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Original Article

Effects of acupuncturing Pishu combined with Ginsenoside Rg3 on the immune function of rats with chronic fatigue

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Abstract: Objective: This study was designed to investigate the effects of acupuncturing Pishu combined with Ginsenoside Rg3 on the immune function of rats with chronic fatigue. Methods: Forty male SD rats were equally randomized into control group, chronic fatigue system group (CFS), Ginsenoside Rg3 (Rg3) group, acupuncture group and acupuncture combined with Ginsenoside Rg3 (A+Rg3) group. Rats with chronic fatigue were established by bounding and forced swimming in cold water once daily for 21 days except control group, then the rats in the acupuncture and A+Rg3 group were treated by manual acupuncture stimulation of bilateral "Pishu" once daily for 7 days. Ginsenoside Rg3 was administered by intravenous to the rats of the A+Rg3 and Rg3 group for 7 days in dosages of 2 mg/kg body weight, and two markers of physical fatigue were evaluated: body weight and blood lactic acid (LA). The percentages of $CD3^+$ lymphocytes, $CD4^+$ lymphocytes, and $CD8^+$ lymphocytes in the spleens of the rats were evaluated using flow cytometric analysis. Serum IFN-gamma (IFN-γ) and IL-4 contents were detected by ELISA. Results: Increased body weight and reduced blood LA concentrations were found in the rat of Rg3 group and A+Rg3 group than that in CFS group. The rat of Rg3 group and A+Rg3 group also showed a significant increase in the percentage of $CD4^+$ lymphocytes and a significant decrease in the percentage of $CD8^+$ lymphocytes and correct $CD4^+/CD8^+$ ratio. Compared with the CFS group, the level of IFN-γ in the Rg3, acupuncture and A+Rg3 groups was reduced and IL-4 was increased. Conclusions: Acupuncture and Rg3 can improve the immune system activity of CFS rats and acupuncturing Pishu combined with Rg3 was significantly superior compared with Rg3 and acupuncture, respectively.

Keywords: Forced swimming test, fatigue, acupuncture, Ginsenoside Rg3, systemic immune response

Introduction

Chronic fatigue syndrome (CFS) is a fatigue syndrome characterized by numerous symptoms appearing in various body systems [1]. CFS was closely related with immunosuppression resulting from physical and psychological fatigues. Recent research has revealed evidence for a disturbance in the immune system of people with CFS. For example, abnormal activation of T-lymphocytes, decreased antibody dependent cell-mediated cytotoxicity, the presence of autoantibodies, reduced T-cell responses to mitogens and other specific antigens and altered cytokine profiles have been described in the patients with CFS [2]. However, the pathophysiological mechanism underlying CFS is still unclear; and current therapy, which is directed toward relieving symptoms, often causes deleterious side effects. To date, pharmacological drugs or therapies have not been effective. Recently, interest has increased in the use of natural substance supplements for the attenuation of exercise induced physical fatigue.

Acupuncture, as an important part of traditional Chinese medicine (TCM) and easily acceptable therapy, with advantages of convenience, safety, efficacy with few side effects, is commonly used in present clinics and is gradually reconsidered in many countries. Recent study indicated pulse current can elevate endurance capacity and facilitate recovery from fatigue [3]. Manual acupuncture can inhibit CFS induced reduction of serum IFN-γ level and the ratio of

IFN-γ/IL-4 in CFS rats, suggesting a favorable adjustment of acupuncture intervention for CFS by balancing the ratio of IFN-γ/IL-4 [4].

Ginseng, an ancient and famous herbal drug in traditional Chinese medicines, has been used in Chinese folklore for more than 4,000 years. Ginsenosides, which are considered to be the biologically active ingredients of ginseng, are the mixture of triterpene glycosides belonging either to the protopanaxadiol or protopanaxatriol groups. Ginsenoside tablets as positive control which is a recognized quite expensive mono-medicine to cure CFS patients in Chinese medicine market. Pharmacological studies have proved that ginsenoside Rg3 has various biological activities, including enhancing immunity [6], antifatigue [7], and anti-oxidant and neuroprotective effects [8], inducing apoptosis in multiple myeloma cells [9], prevention against vascular inflammatory disease [10], and antitumor effects [11, 12].

However, whether acupuncture stimulation of "Pishu" and Rg3 displayed immune regulatory effect in chronic fatigue remains unclear. In the current study, we examined the effects of acupuncture stimulation of "Pishu" and Rg3 in a rat model of immunologically induced fatigue. The effects of acupuncture and Rg3 on splenocyte proliferation and cytokine secretion were evaluated in chronic fatigued rats.

Materials and methods

Chemical and dose formulation

Rg3 was purchased from Beijing yingnazexin institute of chemical technology (MUST-13041211). LA, IL-4 and INF-γ test Kits were purchased from Uscn Life Science, Inc. All other chemicals were purchased from commercial sources.

Experimental animals and establishment of CFS model

Total 40 Sprague-Dawley rats (180-220 g), 6-8 week old, were purchased from Experimental Animal Centre of Liaoning University of Traditional Chinese Medicine Shenyang, China. All animals were acclimatized for at least one week prior to use and maintained in a temperature- controlled environment (23±2°C) with humidity 55%±15% and 12 h light-dark cycle with free access to water and standard rodent chow. All animals were treated in accordance with the Guidelines of the Principle of Laboratory Animal Care (NIH Publication, revised 1985).

Rats were randomly divided into five groups, including control group, chronic fatigue system group (CFS), Ginsenoside Rg3 (Rg3) group, acupuncture group and acupuncture combined with Ginsenoside Rg3 (A+Rg3) group. Rats were established by bounding and forced swimming in cold water once daily for 21 days except control group.

Cold-Water-Swim: The rats were placed individually into a container that was 90 cm high and 60 cm in diameter containing 45 cm of water at 12°C. The depth of the water was adequate to prevent the animals from touching the bottom of the floor with their tails. Swimming endurance capacities were assessed by forcing rats to swim to exhaustion, which was defined as the point at which the rat's nose remained below the water surface for 10 s.

Restraint-stress: After the Cold-Water-Swim, the rats were placed individually in PVC tubes (20.0 cm in length, 5.0 cm in diameter) for 1 h every day. The front wall of each tube was perforated, so that rat could breathe. The rat's tail extended through the rear door of tube and was fixed to the tube by adhesive tape. The experiment was performed once daily for a period of 3 weeks.

At the 22 nd day, rats in the acupuncture group were treated by manual acupuncture stimulation of bilateral "Pishu" once daily for 7 days. Rg3 was dissolved in Saline and given to rats in the respective groups by intravenous for 7 days, and those in control and CFS groups were given the same volume of vehicle alone.

Acupuncture manipulation

Rats were fastened onto an autopsy table for 15 minutes after ether inhalation. Stainless acupuncture needles (12 mm × 7 mm) were inserted perpendicularly into the points of Pishu. The principle of points selection follows the standard on rats recorded in the teaching material of experimental acupuncturology [13].

Rats in the treated groups received daily applications of a pulse current for 20 min over a period of 7 days. In the acupuncture and A+Rg3 groups, frequencies of 4~20 Hz were used at

an intensity of 10 mA. We measured body weight (BW) for all rat from the start to the end of experiment. The BLA content was determined based on the lactate dehydrogenase enzymatic method and the absorbance was read at 530 nm [14].

Sample collection

Rats were deeply anesthetized with 10% chloral hydrate (3.5 mL/kg body weight) through intraperitoneal injection. Blood samples were collected in vacuum tubes from the femoral artery. Serum was centrifuged at 2,500 rpm for 15 min and stored at -80°C until use.

Preparation of spleen-derived lymphocyte populations

Single-cell lymphocyte populations were prepared from the spleen of rats. Spleen was dissociated on a steel mesh in Hanks' balanced salt solution (Beyotime, Nantong, China). The splenocyte that passed through the mesh were centrifuged at 2000 rpm for 5 min. The supernatant was discarded and cells were resuspended in the Hanks' medium. The red blood cell lysis buffer (Beyotime, Nantong, China) was added to lyse the erythrocytes. Then, the cell suspension was filtered and washed to remove cell debris. Finally, cells were resuspended in RPMI1640 [with 10% fetal bovine serum (FBS)] (Hyclone, Victoria, Australia). Cell viability was assessed using the trypan blue exclusion test and routinely found to contain <5% dead cells. The cell density was adjusted to 5×10^6 cells/mL for further assay.

Mitogen stimulated lymphocyte proliferation assay

Lymphocyte proliferation responses to mitogens (Concanavalin A, ConA) were evaluated with the CCK-8 assay. For each spleen sample, lymphocyte was placed into a U-bottom 96-well plate in triplicate at a density of 1×10^6 cells/mL with a total volume of 90 μL. 10 μL ConA (Sigma, St. Louis, MO, USA) at a concentration of 20 μg/mL was added to wells. Supplemented RPMI1640 media were added to control wells. Plates were briefly mixed on a plate shaker and then placed in a 5% CO_2, 37°C incubator for 72 h. Then, CCK-8 was added and incubated for an additional 4 h. Following incubation, spectrophotometric data were measured using an automatic microplate reader (type Anthos 2010, Anthos Labtec Instruments GmbH) at a wave length of 450 nm.

Flow cytometric analysis of T-lymphocyte subsets

One hundred microliters of Spleen sample was washed twice in phosphate buffered saline (PBS) and resuspended to form a suspension of 1×10^6 cells/mL. One hundred microliters of each cell suspension was used for flow cytometric analysis to detect lymphocyte subsets by a double staining technique. To analyse $CD3^+$ (total T-cells), $CD4^+$ (T-helper/inducer) and $CD8^+$ (suppressor/cytotoxicT) cell subsets, the cells were stained with fluorescein (FITC)-labeled anti-CD3, FITC-labeled anti-CD4, and phycoerythrin (PE)-labeled anti-CD8 (BD Bioscience, USA) after erythrocyte lysis with ACK lysis solution. As a control, additional cell suspensions were stained with rat isotope control IgG2a-PE and rat isotope control IgG3-FITC (Jingmei Genevale Technology Co., Ltd, Beijing, China). After antibodies were added, the cells were kept in the dark for 30 min at 4°C. After washing twice with PBS, the double-stained cells were analyzed with a FACScan flow cytometer (BD FACSCalibur), and the data were processed using Cell Quest software (Becton Dickinson, San Jose, CA). Values of total $CD3^+$ T, $CD4^+$ T and $CD8^+$ T cells subsets are expressed as a percentage of total lymphocytes.

Detection of IFN-γ and IL-4 in serum using enzyme linked immunosorbent assay (ELISA)

Detection was performed in accordance with the instructions of ELISA kit manufacturer (Uscn Life Science, Inc). In brief, the primary antibody was (IFN-γ and IL-4) diluted (1-10 μg/mL) in coating solution. Then, 100 μL diluted antibody was added to appropriate wells, and incubated for 2 h at room temperature. The plate was emptied, residual liquid removed, and washed twice with 300 μL 0.05% Tween-20. Then, 300 μL blocking solution was added to each well and incubated for 1 h at room temperature. Then, the plate was washed twice with 300 μL wash solution. Next, 100 μL of diluted biotinylated detection antibody was added to each well and incubated for 1 h at 37°C, then the plate was washed three times. Then, 100 μL diluted AKP conjugated streptavidin was added to each well and incubated for 1 h at room temperature. The plate was emptied and washed three times for 5 min each. The plate was then washed five more times. Then,

Table 1. Effects of acupuncture and Rg3 on body weights and LA in rat (mean ± SE)

Group	n	Initial body weight (g)	End body weight (g)	LA (ng/mL)
Control	8	296.13±7.86	353.63±13.80	3.03±0.73
CFS	8	297.00±9.62	315.63±11.98*	4.60±1.23*
Rg3	8	301.01±7.76	329.63±9.69#	4.12±1.18#
Acupuncture	8	299.90±8.42	338.63±9.93#	3.99±0.89#
A+Rg3	8	289.56±7.45	341.63±10.81#	3.67±1.14#

*P<0.05 as compared to control group, #P<0.05 as compared to CFS group.

Table 2. Lymphocyte proliferation in the spleen from rats exposed to acupuncture and Rg3 (mean ± SE)

Group	n	Stimulate index
Control	8	1.00±0.11
CFS	8	0.45±0.17*
Rg3	8	0.64±0.16#,Δ
Acupuncture	8	0.66±0.18#,Δ
A+Rg3	8	0.77±0.15#

*P<0.01 versus Control group; #P<0.01 versus CFS group; ΔP<0.05 versus A+Rg3 group.

200 μL of substrate was placed into each well and the color developed for 30 min at room temperature. Finally, 0.05 mL 2 M of H_2SO4 was added to each well, and immediately read with a plate reader at 405-410 nm.

Statistical analyses

Values are expressed as mean ± standard error. Normal distribution of all data was examined using the Shapiro-Wilk normality test. One-way ANOVA was used followed by Fisher's least-significant difference (LSD) for the homogeneity testing of variance (Levene's test), and the data were analyzed by Dunnett's T3 for the heteroschedasticity of variance test. P<0.05 was considered statistically significant. All statistical procedures were performed using SPSS 17.0 software for Windows (SPSS Inc., USA).

Results

Effect of acupuncture and Rg3 on body weight and lactic acid

CFS produced a significant reduction in the overall body weight. Rg3, acupuncture and A+Rg3 administration significantly prevented this reduction in body weight. The LA content of the Rg3, acupuncture and A+Rg3 groups was significantly lower than that of the CFS group (**Table 1**). No significant difference on body weight and lactic acid was found in the Rg3, acupuncture and A+Rg3 groups. These results indicated that acupuncture and Rg3 effectively increased body weight and delayed the increase in LA, which ultimately postponed the appearance of physical fatigue. These observations suggest that acupuncture and Rg3 have anti-fatigue effects.

Mitogen stimulated lymphocyte proliferation assay

The effects acupuncture and Rg3 on spleen-derived lymphocyte proliferation in the rats are shown in **Table 2**. The T-lymphocyte proliferation of the Rg3, acupuncture and A+Rg3 groups was significantly higher than that of the CFS group. Significant difference in T-lymphocyte proliferation ability was found in the Rg3 and acupuncture groups compared with A+Rg3 group. These observations suggest that acupuncture and Rg3 improve lymphocyte proliferation. Acupuncturing Pishu combined with Ginsenoside Rg3 exhibit optimal effects.

Effect of acupuncture and Rg3 on T-lymphocyte subset counts

The CFS rats had reduced numbers of $CD3^+$ and $CD8^+$ T-cells and had a significantly higher number of $CD4^+$ T-cells and ratio of $CD4^+/CD8^+$ T-cells compared with the control group. Rats in the Rg3 and A+Rg3 group showed an obvious increase in the number of $CD3^+$ T-cells than in the CFS group. The number of $CD4^+$ T-cells was lower in the A+Rg3 group than in the CFS group. In the Rg3, acupuncture and A+Rg3 group, $CD8^+$ T-cells were significantly higher and ratio of $CD4^+/CD8^+$ T-cells lower than in the CFS group. The number of T-cells in the A+Rg3 rats was slightly ameliorative than that observed in the Rg3 and acupuncture group, but this result did not achieve statistical significance (**Figure 1**; **Table 3**).

Influence of acupuncture and Rg3 on serum levels of IFN-γ and IL-4 in rats

Compared with those in the Control group, IFN-γ serum levels in the CFS group were increased and IL-4 was decreased. Compared with the CFS group, IFN-γ in the Rg3, acupunc-

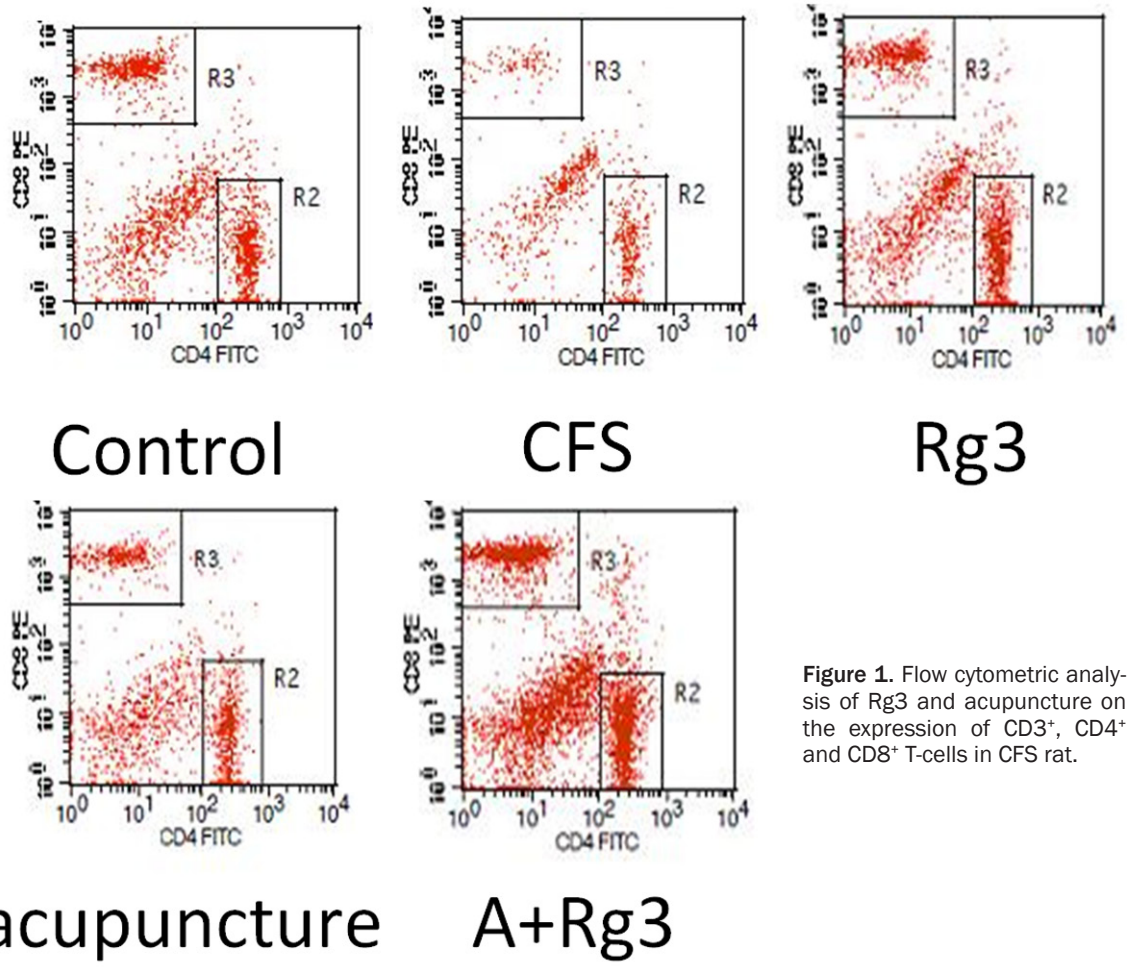

Figure 1. Flow cytometric analysis of Rg3 and acupuncture on the expression of $CD3^+$, $CD4^+$ and $CD8^+$ T-cells in CFS rat.

Table 3. Effects of acupuncture and Rg3 on $CD3^+$, $CD4^+$, $CD8^+$ T cell counts and $CD4^+$ T cell/$CD8^+$ T cell in CFS rats (mean ± SE)

Group	n	$CD3^+$	$CD4^+$	$CD8^+$	$CD4^+/CD8^+$
Control	8	61.41±7.45	32.16±3.66	29.2475±4.09	1.11±0.08
CFS	8	46.86±5.52**	37.01±6.46*	9.8512±1.48**	3.89±1.11**
Rg3	8	57.39±5.26##	35.27±3.68	22.1225±1.86##	1.59±0.10##
Acupuncture	8	53.42±8.49	34.87±5.576	18.5488±2.96##	1.88±0.07##
A+Rg3	8	55.30±5.13#	31.89±3.162#	23.4125±2.20##	1.36±0.08##

*P<0.05, **P<0.01 versus Control group; #P<0.05, ##P<0.01versus CFS group.

ture and A+Rg3 groups was reduced and IL-4 was increased. Compared with the CFS group, Th1/Th2 in Rg3, acupuncture and A+Rg3 groups was decreased, and IL-4 was reduced. Significant difference on serum levels of IFN-γ, IL-4 and IFN-γ/IL-4 was found in the Rg3 and acupuncture groups compared with A+Rg3 group. Calculation of the IFN-γ/IL-4 ratio suggested that acupuncture and Rg3 exerted implicated immunomodulation on TH1/TH2 cytokine balance (**Table 4**).

Discussion

Many CFS animal models were used to study the mechanism of CFS, such as mental stimulation, forced exercise, herbal drugs and restraint stress protocol [15, 16]. The required time for CFS model construction is always more than two weeks [17]. In this study, the time of CFS model construction was 3 weeks and two types of stimulations (Restraint-stress and Cold-water-swim) were adopted for emotional stimulation and energy consumption. In general, the swimming exercise is known to induce blood biochemical changes [18]. The muscle produces a considerable amount of lactic acid when it obtains suf-

Table 4. Cytokine levels in the serum (mean ± SE)

Group	n	IFN-γ (ng/mL)	IL-4 (ng/L)	IFN-γ/IL-4
Control	8	379.10±33.01	19.61±1.38	19.33±1.52
CFS	8	715.41±42.07*	8.62±0.83*	82.99±6.44*
Rg3	8	653.58±28.05#,Δ	10.09±0.75#,Δ	64.78±3.80#,Δ
acupuncture	8	605.45±21.92#,Δ	10.25±0.70#,Δ	59.07±3.09#,Δ
A+Rg3	8	473.60±40.48#	12.38±1.11#	38.26±3.35#

*P<0.01 versus Control group; #P<0.01 versus CFS group; ΔP<0.01 versus A+Rg3 group.

ficient energy from anaerobic glycolysis, and the increased concentration of lactic acid brings about a reduction in the pH of muscle tissue and blood, which could induce various biochemical and physiological side effects, including glycolysis and phosphofructokinase and calcium ion release, through muscular contraction [19]. Therefore, blood LA is a sensitive index of fatigue status. Our results show acupuncture and Rg3 reduced the levels of blood LA and increased body weight in fatigued rats. These results provide evidence that acupuncture and Rg3 exerts beneficial effects and can ameliorate CSF. This was consistent with the study [3, 4, 7].

Being main protectors of the host against diseases, lymphocyte activation and proliferation are vital during onslaught of infection or other pathological conditions. In this assay, Concanavalin A (ConA) are usually used to analyze the proliferation of T-lymphocyte [20]. The acupuncture and Rg3 treatment enhanced the Con A induced lymphocyte proliferation. T-lymphocytes, the vital part of the defensive immune system, are differentiated into two different subsets according to their specific membrane molecule, which are $CD4^+$ and $CD8^+$ T-lymphocytes. To further elucidate the mechanism of Rg3 and acupuncture, the effects of Rg3 and acupuncture on both $CD4^+$ and $CD8^+$ spleen T-lymphocyte populations in fatigue animals were analyzed by flow cytometric assay. Our results showed a reduction in the total T-cell number and a specific reduction in $CD8^+$ T-cells in fatigued rats, which are suppressor/cytotoxic T-cells. These results suggest that Rg3 and acupuncture play a possible role in cell-mediated immune activation in CFS. In addition, the results demonstrate that CFS results in increased $CD4^+$ T-lymphocytes in rats. Several lines of evidence have suggested that the activation of innate immune pathways plays a pivotal role in the pathogenesis of CFS. Papers reporting immunological changes in CFS are numerous but the relationship between markers of immune function and CFS remains controversial [21]. Some researcher reported that $CD4^+/CD8^+$ ratio of CFS patients were decreased [22]. However, in the present study, the results showed that $CD4^+/CD8^+$ ratio was increased in CFS rats. This was consistent with the study [23]. Our results indicate that Rg3 and acupuncture corrects the alterations in $CD4^+/CD8^+$ ratio induced by CFS. Our data suggest that Rg3 and acupuncture may result in decreased fatigue through its regulation of immune function. Acupuncturing Pishu combined with Ginsenoside Rg3 exhibit optimal effects on improving immune functions.

$CD4^+$ T-lymphocytes (helper) cells are responsible for orchestrating and directing an immunity response [24]. After activation, T helper cells can be differentiated into Th1 or Th2 cells that secrete specific subsets of cytokines. The Th1 lymphocytes characterized by the production of IL-2, IFN-γ, and TNF-α add to cellular immunity, while Th2 lymphocytes, mostly involved in humoral immunity, produce IL-4, IL-5, and IL-10. Th1 immunity defends against cancer progression and several intracellular infectious diseases, while Th2 immunity is beneficial against extracellular pathogenic infections and autoimmune diseases [25]. Therefore, the balance among Th1 and Th2 release of cytokine plays an essential role in the determination of direction and outcome of an immune response. It had been reported that many diseases are related to the change in the Th1/Th2 axis. In the present study, the typical cytokines of Th1 and Th2, IFN-γ and IL-4 were observed. We find Rg3 and acupuncture reduced IFN-γ and enhanced IL-4 levels as compared with those in CFS group. Our results showed that animals with CFS had impaired immunoregulatory balance demonstrated by a dominance of TH1 (IFN-γ) over TH2 (IL-4) cytokines secretion, and Rg3 and acupuncture counteracted the disorder TH1/TH2 profile. Acupuncturing Pishu combined with Ginsenoside Rg3 exhibit optimal effects. Our results suggest that Rg3 and acupuncture may enhance humoral immune function of CFS rats.

In conclusion, these results indicate Rg3 and acupuncture have anti- fatigue effect and can obviously elevated T cells, corrected subsets of T-cells and improve the levels of IL-4, reduce the expression of IFN-γ, and regulatory balance the coordination of Th1/Th2 cells. This suggests that the effect of Rg3 and acupuncture in CFS rats may be related to regulating the immune balance. Acupuncturing Pishu combined with Ginsenoside Rg3 was significantly superior compared with Rg3 and acupuncture, respectively. Hence, we postulate that Acupuncturing Pishu combined with Ginsenoside Rg3 treatment may be a more effective therapy for CFS.

Disclosure of conflict of interest

None.

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