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RESEARCH ARTICLE

Comparative Development of Promastigote- and Amastigote-Based Cutaneous Leishmaniasis Models in BALB/c Mice

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Abstract

Cutaneous leishmaniasis (CL) is a significant public health problem caused by parasites of the genus *Leishmania*. Experimental animal models developed for cutaneous leishmaniasis are of great importance for understanding the disease process, the mechanisms of disease development, and evaluating treatment approaches. The aim of our study was to compare lesion development and the clinical course of infection in experimental cutaneous leishmaniasis models created in BALB/c mice using promastigote and amastigote forms of *Leishmania major*. The promastigote form of *L. major* was cultured in NNN and RPMI-1640 media. The amastigote form was previously obtained from the THP-1 cell line and stored by cryopreservation. The parasite forms were applied intradermally to the right foot of the experimental animals. The experimental animals were treated with 10^5 promastigote/mL, 10^5 amastigote/mL, and sterile saline. Lesion development was monitored weekly for 12 weeks. Lesion development was assessed by measuring footpad thickness. Infection was successfully induced in both groups. After 12 weeks, the average footpad thickness was 3.32 mm in the amastigote group and 2.33 mm in the promastigote group. Amastigotes were observed in Giemsa-stained touch preparations, and promastigotes were found to grow from the lesions in inoculation onto NNN medium. The findings of this study demonstrate that the experimental cutaneous leishmaniasis model created with the amastigote form is reliable and applicable, allowing faster results in therapeutic and immunological studies.

Keywords: Amastigote, Cutaneous leishmaniasis, Experimental model, *Leishmania major,* Promastigote

Introduction

Leishmaniasis is an important parasitic infection affecting both humans and animals, caused by protozoa of the genus *Leishmania* and classified as a neglected tropical disease. *Leishmania* has two main morphological forms throughout its life cycle: the flagellated promastigote form, which develops in the gut of female sandflies *(Phlebotomus* spp.), and the non-flagellate amastigote form, which lives intracellularly within macrophages in mammalian hosts [1,2]. Although both forms exhibit different morphological features, they share basic cellular structures such as the nucleus, kinetoplast, and basal body.

Leishmaniasis is transmitted through the bite of infected female sandflies. Three main clinical forms are seen: cutaneous leishmaniasis, visceral leishmaniasis (VL), and mucocutaneous leishmaniasis (MKL). More than 20 *Leishmania* species have been identified that cause disease in humans and animals [3]. Canine leishmaniasis, particularly that caused by *Leishmania infantum*, is prevalent in many endemic regions, including the Mediterranean Basin. Infected dogs play a central role as reservoir hosts in the epidemiology of the disease [4]. Therefore, leishmaniasis is considered an important zoonosis from a veterinary public health perspective.

Türkiye is one of the endemic countries for leishmaniasis. The vast majority of reported CL and VL cases are caused by *L. tropica* and *L. infantum* [5]. Factors such as climate change, inadequate vector control, increased migration, and insecticide resistance have been reported to affect the epidemiology of the disease [5].

The experimental animal models developed are critical for understanding disease pathogenesis, host-parasite relationships, host immune responses, and the development of new treatment strategies. Mouse models are preferred because of their low cost, defined genetic structures, and the ability to macroscopically assess the infection process. BALB/c mice are widely used in cutaneous leishmaniasis studies because of their high susceptibility to *L. major* infection and their ability to develop significant cutaneous lesions [6].

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In experimental cutaneous leishmaniasis models, the promastigote form is the most commonly used because it mimics vector-borne transmission. Studies on experimental cutaneous leishmaniasis induced using the amastigote form also exist. However, the clinical course of infections initiated by promastigote and amastigote forms under the same experimental conditions remains insufficiently characterized.

Accordingly, in this study, experimental cutaneous leishmaniasis models were established in BALB/c mice using *L. major* promastigotes and amastigotes, and the clinical progression of infection was evaluated comparatively. We hypothesized that infection with the amastigote form of *L. major* would result in earlier lesion onset and more rapid disease progression compared to the promastigote form in the BALB/c mouse model.

Material and Methods

Ethical Statement

This study was approved by the Manisa Celal Bayar University Animal Experiments Local Ethics Committee (Approval no: 319, Date: 15/04/2025).

Cultivation of *Leishmania major* Promastigotes

In our study, we used the *L. major* isolate coded MHOM/TR/2013/CBUALA17, which was isolated from a patient living in Türkiye with no history of travel abroad, subsequently cryopreserved after species identification, and stored in the Parasite Bank of Manisa Celal Bayar University, Faculty of Medicine. The isolate was removed from the liquid nitrogen tank and thawed in a 37°C water bath for approximately two min. Before use, 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1% gentamicin were added to RPMI-1640 medium. Five mL of RPMI-1640 medium was added to a sterile Falcon tube. Then, the entire suspension from the cryo tube after thawing was transferred into the Falcon tube. The Falcon tube was centrifuged at 1200 rpm for 5 min. After centrifugation, the supernatant was discarded, and the remaining pellets were transferred to NNN (Novy-MacNeal-Nicolle) medium. The NNN medium was incubated in an oven at 26°C.

The growth status of *Leishmania* parasites was monitored by examining a sample taken from the culture medium every two days using an inverted microscope. Promastigotes from the NNN medium showing growth were inoculated into RPMI-1640 medium, which had been previously supplemented with 10% FBS, 1% penicillin/streptomycin, and 1% gentamicin. The RPMI-1640 medium containing promastigotes was incubated at 26°C. Parasite growth was monitored every two days using an inverted microscope. When the parasites reached the logarithmic phase, they were counted using trypan blue stain on a Thoma slide. A suspension with a final concentration of 10^5 promastigotes/mL was prepared for use in our study.

Preparation of *Leishmania major* Amastigote Suspension

The infected THP-1 cell line [7] was first treated with 0.05% sodium dodecyl sulfate (SDS) for 30 sec to disrupt the cell membrane. Then, PBS was added to neutralize the effect of SDS, yielding a suspension. The resulting suspension was passed three times through a 27 G infector needle. A density gradient was created in the suspension using 45% Percoll and 100% Percoll to obtain pure amastigotes. The prepared density gradient was centrifuged at 3500 × g for 30 min, and amastigotes were obtained [8]. The obtained amastigotes were washed three times with RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1% gentamicin and cryopreserved. In our study, we used a cryopreserved *L. major* strain (MHOM/TR/2013/CBUALA17) stored in liquid nitrogen. The cryo tube, removed from the liquid nitrogen tank, was thawed by holding it in a 37°C water bath for 2 min. The suspension in the cryo tube was transferred to a sterile Falcon tube containing 5 mL of RPMI-1640 medium. The Falcon tube was centrifuged at 1200 rpm for 5 min. After centrifugation, the supernatant was discarded. One mL of medium was added to the remaining pellet to create a suspension. Viability and amastigote count were determined on a Thoma slide using trypan blue staining. A suspension with a final concentration of 10^5 amastigotes/mL was prepared.

Experimental Animals and Group Allocation

In our study, three groups were formed to create an experimental cutaneous leishmaniasis model. Fifteen male BALB/c white mice, aged 3-4 months and weighing 20-25 g, obtained from the Manisa Celal Bayar University Experimental Animal Application and Research Center, were randomly divided into three groups of five. The experimental animals were housed in polycarbonate cages on sawdust bedding under controlled environmental conditions with a 12-h light/12-h dark cycle. The experimental animals were fed a standard laboratory diet and provided with unlimited drinking water.

The experimental groups were defined as follows:

L. major promastigote group

L. major amastigote group

Control group

Establishment of the Cutaneous Leishmaniasis Model

Fifty µL of the prepared 10^5 promastigote/mL *L. major* promastigote suspension was administered intradermally

to the right footpad of each mouse in the promastigote group. 50 µL of the 10^5 amastigote/mL *L. major* amastigote suspension was administered to the right footpad of each mouse in the amastigote group. Mice in the control group received 50 µL of saline intradermally to their right footpads.

Follow-up of the Model, Sampling, and Study Termination

Experimental animals were monitored weekly for 12 weeks. Footpad measurements were taken weekly at the same time of day by the same researcher using the same digital caliper for 12 weeks. To ensure measurement consistency, the same researcher measured the footpad thickness three times consecutively from the same anatomical region of each experimental animal. The average of these measurements was used for statistical analysis. At the end of the study, the experimental animals were sacrificed, and their right hind footpad was removed. Tissue samples were prepared and inoculated into NNN medium to investigate the presence of parasites.

Statistical Analysis

The normality of model residuals was assessed using the Shapiro-Wilk test. Because repeated measurements were obtained from the same experimental animals over 12 weeks, a mixed-design repeated-measures ANOVA was performed using R (version 4.6.0; R Foundation for Statistical Computing, Vienna, Austria). Experimental group (promastigote, amastigote, and control) was included as the between-subject factor, whereas time (week) was included as the within-subject factor. The sphericity assumption was evaluated using Mauchly's test. Since the assumption of sphericity was violated, Greenhouse-Geisser-corrected degrees of freedom and p-values were reported. Partial eta squared (ηp^2) was calculated as the measure of effect size. Pairwise comparisons based on estimated marginal means were performed using Tukey-adjusted P-values where appropriate. As a sensitivity analysis, the longitudinal data were additionally analyzed using a linear mixed-effects model (LMM) with mouse included as a random intercept, group and week as fixed effects, and Kenward-Roger approximation for significance testing. Marginal and conditional R^2 values were calculated to evaluate the explanatory power of the mixed model. Statistical significance was defined as $P<0.05$.

The sample size (n=5 mice per group) was determined in accordance with previous cutaneous leishmaniasis modeling using BALB/c mice and the ethics committee's principle of reducing the use of animals in animal experiments. No a priori power analysis was performed to determine the sample size. A post-hoc sensitivity power analysis was performed using G*Power software (version 3.1.9.7) to assess the statistical sensitivity of the study.

Results

Leishmania major Promastigote Culture

Microscopic examination following the inoculation of the *L. major* promastigote form into NNN medium revealed the initiation of parasite proliferation on the fifth day. Monitoring of liquid cultures using an inverted microscope demonstrated that the promastigotes reached the logarithmic growth phase on the sixth day. Based on counts performed during the logarithmic phase, a suspension with a concentration of 10^5 promastigotes/mL was obtained for use in experimental applications.

Preparation of *Leishmania major* Amastigote Suspension

Based on counts performed after removal of *L. major* amastigotes from the liquid nitrogen tank, a suspension with a concentration of 10^5 amastigotes/mL was prepared for use in experimental infections.

Promastigote Group Findings

Weekly footpad thickness measurements were recorded in the promastigote group following infection. The mean measurement in the first week was 1.39 mm, and values gradually increased in subsequent weeks. By the fourth week, the mean measurement reached 1.67 mm, and the increase continued during the later stages of follow-up in parallel with the progression of inflammation *(Fig. 1, Fig. 2)*.

At the end of the twelve-week follow-up period, the mean footpad thickness in the promastigote group was recorded as 2.33 mm. The gradual increase observed in weekly measurements indicated that the lesion thickness increased regularly throughout the follow-up period *(Table 1)*.

Amastigote Group Findings

In the amastigote group, weekly footpad thickness measurements were recorded to monitor lesion development. A gradual increase in mean footpad

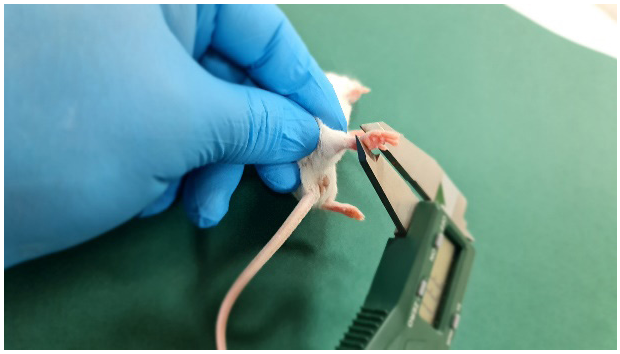

Fig 1. Footpad thickness measurements

Fig 2. Footpad of the *Leishmania major* promastigote group

thickness was observed during the first four weeks of follow-up, with the rate of increase accelerating significantly after the fourth week.

The mean footpad thickness reached 2.90 mm in the fifth week, and measurements continued to increase in the following weeks, reaching 3.32 mm at the end of the twelfth week *(Table 2)*. When comparing the groups, higher footpad thickness values were observed in the amastigote group than in the promastigote group from the fourth week onwards *(Fig. 3)*.

Control Group Findings

In the control group treated only with saline solution, footpad thickness measurements remained unchanged throughout the 12-week follow-up period. Footpad thickness in control group mice varied between 1.33-1.34 mm on average over 12 weeks *(Table 3)*. No significant change in footpad thickness was observed in control group mice during the study period. No increase suggestive of lesion development was detected.

Statistical Comparison of Footpad Thickness Measurements Between Groups

A mixed-effects repeated-measures ANOVA showed significant effects of group ($F(2,12) = 2812.74$, $P<0.001$, $\eta p^2 = 0.998$, 95% CI [1.00, 1.00]), time (Greenhouse-Geisser correction: $F(1.80, 21.58) = 3811.16$, $P<0.001$, $\eta p^2 = 0.997$, 95% CI [1.00, 1.00]) and group × time interaction (Greenhouse-Geisser correction: $F(3.60, 21.58) = 1502.92$, $P<0.001$, $\eta p^2 = 0.996$, 95% CI [1.00, 1.00]). These findings demonstrate that footpad thickness changes significantly over time and that the infection pattern differs between experimental groups.

Almost identical results were obtained when applying the linear mixed-effects (LMM) model. Group ($F(2,12) = 2812.7$, $P<0.001$), week ($F(11,132) = 3811.2$, $P<0.001$) and group × week interaction ($F(22,132) = 1502.9$, $P<0.001$) showed significant fixed effects. The model explained almost all of the observed variability in footpad thickness (marginal $R^2 = 0.998$, 95% CI 0.996-0.998; conditional $R^2 = 0.999$, 95% CI 0.999-0.999).

Tukey-corrected pairwise comparisons based on estimated marginal means revealed no significant difference between the control and promastigote groups during week 1 ($P=0.065$). However, all remaining pairwise comparisons became statistically significant from week 2 onwards (all $P<0.001$). A clear distinction emerged between the promastigote and amastigote groups at week 5, consistent with lesion development observed in amastigote-infected mice *(Fig. 4)*.

A sensitivity power analysis was performed using G*Power software (version 3.1.9.7) for a repeated-measures between-groups interaction design. Considering a significance

Table 1. *Weekly footpad thickness (mm) in the Leishmania major promastigote group*

Week	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5	Mean ± SD
Week 1	1.39	1.40	1.38	1.38	1.39	1.39±0.01
Week 2	1.49	1.49	1.47	1.47	1.48	1.48±0.01
Week 3	1.60	1.59	1.56	1.56	1.58	1.58±0.02
Week 4	1.70	1.68	1.65	1.65	1.67	1.67±0.02
Week 5	1.81	1.78	1.74	1.74	1.76	1.77±0.03
Week 6	1.91	1.87	1.83	1.83	1.86	1.86±0.03
Week 7	2.02	1.97	1.92	1.92	1.95	1.96±0.04
Week 8	2.12	2.06	2.01	2.01	2.04	2.05±0.05
Week 9	2.23	2.16	2.10	2.10	2.14	2.15±0.05
Week 10	2.33	2.25	2.19	2.19	2.23	2.24±0.06
Week 11	2.38	2.31	2.23	2.25	2.26	2.29±0.06
Week 12	2.44	2.35	2.28	2.28	2.32	2.33±0.07

Table 2. Weekly footpad thickness (mm) in the Leishmania major amastigote group

Week	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5	Mean
Week 1	1.44	1.44	1.46	1.44	1.45	1.45±0.01
Week 2	1.56	1.56	1.56	1.56	1.57	1.56±0.00
Week 3	1.68	1.68	1.66	1.68	1.69	1.68±0.01
Week 4	1.80	1.80	1.76	1.80	1.81	1.79±0.02
Week 5	2.92	2.93	2.85	2.87	2.93	2.90±0.04
Week 6	2.99	2.98	2.90	2.92	2.96	2.95±0.04
Week 7	3.06	3.02	2.95	2.97	2.99	3.00±0.04
Week 8	3.13	3.06	3.00	3.02	3.02	3.05±0.05
Week 9	3.20	3.10	3.05	3.07	3.05	3.09±0.06
Week 10	3.27	3.14	3.10	3.12	3.08	3.14±0.07
Week 11	3.30	3.22	3.18	3.22	3.20	3.22±0.05
Week 12	3.33	3.30	3.31	3.32	3.32	3.32±0.01

level of 0.05, statistical power of 80%, three experimental groups, 12 repeated measurements, a total of 15 animals, a correlation coefficient of 0.50 between repeated measures, and the Greenhouse-Geisser sphericity correction factor (ε = 0.1635) obtained from the existing data, the minimum detectable effect size was calculated as Cohen's f = 0.49. According to Cohen's classification, this value represents a large effect size.

Fig 3. Footpad of the *Leishmania major* amastigote group

Tissue Samples and NNN Culture Positivity Findings

Microscopic examination of touch preparations prepared from tissue samples obtained from the right hind footpads of experimental animals sacrificed at the end of the study clearly demonstrated the presence of amastigote forms in tissues from both the promastigote- and amastigote-infected groups.

Following inoculation of the tissue samples onto NNN

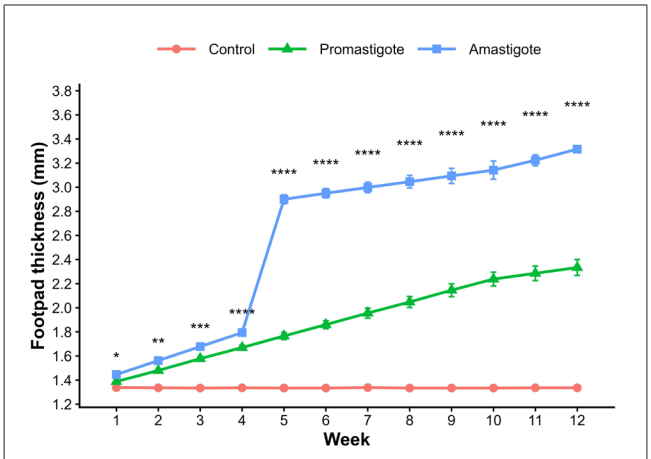

Fig 4. Footpad thicknesses of *Leishmania major* promastigote and amastigote group mice and control group mice over a 12-week follow-up period. Data are presented as mean ± standard deviation (SD; n = 5 mice per group). Asterisks indicate Tukey-corrected pairwise comparisons between promastigot and amastigot groups based on estimated marginal means: *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001

Table 3. *Weekly footpad thickness (mm) in the control group*

Week	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5	Mean
Week 1	1.33	1.34	1.34	1.34	1.34	1.34±0.00
Week 2	1.34	1.33	1.34	1.33	1.34	1.34±0.01
Week 3	1.33	1.34	1.33	1.34	1.33	1.33±0.01
Week 4	1.33	1.33	1.34	1.34	1.34	1.34±0.01
Week 5	1.34	1.33	1.34	1.33	1.33	1.33±0.01
Week 6	1.33	1.34	1.33	1.34	1.33	1.33±0.01
Week 7	1.34	1.33	1.34	1.34	1.34	1.34±0.00
Week 8	1.33	1.34	1.33	1.34	1.33	1.33±0.01
Week 9	1.34	1.33	1.34	1.33	1.33	1.33±0.01
Week 10	1.33	1.34	1.33	1.33	1.34	1.33±0.01
Week 11	1.34	1.33	1.34	1.34	1.33	1.34±0.01
Week 12	1.33	1.34	1.33	1.34	1.34	1.34±0.01

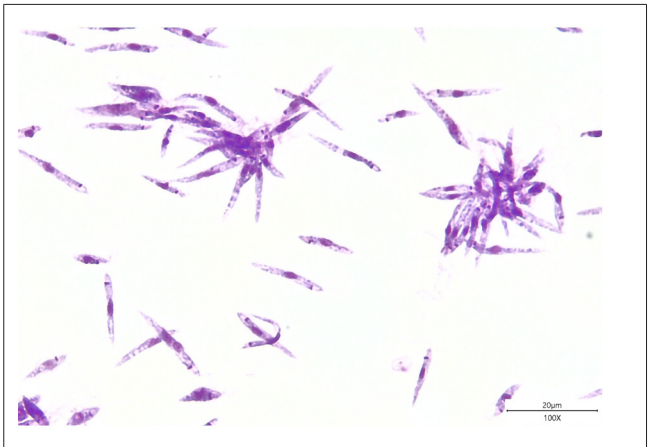

Fig 5. Giemsa-stained microscopic appearance of *Leishmania major* promastigotes growing on NNN medium

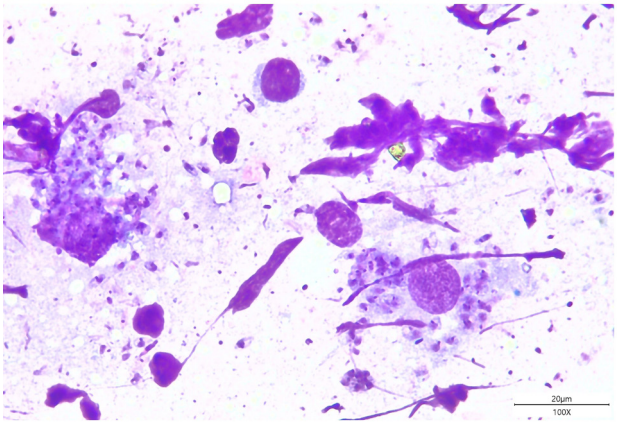

Fig 6. Giemsa-stained microscopic view of a mouse footpad touch preparation from the amastigote group

medium, transformation into the promastigote form and active parasite proliferation were detected in both infected groups, and the cultures were found to be positive (*Fig. 5*, *Fig. 6*). In contrast, no amastigote forms were observed in preparations prepared from tissue samples of the control group, and no promastigote growth was detected in NNN cultures inoculated with control group samples.

Discussion

Cutaneous leishmaniasis is a common parasitic infection both worldwide and in Türkiye. The most important causative agent of cutaneous leishmaniasis in Türkiye is *L. tropica.* In recent years, cases of cutaneous leishmaniasis caused by *L. major* have also been observed. In cutaneous leishmaniasis, where treatment options are limited, treatment success is decreasing and treatment durations may be prolonged due to drug resistance reported in recent years. Therefore, experimental animal models are of great importance for understanding the development mechanism, biology, host-parasite relationship, and developing new treatment strategies for cutaneous leishmaniasis [6,9]. BALB/c mice are one of the commonly used experimental models in cutaneous leishmaniasis research due to their high susceptibility to *L. major* infection and Th2-dominant immune response [10]. This study aims to comparatively evaluate infections induced by promastigote and amastigote forms in the BALB/c model and thus explain the infection dynamics of different life cycle stages of the parasite.

Our findings demonstrated that both promastigote and amastigote forms successfully induced infection in BALB/c mice. The cutaneous leishmaniasis model induced by the amastigote form showed a more pronounced pathological course from the fifth week onwards compared to the cutaneous leishmaniasis model induced by the promastigote form. This difference may be related to the biological characteristics of the amastigote form that are compatible with the mammalian host; however,

further immunological and molecular studies are needed to elucidate these possible mechanisms.

It has been reported that the promastigote and amastigote forms of the *Leishmania* parasite differ not only in shape but also in surface molecules, metabolism, gene expression, and virulence factors [11]. Promastigote forms express high levels of lipophosphoglycan (LPG) and the surface protease gp63, while these are expressed less in amastigote forms. It has also been reported that cysteine protease expression is low in promastigote forms and high in amastigote forms [12,13]. These differences lead macrophages to perceive promastigote and amastigote forms as different targets and to develop different cellular responses to each. In addition, promastigote and amastigote forms do not use the same mechanism to suppress the immune response. Studies have shown that promastigotes activate two phosphatases, SHP-1 and PTP-1B, together to suppress macrophage defense mechanisms upon initial entry into the host. Together, these molecules suppress pro-inflammatory signaling pathways such as JAK2, STAT1, NF-κB, AP-1, and TNF-α, consequently reducing macrophage microbicidal activity [13]. By contrast, amastigote forms evade the immune response by activating only SHP-1 and are reported to suppress NF-κB, STAT-1, and AP-1 more strongly than promastigotes upon initial interaction with macrophages [13]. In our study, the differences observed between promastigote and amastigote groups are thought to stem from their different mechanisms for entry into the host cell and the development of intracellular infection. After inoculation of promastigotes, they first encounter the complement system and phagocytic cells, then enter the cell and transform into the amastigote form. This transformation process, which requires adaptation to the host cell environment, suggests that intracellular proliferation and lesion development may begin later in infections initiated by promastigotes. By contrast, amastigotes are already adapted to the intracellular environment. They can begin intracellular proliferation after being taken up by phagocytic cells. Therefore, it is thought that infections caused by amastigotes lead to an earlier increase in parasite load and faster lesion development compared to infections caused by promastigotes.

The high susceptibility of BALB/c mice to *L. major* infection is associated with their development of a Th2-dominant immune response in which IL-4 and IL-10 production is predominant [10]. In the present study, the acceleration of inflammation and the regular increase in footpad thickness observed particularly from weeks 4-5 onward were consistent with the susceptible phenotype of the BALB/c model described in the literature. The relatively limited increase in footpad thickness during the early weeks and the delayed manifestation of infection in the promastigote-infected group may be explained by the requirement of promastigotes to undergo differentiation into amastigotes following phagocytosis in the mammalian host. Although equal inoculum doses were used numerically in both groups, the requirement for *in vivo* differentiation of the promastigote form into the amastigote stage should be considered a fundamental biological factor that may partially explain the delayed lesion development observed in this group. It has previously been reported that this biological transition process can slow infection kinetics [14].

The observation that the amastigote form induced earlier and more severe pathology is consistent with studies reporting that amastigote inoculation is more effective in initiating infection. It has been demonstrated that axenic amastigotes of *L. infantum* establish infection more rapidly and reach detectable parasite burdens earlier than promastigotes [15]. Similarly, amastigotes have been reported to possess metabolic and proteomic advantages in host adaptation and to proliferate without requiring transition to the intracellular phase [16]. In the present study, the marked inflammation observed in the amastigote group, particularly from weeks 4-5 onward, and the higher footpad thickness measurements recorded at week 12 compared to the promastigote group are consistent with this evidence.

From a practical standpoint, these findings provide researchers with useful information for selecting an experimental model to develop antileishmanial treatments. The earlier onset and faster progression of lesions observed in the amastigote-based model may better reflect the intracellular stage responsible for pathology in human infections. An experimental cutaneous leishmaniasis model created with the amastigote form could provide a practical experimental platform, particularly for studies focusing on compounds that target intracellular amastigotes. The shorter time required for lesion development may contribute to more efficient evaluation of new molecules and active substances.

Although both parasite forms are used in experimental cutaneous leishmaniasis modeling, comparisons conducted under the same experimental conditions remain incomplete. The results show that the choice of parasite form affects infection kinetics, lesion severity, and experimental duration, thereby improving the experimental model. This study can guide researchers in selecting the most suitable experimental model for their research objectives.

The use of repeated-measures analysis controlled within-subject variability in time-dependent measurements obtained from the same animals and allowed for a more reliable evaluation of differences in lesion progression

among groups. As a result of these analyses, both the promastigote and amastigote groups were found to reach significantly higher footpad thickness values throughout the follow-up period compared to the control group.

The demonstration of amastigotes in Giemsa-stained touch-up preparations and the growth of promastigotes from footpad lesions in NNN medium at the end of the study confirmed the successful occurrence of *in vivo* infection. The absence of both microscopic and culture positivity in the control group supports the idea that the observed pathological changes are specific to the experimental infection.

The contribution of this study to the literature can be summarized in two main points: (i) the promastigote and amastigote forms of *L. major* isolate exhibit different kinetics in infection severity and development; and (ii) the amastigote form allows the experimental model to be established in a shorter time, enabling earlier evaluation of therapeutic agents. Amastigote injection has been reported to reliably and reproducibly create lesions in the BALB/c model [17]. Our study offers a feasible and time-efficient approach for testing drugs, vaccines, and immunomodulatory agents.

The low biological variation observed among individual animals in this study is likely due to the good standardization of experimental conditions. The same *L. major* isolate was used in both experimental groups; the same amount of parasite was applied to the footpads; all experimental animals were BALB/c mice of the same age and sex, and they were monitored under the same environmental conditions. Since the footpad thickness measurements were performed by the same researcher, from the same anatomical region, and using the same digital caliper, technical variability between measurements was reduced. The fact that the measurements were not performed blinded can be considered one of the limitations of the study. The genetically homogeneous BALB/c mouse strain and the selection of all experimental animals of the same age and sex may also have contributed to the recorded low biological variation.

Our study was designed as a comparative experimental study. Therefore, immunological evaluations and quantitative parasitological analyses were not included in our study. Our study encompasses the clinical findings of two different models, with microscopic and culture evaluations performed to confirm the infection. For future studies, it is recommended to strengthen the model through further histopathological evaluations, monitoring immune response dynamics using cytokine profiling, quantitative measurement of parasite load, and integration of treatment strategies. Time-course analysis of Th1/Th2 response polarization may help elucidate the interaction between the immune system and the more aggressive course observed in amastigote infections. In addition, comparing different *Leishmania* species with different doses will increase the value of the developed amastigote model.

In our study, an experimental cutaneous leishmaniasis model was created on the paw pads of BALB/c mice using both promastigote and amastigote forms of *L. major*. The created experimental cutaneous leishmaniasis model was monitored for 12 weeks. The experimental cutaneous leishmaniasis model created with both parasite forms was successfully established. In the modeling with the amastigote form, lesion development accelerated significantly from the fifth week onwards, and higher lesion measurements were obtained. In the modeling with the promastigote form, lesion development showed a slower and more stable progression.

Microscopic examination and culture results provided biological confirmation of the infection. The findings show that the developed experimental model has been successfully implemented.

The fact that the experimental cutaneous leishmaniasis model created with the amastigote form appeared approximately 5-6 weeks earlier than the model created with the promastigote form led to a significant shortening of the study period. The earlier initiation of the model provides a significant advantage in reducing animal care time and associated costs. The experimental cutaneous leishmaniasis model created with the amastigote form could be an ethically and practically advantageous alternative, as it can be established approximately 50% faster than the promastigote model and reduces care burden and costs.

Modeling experimental cutaneous leishmaniasis using the amastigote form of the *Leishmania* parasite affords several benefits. This model reduces the time required for lesion development, shortens the evaluation time for candidate molecules or compounds, and consequently increases efficiency in workflow planning. In experimental cutaneous leishmaniasis modeling with the amastigote form, the transformation process from promastigote to amastigote is eliminated, enabling faster observation of the resulting lesion.

In conclusion, the BALB/c cutaneous leishmaniasis model established using the amastigote form appears to be a reliable and applicable experimental model for evaluating the efficacy of drugs, vaccines, and immunotherapeutic agents, while offering notable advantages with respect to time efficiency, cost reduction, and animal welfare.

When all findings are considered, the model created in BALB/c mice with the amastigote form of the *Leishmania* parasite could be a practical and time-efficient

experimental model for future studies evaluating new antileishmanial strategies.

Declarations

Availability of Data and Materials: All the data generated are included in the manuscript.

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Declaration of Generative Artificial Intelligence (AI): The article and/or tables and figures were not written/created by AI and AI assisted technologies.

Ethical Compliance Declaration: The authors declare that the study was conducted in accordance with all applicable animal welfare and research ethics principles, institutional and national/international regulations, and the requirements of the relevant ethics committees and funding/supporting institutions, where applicable. The authors accept full responsibility for the ethical integrity of the study.

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