UNIVERSIDAD SAN FRANCISCO DE QUITO

Colegio de Postgrados

Nueva estrategia terapéutica basada en nanotecnología contra leishmaniasis tegumentaria en Ecuador

New pharmacological strategy based in nanotechnology against cutaneous leishmaniasis in Ecuador.

(El idioma de esta tesis es inglés)

María Fernanda Loayza Villa

Tesis de grado presentada como requisito

para la obtención del título de Magister en Microbiología

Cumbayá, Julio del 2010

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Hoja de Aprobación de Tesis

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RESUMEN

La leishmaniasis es una parasitosis endémica y ampliamente distribuida en zonas tropicales y subtropicales en el mundo. Se considera que la incidencia de esta infección podría incrementarse por la progresiva urbanización, cuya consecuencia es el incremento en la exposición de las personas a la picadura del vector. Actualmente, en países endémicos, incluido el Ecuador, el tratamiento de elección contra leishmaniasis es la aplicación intramuscular de compuestos amoniacales. Sin embargo este tratamiento está asociado a una baja adherencia dado el requerimiento de múltiples aplicaciones de la medicina, la toxicidad y la resistencia desarrollada por los parásitos. Estas características incrementan el interés de desarrollar nuevas estrategias terapéuticas contra la infección. Recientemente, algunos sistemas de suministro de drogas, tales como las nano - partículas, se han utilizado in vivo e in vitro, con diferentes medicamentos de aplicación local. El objetivo del presente trabajo fue evaluar la eficacia de una nanoemulsión tópica de antimonato de meglumina en úlceras de leishmaniasis cutánea en un modelo murino. Se infectó 55 ratones y se destinó aleatoriamente animales infectados a distintos grupos de tratamiento. Seis ratones fueron tratados únicamente con crema, doce ratones recibieron la nanoemulsión sin antimonato de meglumina y 21 ratones recibieron la nanoemulsión con antimonato de meglumina. El grupo control (16 ratones) fue tratado con el medicamento original (sin nanoemulsión) vía intraperitoneal. Los resultados demostraron que administración tópica de antimonato de meglumina en nanoemulsión causa un efecto benéfico en el control de la parasitosis y la curación de la úlcera, pero este efecto no tiene soporte estadístico.

ABSTRACT

Leishmaniasis is an endemic disease with wide distribution in tropical and subtropical regions around the world. It is considered that the incidence of this infection would be increased due to the progressive urbanization process which consequence is the increment of the human exposition to vector bite. Currently, in endemic countries included Ecuador the treatment of choice against leishmaniasis is the intramuscular application of amoniacal compounds. However, this treatment is associated with poor compliance due to the requirement of multiple applications of medicine, toxicity and parasite resistance. These characteristics increase the the interest to develop new therapeutic strategies against the infection. Recently, some drug delivery systems such as nanoparticles have been used *in vivo* and *in vitro*, with different drugs for local application. The aim of this research was to evaluate the efficacy of a topic nanoemulsion with meglumine antimonate applied in ulcers of cutaneous leishmaniasis in a murine model. Fifty five mice were infected and randomly distributed in different groups of treatment. Six mice were treated with cream alone, 12 mice received empty nanoemulsion and 21 mice received nanoemulsion with meglumine antimonate. The control group (16 mice) was treated with the original drug (without nanoemulsion) by intraperitoneal administration. The results showed that the efficacy of topic administration of meglumine antimonate cause a benefit effect on parasitosis control and ulcer healing but this effect do not have statistic support.

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INTRODUCTION

Leishmaniasis is a vector-borne parasitic disease caused by the protozoa *Leishmania spp*. Leishmaniasis currently present in 88 countries throughout the world and afflicts 12 million people, with 350 million at risk. There are several manifestations of leishmaniasis that range from cutaneous to visceral forms. The clinical presentation of this infection depends on the species of *Leishmania* and the host immune response (1).

In Ecuador, leishmaniasis is considered a public health problem. It is widespread in tropical and subtropical areas near the Pacific Coast, Andean valleys and tropical zones in Amazon region. Clinical cases of leishmaniasis have been reported in 22 of 25 provinces, with approximately 3.1 a 4.1 million of people at risk to acquire the infection. Calvopiña et al. estimated that the incidence rates of leishmaniasis in Ecuador probably range from 3, 000 a 4, 500 cases each year (2).

Three forms of leishmaniasis have been reported in Ecuador, cutaneous (CL); mucocutaneous (MCL), and diffuse cutaneous (DCL) leishmaniasis (2). Cutaneous leishmaniasis, the most common form, is caused mainly by *Leishmania (Viannia) panamensis* and *Leishmania (Viannia) guyanensis* species and is more common in the Coast. Other forms of leishmaniasis affect mucosal surfaces and different organs that include bone marrow, liver, and spleen. (3).

The diagnosis of leishmaniasis is done on the basis of clinical presentation and histopathology. The most common diagnosis method used is a smear of the lesion which is stained with Giemsa or Wrigth. In these samples, it is possible to see extracellular parasites in amastigote form and vacuolated macrophages with parasites (1). Other diagnosis methods that have been developed are molecular techniques like multiplex PCR, RFLP and real time PCR (1).

Current therapy protocol consists of 20 daily intramuscular injections of meglumine antimonate (MA) in a dose equivalent to 20 mg of pentavalent antimonial (Sb^{v})/kg/day. The number of injections can be increased until the complete heal of the ulcer (4). MA is distributed by the Public Health Ministry of Ecuador, but the availability of the medicine is limited (2). Despite of the fact that Sb^{v} have been extensively used for more than 60 years, its structure and mechanism of action as leishmanicida remain poorly understood (5).

There are several side effects as a consequence of MA treatment. Leucopenia, agranulocytosis, thrombocytopenia are examples of hematologic effects that have been reported after antimonials application. MA toxicity also results in renal damage and increase in serum hepatic and pancreatic enzymes. Moreover, behavioral changes, and electrocardiographic (ECG) changes have been observed after this treatment (1, 4). Daily treatment with MA injections has a poor compliance due to the chronic use of the drug, intramuscular route of administration, intense pain (related to high volume for intramuscular administration), cost, and toxicity. A research in rural Ecuador reported that only 32.6% of patients completed the treatment (6). In addition, this disease affects mostly people in rural areas who usually have

limited access to health care centers (6). Drug discontinuation is one of the most important hurdles in the treatment and is probably contributing to create parasitic resistance to drug (2, 4). Hence poor compliance, side effects, drug toxicity, and cost lead to the treatment discontinuation and probably affect its effectiveness.

In the rural area, people use unconventional treatments to deal with the infection (6). Alternative fast treatments include topical application of petroleum-based products, burning of the ulcer, and local application of acid products. Major natural treatments are propolis, herbicidal agents and indigenous plants, for example: (*Verbena officinalis*), sangre de drago (*Chroton lechleri*), Llantén (*Plantago major*), etc. (6). Several researches have reported reduction of parasite burden using other drugs such as Amphotericin B, Azithromycin, Pentoxifilline, and Miltefosine Tamoxifeno, but their cost and side effects are similar to antimony drugs (1, 7-11).

Dermal drug application is the least frequently used therapy against *Leishmania* ulcers, paramomicina for example showed contradictory results in different reports maybe it is due for the auto release of the lesion in the experiments done (1). Herbal extracts applied directly over the cutaneous lesion in mouse model (*Thymus vulgaris and Achillea millefolium)* applied daily twice with a cotton applicator over the lesion site showed a better effect in the release of the lesion than MA IP application ($p= 0,008$ and $0,006$) (11).

Recently, new drug delivery systems have been developed in order to improve absorption and minimize side effects, for example, liposomes, niosomes, microspheres, and nanoparticles are vesicles that could be applied topically and that can deliver active principles of medicines. These vesicles allow better absorption with better activity of the drugs from skin (12, 13). Therefore, the effectiveness of MA against *Leishmania* parasites could be improved through a topical application of vesicles with medicine while the side effects are reduced.

Nanoemulsions are dispersions of oil and water which are thermodynamically stable and transparent. The stability of these dispersions is due by an interfacial film of surfactant and co-surfactant molecules. At the end of the process nanoparticles have a droplet size less than 100 nm (13, 14).

Nanoparticles are made in base of spontaneous emulsification process. Currently, different technology developments lead mixing organic and aqueous medicine phases in homogeneous solutions (13). Nanoemulsion ointments have been prepared with anti-inflammatory and antibiotic drugs in order to test their beneficial overcome (14-16).

Experiments in vitro have shown that chemical and physical features of nanoparticles depend on the size of the nanoparticles, the ionic charge in the surface, the surfactant and co-surfactant reagent film (13). Moreover, nanoparticles let deliver drugs with low water solubility easier, in colloidal suspensions (14). For example, there are different selective

cyclo-oxigenase 2 inhibitors like celecoxib, non-steroidal anti-inflammatory drugs like aspirin and aceclofenac and antibiotics like amphotericin B that have been applied with different nanoemulsion formulations (14 -17).

To test the skin permeability of nanoemulsions, Franz diffusion chamber have been used in different experiments. The experiment began with the division of the Franz chamber in donor and receptor sides using a prepared membrane with treated rat abdominal skin. For example, in Faiyaz et al. (2007) research, the experiment consist on a chamber with an effective diffusional area of 0.636 cm^2 and 4mL of receiver chamber capacity with the dermal side of the rat skin faced the receiver compartment. Then, a skin stabilization process was done with ethanolic phosphate buffered saline solution (pH 7.4; 20:80% vol/vol). This solution was replaced every 30 minutes. The fluid that passed to the receiver compartment was stirred with a magnetic rotor (600 rpm) (16).

The Franz chamber was placed in Logan transdermal permeation apparatus at 32°C. After 4.5 hour the absorbance of the receiver fluid must be negligible indicating complete stabilization of rat skin. Later, 1 mL of aceclofenac nanoemulsion (20mg/mL) was placed on donor compartment of the Franz chamber. Then, samples were taken of the receiver chamber at regular time intervals to analyze the drug concentration. The cumulative drug permeated (mg/cm^2) was calculated in each sample showing that in vitro skin permeation was highest in nanoemulsion formulation and lowest for conventional aceclofenac gel ($p>0,05$) (16).

The efficacy of the use of nanoparticle drug delivery systems was demonstrated *in vitro,* using Amphotericin B (AmB) against *Candida albicans* culture (18). Vieira D. and Carmona A. (2008) showed that cationic bilayer nanoparticles with dioctadecyldimethylammonium bromide (DODAB) plus Amphotericin B (AmB) had an inhibitory effect in *Candida albicans* growth (18). They demonstrated the anti microbial effect of a single supramolecular structure. These particles are the result of a self – assemble of inert carboxymethylcellulose (CMC), poly(diallydimethylammonium chloride) (PDDA) and AmB solubilized at the edges of DODAB bilayer fragments (DODAB BF) (18).

The experiment consisted on leading the microorganism (1×10^6 CFU/mL) to be in contact with the particles for 1 hour. Then they plated 0.1mL of a 1000- fold dilution in Mili-Q water for each original culture in Plate Count Agar. The plates were incubated for 48 hour at 37ºC. Results of this experiment showed a 100% of growth inhibition with a concentration of AmB/DODAB BF/CMC/PDDA at PDDA concentration > 2 mg/mL (18).

The efficacy of dermal application of a medicine in nanoemulsion was proved by Subramanian et al. (2008). They developed an inflammation model in the ear lobes of CD-1 mice. The dermal application of aspirin nanoemulsion caused a reduction of 70% of thickness of the mice ear lobes compared with a reduction of 42% of the application of aspirine suspension alone (14). As a result, the analysis showed that the ear tissue reduction was greater when the treatment consisted on nanoemulsion with aspirin vs. aspirin suspension alone ($p<0.05$) (14).

Faiyaz et al. (2007) showed an effective anti inflammatory effect when an aceclofenac nanoemulsion was applied in an edema in the hind paw of female Wistar rats (16). The edema was induced with carregeenan application. After 24 hours of administration of aceclofenac nanoemulsion the inhibition was 82.2% while the convencional aceclofenac gel only inhibited de edema in 41,8% ($p<0.01$) (16). Additionally the authors reported there were no side effects in animal skin by the nanoemulsion application. (16). Data are consistent with an increased efficacy and low side effects of drugs administered topically in a nanoemulsion form (14).

Nanoemulsions are complex mixtures of different compound and drugs that have showed to be best mechanisms to deliver drugs easily though the skin or mucous surface. The advantages of their use include their simple application, high grade of permeability through the skin, low size that let the particle cross the cell membrane and easy release the drug inside cells. At the same time, these characteristics improve the effectiveness of medicine and avoid or decrease side effects that drugs cause when are parenterally administered.

To our knowledge, there are not reports describing the use MA in nanoemulsion preparations for the local treatment of CL. The aim of this study was to test the effect of a nano-emulsion with MA applied topically in the treatment of CL in a murine model of this parasitosis.

2. OBJECTIVES

The principal objective of this research was to evaluate the effect of a topical administration of meglumine antimoniate nanoemulsion in cutaneous leishmaniasis lesion using a murine model.

Specific aims:

- To infect susceptible BALB/c mice with *Leishmania (L.) mexicana MHOM/BE/82/BEL-21* or *Leishmania (L.) amazonensis MHOM/BR/73/M2269.*
- To treat BALB/c mice with CL with topical application of cream alone, nanoemulsion ointment without meglumine antimoniate, meglumine antimoniate in nanoemulsion ointment and intraperitoneal injections of meglumine antimonate.
- To compare the healing rate and parasite load of BALB/c mice with CL treated with topical application of cream alone, empty nanoemulsion ointment, meglumine antimoniate in nanoemulsion ointment and intraperitoneal (IP) injections of meglumine antimoniate.
- To assess the hepatic, pancreatic and renal side effects in the groups of treated animals.

3. HYPOTHESIS

Topical treatment of CL with a meglumine antimoniate nanoemulsion ointment is comparable to the intraperitoneal treatment with the medicine alone.

4. MATERIALS AND METHODS

Parasites

The strains *Leishmania (L.) mexicana MHOM/BE/82/BEL-21 and Leishmania (L.) amazonensis MHOM/BR/73/M2269 parasites* were kindly donated by Jorge Arévalo of Cayetano Heredia University, Tropical Diseases Institute (Lima- Peru). Parasites were cultured in Ushmaru Biphasic Medium supplemented with 15% of rabbit blood and Gentamicin (50 μg/mL) in PBS pH 7.2 with 100 U/ml penicillin and 100 mg/ml streptomycin. (19).

Mice infection

BALB/c mice were used in all experiments (8). Mice were used at 4–6 weeks of age. The initial stock of mice was obtained from Biomedicine Center at Universidad Central del Ecuador (Quito- Ecuador). Animals were bred and maintained at the Animal Facility of the San Francisco University.

BALB/c mice (n=54) were infected subcutaneously with 1 X 10^6 *Leishmania (L.) mexicana MHOM/BE/82/BEL-21* (n = 24) or *Leishmania (L.) amazonensis MHOM/BR/73/M2269* promastigotes (n = 30) in log phase of growth using PBS as vehicle. The inoculation was applied in the base of the tail (8). Cutaneous ulcers developed within 8 weeks after initial inoculum. Infected mice were divided in four groups of treatment as indicated in Table 1.

Treatments started 10 weeks after the initial infection in order to obtain obvious and well-defined lesions. Animals were treated for 20 days (8). Size of ulcers was monitored every week and the measurements at the beginning and at the end of the treatment period were used for analysis. Also, animal weight was monitored every week during the time of the study. Topical treatment was performed with the animal immobilized in a 50 ml high -clarity polypropylene conical tube (BD Falcon^{TM}) for 30 minutes to avoid leaking of the cream.

Lesion morphology and parasite burden

After treatment, both macro and microscopic analysis of the lesion was carried out. Also the parasitic burden was evaluated. Every week cutaneous lesions were measured with a caliper and the widest diameter was recorded (8). Delta index of the lesion was calculated subtracting the final diameter measure minus the initial one.

Animals were sacrificed according to standard operating procedures and strictly following USFQ guidelines regarding research using animal subjects. The presence of *Leishmania* parasites were determined using histological procedures (20). Briefly, 2 mm of the skin lesion of each mouse was preserved in 10% buffered formalin. Subsequently samples were placed in paraffin blocks and cut with a microtome into 4 μm think cuts. Samples were then placed in glass slides that were stained with Hematoxilin – Eosin (HE) following standard procedures. The slides were analyzed by a trained pathologist in order to describe the presence of parasites

in tissue sample. The characterization of histological changes was done following the criteria described by Massom et al. (20).

The following variables were recorded in the histological analysis: presence or absence of ulcer, dermal atrophy, hyperkeratosis. Microscopically the presence of macrophage containing parasites, extracellular parasites, plasma cells, lymphocytes, eosinophils, and neutrophyls (20).

For every variable a semi-quantitative scale was established (Table 2). Infiltrating leukocytes were evaluated by number of each cell observed by optical field (400x). Dermal atrophy was measured by the absence of hair follicles and glands and an increase of collagen surface. Hyperkeratosis was measured by the increase in the thinness of the corneal layer. A second part of skin lesion was conserved at -80^{o}C for *Leishmania* nucleic acids extraction.

DNA extraction for parasitic burden

In order to determine the parasitic burden in infected mice, DNA from skin sample was extracted with High Pure PCR Template Preparation kit (Roche Applied Science) (21). To detect the presence of parasite specific DNA, real time PCR was performed to amplify a 120-bp DNA fragment from *Leishmania* species kinetoplast minicircles (21).

LC Fast Start DNA Master Plus SYBR Green kit (Roche Diagnostics, Quito - Ecuador) was used to quantify parasite DNA with a Light Cycler 2.0 instrument (Roche Applied Science,

Quito - Ecuador). The primers used to amplify the indicated DNA fragment were: forward, JW11: 5´-CCTATTTTACACCAACCCCCAGT-3´ and reverse, JW12 5´-GGGTAGGGGCGTTCTGCGAAA-3 (15). Times and temperatures of qPCR are indicated in Table 3.

Side effects evaluation

In order to determine the side effects of treatments, serum concentrations of AST (Aspartate Aminotrasferase), ALT (Alanine Aminotransferase), Alkaline Fosfatase (Liver Function) and Creatinine (Kidney function) were determined (22). Briefly, at the end of treatment periods, animals were sacrificed as indicated previously and 0.5 to 1mL of blood was collected. Each analysis was done in 10 μL of serum using commercially available reagents (Orthoclinical diagnostics - Johnson & Johnson, Quito - Ecuador) in a Vitros 250 System dry biochemical analyzer (Orthoclinical diagnostics - Johnson & Johnson, Quito - Ecuador). Macroscopic morphology of liver, spleen, and kidneys were evaluated by visual inspection.

Reagents

Glucantime® (Meglumine antimonate) (AVENTIS PHARMA) 300 mg /ml equivalent to 85 mg Sb^{v}/ml was acquired at Hospital General de las Fuerzas Armadas, Quito – Ecuador. The nanoemulsion of MA and the empty nanoemulsions were prepared at Center of Health and Disease Research (University of Massachusetts LOWELL-USA) as described previously by Subramanian et al. with a final concentration of 280mg/mL of Glucantime® (14).

Both empty nanoemulsion and MA nanoemulsion were applied topically as creams. To obtain the cream, nanoemulsion were mixed with hypoallergenic cream at a ratio of 1:1 before application.

5. RESULTS

Effect of topical treatment with MA nanoemulsion or intraperitoneal MA on lesion size

Macroscopic analysis

The clinical course of lesion progression was monitored weekly. Lesions were measured with a caliper in millimeters as reported previously (8). Data from lesions before and after treatments were used for analysis. There were not obvious additional infections on skin ulcers by simple inspection throughout the study. In the control animals that received cream alone or empty nanoemulsion the size of the lesions increased with time of infection (Table 4). As expected, the intra-peritoneal injections of MA caused a significant decrease of lesion size ($p=0.005$). Topical treatment of cutaneous leishmaniasis with the MA nanoemulsion resulted in a decrease in the size of the ulcer but these results were not statistically significant ($p=0.45$).

To better analyze the effects of the different treatments a delta index of the size of the lesions was estimated (8). This index was calculated by subtracting the largest diameter of the ulcers at the end of the treatment period to the largest diameter at the beginning of the study. Delta values greater than cero indicate an increase in lesion size and values lower that cero indicate a decrease. As indicated in figure 1, there was an increase in the size of the lesions in animals treated with cream alone or empty nanoemulsion. Contrary, in animals treated with MA in nanoemulsion or with IP injections of MA there was a decrease in lesion size. A comparison between IP and topical treatments demonstrated that there were differences when delta lesion value between these treatments was compared ($p< 0.05$).

Due to the different size of lesions within the groups before treatments it was important to compare the effect of the different treatments in animals with similar lesion size. A group of 28 mice with initial lesion size between 6.0 - 7.8 mm were separately analyzed. Lesions greater than 6.0 mm allowed us to monitor better the changes during the treatment period. As was observed in all animals treated with empty nanoemulsion the size of the lesions increased **Figure 2**. For this reason these group were considered as negative control group for data analysis. No animals treated with cream alone were selected. On the other hand, MA IP decreased cutaneous lesion size in this subgroup of animals, ($p<0, 01$). Topical treatment of MA nanoemulsion in animals with lesions > 6.00 mm also decreased the size of the cutaneous lesions, but the described effect did not have statistic support ($p=0.12$).

Effect of topical treatment with MA nanoemulsion or intraperitoneal MA on parasite burden

In order to measure the amount of *Leishmania* parasites in the lesions of infected mice the amount of *Leishmania* specific DNA was determined by Real time PCR. It was assumed that there is a direct relationship between the concentration of *Leishmania* DNA and the degree of parasite burden, the greater the concentration of parasite DNA the greater the *Leishmania* present in the lesions. Infected mice treated as indicated in Materials and Methods were euthanized and a piece of approximately 3 mm of cutaneous lesion was collected and stored at -80°C. Total DNA was extracted as indicated in materials and methods.

Data indicate that the concentration of *Leishmania* DNA was greater in the control groups that were treated with cream of empty nanoemulsions than in animals treated with MA IP or MA nanoemulsion. Due to the great variation of *Leishmania* DNA concentrations observed within the groups the differences between treatments were not statistically different. Although data analysis did not show differences in parasitic burden between treatment groups, it is possible to observe a tendency for MA IP or MA nanoemulsion to decrease the parasites burden (Table5 and Figure3).

Histological analysis

In order to determine the pathology of the lesions after treatments, lesion samples were taken at the end of the experiment. Samples were processed and stained as indicated in materials and

methods. For tissue analysis a semi quantitative score was created as described in methods section. Data showed no differences for the histological parameter analyzed among groups (Table 6).

Side Effects

Weight

To determine the general health of the animals, weight was monitor throughout the study. There were not changes in the weight of the animals due to treatments. Figure 4 shows the variation of weight estimated by the delta weight value that was calculated subtracting the final minus initial weight of infected mice. There were not statistic differences ($p>0.05$) in the mean of delta values per treatment group. There was a tendency to lose weight in mice treated with MA IP injections.

Effect of topical treatment with MA nanoemulsion or intraperitoneal MA on blood biochemistry

In order to evaluate the effects of the different MA treatments (IP or topical) on liver, pancreas and kidney functions, serum transaminases, alkaline phosphatase and creatinine were

measured. For this analysis an extra group of uninfected mice (n=13) that did not received any treatment was also included as reference. Serum enzymatic activity of hepatic transaminases of animals in the different treatment groups was similar to the uninfected mice. Similarly, there were not differences in the concentration of serum creatinine levels among treatment groups and uninfected group (Figure 5).

Discussion

The use of liposomes and nanoparticles as means to deliver drugs has produced an improvement in pharmacotherapy. Present results suggest that treatment of cutaneous leishmaniasis lesions with a nanoemulsion of MA may limit the course of the parasite infection. Compared with control treatments cream and nanoemulsion alone, nanoemulsion with MA limited the growth of cutaneous lesions. On the other hand MA administered IP decreased the size of the lesion. At the same time, the parasite burden in the lesion was also controlled in mice that were treated with MA nanoemulsion.

Animals treated with cream and nanoemulsion alone had greater number of parasites (Figure3) compared with animals treated with nanoemulsions of MA or IP MA, although the differences did not show statistical support.

Amphotericin B desoxycholate in nanoemulsion applied IP showed a greater inhibitory effect on *Leishmania* amastigotes growth than the application of Amphotericin B (p=0,005) without

nanoemulsion (17). The authors of these observations indicate that the greater inhibition of the parasites maybe due to the improvement in the absorption of the drug in nanoemulsion (17). It would be interesting to test the topical administration of Amphotericin B desoxycholate in nanoemulsion on cutaneous lesions of leishmaniasis.

Pharmacokinetics studies of intramuscular injections of Sb^V (active ingredient in MA) in hamsters indicate that the drug is rapidly absorbed from the muscle, has a short life in blood of approximately 2 hours and readily accumulates in kidneys> liver> skin>spleen > heart, (23). The half-life of Sb^V in skin after intramuscular administration of Sodium stibogluconate (a MA equivalent medicine) was approximately 3 hours. Several studies showed that *Leishmania* parasites have not complete eliminated in hamster lesions after 20 days of treatment in spite of administered dosis of Sb^V since 20 mg to 120 mg /Kg of animal weigh. Similarly, the present study shows that using a dose of 20mg of Sb^V/Kg of animal weight for 20 days was not able to eliminate all parasites on the lesion (22). In our study, MA in nanoemulsion was applied directly in the ulcer to avoid the inconvenience of IM injections and to directly deposit the drug in contact with the parasite. The limited effect with MA nanoemulsion to decrease lesion size could be the result of an insufficient dose and the liking of the cream after the animal was unstrained (see materials and methods). This reduction of the dose is unlikely to occur in humans. It is of interest to study higher doses of topical MA in nanoemulsions and the pharmacokinetics of the drug in mice infected with *Leishmania*. In addition it will be interesting to test several local applications of MA to have a better effect against the parasite.

The effect on lesion size observed in the different treatment groups, was in agreement with the parasite load measured (figure2 and figure3). Control animals that did not received MA had higher concentrations of the parasite compared with animals that had MA in nanoemulsion or intraperitoneally although the differences were not statistically meaningful. Direct observation of histological samples of the different treatment groups showed similar parasitic load among treatments. These results were confirmed with Real Time PCR analysis (figure6) which is a more efficient method for this evaluation (24).

The mechanism of action of MA to kill *Leishmania sp.* has not been established yet. It has been proposed that Sb^{v} drugs act after their conversion into a more toxic trivalent antimony compound within cells of the host or the parasite itself (25). However others indicate that there is a direct toxicity effect of Sb^{v} by itself on the parasite (25). Despite of contradictory data, it is possible that the effect of Sb^{v} was indirect and results from macrophage – dependent reduction to Sb^{III} (4, 25). In either case, local application of MA could result in a more direct exposure of the parasite to the drug. This effect could explain the observation that the size of the lesion did not increase in infected animals treated with the MA nanoemulsion.

Treatment of leishmaniasis infection with MA IM is associated with several side effects (1, 4). Organs affected by MA treatment include bone morrow, kidneys, liver, heart, and skin (4, 22, 23). Present study shows that concentrations of ALT, AST, ALKP and creatinine that indicate liver and kidney function in animals treated with MA nanoemulsions or IP were similar to those observed in animals without *Leishmania* infection. Also, histological analysis showed similar inflammatory processes in all treated groups. Indicating that the local treatment did not

provoke a local reaction as observed with some topical agents (26). This response is similar to the response reported in other studies that tested topical nanoemulsion application (13-15).

Conclusions

Leishmaniasis is an emergent infectious disease that affects rural areas in tropics and subtropics around the world. Pentavalen antimonials (stibogluconate sodium or MA) are the first line treatments against the leishmaniasis infection, but their long term and parenteral application have resulted on poor compliance, high toxicity and the development of resistantance in parasites to this medicine.

Due to the natural course of cutaneous leishmaniasis infection it is possible to design local treatments for this parasitosis. Cutaneous leishmaniasis affects principally exposed skin in face, arms or legs. Anti fungal, antibiotics and anti cancer drugs among others have been tested to find a new pharmacologic strategy against the leishmaniasis infection. However, different reports have shown contradictory results of the application of these alternative drugs. Furthermore, cost and several side effects related with dosage and toxicity of medicine application result in restrictions of their use and less availability of the medicine. For these statements, pentavalen antimonials have not been completely replaced in the leishmaniasis treatment in spite of their use for more than 60 years. It is possible that higher concentrations and more frequent applications of local nanoemulsions of MA could result in better treatments for this infection.

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Table1. **Groups of mice infected with *Leishmania spp.* classified by applied treatment.** Groups of mice infected with *Leishmania (L.) mexicana MHOM/BE/82/BEL-21* or *Leishmania (L.) amazonensis MHOM/BR/73/M2269* and treated with 1. Cream alone, 2. Empty nanoemulsion ointment, 3. MA nanoemulsion ointment, 4. MA IP administration. n=number of infected animals. BEL-21=*Leishmania (L) mexicana*, M2269 *Leishmania (L) amazonensis.* MA: Meglumine antimonate.

Groups	Treatments (n = number of animals)	*Leishmania* strain (n)	Dose of MA®
1	Cream alone (n = 6)	BEL-21 (n=1) M2269 (n=5)	Control
2	Empty nanoemulsion ointment (n = 12)	BEL-21 (n=4) M2269 (n=8)	Control
3	MA nanoemulsion ointment (n = 21)	BEL-21 (n=8) M2269 (n=13)	20 mg/Kg/d
4	MA IP (n = 15)	BEL-21 (n=11) M2269 (n=4)	20 mg/Kg/d

Table2. **Semi-quantitative scale established for variables in the histological analysis.** Analysis of histology tissues in BALB/c mice infected skin with *Leishmania (L.) mexicana MHOM/BE/82/BEL-21* or *Leishmania (L.) amazonensis MHOM/BR/73/M2269* were performed in a little part of lesion of each mouse after treatment period. The tissue was embedded in paraffin block and then it was cut in 4mm layers in order to perform a hematoxylin – eosin stain. These stained samples were analyzed by an expert pathologist. Table shows score system for histology characteristics classification in mice lesion

Parameter- category		Score classification
Dermal atrophy	Nil	Normal tissue
	Mild	Decrease of a third in the number of follicles and glands
	Moderate	Decrease in two third in the number of follicles and glands
	Severe	Absence of follicles and glands
Hyperkeratosis	Nil	Normal tissue
	Mild	1-4 corneal layers
	Moderate	5-10 corneal layers
	Severe	More than 10 corneal layers
Macrophages with parasites	Nil	Normal tissue
	Mild	1-8 cells
	Moderate	9-20 cells
	Severe	More than 20 cells
Plasma cells, Lymohocytes , polimophonuclear cells and Eosinophils infiltration	Nil	Normal tissue
	Mild	1-8 cells
	Moderate	9-20 cells
	Severe	More than 20 cells

Table3. Real time PCR conditions to determine DNA from *Leishmania spp.*

Analysis Mode	Cycles	Segment	Target Temperature	Hold Time	Slope °C/s
			Preincubation		
None	1		95 °C	10 min	20
			Amplification		
Quantification	45	Denaturation	95°C	10 s	20
		Anneling	56°C	(20uL) 5 s	20
		Extension	72°C	4,8 s	20
			Melting curve		
Melting curve	1	Denaturation	95°C	0 s	20
		Anneling	65°C	30 s	20
		Extension	95°C	0 s	0.1/s
			Cooling		
None	1	40°C	30s	None	20

Table4. **Description of lesion size in infected mice.** Lesion size was measure before and after treatment application. Infected mice were divided in four groups of treatment: 1. Cream alone, 2. Empty nanoemulsion ointment, 3. MA nanoemulsion ointment, 4. MA IP administration. Treatments were started 10 weeks after the initial infection in order to obtain obvious and well-defined lesions. Animals were treated for 20 days. Size of ulcers was monitored every week and the measurements at the beginning and at the end of the treatment period were used for analysis with a T test. n= number of mice in each group, SD. Standard deviation in mm, p. t test p value to evidence the statistic differences between initial and final lesion size in each group of mice.

Treatment Group		N	Mean (mm)	SD	p
Cream alone	Initial lesion	6	3,83	1,36	
	Final lesion	6	6,07	0,94	0,002
Empty nanoemulsion ointment	Initial lession	12	4,99	1,92	
	Final lesion	12	6,93	2,94	0,001
MA nanoemulsion	Initial lession	21	5,96	1,12	
	Final lesion	21	5,61	1,83	0,455
MA IP	Initial lession	15	6,44	1,40	
	Final lesion	15	4,61	1,47	0,005

Table5. Parasite burden in mice skin after treatment period. BALB/c mice infected skin with *Leishmania (L.) mexicana MHOM/BE/82/BEL-21* or *Leishmania (L.) amazonensis MHOM/BR/73/M2269* were performed in a little part of lesion of each mouse after treatment period. The tissue was frozen at -20°C until DNA extraction. DNA was extracted with High Pure PCR Template Preparation kit (Roche Applied Science). To detect the presence of parasite specific DNA, real time PCR was performed to amplify a 120-bp DNA fragment from *Leishmania* species kinetoplast minicircles. Parasite in mice skin were quantified using Light Cycler 2.0 instrument.

TREATMENT GROUP	DNA (ng/uL)	PARASITE BURDEN	MEAN	TREATMENT GROUP	DNA (ng/uL)	PARASITE BURDEN	MEAN
Cream alone (n=5)	23,18	4,36E+04	2,70E+05	Empty Nanoemulsion (n=8)	10,76	1,44E+05	1,41E+05
	45,53	2,82E+04			44,62	1,10E+05	
	26,79	7,32E+05			35,67	1,61E+05	
	43,49	4,50E+04			16,86	1,28E+05	
	38,75	5,03E+05			43,42	1,09E+04	
					31,08	3,33E+05	
					32,88	8,36E+04	
					33,56	1,60E+05	
MA Nanoemulsion (n=15)	45,75	2,16E+02	1,54E+05	MA injected IP (n=14)	44,25	3,28E+05	1,03E+05
	28,82	4,01E+04			45,15	3,12E+05	
	39,43	4,13E+05			28,82	3,28E+04	
	44,17	1,13E+04			45,53	3,23E+03	
	44,77	3,28E+04			38,98	4,72E+05	
	30,10	1,07E+05			43,49	1,25E+05	
	44,70	4,16E+03			46,50	6,34E+02	
	32,88	3,95E+04			40,56	5,28E+03	
	38,30	7,15E+04			39,13	2,32E+04	
	37,47	6,51E+05			45,98	3,96E+01	
	23,93	9,00E+04			44,70	6,26E+04	
	39,28	2,22E+05			25,51	2,69E+03	
	41,09	2,85E+04			32,73	7,18E+04	
	30,55	2,25E+05			22,35	6,53E+03	
	17,16	3,75E+05					

Table6. Histological variations reported in BALB/c mice cutaneous lesions. BALB/c mice infected skin with *Leishmania (L.) mexicana MHOM/BE/82/BEL-21* or *Leishmania (L.) amazonensis MHOM/BR/73/M2269* were performed in a little part of lesion of each mouse after treatment period. The tissue was embedded in paraffin block and then it was cut in 4mm layers in order to perform a hematoxylin – eosin stain. These stained samples were analyzed by an expert pathologist. All samples with reports grouped in nil or mild category was defined like negative reports for histological variations observed. On the other hand, the presence of changes in histology parameters were categorized in groups defines like moderate o severe ones. n= represents number of mice.

Histology issue \ Treatment group		Cream alone n (%)	Empty nano-emulsion n (%)	Glucantime-nanoemulsion n (%)	Glucantime IP n (%)
Dermal atrophy	Positive	1(16.7)	1(4.3)	3(18.8)	7(46.7)
	Negative	5 (83.3)	6(85.7)	13(81.3)	8(53.3)
Hiperkeratosis	Positive	1(16.7)	2(28.6)	1(6.3)	7(46.7)
	Negative	5(83.3)	5 (71.4)	15(93.8)	8(53.3)
Extracelular parasites	Positive	5(83.3)	7(100)	12(75.0)	12(80.0)
	Positive	1(16.7)	0(0.0)	4(25.0)	3(20.0)
Linphocyte cells infiltration	Positive	3(50.0)	0(0.0)	2(12.5)	6(40.0)
	Negative	3(50.0)	7(100)	14 (87.5)	9(60.0)
Plasma cells infiltration	Positive	3(50.0)	2(28.6)	4(25.0)	6(40.0)
	Negative	3(50.0)	5(71.4)	12(75.0)	9(60.0)
Macrophages withparasites	Positive	5(83.3)	7(100)	12(75)	12(80.0)
	Negative	1(16.7)	0 (0.0)	4(25)	3(20.0)
neutrophyl cells infiltration	Positive	2 (33,39	0(0.0)	1(6.3)	3(20.0)
	Negative	4 (66,7)	7(100)	15(93.8)	12(80.0)
Eosinophil cell infiltration	Positive	0(0.0)	0(0.0)	1(6.3)	0(0.0)
	Negative	6(100)	7(100)	15(93.8)	15(100)

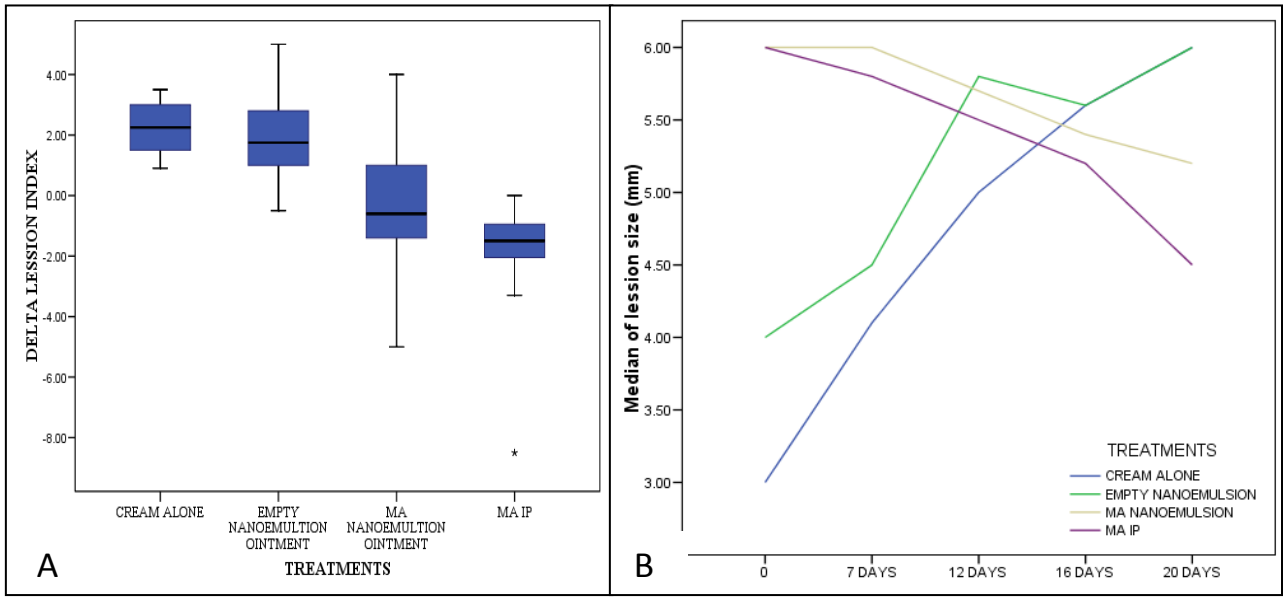


Figure1. Lesion size differences in infected BALB/c mice. BALB/c mice were infected with *Leishmania (L.) mexicana MHOM/BE/82/BEL-21 (n=24)* or *Leishmania (L.) amazonensis MHOM/BR/73/M2269 (n-30)* were divided in four groups of treatment: 1. Cream alone, 2. Empty nanoemulsion ointment, 3. Meglumine antimonate (MA) nanoemulsion ointment, 4. Intraperitoneal injections of meglumine antimonate (MA IP). A) **Delta values of lesion size.** This index was calculated by subtracting the largest diameter of the ulcers at the end of the treatment period to the largest diameter at the beginning of the study. Increase in lesion size is represented by positive values and lesion size decrease is represented by negative values (n=55 mice). * Lesion size differences were statistically meaningful (p<0,05). B) **Median of lesion size:** Lesion size in each infected mouse was measured during treatment application period (0, 7, 12, 16, 20 days). Median of lesion size for each group of treatment was calculated and plotted to observe the changes in lesion trough the treatment period.

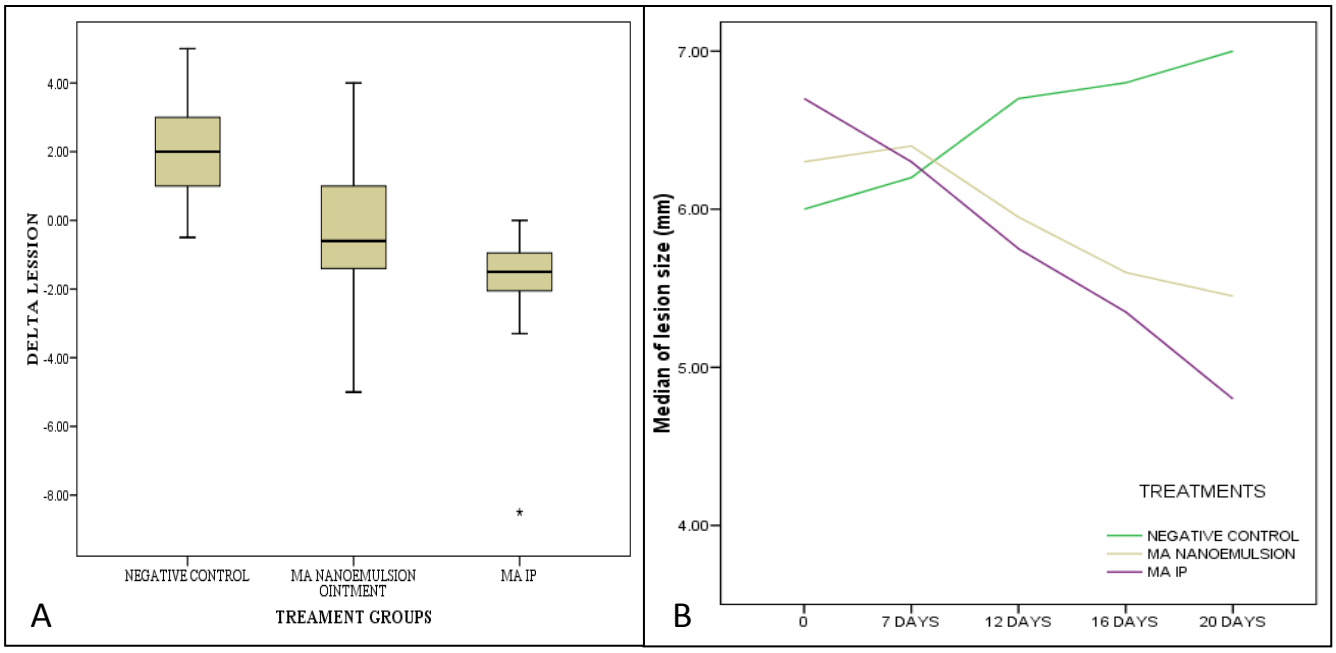


Figure2. **Lesion size differences in infected BALB/c with lesion greater than 6.0mm.**

Group of 28 *Leishmania* infected mice with an initial lesion sizes between 6.0 – 7.8mm was selected for a better data analysis. Groups of treatment application were 1. Empty nanoemulsion ointment like negative control. 2. Meglumine antimonate (MA) nanoemulsion ointment, 3. Intraperitoneal injections of Meglumine antimonate (MA IP). A) The boxes represent 1.5 of interquartile range in the distribution of delta values of lesion size. This index was calculated by subtracting the largest diameter of the ulcers at the end of the treatment period to the largest diameter at the beginning of the study. Increase in lesion size is represented by positive values and lesion size decrease is represented by negative values. * Lesion size differences were statistically meaningful ($p < 0,05$). B) **Median of lesion size:** Lesion size in each infected mouse was measured during treatment application period (0, 7, 12, 16, 20 days). Median of lesion size for each group of treatment was calculated and plotted to observe the changes in lesion trough the treatment period.

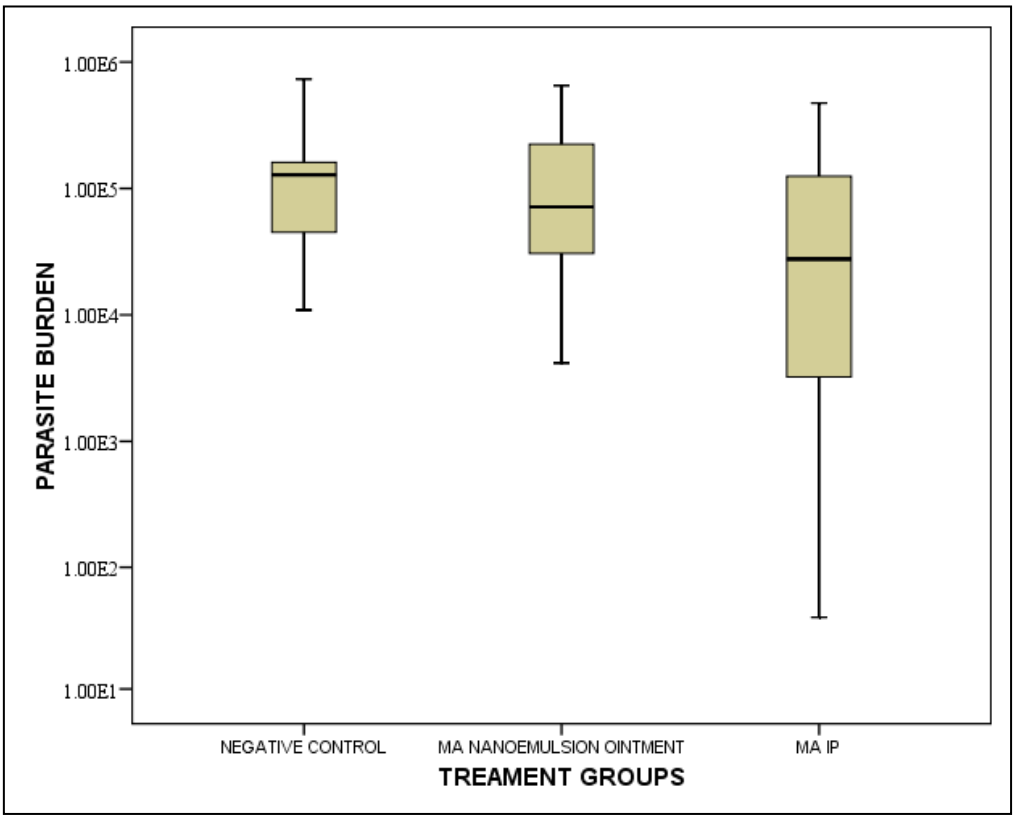


Figure3. **Parasite burden in BALB/c mice skin after treatment period.** At the end of treatment period, a group of 28 Balb/c mice infected with *Leishmania (L.) mexicana MHOM/BE/82/BEL-21 (n=12)* or *Leishmania (L.) amazonensis MHOM/BR/73/M2269 (n-16)* with an initial lesion size greater than 6,00 mm, were sacrificed according international protocols. Parasite burden was quantified by Real time PCR (Light Cycler 2.0, Roche – Diagnostics) in skin samples of each mice cutaneous lesion. Each box represents a different treatment group: 1. Negative control (Empty nanoemulsion ointment). 2. Meglumine antimonato nanoemulsion. 3. Intraperitoneal administration of Meglumine antomonate. Median of parasite burden is represented by an horizontal line inside the boxes. Bars represent the 95% of the data in the distribution. (1,75 interquartile range).

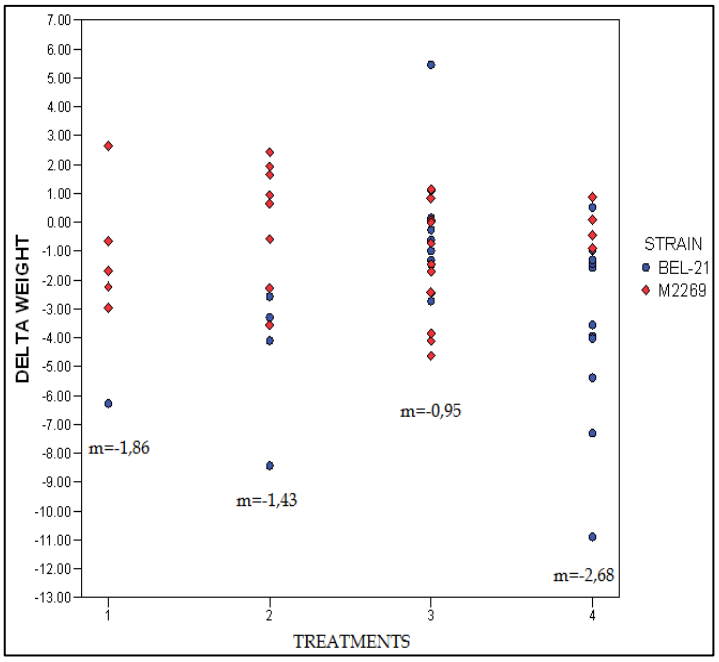


Figure4. Delta weight index for each mouse infected with *Leishmania spp*. 54 Balb/c mice were infected with *Leishmania (L.) mexicana MHOM/BE/82/BEL-21 (n=24)* or *Leishmania (L.) amazonensis MHOM/BR/73/M2269 (n-30).* 10 weeks after initial infection mice were randomly divided in four treatment group. Groups: 1. Cream alone; 2. Empty nanoemulsion ointment; 3. Meglumine antimonate nanoemulsion ointment; 4. Daily IP injection of meglumine antimonate in a dosis of 20mg Sb^{v}/Kg/day. To evaluate the life conditions of mice in the experiment, mice weight was weekly registered. The final weight value minus the initial one in the treatment period was calculated to obtain a delta weight index. Negative values means lose of weight during the treatment period and positive ones means a gain of weight during the treatment period. There were no statistical differences in weight between treatment groups. BEL-21: Mice infected with *Leishmania (L.) mexicana MHOM/BE/82/BEL-21; M2269:* Mice infected with *Leishmania (L.) amazonensis MHOM/BR/73/M2269; m:* mean value of delta weight in each treatment group of mice.

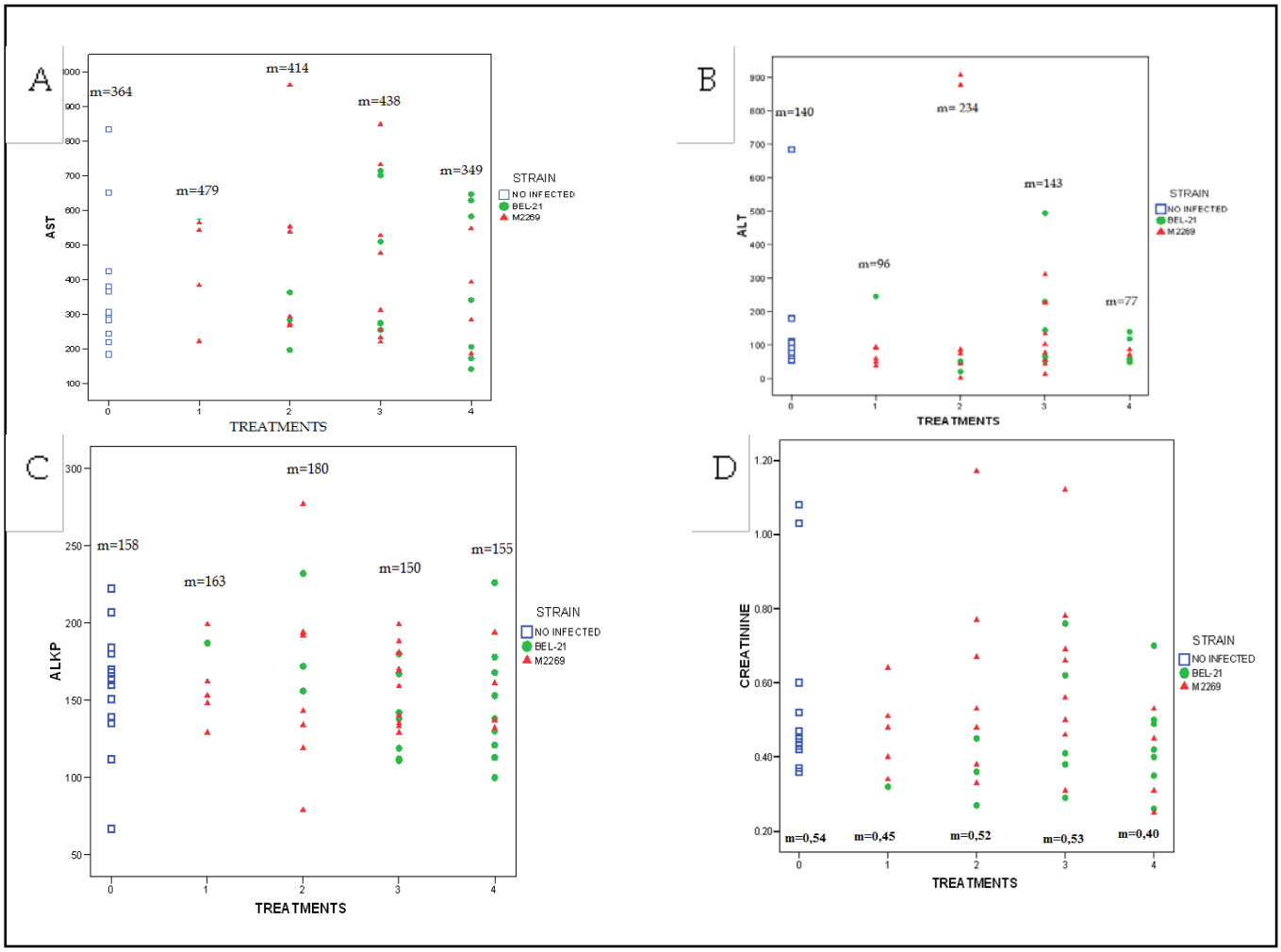
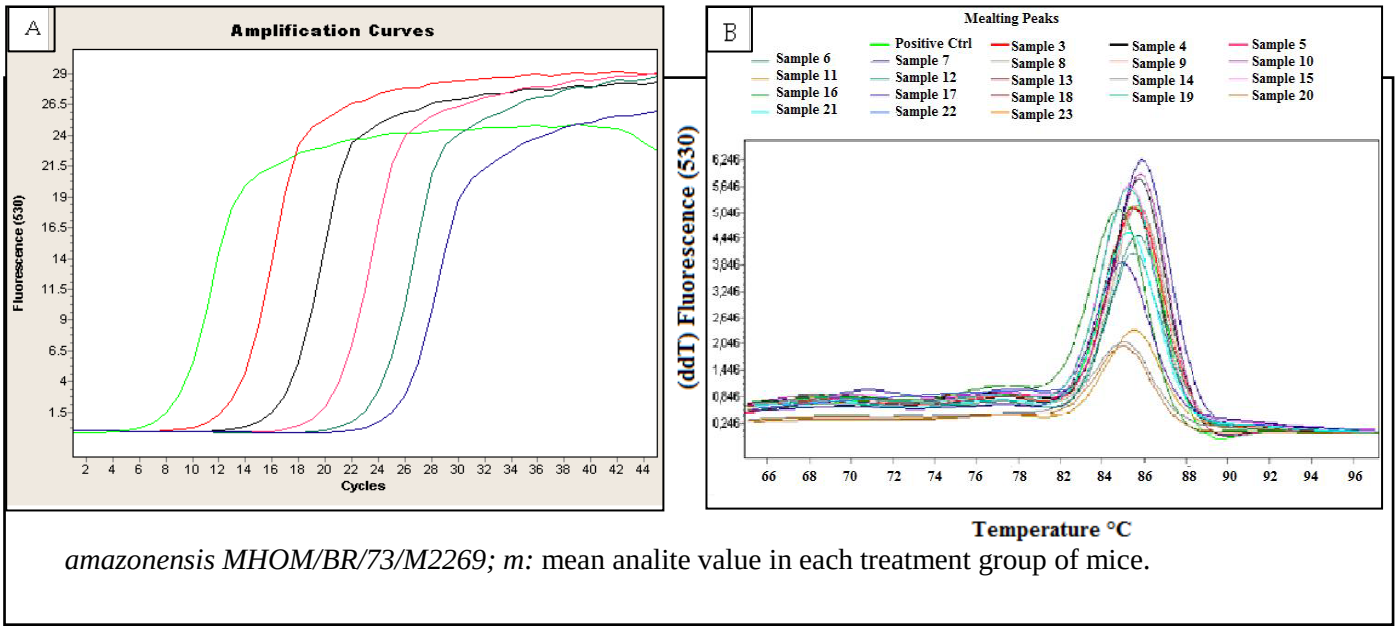


Figure5. Hepatic enzymes and creatinine results in mice blood after treatment period. Fifty four Balb/c mice infected with *Leishmania (L.) mexicana MHOM/BE/82/BEL-21 (n=24)* or *Leishmania (L.) amazonensis MHOM/BR/73/M2269 (n-30)* were randomly divided in four treatment groups: 1. Cream alone; 2. Empty nanoemulsion ointment; 3. Meglumine antimonate (MA) nanoemulsion ointment; 4.Intraperitoneal administration of MA (dosis of 20mg Sb^{v}/Kg/day). To evaluate the toxic effect of MA administration, after treatment period blood mice were collected following standard methods in animal research. Serum were separated and used for A. Alanine aminotranferase (ALT), B. Aspartate aminotransferase (AST), C. Alkaline phosphatase (ALKP) and D. Creatinine analysis (Vitros 250, Johnson & Johnson). As reference 13 mice without infection (Group 0) were slaughtered to obtain serum for enzyme and creatinine reference values. There were no meaningful differences when enzyme and creatinine values were compared between treatment and reference groups. BEL-21: Mice infected

with *Leishmania (L.) mexicana MHOM/BE/82/BEL-21; M2269:* Mice infected with *Leishmania (L.)*



amazonensis MHOM/BR/73/M2269; m: mean analite value in each treatment group of mice.

Figure6. Standard curve for quantification of parasite burden by Real Time PCR and specific amplification of *Leishmania* DNA. At the end of treatment period, a group of 28 Balb/c mice infected with *Leishmania (L.) mexicana MHOM/BE/82/BEL-21 (n=12)* or *Leishmania (L.) amazonensis MHOM/BR/73/M2269 (n-16)* with an initial lesion size greater than 6,00 mm, were sacrificed according international protocols. Parasite burden was quantified by Real time PCR (Light Cycler 2.0, Roche – Diagnostics) in skin samples of each mice cutaneous lesion. A) A standard curve for quantification was determined with parasite DNA extracted of serial dilutions of counted parasites. The quantification of parasites was done through simple count in hemocytometer chamber. B) Mealting peaks resulted of the specific amplification of DNA of mice skin sample. The melting temperature for amplification of 120-bp fragment of the minicircle kDNA of *Leishmania spp.* was 84ºC – 86ºC, unspecific products could be observed with mealting peaks at 79ºC.