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Nitric-oxide releasing chitosan nanoparticles towards effective treatment of cutaneous leishmaniasis

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ABSTRACT

Cutaneous leishmaniasis (CL) is a major public health problem caused by *Leishmania* parasites that produce destructive and disfiguring skin conditions. There is an urgent need for alternative topical therapies due to the limitations of current systemic treatments. Recently, we have synthesized nitric oxide-releasing chitosan nanoparticles (NONPs) and shown their potential *in vitro* against *Leishmania amazonensis*. Herein we evaluated the application of NONPs for the treatment of CL on infected BALB/c mice. Mice were treated with topical administration of increasing concentrations of NONPs and disease progression was investigated regarding parasite load, lesion thickness, and pain score. As a result, we observed a dose-dependent NONPs effect. Parasite burden and lesion thickness were substantially lower on animals receiving NONPs at a 2 mM concentration compared to untreated control. Moreover, the clinical presentation of the lesions did not show any visible signs of ulcer, suggesting clinical healing in these animals. This successful outcome was sustained for at least 21 days after therapy even in one single dose. Thus, we demonstrate that NONPs are suitable for topical administration, and represent an attractive approach to treat CL.

1. Introduction

Cutaneous leishmaniasis (CL) is a widespread vector-borne disease transmitted by infected phlebotomine sandflies [1]. The etiologic agents of CL comprise several species of parasites of the genus *Leishmania* that are infectious to a broad range of mammalian hosts, including humans and animals [2]. Tropical and subtropical countries such as Brazil, Colombia, Peru, Afghanistan, Syrian, Algeria, Iran, Pakistan, and Saudi Arabia are the most endemic regions [3,4].

Cutaneous manifestations and patients' prognosis depend on both, the host immune response and the *Leishmania* spp. involved [5]. There are several clinical manifestations, ranging from a single ulcerative lesion, such as localized cutaneous leishmaniasis up to more severe forms, like disseminated leishmaniasis, mucocutaneous leishmaniasis, or diffuse cutaneous leishmaniasis [6]. In the latter, parasites grow uncontrollably within non-ulcerating nodules across the body [6,7].

The current scenario for CL therapy comprises pentavalent antimonials as a first-line choice [8]. Amphotericin B (liposomal or deoxycholate) or pentamidine are usually administered for unresponsive cases [9]. However, drug resistance and relapse cases have been increasingly reported over the past years [9,10]. Besides, limitations of therapy have been a real concern since they are long-term, expensive, toxic, and require parenteral administration [10].

Despite its huge prevalence, the cutaneous form is not usually life-threatening. However, the life-long scars on exposed areas of the body (*i.e.* face, arms, hands, and legs) bring a serious social stigma, leading to depression, anxiety, and/or other mental health disorders [11]. Since it is a poverty-related disease, it receives little attention from government and healthcare systems [1,10]. Nevertheless, CL is rated as one of the neglected tropical diseases that urgently require new treatments, being on the priority list of the World Health Organization for development and search for new antileishmanial drugs [1,3].

Abbreviations: CL, cutaneous leishmaniasis; CS, chitosan; iNOS, inducible nitric oxide synthase; LaLUC, *L. amazonensis* expressing luciferase gene; MSA, mercaptosuccinic acid; NPs, nanoparticles; NONPs, NO-releasing nanoparticles; NTA, nanoparticle tracking analysis; PV, parasitophorous vacuole; RNS, reactive nitrogen species; RSNOs, S-nitrosothiols; S-nitroso-MSA, S-nitroso-mercapsotosuccinic acid.

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Nitric oxide (NO), an endogenous molecule, has been under the spotlight as a potential compound to target *Leishmania* spp. [12,13]. It has been demonstrated that *in vivo* resistance or susceptibility to *Leishmania* infection is closely related to Th1 or Th2 T cell response, respectively [15]. Th1 T cell subsets release interferon-γ (IFN-γ) that upregulates inducible nitric oxide synthase (iNOS) [14,15]. The synthesis of iNOS in mononuclear phagocytes (primarily macrophages) catalyzes the oxygenation of L-arginine to produce NO and citruline [5,16]. Indeed, NO plays a pivotal role in infection control [17]. It can disrupt *Leishmania* mitochondrial respiration, naturally inducing a lethal metabolic inactivation limiting parasite proliferation [17,18]. In contrast, Th2 cell response leads to NO inhibition promoting susceptibility to infection, allowing parasites proliferation within the host [18].

As a free radical of short-life, NO is likely to be oxidized and inactivated in biological environments when exogenously administered [19]. In this regard, NO donors have arisen to overcome such challenges, providing more stability over NO releasing [20,21]. Indeed, we have recently entrapped a NO donor, S-nitrosothiol (S-nitroso-mercaptosuccinic acid, S-nitroso-MSA), into chitosan (CS) nanoparticles (NPs) to develop S-nitroso-MSA CSNPs (here defined as NONPs). The NO donor encapsulation into CSNPs prevents molecule degradation and allows a more controlled NO-releasing. Also, we have demonstrated the great potential of NONPs in effectively inactivating *in vitro Leishmania amazonensis,* one of the most important causative agents of CL [22]. For that reason, we have been encouraged to further evaluate the susceptibility of *L. amazonensis* to NONPs *in vivo*.

In this work, BALB/c mice were infected in the left paw with *L. amazonensis* parasites and treated topically with increasing concentrations of NONPs. Then, the disease progression was evaluated by measuring lesion thickness, pain score, and parasite burden using real-time bioluminescence.

2. Materials and methods

2.1. Synthesis and characterization of CSNPs

As reported in previous studies, the precursor of NO donor molecule (MSA) was encapsulated into CSNPs by adding MSA in a solution of CS throughout the synthesis of nanoparticles NPs [22–25]. MSA at 66.67 mM and an aqueous solution of CS at 1.0 mg/mL were dissolved in 1% acetic acid at room temperature and stirred for 90 min. Then, 0.6 mg/mL of sodium tripolyphosphate was added dropwise to the CS/MSA solution previously prepared.

The hydrodynamic size and concentration of MSA encapsulated CSNPs were evaluated by nanoparticle tracking analysis (NTA LM-20, NanoSight Ltd., UK). The size distribution of the CSNPs was obtained on a particle-by-particle basis. NTA enables separation of particle population by size and intensity, microscopical visualization of individual CSNPs in suspension and simultaneously determining their Brownian motion [22]. The curve of particle concentration per hydrodynamic size was fitted using a LogNormal model with R^2 higher than 0.9.

2.2. Nitrosation of MSA encapsulated CSNPs

Nitrosation of the thiol group of encapsulated MSA at 50 mM was carried out by the addition of an equimolar concentration of sodium nitrite producing S-nitroso-MSA containing CSNPs, named in this manuscript, NONPs. By using a UV–Vis spectrophotometer (Agilent 8454, Palo Alto, CA, USA) at either 336 nm ($\varepsilon = 980.0$ L mol^{-1} cm^{-1}) or 545 nm ($\varepsilon = 18.4$ L mol^{-1} cm^{-1}), S–NO group absorption bands were detected, confirming then the generation of encapsulated S-nitroso-MSA [22].

2.3. Kinetics of NO release

The NO release profile from free and encapsulated S-nitroso-MSA (at 1 mM) was evaluated by spectroscopic method, by following the spectra changes at λ= 336 nm, associated with the S-NO cleavage and NO release [22]. The samples were transferred to a quartz cuvette and absorbance changes at λ= 336 nm were acquired every 30 min. The sample holder was kept at 37.0±1 °C for 12 h in the dark. The concentration of NO released was calculated from Eq. (1). Each point of the kinetic curve is the average of three independent experiments. The data were expressed as the percentage of total NO that was released.

$$[NO]t = \frac{Abs_{t=0} - Abs_{t=t}}{18.4} \times 100 \quad (1)$$

2.4. Parasite culture and in vivo infection

Promastigotes of L. *amazonensis* recombinant strain expressing the luciferase gene (La-LUC) were grown in M199 medium (Sigma-Aldrich, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco Invitrogen Corporation, USA), hemin (0.005%) (Sigma-Aldrich, USA), HEPES (40 mM pH 7.4) (Sigma-Aldrich, USA), and penicillin/streptomycin 100 μg/mL (Sigma-Aldrich, USA). Parasites were incubated at 25 °C until the stationary growth phase [22].

Animal experimentation was approved by the Ethical Committee on Animal Use from IPEN-CNEN/SP. Sixteen BALB/c mice (6–8 weeks old) were infected by 10^6 stationary phase promastigotes. Parasites were injected in the left hind paw and disease progression was monitored for 4 weeks (28 days) until the development of lesions. Then, they were randomly allocated to four groups ($n = 4$ per group):1) Control-infected and untreated; and other three treated groups that were divided according to the concentration of NONPs applied, respectively: 2) NONPs 0.4 mM, 3) NONPs 1 mM, and 4) NONPs 2 mM. In the present work, the molar concentrations refer to the molar concentrations of S-nitroso-MSA for NONPs.

2.5. Treatment and disease progression

On day 0, nitrosation of encapsulated MSA (50 mM) was performed and NONPs were kept in the fridge at 4 °C, protected from light for 60 min. Then, NONPs were diluted in phosphate buffer saline (PBS) according to the concentrations used for each treated group (0.4 mM, 1 mM, and 2 mM). After nitrosation, mice were treated with one single administration by injecting topically 20 μL of NONPs in the infected paw. The control group received 20 μL of PBS. Then, mice were monitored for the following 3 weeks in terms of parasite burden, lesion thickness, and pain score.

Parasite burden was monitored in real-time by quantifying luciferase activity through bioluminescence imaging (IVIS Spectrum; Caliper Life Sciences, USA). Three days before treatment, imaging was carried out to monitor the presence of parasite burden in all animals. Then, imaging was performed on the treatment day and once a day for 5 consecutive days. Afterward, parasite burden was assessed once a week in the next 3 weeks (or 21 days), as described in Fig. 1.

Before luminescence detection, 100 μL of luciferin (VivoGlo, Promega Corporation, USA) at a dose of 75 mg/kg was intraperitoneally injected in each animal. Then, mice were anesthetized with isoflurane (2.5% induction and 1.5% maintenance). Twenty-min after luciferin injection, images were acquired using 2 min of exposure time in a high-resolution mode, and total photon emission was analyzed over a region of interest and quantified using a living image software version 4.3.1 (Caliper Life Sciences). Results were expressed as photons/second/square centimeter/steradian (ph/sec/cm^2/sr) [26].

Nociceptive sensibility was assessed with von Frey filaments, which are frequently used to evaluate pain upon a given mechanical stimulus [27]. These stiff filaments with calibrated forces were applied directly to the lesion and surrounding areas at 5 different forces: 10, 15, 26, 60, and 100 g. The force is directly proportional to the stiffness, and it is associated with the thickness of filaments [27]. When we observed the paw

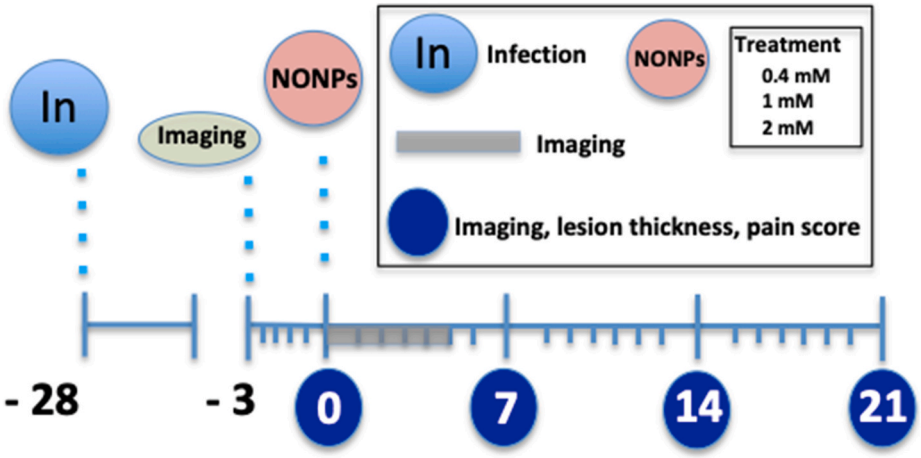

Fig. 1. Chronological timeline and experimental design. Mice were infected in the left hind paw with La-LUC (1 × 10 [6] promastigotes) and monitored for 28 days, until the development of the lesion. Three days before treatment, imaging was performed to assess bioluminescence detection. On day 0, except for control (infected and untreated), animals were treated receiving different NONPs concentrations according to each experimental group (0.4 mM, 1 mM, and 2 mM), in one single dose. Parasite burden was monitored over time by bioluminescence every day for 5 days. Then, parasite burden, lesion thickness, and pain score were carried out once a week for 21 days.

withdraw and/or a sustained leg retraction/reflex, the response to the stimulus was considered positive. The pain was evaluated once a week for 3 weeks. Then, a pain score (1–6) was set to quantify hyperalgesia, in which scores 1 and 6 were associated with the lowest and highest (severe) nociceptive sensibility, respectively, as shown in Table 1.

Lesion thickness was evaluated once a week for 3 weeks. Lesion thickness was obtained by measuring the differences between infected and contralateral non-infected paw with a caliper as follows in Eq. (2):

$$Lesion\ thickness\ (mm) = Pi - Pc \quad (2)$$

In which Pi is the infected paw and Pc is the contralateral uninfected one of the same animal [38].

2.6. Statistical analysis

We used one-way ANOVA of repeated measures to compare outcomes of experimental groups over time. We used Tukey's test to assess statistically significant differences. Statistical analysis was carried out using GraphPad Prism 7 software and results were considered statistically significant when $p < 0.05$.

3. Results

The CSNPs showed a hydrodynamic size of 137.8 ± 1.1 nm acquired by the NTA technique (Fig. 2a). The nanoparticles showed low aggregation and spherical shape. These results are in accordance with other authors, who showed CSNPs containing S-nitrosothiols with a hydrodynamic size around 120–130 nm [22,28]. The CSNPs were then nitrosated to yield NONPs able to release NO.

Fig. 2b shows the NO release profile from free and encapsulated S-nitroso-MSA. As can be observed, the incorporation of the NO donor into the nanoparticles promoted a sustained NO release. The NO release profile shows a linear increase in the first 6 h and a constant influx of NO until 12 h. From 6 to 12 h, the amount of NO released was around 200 to 400 μM. In contrast, free S-nitroso-MSA released higher levels of NO from 3 h (around 790 μM). After 12 h, the NO level increased slightly to 870 μM.

Table 1
Pain score for Von Frey filaments referred to force scale.

Pain Score	Force (g)
1	>100
2	60–100
3	26–60
4	15–26
5	10–15
6	<10

Figs. 3 and 4 display the parasite load over the experimental period. We monitored parasite load every day in the first week to identify whether a second NONP application would be necessary (Fig. 3). Additionally, *Leishmania* parasites can adapt to different environments, evade the host immune system, become resistant to drugs, and proliferate very fast. Hence we also monitored parasite load every week (Fig. 4). As the *Leishmania* strain expresses the luciferase gene, this allowed us to monitor the same animals overtime for the entire period.

Results show an outstanding activity of NONPs at all concentrations applied over the first 5 days post-treatment. Fig. 3a shows that in the control (untreated) group, the parasite population underwent exponential growth, resulting in an average growth rate of one-log (from 3.9 × 10^5 to 3.7 × 10^6 ph/s/cm^2/sr) regarding the days - 3 and 5, respectively.

In contrast, we observed a different pattern for NONPs. Lower rates of growth are shown between days - 3 and 1 at 0.4 mM and 1 mM, reaching a stationary phase until the 4th day. Then, a slight increase occurs on the 5th day for both concentrations. Despite that, parasite load remained lower than control over this period, pointing to statistically significant differences between both treated groups and control. In fact, NONPs antileishmanial effect seems to be dose-dependent, which becomes more evident when we analyze the overall parasite proliferation rate at 2 mM. Interestingly, at 2 mM, NONPs were able to reduce in 48.8% the parasite load (from 3.9 × 10^5 to 2.0 × 10^5) on the first day following treatment, remaining low until the 5th day. As a result, there were differences statistically significant between the control and NONPs 2 mM group, estimated in approximately 1.5-log (Fig. 3a).

This remarkable outcome is also reflected in the presence of a higher bioluminescence signal in the control group in comparison with treated ones. The red color at the center of the control's infected paw refers to a high number of parasites in that region that decreases in the surrounding areas. Besides, the luminescence signal is brighter and the distribution of light covers a larger area as related to that of treated groups. Indeed, groups treated with NONPs at 0.4 mM and 1 mM presented similar signals on the 5th day after therapy. It is noteworthy that no luminescent signal was detected on animals receiving NONPs at 2 mM, leading to a successful result even at only one dose (Fig. 3b).

As a follow-up study, parasite burden was monitored for 21 days after therapy. Fig. 4a shows the overall effect of NONPs throughout this preclinical trial, in which control reaches a stationary phase between 8 and 17 days, showing an upward trend again over the rest of the experimental period. Although groups receiving 0.4 mM and 1 mM

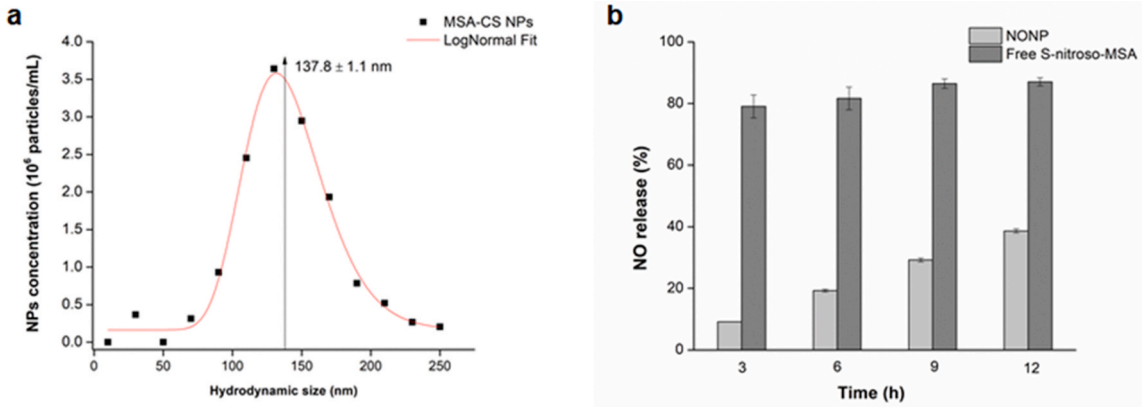

Fig. 2. (a) Characterization of CSNPs hydrodynamic size by NTA; (b) NO release profile from free and encapsulated S-nitroso-MSA (1 mM) for 12 h of monitoring.

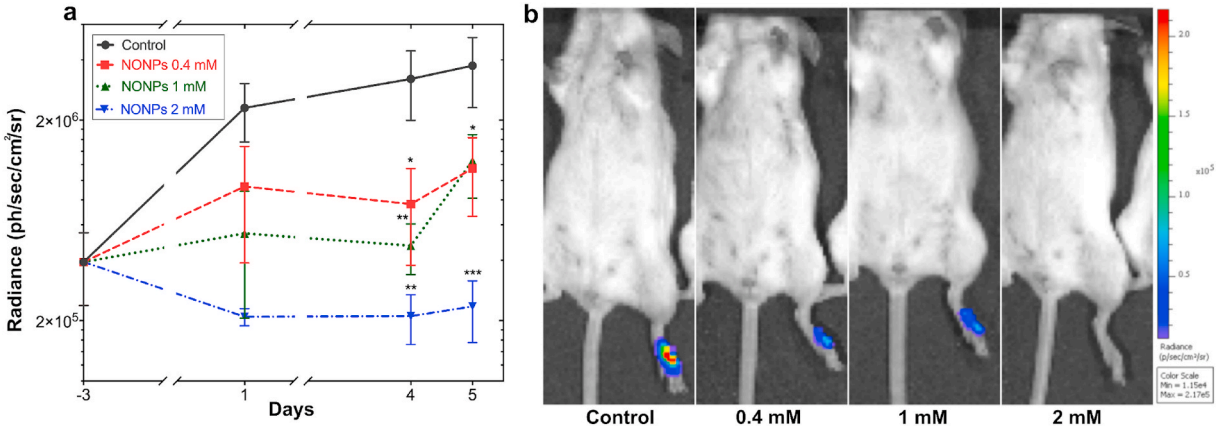

Fig. 3. NONPs antileishmanial activity against La-LUC-infected BALB/c mice. (a) Values represent parasite burden mean ± SEM. Statistically significant differences observed between control and treated groups (*p < 0.05, **p < 0.01, ***p < 0.001). (b) Bioluminescence images of untreated control and NONPs groups 5 days post-treatment. BALB/c mice were infected with 1 × 10 [6] La-LUC stationary promastigotes in the left paw. Treatments were carried out at three different NONPs concentrations (0.4 mM, 1 mM, and 2 mM), in one single dose. Bar on the right side of "b" refers to a color scale representing light intensities expressed as photons/second/square centimeter/steradian (ph/sec/cm^2/sr). n = 4 animals/group.

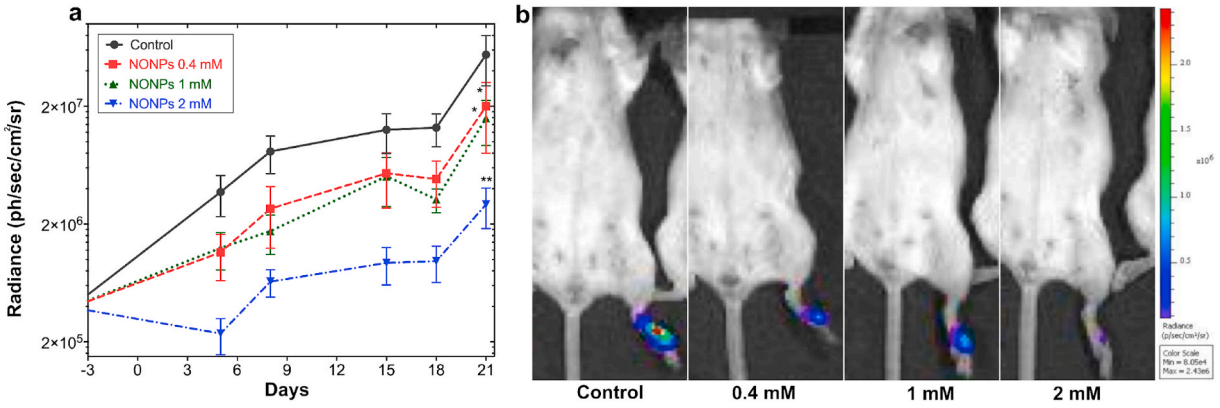

Fig. 4. NONPs antileishmanial activity against La-LUC-infected BALB/c mice. (a) Values represent parasite burden mean ± SEM. Statistically significant differences observed between control and treated groups (*p < 0.05, **p < 0.01). (b) Bioluminescence images of untreated control and NONPs groups 21 days post-treatment. BALB/c mice were infected with 1 × 10 [6] La-LUC stationary promastigotes in the left paw. Treatments were carried out at three different NONPs concentrations (0.4 mM, 1 mM, and 2 mM), in one single dose. Bar on the right side of "b" refers to a color scale representing light intensities expressed as photons/second/square centimeter/steradian (ph/sec/cm^2/sr). n = 4 animals/group.

presented a similar behavior, parasite burden remained 64.9% (1.9 × 10^7 ph/s/cm^2/sr) and 72.3% (1.5 × 10^7 ph/s/cm^2/sr) lower than control (5.4 × 10^7 ph/s/cm^2/sr), respectively, revealing statically significant differences even 21 days following NONPs administration. Impressively, the sustainable NONPs activity against parasites provided a superior outcome for mice treated at 2 mM concentration. Although after 5 days parasites started proliferating again, the growth rate was significantly lower compared to the control group, resulting still in a 1.5-

log difference between these groups even after 21 days (Fig. 4a).

Imaging also provided further substantial information regarding the long-lasting effects of NONPs. As shown in Fig. 4b, at the end of the experimental period, the control group exhibited a high luminescence signal over the entire paw, whereas for 0.4 mM and 1 mM groups, luminescence is detected only at the center of the animal's paw. Additionally, the red colors surrounded by yellow-green ones indicate an increased level of parasitic load within this area of untreated animals. Unlike control, NONPs at 0.4 mM and 1 mM presented only blue-green intensities, referring to a lower light signal. In contrast, bioluminescence signals were barely detected in a very specific area for the 2 mM group. Localized at the center of the paw, the blue-colored region clearly shows that the number of parasites was substantially lower compared to the other groups after 21 days.

Disease progression was also evaluated by measuring lesion thickness and pain score every week. Lesion has nearly doubled in thickness 7 days after treatment in all groups: Control (0.5 mm–0.95 mm), NONPs 0.4 mM (0.44 mm–0.87 mm), NONPs 1 mM (0.42 mm–0.76 mm) and NONPs 2 mM (0.43 mm–0.7 mm). Except for NONPs 2 mM, a similar trend was observed in all groups throughout the experiment. The upward trend remained for control and NONPs 0.4 mM up to the end of the experiment, both showing lesion thickness of an average of around 1.1 mm (Fig. 5a).

However, twenty-one days post-treatment, lesions tended to have a smaller thickness than the control for groups NONPs 1 mM (0.95 mm) and NONPs 2 mM (0.25 mm), reducing in 11.7% and 71.4%, respectively, compared to the 14th day. In fact, animals receiving 2 mM presented statistically significant differences, of which lesion thickness resulted in an average of values similar to a non-infected paw (Fig. 5a).

Pain score revealed similar tendencies for all groups. Seven days after therapy, groups receiving NONPs at 0.4 mM and 2 mM demonstrated to be less tolerant to the nociceptive stimulus provoked by von Frey filaments, where we may observe a strong response, indicating a scale of 6 and 5, respectively. Control and NONPs 1 mM showed a moderated pain with a score of 3 in the first week. However, we noticed a severe (score 5) nociceptive sensibility in all groups on the 14th and 21st days after therapy (Fig. 5b).

The work was also conducted by a clinical analysis of animals during the course of the experiment. Fig. 5c, represents animals' paw at the end of the study, on the 21st day. Non-treated mice of the control group showed massive edema, reflected by a large lesion thickness. The redness of the skin indicates the presence of erythema extending over the paw. There is also the formation of a deep ulcer with a raised outer border and infiltrated plaques. Animals receiving NONPs at 0.4 mM had a similar clinical aspect. Although we might observe an evident paw swelling, the ulcer was smaller and no notable outer border was observed compared to control. In fact, the same trend was observed towards NONPs 1 mM group. Despite the swollen paw, we may perceive the beginning of an ulcer, almost imperceptible and even smaller than control or NONPs 0.4 mM (Fig. 5c).

Indeed, clinical findings support the hypothesis of a dose-dependent effect. The outstanding result was even clearer on animals treated at 2

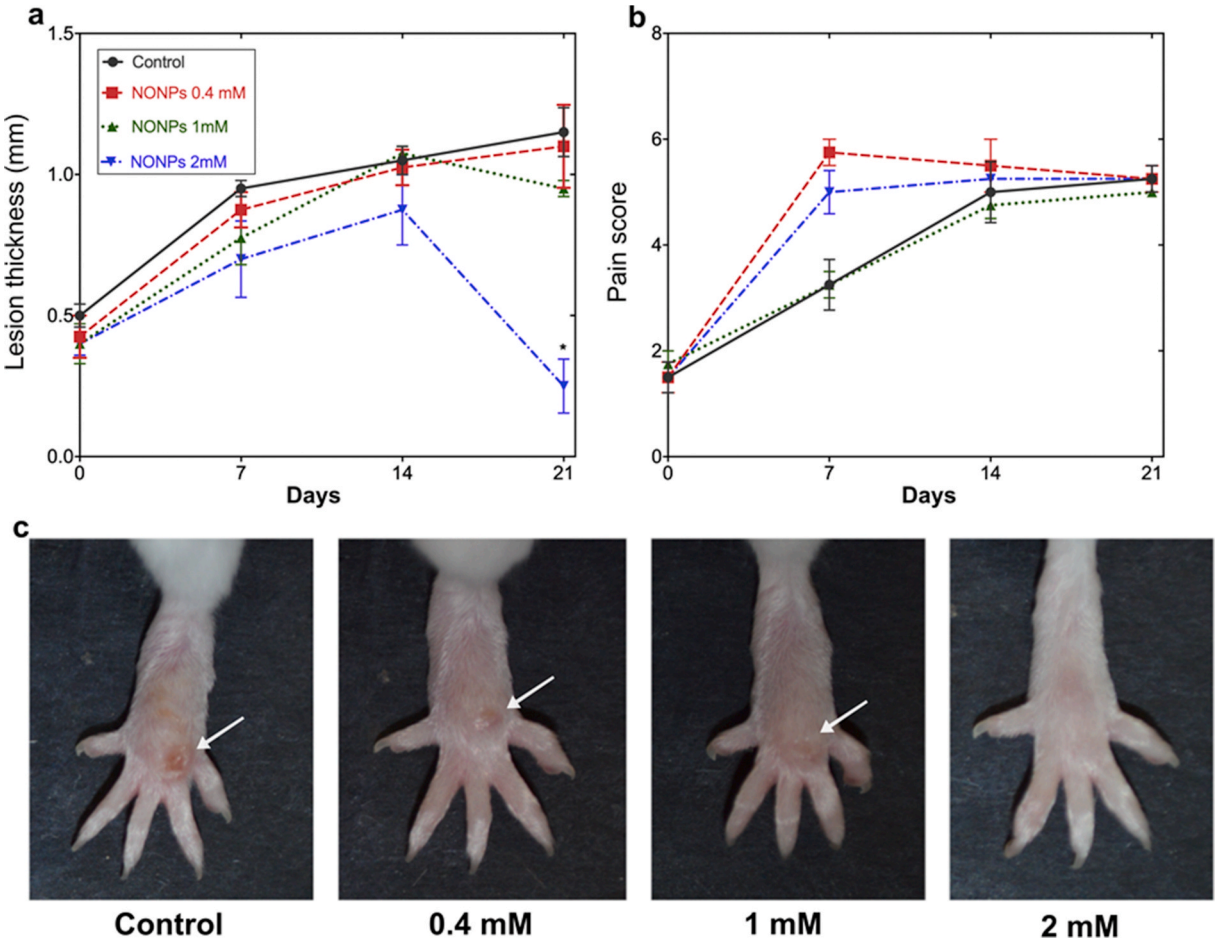

Fig. 5. NONPs activity against La-LUC-infected BALB/c mice. Values represent mean ± SEM of (a) lesion thickness and (b) pain score. Statistically significant differences were observed between control and treated groups (*$p < 0.05$). (c) Clinical presentations of untreated control and NONPs groups 21 days post-treatment. BALB/c mice were infected with 1×10 [6] La-LUC stationary promastigotes in the left paw. Treatments were carried out at three different NONPs concentrations (0.4 mM, 1 mM, and 2 mM), in one single dose. White arrows indicate ulcers developed during the course of infection. n = 4 animals/group.

mM concentration. The sustained NONPs effect led to a remarkable wound healing on animals of this group. Neither erythema nor infiltrated plaques were observed. We also did not find any signs of ulcer or edema. Indeed, paw thickness is very similar to a healthy non-infected one (Fig. 5c).

4. Discussion

The present work aimed to investigate the potential of NONPs in a preclinical trial for CL induced by La-LUC. As a result, we observed an *in vivo* dose-dependent effect. Indeed, at a 2 mM concentration of the NO donor (S-nitroso-MSA), NONPs were able to significantly reduce parasite burden and lesion thickness in one single dose. The positive effect was sustained for at least 21 days after therapy. Additionally, our data indicate that NONPs could be administered weekly.

Recently, we have demonstrated the successful synthesis, characterization, and encapsulation of S-nitroso-MSA into CS NPs to form NONPs. Then, *in vitro* activity was evaluated against La-LUC in both, promastigotes and intracellular amastigotes forms, resulting in an effective killing of parasites at 0.4 mM [22].

Hence we have been motivated to carry on with *in vivo* experiments by starting with the same concentration of 0.4 mM. It is important to note that free chitosan nanoparticles (without NO donor) did not promote pronounced activity against parasites *in vitro* [22]. Therefore, we pursued *in vivo* experiments only with NONPs following the principles of the 3Rs (Replacement, Reduction, and Refinement) for animal experimentation.

As mentioned earlier, all concentrations of NONPs promoted activity against this *Leishmania* strain. However, the highest dose (2 mM) resulted in a remarkable parasite burden (see Figs. 2 and 3) and lesion thickness (see Fig. 5a) reduction, leading to a notable wound healing (see Fig. 5c). Indeed, *in vivo* studies encompass other factors as the higher number of parasites and barriers for the treatment to target the site of infection [29,30]. Besides, the interaction between parasites and the host immune response should be considered [15,30]. Thus, a greater therapeutic response would be expected at a higher dose of NONPs *in vivo* compared to the conditions used for *in vitro* assays.

The susceptibility or resistance of mice to *Leishmania* infection is a balance between the host immune response and *Leishmania* species [15,30,31]. In terms of animal models, BALB/c mice have been widely used for evaluation of antileishmanial drugs in preclinical trials [30]. Cutaneous leishmaniasis induced by *L. amazonensis* in this mouse strain is well known to promote primarily a Th2-type immune response, which makes these animals extremely susceptible to this species [15,29]. Indeed, BALB/c mice present a weak immune response, and lesions are exacerbated. The purpose of using this model for drug screening relies on the idea that if the drug can heal susceptible animals, so it will be a good compound for the resistant ones.

The role of Th1/Th2 signaling pathways is of particular interest regarding CL and NO [4,15]. The production of NO is one of the major mechanisms related to *Leishmania* control [32]. In fact, a Th1 T cell response enables high levels of INF-γ to be released. INF-γ in synergism with tumor necrosis factor-alpha (TNF-α) and lipopolysaccharides activates infected macrophages, which upregulates iNOS to convert L-arginine into NO to kill parasites [14,15,32]. On the other hand, Th2 T cell-mediated immune response induces the conversion of L-arginine to L-ornithine through the synthesis and activity of arginase [32]. Such pathway results in the synthesis of polyamines, which is essential for *Leishmania* growth, replication, and survival within the host [32]. Consequently, the host fails to control infection, developing huge and chronic lesions at the site of infection [18,30]. Thus, BALB/c mice are unable to effectively control *Leishmania* infection induced by *L. amazonensis* [33]. However, using this model for drug evaluation relies on the idea that if the therapy enables wound healing in susceptible animals, it will also be effective for immunocompetent ones [29].

Taking this into account and considering the relevance of NO, this molecule has been explored as an antimicrobial compound to tackle leishmaniasis [12,13]. Nevertheless, due to the gaseous nature and short half-life (1–5 s), exogenous administration of NO is very unlikely [19,20]. To address such issues, NO donors have been used to provide a more sustained NO-releasing [21,34].

It has been demonstrated the huge susceptibility of *Leishmania* spp. to reactive nitrogen species (RNS) [35]. In fact, RNS are of great importance over the role of the innate immune system [36]. Under physiological and pathophysiological conditions, NO as a signaling molecule, is able to react with distinct cellular constituents to form RNS, such as peroxynitrite ($ONOO^-$), nitrogen dioxide (NO_2), dinitrosyl iron complexes, and nitrosothiols. Each of these species has different targets and biological effects [37].

The reaction of NO with zinc or iron metalloproteinase inhibits several enzymes that end up disrupting the mitochondrial respiratory chain [37]. Likewise, peroxynitrite might target lipids as well as DNA replication [37–39]. The interaction of NO with cysteine thiols results in the formation of S-nitrosothiols (RSNOs) through S-nitrosylation [38,40]. As a post-translational protein modification, such process might strongly affect a broad range of proteins [38,39]. All of these are possible mechanisms that have been reported by which NO may result in parasite death [18,37].

RSNOs are endogenous classes of NO donors that have a relevant biological effect against pathogens. The exogenous administration of different low molecular weight RSNOs has been shown to promote positive effects *in vivo* over *Leishmania* inactivation, including in susceptible animal models [41,42]. In those studies, *in vivo* antileishmanial activity was achieved by multiple doses of NO donors in long-term treatment. In contrast, we have successfully attained a great parasite load and lesion thickness reduction in one single dose of NONPs at 2 mM. This remarkable outcome has probably occurred as a consequence of S-nitroso-MSA encapsulation into CSNPs.

In this regard, our study suggests that encapsulation of NO donors into chitosan nanoparticles enhanced the potential activity of NO donors not only because of intrinsic NO characteristics and controlled release but also because of chitosan antimicrobial properties, resulting in a long-lasting response even in one administration[34,43–46].

Other factors probably contributed to this successful outcome. The synthesis of nanoparticles with sodium tripolyphosphate resulted in a positively charged structure, which is very likely to interact with negatively charged *Leishmania* membranes [43,47]. Additionally, it has been reported that small-sized nanoparticles with spherical morphology enhance NPs intracellular uptake by murine macrophages [48,49]. Indeed, it has been shown that chitosan is taken up by pinocytosis, accumulating within the parasitophorous vacuole (PV) in macrophages infected by *L. major* and *L. mexicana* [43]. Likewise, we have reported PV reduction over *L. amazonensis*-infected macrophages treated with NONPs [22], which suggests that PV could be a possible localization of this compound as well.

It worth noting that intralesional administration of NONPs has brought further benefits to the treatment since current drugs are systemic and potentially toxic to the host [50]. Local therapy is very advantageous for allowing high drug concentration directly into the site of infection avoiding systemic toxicity [51]. As a consequence, there might be a short-term wound healing, improving as well patient's compliance [50,51]. In fact, this route of administration was minimally invasive and did not show any clinical side effects to treated animals.

Surprisingly, despite all the clinical benefits, we did not find any improvement in pain score. In humans, pain is not a usual symptom described by patients with CL [52]. However, *Leishmania* has been shown to induce pain in animal models, particularly mice. In chronic infections, there is releasing of inflammatory mediators, growth factors, and cytokines, resulting in increased hyperalgesia [52–55].

Our results indicate that NONPs were not able to reduce hyperalgesia in treated mice. This could be explained by the dual function of NO [56]. Although NO has important antimicrobial properties, it also plays a

pivotal role in the inflammatory process [56,57]. NO is able to induce vasodilation in acute or chronic inflammation, enhancing the sensitivity of peripheral nociceptors in response to a stimulus [57,58].

Despite that, the exciting results observed here were very promising, even though the exact biological mechanisms by which NONPs control *Leishmania spp.* in the host remains unexplored. From this perspective, the long-lasting effect achieved by one dose of NONPs on a susceptible animal model indicates a successful future for this compound. Indeed, it seems that NONPs could be administered weekly and further studies are welcome to evaluate new protocols. Besides, NONPs are suitable for topical administration and could be easily combined with conventional systemic antileishmanials in lower doses. Worth note that to acquire an optimum NO release for the CL treatment is necessary an influx of NO at low and constant concentration, i.e., in a sustained manner, as shown in this work. Thus, we hope our findings inspire further studies to implement this therapy in future clinical trials.

Author contributions

All authors contributed equally to this manuscript.

Declaration of competing interest

The authors declare no conflict of interest.

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