

Therapeutic effect of acupuncture in BALB/c model of cutaneous leishmaniasis

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Abstract

Background: Current research findings demonstrate that acupuncture, a traditional Chinese medicine, has beneficial effects on several acute and chronic infectious and inflammatory diseases. Acupuncture promotes tissue healing and regulates immune response in various disease conditions. Cutaneous leishmaniasis (CL) is a parasitic disease caused by protozoan from genus *Leishmania*. Acupuncture is supposed to accelerate healing of CL because of common mechanisms involved in the cure of the CL lesions.

Materials and Methods: 60 BALB/c mice were experimentally infected with *L. major* strain MRHO/IR/75/ER and divided into three groups: (1) Treatment group received acupuncture 2 times a week for 5 weeks (10 sessions) with intraperitoneal diazepam as a sedative agent. (2) Diazepam control group only received diazepam the same as the treatment group. (3) Control group did not receive any intervention. Size of the lesions was measured before the experiment, on session 5 and 10 and 4 weeks after the experiment. Parasite burden was evaluated by microscopic assay as well as quantitative real-time polymerase chain reaction technique.

Results: Size of the lesions decreased significantly on session 5 in treated group in comparison with session 0 ($P = 0.02$) while the size of the lesions increased significantly in two control groups on session 5 and 4 weeks after treatment ($P = 0.04$ and $P = 0.01$ respectively). Mean parasite burden did not show a significant difference between or within groups on session 0 and 10 by any methods.

Conclusions: This investigation showed that acupuncture decreased size of the CL lesions by session 5 in the BALB/c mice model, but did not cause a significant reduction in parasite burden.

Key Words: Acupuncture, BALB/c, *Leishmania major*, real-time polymerase chain reaction

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INTRODUCTION

Acupuncture is a well-known area of complementary and alternative medicine that has been practiced in China from more than 3000 years ago. Recently a considerable interest in complementary medicine as well as acupuncture therapy is noticed all over the world possibly because of insufficiencies and side effects of

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conventional medicine.^[1] Acupuncture is the insertion of fine needles into specific body sites. According to the theory of Chinese medicine, energy and blood flow through different meridians (channels of energy flow) in the body is balanced by this manipulation.^[2] In acupuncture practice two types of points may be employed for needle insertion: (1) Ashi-points are tender points or points determined by the practitioner over the body, which can be anywhere and are unlimited. (2) Acu-points are more than 300 specific mapped sites on the body with defined properties in Chinese medicine.^[3] Growing scientific literature on acupuncture let several health related organizations to accept this method as an alternative treatment for various diseases. The World Health Organization has indicated acupuncture for more than 40 medical conditions including several acute and chronic infectious and inflammatory diseases.^[1]

Cutaneous leishmaniasis (CL) is a parasitic disease caused by a number of *Leishmania* spp. that is transmitted by the bite of sand flies. The infection is endemic in many countries. Because of long duration of the skin lesions and disfiguring scars, it is regarded as a national problem in most of the affected countries. The standard treatment is intramuscular or intralesional pentavalent antimonials; however, high rates of treatment failure and side-effects are noticed with these agents and still no superior treatment is available. Several alternative and physical therapies have been attempted to accelerate the healing of CL lesions, yet a few have been beneficial.^[4]

Previous experimental and human studies demonstrate that acupuncture efficiently promotes wound healing,^[5-7] Furthermore, it is shown that acupuncture not only enhances innate immunity, but also modulates adaptive immune response specifically T helper 1 (Th1)/Th2 balance.^[8] Acupuncture suppresses the excessive Th2 response in some Th2 dominant disorders as asthma and allergy.^[9,10] Cure of CL lesions depends upon both wound healing mechanisms and effective immune response to infection.^[11,12] Over-expression of Th2 response is known to delay healing of CL lesions,^[12] so the acupuncture might benefit CL patients by both accelerating wound healing and suppression of excessive Th2 response. However, there is no report of acupuncture treatment in human infection or in animal models of CL.

In this study, we evaluate the effect of acupuncture on healing of CL lesions in BALB/c mice. Also we investigate the effects of acupuncture on parasite burden of the lesions using the real-time polymerase chain reaction (PCR) as well as the microscopic assay.

MATERIALS AND METHODS

Animals and experimental infection

Female 6-8 weeks old BALB/c mice were obtained from Pasture Institute (Tehran, Iran). Animals were kept in the animal facility of Animal House of Medicine School, Isfahan University of Medical Sciences, Isfahan, Iran with free access to food and water. Animal care and all experiments were carried out in accordance with a protocol designed by the ethical committee related to animal rights of the department.

L. major strain (MRHO/IR/75/ER) promastigotes were grown in Roswell Park Memorial Institute (RPMI) 1640 (Sigma) supplemented with 10% fetal calf serum (FCS) (Sigma) and were harvested at stationary phase. Mice were inoculated subcutaneously at the base of their tails with 1×10^5 promastigotes suspended in 10 μ l of sterile phosphate buffered saline (PBS). Nodules and subsequently ulcers were developed in 3-4 weeks after inoculation.

60 mice with lesions, 4 weeks after parasite inoculation, were included in the study and were divided into three groups. (1) Treatment group ($n = 20$) was treated with acupuncture according to the study protocol and received intraperitoneal diazepam to sedate the animals during the procedure. (2) Diazepam control group ($n = 20$) only received diazepam the same as the treatment group. (3) Control group was kept as the other two groups without any intervention.

Acupuncture treatment

Acupuncture was performed 2 times a week for 5 weeks in the treatment group (10 sessions). Initially, mice were sedated with intraperitoneal diazepam. After shaving and cleaning of the skin around lesions with 70% ethanol, stainless needles of 0.25 mm in diameter and 30 mm in length (Suzhou Hua Tuo Medical Instruments Co., China) were introduced into the target points and were maintained for 30 min. Needles were inserted into St-36, Sp-6 and Li-11 points bilaterally and around the wound with depth of 1.5 mm [Figure 1].

Measurement of the lesions

Two perpendicular diameters of the lesions were measured by a vernier caliper primarily 4 weeks after parasite inoculation (beginning of the experiment), after session 5 and 10 and 4 weeks after the end of the experiment in three groups. Mean of the two measured diameters was recorded and used for statistical analysis.

Microscopic evaluation of parasite burden

Giemsa stained smears of the lesions were examined under high power field (HPF: $\times 1000$) and were scored

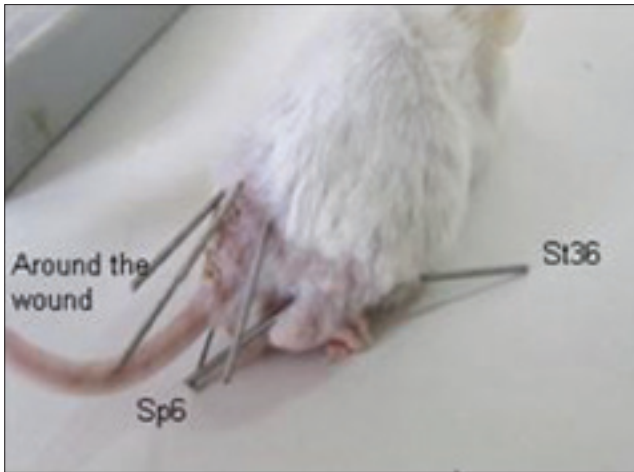


Figure 1: Acupuncture in St-36, Sp-6 points and around the wounds

from 1+ to 4+ depending on the mean number of amastigotes per HPF at the session 0 and 10 as follows:

One to 10 parasites/1000 HPF: 1+, 1-10 parasites/100 HPF: 2+, 1-10 parasites/10 HPF: 3+, and 1-10 parasites/1 HPF: 4+.

Real-time PCR quantification of parasite burden

FTA Elute™ Microcards (Whatman, USA) were used for deoxyribonucleic acid (DNA) extraction. Briefly after lesion scraping by a sterile blade, about 20 µl of serous secretion was collected and absorbed completely to FTA Elute™ Microcards. Cards were allowed to dry for 3 h at room temperature and 3 mm punches were taken from them. DNA was extracted from a 3 mm punch according to the FTA Elute™ standard protocol. 5 µl of DNA extract was used in each real-time PCR reaction. Taqman real-time PCR was performed on an Applied Biosystems (ABI) step-one real-time PCR machine in a 48-well format (ABI, Foster City, CA, USA). The cycling profile was 50°C for 2 min, 95°C for 10 min, 95°C for 10 s and 60°C for 45 s for a total of 40 cycles. A pre-designed kit by Mirhendi *et al.* (unpublished data) was used in this study. The sensitivity and specificity of the kit have been evaluated and optimized by him. In this kit, a conserved region of ribosomal DNA of *L. major* is amplified and detected. One positive control (DNA extracted from pure *Leishmania* culture) and one negative control were run with each PCR [Figure 2].

Statistical analysis

Data analyses were performed by non-parametric tests, Mann-Whitney and Wilcoxon. Data are presented as the mean value ± standard deviation of the mean. $P < 0.05$ was considered to be statistically significant.

RESULTS

Wound size was measured before treatment, after five treatment sessions, at the end of the treatment (session

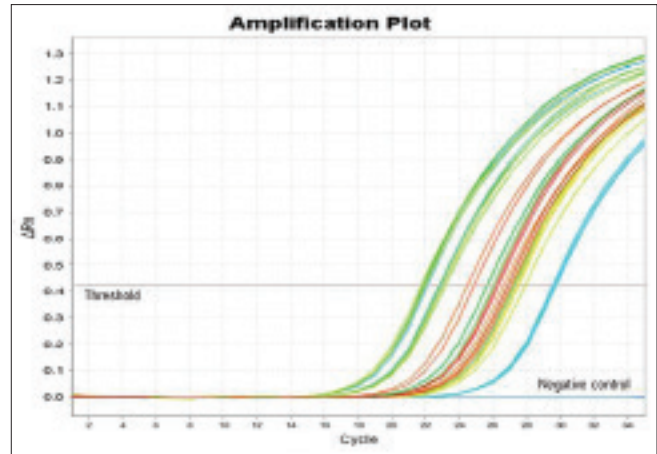


Figure 2: The amplification plot of ribosomal deoxyribonucleic acid (DNA) of *Leishmania major*. After scraping of the cutaneous leishmaniasis lesions, DNA was extracted from serous secretions and Taqman real-time polymerase chain reaction was performed to estimate parasite burden of the lesions

10) and 4 weeks after treatment course. The mean diameter of skin lesions significantly decreased by session 5 (4.27 ± 0.87) in acupuncture treated group in comparison with the session 0 (5.38 ± 0.56 , $P = 0.02$), but the mean lesion size was slightly higher than session 5, on session 10 and 4 weeks after treatment course (5.33 ± 1.28 and 5.70 ± 2 respectively, $P > 0.05$). The mean size of the lesions increased significantly in two control groups in the session 5 and 4 weeks after treatment in comparison with the session 0 ($P = 0.049$ and $P = 0.01$ respectively). Thus, the acupuncture was effective by the session 5 of the treatment [Figure 3].

When acupuncture treated group was compared with diazepam control group, in all measurement points except for pre-treatment, mean lesion size was greater in control than in treated group, however only at session 5 the difference was significant ($P = 0.024$) [Figure 4].

The mean of the lesion size was not significantly different at session 5, 10 and 4 weeks after treatment course between two control groups, thus diazepam treatment might not affect the course of CL in this study.

The mean score of parasite burden in the microscopic evaluation of the lesions was not significantly different between or within groups on session 0 and 10. The same results were obtained for real-time PCR quantification of the parasite burden in the skin lesions [Table 1].

DISCUSSION

Although the mechanism of acupuncture therapy in western medicine is not completely understood, many clinical studies support the beneficial role of it in a variety of disease states.^[1] The results of this study suggest that acupuncture treatment reduces



Figure 3: Measurement of the lesions of cutaneous leishmaniasis by vernier caliper

Table 1: Parasite burden measured by real-time PCR and microscopy

| Parasite burden (group) | Micro scopice valuation | | Real-time PCR | |
|-------------------------|-------------------------|------------|---------------|------------|
| | Session 0 | Session 10 | Session 0 | Session 10 |
| Diazepam control | 1.57±0.41 | 1.62±0.51 | 24.5±2.52 | 23.5±3.31 |
| Treatment | 1.71±1.12 | 1.87±1.26 | 24.62±2.91 | 23.18±2.03 |

PCR: Polymerase chain reaction

the size of the CL lesions by session 5 of the therapy but do not cause significant improvement of the lesions since then. In a model of burn injury Lee *et al.* treated BALB/c mice with two needles inserted around the wound for 30 min daily and found that this treatment accelerates the burn wound healing process in 7 days by reducing inflammation and promoting re-epithelialization.^[6] In another study, Park *et al.* showed that acupuncture of injured rats in eight points around the wounds promotes wound healing probably because of increased angiogenesis and granulation-tissue formation and suppression of inflammatory cytokines such as tumor necrosis factor alpha (TNF- α) and interleukin-1 β (IL-1 β).^[13] Also acupuncture around tender areas causes tissue healing in tendinopathies possibly via nitric oxide induced vasodilatation and increasing local blood flow.^[14,15] Therefore in the current study decreased size of CL lesions in acupuncture treated mice might be due to healing effect of acupuncture around the CL lesions.

Needle insertion around the wounds stimulates Ashi-points, which its benefits are revealed in the discussed studies. However, numerous studies propose that stimulation of specific Acu-points is helpful for management of immune-related diseases, including allergic disorders, infections and autoimmune diseases.^[9,16,17] Among the most popular of these points are St-36, Sp-6 and Li-11, which were stimulated in this study too.^[18] Interestingly acupuncture in

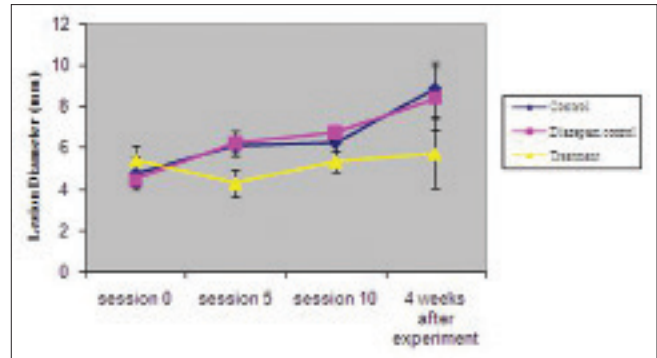


Figure 4: Lesion diameter (mm) in diazepam control, treatment and control group on session 0, 5, 10 and 4 weeks after the end of the experiment

Acu-points is advantageous for both Th1 dominant and Th2 dominant immune disorders; for example asthma and allergic rhinitis resulting from Th2 hyper activation are relieved by acupuncture possibly via reduction of antigen specific IgE, IL-4 and IL-10.^[9,10,19] On the other hand, some clinical and experimental studies report that acupuncture positively affects rheumatoid arthritis probably by reducing TNF- α and interferon gamma which are linked to Th1 response.^[16,17] It is supposed that the acupuncture balances Th1/Th2 response as well as the immune cells and antibody response in disease states to maintain homeostasis.^[8]

Healing of CL lesions is a complex process that depends on the fine balance between pro-inflammatory Th1 responses with immune-regulatory, Th2 and humeral responses which reduce inflammation, but increase parasite replication.^[20,21] Different clinical forms of CL result from disruption of this balance for example in non-healing forms of old world CL a Th2 dominant response is reported,^[22] and in mucocutaneous forms possibly an overwhelming cellular response destroys affected tissues.^[23] Therefore acupuncture might promote cure of CL lesions in different forms of the disease by regulating the ongoing immune responses. However, appropriate duration of treatment and correct acupuncture points have to be determined via subsequent studies. In the current study, parasite burden did not show a significant difference before and after acupuncture treatment, thus it seems that acupuncture does not affect parasite survival and also may not induce a potent Th1 response in this model of CL.

We did not investigate the underlying mechanisms of the beneficial role of acupuncture in this study, however understanding these mechanisms might help to design superior experiments.

Indeed combination of pharmacologic treatments with acupuncture can accelerate cure of CL lesions and lower dose of chemical drugs used by patients. Side-effects with standard acupuncture are uncommon and it is well-tolerated. Certainly, optimization of the procedure is needed and will improve the outcome.

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