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A Controlled, Randomized-Blinded Clinical Trial to Assess the Efficacy of a Nitric Oxide Releasing Patch in the Treatment of Cutaneous Leishmaniasis by *Leishmania* (*V.*) *panamensis*

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Abstract. A topical nanofiber nitric oxide (NO) releasing patch (≈3.5 μmol NO/cm^2/day for 20 days, NOP) was compared with intramuscular meglumine antimoniate (Glucantime, 20 mg/kg/day for 20 days) for the treatment of cutaneous leishmaniasis (CL) caused by *Leishmania* (*V.*) *panamensis* in Santander and Tolima, Colombia. A double-blind, randomized, placebo-controlled, clinical trial was conducted to determine whether the NOP is as effective as Glucantime for the treatment of CL. Patients were randomly assigned to Glucantime and placebo patches or NOP and placebo of Glucantime. The cure rates after a 3-month follow-up were 94.8% for the group that received Glucantime compared with 37.1% in the NOP group. Despite the lower efficacy of the NOP versus Glucantime, a significantly lower frequency of non-serious adverse events and a reduced variation in serum markers were observed in patients treated with NOP. Treatment of CL with NOP resulted in a lower effectiveness compared with Glucantime; however, the low frequency of adverse events and the facility of topic administration justify the development of new generations of NOP systems for the treatment of CL.

INTRODUCTION

Cutaneous leishmaniasis (CL) is an endemic disease in several countries around the world.[1] It is estimated that 1.5 million people suffer from the disease annually and more than 350 million are at risk of infection.[2,3] In Latin America 62,000 new cases are reported every year, being endemic in 18 countries.[4] In the last two decades despite the prevention programs, the incidence of leishmaniasis in the Americas continues as a public health problem. Colombia ranks now second after Brazil with more than 30,000 and 15,000 reported cases per year, respectively.[5] Cutaneous leishmaniasis is caused by intracellular protozoan parasites of the genus *Leishmania*, which are transmitted by the bite of the female *Phlebotomus* (in Africa, Asia, and Europe) and *Lutzomyia* (in America) sandflies.[1,6] The typical lesions of this disease are ulcers that may heal spontaneously over a period of 3 months to a year depending on the strain of the parasite, leaving a flat, atrophic, and depigmented scar.[7,8] For more than 60 years the pentavalent antimony compounds sodium stibogluconate (Pentostan) and meglumine antimoniate (Glucantime) have been the treatments of choice for this entity.[9] Meglumine antimoniate treatment has shown a cure rate greater than 85% in Colombia.[10,11] However, this drug has been associated with several disadvantages such as the need for a parenteral route, discomfort, and the presence of various side effects (fatigue, vomiting, muscle and abdominal pain, cardiac abnormalities, increased hepatic aminotransferase, and pancreatitis).[12,13] Thus, new safe therapeutic options are needed.

Nitric oxide (NO) plays a key role in the elimination of *Leishmania* intracellular amastigotes.[14–19] The first topical treatment with NO used against CL in humans showed encouraging results and minimal side effects but NO release was in peaks, requiring multiple applications and hindering the adherence of the patients.[20,21] In this study, the purpose was to determine the efficacy of transdermal patches of continuous delivery of NO (NOP), produced by the technique of electrospinning,[22–24] in comparison to meglumine antimoniate for treatment of CL.

MATERIALS AND METHODS

Study design and protocol. This study was a double-blind, randomized clinical trial comparing NOP versus meglumine antimoniate (Glucantime) in the treatment of CL caused by *Leishmania panamensis.* Study patients were admitted to the local hospitals in Santander (El Carmen de Chucuri, San Vicente de Chucuri, Rionegro, El Playon, Lebrija, Cimitarra, and Landazuri), and Tolima (Rovira, Ortega, and Rio Blanco), Colombia, during the period March 2007 and August 2008. Patients who fulfilled the following criteria were enrolled: males and females ≥ 10 years of age; a parasitological diagnosis of CL with demonstration of *Leishmania* amastigotes on smears or promastigotes in culture. The exclusion criteria included any history of anti-*Leishmania* therapy in the last 3 months, presence of > 5 lesions, or presence of lesions in the perimeter (< 2 cms) of mucosal areas, eyes, nose, mouth, or genitals. One hundred seventy-eight (178) patients with confirmed CL were randomly allocated to one of the two study arms: One group received meglumine antimoniate (Glucantime) 20 mg/kg/day plus a placebo patch for 20 days. The other group received intramuscular placebo (5–20 cc/day), and an active NOP for 20 days and were monitored closely by trained medical doctors during the course of the study. A randomization list was prepared using a computer program. The randomization process was blinded and centralized: once eligibility of a patient was established, the investigators informed the study headquarters. The assigned code was reported to the monitoring nurse who had no contact with the participants. Informed consent was obtained from all patients or parents of minors before enrollment. This study was approved by the institutional review board at the Cardiovascular Foundation of Colombia, and the Health Departments of Santander and Tolima localities.

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Production of the electrospun nitric oxide releasing patches. To ensure a constant release of the NO and to stabilize the main components of the reaction, in this study we used a nanofiber polymer produced by the electrospinning technique that provides non-wovens to the order of a few nanometers with large surface areas, ease of fictionalization for various purposes, and superior mechanical properties.[22–26]

The NO was generated using acidified nitrite with ascorbic acid (ASC); however, to ensure a controlled release of NO, ASC and nitrite were encapsulated in Tecophilic polymer (TP) (Noveon, Cleveland, OH) nanofibers produced using the electrospinning technique. The ASC (pKa_1 ≈4.6) was stabilized using microcrystalline cellulose (MCC). Potassium nitrite was bound to the ion-exchange resin DOWEX 1–4 × 400 (Sigma-Aldrich Chemical Company, Milwaukee, WI) before encapsulation. A four-layer device was constructed by electrospinning, as shown in Figure 1. All the solutions prepared were spun at voltages ranging from 20 to 30 kV. The first layer was composed of TP solution + Waterlock superabsorbent (Grain Processing Corporation, Muscatine, IA) + ethanol (EtOH) (66:10:24 [w/w]). The second layer was prepared by mixing TP solution + ASC/MCC + EtOH (50:14:36 [w/w]). The third layer was achieved as the first layer by mixing TP solution + Waterlock superabsorbent + EtOH (66:10:24 [w/w]). Finally, the fourth layer (facing the wound) was prepared by mixing TP solution + DOWEX and EtOH (69:6:25 [w/w]). Waterlock superabsorbent/TP layers were added to help absorb exudates from the wound, keep a moist environment, and allow the reactants to encounter and react in a controlled fashion on hydration of the patch. The nitric oxide release from the nanofibers (1 cm^2, ≈40 mg) was determined using a Sievers Nitric Oxide Analyzer Model 280i (Sievers Instruments, Boulder, CO), interfaced with a Dell Pentium III PC (Dell Inc., Round Rock, TX), in phosphate buffer (pH 7.4/37°C).

Treatment outcome assessment. At baseline (Day 0) a complete clinical history and physical examination was performed to each subject. The distribution, appearance, and severity of lesions were documented. Treatment progress was assessed on Days 21, 45, and 90 by clinical evaluation of the distribution and severity of the skin lesions. Photographs of the lesions were taken on Days 0, 21, 45, and 90.

Patients were scheduled for three follow-up visits at Day 21 (one day after the end of treatment) and at Days 45 and 90 after the beginning of the treatment. In each visit a complete physical examination was performed; lesions were measured to calculate the variation of the lesion size.

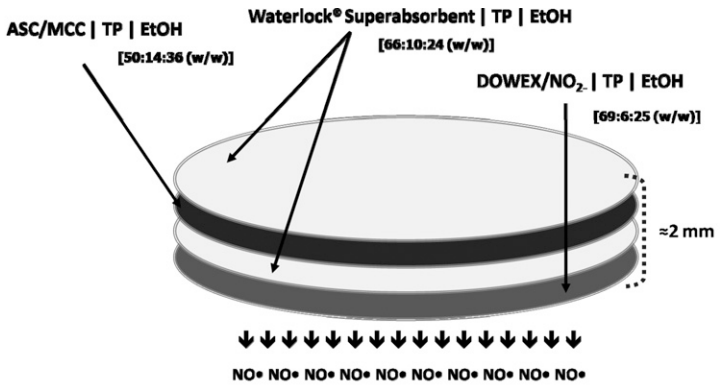
The considered outcomes were *clinical response* when complete re-epithelization of the ulcer was observed. *Clinical improvement* was determined when a decrease in the lesion size ≤ 100% ≥ 50% was observed. *No response* was registered when a decrease in the lesion size was < 50% or an increase of up to 50% of the initial size was observed. Finally, *therapeutic failure* was registered when an increase ≥ 50% in the lesion size, lack of re-epithelization at Day 90 of follow-up, or no response in two consecutive visits was observed. When therapeutic failure was reported, the presence of *Leishmania* amastigotes in lesions was confirmed by Giemsa stain of smears and patients were treated with intramuscular Glucantime at doses of 20 mg/kg/d for 20 days. Two additional secondary outcomes considered were *relapse*, when a lesion appeared at the edges or center of the scar after complete re-epithelization of the lesion, and *reinfection* when a lesion appeared in a different site from the initial injury.

Determination of toxicity. Each day during the treatment period, patients were questioned about symptoms suggesting possible drug side effects, including fever, myalgia, arthralgia, nausea, vomiting, abdominal discomfort, hyporexia, local rash or pain, and headache. In addition, vital signs were recorded and examined. Laboratory tests to monitor blood creatinine, amylase, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) concentrations were taken at baseline (Day 0) and at Day 1 after treatment (Day 21).

Statistical analysis. A descriptive analysis including means and proportions with 95% confidence intervals was carried out. Comparison between groups using the student *t* and non-parametric Mann-Whitney tests were done for variables with normal and non-normal distribution, respectively. Categorical variables were compared using a χ^2 test or Fisher's exact test. Data analyses were made using STATA version 8.0 (STATA Corp., College Station, TX).

RESULTS

Baseline characteristics of patients. Of the 178 patients in the analysis, 90 patients were assigned to receive Glucantime and 88 patients to NOP (Figure 2). Sixty-nine (38.8%)



ASC: ascorbic acid, **MCC:** microcrystalline cellulose, **TP:** Tecophilic® polymer, **EtOH:** ethanol, **NO:** nitric oxide

FIGURE 1. Production of the electrospun nitric oxide releasing patch (NOP).

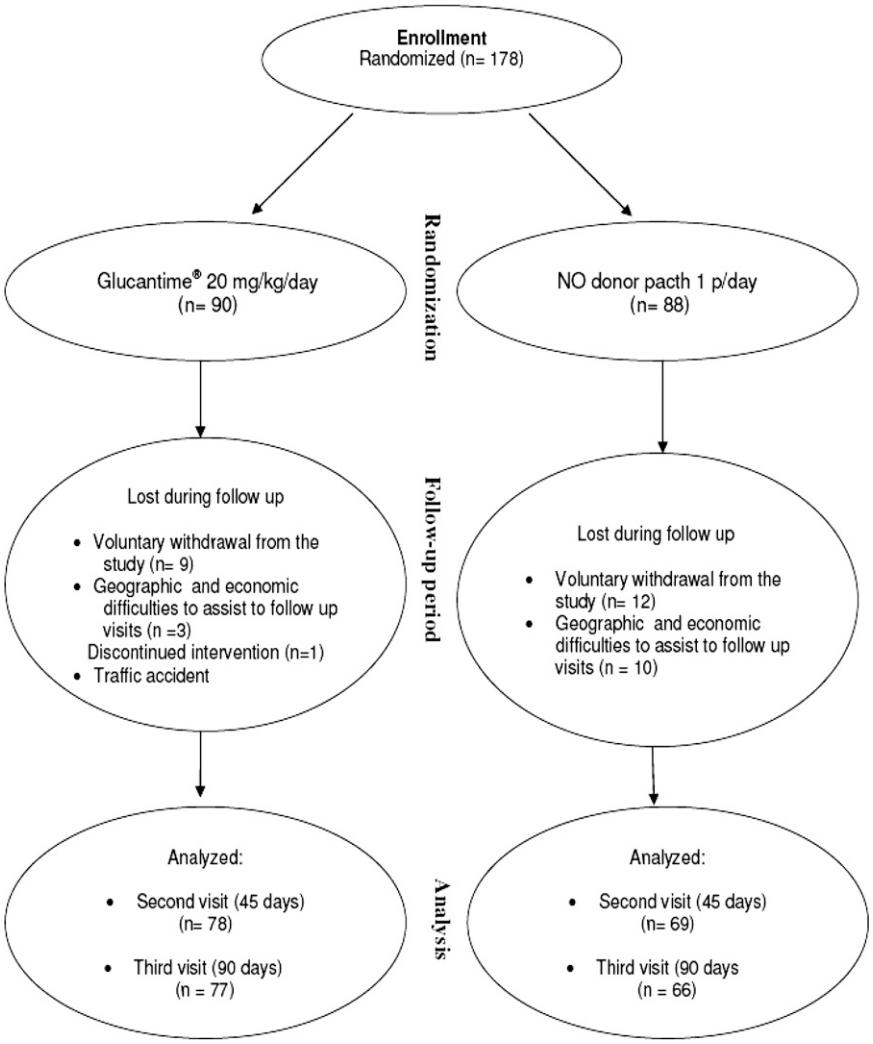


FIGURE 2. Study flow chart.

women and 109 men (61.2%) were included. Demographic and anthropometric variables were similar between the two study groups (Table 1). The 43.8% of patients of the Glucantime group had two or more lesions, in comparison with 46.5% of patients in the NOP group ($P = 0.9$). There were no significant differences between groups with respect to the initial size (26.4 ± 31.3 cm^2 versus 21.7 ± 33.4 cm^2, $P = 0.35$) or the evolution time (48.8 ± 28.6 days versus 49.0 ± 35.2 days, $P = 0.67$) of the lesions.

TABLE 1
Characteristics of the patients included in the study*

	Glucantime ($N = 90$)		NOP ($N = 88$)		
	n.	%	n	%	*P*
Gender					0.37
Male	58	64.4	51	57.95	
Female	32	35.5	37	42.05	
Age (years)					0.11
< 18	16	18	25	28	
19–50	57	64	61	69	
> 50	6	7	11	12	
Body mass index* (Kg/m^2)					0.39
< 19.9	17	20	13	16	
20.0–24.9	52	61	48	58	
> 25.0	16	19	22	26	
History of leishmaniasis	3	3.7	3	3.95	0.94

*Data shown as mean value.

Efficacy. The initial cure rate after 90 days of follow-up for the two treatment groups was 94.8% and 37.1%, for Glucantime and NOP ($P = 0.0001$), respectively (Table 2). Therapeutic failure was 5.2% and 62.9% after the same time of follow-up for Glucantime and NOP, respectively ($P = 0.0001$). No significant differences were observed in the percentage of clinical improvement and absence of clinical response between groups at any visit (Table 2). Additionally, reactivation of the initial lesion was observed in five patients: three of them (3.75%) were treated with Glucantime and two patients (2.63%) were treated with NOP ($P = 0.5$). Reinfection was similar in Glucantime and NOP groups (1.25% versus 3.95%, $P = 0.35$).

Toxicity. Patients treated with NOP presented a significant lower proportion of non-serious adverse events such as fever, headache, myalgia, and arthralgia. Nevertheless, the proportion of patients having symptoms at the lesion site (itching or pain) was higher in the NOP group compared

TABLE 2
Clinical response after treatment with NOP vs. Glucantime

	Visit 1 (Day 20)			Visit 2 (Day 45)			Visit 3 (Day 90)		
	Glu* (*N* = 80)	NOP (*N* = 78)	*P*	Glu* (*N* = 78)	NOP (*N* = 69)	*P*	Glu* (*N* = 77)	NOP (*N* = 66)	*P*
Complete clinical response n (%)	55 (68.7)	20 (25.6)	**0.0001**	75 (96.1)	24 (34.8)	**0.0001**	73 (94.8)	25 (37.8)	**0.0001**
Therapeutic failure n (%)	0	29 (37.2)	**0.0001**	0 (0)	36 (52.2)	**0.0001**	4 (5.2)	41 (62.1)	**0.0001**
Clinical improvement n (%)	13 (16.3)	10 (12.8)	0.66	3 (3.8)	7 (10.1)	0.14	–	–	–
No response n (%)	12 (15)	18 (23.1)	0.19	0	2 (4.8)	0.06	–	–	–

*Glucantime.

with those receiving Glucantime (Table 3). Blood levels of creatinine, ALT, AST, and amylase were similar in patients at the time of the enrollment; however, a significant increase in AST, ALT, and amylase levels at the end of treatment was observed in patients who received treatment with Glucantime. After treatment with NOP, the changes were not statistically significant (Table 4).

DISCUSSION

In the last decade clinical trials in the search of new treatments for CL have been developed, including mefloquine, itraconazole, ketoconazole, paromomycin, allopurinol, and dapsone, but some of them have not been effective and there is not enough evidence about the effectiveness for others.[12,13,27–29] A double-blind, randomized clinical trial comparing NO controlled release patches (NOP) versus meglumine antimoniate (Glucantime) in the treatment of CL caused by *L. panamensis* in Colombia was carried out.

A patch of four layers < 2 mm thick and capable of releasing topical levels of NO of ~3.5 µmol NO for 12 h was used.[23,24] Percentages of cure near 40% at 90 days follow-up was observed in patients treated with NOP. This cure rate was not as high as that showed by Glucantime. Rather, the efficacy shown by NOP treatment was similar to the placebo cure rate for *L. panamensis* in Colombia, observed by Velez and coworkers[29] who reported in a study using allopurinol versus placebo, cure rates in the placebo group near 37%.

Because NO is able to diffuse into the dermis, enough concentration to achieve stability and eliminate *Leishmania* parasites without affecting the healthy tissue is needed. A cream with NO generation by S-nitroso-*N*-Acetyl penicyl amine (SNAP) was tested in Ecuador showing a potential benefit in the management of CL, without any serious adverse events. Nonetheless, because of the irregular release of NO (from a s-nitrosothiol), the substance needed to be applied consistently affecting the adherence to treatment.[21] In another study, a potassium nitrate acidified with salicylic acid and ascorbic acid mixture with some activity against intracellular amastigotes and promastigotes of *Leishmania major in vitro*, was used for topical treatment of CL caused by *Leishmania tropica.*[20] After treatment, cure was observed in 12% of the patients and the lesion size was decreased in 28%. The difference between these two studies was caused by the technique used to obtain the NO, because acidification of nitrite produces an instant blast of NO but its release is not maintained over a long period.[20] However, with the NOP we aimed to solve these problems by encapsulating the components of the acidified nitrite approach in electrospun nanofibers, and we achieved a controlled release of NO of 3.5 µmol/cm^2 after 12 h. The choice of delivering µmolar concentrations of NO was based on the fact that leishmaniasis promastigotes are known to compromise macrophages, which are one of the cells of the innate immune system that engulf and destroy pathogens through the generation of a "respiratory burst" and are known to produce massive concentrations of NO in a cytokine mediated process.[14,30–34] Thus, endogenous NO release from NOP was expected to supply the lack of NO coming from infected macrophages from the inducible nitric oxide synthase (iNOS). Although the efficacy of the NOP at the used formulation was shown not to be effective as expected, studies are currently in place to fine-tune the NOP to increase the amount of NO released to the infected area and to mimic and supplement the NO burst released from innate immune response cells in response to pathogen infection. To mimic the NO burst released from innate immune response cells, a sustained release of micromolar concentrations of NO is required from NOP even at shorter periods of time.

Despite the effectiveness obtained with NOP, a significantly lower frequency of non-serious adverse events and a reduced variation in serum markers were observed in patients treated in the NOP versus the Glucantime group. In general, the use of the pentavalent antimony compounds sodium stibogluconate (Pentostan) and Glucantime have been the most effective treatments of choice for CL.[9] Glucantime treatment trials have reported cure rates greater than 85% in Colombia and other places in the world.[10,11] On the other hand, this drug has been associated with several disadvantages such as the need for a parenteral route, discomfort, and the presence of various side effects (fatigue, vomiting, muscle and abdominal pain, cardiac abnormalities, increased hepatic aminotransferase, pancreatitis).[12,13] Thus, new safer therapeutic options are needed. For special populations such as pregnant women, children, or patients to whom Glucantime is contraindicated, the development of a next generation NOP system that mimics the innate immune cell system should be investigated.

TABLE 3
Adverse events observed in the study

	Glucantime (*N* = 80)			NOP (*N* = 76)			
	n	%	Days*	n	%	Days	*P*
Fever	25	31.6	5.6 ± 3.3	10	13.7	3.5 ± 5.1	**0.009**
Myalgia	49	62.1	7.3 ± 4.2	16	21.9	7.6 ± 4.2	**0.001**
Arthralgia	41	51.9	7.3 ± 4.9	5	6.8	8.8 ± 5.5	**0.001**
Headache	42	53.2	6.5 ± 4.4	24	32.8	5.1 ± 3.9	**0.01**
Vomiting	5	6.3	1.7 ± 0.9	3	4.1	1.3 ± 0 0.6	0.54
Nausea	28	35.4	5.3 ± 4.4	10	13.7	4 ± 3.4	**0.002**
Hyporexia	25	31.6	5.9 ± 4.4	3	4.2	9.6 ± 7.2	**0.001**
Local Rash	8	10.1	6.4 ± 5.7	21	28.8	8.4 ± 6.1	**0.003**
Local Pain	4	5.1	7.5 ± 8.1	11	15.1	10.4 ± 7.5	**0.03**

*Data shown as mean ± SD values.

TABLE 4
Blood levels of creatinine, AST, ALT and amylase in patients treated with NOP vs. Glucantime

	Glucantime*			NO donor patch*		
	Basal ($N = 80$)	Day 20 ($N = 80$)	P	Basal ($N = 76$)	Day 20 ($N = 76$)	P
Creatinine (mg/dL)	0.92 ± 0.15	0.91 ± 0.17	0.69	0.89 ± 0.16	0.87 ± 0.14	0.4
AST† (U/L)	28.47 ± 9.17	41.02 ± 27.87	**0.0002**	26.92 ± 8.24	26.93 ± 8.61	0.99
ALT‡ (U/L)	25.46 ± 12.95	39.06 ± 35.89	**0.001**	23.46 ± 9.76	25.05 ± 8.79	0.28
Amylase (U/L)	74.13 ± 26.85	90.87 ± 34.62	**0.0008**	73.48 ± 24.67	77.13 ± 21.1	0.32

*Data shown as mean ± SD values.
†AST = aspartate aminotransferase.
‡ALT = alanine aminotransferase.

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