			
Diabetes	3	7	0.16
Hypertension	21	17	0.27
Ischemic heart disease	7	5	0.52
Stroke	11	15	0.29
Tumor	5	9	0.22

(*P*>0.05). APACHE-Ⅱ scores of both groups at the 3rd day and 7th day of treatment were higher than those before treatment (*P*<0.05). APACHE-Ⅱ scores of the intervention group was higher than the control group (*P*<0.01; Table 2).

Mortality at the 28th Day after Onset

There were 5 patients died and 24 survived in the intervention group, while 9 died and 20 survived in the control group. The mortality at the 28th day of the intervention group was lower than the control group (5/29 *vs*. 9/29) without significant difference (*P*>0.05).

Table 2. Comparison of APACHE-Ⅱ Scores between Groups (Score, $\bar{x} \pm s$)

Group	Case	Before treatment	3rd day of treatment	7th day of treatment
Control	29	19.93 ± 5.79	18.03 ± 4.83*	17.10 ± 5.83*
Intervention	29	17.79 ± 6.67	15.66 ± 7.38*	13.28 ± 7.07*△

Notes: *P<0.05, compared with before treatment in the same group; △P<0.01, compared with the control group at the same time

Comparison of T Lymphocyte Subsets between Groups

The levels of CD3+, CD4+,CD8+ and CD4+/CD8+ ratio of the two groups showed no significant difference(*P*>0.05). At the 7th day of treatment, the levels of CD3+, CD4+ and CD4+/CD8+ ratio of the intervention group were increased significantly (*P*<0.01); the levels of CD8+ of control group were decreased (*P*<0.05); the levels of CD3+, CD4+, CD8+ and CD4+/CD8+ ratio of the intervention group was higher than the control group (*P*<0.05; Table 3).

Comparison of Expression of HLA-DR between Groups

The expressions of HLA-DR in both groups increased after treatment. The expression of HLA-DR of the intervention group at 7th day of treatment was better than those before treatment (*P*<0.05); improvement of expression of HLA-DR in the intervention group was higher than that in the control group at 7th day of treatment (*P*<0.01; Table 4, Figure 2).

DISCUSSION

Sepsis is the main cause of death in ICU. APACHE-Ⅱ score is an indicator of severity of sepsis, The higher scores represents the heavier disease.[8] Our study found that electro-acupuncture reduced APACHE-Ⅱ score of patients with sepsis,

Table 3. Comparison of T Lymphocyte Subsets between Groups (%, $\bar{x} \pm s$)

Group	Case	Time	CD3+	CD4+	CD8+	CD4+/CD8+
Control	29	Before treatment	50.66 ± 19.71	29.91 ± 14.62	18.86 ± 8.70	2.19 ± 1.99
		3rd day of treatment	52.80 ± 13.90	32.44 ± 8.87	20.18 ± 9.48	2.04 ± 1.20
		7th day of treatment	52.54 ± 11.86	31.58 ± 10.23	23.07 ± 7.30*	1.54 ± 0.80
Intervention	29	Before treatment	49.88 ± 11.78	28.50 ± 8.20	20.35 ± 9.73	1.79 ± 1.06
		3rd day of treatment	51.44 ± 13.33	29.02 ± 12.13	19.85 ± 9.18	1.77 ± 0.99
		7th day of treatment	59.71 ± 11.94**△	36.46 ± 7.60**△	18.40 ± 8.82△	2.38 ± 1.14**△

Notes: *P<0.05, **P<0.01, compared with before treatment in the same group; △P<0.05, compared with the control group at the same time

Table 4. Comparison of HLA-DR Expression between Groups (%, $\bar{x} \pm s$)

Group	Case	Before treatment	3rd day of treatment	7th day of treatment	Changes after treatment
Control	29	36.26 ± 7.17	36.51 ± 8.93	37.53 ± 9.98	1.27 ± 7.00
Intervention	29	37.46 ± 9.62	36.89 ± 11.93	44.74 ± 11.22*△	7.28 ± 9.26△△

Notes: *P<0.05, compared with before treatment in the same group; △P<0.05, △△P<0.01, compared with the control group at the same time

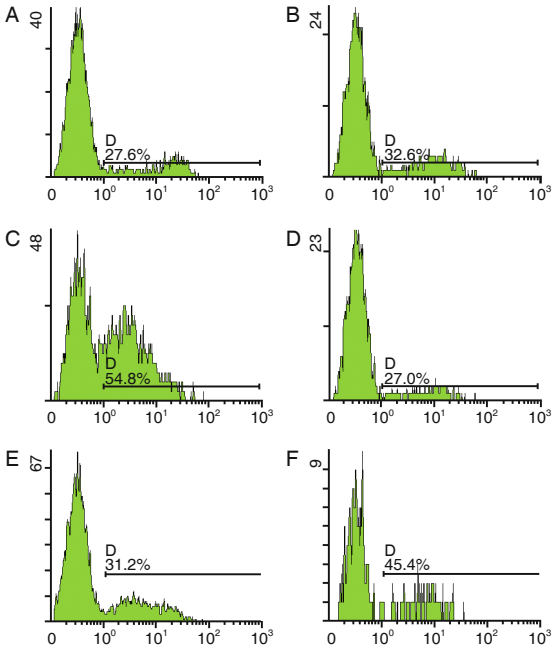

Figure 2. Comparison of HLA-DR Expression by Flow Cytometry

Notes: A: intervention group before the treatment; B: intervention group at the 3rd day of treatment; C: intervention group at 7th day of treatment; D: control group before the treatment; E: control group at the 3rd day of treatment; F: control group at 7th day of treatment

which suggested that electro-acupuncture at Zusanli and Guanyuan has clear curative effect at sepsis.

Patients with severe sepsis often display severely compromised immune dysfunction. During sepsis, not only the apoptosis of lymphoid and myeloid cells increased which depletes critical components of the immune system, but also the function of the remaining immune cells significantly decreased. Studies in animals and humans suggested that the immune defects occurred during sepsis may be critical to its pathogenesis and subsequent mortality.[9] Previous clinical study has confirmed that acupuncture could bi-directionally regulate the nerve-endocrine-immune network in the human.[10] T lymphocytes are important cells in the immune system, which not only serves as the cell-mediated immune effector cell, but also as immune regulating cell. According to the expression of different CD molecule, T-lymphocyte cells are divided into CD3+, CD4+, CD8+, etc. The formation of CD3 molecule T-lymphocyte receptor complexes can induce the activation of T-lymphocyte. CD4+ T-lymphocytes was one member of T helper/inducer (Th/Ti) lymphocyte subsets, which has a core position in the immune response. CD8+ T-lymphocytes, as a member of T suppressor/cytotoxic (Ts/Tc) lymphocyte subsets, plays a negative role in the immune response. It also can inhibit cellular immune function in sepsis.[11] Therefore, the CD4+/CD8+ ratio is generally considered as a good parameter reflecting the host immune balance. As shown by the above results, patients with sepsis were of low levels of CD3+, CD4+ before the treatment, which indicates the immune dysfunction of these patients, and after electro-acupuncture treatment, the expression of CD3+ and CD4+ was up-regulated significantly, CD8+ decreased, CD4/CD8+ ratio was significantly higher in intervention group, all of which suggested the electro-acupuncture at the acupoints of Zusanli and Guanyuan has beneficial influences on cell immune function in patients with sepsis, and can positively regulate their immune function.

Previous study also suggested that the electro-acupuncture at Zusanli and Guanyuan in rats may increase the rate of CD4+ cells, and reduce the ratio of CD8+ cells, thus improve the ratio of CD4+/CD8+, which is consistent with the results of this study.[12] The mechanism of acupuncture can raise the immunity of the body may be closely associated with the increased synthesis and release of substance P and vasoactive intestinal peptide in the pituitary gland and peripheral blood, facilitating modulation of the nerve-endocrine-immune regulatory network.[13] This regulation in human physiology were recognized as the balance physiological functions between yin and yang and between qi and blood in the human body according to Chinese medicine.

Todd, et al[14] found HLA-DR could mediate not only the process of cells recognition and phagocytosis on LPS, but also a series of post-inflammatory factor release, which is a key effector molecules in the process of cellular

immunity. Previous study also demonstrated that the decreased expression of HLA-DR was an important indicator of immune suppression in sepsis.[15] Our study, as well, found that HLA-DR expression levels significantly increased after electro-acupuncture treatment, which indicated the electro-acupuncture at Zusanli and Guanyuan can up-regulate the expression of HLA-DR.

It was also elucidated in a previous study that immune stimulation treatment improved prognosis of patients with sepsis with reversing immune suppression.[16] And this study reflected that the electro-acupuncture at Zusanli and Guanyuan has obvious clinical effect in patients with sepsis and its mechanism may be involved in immune stimulation, so as to improve low immune status and correct immune disorders in patients with sepsis. Previous animal studies showed that the immune indexes of sham electro-acupuncture group were not significant different compared with control group.[12,13] Considering the compliance and stability of sham electro-acupuncture, our study did not set up sham electro-acupuncture group.[17]

Whereas this study still exist limitations, such as the small sample size. Therefore, future research with larger sample size is warranted to further identify this effect.

Conflict of Interests

The authors declare that they have no conflict of interests.

Author Contributions

Yang G, Deng AJ and Li J were involved in study design and writing the manuscript. Yang G, Hu RY and Huang Y contributed study conduct and data analysis. All authors read and approved the final manuscript.

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