Reduction in skin/systemic parasite burdens as a combined effect of topical paromomycin and oral miltefosine treatment of mice experimentally infected with *Leishmania (Leishmania) amazonensis*

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Abstract

This study aimed to investigate the activity of the combination of topical paromomycin gel and oral miltefosine for the treatment of experimental cutaneous leishmaniasis caused by *Leishmania (Leishmania) amazonensis*. The efficacy of the combination, evaluated by measuring lesion size and parasite burden in the skin and spleen, was assessed in BALB/c mice infected by *Leishmania (L.) amazonensis*. The miltefosine was administered orally at 10 mg/kg/day for 10 days, while 10% paromomycin gel was applied topically twice a day for 20 days. Treatment of the experimentally infected animals with topical paromomycin + oral miltefosine combination induced a statistically significant reduction in lesion size and parasite burden in the skin and spleen, with complete healing of ulcers, as compared with those treated with placebo group. Combination of topical paromomycin gel + oral miltefosine provides an enhanced efficacy in the treatment of *L. (L.) amazonensis*-infected mice, thus presenting a higher activity than that observed for the monotherapeutic regimens.
The World Health Organization (WHO) estimates that the annual incidence of cutaneous leishmaniasis (CL) is 1.5 million, with ninety percent of these cases occurring in seven countries: Afghanistan, Algeria, Brazil, Iran, Peru, Saudi Arabia and Syria. The New World CL is caused mainly by *Leishmania (Vianna.) peruviana*, *Leishmania (Vianna) guyanensis*, *Leishmania (Vianna) braziliensis*, *Leishmania (Leishmania) mexicana*, *Leishmania (Leishmania) amazonensis*. This last specie is also responsible for diffuse cutaneous leishmaniasis (DCL) in South America.\(^4\,17\)

Parenteral administration of pentavalent antimony organic compounds remains as the first choice therapy for all leishmaniasis syndromes, including CL. However, resistance and high frequency of side effects (anorexy, myalgias, arthralgias, chemical pancreatitis, leucopenia, cardiotoxicity, etc.) are still relevant problems associated with this treatment.\(^7\,13\)

Over the past few decades, major emphasis has been given to the development of alternative therapies, including the identification of formulations for both oral and topical treatment of CL.\(^3\,7\) Topical treatment represents an interesting approach, offering several advantages in comparison with the parenteral administration: easy administration, lower adverse reaction incidence, and an attractive cost-benefit ratio.\(^10\) Nevertheless, systemic metastasis, that frequently occurs in cases of New World CL may require a systemic therapy.

Paromomycin, an aminoglycoside antibiotic, is the most commonly studied drug for the topical treatment of CL. Recent studies have shown that a new paromomycin formulation
was effective in *Leishmania (L.) major*, *L. (L.) amazonensis*-infected mice, or *L. (V.) braziliensis*-infected hamsters.\(^{12, 18}\) This formulation was recently tested in patients in which CL was caused by *L. V. braziliensis* and who could not be submitted primarily to meglumine antimoniate therapy.\(^{21}\) A new agent – miltefosine – has been successfully implemented in the oral treatment of New World CL.\(^{25}\) However, therapies based on the application of miltefosine are extensive, lasting in general 28 days, and raising concerns regarding toxicity, resistance, and teratogenicity.

Combined therapy, as compared to monotherapeutic regimens, also represents an exciting alternative in the treatment of CL, joining new therapeutic modalities that offer several advantages, such as preventing the emergence of resistance, increasing efficacy, or shortening the course of treatment.\(^{23}\) The present study took one step further in this approach and investigated the efficacy of the combination of topical paromomycin with oral miltefosine in the treatment of mice experimentally infected with *L. (L.) amazonensis*, another etiological agent of New World CL.

**Materials and methods**

**Materials**

Paromomycin, as sulfate 757 µg/mg (Antibioticos, Milan, Italy), hydroxethylcellulose (HEC, Natrosol 250 HR, Aqualon), methylparaben (MP), and propyleneglycol (PG, Basf, Ludwigshafen, Germany) were used to prepare the hydrophilic gel. Miltefosine was donated by Zentaris GmbH (Frankfurt, Germany).
Methods

Preparation of formulations

The paromomycin hydrophilic gel was prepared by heating 1.5% HEC, 10% PG, and 0.2% MP in water to 60–70°C, under constant agitation, until a homogeneous and transparent gel had been obtained. After cooling, paromomycin, previously dissolved in water, was incorporated to the gel at a 10% concentration. Subsequently, the mixture was agitated until a homogenous preparation has been attained. For oral treatment, miltefosine was dissolved in distilled water.

Parasites and infection of animals

*L (L.) amazonensis* (strain IFLA/BR/1967/PH8) amastigotes were isolated of dorsal nodules from Golden hamsters. Nodules were homogenized with an Ultra-Turrax (Ika, Germany) in Schinleider’s modified medium supplemented with 10% bovine fetal serum and 1% of a 100 U/mL penicillin and 100 µg/mL streptomycin solution. The tissue was centrifuged at 50 g for two minutes for sedimentation (Hitachi, Himac). The supernatant was separated, centrifuged at 1700 g for 15 minutes (Express, Jouan) and resuspended in Schinleider’s at 5.0 x 10^7 amastigotes/mL.

BALB/c mice (females, 6–7 weeks old) were inoculated with approximately 1×10^6 amastigotes of *L (L.) amazonensis* through subcutaneous injections at the base of the tail, after trichotomy. The study was approved by the Ethics Committee for Animal Experimentation from Federal University of Minas Gerais (CETEA/UFMG: 181/2006).
Treatment of infected animals

Initially, a dose-effect study of oral miltefosine was carried out. After the development of ulcerated lesions (average diameter of 7 to 9 mm), BALB/c mice were divided into four groups (n = 5), according to lesion size, to assure similar average lesion size among treated groups. The miltefosine was administered by oral gavage (200 µl) at 5, 10 or 25 mg/kg/day for 5 days a week, over a 2–week period. The control group received distilled water. The animals were maintained in abstinence from food 3 h pre-treatment and 1 h post-treatment. For this first study, the treatment efficacy was evaluated through parasite quantification at the site of infection (see below).

The second study evaluated the efficacy of the combination of topical paromomycin (gel containing 10% drug) and oral miltefosine at doses of 5, 10, or 25 mg/kg/day. After the development of ulcerated lesions, BALB/c mice were divided into four groups (n = 5). For the topical paromomycin + 5 mg/kg/day miltefosine group, lesions were covered with 50 µl of 10% paromomycin gel, twice a day, for 20 days. The gel was topically applied using a semi-solid pipette. The animals were also treated with miltefosine administered by oral gavage (5 mg/kg/day) on alternate days for 20 days. For the remaining groups, the animals were treated similarly with 10% paromomycin gel for 20 days and miltefosine by oral gavage at doses of 10 or 25 mg/kg/day, on alternate days, for 20 days. For the control group, animals were treated with a gel that did not contain paromomycin (placebo). Treatment efficacy was evaluated by determining parasite loads at the site of infection (see below).
The third study evaluated the efficacy of the combination of topical paromomycin (gel containing drug at 10%) + 10 mg/kg/day miltefosine in comparison to the monotherapeutic regimens (10% paromomycin gel or 10 mg/kg/day oral miltefosine alone). After the development of ulcerated lesions, BALB/c mice were divided into four groups (n = 5). For the paromomycin group, lesions were covered with 50 µl of 10% paromomycin gel, twice a day, for 20 days. For the miltefosine group, the animals were treated with miltefosine by oral gavage (10 mg/kg/day) on alternate days for 20 days. For the topical paromomycin + 10 mg/kg/day miltefosine group, the lesions received a topical treatment (10% paromomycin gel) as previously described for the paromomycin group. In addition, the animals were also treated with miltefosine, which was administered orally in the same manner as the miltefosine group. For the control group, animals were treated with a gel that did not contain PA (placebo). Treatment efficacy was evaluated by measuring the size of the lesions as well as by determining parasite loads, both at the site of infection (skin) and in the spleen, after the interruption of treatment.

### Parasite quantification

Three days after the interruption of treatment, the number of viable parasites at the site of infection was determined by a limiting-dilution assay. Skin fragments, consisting of ulcerated lesions, were weighed and homogenized with an Ultra-Turrax (Ika, Germany) in Schineider’s modified medium supplemented with 10% bovine fetal serum and 1% of a 100 U/mL penicillin and 100 µg/mL streptomycin solution. Next, the tissue was centrifuged at 50 g for two minutes for sedimentation (Hitachi, Himac). The supernatant was separated and centrifuged at 1700 g for 15 minutes (Express, Jouan). The pellet formed was
resuspended in 1 mL of Schneider’s modified medium supplemented with 10% bovine fetal serum and 1% of a 100 U/mL penicillin and 100 µg/mL streptomycin solution. The homogenate was submitted to serial dilutions in duplicates in sterile 96 well culture plates and incubated at 23°C. Each well was examined for the presence of parasites, and the number of parasites was determined by the highest dilution at which parasites could grow over a 7-day period. The number of viable parasites was also determined in spleens, as previously described. The supernatant was separated and centrifuged at 1700 g for 2 minutes (Express, Jouan).

Lesion size measurements

During and after treatment, lesion size was followed up weekly using a caliper to measure its diameter (Mitutoyo, Brazil). The lesion size was determined by obtaining the average value between the longest line that can be traced from one border of the lesion to another and the line that bisects this distance at a 90° angle. Further evaluations, through careful observation of paws and tails, included the appearance of relapses, nodules, and metastasis in other locations on the animals’ skin. Mice infected were observed for an additional 49-day period after the interruption of treatment. Animals were considered cured only if nodules and ulcers were completely absent (end of experiment).

Statistical analysis

The statistical significance of differences in the parasite quantification and average lesion diameter among groups was evaluated using the one-way analysis of variance (ANOVA)
test followed by Tukey’s test. The difference was considered significant when the P value was less than 0.05.

Results

Dose-effect study of oral miltefosine

The quantification of parasites within lesions was used to evaluate the efficacy of different doses of oral miltefosine in animals infected with *L. (L.) amazonensis*. As shown in Figure 1, the number of parasites within the lesion decreased when doses of oral miltefosine were increased. The number of parasites in the control group (1.4 x 10^6) was higher than that observed in the groups treated with miltefosine at 5 mg/kg/day (6.4 x 10^5), 10 mg/kg/day (2.5 x 10^5), or 25 mg/kg/day (3.3 x 10^2). However, statistical analysis showed a significant reduction in parasite numbers only in the animals treated with the dose of 25 mg/kg/day, when compared with the control group. The animals treated with the doses of 5 mg/kg/day or 10 mg/kg/day showed an insignificant reduction when compared to the control group (p>0.05). All doses (5, 10 or 25mg/kg/day oral miltefosine) were selected to evaluate the efficacy of the combination of topical paromomycin and oral miltefosine.

Efficacy of combination of topical paromomycin and oral Miltefosine

The second study was carried out to evaluate the efficacy of the combination between oral miltefosine and topical paromomycin. The parasite burdens in the lesion were assessed three days after the interruption of the treatment (Figure 2). The number of parasites in the paromomycin + 10 mg/kg/day oral miltefosine group (1.3 x 10^4) was significantly lower than that observed in the control group presenting a reduction of 92.6% in the parasite load.
Interestingly enough, the treatment of animals using the combination of topical paromomycin + 25 mg/kg/day oral miltefosine completely eliminated the parasites at the site of infection, thus presenting a reduction of 100%. The animals treated with the combination of topical paromomycin + 5 mg/kg/day oral miltefosine showed an insignificant reduction when compared to the control group (p>0.05). Thus, the combination of topical paromomycin + oral miltefosine at doses of 10 mg/kg/day or 25 mg/kg/day provided an enhanced efficacy in the treatment of *L. (L.) amazonensis*-infected mice.

To further investigate the efficacy of drug combination, a third study evaluated the efficacy of the association of topical paromomycin + miltefosine in comparison to the monotherapeutic regimens (topical paromomycin gel or oral miltefosine alone). Oral miltefosine at a dose of 10 mg/kg/day was selected for this investigation. The parasite burdens in the lesion and spleen were assessed three days after the end of the treatment. The data are presented in Figure 3. The animals treated with the monotherapeutic regimens (topical paromomycin or 10 mg/kg/day oral miltefosine alone) showed an insignificant reduction when compared to the control group (p>0.05). As expected, the parasite burden at the site of infection (lesion) was significantly reduced (p < 0.05) in animals treated with the combination of topical paromycin + oral miltefosine (3.4 x 10^4), as compared to the placebo gel (1.1 x 10^7), presenting a reduction of 99.99% in the parasite load (Figure 3a).

To investigate the systemic efficacy of the paromomycin + miltefosine combination, the spleen parasite burden was also evaluated (Figure 3b). Treatment of infected animals with topical paromomycin alone did not change the parasite load in the spleen, while oral miltefosine, as compared to the control group, induced an insignificant reduction. However,
the parasite burden in the spleen was significantly reduced (P < 0.05) in animals treated with the topical paromomycin + oral miltefosine combination as compared to those that received either monotherapeutic regimens or the placebo gel (control group).

Figure 4a shows the evolution of the lesion size after the beginning of the treatment as a function of time. At the beginning of the treatment, the animals treated with miltefosine alone and with the topical paromomycin + oral miltefosine combination presented lesions with an average diameter of 7.9 and 8.4 mm, respectively. The lesion size of these animals significantly diminished during the evaluation period until complete healing had been established, which could be observed 28 days after the onset of therapy for both groups. All animal lesions treated with the topical paromomycin + oral miltefosine combination remained cured throughout the entire observation period (49 days) and no relapse, characterized by the reappearance of ulcers, could be observed during this time interval. In contrast, for those treated with oral miltefosine alone, relapse was observed 42 days after the onset of therapy. Animals treated with topical paromomycin alone presented a slight and gradual reduction in lesion size. However, when compared to the control group, significant differences could be observed only 42 days after the onset of therapy. In addition, a gradual increase in the average lesion size could be observed for the control group.

Data concerning the percentage of cured animals are shown in Figure 4(b). The cure criterion adopted was the complete healing of lesions and the absence of nodules. In the groups treated with oral miltefosine alone or topical paromomycin + oral miltefosine combination, complete healing (100%) was observed in all animals 28 days after the
beginning of the treatment. No relapse, characterized by the reappearance of nodules or ulcers, could be observed for animals treated with the combination throughout the evaluation period (49 days) and hair growth could be detected in all cured animals. However, 49 days after the beginning of treatment, relapse, characterized by the reappearance of ulcers, was observed in four-fifths of the animals treated with miltefosine alone, producing a reduction in the cure percentage (20% cure rate). In the group of animals treated with topical paromomycin, no cure was found for any animal (0% cure rate) up to 42 days after the beginning of treatment, which was similar to that observed in the placebo (control) group. In addition, a low cure rate (20%) was observed 49 days after the onset of treatment. Therefore, under these conditions, the activity of topical paromomycin + oral miltefosine combination was significantly higher than that observed for monotherapeutic regimens.

Discussion

Recently, our research group reported that the combination of topical PA gel + oral miltefosine provides an enhanced efficacy in the treatment of *L. (L.) major*-infected BALB/c mice\(^1\). A positive interaction between miltefosine (oral) with paromomycin (subcutaneous) was observed in BALB/c mice infected with *L. (L.) donovani*.\(^23\) Since the sensitivity to both paromomycin and miltefosine varies considerably among *Leishmania* species,\(^9, 19, 20\) the present study sought to investigate the activity of the topical paromomycin + oral miltefosine combination in mice infected by *L. (L.) amazonensis*, a species causing New World CL.
Since there are no previous reports on the activity of oral miltefosine against *L. (L.) amazonensis* in animal models, the first step of this study was to evaluate its efficacy in experimentally infected mice through a dose-effect analysis. Previous findings indicated a significant toxicity in animals that received oral miltefosine at a dose of 50 mg/kg/day, expressed as a significant loss of body weight. On the other hand, *in vitro* the IC$_{50}$ of miltefosine against *L. (L.) major* was higher than that observed for *L. (L.) amazonensis*. Therefore, the doses selected in present study ranged from 5 to 25 mg/kg/day.

The parasite burden in the lesion was significantly reduced when miltefosine was orally administered at a dose of 25 mg/kg/day. However, lower doses (5 or 10 mg/kg/day) were not associated with a significant reduction in parasite burdens in lesions. Subsequently, the treatment efficacy of the topical paromomycin + oral miltefosine combination was evaluated by measuring the parasite burden on the skin. Treatment of the experimentally infected animals with the combination of paromomycin and 10 or 25 mg/kg/day miltefosine, as compared with those treated with a paromomycin + 5 mg/kg/day miltefosine combination or a placebo, led to a statistically significant reduction in the lesion parasite burden. It is interesting to note that the combination of paromomycin + 25 mg/kg/day miltefosine completely eliminated the parasite burden at the site of infection. However, due to concerns of toxicity associated with high doses of miltefosine, the dose of 10 mg/kg/day was selected to compare the combined therapy to the monotherapeutic regimens.

A low cure percentage in animals treated with topical paromomycin alone could be observed. This runs in contrast with prior studies which reported that the activity of the topical paromomycin gel in mice infected by *L. (L.) amazonensis* was in fact higher than...
that observed for parenteral antimony, thus presenting a complete healing of ulcers in all animals. This finding may well be attributed to differences in experimental procedures between the two studies, since mice were infected with promastigotes and amastigotes in previous and present studies, respectively. The disease proved to be more aggressive in animals infected with amastigotes, when compared to those infected with promastigotes, and the outcome of the therapy may be markedly influenced by this. Nevertheless, the findings of the present study suggest that the paromomycin and miltefosine combination represent an interesting alternative in the treatment of CL.

The systemic efficacy of the topical paromomycin + oral miltefosine combination was also evaluated by measuring the parasite burden in the spleen. Treatment of the experimentally infected animals using the paromomycin + miltefosine combination, as compared to those treated with monotherapeutic regimens or a placebo, led to a reduction in the lesion parasite burden, consequently presenting a complete healing of ulcers. The combination proved to be as effective as oral miltefosine alone in reducing the lesion size; however, relapses could clearly be observed in animals receiving the monotherapy, which did not occur in the group treated with the drug combination. In addition, the efficacy of the combination was higher than that observed for the other treatments, including oral miltefosine alone, in reducing the parasite burden in the spleen.

The improved systemic efficacy of the topical paromomycin + miltefosine combination may well be attributed to the effects of both drugs, since an enhanced percutaneous absorption of paromomycin after topical application could be observed. Our previous studies showed that the paromomycin can be absorbed when administered topically. This
finding is consistent with previous observations, which showed that the in vitro permeation of paromomycin, from a hydrophilic gel applied to stripped mice skin was high. On other hand, miltefosine was found to be efficiently absorbed by the intestinal tract after oral administration. In addition, the remarkable activity of the drug in the spleen might be explained, at least in part, by a favorable distribution of the compound in the reticuloendothelial system.\textsuperscript{5,14}

Investigations on the mode of action of paromomycin in Leishmania are scarce, and several targets have been implicated as suggested mechanisms.\textsuperscript{15,16} The action of miltefosine includes the perturbation of ether-lipid metabolism, glycosylphosphatidylinositol anchor biosynthesis, and signal transduction.\textsuperscript{23} Recently, it has been demonstrated that miltefosine may also affect immune responses due to its ability to induce INF-$\gamma$ production and to enhance INF-$\gamma$ responsiveness by inducing the INF-$\gamma$ receptor in macrophages found in BALB/c mice infected with L. (L.) donovani.\textsuperscript{27} Although confirmation of the presence of this mechanism of action during the treatment of L. (L.) amazonensis infection in BALB/c mice requires further investigation, this could be considered a very interesting additional effect, considering that BALB/c mice are very susceptible to infection by L. (L.) amazonensis. The underlying immune responses linked to these extreme susceptibilities are, at least in part, associated with increased IL-4 and IL-10 levels, which suppress the development of Th1 cells and inhibit the INF-$\gamma$ secretion.\textsuperscript{2,26} Indeed, these mice have been considered a rigorous non-cure model in which only the most active drugs are effective. Cure of the infection in this model, even if temporary, is usually attributed to effects from chemotherapy.\textsuperscript{8} However, it is tempting to hypothesize that, besides the anti-leishmania
activity, miltefosine may also improve Th1 immune responses and macrophage microbicidal activation, as it has been observed during *L. (L.) donovani* infection.

Comparing the data of our previous studies\(^1\), in which animals experimentally infected by *L. (L.) major* were treated with combination and monotherapeutic regimens, with those of the present study, some important additional considerations can be raised. Miltefosine alone showed a high efficacy in animals experimentally infected by *L. (L.) amazonensis* and a low efficacy in mice infected by *L. (L.) major*. In contrast, the topical paromomycin alone presented an inverse behavior. Interestingly, the miltefosine and topical paromomycin combination presented a high efficacy in animals infected either with *L. (L.) major* or *L. (L.) amazonensis*), leading to statistically significant reduction in parasite burdens in the lesion and in spleen, in comparison to the control group, while the monotherapeutic regimens were not associated with a significant reduction. These findings suggest that the combined therapy of miltefosine and paromomycin may circumvent the well documented differences in susceptibility to these drugs observed for these *Leishmania* species.

Finally, the present study was performed on mice infected by *L. (L.) amazonensis*, one of the etiologic agents of New World CL. Human CL in the New World can be associated with the dissemination of parasites, a situation that may require systemic treatment. Moreover, infection by *L. (L.) amazonensis* is associated with all clinical syndromes of leishmaniasis, including DCL. Patients with DCL are usually not responsive to treatment and present a specific anergy to parasite antigens.\(^2\) It has been demonstrated *in vitro* that New World *Leishmania* species are more refractory to paromomycin\(^19\) and more sensitive to miltefosine.\(^9\) However, therapies recommended to treat human New World CL with oral
miltefosine are extensive (28 days) and raise concerns regarding the emergence of toxicity and resistance. On the other hand, a sole topical treatment of New World CL may lead to concerns regarding the elimination of disseminated parasites. Therefore, a therapy including a topical paromomycin + oral miltefosine combination may circumvent the requirements for prolonged treatment, with favorable impacts regarding toxicity and the efficient elimination of disseminated parasites. Thus, our data suggest that the topical paromomycin + oral miltefosine combination may also present an interesting alternative in the treatment of New World CL.

In summary, our data showed that the combination of topical paromomycin gel + oral miltefosine provides an enhanced efficacy in the treatment, in turn presenting a significantly higher activity than that observed for the monotherapeutic regimens, in mice infected by *L. (L.) amazonensis*, one of the main etiologic agents of New World CL. These findings suggest that the topical paromomycin + oral miltefosine combination represents a promising alternative in the treatment of CL.

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References


**Figure 1:** In vivo efficacy of oral miltefosine in *L (L.) amazonensis*-infected mice. Female BALB/c mice were infected with *L (L.) amazonensis* amastigotes in base of the tail; 6 weeks after inoculation, the animals were treated with distilled water (Control) and administered orally miltefosine at 5 mg/kg/day (Milt 5), 10 mg/kg/day (Milt 10) or 25 mg/kg/day (Milt 25) for 5 days a week, over a 2–week period. Three days after interruption of treatment, parasite numbers recovered from lesions were evaluated by limiting dilution assay (*p <0.05 when compared to control group) (n = 5).
Figure 2: In vivo efficacy of the combination 10% paromomycin gel (PA) with oral miltefosine in *L. (L.) amazonensis*-infected mice. Female BALB/c mice (n = 5) were infected with *L (L.) amazonensis* amastigotes in base of the tail; 6 weeks after inoculation, the animals were treated with combination of topical PA + 5 mg/kg/day oral miltefosine (Milt 5 + PA), topical PA + 10 mg/kg/day oral miltefosine (Milt 10 + PA), topical PA + 25 mg/kg/day oral miltefosine (Milt 25 + PA) or placebo gel (Control) for 20 days. Three days after interruption of treatment, parasite numbers recovered from lesions were evaluated by limiting dilution assay (*p <0.05 when compared to control group) (n = 5).
Figure 3: In vivo efficacy of the combination 10% paromomycin gel (PA) with oral miltefosine as compared to isolated treatments in *L. (L.) amazonensis*-infected mice. Female BALB/c mice (n = 5) were infected with *L. (L.) amazonensis* amastigotes in base of the tail; 6 weeks after inoculation, the animals were treated with combination of topical PA + 10 mg/kg/day oral miltefosine (Milt 10 + PA), topical PA (PA), 10 mg/kg/day oral miltefosine (Milt 10) or placebo gel (Control) for 20 days. Three days after interruption of treatment, parasite numbers recovered from lesions and spleen were evaluated by limiting dilution assay. (a) Parasite burden quantified in lesion (*p <0.05 when compared to control) and (b) parasite burden quantified in spleen (*p <0.05 when compared to control).
Figure 4: Evaluation of treatment efficacy in female BALB/c mice infected by *L. (L.) amazonensis*. Animals (n = 5) were treated topically with 10% paromomycin gel (PA), 10 mg/kg/day oral miltefosine (Milt 10), combination of 10 mg/kg/day oral miltefosine + topical PA (Milt 10 + PA) or placebo gel (Control) for 20 days. In (a), dots and vertical bars represent, respectively, average lesion diameter and standard deviation (SD). In (b), percentage of cured animals (complete healing of lesions and absence of nodules) evaluated for 49 days.