Cryotherapy of cutaneous leishmaniasis caused by *Leishmania major* in BALB/c mice: A comparative experimental study

Minoo Shaddel¹,², Iraj Sharifi², Mehran Karvar¹, Alireza Keyhani² & Zahra Baziar²

¹Department of Medical Parasitology and Mycology, School of Medicine, AJA University of Medical Sciences, Tehran; ²Kerman Leishmaniasis Research Center, Kerman University of Medical Sciences, Kerman, Iran

**ABSTRACT**

*Background & objectives:* Leishmania parasites are sensitive to very low temperature. Cryotherapy is considered as an alternative to the existing pentavalent antimonials, for local treatment of cutaneous leishmaniasis (CL). Normally, liquid nitrogen (N₂) at a temperature of –196 °C, is used in cryotherapy of CL, but its efficacy is not consistent. Recently, few studies have also reported the use of carbon dioxide (CO₂) slush at –78.5 °C in CL cryotherapy. This study was aimed to evaluate the effectiveness of N₂ vs CO₂ cryotherapy for CL treatment in mice.

*Methods:* In total, 21 BALB/c mice were infected with *Leishmania major* strain [MRHO/IR/74/ER]. Samples were divided into three groups based on the intervention provided—Solid CO₂ cryotherapy, liquid N₂ cryotherapy and control group; with seven mice randomly assigned to each group. Control group received no intervention, and in the other two groups cryotherapy was used every two weeks for maximum of three months. Follow up examinations were scheduled at the time of cryotherapy, during which the size of each lesion was measured. For three mice in each study group, the spleen parasite DNA load was quantified using real-time PCR.

*Results:* After treatment, the liquid N₂ cryotherapy showed significant reduction in size of the lesions \((p = 0.029)\) as compared to the solid CO₂ cryotherapy and control group. Also, *Leishmania* DNA load in spleen was significantly lower in the mice receiving liquid N₂ cryotherapy \((p <0.001)\).

*Interpretation & conclusion:* Liquid N₂ cryotherapy is superior to CO₂ cryotherapy, and it can be an effective method for controlling *L. major* infection. Further investigations are essential to find optimal number of treatment sessions and time intervals.

**Key words** Cryotherapy; cutaneous leishmaniasis; *Leishmania major*; liquid nitrogen; solid carbon dioxide

**INTRODUCTION**

Leishmaniasis is a parasitic disease caused by various species of *Leishmania* protozoan. The parasite is transmitted to humans through the bite of an infected sandfly¹. In recent years, the prevalence and geographical distribution of the disease have increased and expanded to new regions of the world²,³. Furthermore, due to high immigration, international travel and military operations in endemic areas, leishmaniasis has received more attention in developed countries⁴,⁶. It is a serious health problem in the Middle East, particularly Iran⁷.

Although, some promising attempts have been done to develop a vaccine⁸-¹⁰, treatment is still the cornerstone modality for controlling leishmaniasis infection. The first-line treatment for cutaneous leishmaniasis (CL) include intramuscular or intralesional injection of pentavalent antimonial compounds particularly Glucantime⁶,⁷. However, due to long duration of use, several side-effects, low efficacy and increasing resistance to these drugs, tremendous efforts have been made in order to develop alternative systemic or topical treatments¹¹-¹³.

Given the sensitivity of *Leishmania* parasites to freezing, cryotherapy is now used as an adjuvant therapy along with antimonial compounds and sometimes used alone as an alternative treatment to cure CL, in certain regions such as Iran, Yemen, and Jordan²,⁷,¹³-¹⁹.

Normally, liquid nitrogen (N₂) at a temperature of –196 °C is used in cryotherapy of cutaneous lesions. This therapy has been reported to be successful in >95% of cases, however, in some controlled trials, its efficacy was as low as 27%¹⁴-¹⁸. The ambiguity in results have caused controversy about the efficacy of cryotherapy on CL. Recently, a study showed that cryotherapy with CO₂ slush (dry ice) at –78.5 °C was effective in treating CL in Yemen¹⁹. The effectiveness of the two types of cryotherapy regimens (one with liquid N₂ and another with solid CO₂) have not been studied and compared in any published study.

Liquid nitrogen, compared to dry ice, is not easily available and requires higher cost of maintenance. This study was aimed to evaluate the effectiveness of liquid N₂ cryotherapy, and compare its efficacy with the solid CO₂ cryotherapy in the treatment of CL. To investigate the ef-
fect of cryotherapy in two different temperature ranges (−196 and −78.5 °C) in treating CL, a standard animal model of the disease was used.

MATERIAL & METHODS

Setting
The study was conducted in the Kerman Leishmaniasis Research Center, Kerman University of Medical Sciences, Kerman, Iran during summer and fall of 2015.

Study sample and design
Pasteur Institute of Iran, Tehran provided male BALB/c mice (6–8 wk old and weighing approximately 20 to 22 g) for the study. Given the heterogeneity of mice and the similarity of their maintenance conditions, 21 mice were included in the study; and divided into three groups (of seven mice each), namely control, cryotherapy with solid CO2, and cryotherapy with liquid N2. This study was conducted as an experimental controlled animal trial.

Ethical considerations
The animal care and the experimental protocols were in accordance with the Ministry of Health and Education of Iran animal care guidelines. The study procedures were approved by the Ethics Committee, AJA University of Medical Sciences, Tehran, Iran (the assigned ethical code is IR.AJAUMS.REC.1396.109). Mice in all the three groups were maintained in similar settings in terms of storage conditions, ambient temperature, drinking water, food and light–dark cycle. At the end of the experiments, all mice were buried according to standard protocols to prevent the spread of the infection.

Cultivation of L. major and infection of mice

The required parasites for inoculation in mice were cultured in a suitable environment in the Kerman Leishmaniasis Research Center, Kerman, Iran. Leishmania major strain (MRHO/IR/74/ER) used in this study, was kindly provided by Prof. A. Khamesipour (Center for Research and Training in Skin Diseases and Leprosy, Tehran University of Medical Sciences, Tehran, Iran). The isolation and mass production of the parasites were carried out via culturing them in Novy-MacNeal-Nicolle medium (NNN) and then sub-culturing in RPMI 1640 medium (Biosera, France) supplemented with 10% fetal bovine serum (Sigma-Aldrich, USA), 2 mM L-glutamine (Gibco BRL), 100 IU/ml penicillin and 100 μg/ml streptomycin (Sigma, Germany) followed by incubation at 25 °C.

Before inoculation, the base of 30 mice tails was shaved by scalpel and was disinfected by 70% ethanol. Then 0.1 ml of suspension containing 2.5 × 10⁶ L. major (MRHO/IR/74/ER) promastigotes in stationary stage was injected subcutaneously at the base of the tails by using insulin syringe. Till the appearance of CL lesions, the mice were kept in cages for six weeks. When they developed cutaneous lesions, 21 of them were selected for the study. Remaining infected mice were killed and buried as per standard protocols.

Study arms, intervention, and follow-up protocol
The 21 infected mice were randomly divided into three groups and a number was assigned to each mouse. The clinical status and size of the induration were recorded in a structured form.

In the control group, the mice were maintained in their settings without any intervention. In cryotherapy with solid CO2 group, dry ice pellet, based on the lesion size, was placed on the lesion (including a margin of 2 mm on the healthy skin) with gentle pressure for 1 min in order to freeze the lesion completely (with a margin of 2 mm of healthy skin). In cryotherapy with liquid N2 group, liquid N2 was placed on the lesion using a swab (including 2 mm margin out of the lesion on the healthy skin), with gentle pressure for 20 sec to freeze the lesion.

The follow-up examinations were scheduled every two weeks for maximum of three months. At the follow-up visits, the lesions of all three groups were evaluated and if a lesion was found active (presence of induration), another session of the corresponding therapy was applied to the lesion. The treatment sessions were continued for maximum of three months or until the lesions were cured, whichever was earlier. At the time of follow-up, longitudinal and transverse lesion diameters were measured by using a Vernier caliper, and the size of the lesion was calculated by dividing the total by two, as described earlier by Soka³⁰.

At the end of the three-months period, all of the mice were killed painlessly. Three mice from each group (total nine) were randomly selected and their spleens were aseptically removed for DNA extraction. In this study, we developed and validated a SYBR green based real-time PCR in order to detect and quantify L. major parasite load with high sensitivity in spleen biopsy specimens.

DNA extraction and real-time PCR
Genomic DNA was extracted from 10 mg of spleen tissue with TIANamp Genomic DNA kit (Tiangen Bio-tech, China) according to the manufacturer’s protocol.

Reaction was performed using 200 ng of the DNA, 5 pmol of RV1 (Forward: 5'-CTTTTCTGTCCTGGCAGG-3') and RV2 (Reverse: 5'-CCACCTG-
GCCTATTTTACACCA-3') primers (which were used to amplify a 140 bp conserved region of the *Leishmania* kinetoplast DNA minicircle as described by Lachaud *et al*21), and 5 μl SYBR Green Master mix (Takara Bio Inc, Japan) in a total volume of 12 μl with the following amplification program: 95 °C for 5 min, and then 40 cycles at 95 °C for 10 sec, 60 °C for 20 sec, and 72 °C for 20 sec. After PCR amplification, a melting curve was generated to check the amplicon specificity; consisting one cycle at 95–72°C. Reactions were run in Rotor Gene Q real time system (Qiagen, Germany).

The DNA of a normal mouse skin from the base of the tail and wild-type *L. major* DNA were used as negative and positive controls, respectively. For drawing the standard curve, the genomic DNA of *L. major* was used in dilution series 0.1–10⁵ /ml parasites. All tests were performed in duplicate.

Statistical analysis

One-way ANOVA was used to compare the size variables of the lesion and spleen parasite load in the three groups. Paired t-test was used to compare lesion size at the beginning and the end of treatment in each group. GraphPad Prism software version 7 was used for data analysis. The *p*-value ≤ 0.05 was considered significant.

RESULTS

The mean sizes of the lesion’s induration at the beginning and end of the treatment in the three groups are presented in Table 1. The ANOVA test, revealed that the mean size of induration of the lesions pre-treatment was not significantly different in the three groups. However, after the treatment, the mean size in the liquid N₂ group was significantly smaller than the other two groups (*p* = 0.013 as compared to control and *p* = 0.046 as compared to solid CO₂ group). No significant difference was observed in the mean size of lesions at the end of follow ups between the control and cryotherapy with solid CO₂ group. The paired t-test showed that the lesion’s size in mice receiving liquid N₂ cryotherapy significantly decreased after the treatment (*p* = 0.029), though this was not significant for the control and solid CO₂ group. Figure 1 illustrates the mean spleen parasite DNA load in the study group. The comparison of the mean parasite load in the three groups by one-way ANOVA showed that the spleen parasite load in liquid N₂ was significantly less than the other two groups (*p* < 0.001). However, this value was not significantly different between the solid CO₂ and control group. Few side effects such as erythema and swelling were seen in both the intervention methods.

DISCUSSION

So far, there is no comprehensive consensus on CL treatment method, dose and duration of therapy. Since half century, the first-line treatment of leishmaniasis has been pentavalent antimonial compounds. However, due to the significant side-effects, low efficacy, painful injections and the emergence of drug resistant parasites, many attempts have been carried out to find an effective adjuvant therapy or alternative regimen. Finding an alternative cure for CL is still a big challenge for countries where the disease is endemic²–⁷, ¹¹–¹³.

*Leishmania* parasite is sensitive to freezing as very low temperature causes the parasites to break apart in the connective tissue of the dermis. Even, cryotherapy can cause tissue damage, rupture of the membranes of macrophages, and release of Leishman-Donovan bodies within the tissue, along with the possibility of increase in antigen presentation to the immune system¹⁵, ²².

Considering the sensitivity of *Leishmania* parasites to freezing, cryotherapy is practiced in some regions, such as Sri Lanka, Jordan, Turkey, Yemen and notably in Iran as adjunctive therapy along with pentavalent antimonial compounds, and even sometimes as replacement therapy in the treatment of CL⁷.
Different studies with various findings have reported the efficacy of cryotherapy with liquid $\text{N}_2$. The degree of effectiveness in these studies varied between 27 to 100%\(^{14-18}\). Factors such as different species, location and size of lesions, demographic changes (population variations), cryotherapy regimen, frequency, accuracy, and study type could be the possible reasons for such differences in the efficacy of the therapy. This study aimed to evaluate the effectiveness of cryotherapy in treating CL by using liquid $\text{N}_2$ and compared it with the effectiveness of solid CO\(_2\) in BALB/c mice, all of which were infected with a similar parasite species in similar site of their body (tail). This was in order to minimize the impact of population variations and different locations of the lesions on treatment results.

With respect to lesion size, the results showed that the effectiveness of cryotherapy with liquid $\text{N}_2$ was significantly higher than the effectiveness of cryotherapy with dry ice. On molecular level, liquid $\text{N}_2$ group had significantly less spleen parasite load as compared to the control group. This also confirmed the desirable effect of liquid $\text{N}_2$ cryotherapy for control of *Leishmania* infection. At the end of the treatment, parasite load and size of lesions in dry ice cryotherapy group did not show any significant difference in comparison to the control group.

Several studies have evaluated the efficacy of cryotherapy in treatment of CL. In Turkey, Gurei et al\(^{14}\) reported that the efficacy of liquid $\text{N}_2$ cryotherapy in the treatment of CL was 78%, following three-month treatment period. Asilian et al\(^{16}\) have reported response rate of 57.3% for cryotherapy. Recently, Mosleh et al\(^{17}\) found that the effectiveness of cryotherapy with liquid $\text{N}_2$ spray, for one to four sessions in 120 patients consisting 375 CL lesions caused by *L. major* was 84%; the remaining lesions (16%) were cured after an additional one to three session(s). In a controlled trial carried out by Al-Gindan et al\(^{18}\) in Saudi Arabia, on 600 patients with CL, 27% efficacy was reported for $\text{N}_2$ cryotherapy as compared to 41% with Sb and 30% with ketoconazole.

Despite the differences listed in the above studies, the majority of researches demonstrated that the efficacy of liquid $\text{N}_2$ cryotherapy in the treatment of CL was significant. The results of this study also indicated a favourable impact of liquid $\text{N}_2$ cryotherapy to control the parasitic load of *L. major* in the host body.

Cryotherapy may cause bulla and pain at the site of its application which would last for several days. Hence, there should be a time interval between the treatment sessions in order to minimize the side effects. Different treatment intervals of cryotherapy have been used for treating CL. Studies have used single dose\(^{22}\), weekly\(^{15,17}\), or once every two weeks\(^{16,18}\) regimens. These studies have applied different number of treatment sessions, for example one\(^{22}\), two\(^{14-15,18}\) or more\(^{16-17}\). The number of treatment sessions is usually based on the clinical response. The researchers may use the guidelines of their country or they may decide about the duration of treatment based upon their own clinical experience. In the present study, according to the national protocol\(^7\), cryotherapy was performed once every two weeks for maximum of three months. However, further studies with large sample sizes are needed to determine the appropriate time interval and duration of CL treatment with cryotherapy, to obtain higher efficacy in future.

Few studies have focused on the effectiveness of solid CO\(_2\). Al-Qubati et al\(^9\) reported that cryotherapy with CO\(_2\) slush with a temperature of –78.5 °C was effective in the treatment of CL. However, considering the limitations of their study, they stated that additional randomized clinical trials are needed to further analyze the efficacy of CO\(_2\) slush in CL therapy. In a non-controlled study on 30 patients with CL in Egypt, Bassiouny et al\(^{22}\) reported that the efficacy of cryosurgery with CO\(_2\) cryomachine in the treatment of CL was 100%. Studies comparing the effectiveness of the two methods of cryotherapy with liquid $\text{N}_2$ and solid CO\(_2\) are not available in literature. In addition, there is no any study on the effectiveness of cryotherapy in treatment of CL using molecular diagnostic assays either.

With respect to the effectiveness of cryotherapy with solid CO\(_2\) at a temperature of –78.5 °C, the findings of this study are not consistent with the results reported by Al-Qubati et al\(^9\) and Bassiouny et al\(^{22}\). This could be due to differences in the species causing infection, the study design, lack of control group etc. Further, studies are suggested targeting efficacy of this method with more frequent treatment sessions (e.g. once every week).

The results of the present study showed that use of liquid $\text{N}_2$ for cryotherapy is more effective than solid CO\(_2\) in treatment of CL.

**Limitations of the study**

It has been demonstrated that in an experimental group not all animals achieve the same levels of infection. Therefore, it is suggested that further studies with larger sample size should be designed in which the levels of parasitaemia pre-treatment can be determined.

**CONCLUSION**

$\text{N}_2$ cryotherapy showed better results than CO\(_2\) cryotherapy. Since cryotherapy is a topical treatment modality
and is well tolerated due to its few side-effects, it could be an effective method for controlling *Leishmania major* infection. Further clinical trials using larger sample size in volunteer humans is essential for future CL treatment policies.

**Conflict of interest**

The funder had no financial or proprietary interest in any material or method used in this study and had no role in study design, data collection and analysis, or preparation of the manuscript. The authors declare that there is no conflict of interests.

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Correspondence to: Dr Mehran Karvar, Kerman University of Medical Sciences, Emam High Way, Kerman 7616914115, Iran. E-mail: mehrankarvar@yahoo.com

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