EUROPEAN ACADEMIC RESEARCH
Vol. VIII, Issue 9/ December 2020

Impact Factor: 3.4546 (UIF)
DRJI Value: 5.9 (B+)

ISSN 2286-4822
www.euacademic.org

Effects of Glucantime® intralesional treatment for cutaneous leishmaniasis in hamsters[1]

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Abstract

In Brazil, the recommended treatment for integumentary leishmaniasis involves therapeutic approaches involving meglumine antimoniate, amphotericin B and pentamidine. However, these drugs

[1] **Efeitos do tratamento com Glucantime® por via intralesional para leishmaniose cutânea em hamsters**

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exhibit a high degree of toxicity and undesirable effects. Faced with this problem, the intralesional application (IL) of Glucantime®, with lower doses of antimoniate has been discussed as an alternative treatment method in order to avoid cumulative toxic effects and has the purpose of improving the absorption of the drug through a systemic route. In the present study, the objective was to evaluate the efficacy of a protocol of intralesional treatment with Glucantime® in hamsters (Mesocricetus auratus) infected with Leishmania braziliensis and Leishmania guyanensis. The animals (n=25) were infected, experimentally, on the snout with the L. braziliensis and L. guyanensis, and separated into the groups of positive control (treated orally with IL with Glucantime®) and the negative control (no treatment given). After the follow-up period (60 days), euthanasia was performed to remove fragments of the lesion, the liver and spleen, which were subsequently used for parasitological (NNN and imprinting) and histopathological analyses. The results showed that the positive control infected with L. braziliensis showed clinical and parasitological cure. Animals from the positive control infected with L. guyanensis showed improvements in the clinical course of lesions through the tissue reepithelization process, however, they still exhibited viable parasites. Statistical significance was verified ($p = 0.0028$) and the Tukey test presented a parameter of $p<0.05$ between the groups regarding the evolution of wound healing. In the histopathological evaluation of the organs, signs of healing and tissue healing were found. Thus, it was concluded that the IL protocol with the drug and the route used gave positive results, since clinical cure was demonstrated and the deep tissue regions of the organs were not affected.

Keywords: American cutaneous leishmaniasis, experimental treatment, *Mesocricetus auratus*.

Resumo

No Brasil, o tratamento preconizado para leishmaniose tegumentar apresenta abordagens terapêuticas envolvendo antimoniato de meglumina, anfotericina B e pentamidina. No entanto, esses medicamentos exibem um alto grau de toxicidade e efeitos indesejáveis. Diante dessa problemática, vem se discutindo como alternativa a

aplicação intralesional (IL) do Glucantime®, com menores doses do antimoniato afim de evitar efeitos tóxicos acumulativos e com a finalidade de melhorar a absorção da droga por via sistêmica. O objetivo do presente estudo foi avaliar a eficácia do protocolo de tratamento intralesional com Glucantime® em hamsters (Mesocricetus auratus) infectados com Leishmania braziliensis e Leishmania guyanensis. Os animais (n=25) foram infectados experimentalmente no focinho com L. braziliensis e L. guyanensis e, separados nos grupos controle positivo, tratados por via IL com Glucantime® e controle negativo. Após o período de acompanhamento (60 dias), realizou-se eutanásia para retirada de fragmentos da lesão, fígado e baço, posteriormente, utilizados para análises parasitológica (NNN e imprinting) e histopatológica. Os resultados demonstraram que os animais de experimentação infectados por L. braziliensis apresentaram cura clínica e parasitológica. Os animais infectados por L. guyanensis mostraram melhora no curso clínico das lesões com processo de re-epitelização tecidual, contudo, exibiram parasitas ainda viáveis. Foi verificado significância estatística (p = 0,0028) e o teste de Tukey apresentando um parâmetro de p<0,05 entre os grupos quanto à evolução de cicatrização das lesões. Na avaliação histopatológica dos órgãos, verificou-se sinais de cicatrização e cura tecidual. Dessa forma, conclui-se que o protocolo IL com a droga e a via utilizada possuem resultados positivos, uma vez que se demonstrou cura clínica e as regiões teciduais profundas dos órgãos não foram atingidas.

Palavras-Chave: Leishmaniose tegumentar americana, tratamento experimental, *Mesocricetus auratus*.

INTRODUCTION

Leishmaniasis is a non-contagious infectious disease, with a zoonotic character, and affects humans and animals of various species. It is caused by the parasitic protozoa of the genus *Leishmania*, which are transmitted through the bite of some of the species of the subfamily Phlebotominae (Alvar *et al*. 2012; Pigott *et al*. 2014; Morais *et al*. 2016).

Leishmaniasis, which was previously characterized as a strictly rural zoonosis, has seen an expansion to urban centers due to increasing deforestation (Brasil 2017). However, according to the World Health Organization (WHO), leishmaniasis is classed among the neglected tropical diseases (NTDs) and is in the category of emerging and uncontrolled diseases, due to the complexity in its control and treatment (Andrade-Filho *et al.* 2001; WHO 2018).

Leishmaniasis is widely distributed around the world, and about 95% of cases of the cutaneous form occur in the Americas, the Mediterranean basin, the Middle East and Central Asia. Worldwide, it is estimated that about 600, 000 to 1 million new cases of the disease occur per year (WHO 2019). In 2017, 17,528 new cases of the disease were reported in Brazil, with 7,832 cases in the northern region and 1,865 cases in the Amazonas state (Brasil 2019).

The main clinical forms of American tegumentary leishmaniasis (ATL) are cutaneous, mucocutaneous and diffuse cutaneous. Cutaneous leishmaniasis (CL) is characterized by ulcerated, papular, nodular, verrucous lesions, among other dermatological aspects. These lesions are painless and can be single, multiple, disseminated or generalized by the patient's body (Paes *et al*. 2000). In Brazil, the following seven species are known to cause the disease: *Leishmania (Leishmania) amazonensis, L. (Viannia) braziliensis, L. (V.) guyanensis, L. (V.) naiffi, L. (V.) shawi, L. (V.) lainsoni,* and *L. (V.) lindenbergi* (six are of the subgenus *Viannia* and one of the subgenus *Leishmania*). The most predominant species in the Amazonas state are *L. amazonensis, L. braziliensis, L. guyanensis* and *L. naiffi* (Brasil 2017).

The treatment recommended in Brazil for ATL presents therapeutic approaches involving meglumine antimoniate–Sb^{5+} (Glucantime®), amphotericin B and pentamidine. However, these drugs are limited to the treatment of patients with Leishmaniasis due to a variety of side and adverse effects, such as arthralgia, myalgia, nausea, vomiting, abdominal pain, pancreatitis, pruritus, fever, headache, dizziness, palpitations, insomnia, nervousness, edema and acute renal failure (ARF), inappetence, bloated stomach, heartburn, weakness, hepatitis with increased transaminases and alkaline phosphatase, as

well as alterations in electrocardiograms, such as alteration of the ventricular repolarization with ST segment inversion, and QT interval prolongation, among others (Sampaio *et al*. 1988; Gontijo; Carvalho 2003). These factors, which are related to high toxicity and prolonged duration of treatment, make adherence and regularity of treatment difficult in rural areas (Neves *et al*. 2018).

An alternative that could reduce the systemic absorption of Glucantime® and its adverse and side effects would be its intralesional (IL) administration (Soto *et al*. 2013). The recommendation for IL use is restricted to the clinical form of localized cutaneous leishmaniasis, in which mild or possibly moderate adverse clinical, laboratory and electrocardiographic effects can be observed, without the need to interrupt treatment (Brasil 2017).

The Brazilian Ministry of Health (BMH) recommends the use of intralesional administration of Glucantime® as one of the preferred treatment options for the localized cutaneous form, in lesions of up to 3 cm caused by *L. braziliensis* and *L. guyanensis* (Brasil 2017). The BMH cites pentamidine as the recommended drug for the treatment of CL caused by *L. guyanensis*, but this medication is expensive and its administration needs to be in an outpatient setting, due to this being via the deep intramuscular (IM) route, and due to it causing immediate colateral effects, such as hypoglycemia (Neves *et al.* 2011).

Given the high degree of toxicity caused by conventional treatment and the lack of adherence by patients, this study aimed to evaluate the effectiveness of the intralesional treatment protocol with Glucantime® using hamsters (*Mesocricetus auratus*) infected with *Leishmania* (*Viannia*) *braziliensis* and *Leishmania* (*Viannia*) *guyanensis*.

MATERIALS AND METHODS

Ethical aspects

This work is part of a project at the Leishmaniasis and Chagas disease Laboratory, and was approved by the Commission on Ethics in the Use of Animals (CEUA) at the National Institute of Amazonian Research (INPA) – nº 059/2018.

Cultivation of *Leishmania* sp.

Promastigote forms of *L. braziliensis* (MHOM/BR/1975/M2903) and *L. guyanensis* (MHOM/BR/1975/M4147), cryopreserved in the cryobank of the Laboratory of Leishmaniasis and Chagas disease at INPA, were used. The parasites were cultured in biphasic NNN medium and the culture was expanded in RPMI 1640 medium with HEPES and L-glutamine (LGC Biotechnology®), supplemented with 10% inactivated bovine fetal serum (iBFS) and gentamicin (50 µg/mL) and incubated at 25 ºC, with verification of the media under an inverted optical microscope every 3 days until the growth of the parasites was achieved. After this period, the media with the promastigote cultures of *L. braziliensis* and *L. guyanensis* were centrifuged for 10 minutes at 3000 x g, the discarded supernatant and the pellets were resuspended in RPMI 1640 medium for the quantification of promastigotes under optical microscopy in a Neubauer chamber.

***In vivo* experiments**

We used 25 adult males golden hamsters (*M. auratus*) (age ≥ 90 days, weight ≥ 150 g), which were provided by the central vivarium at INPA. The treatment and care of the animals took place on the premises of the vivarium, and they were kept in polypropylene cages in conditions suitable for their maintenance, with food and water *ad libitum*, were free of pathogens, at a temperature of 21 ºC (± 3), relative humidity of 55% (± 15) and free of external sources of noise and/or ultrasound, and under the supervision of a veterinarian.

The animals in the experiment were organized into three groups as described in Table 1. The hamsters of the positive and experimental control group were previously anesthetized with lidocaine 1% and were infected in the snout and the concentration of the parasites was calculated according to the cultures obtained.

Table 1. Experimental design for intralesional Glucantime® treatment in experimentally-infected hamsters (*M. auratus*).

Experimental groups of golden hamsters (*Mesocricetus auratus*)			
Groups	**Infected with *Leishmania* spp.**	**Drug treatment Glucantime® via IL**	**N**
Positive control	*L. braziliensis*	-	5
	L. guyanensis	-	5
Negative control	-	-	5
Experiment	*L. braziliensis*	Dose 20 mg Sb^{5+}/Kg/dia	5
	L. guyanensis	Dose 20 mg Sb^{5+}/Kg/dia	5
Total			**25**

After inoculation with the parasites, the animals remained under observation and were monitored for the following 33 days until the lesions of CL were apparent. The treatment was performed through injections of Glucantime® via IL (20 mg Sb^{5+}/Kg/day) in the snouts of the animals of the positive control group and experimental group, whose lesions were previously cleaned with sterile saline and the were animals anesthetized with lidocaine 1%. The duration of the treatment with Glucantime was 30 days, during which three applications were performed at intervals of 15 days. The post-treatment had follow-up extended for another 30 days, making 60 days in all. (Table 2), following the recommendations of the tegumentary leishmaniasis surveillance manual published by the BMH (Brasil 2017). After seven days of the experiments, the hamsters were euthanized with Xylazine in accordance with the recommendations of the Animal Ethics Committee at INPA.

Table 2. Intralesional Glucantime® treatment and post-treatment period in experimental models using golden hamsters (*Mesocricetus auratus*) infected with *Leishmania* spp. Treatment according to the protocol of the BMH (Brasil 2017).

Experimental follow-up period				
Drug treatment			**Post-treatment**	
1st application Day 1	2nd application Day 15	3rd application Day 30	1st application Day 45	2nd application Day 60

The morphological aspects of the lesions were measured with a digital caliper (Zaas Precision®), and photographed throughout the treatment

and post-treatment in order to monitor possible adverse effects, secondary infections and signs of clinical healing of the lesion.

Parasitological Assessment

Analysis of parasitic viability by culture in NNN medium (Novy and McNeal 1904; Nicolle 1908): after the euthanasia of the hamsters, samples of the fragments of snout lesions were collected. These materials were sown in tubes with NNN culture medium, incubated in a heating chamber at 25 ºC for six days and, afterwards, a slide was prepared. We investigated the presence of viable promastigotes by using an optical microscope (1000X). The following estimate was used: score 0 = absence; score 1 = 1 to 10 parasites/field; score 2 = 10 to 100 parasites/field and score 3 = > 100 parasites/field (Comandolli-Wyrepkowski *et al*. 2017).

Analysis of parasitic viability using the method of imprinting on slides (*imprints*): part of the samples of the snout fragments were used to make the imprints. The slides were stained with the rapid panotic method (Laborclin®) and analyzed under optical microscopy (1000x) to investigate the presence of extracellular amastigotes and macrophages for every 100 fields of the slides.

Processing and histopathological analysis

Fragments of the CL lesion of the muzzle, and the liver and spleen of hamsters were subjected to histological processing for light microscopy. The preparation and reading of histological slides were carried out in the Histopathology Laboratory of the Department of Morphology of the Federal University of Amazonas (UFAM), Manaus, and stained with hematoxylin and eosin (H&E).

Subsequently, the samples were fixed in 10% formaldehyde and buffered for 72 hours. They were then registered, stored in histological cassettes and progressively dehydrated in 30-minute baths in four concentrations of ethyl alcohol (70%, 80%, 96% and 100%). The samples were then diaphanized by a 30-minute bath in xylol. In the heating chamber, at a temperature of 60 ºC, the tissues were maintained in two

baths of histological paraffin for 40 minutes each. The blocks were cut using a rotating microtome to obtain 5 µm thick slivers and mounted on clean and degreased slides.

After the assembly of the slides, paraffin removal was followed by hydration of the tissue slivers. For this, the slides with the slivers remained in a heating chamber at 60 ºC for 24 hours and then subjected to a sequence of two baths in xylol for 5 minutes, two baths of absolute alcohol for 3 minutes, alcohol 96% for 3 minutes, alcohol 80% for 3 minutes, alcohol 70% for 3 minutes and distilled water for 1 minute.

After hydration, the slides with the slivers were stained in Harris hematoxylin for 4 minutes and washed in distilled water for 1 minute, placed in eosin for 30 seconds, quickly washed in distilled water and dehydrated in 100% alcohol for 3 minutes. Finally, the slides were assembled using Canada synthetic balm and a glass slide. Photometric analyses were performed using an image capture system, consisting of a binocular microscope with an attached camera.

Statistical Analysis

The statistical analyses were performed using the GraphPad Prism® 6.0 software and then analyzed by the analysis of variance test (ANOVA) ($p<0.05$) and Tukey test, using a 95% confidence limit. The data is shown in tables and graphs.

RESULTS

Intralesional treatment with Glucantime®

All 25 hamsters infected with *L. braziliensis* and *L. guyanensis* in the snout had the onset of ulceration around 48 days after infection. Treatment with Glucantime® via IL began 33 days after infection of the animals. The lesions began with small nodules and the development of histiocytoma, which is a common symptom of CL.

Both groups of infected and pharmacologically treated animals presented histiocytoma up to about 20 days after the first application of the drug. A reduction in the volume of the lesions was observed after 30 days of treatment for both groups of infected and treated animals. The animals infected with *L. guyanensis* and treated showed signs of

lesions and intense erythema in the snout during the three treatment sessions, clinical healing with total re-epithelialization of the lesions occurred about 50 days after the start of treatment (Figure 2).

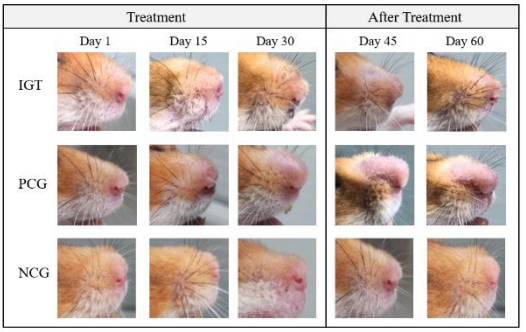

Figure 1. Clinical evolution of skin lesions in the snouts of hamsters (*Mesocricetus auratus*) infected with the promastigotic form of *Leishmania* (*Viannia*) *braziliensis* during treatment and 30 days after beginning intralesional glucantime treatment®. Key: IGT = intralesional glucantime treatment; PCG = positive control group; NCG = negative control group.

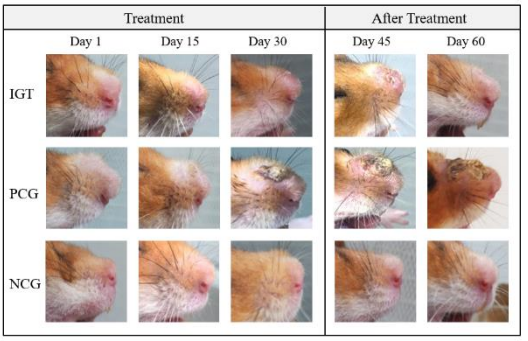

Figure 2. Clinical evolution of skin lesions in the snouts of hamsters (*Mesocricetus auratus*) infected with the promastigotic form of *Leishmania* (*Viannia*) *guyanensis* during treatment and 30 days after beginning intralesional glucantime treatment®. Key: IGT = intralesional glucantime treatment; PCG = positive control group; NCG = negative control group.

A significant reduction in the volume of lesions was observed after 30 days of treatment for both groups of infected and treated animals ($p = 0.0028$) (Figure 3), with a statistically significant value . For the species *L. guyanensis*, the differences between the evolution of the lesions in

the untreated group compared to the treated groups were observed 15 days post-treatment ($p < 0.05$).

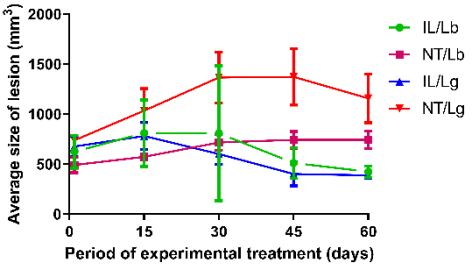

Figure 3. Clinical evolution of lesion volume during local treatment in hamsters (*Mesocricetus auratus*) infected in the snout by *Leishmania (Viannia) braziliensis* and *Leishmania (Viannia) guyanensis*. IL/Lb-intralesional treatment with Glucantime®/ *Leishmania (Viannia) braziliensis*; NT/Lb - untreated/*Leishmania (Viannia) braziliensis*; IL/Lg – intralesional treatment with Glucantime®/*Leishmania (Viannia) guyanensis*; NT/Lb-positive control, untreated/*Leishmania (Viannia) guyanensis*.

Parasitic evaluation

Samples from fragments of lesions of hamsters infected with *L. braziliensis* treated with Glucantime® grown in NNN medium did not reveal the presence of active parasites. This was only observed in the samples of the positive control groups, where more than 100 parasites per field were counted (score = 3), and in animals infected with *L. guyanensis* treated with Glucantime®, which numbered from 10 to 100 parasites per field (score = 2) (Figure 4).

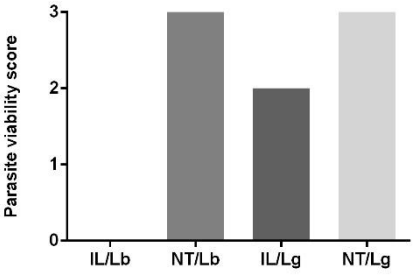

Figure 4. Parasite viability score visualized though NNN culture media, seven days after sowing fragments of hamster lesions (*Mesocricetus auratus*) infected with *Leishmania (Viannia) braziliensis* and *Leishmania (Viannia) guyanensis*, and treated intralesionally with Glucantime®.

The evaluation of parasitic viability using the imprint method is shown in Figure 5. Absence of internalized amastigotes in macrophages and extracellular amastigotes was observed in animals infected with *L. braziliensis* (Figure 5A) and treated. The results presented statistical significance when compared with the positive control group ($p = 0.0336$; $p \geq 0.05$). For animals infected with *L. guyanensis*, viable forms of extracellular and macrophage amastigotes were identified, and showed no significant difference with the positive control group ($p = 0.1631$; $p \geq 0.05$) (Figure 5B).

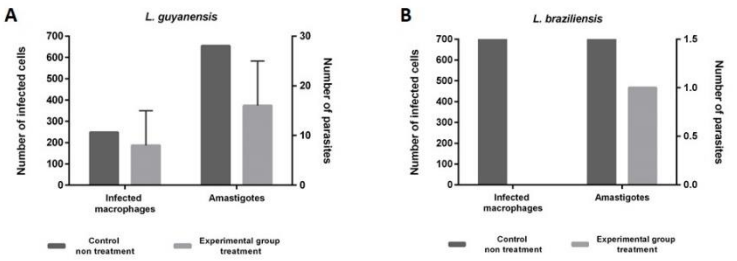

Figure 5. Percentage relative to the number of amastigotes and abundance index of infected macrophages (parasitic viability) visualized and quantified by optical microscopy (1000x) in the slide imprints of hamster skin lesion fragments (*Mesocricetus auratus*) infected with *Leishmania (Viannia) braziliensis* **(A)** and *Leishmania (Viannia) guyanensis* **(B)**.

Histopathological evaluation

In the histopathological evaluation of the fragments of the lesions of animals infected with *L. guyanensis* and *L. braziliensis* treated via IL with Glucantime®, inflammatory infiltrate compatible with mild dermatitis was observed. In the papillary dermis, low mononuclear infiltrate extending to the hair follicles was noted, and sebaceous adenites were not observed. In infected animals that did not receive treatment, the most common lesion pattern was perifollicular dermatitis, especially around the capillary isthmus. In some animals, the diffuse or perivascular infiltrate extended to the region of the sebaceous gland, and was usually accompanied by perifollicular inflammation and some epithelial cells of permeation.

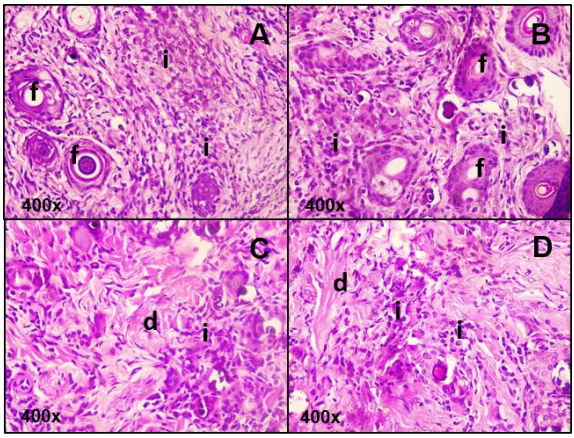

Figure 6. Photomicrography of the fragment of the lesion of hamsters (*Mesocricetus auratus*). (**A**) Animals infected with *Leishmania (Viannia) guyanensis*, and treated with intralesional applications of Glucantime®); (**B**) Animals infected with *Leishmania (Viannia) braziliensis* and intralesionally treated with Glucantime®; (**C**) In the hamsters infected with *Leishmania (Viannia) guyanensis* and not treated; (**D**) In the hamsters infected with *Leishmania (Viannia) braziliensis* and not treated; (**A** and **B**) Inflammatory infiltrate (i), perifolicular composed of macrophages, dispersed lymphocytes, plamocytes, and neutrophils; follicles in cross section (**f**); (**C** and **D**) Diffuse dermatitis with inflammatory infiltrate (i) reaching deep into the dermis (**d**); Staining with HE. 400x magnification.

In the histopathological analysis of the liver of animals infected with *L. guyanensis* and treated with Glucantime® via IL, abundant inflammatory infiltrate, hypercellularity, mild steatosis (microvesicular), abundant periportal infiltrate and Kupffer cells were observed. In the positive control group, hypercellularity, periportal inflammatory infiltrate, anisocytosis and apoptosis cells were found. Animals infected with *L. braziliensis* and treated presented abundant periportal infiltrate, mild steatosis (mixed, macrovascular, microvascular and at zone 3 level) and binuclear hepatocytes. Animals infected by the same species and untreated presented abundant inflammatory infiltrate, hepatocyte hypercellularity, absence of steatosis, some binuclear hepatocytes and areas with foci of necrosis.

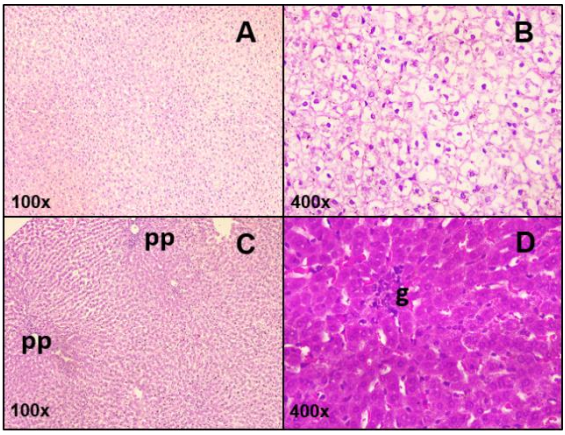

Figure 7. Photomicrography of the liver of hamsters (*Mesocricetus auratus*). (**A**) Animals infected with *Leishmania* (*Viannia*) *guyanensis*, and treated with intralesional applications of Glucantime®); (**B**) Animals infected with *Leishmania* (*Viannia*) *braziliensis* and treated intralesionally with Glucantime®; (**C**) Hamsters infected with *Leishmania* (*Viannia*) *guyanensis* and not treated; (**D**) Hamsters infected with *Leishmania* (*Viannia*) *braziliensis* and not treated (**A** and **B**) Intumescent hepatocytes with no apparent parasites; (**C**) mild periportal histiolymphocytic infiltrate (pp) and (**D**) small intralobular granuloma (g), bordered by lymphocytes and macrophages. Staining with HE. **A-C** 100x magnification and **B-D** 400x magniifcation.

Through the histopathological study of the spleen of animals infected with *L. guyanensis* and treated with Glucantime® via IL, preservation of the splenic parenchyma, without thickening of the splenic capsule, good visualization of the areas of white pulp (WP) and red pulp (RP) was observed. In the positive control group, hyper-reactivity of WP and RP and thickening of the central arteriole were detected. In hamsters infected with *L. braziliensis* and treated, the preservation of splenic architecture and splenic parenchyma, as well as small foci of mononuclear infiltrate (reactivity), was identified. In the positive control group, it was found that the splenic capsule remained thin, but there was a presence of great reactivity of the subcapsular WP.

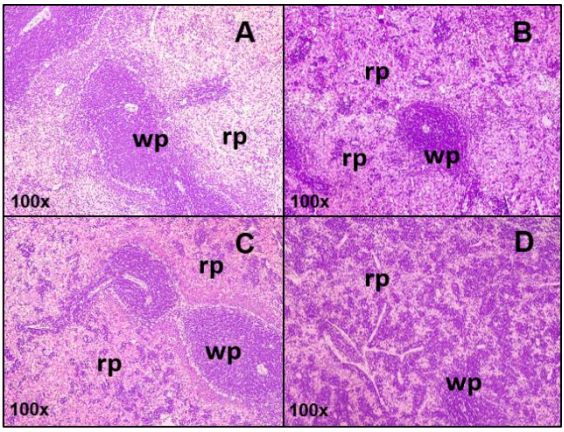

Figure 8. Photomicrography of the spleen of hamsters (*Mesocricetus auratus*). (**A**) Animals infected with *Leishmania* (*Viannia*) *guyanensis*, and treated with intralesional applications of Glucantime®); (**B**) Animals infected with *Leishmania* (*Viannia*) *braziliensis* and treated intralesionally with Glucantime®; (**C**) Hamsters infected with *Leishmania* (*Viannia*) *guyanensis* and not treated; (**D**) Hamsters infected with *Leishmania* (*Viannia*) *braziliensis* and treated. (**A**) Normal histological aspect evidencing the nodular organization of the white pulp (**wp**). (**B** and **C**) Splenic histological disorganization with moderate red pulp macrophage hyperplasia (**rp**). (**D**) Intense macrophage hyperplasia (**h**) in red pulp (**rp**) and atrophy of the white pulp (**wp**). Staining HE. 100x magnification.

DISCUSSION

For the treatment of skin lesions, the pharmacotherapeutic arsenal is limited. New alternative treatments (Jensen *et al*. 2017; Comandolli-Wyrepkowski *et al*. 2017) have been tested for the desired clinical and parasitic cure, however, pentavalent antimonials – Sb^{5+} remain the preferred drugs for the treatment of CL (Brasil 2017). Treatment with pentavalent antimonials trigger a high degree of toxicity, with serious side effects such as hepatoxicity, nephrotoxicity, arrhythmias and pancreatitis (Ouellette *et al*. 2004; Brazil 2017). To reduce the adverse effects caused by conventional treatment with Sb^{5+}, the WHO included intralesional treatment in their recommendations (Alvar *et al*. 2012).

Most of the scientific projects use mice (*Mus musculus* BALB/c) as an experimental model, and few studies are related to the use of hamsters (*M. auratus*) (Mears *et al*. 2015). However, the golden

hamster is highly susceptible to infection by species of the *Leishmania* of the subgenus *Viannia* (Gomes-Silva *et al*. 2013). In a study by Gomes-Silva *et al*. (2013), the authors observed that lesions in hamsters infected with *L. braziliensis* are very similar to CL infections in humans, since they presented inflammatory signs and evolution of lesions, nodulations and skin ulceration, elevated and erythematous edges, granulose appearance and necrotic surface, and without manifestations of spontaneous healing. This corroborates the results of this project, in which nodulations of lesions that presented bulky and no apparent reduction, characterizing chronification and absence of spontaneous healing were observed in animals infected with *L. braziliensis*.

According to the BMH, the clinical cure of cutaneous leishmaniasis is defined when there is re-epithelialization of previously ulcerated lesions, and total regression of infiltration and erythema (Brasil 2017). The clinical aspect is one of the main parameters of preclinical studies for new therapeutic techniques for treatment of ATL (Mears *et al*. 2015).

Different studies show interesting results when correlating different methods of intralesional antimony application (Robledo *et al*. 2012; Soto *et al*. 2013). Eissa *et al*. (2011) observed CL lesions in the paws of BALB/c mice infected with *Leishmania major* with an exacerbated clinical aspect, with evolution of ulceration lesions to crusts, which was similar to necrosis. Nodular and bulky lesions with intense crustal chronification were observed in animals infected with *L. guyanensis* and treated with Sb^{5+} (Figure 2).

In our experiments with hamsters (*M. auratus*), animals infected with *L. braziliensis* and *L. guyanensis* treated with Glucantime® via IL demonstrated a reduction of the CL lesion in the first 30 days of treatment (Figures 1 and 2, respectively). In the study of Comandolli-Wyrepkowski *et al*. (2017), it was observed that hamsters infected with *L. amazonensis* in the snout and treated with Glucantime® via an IM route at a dose of 20 mg Sb^{5+}/kg/day for 40 days showed reduction of lesions after 20 days of treatment, which was a statistically significant difference ($p < 0.001$) when compared to the

positive control group (infected - untreated) of the study during the same period.

The aforementioned study also presented viable parasites 60 days after the start of treatment, and showed parasitic viability corresponding to a score = 2. This observation was also noticeable in our study, where the IL/Lg Group presented a similar score, with a concentration of 10 to 100 parasites (Figure 5). The resistance presented by *L. guyanensis* that was observed in the present study is a concern that has already been reported in the literature (Bourreau *et al*. 2015).

In the evaluation of the parasitic load, the animals of the IL/Lb group were negative for the presence of amastigotes in macrophages. On the other hand, the IL/Lg group presented viable forms of amastigotes, thus it is perceived through findings from the literature that the small presence of parasitic load by infection of *Leishmania* of the subgenus (*Viannia*) stimulates the immune response of patients to control the infection, even after clinical cure (Mendonça *et al*. 2004; Scott 2005; Gollob *et al*. 2005).

The parasitic resistance of the species previously described in the IL/Lg group is considered a concern in cases of infection in humans in the New World, since it is linked to treatment failure. These findings are described in the literature regarding patients with distinct responses, who presented chronic cutaneous leishmaniasis even after first-line treatment for the disease (Borges *et al*. 2018). Such observations are also depicted in a study of a German repatriate from Ecuador, who presented a deep skin infection caused by *L. guyanensis* with relapse after treatment with Glucantime® (Wollina *et al*. 2019). In the histopathological evaluation of the lesions of the group of animals infected with *L. guyanensis* and animals infected with *L. braziliensis* and treated (Figure 7), mild dermatitis and mononuclear infiltrate in the region of the papillary dermis, extending to the hair follicles was observed. Sebaceous adenitis was not observed in either of the groups. These histopathological findings were similar to the work of Comandolli-Wyrepkowski *et al*. (2017), in which the dermal inflammatory pattern and the cell population, mild dermatitis, papillary dermis with low mononuclear infiltrate that did not extend to

the hair follicles and absence of sebaceous adenitis in hamsters infected with L. amazonensis were also observed. Thus, it is possible to see that both treatments (IL and IM) have similar responses when compared histologically. It is noteworthy, however, that the IL route of administration should be considered for use in patients with contraindications to systemic treatment (Vasconcellos *et al*. 2012; Silva *et al*. 2016), due to it presenting lower toxicity.

The work of Moreira (2012) showed that hamsters infected with *L. infantum* (strain PP75) presented normal aspects in the histological evaluations of the liver after infection (1 to 3 months) via intradermal (ID), intraperitoneal (IP) and intracardiac (IC) routes. However, after evolution (6 to 9 months) of the infection, the ID and IP groups demonstrated discrete inflammatory infiltrate and, in the ID group, the presence of lymphocytes and macrophages in small intralobular granulomas well delimited (6 to 9 months) was also observed. In this study, identical findings were perceived by comparing the shorter time (60 days) for histopathological analysis after infection. These findings included well-delimited, small, intralobular granulomas, abundant periportal infiltrate and abundant inflammatory infiltrate (Figure 7).

In the histopathological analysis of the liver of hamsters infected with *L. braziliensis*, the predominant presence of mononuclear cells was observed, mainly around the perivascular area, which is characteristic of a mixed inflammatory infiltrate (Gomes-Silva *et al.* 2013). In our study, observations of the group of animals infected by this same species and treated showed more abundant characteristics in regards to periportal infiltrate, as well as the presence of mixed mild steatosis and binuclear hepatocytes.

Gomes-Silva *et al*. (2013) performed the histopathological analysis of the spleen of hamsters infected by *L. braziliensis* and described the presence of nodules, a granulomatous reaction typified by the presence of epithelioid macrophages, mixed inflammatory reaction, as well as the presence of granuloma and infiltrate. Moreira (2012) described in their study that hamsters infected with *L. infantum* (strain PP75) by the ID and IP routes (6 or 9 months of evaluation) demonstrated an appearance without nodular disorders of splenic PB, and PB was maintained in primary and secondary lymphoid follicles in

all the periods evaluated. They also found changes in RP 3 months after infection due to the high presence of macrophages.

An analysis of the study of Moreira (2012) reveals that its findings corroborate those observed in our group of animals infected by *L. braziliensis* and treated, since a splenic histological disorganization in RP was also identified, in addition to foci of mononuclear infiltrate. However, in animals infected with *L. guyanensis* and treated, preservation of the splenic parenchyma was observed.

CONCLUSION

The present study, with experimental animals infected with *L. braziliensis*, with a follow-up period of 60 days, demonstrated clinical and parasitological cure through the use of the biweekly protocol of Glucantime® with administration via IL. However, animals infected with *L. guyanensis* and treated showed clinical cure, but, in the analysis of parasitic viability, the results were not so satisfactory.

Treatment using an IL protocol with Glucantime® showed greater efficacy for the species of *L. braziliensis* compared to *L. guyanensis*. Although the latter showed total healing of lesions, there was parasitic resistance. However, the BMH recommends that the tissue epithelialization process be evaluated for up to 120 days and, if total healing does not occur after this period, the therapeutic scheme may be restarted.

In the histopathological evaluation of the fragments of the lesions, it was identified that animals infected with *L. braziliensis* and *L. guyanensis* and treated with Glucantime® via the IL route showed signs of tissue healing and cure, which demonstrates that the drug and route used are effective. In the histological findings of these groups, it was evidenced that deep tissue regions were not reached.

Observations of the results of the histopathological analyses of the liver revealed that, even with the presence of abundant inflammatory and periportal infiltrate in the treated groups, the presence of parasites was not identified. The results demonstrated in this study show that the response of infections to fortnightly treatments with Glucantime® via IL were satisfactory.

The histopathological data of the spleen showed preserved structures, such as the splenic parenchyma, splenic capsule and the areas of WP and RP, thus demonstrating that the IL Glucantime® treatment against infection of the species used in this study were effective, without spread of the infection to the organ.

ACKNNOWLEDGMENTS

The authors would like to thank Fundação de Amparo à Pesquisa do Estado do Amazonas – FAPEAM for its financial support, to Leonardo Brandão and his team at the Biotério Central - INPA for all assistance given to the researchers and maintenance of the animals, to the technician Lourival Maciel Castro for the help at Laboratório de Leishmaniose e Doença de Chagas, to Mayla Silva Leão Ferreira and Maiza Conceição Ferreira da Cunha for all support and time made available during experiments in the Laboratório de Histopatologia - UFAM.

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