

1 **Application of Cryotherapy in the Treatment of Cutaneous Leishmaniasis in**
2 **BALB/c Mice Compared with Commercial Injectable Treatments**

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11 **Abstract**

12 Cutaneous leishmaniasis (CL), caused by *Leishmania* species, is a parasitic disease prevalent in
13 tropical and subtropical regions. Currently, pentavalent antimonial compounds such as pentostam
14 (sodium stibogluconate) and glucantime (meglumine antimoniate) are considered first-line
15 treatments for CL. In this study the efficacy of cryotherapy administered alone and in combination
16 with commercial drugs for treating CL compared to monotherapy with commercial treatments
17 (glucantime and amphotericin B) in BALB/c mice was investigated. Following lesion
18 development, glucantime was administered daily via direct intralesional injection, while
19 amphotericin B was administered intraperitoneally as a single daily dose for three weeks.
20 Cryotherapy was performed six times over a maximum period of three weeks. The results
21 demonstrated a significant reduction in lesion size and accelerated wound healing in groups treated
22 with cryotherapy combined with glucantime or amphotericin B compared to the control group. In
23 the cryotherapy-glucantime group, complete wound closure was achieved by the third week, with
24 no residual nodules or lesion expansion, as observed in the untreated control group. Notably, at
25 the end of the treatment period, no parasites were detected in the spleens of any treatment group.

1 This study supports cryotherapy as an effective adjunctive strategy for enhancing the efficacy of
2 conventional drugs in cutaneous leishmaniasis.

3 **Keywords:** amphotericin B, cryotherapy, cutaneous leishmaniasis, glucantime, *Leishmania major*

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5 **1. Introduction**

6 Leishmaniasis represents a significant parasitic disease affecting various tropical and subtropical
7 regions worldwide, including Iran. The World Health Organization estimates that 350 million
8 people are at risk of infection, with approximately 12 million people currently infected and an
9 additional 1.5 million new cases occurring annually (1). Over time, several treatment modalities
10 have been employed for cutaneous leishmaniasis (CL), including localized radiation therapy,
11 lesion cauterization, cryotherapy, and local drug injections (2-4). Currently, pentavalent
12 antimonial compounds such as pentostam (sodium stibogluconate) and glucantime (meglumine
13 antimoniate) are considered first-line therapies for CL treatment (5). However, the use of these
14 compounds presents several limitations, including prolonged treatment duration, high drug costs,
15 treatment failure in approximately 10-15% of cases, and severe toxicity affecting the heart, liver,
16 and kidneys (6). Consequently, extensive research continues in the development of alternative
17 leishmaniasis treatment strategies.

18 Various therapeutic approaches have been employed, typically based on host and parasitic factors,
19 although current treatment data remain variable and often provide limited guidance for specific
20 protocols. Given the thermosensitivity of *Leishmania* (7), cryotherapy has emerged as a viable
21 therapeutic option, demonstrating high efficacy, particularly in cutaneous leishmaniasis cases
22 caused by *L. tropica*, *L. aethiopica*, *L. infantum*, and *L. braziliensis* (8,9). By destroying
23 amastigotes and triggering the host's immune response through antigenic release, cryotherapy
24 promotes cryonecrosis (10). Meta-analyses have demonstrated that cryotherapy exhibits similar
25 efficacy to sodium stibogluconate for smaller lesions while producing fewer adverse effects (11).
26 However, this method is limited to lesions under 4 cm in diameter and fewer than four lesions per
27 patient. Combination therapy utilizing cryotherapy and sodium stibogluconate appears to yield
28 superior outcomes, with evidence supporting synergistic effects (8,11,12).

1 The increasing incidence of therapeutic failure, recurrence, and drug resistance in *Leishmania*
2 species indicates an urgent need to reassess current treatment protocols. Combination therapies
3 utilizing synergistically active compounds could reduce treatment duration, dosage requirements,
4 adverse effects, and costs while potentially mitigating drug resistance development (13).
5 Combination therapy incorporating antimonials and cryotherapy has demonstrated statistically
6 significant improvement rates compared to monotherapy with either treatment modality for *L.*
7 *tropica* or *L. major* infections across various geographic regions (9,14,15).

8 The objective of this study was to evaluate the efficacy of cryotherapy administered alone or in
9 combination with commercial drugs for treating CL caused by *Leishmania major* in BALB/c mice.

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11 **2. Materials/Methods**

12 This experimental study was conducted in the Parasitology Laboratory of Kurdistan University of
13 Medical Sciences and received approval from the Research Committees of Islamic Azad
14 University Sanandaj Branch (approval ID: IR.IAU.SDJ.REC.1403.003).

15 **2.1 Parasite**

16 The *Leishmania major* strain (MRHO/IR/75/ER) was obtained from the Education and Research
17 Center for Skin Diseases and Leprosy of Tehran University of Medical Sciences. Parasites were
18 cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) in culture flasks
19 and incubated at 25°C.

20 **2.2 Animals**

21 Thirty female BALB/c mice aged 6-8 weeks were randomly allocated into six groups: control (no
22 treatment), cryotherapy alone, glucantime alone, amphotericin B alone, glucantime-cryotherapy
23 combination, and amphotericin B-cryotherapy combination (n=5 per group).

24 **2.3 Parasite Inoculation**

1 Stationary-phase parasites were utilized for inoculation. The culture medium containing
2 stationary-phase parasites was centrifuged at 1500 rpm for 10 minutes, washed three times with
3 sterile phosphate-buffered saline (PBS), and concentrated to 2×10^6 promastigotes/mL.
4 Subcutaneous injections were administered at the tail base (0.2 mL). Lesions appearing after 3-5
5 weeks (16).

6 **2.4 Drug Administration**

7 Following lesion development, glucantime (20 mg/kg) was administered daily via direct
8 intralesional injection (17), while amphotericin B was administered intraperitoneally as a single
9 daily dose (4 mg/kg/day) for three weeks (18). For cryotherapy, a cotton swab saturated with liquid
10 nitrogen was applied with gentle pressure to the lesion until the lesion and a 1-2 mm margin were
11 blanched. Treatment duration varied according to lesion size, thickness, and location (ranging from
12 10 to 30 seconds). For thicker lesions, the freeze-thaw cycle was repeated six times over a
13 maximum period of three weeks (18). Mouse weights were recorded before and after treatment.
14 At the conclusion of the treatment period, two mice from each group were euthanized under sterile
15 conditions, and their spleens were harvested. Spleen weights were recorded, after which samples
16 were homogenized in 2 mL of RPMI medium containing 10% FBS. Seven serial 10-fold dilutions
17 were prepared, and 200 μ L of each dilution was added to 96-well plates in duplicate. Plates were
18 sealed with parafilm and incubated at 26-28°C for 7-15 days. Plates were examined periodically
19 under an inverted microscope to detect promastigotes. Parasite load was calculated using the
20 following formula (19):

$$21 \quad \text{Parasite load} = -\log^{10} (\text{dilution/spleen weight})$$

22 **Statistical Analysis**

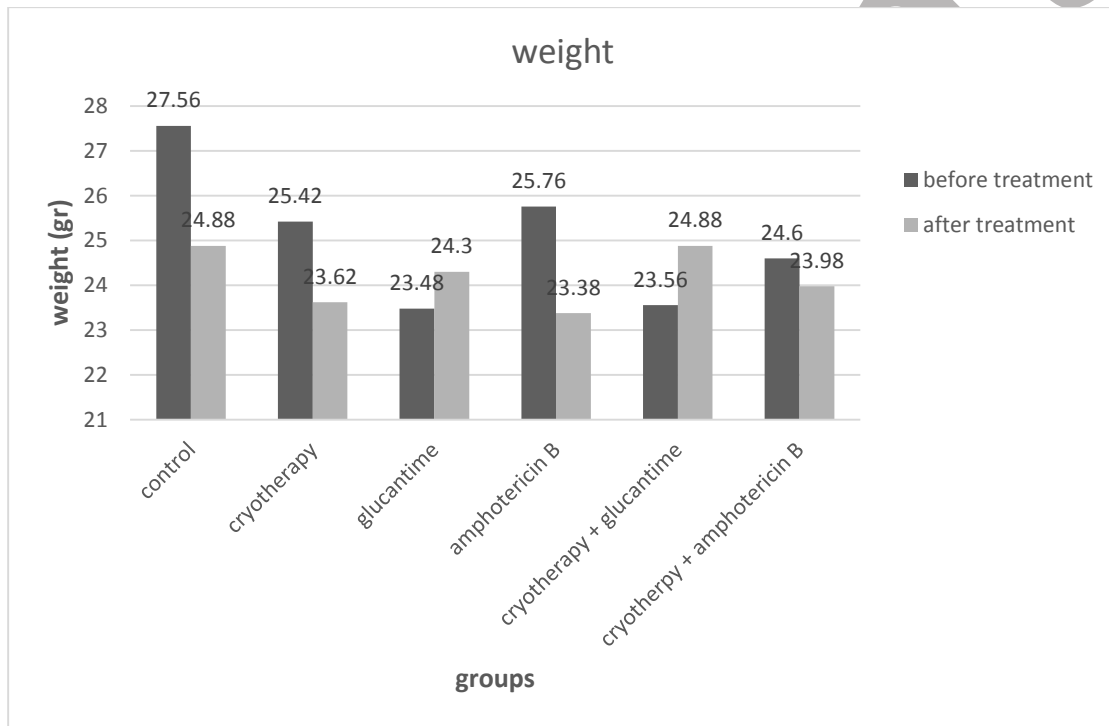
23 Data were analyzed using SPSS software version 24. Descriptive statistics, including frequencies,
24 percentages, means, and standard deviations, are presented graphically. Comparisons of
25 quantitative data were performed using t-tests and Tukey's post hoc tests. Statistical significance
26 was set at $P < 0.05$ (95 percent confidence interval).

27 **3. Results**

1 **3.1 Weight Changes**

2 Figure 1 displays the mean weights of mice before and after treatment. The mean weights in the
3 glucantime treatment group (24.30 g) and the glucantime-cryotherapy group (24.88 g) exceeded
4 their pretreatment weights. This increase was more substantial than observed in other treatment
5 groups, indicating a beneficial effect on mouse health and recovery.

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8 Figure 1: Mouse weights in different treatment groups during the study period

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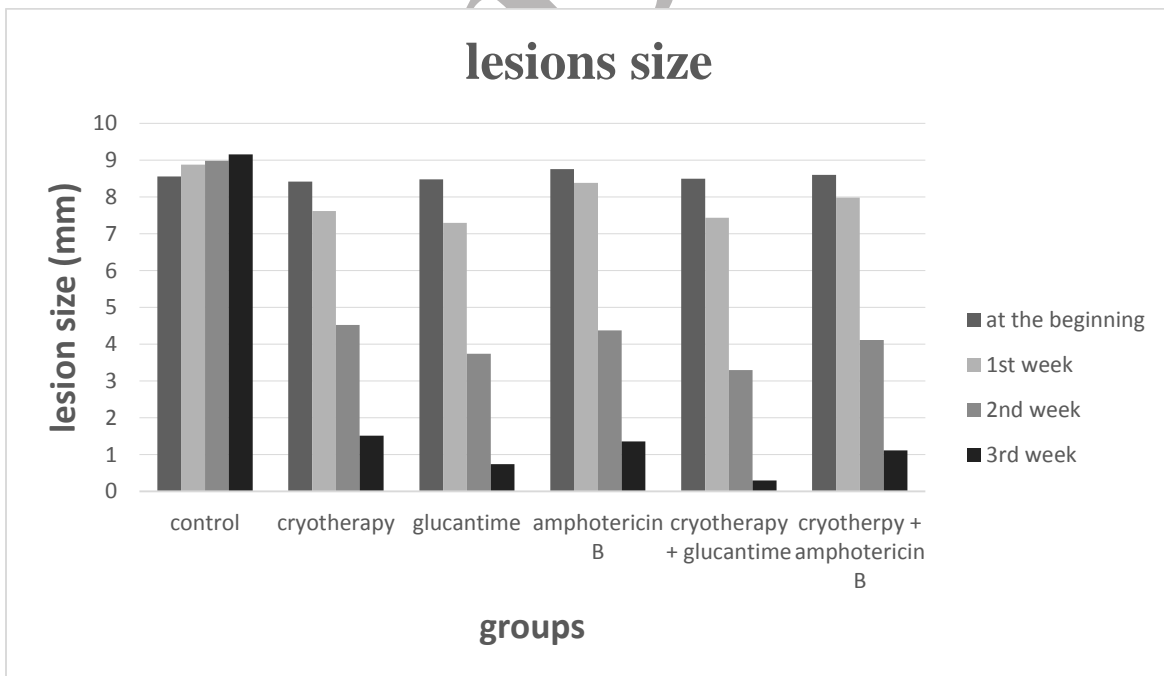
10 **3.2 Lesion Size**

11 As demonstrated in Figure 2, treatment effectiveness was evaluated based on lesion size,
12 induration, and location. Results revealed no significant differences in average lesion diameter
13 among groups at baseline (day zero). During the first week, a decreasing trend in lesion diameter

1 was observed across all treatment groups; however, these reductions were not statistically
2 significant.

3 By the second week, a significant reduction in lesion diameter was observed in the cryotherapy
4 group compared to the control group. Significant differences were also noted between the
5 glucantime-only group and the glucantime-cryotherapy group compared to both control and
6 cryotherapy-only groups. For amphotericin B alone and amphotericin B combined with
7 cryotherapy, lesion reduction was significant only when compared to the control group.

8 In the third week, lesions in treatment groups progressively resolved, leaving no residual nodules
9 or lesions, whereas lesions in the control group continued to expand, ultimately resulting in animal
10 mortality. Compared to the control group, wound healing in the cryotherapy group was
11 significantly enhanced. Notably, the glucantime-only and glucantime-cryotherapy groups
12 demonstrated significantly superior healing compared to both control and cryotherapy-alone
13 groups. Similarly, while amphotericin B alone or combined with cryotherapy resulted in decreased
14 lesion size, this reduction was statistically significant only compared to the control group.



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16 Figure 2: Average changes in lesion diameter (mm) among treatment groups during the study

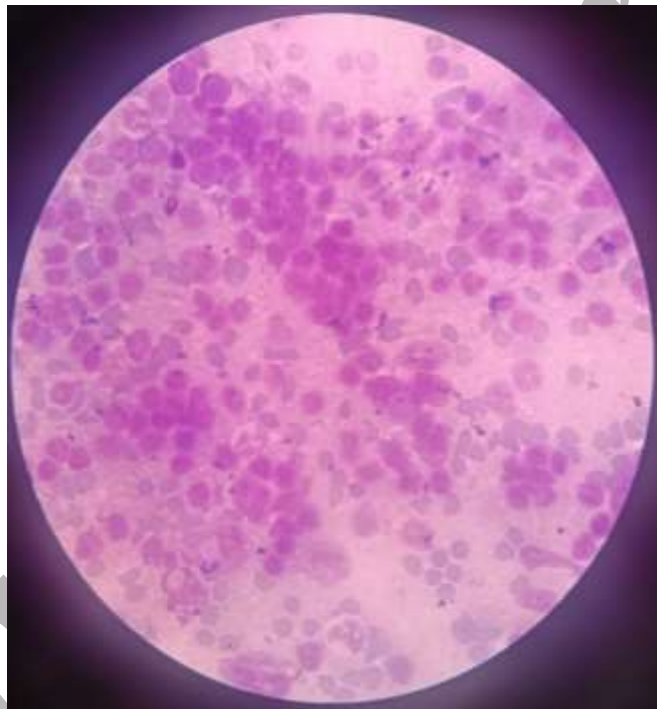
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period

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2 **3.3 Parasite Load in Spleen**

3 Study findings revealed complete absence of parasites in the spleens of all treatment groups by
4 study completion (Figure 3). A critical parameter in evaluating therapeutic system efficacy for
5 treating and healing cutaneous leishmaniasis is the ability to prevent systemic spread and
6 proliferation of *Leishmania* within the host. Examination of spleens from treated mice confirmed
7 that no mice exhibited detectable parasite burden.



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9 Figure 3: Microscopic examination of splenocytes using inverted microscopy demonstrating
10 absence of amastigotes

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12 **4. Discussion**

13 In the present study, mean weight in the glucantime-treated group (24.30 g) and the glucantime-
14 cryotherapy-treated group (24.88 g) increased following treatment compared to baseline and other

14

1 treatment groups. The weight gain was due to increased appetite in both groups, post-treatment.
2 No significant differences in mean lesion diameter were observed among groups at baseline or
3 during the first week. By the second week, however, a statistically significant reduction in lesion
4 size was observed in the cryotherapy group compared to the control group, with further
5 improvement in the glucantime and glucantime-cryotherapy groups relative to control and
6 cryotherapy groups. By the third week, lesions in all treated groups had completely healed without
7 remaining nodules or lesions. Conversely, untreated lesions in the control group continued to
8 expand, ultimately resulting in mortality.

9 Saghafipour reported that intralesional glucantime injections achieved 48.1% recovery, while
10 cryotherapy combined with intralesional glucantime achieved 72.2% recovery after seven
11 treatments, with 100% recovery after 12 treatments for the combination group and 91% recovery
12 for glucantime monotherapy (20).

13 In summary, combining glucantime with cryotherapy improved weight gain and significantly
14 reduced lesion diameter in BALB/c mice. Although no statistically significant differences were
15 observed early in treatment, lesion size decreased substantially by the second week in cryotherapy
16 and glucantime-treated groups. By the third week, lesions in treated groups had completely healed,
17 while those in the control group had deteriorated. Moreover, amphotericin B alone and combined
18 with cryotherapy resulted in reduced lesion sizes, with significant differences only relative to the
19 control group. Importantly, spleen examination revealed that all treatments successfully prevented
20 parasite dissemination to the spleen, underscoring the efficacy of these therapies in controlling
21 systemic proliferation of *Leishmania major*.

22 These findings emphasize the superior efficacy of combination therapies, particularly glucantime-
23 cryotherapy, in promoting accelerated and more complete wound healing.

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3 of Veterinary Medicine, Islamic Azad University of Sanandaj (IR.IAU.SDJ.REC.1402.112). The
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6

7 **Authors' Contributions**

8 Study concept and design: Gh. A.

9 Data acquisition: S. K. S.

10 Data analysis and interpretation: Y. M & Gh. A.

11 Manuscript drafting: Y. M & S. K. S.

12 Critical manuscript revision: Y. M.

13 Statistical analysis: Y. M. & Gh. A.

14 Administrative, technical, and material support: Gh. A. & S. K. S.

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16 **Ethics Statement**

17 The study design received approval from the ethics committee of Islamic Azad University,
18 Sanandaj, Iran (Approval ID: IR.IAU.SDJ.REC.1403.003).

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20 **Conflict of Interest Statement**

21 The authors certify no conflicts of interest exist.

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1 **Data Availability Statement**

2 Data supporting the findings of this study are available upon request from the corresponding
3 author.

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