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IN VIVO ANTILEISHMANIAL EFFICACY OF MILTEFOSINE AGAINST *LEISHMANIA* (*LEISHMANIA*) *AMAZONENSIS*

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?1 ABSTRACT: Leishmaniasis, a disease caused by parasites of the *Leishmania* genus, constitutes a significant health and social problem in many countries and is increasing worldwide. The conventional treatment, meglumine antimoniate (MA), presents numerous disadvantages, including invasiveness, toxicity, and frequent therapeutic failure, justifying the attempts at finding alternatives to the first-line therapy. We have studied the comparative long-term efficacy of MA against miltefosine (MF) in *Leishmania* infection in experimental mice. The criteria for efficacy evaluation were footpad lesion size, anti-*Leishmania* antibodies level, histopathology of the site of inoculation (right footpad, RFP), splenic index (SI), and the presence of parasites in RFP, spleen, and liver, determined by polymerase chain reaction (PCR). Swiss mice, infected with *Leishmania* (*Leishmania*) *amazonensis* were treated, at different time points (5 and 40 days after infection) with either MA or MF. The efficacy of MF was better than that of MA for inhibiting lesions and for reducing tissue damage and presence/load of amastigotes in spleen and liver. Moreover, early administration of MF produced a clear reduction in splenomegaly and was equal in reducing antibody titles in comparison with MA. Our results demonstrated that MF is an effective and safe therapeutic alternative for leishmaniasis by *L.* (*L.*) *amazonensis* and is more efficacious than MA.

Leishmaniasis is a zoonotic disease caused by unicellular parasites of the *Leishmania* genus, which infects different mammals, including humans. This disease constitutes a health and a social problem in many countries, primarily affecting the poor and in socio economically underdeveloped populations. However, the incidence also is increasing worldwide due to imported cases (tourism and job-related travels) (Boecken et al., 2011). Within Argentina, the province of Salta displays the highest endemicity, *Leishmania* (*Viannia*) *braziliensis*, *Leishmania* (*Leishmania*) *amazonensis*, and *Leishmania* (*Viannia*) *guyanensis* being so far the main circulating species (Marco et al., 2005; Barrio et al., 2009; García Bustos et al., 2011). *Leishmania* (*L.*) *amazonensis* stands out for its ability to cause a broad spectrum of clinical manifestations, from localized cutaneous leishmaniasis to more severe forms. These include mucosal leishmaniasis and diffuse cutaneous leishmaniasis (DCL), both of which are mutilating diseases and, in the case of DCL, resistant to treatment (Lucas et al., 1998).

Treatment, at present, has several drawbacks; the first-line drug, meglumine antimoniate (MA), must be administered IV or IM for 20–28 days. It displays a wide range of adverse effects affecting the heart, liver, pancreas, and kidney. Moreover, parasite resistance to the drug and therapeutic failure is increasing (Llanos-Cuentas et al., 2008), and there exist a number of contra indications for its administration. Other second line treatments are available, including amphotericin B. This drug has low efficacy, needs intravenous administration, and has well documented adverse effects, mainly affecting kidney function. Alternatively, a liposomal form of amphotericin is superior in efficacy and safety (Laniado-Laborin and Cabrales-Vargas, 2009), but it's not affordable for most patients. For these reasons, the development of new therapeutic strategies remains a priority.

Miltefosine (MF) is an alkyl-phosphoryl-choline (alkyl analog of a lysophospholipid), active by both topical and oral routes, which was originally developed as antineoplastic drug. Its activity is mainly linked to apoptosis and disturbance of lipid-dependent cell signaling pathways (Dorlo, 2012). It has been postulated also that this drug alters calcium homeostasis, producing an increase in the intracellular concentration of ions, reducing parasite viability (Serrano-Martín et al., 2009). Several preclinical and clinical studies (Sindermann and Engel, 2006; Soto and Berman, 2006; Sundar et al., 2006; Soto et al., 2007) suggest that MF could constitute an effective and safe treatment for different clinical forms of leishmaniasis, caused by different species of *Leishmania*. Particular advantages are oral administration and the good balance between efficacy and adverse effects (Soto and Berman, 2006). For these reasons MF could constitute a good therapeutic alternative.

In this study, we examined the efficacy of MF, compared with MA in a Swiss mouse model of infection by *L.* (*L.*) *amazonensis*.

MATERIALS AND METHODS

This study was approved by the Institutional Committee for the Care and Use of Laboratory Animals, School of Medical Science, National University of Cuyo (Argentina). This work complies with institutional regulations and with national and international legislation concerning the use of animals in research.

Drugs

Commercial forms of MA (Glucantime®, Aventis, Paris, France), and MF (Mitenox®, Lazar, Buenos Aires, Argentina) were used.

Parasites

Leishmania (*L.*) *amazonensis* (strain MHOM/VE/84/MEL) were obtained from the Biomedicine Institute (Central University of Venezuela). The strain was characterized by polymorphism specific–polymerase chain reaction (PS-PCR) (Mimori et al., 2002) and maintained by serial passages in the right footpad (RFP) of BALB/c mice. Isolation was performed by seeding lesion homogenates in modified USMARU medium (blood-agar base overlayed with 1% glucose-saline solution) at 24 C. Subcultures first to eighth were used. During the exponential phase, parasites were harvested, washed in buffered saline, pH 7.2 with 100 IU/ml penicillin/streptomycin, and resuspended in PBS at the concentration required for inoculation into mice (see below).

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Mice

Swiss male mice, 1.5-mo-old, weighing 17–20 g were used. Each treatment group consisted of 10 mice.

Minimal infective intake

To determine the minimal intake of *L.* (*L.*) *amazonensis* that induces measurable lesions in all mice, 5 groups of 5 animals each were inoculated with serial dilutions of the parasite as follows: 10^5, 10^4, 10^3, 10^2, and 10 promastigotes in 50 μL of PBS by subcutaneous inoculation into the RFP. Between days 15 and 176 post infection (pi) the progressive growth of the lesion was measured weekly with a submillimetric caliper. The difference between the thickness of the RFP and left footpad (delta value, Δ) was recorded. A difference in lesion size was considered measurable when $\Delta \geq 0.2$ mm.

Therapeutic dose of miltefosine

The administered MF dose was decided following previous clinical determinations. Groups of 5 animals each, infected with the predetermined minimal amount of parasites, were treated with different MF doses (8, 16, or 25 mg/kg/day) at 5 days pi. The drugs was dissolved in aqueous solution containing 5% NaCl and 9% Tween 80 and administered through an intragastric cannula, in a volume of 0.1 ml. Controls received the vehicle only. Between days 27 and 132 pi the growth of the lesions was measured weekly.

Treatment schedules

MF was administered through an intragastric cannula, in a volume of 0.1 ml, at a dose of 16 mg/kg/day. MA was administered by intraperitoneal route, in a volume of 0.1 ml, the dose being 400 mg/kg/day (100 mg SbV/kg/day). The MA dose was based on previously published data (Velloso da Costa et al., 2008). Drugs were dissolved in aqueous solution containing 5% NaCl and 9% Tween 80. MF and MA were each administered following 2 different schedules: early (MF5 and MA5 groups), before appearance of macroscopic lesions (day 5 pi), and late (MF40 and MA40 groups), after appearance of macroscopic lesions (day 40 pi), in order to assess whether the time of administration influences its effectiveness. Controls received the vehicle only. All treatments were administered daily from Monday to Saturday, until 30 doses were administered.

Evaluation of treatment efficacy

Five variables were examined in order to assess treatment efficacy: size of the lesions, anti-*Leishmania* antibody level, histopathology, splenic index, and the presence of parasites (determined by PCR).

Measurement of lesions: We measured the size of the lesions on both hind footpads weekly, from day 27 to day 139 pi, and then we calculated the delta value.

Dosage of anti-Leishmania antibodies: ELISA tests were performed at day 70 pi. Polystyrene microplates were coated with 2 μg per well of a homogenate of *L.* (*L.*) *amazonensis* (MHOM/VE/84/MEL) dissolved in carbonate buffer (pH 9.6). The plates were incubated at 4 C overnight. Wells were blocked with PBS with 5% skimmed milk for 1 hr at room temperature. Anti-mouse immunoglobulin biotin-labeled and peroxidase-avidin was diluted to 1:2,500 and 1:2,000, respectively. The reaction was developed using o-phenylenediamine in H_2O_2 diluted in citrate buffer (pH 5.3) and incubated for 30 min at room temperature. In order to stop the reaction, 2 M H_2SO_4 was used, and absorbance read at 490 nm using a BioTeck™ ELx800™ apparatus (BioTeck™, Winooski, Vermont). All samples were in duplicate.

Histopathology: At day 139 pi, mice were weighed and sacrificed. Spleens were excised and weighed. The RFP granulomas and livers were also excised. To check visually for the presence of the parasite, impression smears from liver and spleen were stained with May Grünwald–Giemsa. All tissues were fixed in 10% formalin; histological preparations were made after 12–24 hr and finally stained with hematoxylin-eosin (HE). Each slide was examined under the microscope at the highest magnification (×100) by 2 individuals. The number of amastigotes per histiocyte was determined in 100 microscopic fields and expressed as the average for each group. According to the number, cases were classified as abundant (≥5 parasites/histiocyte); scarce (≤4 parasites/histiocyte); or absent (0 parasites/histiocyte).

Splenic index: To estimate the parasite load in the spleen we determined the weight of the organ and calculated the ratio with respect to the body weight according to previously published protocols (Fechio et al., 1999).

Parasite infection: To determine the presence of parasites in RFP, spleen, and liver, we performed PCR analysis. The spleen and liver impression smears were scraped off the slide with a sterile scalpel (Pourmohammadi et al., 2010). The material was transferred to a 1.5 ml microcentrifuge tube containing 100 μl of TE buffer, heated at 100 C in a boiling water bath, and then stored at –20 C until use. Paraffin-embedded right footpads were washed 3 times with ethanol 100%. For each sample, 2 (15 g) sections of tissue were separated, and DNA extraction was made using Biostic® FFPE Tissue DNA Isolation Kit (MO BIO Laboratories, Inc, Carlsbad, California). The PCR reaction was performed according the procedure described by Barrio et al. (2007).

Statistical analysis

Delta values were analyzed via 2-way classification by repeated measurement variance analysis (ANOVA), which renders the statistical significance of the treatment effect, as to the RFP lesions progression, and also the interaction of both measurements. After processing the data, the Bonferroni test was applied to detect where the most significant differences in lesion development between treatment groups were concentrated. The data from SI, anti-*Leishmania* antibody level, and tissue damage in the RFP were analyzed using the non parametric, Kruskal–Wallis ANOVA. To detect where the differences between treatment groups were concentrated, the Dunn multiple comparison test was applied. The data from PCR analysis, mortality, and load of amastigotes were subjected to a chi-square test for homogeneity and to determine statistical differences between both treatment groups. In this case, to identify the variables concentrating differences, the Fisher's exact test or the table partition method was used (Zar, 1999). The level of significance was set at $P = 0.05$. The statistical packages used were GraphPad Prism 5 and InfoStat, version 2008.

RESULTS

Minimal infective intake

We determined that 10^4 parasites were able to produce macroscopically visible lesions ($\Delta \geq 0.20$ mm). However, and in order to ensure infections giving measurable lesions, we inoculated 2×10^4 promastigotes in each experiment.

Therapeutic dose of miltefosine

To determine the therapeutic dose of miltefosine, we assayed 3-+ different concentrations of the drug (8, 16, and 25 mg/kg/day). Figure 1 shows the evolutions of the RFP lesions in treated animals. The lowest concentration was effective in inhibiting the development of the lesions. However this clinical recovery of the animals was only transient, at day 111 pi the mice again presented very evident lesions. Instead, a 16 mg/kg/day dose showed adequate clinical effectiveness, with complete inhibition in lesion development, and therefore was chosen to continue the experiment. Also, animals receiving the 25 mg/kg/day dose did not develop lesions, but at this concentration there was high mortality (60%). During treatments with 8 or 16/mg/kg/day and follow up, no symptoms of distress or morbidity were observed, suggesting low toxicity of the drug at these doses.

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Lesion development

Significant differences were found in the development of lesions between treatment groups. Both groups receiving MF, starting on days 5 or 40, displayed highly significant inhibition of lesion

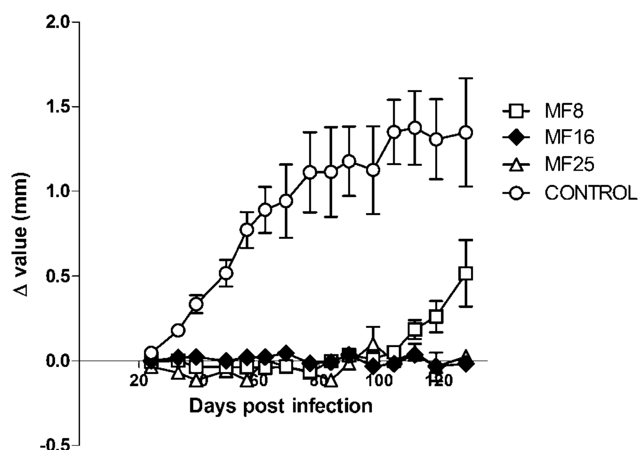

FIGURE 1. Progression of Δ values in mice treated with different miltefosine (MF) doses (MF8, 8 mg/kg/day; MF16, 16 mg/kg/day; MF25, 25 mg/kg/day) and controls (5% NaCl and 9% Tween 80). MF8 showed an initial inhibition in the development of lesions, with subsequent recovery (Δ ≥ 0.20 mm). MF16 showed complete inhibition in lesions development. MF25 proved to be effective, but displays a high mortality (60%).

development as compared with controls after day 55 pi ($P < 0.001$). As shown in Figure 2, none of the mice of MF5 group developed measurable lesions during the experiment. Mice of the group MF40 started to develop lesions after day 27 pi, but from day 83 and on, the Δ values remained <0.20 mm. Mice receiving MA on day 5 (MA5) and on day 40 pi (MA40) also displayed significant differences as compared with the placebo group. The statistical comparison between the 2 types of treatment (MF vs. MA), showed that both MF5 and MF40 groups displayed significant inhibition of lesion growth with respect to the MA groups: these differences were significant from day 83 for MA40 and from day 111 for the MA5 group. Moreover, significant differences were only detected on day 104 in the MA5 with respect to the MA40 group. On the contrary, we did not detect significant differences between the MF5 and MF40 groups at any time after treatment. Of note, animals treated with MF developed fewer lesions.

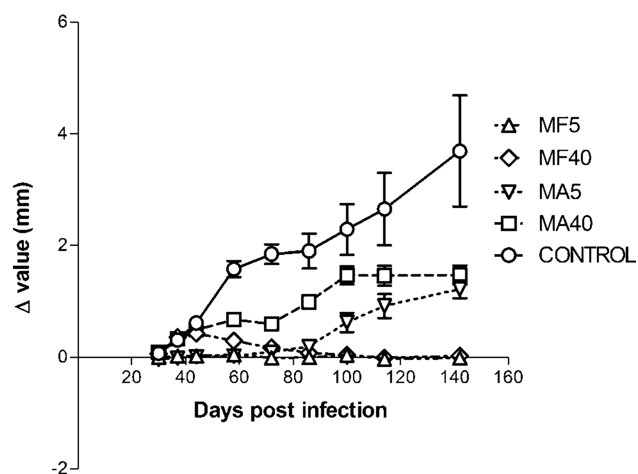

FIGURE 2. Progression of Δ values at different times in the 5 treatment groups. None of the mice of MF5 group developed measurable lesions during the experiment.

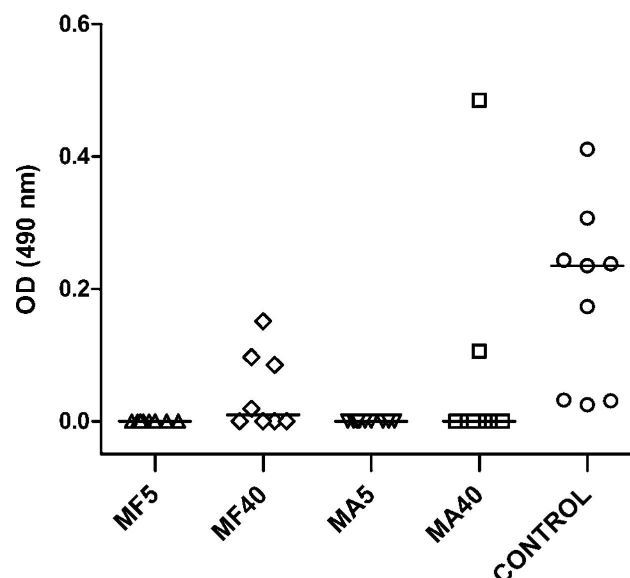

FIGURE 3. Concentration of anti-*Leishmania* antibodies in serum, expressed as optical density (OD) values, as found in the different treatment groups on day 70 post infection. No detectable antibodies in mice treated early (MF5 and MA5).

Anti-*Leishmania* antibodies

Analyzing serum samples of day 70, the Kruskal–Wallis test demonstrated that the median optical density (MOD) values were significantly different ($P < 0.0001$) between some groups. The Dunn test showed that the MOD of samples from mice receiving the drugs early (MF5 and MA5) was significantly lower (MOD = 0) than controls (MOD = 0.23), as well as the MA40 group (MOD = 0). Statistics showed that the MF40 group (MOD = 0.009) did not differ from that of the placebo group (Fig. 3).

Histopathology

The histological preparations from RFP were examined under the microscope, and the degree of tissue damage was classified as follows: (0) absent tissue damage, no inflammation detected (Fig. 4A); (+) slight tissue damage, presence of granulomatous inflammation without necrosis or ulceration (Fig. 4B); (++) moderate tissue damage, presence of granuloma and necrosis, without ulceration (Fig. 4C); (+++) severe tissue damage, presence of granulomatous inflammation with necrosis and ulceration (Fig. 4D). The microscopic analysis showed that all animals from the MF5 group had no tissue damage. The Kruskal–Wallis test demonstrated that the magnitude of the tissue damage in the MF5 group was lower than that of mice from the MA5 and MA40 groups and animals treated with placebo. Furthermore, there were not significant differences between animals from the MF5 and MF40 groups, but marked differences with the control group, in which there was severe damage in 67%, moderate in 22%, and slight in 11% of mice. There was no significant difference between MA-treated groups regarding tissue damage (Fig. 4e). ?3

Presence of amastigotes: The presence or absence of detectable amastigotes in HE stained samples is displayed in Table I. Our analysis revealed that the number of mice with amastigotes at the lesion site is not homogeneous between groups ($\chi^2 = 25.45$; df = 5; $P = 0.0001$). The largest percentage (100%) of animals without

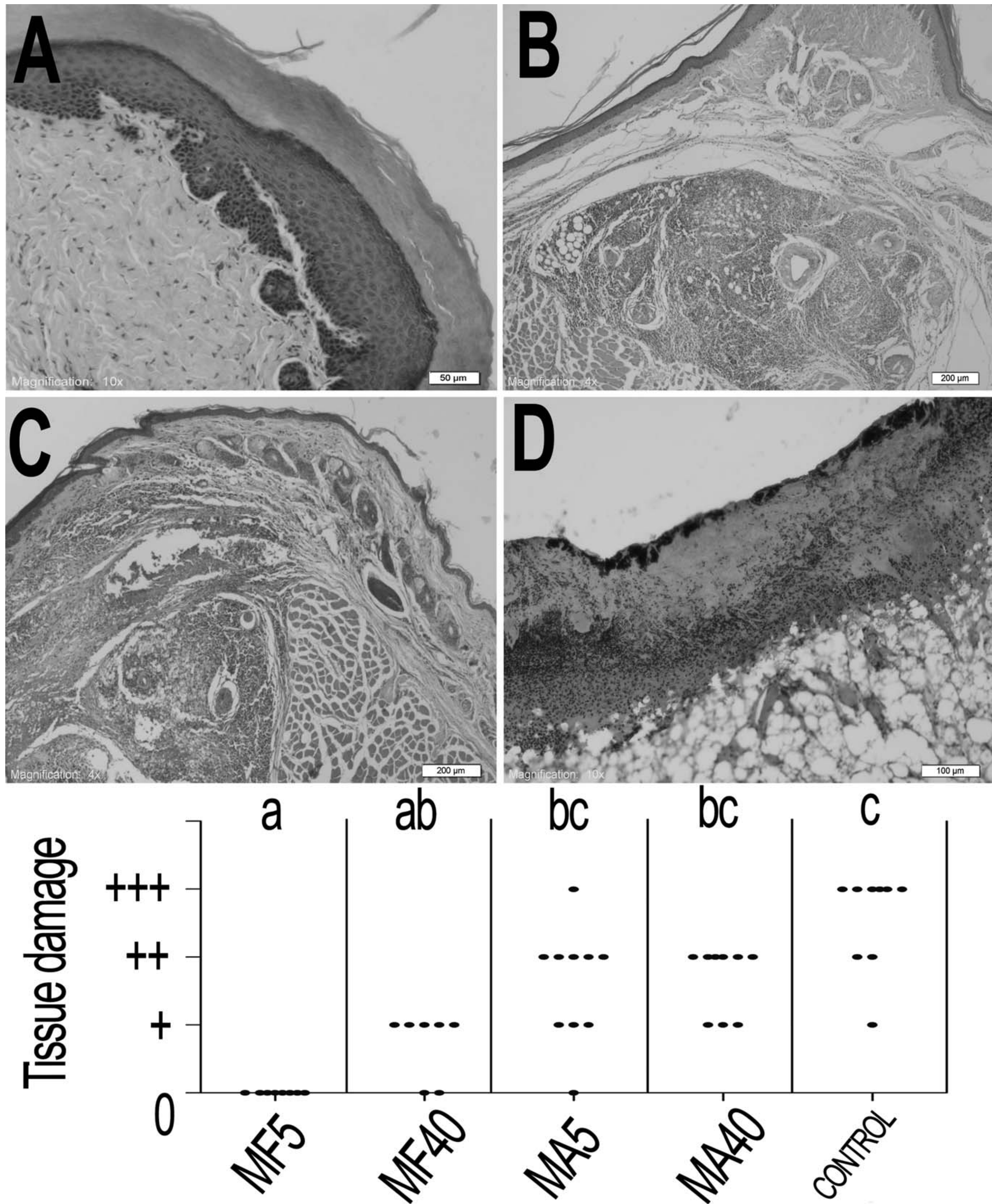

FIGURE 4. Right footpad (RFP) histopathology: quantification of tissue damage. (**A**) Normal skin (absent tissue damage). (**B**) Granuloma, located in deep dermis (slight tissue damage). (**C**) Granuloma in deep dermis, with necrotic tissue (moderate tissue damage). (**D**) Ulceration of epidermis (severe tissue damage). HE, X25. The graphic shows: (0) = absent tissue damage; (+) = slight tissue damage; (++) = moderate tissue damage; (+++) = severe tissue damage. See absence of tissue damage in the group with early miltefosine treatment (MF5). Different letters indicate significant differences ($P < 0.05$).

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TABLE I. Results of amastigotes present in the right footpad (RFP) sections (HE stained samples) of the different treatment groups. Values indicate the number of mice with microscopically visible amastigotes at the lesion site.

	Amastigote search in RFP*		
Groups	Positive (No.)	Negative (No.)	Total (No.)
MF5	0	8	8
MF40	3	4	7
MA5	9	1	10
MA40	9	0	9
Control	9	0	9
Total	30	13	43

* $\chi^2 = 25.45$; $P = 0.0001$.

detectable amastigotes corresponds to the MF5 group, followed by the MF40 group (71.43%). However, the difference was not statistically significant (χ^2 = 2.64; df = 1; P = 0.104). On the contrary, the animals treated with MA at 5 and 40 days, as well as those from the control group, exhibited parasites in their RFP; although statistics showed that the level of infestation across the different groups was similar, the number of mice with amastigotes was homogeneous (MA5 vs. MA40 χ^2 = 1.94; df = 3; P = 0.584). Finally, grouping the absolute frequencies of both MF groups and comparing them with all other groups, a significantly lower number of mice with detectable amastigotes was found in the MF groups (χ^2 = 23.43; df = 1; P = 0.001). The amount of amastigotes in tissues was assessed quantitatively (Table II). The parasite load was not homogeneous among groups (χ^2 = 37.99; df = 8; P = 0.0001). The animals from the MF5 group had no amastigotes, whereas the animals from the MA40 and placebo groups had the highest load. Statistics demonstrated that both MF groups displayed significantly lower amount of amastigotes as compared with both MA groups (χ^2 = 20.08; df = 2; P = 0.0001) and with the placebo group (χ^2 = 16.00; df = 2; P = 0.0003). Conversely, treatment groups MA5 and MA40 did not differ from the placebo group (χ^2 = 1.79; df = 2; P = 0.4095).

Microscopic inspection of liver and spleen preparations showed that all experimental animals, both control and treated groups, presented hepatocyte swelling, and also chronic inflammatory infiltrate foci around the portal tracts, composed of lymphocytes and eosinophils, and in some animals foci of necrosis. The analysis of spleen sections allowed us to detect lymphocytic reaction, although there were no differences between treatment groups in this regard.

TABLE II. Quantification of amastigote density in right footpad (RFP) sections (HE stained samples) of the different treatment groups. Scoring is as follows: Abundant amastigotes when 5 or more parasites per histiocyte were counted; scarce amastigotes when fewer than 5 parasites per histiocyte were counted; absence of amastigotes when are there no visible amastigotes. Values indicate the number of mice with abundant, scarce, or absent amastigotes.

	Amastigote density in RFP*			
Groups	Abundant (No.)	Scarce (No.)	Absent (No.)	Total (No.)
MF5	0	0	8	8
MF40	1	2	4	7
MA5	3	6	1	10
MA40	7	2	0	9
Control	7	2	0	9
Total	18	12	13	43

* $\chi^2 = 37.99$; $P = 0.0001$.

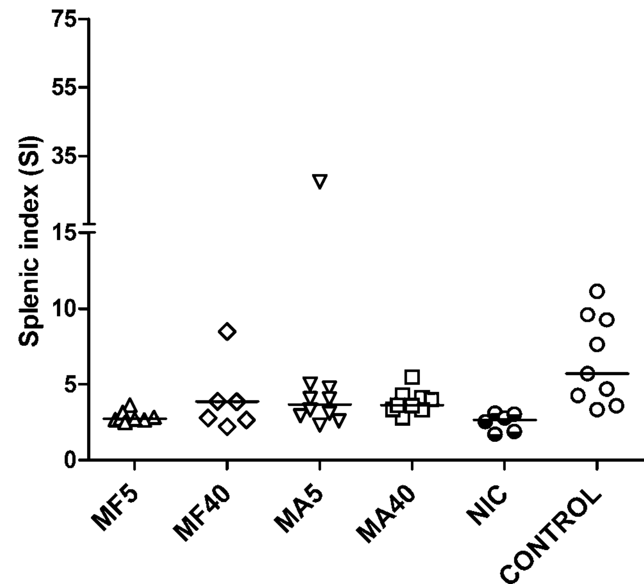

FIGURE 5. Splenic index (SI) values found in different treatment groups. NIC refers to non-infected mice. Note the absence of splenomegaly in the group with early treatment with miltefosine (MF5).

Splenic index

Splenic index (SI) data were compared among different treatment groups, including non-infected control mice (NIC) of the same sex and age (Fig. 5). Applying the Kruskal–Wallis test, significant differences (P = 0.0006) were found between the average SI (ASI) of different study groups. Dunn's test indicated that this difference was evident between MF5 (ASI = 2.9) plus NIC (ASI = 2.5), as compared with infected, non-treated controls (ASI = 6.6).

Spleen and liver imprints PCR

Chi-square homogeneity tests showed that the positive results were not homogeneous between groups (χ^2 = 10.14; df = 4; P = 0.0382). Fisher's exact test found that positivity was significantly higher in controls (n = 7/9) than in the groups treated with MF5 (n = 2/8; P = 0.0445) and MF40 (0/9; P = 0.0011). No significant differences were found between controls and MA5 (n = 4/10; P = 0.1149) and MA40 (n = 3/9; P = 0.0767) groups.

Right footpad PCR

The PCRs performed on inoculation sites (RFP) were positive for most of the mice in all treatment groups and the control group, except for 1 mouse treated with MF40 group.

Mortality

Death occurred during treatment administration in 2 mice from the MF5 group, 1 from the placebo group, 2 from the MF40 group, and in 1 from the MA40 group. The chi-square test for

homogeneity indicated that mortality was similar among all groups ($\chi^2 = 1.82$; $P = 0.61$).

DISCUSSION

In the present work, we investigated the efficacy of MF, as compared with the conventional treatment with MA, against *L.* (*L*) *amazonensis* in a murine model, using a series of clinical and laboratory parameters, such as the size and clinical evolution of RFP lesions, the level of anti-*Leishmania* antibodies, histopathology, determination of the SI and the presence of parasites in different tissues by PCR analysis.

In contrast with other studies (Velloso da Costa et al., 2008; Aguiar et al., 2010; Godinho et al., 2012), our model resembles the natural conditions of infection to a larger extent. Velloso da Costa et al. (2008) used C57BL/6 mice, inoculated with 3×10^6 promastigotes, whereas Aguiar et al. (2010) and Godinho et al. (2012) infected BALB/c mice with 10^6 amastigotes and 10^7 promastigotes, respectively. In this work we used Swiss outbred mice infected with a promastigote dose (2×10^4), which is similar to that occurring in natural infections (Rosas et al., 2005). Previous studies from our group (Falú et al., 2009) demonstrated that genetic susceptibility of these 3 mice strains to *L.* (*L*) *amazonensis* is different. C57BL/6 strain is highly resistant, BALB/c mice are highly susceptible, and Swiss mice present an intermediate susceptibility. Moreover, Swiss outbred mice display genetic variability, a desirable feature for animal models because human populations have this feature. Vectorial transmission of *Leishmania* occurs in nature with very low parasite numbers. In fact, under normal conditions the sandfly inoculate into mammalian hosts a small number of parasites (<1,000 metacyclic promastigotes) (Rosas et al., 2005). With respect to the size of the inoculum, the vast majority of studies performed so far were made with larger amounts of parasites to ensure the infection of the animals, but this strategy showed that there is not always correlation between the challenge and the degree of disease. For example, NBZ mice develop progressive lesions when inoculated with low numbers of parasites, but they are resistant to a high dose (10^7) of parasites of *Leishmania* (*Leishmania*) *mexicana* (Dorea et al., 1991). In our study, we determined that the minimum infective dose was 10^4; however, we decided to inoculate with 2 times this amount to ensure infection in all mice.

We also performed a preliminarily experiment with respect to the effectiveness and tolerance of Swiss mice to different doses of MF. The middle dose (16 mg/kg/day) was well tolerated and effective for the time tested, and it is consistent with that used by other authors (Velloso da Costa et al., 2008; Aguiar et al., 2010). The lower dose (8 mg/kg/day) was not satisfactory, as there was a relapse in the infection. These results agree with those of Godinho, who discloses that at doses $\leq$10 mg/kg/day there is an initial inhibition of injuries, with subsequent recurrence; but at doses $\geq$20 mg/kg/day, long-term evaluated animals showed no macroscopically visible lesions. However, the author presents the results of treatment with doses of 30, 40, and 50 mg/kg/day, describing that none of the animals died during treatment. This conflicts with the results of our work, because the maximum dose we used was effective (25 mg/kg/day), but there was high mortality (60%), which did not allow us the assessment of the results with this therapeutic regimen. This shows that the long-term effectiveness of miltefosine is dose-dependent, but high doses ($\geq$25 mg/kg/day) are very toxic. In fact, the recommended human MF oral dosage (OPS, 2013) is much lower than those described here (2.5 mg/kg/day).

In spite of the differences in the experimental conditions and in the mouse strains used, our results are consistent with those of other authors regarding the efficacy of MF against *L.* (*L*) *amazonensis*. They reported that MF is efficacious for the treatment of leishmaniasis but based only on clinical and parasitological criteria. Here, efficacy was evaluated and confirmed using 4 different parameters: clinical (lesion measurement), pathological (histopathology of primary inoculation sites and SI), parasitological (presence and amount of amastigotes in histological preparations from RFP, and PCR analysis in RFP, spleen, and liver), and immunological (antibody anti-*Leishmania* level).

MF has been shown to be clinically effective both administered at 5 and at 40 days pi. In delayed treatment, when the infection is well established and the lesions are macroscopically evident, MF induced complete regression of the swelling in RFP. The effect was sustained over time, since the mice were observed until day 139 pi. For early administration, meanwhile, when any injury is not visible, the drug shows complete inhibition of the clinical development of the disease.

The clinical response was correlated with the levels of anti-*Leishmania* antibodies at day 70 post-inoculation in early MF treated animals (Fig. 3), but there was no such correlation for late MF treated animals and MA-treated animals. The MOD values for animals in the MA40 group (with active cutaneous lesions) are indistinguishable from those of the MF5 (with no apparent lesions) and MA5 groups. This fact indicates that the humoral response was not necessarily correlated with the observed pathology, such as occurs in patients during the cutaneous manifestation of the disease. Probably, to detect a significant decrease of antibodies, the determination should be made of different subtypes of IgG (J. D. Marco et al., unpubl. data).

On the other hand, the histopathological findings at 139 days post-inoculation (Fig. 4) do correlate with lesion size, indicating that the cellular response is an appropriate indicator of parasite persistence. Moreover, the presence of intracellular amastigotes in the footpad lesions (assessed by microscopy, Tables I and II) correlated well with lesion size and histopathology.

Leishmania (*L.*) *amazonensis* may cause disseminated lesions and visceralization in susceptible animals (Abreu-Silva et al., 2004). As such, when testing the efficacy of a treatment in an experimental model, it is useful to consider those parameters that evaluate both pathology and parasitology in the spleen and liver. Splenic hypertrophy is a classical sign of the disease both in human and experimental kala-azar and has been attributed to both monocyte recruitment and polyclonal activation (Fechio et al., 1999). The splenic index measurement, however (Fig. 5), did not correlate very well with clinical and histopathology findings: only early treatment prevented the development of an inflammatory response in the spleen, since in this group we found a SI similar to uninfected mice. Conversely, PCR analysis of liver and spleen showed that the amount of PCR-positive mice was significantly lower in both groups treated with MF. The inclusion of this parameter in our study advocates in favor of the effectiveness of MF for the treatment of leishmaniasis.

Despite the greater efficacy of MF demonstrated by clinical and histopathological parameters, we detected parasites by PCR at the site of inoculation of all animals and in all treatment groups on

day 139 pi (except in 1 animal of MF40 group, in which antibodies or parasites in the spleen were not detected). This is not surprising, considering that even after treatment, in healing lesions with the presence of a scar for at least 6 mo, *Leishmania* parasites have been detected by PCR (Mendonça et al., 2004). Moreover, in this regard, the World Health Organization currently recognizes that none of the available treatments has proved to completely eradicate the infection and therefore recommends the use of therapeutic alternatives that have lower toxicity and greater acceptance by patients (PAHO, 2013). However, in the present work, the detection of parasite DNA in most of the treatment groups also suggests the need for combination therapy.

The cutaneous lesions produced by *L.* (*L.*) *amazonensis* in humans are generally more severe than those caused by other species (Silveira et al., 2004). Healing time has been shown to be longer than that described for lesions caused by the *Viannia* subgenus, and this was attributed to particular antigens of the parasite that might inhibit the host's cellular immune responses (Silveira et al., 1998). Here we demonstrate the in vivo efficacy of MF upon well-developed lesions caused by *L.* (*L.*) *amazonensis*; this is particularly relevant because this species circulates in northern Argentina as well as in many other South-American countries. Of note, some authors have reported therapeutic failure with MF on DCL (Zerpa et al., 2007); this is probably due to deficiencies in protective host immunity, which are characteristic of this clinical presentation, including the absence of specific, cell-mediated immunity. In addition, we have found no reports on clinical trials with MF with other clinical presentations of *L.* (*L.*) *amazonensis* infection.

In summary and in agreement with other studies, this therapeutic trial confirms the efficacy of MF for the treatment of experimental leishmaniasis caused by *L.* (*L.*) *amazonensis* in Swiss mice. The encouraging results of this work justify the use assessment of this drug, alone and/or in combined therapy to reduce the therapeutic dose, in clinical trials in regions where infections with *L.* (*L.*) *amazonensis* are a devastating cause of morbidity. Moreover, we suggest that further preclinical studies with MF should be conducted with other *Leishmania* species.

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