Therapeutic Efficacy of Glucantime-Loaded PLGA Nanoparticles Against Cutaneous Leishmaniasis in BALB/c Mice

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ABSTRACT

Cutaneous leishmaniasis (CL) remains a significant global health challenge, particularly in endemic regions. Current first-line therapies, such as Glucantime® (meglumine antimoniate), are frequently limited by systemic toxicity, demanding administration routes, and variable clinical

efficacy, underscoring the urgent need for improved treatment strategies. This study investigated a alternative approach to overcome these limitations: nanoparticle-enhanced delivery of Glucantime utilizing biodegradable poly(lactic-co-glycolic acid) (PLGA) carriers designed to improve therapeutic outcomes and reduce adverse effects. Glucantime was successfully encapsulated within ~200 nm PLGA nanoparticles (PLGA-Glu NPs) using a double-emulsion (W/O/W) solvent evaporation technique. Therapeutic efficacy was comprehensively evaluated in a stringent *Leishmania major*-infected BALB/c mice (n=6 per group). Groups received subcutaneous injections every 3 days for 4 weeks as follows: 1) PBS (negative control), 2) Free Glucantime (10 mg/kg), 3) Blank PLGA NPs (vehicle control), 4) Low-dose PLGA-Glu NPs (100 pg), and 5) High-dose PLGA-Glu NPs (300 pg). PLGA-Glu NPs demonstrated potent and significant immunomodulatory effects. levels of cytokine IL-10 were markedly suppressed in mice treated with the nanoformulations ($p<0.01$). Subsequent splenocyte analysis confirmed a profound reduction in IL-10 secretion ($p<0.001$) and revealed robust Th1-promoting activity, evidenced by significantly elevated production of IL-2 ($p<0.05$). Most notably, treatment with high-dose PLGA-Glu NPs achieved near-complete clinical resolution of cutaneous lesions, supported histologically by minimal residual inflammation and tissue architecture restoration. Furthermore, antigen-specific lymphoproliferative responses were significantly attenuated in PLGA-Glu NP-treated mice ($p<0.001$). In conclusion, these compelling findings position PLGA nanoencapsulation of Glucantime as alternative therapeutic strategy for cutaneous leishmaniasis, though future studies must prioritize comprehensive toxicological profiling, and long-term efficacy assessment in chronic infection models to facilitate clinical translation.

Keywords: Cutaneous Leishmaniasis, Glucantime, Poly (Lactic-co-glycolic acid), Nanoparticles

1. Introduction

Cutaneous leishmaniasis (CL), a neglected tropical disease impacting 12 million individuals worldwide and resulting in 0.9–1.3 million new cases each year, represents a significant public health challenge in tropical and subtropical areas. *L. major* is endemic to rural areas of the Middle East, Iran, Afghanistan, and Southern Russia (1, 2).

CL shows as painful cutaneous lesions capable of causing psychological suffering and disfigurement. The disease mostly spreads via the biting of infected sandflies, and urbanization, climate change, and more human-animal interaction have been driving up its frequency (3). Current options for treatment for cutaneous leishmaniasis, such as pentavalent antimonials like Glucantime (meglumine antimoniate), exhibit limitations including toxicity, uneven effectiveness, and the emergence of resistant to drugs strains, necessitating the exploration of alternative therapeutic strategies (4).

In recent years, nanotechnology has emerged as an intriguing strategy to enhance medicinal delivery systems, particularly for infectious diseases (5). Utilizing biodegradable polymers like poly(lactic-co-glycolic acid) (PLGA) for nanoparticle formulation provides a flexible framework for enhancing the pharmacokinetics and bioavailability of current treatments. PLGA-NPs can encapsulate both hydrophilic and hydrophobic pharmaceuticals, facilitating regulated release mechanisms and targeted distribution to afflicted tissues, potentially improving therapeutic efficacy while reducing systemic side effects (6, 7). These nanoparticles can be internalized by professional antigen-presenting cells (APCs). Encapsulated PLGA antigens possess a superior ability to elicit humoral and cellular immune responses compared to their soluble counterparts. Reduced concentrations of antigens and excipients encapsulated in PLGA-NPs can elicit strong T cell responses (8, 9).

While PLGA encapsulation of antileishmanial drugs has been explored, critical gaps persist in understanding how specific NP characteristics (e.g., precise particle size) influence immunomodulatory outcomes *in vivo*. This study addresses these gaps by engineering 200 nm Glucantime-loaded PLGA-NPs and assessing their therapeutic effectiveness in BALB/c mice of CL.

2. Material and methods

2.1. Materials

Poly (lactic-co-glycolic acid) (PLGA; 50:50 lactic acid:glycolic acid ratio; Mw 30,000–60,000 Da) and polyvinyl alcohol (PVA; Mw 31,000–50,000 Da, 87–98% hydrolyzed) were sourced from Sigma-Aldrich (St. Louis, MO, USA). Acetone (Mw 58.08 g/mol) and dichloromethane (DCM; Mw 84.93 g/mol) were purchased from Merck (Darmstadt, Germany). Phosphate-buffered saline (PBS, pH 7.4) was prepared according to standard protocols. A protein quantification kit was obtained from Parstous (Mashhad, Iran). Cytokines (IL-4, IL-10, IFN-γ) were measured using ELISA kits from eBioscience (San Diego, CA, USA). All other compounds were of analytical quality and utilized without additional purification.

2.2. Methods

2.2.1. Synthesis of nanoparticles containing Glucantime®

PLGA-Glu NPs were synthesized via a double emulsion (W/O/W) solvent evaporation method. Briefly, 50 mg of PLGA was dissolved in 12.5 mL DCM with magnetic stirring. For the internal aqueous phase (W1), 300 μL containing either 100 pg (low dose) or 300 pg (high dose) of Glucantime was emulsified into the PLGA-DCM solution using a Hielscher UP200St probe sonicator (70 W, 1 min, 0.6°C) to form the primary W/O emulsion. This primary emulsion was then injected into 25 mL of 5% (w/v) PVA aqueous solution (W2) under continuous sonication

(70 W, 4 min, 0.6°C). The resulting W/O/W emulsion was stirred overnight at room temperature to evaporate residual DCM. Nanoparticles were collected by centrifugation (14,000 rpm, 30 min, 4°C; Sigma 3-30KS), washed three times with distilled water to remove excess PVA and unencapsulated drug, and resuspended in a minimal volume of water. The suspension was frozen at -80°C and lyophilized (Operon freeze dryer, Labconco, Kansas City, MO) to obtain a free-flowing powder (10).

2.2.2. Nanoparticle Characterization

Size and Zeta Potential: Lyophilized nanoparticles (1 mg) were dispersed in 1 mL deionized water and sonicated for 15 min. Hydrodynamic diameter, polydispersity index (PDI), and zeta potential were measured using dynamic light scattering (DLS; Malvern Zetasizer Nano ZS, UK).

Drug Loading and Encapsulation Efficiency: The amount of Glucantime encapsulated within the PLGA NPs was quantified using atomic absorption spectroscopy (AAS) on washed nanoparticle pellets, adapted from Frezard et al. (11). Encapsulation Efficiency (EE%) and Loading Capacity (LC%) were calculated as:

EE% = (Mass of encapsulated Glucantime / Total mass of Glucantime added) × 100

LC% = (Mass of encapsulated Glucantime / Mass of nanoparticles) × 100

2.2.3. Parasite Culture

L. major promastigotes (strain MRHO/IR/75/ER) were obtained from the Parasitology Laboratory, the Faculty of Medicine at Isfahan University of Medical Sciences in Isfahan, Iran. Parasites were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) and 1% penicillin/streptomycin at 26°C. Soluble *Leishmania* antigen (SLA) for splenocyte stimulation was prepared by subjecting stationary-phase promastigotes to five freeze-thaw cycles

followed by centrifugation (8,000 × g, 30 min, 4°C); the supernatant was collected and stored at 80°C (12).

2.2.4. Animal Model and Infection

Thirty female BALB/c mice (6-8 weeks old) were acquired from the Razi Vaccine and Serum Research Institute (Theran, Iran). Mice were housed under specific pathogen-free conditions with a 12-h light/dark cycle and access to food and water ad libitum. Cutaneous leishmaniasis was established by subcutaneous injection of 1×10^6 stationary-phase *L. major* promastigotes in 50 µL PBS into the right hind footpad. Infection progression was monitored by measuring footpad swelling weekly using a digital caliper (**Fig. 1**).

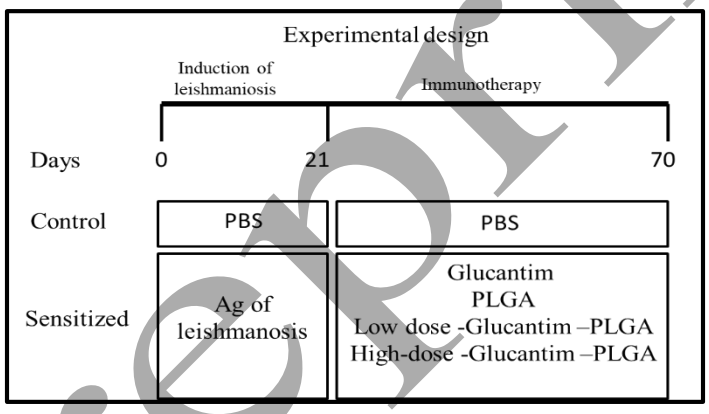

Fig 1. Five groups of mice (n=6 per group) were used in the experiment. PLGA (poly lactic-co-glycolic acid).

2.2.5. Treatment Groups

Three weeks post-infection (confirmed by lesion development and histopathology on pilot animals), mice were randomly assigned to five treatment groups (n=6/group): Group 1 (PBS): Negative control, Group 2 (Free Glu): Received unencapsulated Glucantime (10 mg/kg), Group 3 (Blank NPs): Received empty PLGA nanoparticles, Group 4 (PLGA-Glu Low): Received low-dose Glucantime-loaded PLGA NPs, and Group 5 (PLGA-Glu High): Received high-dose Glucantime-loaded PLGA NPs. Treatments were administered subcutaneously near the lesion site every 3 days for 4 weeks. Lesion size was monitored throughout the treatment period.

2.2.6. Cytokine Analysis

At the study endpoint, mice were euthanized. Spleens were aseptically removed, and splenocytes were isolated. Single-cell suspensions (2×10^6 cells/mL) were cultured in 24-well plates in RPMI-1640 medium with 10% FCS and antibiotics. Cells were stimulated with either SLA (10 µg/mL) or phytohaemagglutinin (PHA, 5 µg/mL; positive control) for 72 h at 37°C in 5% CO_2. Supernatants were collected and stored at -80°C. Levels of IFN-γ, IL-2, IL-4, and IL-10 were quantified using specific sandwich ELISA kits (eBioscience) according to the manufacturer's instructions.

2.2.7. Splenocyte Proliferation Assay (MTT)

Splenocyte proliferation in response to SLA was assessed using the MTT assay. Splenocytes (2×10^5 cells/well) were seeded in 96-well plates and stimulated with SLA (10 µg/mL) for 72 h. MTT reagent (0.5 mg/mL) was added for the last 4 h of culture. The formazan crystals were dissolved in DMSO, and absorbance was measured at 570 nm using a microplate reader. Results are expressed as a stimulation index (SI = Absorbance of stimulated cells / Absorbance of unstimulated cells).

2.2.8. Statistical Analysis

All data are shown as mean ± standard deviation (SD) or standard error of the mean (SE). Statistical significance was assessed using one-way analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) post-hoc test for multiple comparisons, conducted with GraphPad Prism 9.0 (GraphPad Software, San Diego, CA). A p-value less than 0.05 is considered statistically significant.

3. Results

3.1. Characterization of Glucantime-Loaded PLGA Nanoparticles

Glucantime was effectively encapsulated in PLGA nanoparticles via the double-emulsion solvent evaporation technique. The resulting nanoparticles exhibited a negative zeta potential and a monodisperse size distribution (PDI < 0.2). As detailed in **Table 1**, the mean hydrodynamic diameter of Glucantime-loaded NPs was 200 nm, confirming optimal sizing for cellular uptake.

Table 1. Characterization of nanoparticle sizes, PDI and Zeta potential (Mean±SD)

Formulation	Mean Particle size (nm)	Mean Polydispersity (PDI)	Mean Zeta potential
	219.33±18.63	0.116±0.061	-17.21±0.003

3.2. Drug Loading and Encapsulation Efficiency

Quantitative analysis revealed high encapsulation efficiency for both nanoparticle formulations. As presented in **Table 2**, the 200 nm NPs demonstrated excellent drug-loading capacity, with no significant differences observed between low- and high-dose formulations.

Table 1. Encapsulation and Loading efficacy (Mean±SD)

Formulation	Mean EE (%) *	Mean LC (µg/mg) (%) **
	43.6±1.55	3.14±0.255

*Mean EE: Encapsulation

**Mean LC: Loading Capacity

3.3. Serum and Splenocyte Cytokine Profiles

Systemic cytokine analysis revealed distinct immunological patterns across treatment groups. Serum IFN-γ levels were highest in the free Glucantime group (Group 2), with no significant elevation observed in PLGA-encapsulated formulations (**Fig. 2A**). In contrast, serum IL-2 levels showed marked increases in both blank PLGA nanoparticles (Group 3) and Glucantime-loaded NP groups compared to PBS controls ($p < 0.05$; **Fig. 2B**), suggesting intrinsic nanoparticle-mediated immunostimulation. While PLGA-Glucantime groups exhibited a modest reduction in serum IL-4 relative to free drug administration, this trend did not reach statistical significance (**Fig.**

2C). Notably, PLGA-encapsulated Glucantime significantly suppressed serum IL-10 production ($p < 0.01$ vs. all other groups; **Fig. 2D**), indicating systemic modulation of immunosuppressive responses.

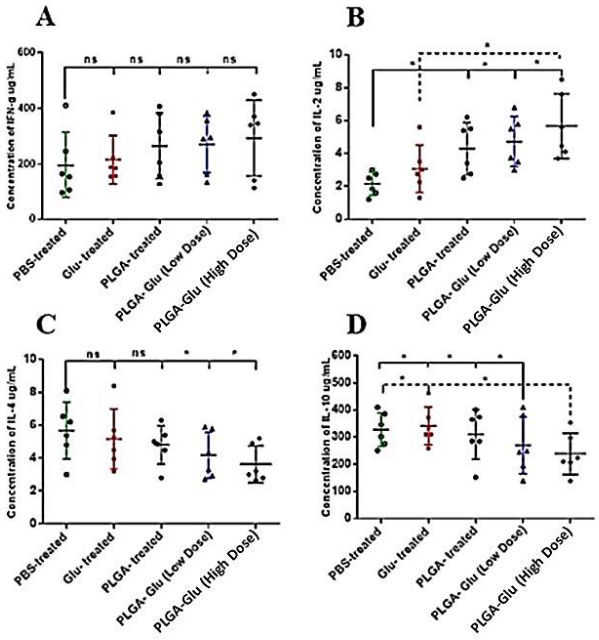

Fig 1. Cytokine level in serum (A: IFN-γ, B: IL-2, C: IL-4 and D: IL-10). PBS-treated, glutamine-treated, PLGA-treated, Glucantime loaded-PLGA (low dose), Glucantime loaded-PLGA (high dose).

Complementing these systemic observations, *ex vivo* splenocyte analysis demonstrated potent local immune modulation. Free Glucantime induced the strongest IFN-γ response in splenocyte cultures ($p < 0.001$ vs. PBS; **Fig. 3A**), though PLGA-encapsulated formulations still showed significant elevation over PBS controls ($p < 0.01$). Mirroring serum findings, both blank and drug-loaded nanoparticles significantly enhanced IL-2 production in splenocytes (**Fig. 3B**). Importantly, PLGA-Glucantime NPs substantially reduced IL-4 levels compared to both free drug and PBS treatments ($p < 0.05$; **Fig. 3C**). Most strikingly, high-dose PLGA-Glucantime (Group 5) profoundly suppressed splenocyte IL-10 secretion ($p < 0.001$ vs. PBS and free drug; **Fig. 3D**), confirming targeted downregulation of immunosuppressive signaling at the cellular level.

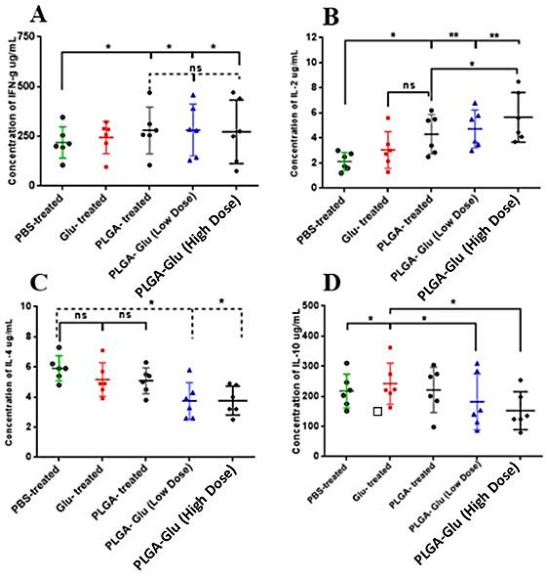

Fig 3. Cytokine levels in supernatant of spleen cell culture. (A: IFN-γ, B: IL-2, C: IL-4 and D: IL-10). PBS-treated, glutamine-treated, PLGA-treated, Glucantime loaded-PLGA (low dose), Glucantime loaded-PLGA (high dose).

3.4. Lymphoproliferative Response

Splenocytes from PLGA-Glucantime-treated mice exhibited significantly reduced proliferation upon *Leishmania* antigen challenge compared to PBS controls ($p < 0.001$; **Fig. 4**). Notably, Free Glucantime showed moderate suppression, and no dose-dependent effect was observed between low/high PLGA-Glucantime groups.

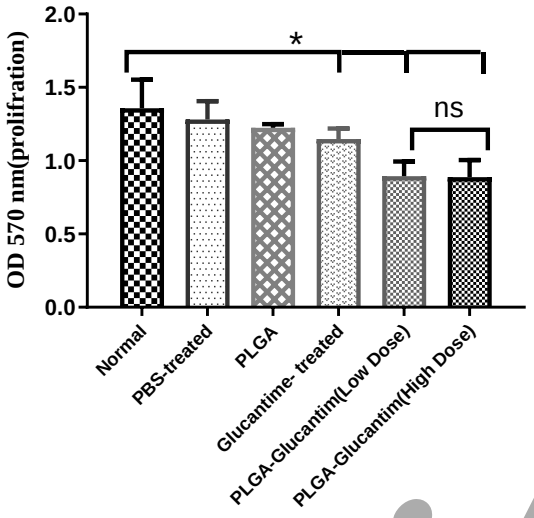

Fig 4. In vitro proliferation assy. ns, nonspecific. *P < 0.05, versus normal and treated Glutamine model group; Normal, healthy control group, PBS-treated, Glutamine-treated, PLGA-treated, PLGA-Glutamine (low dose), PLGA-Glutamine (high dose).

3.5. Histopathological Evaluation

Footpad histology correlated with immunological findings; PBS & Blank PLGA Groups extensive myofiber degeneration with dense macrophage/epithelioid infiltrates (**Fig. 5A, 5E**). Free Glucantime, moderate lymphocytic infiltration with persistent inflammation (**Fig. 5B**). Low-Dose PLGA-Glucantime: Significant lymphocyte-dominated inflammation (**Fig. 5C**), and High-Dose PLGA-Glucantime: Minimal inflammatory infiltration and near-complete resolution of ulceration (**Fig. 5D**).

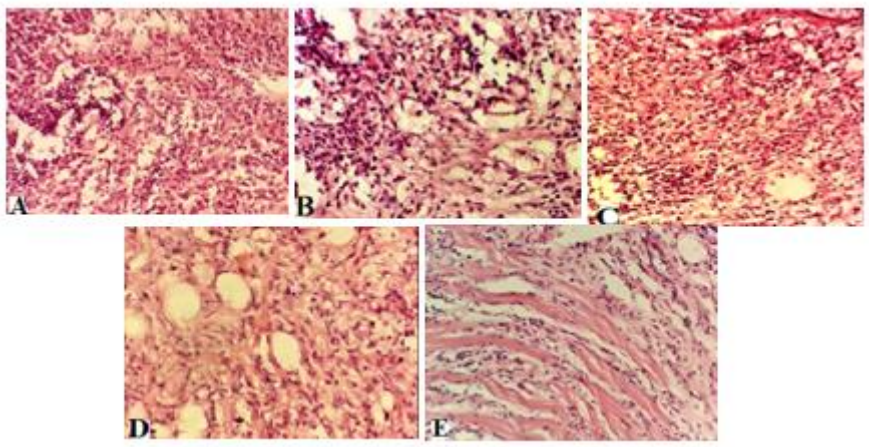

Fig 5. Histological analysis of Leishmania wounds. PBS-treats group (A) and PLGA-treated, Sever mixed inflammation predominately epithelioid macrophages (E); Glutamine-treated, Moderate mixed inflammation predominately lymphocytes(B); PLGA-Glutamine (low dose), Moderate mixed inflammation predominately lymphocytes(C); PLGA-Glutamine (high dose), Mild mixed inflammation with some fibroplasia(D); (haematoxylin eosin, A: ×100, B: ×400).

4. Discussion

This study emphasized on the development and evaluation of Glucantime-encapsulated PLGA NPs as a possible therapeutic approach to improve leishmaniasis treatment. Our findings show good encapsulation of Glucantime in PLGA NPs as well as notable changes in cytokine profiles after treatment, therefore suggesting the possible immunomodulating properties of this formulation. This study presents a notable success: effective encapsulation of Glucantime in PLGA NPs via the double-emulsion solvent evaporation technique. Within the ideal range for nanoparticle delivery systems, the average diameter of 200 nm improves cellular absorption and biodistribution while lowering system toxicity. Crucially for preventing agglomeration and guaranteeing constant release profiles of the encapsulated medication, the negative zeta potential seen suggests a stable colloidal system (13).

Mediating immune responses, cytokines could be involved; their assessment helps one understand the immunomodulating properties of treatments (14). In this study, after Glucantime-encapsulated PLGA therapy in all groups by Glucantime-encapsulated PLGA, reduced production of IL-4 and increased production of IFN-γ. These alterations show a decrease in Th2 immune responses. Like other studies, our investigation also revealed a more outstanding reduction of Th2 responses by the Glucantime-encapsulated PLGA group than the control group (15).

Previous research indicates that this kind of Glucantime-encapsulated PLGA treatment depends critically on the conversion of immunological responses from Th2 to Th1 (16). Our study revealed that PLGA NPs uptaking and transferring to lymphatic tissues would be generally high and successful when their size is about 200 nm. The 20-200 nm particles are usually endocytosed by a receptor-dependent mechanism and stimulate CD4, CD8, and Th1-type cellular immune responses (17). The power of uptake depends on size. Although bigger particles carry more parasite, smaller particles are more effective than large particles and act as adjuvants to mature dendritic cells (17). Dendritic cells uptake particles with a size of 20-200 nm, while macrophages tend to take up larger particles with sizes of 0.5 to 5 μm (10). PLGA NPs were used for different antigen vaccinations and resulted in a change in the balance between the Th1/Th2 response, increasing the cytokines related to the Th1 response (18).

This study's investigation of cytokines produced by Th1, Th2, and regulatory T cells indicated that smaller nanoparticles generated higher levels of IL-2, IFN-γ, and IL-10 compared to larger ones. Despite the lack of statistical significance in our data for IL-4 and IFN-γ, we observed a decrease in IL-4 and a considerable increase in IFN-γ, indicating a transition from Th2 to Th1 responses, analogous to the findings of previous researches (19, 20). The low encapsulation efficiency or anionic charges of Glucantime-encapsulated PLGA NPs may have inhibited their uptake by

antigen-presenting cells. Treatment with Glucantime-encapsulated PLGA nanoparticles significantly suppressed IL-10 levels compared with the control groups, indicating inhibition of regulatory and anti-inflammatory responses. As IL-10 is secreted by various immune cells, including Tregs, Th2, and macrophages, its reduction suggests a shift toward a Th1-dominant immune profile. This finding is consistent with the observed increase in IL-2 production, confirming the strong immunomodulatory and Th1-promoting effects of PLGA-Glu NPs (9, 13).

Using the MTT approach, lymphocyte proliferation was evaluated and the rates of proliferation among the treatment groups were clearly different. Especially, the PBS-treated group showed a much higher proliferation rate than both the Glucantime-treated group and the Glucantime-encapsulated PLGA groups (both low and high dosages). This result implies that *Leishmania* antigens present in the PBS-treated group would have triggered a strong immunological response, hence raising lymphocyte activation and proliferation (21). By contrast, the reduced proliferation rates shown in the Glucantime and Glucantime-encapsulated PLGA groups suggest that these therapies might alter immune cell responses, hence perhaps reducing lymphocyte activation. The lack of significant differences between the low (100 pg) and high (300 pg) doses of Glucantime-encapsulated PLGA suggests a plateau effect in lymphocyte proliferation, indicating that beyond a certain dosage, increasing the amount of Glucantime-encapsulated PLGA does not further enhance immune cell activation. This could imply that the formulation's capacity to stimulate lymphocyte proliferation is limited, possibly due to factors such as drug release kinetics or the immunomodulatory effects of PLGA as a delivery vehicle (22).

Pathological studies using lymphocyte proliferation assays provided understanding of immune responses. Indicating possible tissue damage or inflammation resulting from the immunological response to Leishman infection, both PBS-treated and PLGA-treated groups showed substantial

myofiber degradation and a presence of macrophage-epithelioid cells in the dermis and subcutaneous tissue. In the Glucantime-treated group, mild to moderate inflammatory infiltrates, primarily lymphocytes, were observed, suggesting some therapeutic effects through lymphocyte promotion but a weaker inflammatory response compared to the PBS control. This ongoing adaptive immune response is crucial for controlling *Leishmania* infections, yet its mild nature may limit Glucantime's efficacy as a monotherapy (23). The Glucantime-encapsulated PLGA groups showed varying inflammation levels; the low-dose group exhibited moderate inflammation with lymphocyte infiltration, indicating an enhanced immune response relative to the Glucantime group. Conversely, the high-dose group displayed only mild inflammation, suggesting that excessive dosing might lead to immunosuppression or tolerance, potentially reducing the nanoparticle formulation's therapeutic benefits (24). The major limitations of this study included the following: (a) during the cutaneous injury process, there were differences between them, (b) the uptake of PLGA NPs was not evaluated by incubating with the DC cells, (c) flow cytometry assays for upregulation of CD80 and MHCII were not used in mouse macrophages. In conclusion, this study offers preliminary proof that Glucantime-encapsulated PLGA NPs can modify immune responses in a model of Leishmaniasis. Our results indicated that compared to the control group, Glucantime-encapsulated PLGA NPs with an estimated size of 200 nanometers suggested a decrease in systemic reactions and tissue symptoms and enhanced the TH1 response. More efficient treatment plans against this infectious disease can be made possible by optimizing formulation tactics and knowing their consequences on lymphocyte proliferation and tissue pathology.

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Conflict of interest

The authors declare no conflict of interest, financials or otherwise.

Ethics approval

This work was permitted by the ethical committee of Islamic Azad University, Mashhad Branch, Iran with code number 1119508190006.

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Authors' Contribution

Conceptualization and Data curation: M.R.Kh., Formal analysis: H.Y., M.H., A.M., J.H., A.H., Investigation: H.Y., Project administration: M.R.Kh., Resources: M.R.Kh., Software: A.M., Supervision: M.R.Kh., Validation: M.R.Kh., Visualization: M.H., A.M., Writing–original draft: M.H., and M.R.Kh., and Writing–review & editing: M.H., and M.R.Kh.

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