EFFECTIVENESS OF WET-CUPPING IN TREATMENT OF BALB/C MICE INFECTED BY *LEISHMANIA MAJOR*; PILOT RANDOMIZED TRIAL

Ali Gorgin Karaji[1], Hamid Alipour [2], Alireza Ahmadi[2, 3, *], Reza Mohammadi[3]

[1.]Department of Immunology, School of Medicine, Kermanshah University of Medical Sciences, Iran.[2.] Department of Anesthesiology, Critical Care, Kermanshah University of Medical Sciences, Iran.[3.] Karolinska Institute, Stockholm, Sweden.
*Corresponding author's E-mail: ahmadiar1012@yahoo.com

Abstract

Background: Cutaneous leishmaniasis (CL) caused by *Leishmania major* is common in Middle East countries. Most therapies are limited and there is no effective vaccine for leishmaniasis. It has been reported that the ancient practice of wet cupping has been effective in treatment of drug resistant CL in humans. The purpose of this present study was to evaluate the efficacy of wet-cupping in treatment of *L. major* infected BALB/C mice.
Methods: We designed a randomized clinical trial using 12 male BALB/c mice, aged 8-10 weeks (six mice for each experimental and control group). Each mouse was infected with *L. major* on the left hind footpad. Mice in the experimental group underwent wet-cupping once a week, up to 6 weeks. The appearance of local reaction in the parasite inoculated paw was monitored and footpad thickness was measured for 7 weeks in both groups. Then all mice were killed and their spleen and lymph node cells were cultured and the level of IFN- γ and IL-4 of cultured cells supernatant were measured as the markers of TH1 and TH2, respectively.
Results: Lesion size thickness in the intervention mice seemed to grow faster than control ones. There were no significant differences in animal weight, spleen and lymph node weight and total cell number. The level of INF-γ and IL-4 produced by spleen cells of intervention mice was not significantly different from control mice (*p*=NS).
Conclusion: Our study showed that wet-cupping has no significant treatment effect on coetaneous leishmaniasis in BALB/c mice. Interestingly, the disease was more severe in the intervention group.

Keywords: Leishmania, wet-cupping

Introduction

The protozoan parasite Leishmania causes three main forms of human diseases including cutaneous leishmaniasis, mucocutaneous leishmaniasis and visceral leishmaniasis. Cutaneous leishmaniasis (CL) which is caused by *Leishmania major* (*L. major*) is a well-established experimental model of human disease caused by an intracellular parasite (Yavar et al., 2013).
The protozoan *L. major* is an obligate intracellular parasite, which can infect various mammalian hosts such as rodents, dogs, and humans. Infection in humans with this protozoan parasite causes a spectrum of clinical manifestations, ranging from limited cutaneous lesion to life-threatening systemic form (Rai et al., 2013; Badiee et al., 2013).
Currently there is no effective and safe vaccine for leishmaniasis, and most therapies are limited by toxicity, long-term courses of treatment and development of drug resistance (Blum et al., 2012). As a method of countering these limitations, efforts have been made to develop new drugs and new methods to augment the immune system or improve the ability of the immune responses (Badiee et al., 2013).
Wet-cupping has been a treatment technique for some diseases for many thousands of years. There are some non-published reports from the Iranian wet-cupping research center with regard to the successful effect of wet-cupping on drug resistant type of CL. To evaluate this effect we conducted an experimental study on *L. major* infected BALB/C mice (Ahmadi et al., 2008).

Methods
Mice

In this randomized clinical trial 12 male BALB/c mice, aged 8-10 weeks, were used (six mice for each experimental and control group). Animals were allowed to acclimatize to the laboratory conditions for at least 2 weeks before experimental manipulation. All mice were maintained on a 12-hour light/dark cycle with free access to water and food *ad libitum* and were housed 6 mice per cage.

Infection protocol and lesion size measuring

L. major parasites were prepared from the Pasteur Institute of Iran and were grown in an RPMI1640 medium supplemented with 20% heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 40 mM HEPES. For infection, mice were inoculated subcutaneously (SC) in the left hind footpad with 1×10^6 stationary phase *L. major* promastigotes at a volume of 50 μl.
The thickness of the infected footpad and the contralateral uninfected footpad were measured once a week with a venire caliper. The increase in footpad thickness was calculated with the Footpad Thickness Index (FTI):

$$FTI = \frac{\text{Thickness of infected footpad} - \text{Thickness of uninfected footpad}}{\text{Thickness of uninfected footpad}} \times 100$$

Thickness measurements were begun at the time of local reaction appearance and continued for 7 weeks.

Wet-cupping

Starting at the time of *L. major* inoculation, wet-cupping was applied on the experimental group (n = 6) (Figure 1), once a week as explained. First, mice were anesthetized by ether. Then an area of 2.5 cm on the backs of the mice, between the shoulders, was shaved. The skin of the shaved area was vacuumed into the cup (1 ml) for 5 min by manual suction. Next the suctioned area which now had become indurated with a dark blue color was punched by a needle and vacuumed for 5 min, to drain the blood accumulated in the area.

Spleen and lymph node cell culture

Nine weeks after parasite inoculation (7 weeks following appearance of footpad reaction), all mice were killed and their spleen was aseptically removed. Single-cell suspension was made by forcing the tissues through a cell strainer with a sterile syringe plunger. Erythrocytes were removed using ACK buffer (0.15 M NH4Cl, 1 M KHCO3, 0.01 M Na-EDTA, PH= 7.4), and spleen cells were counted. Popliteal lymph nodes (PLN) draining the infected footpad were also harvested and their single cell suspensions were washed and counted. Spleen and PLN cells were separately suspended in RPMI containing 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 40 mM HEPES. Cells were cultured on 96-well culture plates at 5×10^5 cells per well in triplicate in the presence of concanavalin A (Con A) or leishmanial antigen for 48 hr. Parasite antigen was prepared by freeze-towing of the parasite five times in liquid nitrogen. Cell culture supernatants were removed after 48 hr and stored at -20 for measurement of cytokines.

Cytokine assay

To evaluate the effect of wet-cupping on the pattern of cytokine production, draining lymph node cells of mice were cultured in the presence of Con A and the culture supernatants were assayed for the levels of IFN-γ and IL-4 production. The levels of IL-4 in the supernatant were determined by using double-sandwich monoclonal antibody enzyme-linked immunosorbent assay (ELISA) protocol as standardized by BD Pharmingen (San Diego, CA). In brief, the anti-IL4 capture monoclonal antibody (mAb) was absorbed on a polystyrene 96-well plate, and the IL-4 present in the sample was bound to antibody-coated wells. The biotinylated anti-IL-4 detecting mAb was added to bind the IL-4 captured by the first antibody. After washing, streptavidin-alkaline phosphatase (Sigma) was added to the wells to detect the biotinylated detecting mAb and finally substrate (PNPP, Sigma) was added and a colored product was formed in proportion to amount of IL-4 present in the sample, which was measured at an optical density (OD) of 450 nm. Recombinant mouse IL-4 (Pharmingen BD, cat 550067) was used as standard. IFN-γ was measured in 48-hour supernatant using a commercial kit (R&D Company). The instruction was similar to the above-mentioned protocol for IL-4, except for horse-radish peroxides and TMB substrate, which was used instead of streptavidin-alkaline phosphatase and PNPP, respectively, and the resulting color, was read at 405 nm.

Statistic analysis

Data analysis was performed using the SPSS 22.0 and STATA statistical programs. The following tests were used when appropriate, to compare spleen parasite load at both points in time within each mice group and between the two mouse groups, and the relative cytokine levels in stimulated and non-stimulated CD4+ and CD8+ T cells, within each group of mice: the Kruskal–Wallis test for *k* independent samples ($k > 2$), the Wilcoxon signed-ranks test for two matched samples and the Mann–Whitney test for two independent samples. The student *t*-test and analysis of variance (ANOVA) were used for statistical analysis of the footpad lesion size data and the cytokine (IL-4 & IFN-γ) concentration. In all statistical tests, a 5% level of significance was used.

Results

The main result of this study is outlined in Table 1 and Figure 2. There were no significant differences in lesion size and kinetics of development between wet-cupping-treated mice and control mice. Meanwhile, FTI (lesion size) in the experimental group seemed to grow faster than controls, and it became necrotic sooner. On the other hand, there was no significant difference in animal weight, spleen and PLN weight and total cell number of spleen and PLN between intervention and control groups (data not shown).

Table 1 outlined the result of mean and standard deviation of INF-γ and IL-4 in experimental and control groups. It shows that the level of INF-γ and IL-4 produced by spleen cells of wet-cupping-treated mice was not significantly different from those of control mice.

Table 1: Means (standard deviations), Mean difference, 95% confidence interval (CI) and *p*-value for experimental and control groups and comparing both groups (two-tailed tests)

Measure	Intervention group	Control group	Mean difference	95% CI		P-Value
				Lower	Upper	
FTI*	163(71)	113(42)	50	-27	128	0.179
IFN-γ	2325(2745)	4895(2834)	-2570	-9225	4084	0.344
IL-4	113(83)	70(33)	43	-134	221	0.536

*

$$\text{Footpad Thickness Index (FTI)} = \frac{\text{Thickness of infected footpad} - \text{Thickness of uninfected footpad}}{\text{Thickness of uninfected footpad}} \times 100$$
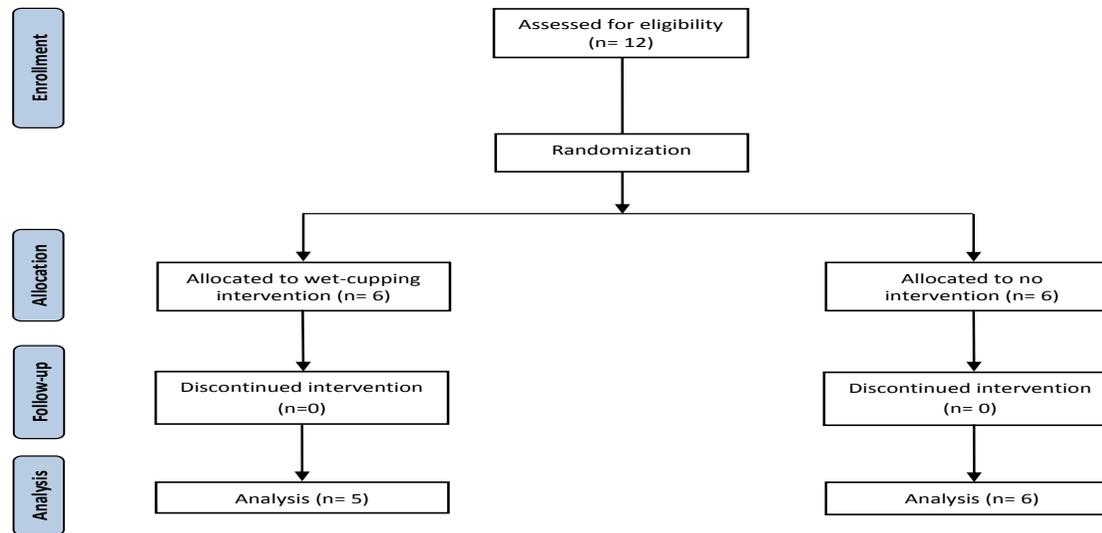
Figure 1: CONSORT flow diagram

$$*\text{Footpad Thickness Index (FTI)} = \frac{\text{Thickness of infected footpad} - \text{Thickness of uninfected footpad}}{\text{Thickness of uninfected footpad}} \times 100$$
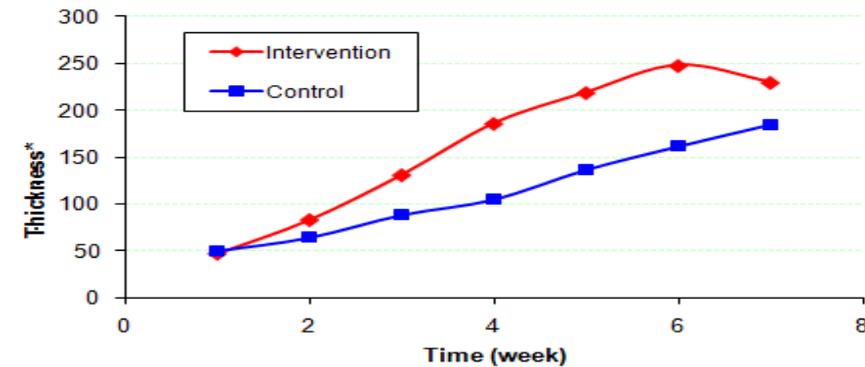
Figure 2: The trend of paw thickness in intervention vs control groups

Discussion

Our study showed that wet-cupping has no significant treatment effect on cutaneous leishmaniasis in BALB/c mice, and we interestingly observed that the disease was more severe in the experimental group, which was contrary to our expected results.

Wet-cupping has been reported as an effective treatment for many diseases and disorders such as low back pain (Farhadi et al., 2009; Kim et al, 2011), neck pain (Kim et al., 2013), tension headache and migraine (Ahmadi et al., 2008), metabolic conditions and LDL level (Niasari et al., 2007), Herpes Zoster infection and post herpetic neuralgia (Cao et al., 2010). In a study conducted at Harvard University Kwong et al. showed that a type of wet-cupping (Gua Sha) has an effect on Heme Oxygenase-1 (HO-1) upregulation. HO-1 is a cell protective agent with some anti-inflammatory, anti-proliferative, and anti-apoptotic effects. This upregulation is induced by factors such as hydrogen peroxide, hypoxia, UV irradiation and physical stresses (see below) (Bach et al. 2005; Lee and Chau, 2002; Otterbein et al., 2003).

As was explained above, in some non-published reports from Iranian's wet-cupping research center, wet-cupping had a significant treatment effect on drug resistant cutaneous leishmaniasis in humans. But, our study result did not show the same effect in L. major infected mice. It is of great interest to mention that there is a persuasive theory with regard to the mechanism of wet-cupping that is known as the "stress response" reaction. It has been suggested that most of the wet-cupping effects are caused by this reaction. Stress response is a complex reaction in the body after injury and stress. It causes two contrasting effects in the immune system. In some cases, stress response suppresses the immune system, but in some others, stress response leads to immune system excitement.

We suggest two reasons to explain why wet-cupping had no treatment effect on cutaneous leishmaniasis in mice and, in contrast, the disease was more severe in the experimental group. First, it seems that stress response suppressed the mice's immune system, thus the severity of the disease was increased in these mice. As we mentioned, a previous study showed that HO-1 that is upregulated by wet-cupping has anti inflammatory effects. This mechanism may be a reason for more progressive leishmaniasis in the wet-cupping group of mice (Cao et al., 2010). Second, we used ether for anesthesia but its effect on the immune system is unknown and it may be a confounding agent. As we know, pain has a significant role in stress response reaction. In humans, no anesthesia is used during wet-cupping, but in our study, we had to anesthetize mice to prevent unwanted movements. Studies on other volatile anesthetics have showed no significant effect on stress response (Ihn et al., 2009), but there is no study on ether. As ether suppresses pain like opioids, it may suppress immune response and cause more severe disease in the intervention group.

Strengths, limitations, and future directions

This study has several methodological strengths. This is the first study in mice to find the effect of wet-cupping in leishmaniasis. We used BALB/c mice that are mice strain sensitive to leishmaniasis. Our variables include some immune cytokines similar to IFN-γ and IL-4. And we used the RCT method in our study.

One of the weaknesses of our study is the use of ether as anesthetic agent. Its effect on the immune system is unclear. We suggest Isoflurane for future studies with regard to wet-cupping in mice, because it has no effect on stress response. The other limitation of our study was the smallness of the sample size with only 6 mice in each group, so we suggest the use of more mice in future studies.

It seems that the effect of wet-cupping in humans differs from mice. We suggest that future studies are conducted on drug resistant human leishmaniasis.

Conclusion

Our study showed no significant effect of wet-cupping in treatment of cutaneous leishmaniasis in BALB/c mice. More wet-cupping studies and interventions in human and primates are proposed. Induction of anesthesia by halogenated anesthetics like Isoflurane which has no significant effect on stress response can estimate the effect of wet-cupping more accurately.

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