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Clinical and parasitological evaluation of cutaneal lesions in *Mesocricetus auratus* infected with *Leishmania amazonensis*

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Cutaneous leishmaniasis is an infectious-parasitic disease that is vector-transmitted by female Phlebotomus species through a blood meal. The drugs recommended by the Ministry of Health show moderate efficacy and numerous adverse reactions, making it necessary to develop new drugs. However, it is essential to establish new and vibrant diagnostic techniques detecting and quantifying the parasites in experimental animals in the pre-clinical phase. Therefore, the aim of this project was to evaluate clinical and parasitological aspects in *Mesocricetus auratus* infected with *Leishmania amazonensis*, thus making it possible to compare the diagnostic methods used in *in-vivo* trials for new therapeutic agents for cutaneous leishmaniasis. *L. amazonensis* promastigotes were inoculated into 12 adult hamsters. The trial was divided into two groups, one treated with Glucantime® and the other untreated. During treatment, the animals were monitored clinically and the lesion area was measured. After the 40th day, the animals were euthanized to obtain fragments of the lesions for parasitological, histopathological, and molecular analysis. The GLUC group had nodular lesions, while the CONT-group had ulcerated lesions with a 445.9% increase in volume. The parasitological and histopathological analyses corroborated the results obtained from real-time PCR, showing a lower parasite load, mild dermatitis and a significant reduction in the concentration of parasite DNA compared to CONT-group. The study confirms that clinical assessment and application of parasitological, histopathological, and molecular diagnostic techniques are essential in determining the clinical and parasite profile more accurately in experimental animals used for pre-clinical which can be used to test and recommend new therapeutic drugs for cutaneous leishmaniasis.

Key words: Cutaneous leishmaniasis, *mesocricetus auratus*, Glucantime®, *Leishmania amazonensis,* diagnostic tests.

INTRODUCTION

American Cutaneous Leishmaniasis (ACL) is a parasitic, non-contagious, zoonotic disease that affects wild and domestic animal species, as well as humans. It is transmitted by vector through blood meal by female

phlebotomine of the genus *Lutzomyia* (OPAS, 2021). There can be various clinical forms of this disease, depending on the species of *Leishmania* infected and the relationship between the parasite and its host (Brasil, 2017).

American Cutaneous Leishmaniasis is a global public health problem, with 1,105,545 cases recorded between the years 2001 and 2021, which corresponds to an annual average of 52,645 cases per year. Brazil is among the countries with the highest number of cases of this endemic disease, with 16,432 cases recorded in 2021, the highest number among the countries in America (OPAS, 2021). It is a disease considered to be neglected because it mainly affects a vulnerable, low-income population that does not have access to quality public health care (Brasil, 2017; Maia-Elkhoury et al., 2019).

Among the protozoan infections that affect humans in the state of Amazonas, ACL occupies a high position in the ranking, with 879 cases recorded in 2022, given that one of the biggest impacts on the best epidemiological parameter for this disease is the fact that it is underreported, preventing a reliable analysis (OPAS, 2021; Brasil and Franco, 2023).

The drugs recommended by the Ministry of Health against ACL are pentavalent antimonial (Sb5+), pentamidine isethionate and amphotericin B (Brazil, 2017). However, this therapy has shown major problems that contribute to patients' resistance against treatment, such as the parenteral route of administration, causing many patients to abandon treatment, and limited efficacy, with numerous adverse reactions, such as acute renal failure, hypotension and hypopotassemia (Ferreira et al., 2012; Brasil, 2017; Carvalho et al., 2019).

One way of adapting pharmacotherapy for ACL would be through research into possible new drugs, but this is a costly, long-term process that requires the participation of multidisciplinary teams, thus necessitating large investments. There is also a need to carry out a pre-clinical phase, in which the pharmacological activity of these new substances will first be assessed using experimental laboratory models, which can be *in silico*, *in vitro* or *in vivo* (Scarabelot et al., 2023; Stanley, 2024).

The animal experimentation model for *in vivo* biological trials to assess antileishmanial activity is usually young and prepubertal mice (Balb/C) or hamsters (*Mesocricetus auratus*), as they are more susceptible to the infection (Comandolli-Wyrepkowski et al., 2017).

During pre-clinical trials, to assess the effect of a given drug on animals infected with *Leishmania* sp., it is necessary to use different diagnostic methods to detect and quantify parasites after treatment. Although there is still no method considered the gold standard, the method commonly used in the literature is the direct parasite method (Brasil, 2017; Ramírez et al., 2000; Reimão et al, 2020). However, there is a need to standardize or indicate other methods for application in preclinical trials for research into new drugs with antileishmanial action.

In this context, the aim of this study was to evaluate clinical and parasitological aspects in hamsters experimentally infected with the *Leishmania amazonensis* species, making it possible to compare the diagnostic methods used in preclinical trials of possible therapeutic targets for cutaneous leishmaniasis.

MATERIALS AND METHODS

Maintenance of *Leishmania* sp.

An assay of *Leishmania amazonensis* promastigote forms were used (MHO/BR/2009/IM5584), maintained and cryopreserved at the Leishmaniasis and Chagas Disease Laboratory, COSAS/INPA. The parasites were grown in complete RPMI 1640 medium (Himedia®) supplemented with 10% inactivated fetal bovine serum (IFBS) and kept in an oven at 24°C.

Origin of the animals

The experimental biological model for the study were 12 adult (over 90 days old) male hamsters (*Mesocricetus auratus*) weighing 200 g each. The animals came from the Central Bioterium of the National Institute for Research in Amazonia – INPA, and the treatment of the infected animals took place on its premises. The animals were kept in polypropylene cages in conditions suitable for their maintenance, with diet and water *ad libitum* and free of pathogens. The project was approved by the Ethics Committee for Research Using Animals - ECRUA/INPA, under protocol number 009/2015.

Inoculation and Design

A solution of 0.1 mL containing 10^6 cells/mL of the parasite in the promastigote form of *L. amazonensis* freshly isolated in amastigote culture was inoculated onto the snouts of the experimental hamsters. After inoculation, the animals were separated and grouped in identified cages. After 24 days of inoculation with *L. amazonensis*, the animals were separated into the following groups:

1. GLUC Group (6 experimental animals): infected and treated with Glucantime® intramuscularly (IM), 20 mg SbV/kg/day for 40 days.
2. CONT- Group (6 experimental animals): infected and not treated during the experiment.

Measurement and morphological aspect of the lesions

The total volume of the lesions was measured daily with a digital caliper (Zaas® Precision) and recorded for analysis of lesion

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progression. The morphological aspect of the lesions was analyzed and monitored daily and recorded in a photo document for analysis of macroscopic morphological evolution. The animals were weighed on a semi-analytical scale (Toledo®) to monitor their body mass over the 40 days of the experiment.
The animals were euthanized with Euthanyle® (Pentobarbital sodium - Diphenylhydantoin sodium), as indicated by CEUA/INPA.

Parasitological diagnosis

Direct method: Imprint smears were taken immediately after collecting small fragments of the snout and Giemsa Kit staining was used. The density of parasites (amastigotes) and infected macrophages was estimated by optical microscopy under immersion oil (1.000 X) and at least 25 fields from each slide were counted and recorded (Comandolli-Wyrepkowski et al., 2017). Cultivation: After euthanizing the animals, the lesions were sectioned and disinfected with saline and Gentamicin®. The snout fragments were then sown in NNN culture medium (Novy and McNeal, 1904; Nicolle, 1908). The culture tubes with NNN medium were incubated at 25°C for eight days.

The cultures were determined negative when no parasites were present after the incubation period. If positive, the concentration of parasites present was semi-quantified according to the logarithmic number of cells present, with values 1 being from 1 to 10 parasites; 2, between 10 and 100 parasites; and 3, greater than 100 parasites per field (Comandolli-Wyrepkowski et al., 2017).

Histopathological diagnosis

After the end of treatment, the animals were euthanized and fragments of the snout were obtained. The fragments were then transferred to 10% buffered formaldehyde and subsequently subjected to histological processing for light microscopy. The histological slides obtained were stained with hematoxylin-eosin (HE). The histological slides were prepared and read at the Histopathology Laboratory of the Morphology Department of the Federal University of Amazonas (UFAM). The descriptive histopathological diagnosis was made according to Magalhães et al. (1986).

The dermal inflammatory pattern and cell population were estimated histologically in HE stained sections. The inflammatory infiltrate was measured according to Solano-Gallego et al. (2004) in crosses as follows: (-), no inflammatory infiltrate; (+), isolated focus of inflammatory cells; (++), areas of isolated or coalescing inflammatory infiltrate; (+++), areas of diffuse inflammatory infiltrate.

Molecular diagnosis

The fragments collected were processed at the Leishmaniasis Surveillance Laboratory of the Evandro Chagas National Institute of Infectious Diseases - IPEC FIOCRUZ/RJ. The samples were stored at -80°Cand sectioned using a scalpel blade and weighed, obtaining fragments of approximately 10 mg. They were then transferred to previously identified Eppendorf tubes and subjected to the DNA extraction procedure using the Wizard® Genomic DNA Purification Kit (Promega) (Comandolli-Wyrepkowski et al., 2017; Fagundes et al., 2010).

To carry out the q-PCR, reactions were prepared with a final volume of 25 µL with 100 nM oligonucleotides and ROTOR GENE® SYBR Green PCR Master Mix (QIAGEN). 5 µL of DNA were added per sample and each sample was run in triplicate. The thermal cycler used was the Rotor-Gene Q model (QIAGEN). The absolute copy numbers for samples from each assay were calculated considering the efficiency (E = 1.19248 (* = $10^{(-1/m)}$ -1)) of the reaction.

Statistical analysis

The data recorded from the results of the lesion measurements were inserted in a database previously prepared in GraphPad Prism® version 6 and then statistically analyzed using the student's t-test and Tukey's test, using a 95% confidence limit to verify the difference between the treatment groups for these two analyzed parameters.

RESULTS

After forty days of treatment, the clinical appearance of the lesions in hamsters from both groups can be evaluated and a comparison made between them. The clinical evolution of lesion volumes is an important aspect to evaluate, since cutaneous leishmaniasis (CL) can cause infected hamsters to develop histiocytic lesions, with extensive tissue growth like benign lesions that evolve chronically into ulcers or nodular lesions.

The animals in the GLUC group had nodular lesions at the beginning, during and after treatment. The animals in the CONT- group had nodular lesions at the start of treatment and these progressed to ulcerations with a meliceric crust in all the animals, but with varying diameters, as can be seen in Figure 1. As for the proportion of lesions, this experiment showed a 445.9% increase in the total volume of lesions on the snouts of hamsters in the control group compared to day 0 of the treatment (24 days post-infection).

Daily measurements of the snouts, recorded throughout the treatment of the animals, made it possible to calculate the volumes of the lesions and, consequently, to assess statistical significance. It was observed that from the 14th day onwards, the GLUC group showed statistical significance, with $p = 0.0138$, according to the student's t-test and Tukey's test with a parameter of $p<0.05$ compared to the CONT- group (Figure 2). The animals continued to receive treatment with Glucantime® until the 39th day of treatment, even though there was a reduction after 20 days. At the end of treatment, there was a 43.4% reduction in the volume of the lesions compared to the start of treatment.

A Summarizing these results in relation to the clinical evolution during treatment, the lesions of the animals in the CONT- group gradually increased during the experimental period, reaching an increase of more than 400%. The lesions in the GLUC group reduced their total volume by more than 40%.

As for the quantitative parasitological method, there was a discrepancy in the results between the experimental groups, with the GLUC group showing a lower parasite load compared to the CONT- group

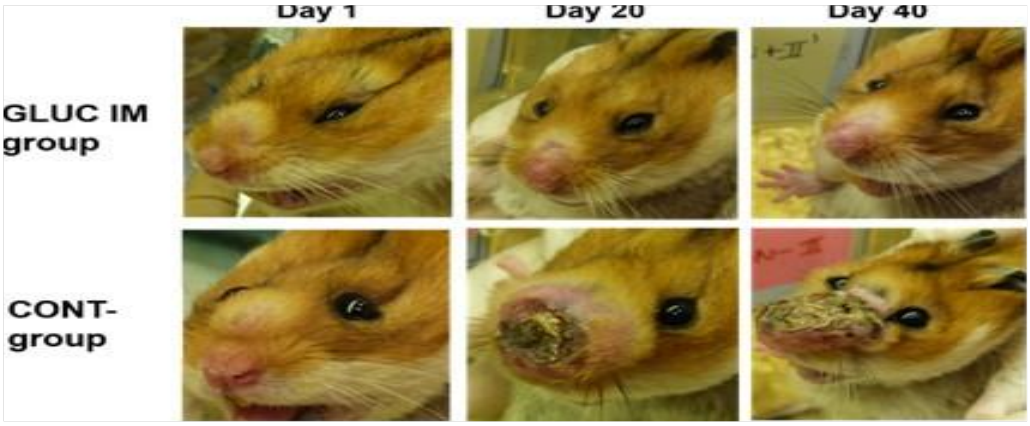

Figure 1. Macroscopic clinical evolution of lesions on the snout of *Mesocricetus auratus* infected with *L. amazonensis* on different days of the topical treatment study.

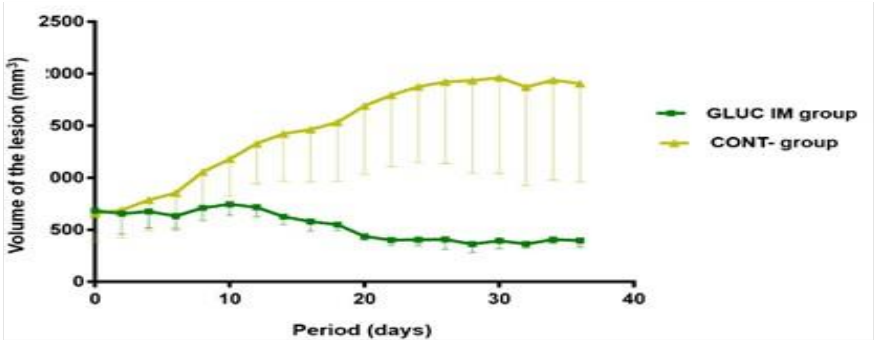

Figure 2. Clinical evolution of lesion volume in *M. auratus* infected with *L. amazonensis* during treatment with Glucantime®.

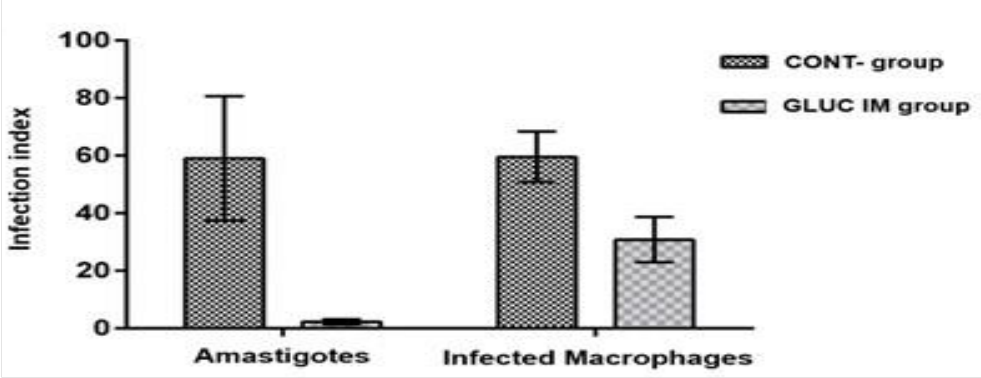

Figure 3. Ratio of infected macrophages to non-infected macrophages present in the lesions analyzed using the printing method on slides and stained with Giemsa.

(Figure 3). The infection values for both groups showed a statistical difference of $p = 0.0086$. In addition, the semi-quantitative parasitological method (culture in NNN medium) revealed the presence of viable parasites (score

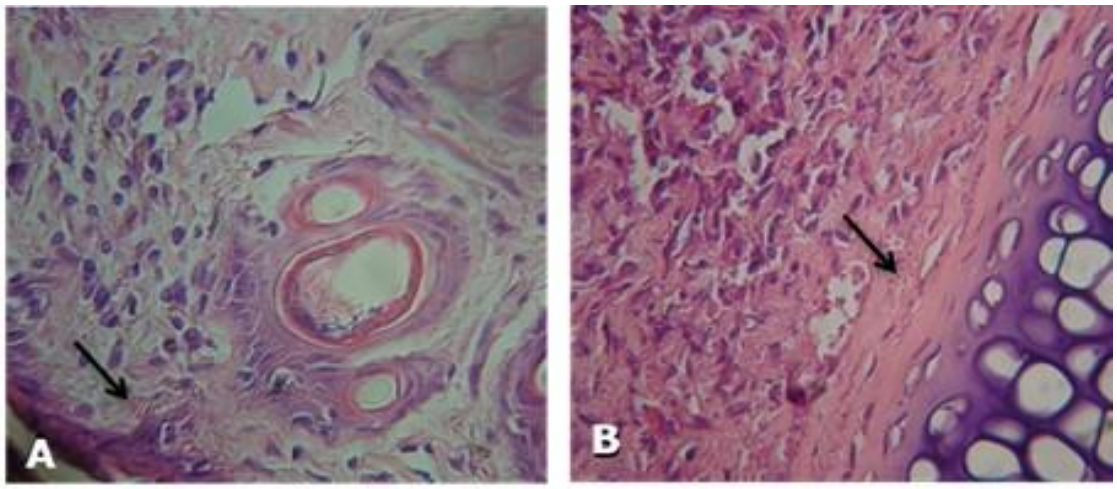

Figure 4. Photomicrograph of the skin of hamsters infected with Leishmania amazonensis. A- Animal treated with Glucantime® with diffuse perifollicular inflammatory cell infiltration in the dermis (HE stains- Magnification 112x). B- Untreated animal with diffuse inflammatory cell infiltration with some epithelioid cells in between. Numerous amastigotes amidst mononuclear infiltrate up to the edge of the nasal cartilage (HE stains- 160x magnification).

> 2) only for the CONT- group, after 8 days of incubation in the culture medium.

The histopathological evaluation showed that the animals in the GLUC group had mild dermatitis. A low mononuclear infiltrate (+) was observed in the papillary dermis, extending to the hair follicles. Sebaceous adenitis was not observed in the samples analyzed (Figure 4A). In animals from the CONT- group, the most common lesion pattern was perifollicular dermatitis, mainly around the hair isthmus. In some animals, the infiltrate extended to the sebaceous gland region. The diffuse (+++) or perivascular infiltrate usually accompanied the perifollicular inflammation where its invasion reached the fibrous perichondrium of the nasal cartilage (Figure 4B).

For molecular diagnosis, the standard curve was prepared beforehand from serial dilutions of promastigote forms of *L. amazonensis*, in triplicate. During the exponential phase of the reaction, in the linear portion of the curve, the equipment's software defined the threshold. The point on the curve that intersects this threshold is called the threshold cycle (Ct). The emitted fluorescence was plotted against the Ct, generating the standard curve graph.

From the standard curve, it was possible to amplify the samples from the two experimental groups, where the Ct values were calculated and used to determine the DNA concentration, since the Ct value has a linear proportion with the logarithm of the concentration. The average Ct value for the GLUC group showed a DNA concentration of 10.7 ng/µL (Ct equal to 25), and for the CONT- group a concentration of 2198.9 ng/µL (Ct equal to 10), showing a reduction in parasite DNA in the animals that received treatment, with a value around 205 times lower after the action of Glucantime®.

DISCUSSION

The clinical aspect has been a frequent parameter presented in pre-clinical studies of new therapeutic targets for tegumentary leishmaniasis, but most studies use the Balb/C mouse (*Mus musculus*) as an experimental model, with few studies using the Golden Hamster (*Mesocricetus auratus*), although the latter is susceptible to infections caused by species of *Leishmania* sp.

Hamsters are commonly used for studies with different species of *Leishmania* sp. due to the fast proliferation of parasites in their system, which demonstrates their susceptibility to developing the infection, and consequently the appearance of lesions (da Costa and do Valle, 2014). Experiments using hamsters infected with the *L. amazonensis* species include the ability to produce focal lesions with numerous macrophages containing large vacuoles filled with amastigotes in the cytoplasm, in which this large and bulky histiocytomatoid aspect causes exacerbated skin lesions.

According to Gomes-Silva et al. (2013), lesions with inflammatory signs were detected after infection with *Leishmania braziliensis* in Golden hamsters. Over the observation period, the lesions evolved, showing nodules and skin ulceration, raised erythematous borders, a granular appearance, with a necrotic surface, a fact that characterizes similar observations in this study, mainly

because no animal was spontaneously cured. Thus, it can be observed that lesions in Balb/C infected with *Leishmania major* can have a more pronounced clinical appearance, with lesions evolving from ulceration to crusting in the paw area, leading to necrosis and self-amputation, results which justify the clinical criticality of the lesions developed in the animals in the CONT- group (Eissa et al., 2011).

Although it is possible to evaluate the clinical aspect of the lesions in experimental animals, this does not guarantee a correlation with the number of parasites inside them, as studies have shown that in Balb/C mice infected with *L. major* experimentally, the size of the lesions caused by the parasites was not proportional to the amount of *Leishmania* present inside the lesions (Titus et al., 1985).

The presence of viable parasites in mice inoculated with *L. amazonensis* submitted to treatment with N-methyl glucamine antimoniate generates controversy since a clinical and parasitological cure is expected from a drug recommended by the Ministry of Health for the treatment of TL (Brasil, 2017; Costa Filho et al., 2008). This corroborates the results of this project, where all the samples showed viable parasites, even those treated with the standard drug, Glucantime®.

Another study also shows this characteristic with viable parasites in cultures from fragments of C57BL/6 mice infected with *L. amazonensis* after monotherapeutic treatment with N-methyl glucamine and combined treatment (N-methyl glucamine plus azithromycin), and there was no statistical difference between the groups (Sampaio et al., 2009). It is suggested that, due to the high susceptibility of these animals to developing the disease, this leads to complications in terms of curing the disease (Lima et al., 2017).

Through the direct parasitological method of lesions from mice inoculated with *L. amazonensis* in different studies, it is possible to detect mostly macrophages infected by *Leishmania* sp., rare lymphocytes, as well as numerous extracellular amastigotes, results which agree with the findings of the slide impressions in the present study (Almeida et al., 2013; Rodrigues et al., 2006).

The classification of the inflammatory infiltrates' degrees can contribute to the analysis of the histological findings of the experimental animals in the study and characterize the inflammatory profile (Solano-Gallego et al., 2004). The general histological profile found in hamsters infected with *Leishmania* sp. is abundant and diffuse dermal infiltrates of macrophages, plasma cells, lymphocytes, and moderate fibroplasia (Ecco et al., 2000).

Other histological findings in hamsters infected with *L. zonensis* that were treated with 5-hydroxy-2-hydroxymethyl-gana-pyrone (HMP) show a decrease in the number of amastigotes and cell infiltrate culminating in the initial healing process and the number of parasites inside vacuoles, observations similar to the present experiment which showed a mild inflammatory infiltrate in the animals in the GLUC group (Rodrigues et al., 2014).

With the results obtained from the analysis of cells from the dermis of Balb/C infected with *L. amazonensis*, it was possible to identify hyperplasia and hypertrophy of the cells, focal areas of necrosis in the skin, cellular infiltrates composed of lymphocytes, plasma cells, neutrophils, and eosinophils, presenting a chronic granulomatous inflammatory process, with intense parasitism (Rodrigues et al., 2006). This corroborated the histological characteristics described for the CONT- group of animals, with diffuse infiltration of inflammatory cells and an abundance of amastigotes amid the nasal infiltrate.

When the real-time PCR data was analyzed, DNA concentration was positive in both groups, but the CONT- group had higher parasite concentrations, as expected. In research with Balb/C infected with different species of *Leishmania* sp., the concentration of parasite DNA load was found to be equal to 15 for *L. amazonensis*, compatible with the result of the group that did not receive treatment with a Ct equal to 10 (Nicolas et al., 2002).

Although there is little use of this molecular method for detecting and quantifying parasites within *in vivo* trials for Cutaneous Leishmaniasis, the method should be applied more frequently, which could guarantee a more successful diagnosis, since it's already used mainly for diagnosing patients and animals with visceral leishmaniasis (Lima Junior, 2011; Nunes et al., 2018), and is a technique of great interest because it is known for its sensitivity and specificity.

Thus, this work also made it possible to verify that the Golden Hamster (*M. auratus*) animal model is susceptible to infection by *Leishmania amazonensis* and could be used in pharmacological trials in the search for treatment for American Cutaneous Leishmaniasis, but due to the uniqueness of the diagnostic methods, a set must be pre-established to detect clinical and parasitological changes.

CONCLUSION

Using the methods employed in this study, it was possible to evaluate the action of one of the drugs recommended by the Brazilian Ministry of Health, Glucantime®. The animals that received it as an IM treatment had a moderate reduction in the volume of the lesions of 40%, a slight inflammatory infiltrate and a reduction in the parasite load when compared to the group that did not receive treatment. This study therefore concludes that clinical assessment and the application of parasitological, histopathological, and molecular diagnostic tests are essential for determining the clinical and parasite profile more accurately and assertively in experimental animals used for pre-clinical trials with the

aim of seeking new therapeutic drugs for cutaneous leishmaniasis.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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