A double-bind and randomized trial to evaluate Miltefosine and topical GM-CSF in the treatment of cutaneous leishmaniasis caused by *Leishmania braziliensis* in Brazil

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Summary:

This is a randomized and double-blind trial to evaluate the efficacy of oral Miltefosine combined to topical GM-CSF compared to monotherapy with Miltefosine and standard pentavalent antimony in the treatment of cutaneous leishmaniasis caused by *L. braziliensis* in Brazil.

ABSTRACT

Background:

The treatment of cutaneous leishmaniasis (CL) in Brazil by pentavalent antimony (Sb^v) is associated with a high rate of failure. Oral miltefosine in monotherapy has proven high efficacy in CL caused by *L. braziliensis* with a cure rate of 75%. A combined treatment with GM-CSF and miltefosine was tested to increase the cure rate and decrease the healing time.

Methods: This is a randomized and double-blind clinical trial to evaluate the efficacy of miltefosine combined with topical GM-CSF (M+GM) versus miltefosine and placebo (M+P) versus standard Sb^v in the treatment of 133 patients with CL caused by *L. braziliensis* in Bahia, Brazil.

Results: The final cure rate at 180 days after the initiation of treatment was 44.4% in the Sb^v group, 76.6% in the M+P group (P= 0.003 versus Sb^v), and 75.6% in the M+GM group (P= 0.004 versus Sb^v). By survival curve analysis median healing time for cure was 102 for Sb^v group and 60 days for both miltefosine groups (P= 0.0009). During the 6 months follow-up period, four relapses were documented, one in the Sb^v group (2%), one in the M+P group (2%) and two in the M+GM group (5%). Adverse events were documented in 65% of subjects from Sb^v group, 79% of M+GM group and 76% of M+P group.

Conclusions: Miltefosine is more effective than standard Sb^v for the treatment of CL caused by *L. braziliensis* in Brazil and accelerate the healing time of CL. The association of Miltefosine with GM-CSF do not improve therapeutic outcome.

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Keywords: 1. Cutaneous Leishmaniasis. 2. Miltefosine 3. GM-CSF 4. Pentavalent antimony 5. *Leishmania (V.) braziliensis*

INTRODUCTION

Cutaneous leishmaniasis (CL) in Brazil is an endemic, neglected and difficult to treat disease. The most frequent CL causal agent in the country, *Leishmania V. braziliensis* is associated to localized ulcerated lesions, but may cause severe disease like mucosal leishmaniasis (ML), disseminated leishmaniasis (DL) and atypical forms [1-4]. The CL burden in the public health is poorly investigated but certainly heavy, due to costs associated to daily transportation to use injectable medication for at least 20 days, absenteeism from work, long time to heal and cure, physical deformities with social and psychological impact [5]. The standard CL treatment for more than five decades in Brazil remains pentavalent antimony (Sb^v), a parenteral and toxic medication contraindicated in subjects older than 50 years-old and also in patients with heart disease [6,7]. Furthermore, the frequency of therapeutic failure in up to 50% of CL in *L. braziliensis* endemic regions to Sb^v is another important challenge [8,9]. Alternative treatment are also parenteral and toxic drugs like pentamidine and amphotericin B [10,11]. Therefore, Miltefosine by oral route should be a logical alternative to the substitution of Sb^v, due to its easier and domiciliary use, less toxicity, and previous data showing its effectiveness in CL caused by *L. braziliensis* and *L. guyanensis* in Brazil [12,13]. Although miltefosine is superior to Sb^v with a cure rate of 75% in CL caused by *L. braziliensis*, there is a clear need to increase its cure rate, accelerate the healing time, and lower side effects like vomiting and nausea. Previous data shows that topical granulocyte macrophage colony-stimulating factor (GM-CSF) associated to Sb^v therapy increases cure rate and accelerates healing time of CL [14,15]. Besides these advantages, combined therapy if successful, could prevent resistance development and provide a lower daily dosage of miltefosine with less costs and side effects. Therefore this randomized, placebo controlled and double-blind clinical trial aimed to compare the efficacy of three arms of treatment in CL: standard Sb^v, miltefosine combined with topical GM-CSF, and miltefosine combined with topical placebo, in a highly endemic region of CL caused by

L. braziliensis. Moreover we evaluate if miltefosine alone or associated with GM-CSF was able to decrease the healing time of CL.

PATIENTS AND METHODS

Endemic area and case definition of CL

The patients were recruited at the Health Clinic of Corte de Pedra (85% of cases) and Health Post of Jequiriçá (15%), in Bahia, northeast Brazil, an endemic area of *L. braziliensis* infection. The diagnosis of CL was performed by the presence of one or more ulcerative lesion(s) at a skin site, with laboratory confirmation by detection of DNA of *L. braziliensis*, by PCR or by histopathology showing amastigotes in the tissue.

Patient selection

Inclusion criteria were age between 18 and 65 years, one to 3 ulcers in any location of the body, lesion size between 10 and 50 mm in a single dimension and a period between 30 to 90 days from the onset of the skin lesion.

Patients with previous CL treatment, patients with evidence of ML, DL or CL, pregnant or breastfeeding mothers, and patients with HIV or any chronic disease were not included.

Sample size, randomization and group assignment

The total sample size of 120 patients was obtained considering a variation of 30% of the cure rate in the control group compared to the interventions groups, with an alpha of 0.05 (two-tailed) and 85% of power.

Randomization was done according to a computer list obtained in www.randomization.com and allocated at a rate of 1:1 into 3 groups: Sb^{v} (control), miltefosine combined with topical GM-CSF (M+GM), miltefosine and placebo (M+P). Two blinded clinicians to the assignment group determined the physical examination and therapeutic outcome. Due to the

impossibility of blind subjects that were in the control group and used parenteral Sb^{v}, patients and clinicians were instructed to do not exchange any information regarding the treatment.

Histopathology and PCR

All patients were biopsied in the edge of the ulcer, and two skin fragments were obtained for histopathological analysis and PCR. DNA isolation, purification and amplification were performed as described elsewhere [12]. Detection of the subgenus Viannia applied the primers GGGGTTGGTGTAATATAGTGG and CTAATTGTGCACG. The *Leishmania* specific band consists of 120 base pairs, and that for Viannia of 750 base pairs.

Drug Administration

Miltefosine (Impavido™, Paesel + Lorel GmbH & Co) was administered in capsules containing 50 mg at a dosage of 2.5 mg/kg of body weight (maximum daily dose of 150mg) for 28 consecutive days. Daily dose was divided in two or three intakes, given always with meals. Patients were asked to return the blisters for verification of regular use and adherence. The control group was treated by intravenous route with Sb^{v} (Glucantime™, Aventis) at a dosage of 20mg/kg/day for 20 days. Health care providers that registered dosage and date administered Sb^{v} in health posts near from patient's home.

Women in childbearing age were included only after a negative beta HCG test to exclude pregnancy and used a parenteral contraceptive during 3 months.

Topical GM-CSF 0.01% and placebo at gel creams were produced by 4G Company, Porto Alegre, Brazil. The rhGM-CSF was purified according to Schwanke et al 2009 [16]. Ointments were prepared on 1.5% (w/w) aqueous polycarbophil gel containing 10 µg/g of rhGM-CSF. Placebo was prepared on the same way, without the rhGM-CSF. Patients were oriented to cover the lesions with topical placebo or GM-CSF twice daily during the period of use of miltefosine (28 days).

Study Procedures

A complete hemogram, aminotransferases (AST, ALT), urea, creatinine and blood sugar levels were determined before and on day 15 of therapy. Patients were seen for follow-up every 2 weeks in the first month, every month up to day 90 and 6 months post-therapy. Subjects who did not return for follow-up were asked to come or were visited at home within 7 days of the missed appointment.

The ulcers were measured with a standardized caliper and photographed at the initial visit, and at each follow-up visit. Clinical and laboratory adverse events were graded according to the Common Terminology Criteria for Adverse Events (CTCAE) [17].

Clinical endpoints criteria

Primary end point: final cure at 180 days after initiation of therapy.

Secondary end-points: a) initial cure at 90 days after the initiation of therapy; b) healing time in days; c) clinical and laboratorial adverse events.

Cure was defined by complete re-epithelialization without raised borders, infiltrations or crusts of all lesions. Failure was defined the presence of active ulcer or a healed lesion but with raised borders.

All patients who failed at day 90 received Sb^{v} at 20 mg/kg/ day for 30 days or Amphotericin B (0.5 to 1mg/kg total dose).

Statistical Analysis

The data are presented as proportions, 95% confidence intervals (95% CI), means and standard-deviations (SD). The normally distributed variables were compared using the T test. The proportions were compared with the Unpaired t test,or Fisher´s exact test for categorical variables. Survival curve was analyzed by Logrank test for trend. The intention-to-treat (ITT) analysis was used to calculate the cure rates. All statistical analyses were performed with the software GraphPad Prism 7.02 for Windows. A value of $P < 0.05$ was considered statistical significant.

Ethical Aspects

Prior to enrollment in the study, a written informed consent was obtained from all patients. The study was approved by the Ethics Committee of the Medical School from Federal University of Bahia, Brazil (number 1.559.552).

RESULTS

We have included 133 CL patients from May 2016 and August 2019 and 127 (95.5%) had a positive PCR for *L. braziliensis*. All subjects with negative PCR, had the diagnosis confirmed by the presence of amastigotes in histopathology. Among the 133 patients the age ranged from 18 – 61 years (34 ± 12.1) with a predominance of male subjects (69%), with no difference between the 3 groups (table 1). A single ulcer was presented by 76% of patients, 2 ulcers by 18% and the remaining presented 3 lesions. The main lesion was considered the one with the biggest diameter and was located in the lower limbs in 73% of the cases. The demographic, clinical and PCR data of the three groups are shown in Table 1.

Efficacy

By intention-to-treat analysis, the initial cure rate at 90 days in the M+G group was 80.5%, compared to 78.7% in the group M+P and 46.6% the control group (Sb^v). The final cure rates at 6 months were 75.6% in the M+G group, 76.6% in the M+P group and 44.4% in the Sb^v) group. The healing time was longer in the Sb^v group (112 ± 69.7) days compared to M+P (72 ± 42.8) days and M+GM group (70 ± 37.8) days, P = .0013 and P = .001 respectively (Table 2).

At day 90 after initiation of therapy, 51% of the subjects of the Sb^v group remained with active ulcers, compared to 20% in the M+P group and 18% in the M+GM group (P = 0.0009 Logrank test for trend), Figure 1.

Relapses due to reactivation of the ulcers and/or infiltration of borders during the 6 month follow-up period were documented in 4 subjects: one from Sb^v group, one from M+P group and 2 from M+GM group. Six patients did not complete treatment due to irregular use or abandon. One of the patients was in the Sb^v group, 2 in the M+GM group and 3 in the M+GM group (Table 2).

Tolerability and toxicity

Adverse events (AE) were documented in the majority of subjects, irrespective of the group of treatment: 65% in Sb^v group versus 76% in M+P group versus 79% in the M+GM group. The AEs that were more frequent in the two miltefosine groups were nausea (43%), vomiting (29.5%), and headache (8%). In the Sb^v group, arthralgia and/or myalgia (31%), fever (17.7%) and headache (13.3%) were the more frequent. Two patients using miltefosine stopped treatment due to intense vomiting, despite the use of ondansetron 4mg (Vonau™, Biolab Sanus). One patient in the Sb^v group also discontinued treatment due to intense arthralgia and myalgia. The others patients in the three groups had mild and transient symptoms.

DISCUSSION

Treatment of CL remains a challenge yet today due to growing ineffectiveness, long time to heal and toxicity associated with the drugs available [5,6,7,9]. Those negatives outcomes may be associated to several factors. One of the most important is the use of an ancient, parenteral and toxic drug in monotherapy for decades, due to the paucity of studies to test new drugs and combination schemes in order to improve cure rates and accelerate the healing time. A successful combined therapy could also be tested with lower dosages of drugs and therefore provide less toxicity and lower costs.

Parenteral Sb^v remains the standard CL treatment in Brazil [6,12]. We have shown that the association of GM-CSF to Sb^v in CL was able not only to increase the cure rate but also shortened the healing time [14,15]. More recently, miltefosine treatment of CL showed superiority over Sb^v in disease caused by *L. braziliensis* and *L. guyanensis* in Brazil, due to easier administration by oral route and higher cure rates [12,13,18]. However, about 20% of patients remained with active disease and needed another treatment in order to be cured. Although evidences are growing indicating that miltefosine is a better therapeutic approach than Sb^v for CL and should be provided as first choice as standard treatment [18], some concerns related to its use should be kept in mind. Side effects like nausea and vomiting occurs in at least 40% of subjects and may interfere with adherence and regular use by auto administration [12,13]. Monotherapy after years of use may decrease its cure rate as already observed with Sb^v in CL, as well as with miltefosine in visceral leishmaniasis [19]. Therefore, an increase in miltefosine actual cure rate of about 80% in CL caused by *L. braziliensis* is highly desirable, due not only to the aggressiveness of this parasite, but to shorter the morbidity associated with long time to heal and additional treatment courses.

A decrease in the healing time of CL is necessary. The treatment with Sb^v and miltefosine last 20 and 28 days, respectively, but cure or failure is only determined up to 90 days of initiation of therapy. The rational to choose GM-CSF to evaluate its association with an anti-Leishmania agent in CL therapy was based on several evidences: GM-CSF increases

parasite killing by direct activation of macrophages [20,21,22], enhances tissue healing and scar formation as shown in chronic venous leg ulcers [23], and previous studies showing the efficacy of GM-CSF associated to antimony therapy in CL. For instance, intralesional GM-CSF associated to Sb^v achieved 70% of cure 40 days after therapy onset, compared with 10% in the placebo group (Sb^v and intralesional saline) and a final cure rate of 80% versus 50% respectively [14]. We also showed that topical GM-CSF diluted in saline plus Sb^v cured 100% of CL subjects compared with 50% of patients in the control group [15]. Moreover in five CL subjects refractory to Sb^v (failure to at least 2 previous courses), cure was achieved with topical GM-CSF combined with another course of Sb^v [24].

The data from this clinical trial did not show any benefit in the combination of topical GM-CSF with Miltefosine to increase the cure rate or in accelerate the healing time of CL. This may be due to several factors. The GM-CSF vehicle used may have had less tissue final concentration of GM-CSF than intralesional applications or dressings diluted in saline like reported before [14,15]; the incorrect use of topical GM-CSF by subjects; as miltefosine monotherapy achieves nearly 80% of cure, the benefit of its combination with GM-CSF to improve this cure rate may require higher concentrations of GM-CSF in the topical preparation. We choose the topical use by auto application due to its practical approach in an endemic rural area, where subjects have difficult to come back to the health post to change dressings three times a week during three weeks [15].

The results of this trial confirm the superiority of miltefosine against Sb^v in the treatment of CL caused by *L. braziliensis* in Brazil. Additionally, we showed that miltefosine accelerate the healing time of CL ulcers. We conclude that miltefosine should be implemented as the first therapeutic choice for CL and that efforts should be made to evaluate combined therapies in order to increase cure rates and shorter the healing time of CL, preventing morbidities and development of drug resistance.

NOTES

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Table 1. Demographic, Clinical and Laboratorial Features in 133 Subjects with Cutaneous Leishmaniasis Included in the Trial.

Characteristics	Sb^v (45)	Miltefosine + Placebo (47)	Miltefosine + GM-CSF (41)	P value
Age, Mean ± SD (years)	33 ± 13.1	36 ± 11.8	33 ± 12.1	ns*
Male/Female ratio	35/10	34/13	25/16	
Number of lesions, Mean ± SD	1.4 ± 0.65	1.3 ± 0.79	1.5 ± 0.71	ns*
Biggest ulcer diameter, Mean ± SD (mm^2)	22 ± 8.9	21 ± 7.8	18 ± 9.0	ns*
Positive PCR for *L. braziliensis*	43/45 (95%)	44/47 (94%)	40/41 (98%)	ns**

SD= standard deviation

* Unpaired t test

** Fisher´s exact test

Table 2. Therapeutic outcome of Cutaneous Leishmaniasis patients in the three arms of the trial: Sb^v, M+P, M+GM.

	Sb^v (45)	M+P (47)	M+GM (41)	P value
Initial cure rate (90 days) (%)	21/45 (46.6)	37/47 (78.7)	33/41 (80.5)	.0023*; .0017#
Final cure rate (6 months) (%)	20/45 (44.4)	36/47 (76.6)	31/41 (75.6)	.004**
Healing time (days) range (mean ± SD)	17–313 (112.3 ± 69.7)	15–198 (72.1 ± 42.8)	30–180 (70.1 ± 37.8)	.009##
Relapses (%)	1/21 (4.7)	1/37 (2.7)	2/33 (6)	ns***
Irregular use (%)	1/45 (2.2)	3/47 (6.4)	2/41 (4.9)	ns***

SD= standard deviation

* Unpaired t test Sb^v versus M+P; # Unpaired t test Sb^v versus M+GM

** Unpaired t test Sb^v versus M+P and Unpaired t test Sb^v versus M+GM

Logrank test for trend

*** Fisher´s exact test

Figure 1. Kaplan Meier curve comparing time to cure* in the three arms of Cutaneous Leishmaniasis treatment

* Days required for complete healing of the ulcers without any sign of clinical activity like inflammation or raised borders.

P= 0.0009; Log rank test for trend.

Figure 1