14

Development of Topical Miltefosine formulations for the treatment of Cutaneous Leishmaniasis

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Cutaneous leishmaniasis (CL) is a neglected tropical disease endemic in ~90 countries, with an increasing incidence. Presently available pharmacotherapy implies the systemic administration of moderately/very toxic drugs. Miltefosine (Milt) is the only FDA approved drug to treat CL via the oral route (Impavido®). It produces side effects; in particular, teratogenic effects are of concern. A topical treatment would have the great advantage of minimising the systemic circulation of the drug, preventing side effects. We prepared dispersions containing Milt and liposomes to modulate trans-epidermal penetration and evaluated *in vivo* efficacy. Treatments were topically administered to BALB/c mice infected with *Leishmania (Leishmania) amazonensis*. The dispersions containing 0.5 % Milt eliminated 99 % of the parasites and cured the lesions with a complete re-epithelisation, no visible scar and re-growth of hair. Fluid liposomes decreased the time to heal the lesion and the time needed to eliminate viable amastigotes from the lesion site. Relapse of the infection was not found one month after treatment in any case. A topical Milt formulation including fluid liposomes seems a promising treatment against CL.

■ INTRODUCTION

Leishmaniasis is an orphan tropical disease caused by *Leishmania* parasites; transmitted by hematophagous vectors of the genus *Lutzomyia* in the New World and *Phlebotomus* in the Old World. The progress of leishmaniasis depends on the parasite species and the immune state of the host. Cutaneous leishmaniasis (CL) is the most prevalent form in all the endemic countries and its incidence is increasing worldwide, with 0.7-1.2 million cases per year (ALVAR *et al.*, 2012). Formerly a disease typical of rural, isolated areas, CL is appearing more frequently in periurban areas (PIZZI *et al.*, 2015). There are more than 20 species of *Leishmania*, all of them with different sensitivities to each pharmacological treatment. In the case of patients with a healthy immune system, CL resolves spontaneously after few weeks/months, leaving a scar. It can however complicate into the muco-cutaneous or even the visceral form, especially in immunocompromised patients (DESJEUX e ALVAR, 2003).

Present pharmacotherapy involves toxic drugs administrated intravenously, intramuscularly or orally. Systemic toxicity of the first line treatment with pentavalent antimonials makes it sometimes mandatory for the patient to be either hospitalized or frequently monitored for renal/hepatic function (DESJEUX, 2004), producing an important increase in treatment costs (BRITO *et al.*, 2019; CARDONA-ARIAS *et al.*, 2018; EID RODRÍGUEZ *et al.*, 2019) and often forcing the patient to move to a city with laboratory/hospital infrastructure. This produces extra costs in transportation, food and lodging to the patient and his family, while in the meantime he/she cannot work (EID *et al.*, 2019).

Available local treatments (intra-lesional injections with antimonials, cryotherapy, thermotherapy) require highly trained medical personnel, are painful and/or require special equipment (MINODIER e PAROLA, 2007). Of note, thermotherapy, intralesional injections and oral Miltefosine capsules are currently not available in Argentina, where the disease is endemic and affects at least 400 people per year (MINISTERIO DE SALUD Y DESARROLLO SOCIAL, 2018).

A topical formulation, by minimising or avoiding the systemic circulation of the drug (ADAMS e KASHUBA, 2012), would solve many of the above problems. It would not produce pain enhancing patient compliance; it would avoid gastrointestinal / renal / hepatic / neurological side effects thus allowing its use in regions where no hospital infrastructure is available; it could be used in children or even pregnant women; it could be self-administered by the patient. If it could also produce a good, rapid cicatrisation, it would minimize the probability of secondary bacterial infection (the most common complication of CL) and it would prevent the scar that usually remains in untreated lesions that heal spontaneously (HEPBURN, 2000). In addition, the production of a topical treatment would be simpler than the production of a parenteral treatment. This should lower production costs and therefore allow for a lower

final market price of the formulation. The development of topical formulations of different drugs to treat CL is presently a subject of very active research.

A topical formulation including amphotericinB 3 % (Anfoleish) has recently failed in a phase II clinical trial due to its low efficacy (LÓPEZ *et al.*, 2018). Topical paromomycin has been reported as effective against some species of *Leishmania*, but the information on effectivity against American species is contradictory and scarce. Besides, the treatment needs to be long, local reactions occur in all cases and the formulations are not commercially available (ARMIJOS *et al.*, 2004; FAGHIHI e TAVAKOLI-KIA, 2003).

Hernandez and co-authors (PAOLA HERNANDEZ *et al.*, 2014) have reported the *in vitro* activity of Miltefosine (Milt) incorporated in liposomes being more active against *L. (V.) braziliensis* than *L. (V.) panamensis.* Momeni and co-authors (MOMENI *et al.*, 2013) reported a series of liposomal formulations with different drugs against cutaneous leishmaniasis induced by *L. (L.) major in vivo*. The formulations, applied as injections in the sites of the lesions, gave the best results when they contained Milt, although none produced a 100 % cure or complete elimination of parasites in the lesions.

Miltefosine is an alkyl-phosphoryl-choline with a high solubility in water (>2.5 mg/ml). It was originally developed as an anti-cancer drug and later found to be effective against various forms of *Leishmania*, including *L. (L.) amazonensis* (AYRES *et al.*, 2008; MARINHO *et al.*, 2011; VARELA-M *et al.*, 2012). Milt was first approved for use in human visceral leishmaniasis in India in 2002. In 2014, the FDA approved Milt oral capsules to treat human visceral, cutaneous and muco-cutaneous leishmaniasis in patients older than 12 years of age (SUNYOTO *et al.*, 2018).

Miltefosine still produces side effects (mainly gastrointestinal) and the record of teratogenicity in pre-clinical trials limits its use in children and fertile/pregnant women (SCHLOSSBERG e SAMUEL, 2011). Importantly, children comprise between 7 and 70 % of all CL patients, depending on the region (AL-TAWFIQ e ABUKHAMSIN, 2004; OLIVEIRA *et al.*, 2004). Fertile women must use a contraception that should not be orally administered due to the frequent vomiting and diarrhoea produced by oral Miltefosine, further complicating treatment. *In vivo,* a commercial form of Milt (Miltenox®) administered orally showed efficacy against *L. (L.) amazonensis* but damage in the liver and spleen was found (GARCÍA BUSTOS *et al.*, 2014).

A commercial topical formulation (Miltex®) contains Milt at 6 % and is used in breast cancer lesions. Schmidt-Ott and collaborators topically tested this formulation in mice infected with *L. (L.) major* and *L. (L.) mexicana* and found an important reduction in parasite burden against *L. (L.) major* but relapse in animals infected with *L. (L.) mexicana* (SCHMIDT-OTT *et al.*, 1999). On the other hand, Van Bocxlaer and collaborators topically applied formulations of Milt 6 % in different solvents (including water) to mice infected with *L. (L.) major* and found
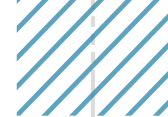
irritation and low anti-*Leishmania* activity (VAN BOCXLAER *et al.,* 2016). The information on the therapeutic value of topical formulations of Milt 6 % is therefore contradictory or negative.

Since during the development of CL the amastigotes are localized in the dermis below the ulcer, it is of the outmost importance that the active compounds of a topical formulation are able to penetrate the skin and reach the dermis. Since the literature indicates that Milt penetrates in very small amounts through the skin (VAN BOCXLAER *et al.,* 2016), the use of a penetration enhancer is probably necessary in order to increase the drug´s concentration near the parasites.

Liposomes in a topical formulation modulate the penetration of drugs through the skin (PERALTA *et al.,* 2018). Ultraflexible/fluid liposomes enhance the penetration of hydrophilic drugs of high molecular weight in healthy or damaged skin (CARNEIRO *et al.,* 2012). In open lesions, it has been shown that different active compounds have an enhanced ability to improve wound healing when incorporated in liposomes (CARNEIRO *et al.,* 2012; CHIANG *et al.,* 2007).

With this scenario, the goal of this work was to find a topical formulation containing Milt and liposomes that is able to give a good therapeutic result *in vivo*. We designed fluid liposomes with incorporated Milt. Topical *in vivo* efficacy was evaluated in an animal model of CL caused by *L. (L.) amazonensis* and was found to be extremely good.

■ METHODS

Ethics

Animal experimental protocols were approved by the Comité Institucional para el Cuidado y Uso de Animales de Laboratorio from Universidad Nacional de Salta (Res. CD N° 745-17). Procedures followed the Guide for the Care and Use of Laboratory Animals of NIH, and complied with the ARRIVE guidelines and with Argentinian Law 14346 on Animal Protection.

Preparation of liposomes

Fluid liposomes (FL) were prepared by the thin film method (CARRER e MAGGIO, 2001). Briefly, an appropriate amount of unsaturated phospholipids (Avanti Polar Lipids, Alabama, USA) was dissolved in methanol:chloroform (Emsure, Germany) 1:2 v/v. The solvent was evaporated with N_2 under constant rotation in a glass vial and then 2 hours of vacuum was applied to eliminate any residues of solvent. The resulting lipidic thin film was hydrated by adding HEPES buffer 30 mM, pH 7.4 at room temperature up to a final lipid concentration of 50 mg/ml. Multi-lamellar liposomal dispersions thus produced were extruded 17 times through 100 nm pore polycarbonate membranes using a 1 ml extruder (Avanti Polar Lipids,

Inc., Alabama, USA) at room temperature to obtain a homogeneous dispersion of 100 ± 30 nm uni-lamellar liposomes.

Preparation of Miltefosine and lipid dispersions

In order to facilitate topical application of the suspensions, a Carbomer 934-P NF (a carboxypolymethylene polymer) (Saporitti®, Bs. As., Argentina) hydrogel was prepared by hydration with Mili-Q water at a concentration of 1 % w/v and thoroughly mixed with either a Miltefosine (Milt) solution or both the Milt solution and the liposomal dispersions. The final Milt concentrations in the gels was 0.5 % w/v. For gels containing only Milt, the drug was dissolved in HEPES buffer 30 mM, pH 7.4 and this solution was mixed, at 1:1 volume ratio, with Carbomer 934-P NF 1 % w/v, pH 6 at room temperature. For gels containing FL, 5 % of the HEPES buffer used to prepare the gel was replaced by the liposomal dispersion. The formulations were stored at 4 °C and visually inspected for homogeneity and stability.

**_In vivo_ efficacy in a murine model of cutaneous leishmaniasis**

BALB/c mice were intradermally injected with $1\text{-}2 \times 10^7$ stationary phase _L. (L.) amazonensis_ (MHOM/BR73/M2269) promastigotes (50 μl) in the rump above the tail. Five weeks after infection, when the ulcer was visible, the treatments were started. Mice were randomly allocated in groups of five as: control group (without treatment), Milt group and Milt-FL group. The formulations (100 μl) containing 0.5 % of Milt were topically administered covering both the border and the centre of the ulcer, twice a day, 5 days a week, during 21 days. Mice were observed after the gel administration for approx. 30 minutes. By visual inspection, the gel was absorbed during this time and animals did not lick the wounds. At the end point of experiments, animals were euthanized with a ketamine/xilacine overdose. The efficacy of the treatments was assessed by the following means:

Lesion areas: Each lesion was measured weekly with a digital calliper during treatments. The area (mm^2) was estimated as: $(d_1/2) \times (d_2/2) \times \pi$, where d_1 is the larger diameter and d_2 is the smaller diameter.

Histology: Half of each lesion was immersed in 25 ml of 10 % formol buffer. After 48 h, the lesions were embedded in paraffin, cut transversally and dyed with haematoxylin eosin.

Parasitic burden: Skin tissue samples (ulcers or cicatrized ulcers) were aseptically removed and weighed. To obtain the amastigotes from tissue, we followed the protocol described by Lima (LIMA _et al.,_ 1997). Briefly, skin tissue samples were homogenized in 25 ml of sterile proline balanced salts solution (PBSS) containing 100 U/ml penicillin and 50 mg/L streptomycin (P-S); the amastigotes were separated through mechanical tissue disruption using a glass grinder. The homogenates were seated for $\geq$ 1 min on ice to allow chunk to

180
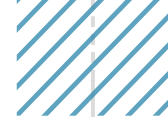
drop to the bottom of the tube. The concentration of parasites (amastigotes/ml) of each homogenate was determined by direct counting under a Zeiss optical microscope at 40X in a Neubauer chamber (PENICHE *et al.*, 2014). The total number of parasites in each homogenate was calculated as amastigotes/ml x 25 ml, then the results were adjusted per milligram of tissue (DIAS *et al.*, 2018). The percentage of parasite inhibition with regard to controls was calculated as (Average total number of amastigotes/mg of tissue in treated group) x 100 / (Average total number of amastigotes/mg of tissue in control group).

Amastigote viability: A sample of each homogenate (1 ml) was cultured in Difco blood agar (USMARU) medium containing 20 % of defibrinated rabbit blood plus P-S (MARCO *et al.*, 2005) since it is the most appropriate culture medium for parasite isolation. In order to determine if visceralization of infection occurred in infected mice, samples of spleens were also cultured in USMARU medium plus P-S. The cultures were examined for the presence of *Leishmania* promastigotes after one week. Negative cultures were examined during one month.

Leishmania-specific antibodys: IgG1 and IgG2a were measured by ELISA in serum (samples taken at the end of the treatment or one month later). Plates were sensitized with *L. (L.) amazonensis* antigen (1.75 mg/L), blocked with PBS-Tween + 5 % milk and treated with mouse serum samples (1/50). IgG1 and IgG2a (BD Pharmigen Biotin Rat Anti Mouse IgG1 and IgG2a, BD Biosciences) and Peroxidase Avidine were added in 1/3000 and 1/5500 dilutions respectively. The plate was revealed with 1 ml of 10X phosphate citrate buffer, 1 ml of TMB in DMSO, 8 ml of bi-distilled water and 10 μL of peroxide hydrogen. The reaction was stopped with H_2SO_4 (0.5 N) and read at 405 nm in a plate reader TECAN Infinite 200 PRO.

Hepatic enzymes: GOT (AST) and GPT (ALT) enzymes were determined by Aspartate Aminotransferase and Alanine Aminotransferase acc. to IFCC without pyridoxal phosphate activation according to the guidelines of the manufacturer. A Roche/Hitachi Cobas® c 701/702 analyser was used to quantify the results.

Statistical analysis: Statistical analyses were performed with a nonparametric Mann-Whitney test at different p values. The software GraphPad (v.6) was used. Results are informed as mean and standard error.

RESULTS

Both treatments (gels containing Milt and Milt-FL) decreased the lesion size compared with the control group (Figure 1A). Mice treated with Milt-FL and Milt showed a parasite inhibition of 99.7 / 99.8 %, respectively (Figure 1B). At day 21 of treatment, we observed clinical cure (ulcer closure) in all the animals; however, a small cicatrix remained in 7 of 10 ulcers treated with Milt and 4 of 10 in lesions treated with Milt-FL. Also, more hair had grown in the

lesion site of animals treated with Milt-FL than in animals treated with Milt (see photographs in Figure 1).

Figure 1. *In vivo* efficacy of 0.5 % Milt hydrogels. **(A)** Lesion area as a function of time. **(B)** *Leishmania* amastigotes count reduction in lesion regions at the end of treatment. Symbols +/- indicates de viability of the remaining parasites. Photographs of the lesions are representative of each group at the end of treatment. N = 5. **p < 0.004.
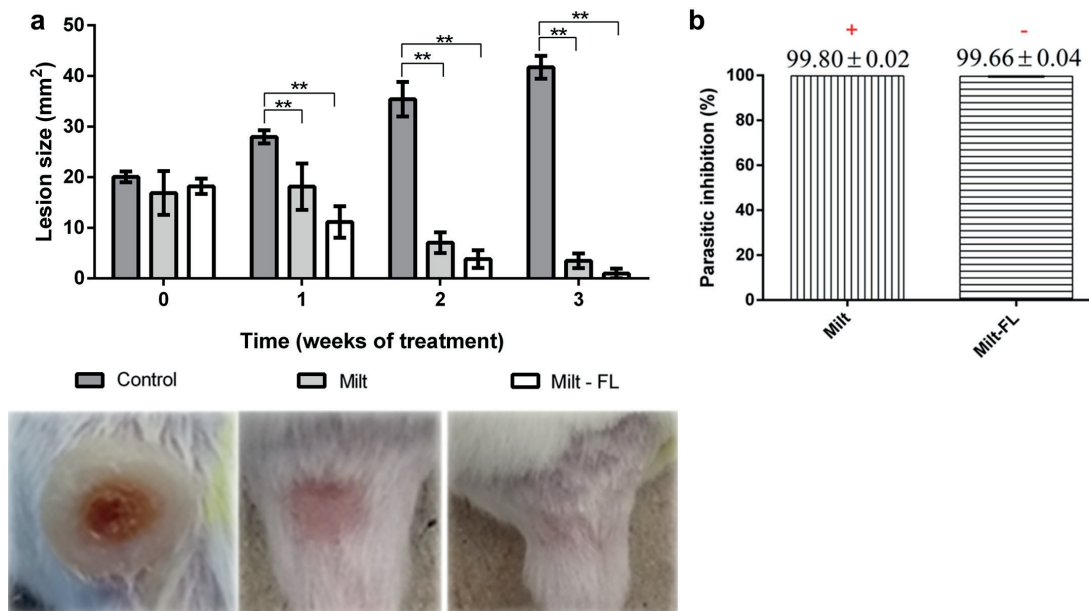
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Notice the high therapeutic efficacy of Milt at 0.5 %. The lesion practically disappears (Figure 1A, light grey bars), and the parasitic inhibition is very high (99.8 %) (Figure 1B). In these mice however, the culture of the tissue in USMARU medium was positive, indicating that immediately after the end of treatment, the little number of parasites left in the skin were still viable (able to transform into promastigotes). The addition of fluid liposomes increased the efficacy of Milt, to the point that no viable parasites were found in the lesion region immediately after the end of treatment (culture in USMARU was negative, Figure 1B). The statistical comparison of lesion sizes with the control group shows highly significant differences (p < 0.004) for Milt and Milt-FL at every tested point. Also, at every point during treatment, lesions were smaller in the Milt-FL group compared to the Milt group, although the differences are not statistically significant (p < 0.016).

To study reactivation (the appearance of a lesion within or at the border of a previously healed lesion (ARANA *et al.*, 2001)), two groups of animals, one treated with Milt and one with Milt-FL, were kept alive for one month after finishing the treatment. We found parasites in the healed skin of mice from the Milt group but they were not able to transform into promastigotes (Figure 2A). No parasites were observed in skin samples of the Milt-FL group. In addition, no visceralization was observed in any of these animals since spleen cultures were negative.

Figure 2. *In vivo* efficacy of 0.5 % Milt hydrogels one month after finishing treatment. **(A)** Number of parasites per lesion. Symbols +/- indicates de viability of the remaining parasites. **(B)** Representative photographs of the lesion site one month after finishing treatment. Notice complete re-epithelization, lack of scarring and re-growth of hair.
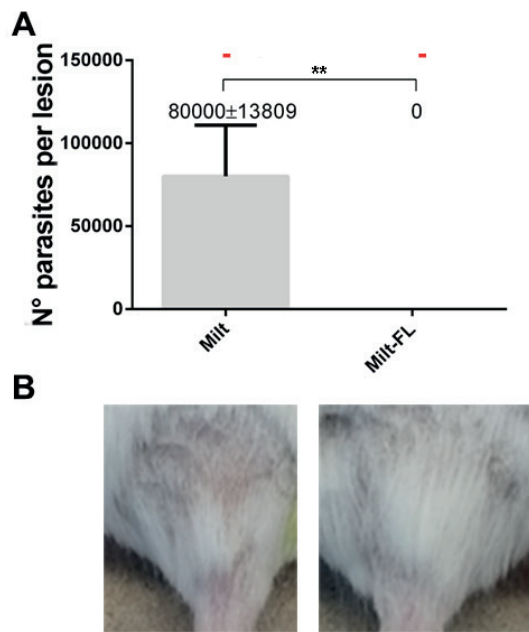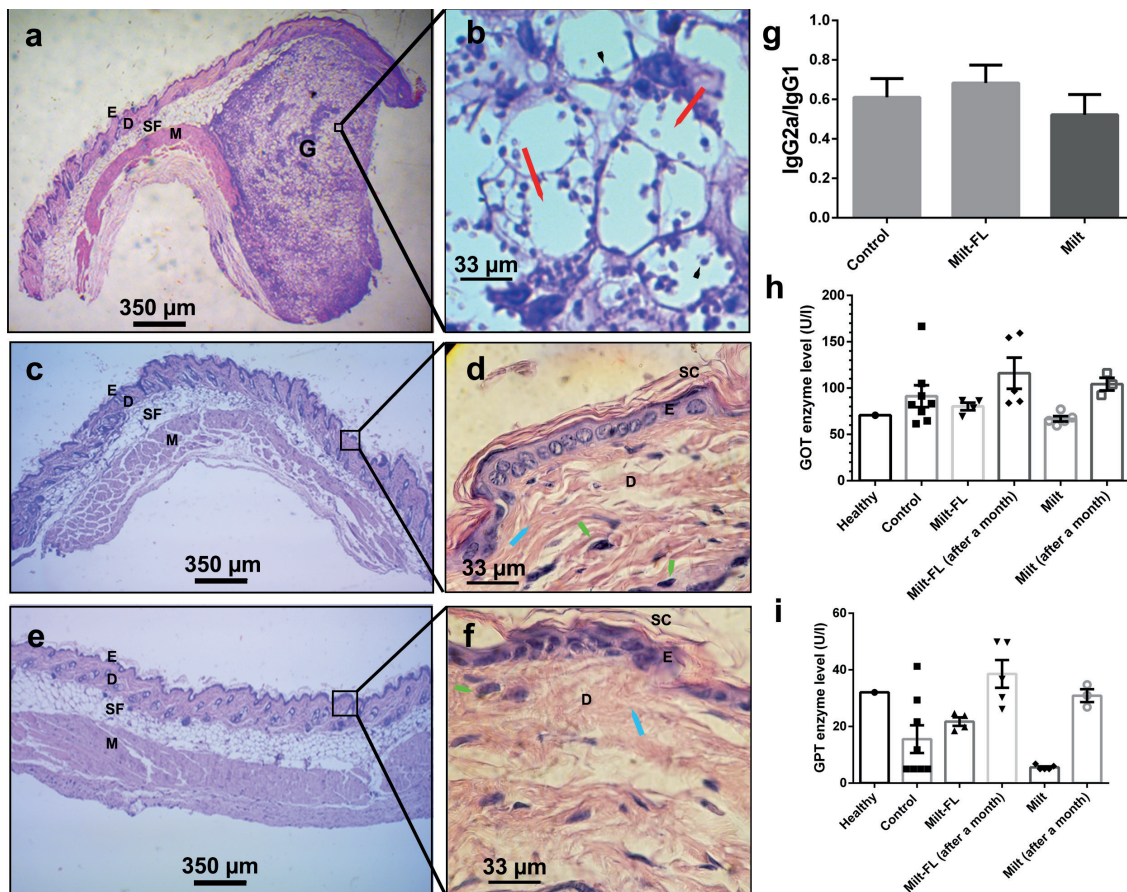

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One month after end of treatment all the animals recovered the hair (Figure 2B) and had a normal-looking skin at the site of the lesion. It is also notable that mice treated with Milt-FL recovered the hair faster, since the length of hair at the lesion site was greater than in the mice treated with Milt. Figure 3 shows histological cuts of the lesion sites of an untreated control animal and of animals treated with Milt 0.5 % with or without liposomes. Figure 3A shows how, in an untreated animal, the border of the ulcer is thickened and the tissue below the skin is filled with a granuloma formed by infected macrophages. The great amount of amastigotes present can be observed in Figure 3B (black arrows). Lesions treated with Milt and Milt-FL showed complete re-epithelisation, without remaining infiltration. The epidermis looks normal and no evident fibrosis can be observed (Figure 3C-F).

Figure 3. (A-F) Histology of mouse skin one month after the end of treatment. (A) lesion of a control animal (without treatment); (C) skin taken from the lesion site of an animal treated with Milt, (E) skin taken from the lesion site of an animal treated with fluid liposomes + Milt. (B) is a magnification of (A), where parasites (black arrows) can be seen inside parasitophorous vacuoles (red arrows). (D) and (F) are magnifications of (C) and (E), respectively, depicting the absence of parasites or signs of inflammatory infiltration. Green arrows point to nucleus of dermal cells, light blue arrows point to the extracellular matrix of the dermis. G = granuloma. E: epidermis, D: dermis, SF: subcutaneous fat, M: muscle, SC: stratum corneum. **(G)** Serum immunoglobulin levels. Measurements made one month after the end of treatment. **(H)** GOT; **(I)** GPT hepatic enzymes measured immediately at the end of treatment and one month later. N = 5.

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Serum immunoglobulins were measured one month after the end of treatment with Milt to see the response of the immunological system to the infection. IgG1 is associated to a Th2 response and with the progression of the disease. On the other hand, IgG2a is associated to a Th1 response and is related to the cure and the elimination of the parasites (CAMPOS, 2015; ROSTAMIAN *et al.*, 2017). The ratio IgG2a/IgG1 considers both responses: a higher value indicates a tendency to cure. One month after the end of treatment, the animals show IgG2a/IgG1 ratios that are not significantly different to control (untreated) animals, although animals treated with Milt plus liposomes have a slightly higher ratio than animals treated only with Milt (Figure 3G).

To check for possible damage to the liver caused by the treatments, hepatic enzymes GOT and GPT were measured (Figure 3H and I). The control group showed normal values for BALB/c mice: 100 U/l for GOT and 30 U/l for GPT (OGASAWARA *et al.*, 1993; SUCKOW *et al.*, 2001). Serum from animals treated with Milt or Milt-FL contained similar enzyme values.

■ DISCUSSION

We studied the *in vivo* efficacy of Milt with and without liposomes at a Milt concentration of 0.5 % w/v. In order to facilitate its topical application, we included the dispersions in a hydrogel. We found that the formulations were extremely efficacious.

Notably, the gel containing only Milt (without any lipid) produced the apparent clinical cure of the animals after a three-week treatment. While this group of animals still had viable parasites in the skin immediately after the end of treatment, a month later the animals were entirely cured, with no scar, regrowth of hair, and no (viable) parasites left at the lesion site. The histology showed an extraordinarily good skin healing, with no apparent fibrosis and no residual granuloma/inflammation.

The addition of fluid liposomes make the treatment equally efficient but faster. Already at day 8 of treatment, there is a difference in lesion size compared to animals treated with pure Milt, although the difference is not statistically significant. Furthermore, immediately after the end of treatment, no viable parasites can be recovered from the lesions, while this result can only be found with pure Milt a month after finishing the treatment. Animals treated with Milt + liposomes also show macroscopical differences in healing speed. While both with and without liposomes the animals show complete re-epithelisation, immediately after the end of treatment those treated with liposomes show no scars at all and regrowth of hair, while animals treated with pure Milt still show small scars, that will take one more month to heal and become invisible due to hair regrowth.

Regarding systemic infection and toxicity, we did not find parasites in the spleen of treated animals. Also, hepatic enzymes were not elevated, which indicates there is no injury in the liver of the animals.

Milt has been shown to act both on the parasite and on the immune system of the host: in the parasite, it interferes in the synthesis of lipids (RAKOTOMANGA *et al.*, 2007), impairs the metabolism of arginine (CANUTO *et al.*, 2014) and produces cell death by oxidative stress (MISHRA e SINGH, 2013). In the host, it has been shown to have an immunomodulatory effect involving Th1 response, especially in animal models of visceral and diffuse cutaneous leishmaniasis (PALIĆ *et al.*, 2019). In our experiments, Milt-FL did not produce a significant Th1 response, although IgG2a/IgG1 levels tend to be higher than in the control cases. Animals treated only with Milt show IgG2a/IgG1 levels lower than the control animals, although again the difference is not statistically significant. This lack of clear effect of Milt may be due to our model. The Th1/Th2 balance is more complex in localised CL models than in visceral models, with high levels of Th2 cytokines present in healing localised lesions (PALIĆ *et al.*, 2019). A future *in vitro* study of Th1 cytokines production like IFN-□ and IL-12 by infected macrophages should help to better understand the immunomodulatory response of Milt-FL.

Van Bocxlaer *et al.* reported that topical formulations of Milt at 6 % induced skin irritation (VAN BOCXLAER *et al.*, 2016). This fact could be related with the concentration used. In that sense, it is important to mention that in our experiments we did not observe signs of irritation in the animals, neither in Milt nor in Milt-FL groups.

Simultaneously with our work and corroborating our results, two very recent reports have been published on the activity of topical Milt formulations on CL induced by other species of *Leishmania* (KAVIAN *et al.*, 2019; NEIRA *et al.*, 2019). Neira *et al.* describe the efficacy of a Carbopol gel containing Milt in BALB/c mice infected with *L. (V.) braziliensis* and *L. (V.) panamensis*. These authors used 10 % DMSO as a penetration enhancer. This is a rather high amount of DMSO, raising concern for irritation/toxicity. Kavian *et al.* report the effect of topical formulations containing Milt on BALB/c mice subcutaneously infected with *L. (L.) major* (KAVIAN *et al.*, 2019). These formulations contained PC and cholesterol liposomes (our liposomes contained no cholesterol) and were dissolved in buffer (a low-viscosity medium). The formulation containing 0.5 % Milt failed to completely inhibit parasite burden, and only the formulations containing 2 and 4 % Milt produced good parasite inhibition. No mention is made to the possible irritation produced by the higher Milt concentrations. The presence of cholesterol induces an increase in microviscosity and bending rigidity in phospholipid membranes, inducing a liquid-ordered state in the membrane (SONG e WAUGH, 1993). It is possible that the presence of cholesterol acts against the trans-epidermal penetration of Milt. This would explain the need for higher concentrations of Milt to obtain a good therapeutic effect.

In the gel containing liposomes, most of Milt molecules will be partitioned into the liposomes membranes. There is a certain agreement in the literature that, when in contact with skin, liposomes can only penetrate intact the first few microns of the Stratum Corneum (SC), thereafter breaking down, and the molecular components diffusing into the skin by themselves, rather than penetrating the skin intact (DREIER *et al.*, 2016; PERALTA *et al.*, 2018; ROBERTS *et al.*, 2017). In our view, it is not the presence of liposomes as intact carriers to the dermis what aides the efficacy of Miltefosine, but rather the effect of the particular phospholipid molecules used to produce the liposomes: unsaturated phospholipids make skin lipids more fluid, therefore diminishing the barrier function of the skin and acting as a penetration enhancer (WILLIAMS e BARRY, 2004).

However, a more nuanced effect may be at play here. As shown before (PERALTA *et al.*, 2018), liposomes can both increase a drug´s penetration into the skin and make the penetration slower. We speculate that in the present case, liposomes accumulate on the first few microns of the SC, acting as a depot of material slowly releasing Milt and phospholipids. The lipids would aid the penetration of free Milt molecules by fluidizing the SC membranes. At the same time, phospholipids could have an effect by themselves on the parasite's survival.

■ CONCLUSION

The present work demonstrates that a topical treatment with pure Milt at a concentration of 0.5 % w/v is highly effective against cutaneous leishmaniasis induced by *L. (L.) amazonensis in vivo,* and that the addition of fluid liposomes enhances the anti-parasitic effect of Milt, accelerating the clinical cure and the complete elimination of parasites. The combination of the use of a gel to enhance viscosity and of fluid liposomes as penetration enhancers/ release modulators seems promising, since it avoids the risk of losing the formulation applied on the skin due to low viscosity and also avoids the need to use a harsh penetration enhancer such as DMSO.

Another important issue is whether this formulation will work in animals with a skin thicker than that of mice, such as humans. Human skin is approximately 4 times thicker than mouse skin. It is therefore of the outmost importance to try the formulation in other mammals with thicker skin in order to evaluate the need of an adjustment of Milt or liposomes concentration, the length of treatment, etc.

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187

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