S-nitrosoglutathione (GSNO) is cytotoxic to intracellular amastigotes and promotes healing of topically treated *Leishmania major* or *Leishmania braziliensis* skin lesions

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**Objectives:** This study was designed to verify the cytotoxic activity of S-nitrosoglutathione (GSNO) against intracellular *Leishmania* amastigotes and to test its efficacy as a topical treatment of localized cutaneous leishmaniasis (LCL) in *Leishmania major*-or *Leishmania braziliensis*-infected mice.

**Methods:** Cytotoxic activity of GSNO was verified in *L. major*-infected THP-1 macrophages. S-nitrosated proteins were detected by immunofluorescence. Topical treatment was done by daily application of a solution of GSNO in PBS to the skin ulcer of *Leishmania*-infected mice. BALB/c and interferon-γ-knockout (IFN-γ-KO) C57BL/6 mice were infected with *L. major* and *L. braziliensis*, respectively. Ulcer size was measured weekly and the parasite loads were determined in the lesion and lymph nodes. Controls received PBS topically or amphotericin B (AMB) intravenously.

**Results:** The number of intracellular *L. major* amastigotes was markedly reduced in GSNO-treated cultures; in these, staining for S-nitrosated proteins was present in the cytoplasm and colocalized with intracellular amastigotes. Topical treatment with GSNO of *L. major* ulcers in BALB/c mice suppressed lesion growth, reduced the parasite load and induced healing comparable to the effect of intravenously administered AMB. Topical GSNO treatment was also efficient at suppressing lesion growth in IFN-γ-KO mice infected with *L. braziliensis*.

**Conclusions:** GSNO is cytotoxic to intracellular *L. major* amastigotes in vitro and had a healing effect on LCL caused by *L. major* and *L. braziliensis* in mice. These positive results on the topical therapeutic effect of GSNO in mouse leishmaniasis infections provide the experimental basis for a possible future trial in the treatment of human LCL.

**Keywords:** localized cutaneous leishmaniasis-LCL, S-nitrosothiols, therapy, nitric oxide donor molecules

**Introduction**

Many species of the protozoan *Leishmania* can cause infection manifested by cutaneous lesions. The most common clinical form of leishmaniasis is that of a single ulcerated lesion, called localized cutaneous leishmaniasis (LCL). Less frequent are mucosal or mucocutaneous forms and, much rarer still, diffuse cutaneous leishmaniasis. Skin ulceration can also appear after treatment of visceral leishmaniasis and is called post-kala-azar cutaneous leishmaniasis (reviewed in¹–⁵). The most widely used and still first choice therapy for cutaneous leishmaniasis are the pentavalent antimonial drugs Pentostam® and Glucantime® administered as one or more series of 10 or 20 intravenous (iv) or intramuscular injections to ambulatory patients, generally over a period of ≥20 days.²⁶ The polyene antibiotic amphotericin B (AMB) is indicated in cases of parasite resistance to antimonials, but is expensive and also requires parenteral injection.²⁶ Because of the difficulty of getting to the medical service, the often painful injections, the relative delay before lesion regression and drug toxicity, many patients with LCL receive suboptimal doses or abandon the treatment.⁵–⁷

Such difficulties have led to a continuous search for more convenient and efficient therapies for LCL. Treatment of LCL patients with orally administered drugs, such as miltefosine and antifungal azoles, has had variable success rates.²⁵–⁹ Topical treatment of LCL lesions with ointments has also met with variable success rates.²⁵–⁹
success. A consultative meeting on strategies for the treatment of LCL considered the search for new topical treatments for LCL as an important strategy among the several approaches to therapy. Topical therapy could also be beneficial in association with systemic therapy, in order to accelerate the healing process.

In a mammalian host, *Leishmania* parasites reside as amastigotes inside macrophages. The promastigote forms inoculated by the insect enter the local macrophages, become amastigotes, multiply, and, upon host cell death, are released to be captured by neighbour macrophages; they are disseminated by lymph and/or blood to other organs. The infection is controlled by macrophages activated by innate and adaptive immune mechanisms. Reactive oxygen or nitrogen intermediates produced by activated macrophages are toxic to the parasites and have a major role in parasite control. Among them, nitric oxide (NO) is one of the species known to be very toxic to *Leishmania.*

Several molecules of a group commonly known as NO donors have been tested and/or are used in humans to treat diseases of various aetiologies. Topical treatment of cutaneous *Leishmania* lesions with traditional NO donor molecules was reported to improve healing or to have a significant effect. In addition, a clinical trial found NO-releasing patches containing nitrite and ascorbic acid as a ‘not effective enough treatment’ of LCL.

These variable results could possibly be related to the instability of NO release from these formulations, as well as to the nature of the active nitrogen-containing species. -nitrosothiols (RSNOs) are endogenous molecules possessing the SNO moiety, which are capable of releasing free NO or transferring it to other substrates. Among them, *N*-nitroso-albumin and *N*-nitrosogluthatine (GSNO) have been considered to be endogenous NO carriers and donors in mammals. One of the key characteristics of RSNOs is their ability to undergo transnitrosation reactions with other molecules possessing reactive cysteine residues. A possible consequence of these reactions is the inactivation of enzymes leading to cytotoxic effects. The synthetic tertiary RSNOs *N*-nitroso-N-acetylpenicillamine (SNAP) and *N*-nitroso-albumin were shown to be toxic to *Leishmania* promastigote and axenic amastigote forms, respectively. Topical treatment with a SNAP-containing cream was reported to accelerate healing of LCL lesions in human patients.

More recently, GSNO and *N*-nitroso-N-acetylcystein were shown to have potent growth-inhibitory activity on promastigote forms of *Leishmania major* and *Leishmania amazonensis* at neutral pH and in parasite liquid culture conditions. Because of its potential advantages, such as increased stability and availability in the solid dry form, we chose to investigate the activity of GSNO on intracellular *L. major* amastigotes in macrophage cultures and as a topical treatment of ulcerated *Leishmania* lesions in mice infected in the dorsal skin with *L. major* or *Leishmania braziliensis*.

### Materials and methods

**Cell cultures and parasites**

The human macrophage cell line THP-1 (202 TIB; ATCC, Rockville, MD, USA) was grown at 37°C in a 5% CO₂ humid atmosphere in RPMI medium supplemented with 10 mM HEPES, 2 mM glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin and heat-inactivated fetal bovine serum (FCS; Gibco, Life Technologies, Sao Paulo, SP, Brazil) at 10% (v/v). *Leishmania* parasites were grown in Grace’s insect cell culture medium supplemented with penicillin/streptomycin, 10%–20% FCS and 5 mg/L haemin (designated as Grace-Leish medium). The culture media and supplements were bought from Sigma Chemical Co., St Louis, MO, USA.

All *in vitro* and most *in vivo* experiments were done with axenically grown *L. major* promastigotes (MHOM/IL/80/Friedlin clone) maintained *in vitro* for a maximum of six passages before resuscitation from infected BALB/c mice. *L. braziliensis* axenic promastigotes (strain RPL5, isolated from patients in the central-west region of Brazil and donated by Dr Milton A. P. Oliveira, University of Goiania, Goiás, Brazil) were used to infect interferon-γ-knockout (IFN-γ-KO) C57BL/6 mice.

**GSNO synthesis**

GSNO was synthesized by the equimolar S-nitrosation reaction of reduced glutathione (Sigma) with acidic (HCl) sodium nitrite (Sigma), under stirring, in an ice bath for 40 min, protected from light with aluminium foil. GSNO formed in the reaction was precipitated with acetone, vacuum filtered, washed with cold water and acetone and freeze-dried for 18 h. The reddish GSNO crystals obtained were stored at −20°C and used to prepare fresh buffered solutions at the desired concentrations.

**Infection of THP-1 cells with *L. major* and in vitro treatment with GSNO**

THP-1 cell cultures were pre-treated with GSNO (100–300 μM) overnight before infection. The cells were then washed twice (280 g/8 min/15°C) in RPMI + 1% FCS and resuspended in RPMI + 20% FCS to 3.3 x 10⁵ cells/mL. Stationary-phase *L. major* promastigotes grown in Grace-Leish medium were washed twice (2000 g/8 min/15°C) in RPMI + 1% FCS and resuspended in RPMI + 20% FCS. Each well of 48-well culture plates received 300 μL of THP-1 suspension and 100 μL of parasite suspension containing 1 x 10⁷ parasites (ratio parasites:cell, 10:1). The plates were incubated for 4 h at 37°C and 5% CO₂. To remove extracellular parasites, the plates were washed twice in RPMI + 1% FCS by centrifugation at 280 g and the suspension was submitted to a 10 s hypotonic shock with distilled water followed by isotonic reconstitution; the cells were then resuspended in RPMI + 1% FCS to which GSNO (100–300 μM) or amphotericin B deoxycholate (Cristalnia, Campinas, Sao Paulo, Brazil) (2 x 10⁻³ μM) was added. This moment was considered as time zero of the experiment. GSNO was added again to the cultures 48 h later. At the low concentration of FCS (1%) in RPMI, the THP-1 cells remain viable but do not significantly divide. The cells were harvested at 0, 24, 48, 72 and 96 h of culture, diluted, transferred to cytospin tubes and cytocentrifuged onto glass slides (100 g, 4 min and 23°C). The preparations were stained by indirect immunofluorescence to detect the parasites and with the stain 4',6-diamidino-2-phenylindole (DAPI) (Sigma) to counterstain the cellular nuclei (detailed in the next section).

The number of infected cells and the number of parasites were counted in ≥200 cells per slide from randomly captured fields. The index of infection (percentage of infected cells × mean number of parasites per cell) was calculated and the results expressed as the mean and standard error of the mean (SEM) from triplicate slides. GSNO concentrations were tested in the range of 100–400 μM for toxicity to cultured THP-1 cells by the trypsin blue exclusion method. At up to 300 μM, the viability of THP-1 24 h cultures was between 85% and 95% compared with untreated cultures; by 96 h of culture, all cultures (including the untreated) had 75%–80% viable cells. Concentrations ≥350 μM were found to be more toxic.

**Parasite staining and detection of S-nitrosated proteins by immunofluorescence**

The slides with the spun cells were fixed in chilled methanol for 3 min, washed in PBS (0.01 M phosphate-buffered 0.15 M NaCl) and the cell-rich area was covered with pooled sera (diluted 1:5 in PBS) obtained from *L. major*-infected mice. After 30 min incubation at room temperature, the
slides were washed three times by immersion in PBS and covered with FITC-labelled goat antimouse immunoglobulin (Ig) (Amersham, GE Healthcare of Brazil, SP, Brazil) to detect the parasites and with the fluorescent stain DAPI to stain the nuclei. After 30 min incubation, the slides were washed in PBS and glass coverslips were mounted using Mowiol (Calbiochem, Merck Millipore, MA, USA) containing 0.1% p-phenylene diamine dihydrochloride (Sigma). The slides were observed under a fluorescence microscope (Nikon Eclipse E-600), photographed at ×40 magnification and the images were captured by a computer and saved for cell and parasite counting.

In order to detect S-nitrosated proteins, the slides were prepared in a cytocentrifuge as above and processed as previously described.33 They were fixed and permeabilized for 30 min at 4 °C in a solution of 4% paraformaldehyde containing 0.2% Triton-X. In sequence, the slides were washed twice in PBS and incubated for 1 h at 4 °C with blocking solution (5% normal goat serum, 3% BSA and 0.2% Triton-X in PBS). The slides were washed twice in PBS and covered with the first antibody (Ab), rabbit anti-S-nitrosocysteine (Sigma), adequately diluted in PBS or with its control, normal rabbit serum, and incubated overnight at 4 °C in a humid box. After two washings in PBS, the slides were covered with serum obtained from L. major-infected mice (or with normal mouse serum as control) diluted in PBS and incubated for 30 min at 4 °C. After three washes in PBS, the slides were incubated for 30 min at 4 °C with Alexa Fluor 488-labelled goat antimouse Ig (Santa Cruz Biotechnology, CA, USA) and FITC-labelled goat antimouse Ig (Caltag Laboratories, Burlingame, CA, USA) antibodies diluted in PBS. Following three PBS washes, the slides were stained with diluted DAPI, washed and mounted and photographed at ×60 magnification.

**Infection of mice with L. major or L. braziliensis and treatment of the infected animals**

Infections with L. major were carried out in female BALB/c mice aged 4–6 weeks. The mice had the lower dorsal region shaved and were inoculated i.p. with 1×10^7 stationary phase promastigotes in 50 μl of PBS. When most animals had developed a single open ulcer of 2–3 mm diameter (5–6 weeks of infection), treatment was started. Groups of six to eight mice were treated topically with 200 μl of GSNO (50, 100 or 150 mM) diluted in PBS applied with a micropipette directly on the ulcer once a day. Other groups of mice received AMB at 1 μg/g body weight iv, three times per week. Control groups were similarly handled and received only the diluent. Treatments were carried out for a period of 5 weeks.

The lesion width was measured twice a week with a calliper; two measurements at right angles were taken and multiplied to express the estimated area of the lesion. The Δ lesion size was calculated for each mouse and expresses the variation in the size of the lesion compared with its original size when treatment was started. Δ Lesion size was calculated as the lesion area on day x minus the lesion area when treatment was started. The means and SEM of each group of six to eight mice are presented.

Infections with L. braziliensis were carried out by injecting 2×10^6 promastigotes of the RPLS strain (derived from 5-day-old axenic cultures) intradermally in female IFN-γ KO C57BL/6 mice. Lesions became ulcerated 2–3 weeks after inoculation and treatment was started 1–2 weeks later for a period of 4 weeks.

All procedures involving animals were approved by the ICB/USP Committee on the ethical treatment of research animals.

**Quantifying parasites in the lesions**

After the lesion treatment was finished, the mice were sacrificed for aseptic skin biopsy and draining lymph node (LN) removal. A 10 mm diameter fragment containing the lesion was removed using a skin punch biopsy. Quantifying the parasites was done by the technique of endpoint dilution.14,34 Briefly, the tissues were minced and homogenized in Grace-Leish medium containing 20% FCS and haemin in plastic tubes followed by serial dilutions in the same medium. From each dilution tube, triplicate cultures were dispensed in flat-bottomed 96-well plates. Cultures were inspected daily under an inverted microscope and scored for the appearance of motile promastigotes for 7 days. The log_{10} of the reciprocal of the last dilution yielding Leishmania was used as an estimate of the parasite load in the organs.

It should be noted that the endpoint dilution technique yields estimates and not absolute numbers of parasites present in the original cell suspensions. In fact, the serial dilutions of the parasite-containing cell suspensions and their subsequent culture may originate estimates that significantly exceed the number of parasites present in the original tissue sample, in spite of the care taken in performing the assays. In our experiments, the samples from the experimental and respective control groups were processed for endpoint dilution on the same day in order to minimize assay variations and enable comparative statistical analysis between groups.

**Statistics**

The data are presented as means accompanied by their SEM. The GraphPad Prism 4 statistical program (GraphPad Software Inc., San Diego, CA, USA) was used to analyse the data by analysis of variance with repeated measurements; P < 0.05 was considered as significant.

**Results**

**GSNO inhibits L. major growth in human THP-1 macrophage cultures**

Treatment with GSNO of L. major-infected THP-1 cells caused a marked reduction in the infection index at 24 h of culture that was comparable to the effect of AMB (Figure 1). However, by 48 h the infection index in GSNO-treated cultures had risen in comparison with that of 24 h, suggesting that GSNO had either been consumed or lost its activity. The readddition of GSNO to the cultures (at 48 h after the lesion treatment was finished) did not lead to significant suppression of the infection index. The mean infection index at 48 h of culture in GSNO-treated cultures was comparable to the effect of AMB (Figure 1). However, by 72 h the infection index at 48 h of culture in GSNO-treated cultures had risen in comparison with that of 48 h, suggesting that GSNO had either been consumed or lost its activity. The readddition of GSNO to the cultures (at 72 h after the lesion treatment was finished) did not lead to significant suppression of the infection index. The mean infection index at 72 h of culture in GSNO-treated cultures was comparable to the effect of AMB (Figure 1). However, by 96 h the infection index at 72 h of culture in GSNO-treated cultures had risen in comparison with that of 72 h, suggesting that GSNO had either been consumed or lost its activity. The readddition of GSNO to the cultures (at 96 h after the lesion treatment was finished) did not lead to significant suppression of the infection index. The mean infection index at 96 h of culture in GSNO-treated cultures was comparable to the effect of AMB (Figure 1).

![Figure 1](https://example.com/figure1.png)  
*Figure 1.** Effect of GSNO on the parasitism of THP-1 cells infected with L. major. THP-1 cells were incubated for 4 h with L. major stationary-phase culture promastigotes (10 parasites per cell) and washed. At this point (0 h of infection), the cultures received GSNO (300 μM) or amphotericin B (2×10^{-3} μM) (both added again at 48 h). At the indicated timepoints, the cells were processed for fluorescence staining. The infection index was obtained by multiplying the percentage of infected cells by the mean number of parasites per infected cell in a given culture. The means and SEM of triplicate cultures are shown. The data are from a representative experiment out of three that were performed with similar results. *p < 0.05 compared with untreated cultures; **p < 0.05, GSNO versus amphotericin B.
counted from time zero) resulted anew in a reduction in the infection index by 72 h. By 96 h of culture, the infection index of GSNO-treated cultures did not differ from that obtained in AMB-treated cultures.

Staining of GSNO-treated cultures with antinitrocysteine Ab indicates the presence of nitrated proteins in the cytoplasm of THP-1 host cells and colocalized with intracellular L. major amastigotes

Because GSNO is added to the extracellular milieu of the cells infected with Leishmania and the parasites reside in intracellular vacuoles, it was of interest to know whether GSNO could reach the parasites lying inside the organelles. The antibody antinitrocysteine detects proteins that underwent S-nitration at the cysteine residue. The preparations were simultaneously stained with red Alexa-labelled antinitrocysteine antibody and FITC-labelled anti-Leishmania antibody and DAPI to stain the cell nuclei (Figure 2). The pattern of fluorescence staining obtained for the red antinitrocysteine antibody indicates the presence of S-nitrated proteins in the hosts cell cytoplasm, whereas the parasites stain green. By merging the photos of the same field obtained with the appropriate filters for red or green/blue fluorescence, it is possible to observe that there is red fluorescence staining coincident with many intracellular amastigotes.

Topical treatment with GSNO markedly reduced the size of the L. major skin ulcer in BALB/c mice and reduced the number of parasites present in the lesion

The topical application of GSNO to the L. major skin ulcer markedly reduced progression of the lesion; after 5 weeks of treatment, the lesions were much smaller than those that had developed in PBS-treated control mice (Figure 3a and b). Dose–response experiments showed that the concentration of 150 mM was not more effective than 50 mM (Figure 3a) and had a slight irritating effect, because the animals immediately tried to bite or rub the lesion. The concentration of 100 mM did not cause irritation and was very effective at reducing lesion size over a period of 5 weeks (Figure 3b), whereas it took longer for the treatment with 50 mM GSNO to reduce the lesion to sizes similar to those obtained with 100 mM GSNO (data not shown). The group of mice treated with 100 mM GSNO was compared with mice treated with AMB; as shown in Figure 3(b), the marked wound-healing effect of GSNO was comparable to that of AMB alone and significantly different from the PBS-treated control group. The estimated mean number of parasites in the lesions removed from 100 mM GSNO-treated mice was significantly lower when compared with those from PBS-treated controls, but still parasites persisted in the cutaneous lesions in all cases (Figure 3c). Two other independent experiments in mice topically treated with 50 mM GSNO confirmed the reduction of parasite loads in the skin lesion (data not shown). However, quantifying the parasites in the draining LN (Figure 3d) did not yield any reduction in either the AMB-treated group or in the GSNO-treated group in comparison with the PBS-treated control group.

Topical treatment with GSNO was also effective at reducing the size of the skin ulcer caused by L. braziliensis in IFN-γ-KO mice

Because L. braziliensis is the main cause of LCL in Brazil, it was of interest to verify whether topical GSNO treatment would also have an ulcer-healing effect in this infection. Two independent experiments were performed in IFN-γ-KO C57BL/6 mice infected with L. braziliensis in IFN-γ-KO mice. These mice developed a lesion that in ~2 weeks became a shallow ulcer that quickly enlarged and became adherent to the underlying muscle. In both experiments (Figure 4, Exp 1a and Exp 2a), topical treatment

Figure 2. Immunofluorescence staining for proteins S-nitrosated at cysteine residues and for parasites in L. major-infected THP-1 cultures treated with GSNO. The cultures were treated with 300 μM GSNO (A series) or kept only in medium (B series) for 24 h. Preparations were stained with rabbit anti-S-nitrosocysteine antibody followed by Alexa Fluor-labelled goat antirabbit immunoglobulin (red fluorescence) and with mouse anti-Leishmania antibody followed by FITC-labelled goat antimouse Ig (green fluorescence). DAPI (blue fluorescence) was used as a counterstain for the nuclei. The fields were photographed with an ×60 objective lens. The arrows point to intracellular parasites. A1: FITC-stained parasites; A2: the same field as A1 showing red staining for S-nitrosocysteine of intracellular structures; A3: the merging of A1 and A2 fields show parasites that are stained by the anti-S-nitrosocysteine antibody; B1: FITC-stained parasites; B2: very faint red staining in some cells; and B3: only green stained parasites are visible. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.
with GSNO markedly suppressed the growth of the *L. bazieliensis* lesion but did not heal the cutaneous ulcer. However, the beneficial effect of GSNO treatment was comparable to that obtained with iv-administered AMB, which also failed to heal the lesion (Figure 4, Exp 1a and Exp 2a). The characteristics of the skin lesion made it impossible to obtain a standardized biopsy of tissue; therefore, quantifying the parasite load was done only in the draining LN. As shown in Exp 1(b), a significant and comparable decrease in the parasitism of LNs was seen in the groups treated with GSNO or AMB in comparison with the PBS-treated control group. However, such a reduction did not happen in either treatment group in the experiment shown as Exp 2(b). It should be noticed that, in this experiment, the parasite loads of LNs were several orders of magnitude higher than those observed in Exp 1.

**Discussion**

We report on the activity of GSNO on *Leishmania* parasites causing cutaneous leishmaniasis. Initially, we have shown that GSNO inhibited the intracellular growth of *L. major* amastigotes in cell culture. Next, GSNO was used to topically treat the ulcerated skin lesion of infected mice. The effect on BALB/c mice infected with *L. major* and on IFN-γ-KO C57BL/6 mice infected with *L. bazieliensis* was a marked reduction of lesion growth.

In relation to the *in vitro* inhibition of intracellular amastigote survival and growth by GSNO, the question arose whether the parasite itself would be a target of the toxic effect of GSNO or whether it would indirectly suffer from the GSNO inhibitory action on the host cell’s metabolism.

The toxic effects of NO donor molecules can be due to the release of free NO and its subsequent reactions and/or direct S-nitrosation of parasite or host cell proteins. There is indication that the leishmanicidal activity of GSNO on promastigotes is due mainly to the transnitrosation of proteins. We found that the cytoplasm of the host cells and also intracellular parasites stained positive for the presence of S-nitrosocysteine groups, indicating a transnitrosation action on the host cell and also on the intracellular parasites lying inside the parasitophorous vacuoles. Several cytotoxic targets of NO or NO donors have been identified in *Leishmania* axenic culture forms, such as the inhibition of glucose, proline and adenine incorporation and of the enzymes glyceraldehyde 3-phosphate dehydrogenase, aconitase and cysteine proteinase activities. In addition, the exposure of *L. amazonensis* axenic parasites to several NO donors triggers apoptosis-like DNA fragmentation. Axenic amastigotes are less susceptible than promastigotes to nitroprussate-induced death that was attenuated by glutathione, suggesting multiple oxidative mechanisms for that molecule. The various toxic mechanisms of GSNO are also effective against *Acanthamoeba castellanii* and keratitis-inducing bacteria.

Topical treatment with GSNO of the *L. major* ulcer was nearly as effective as systemic AMB at reducing the size of and healing *L. major* skin lesions of mice. The dose of AMB chosen for the systemic...
treatment of mice (1 μg/g body weight iv, three times per week) was comparable to the dose indicated by the Brazilian Ministry of Health for the treatment of adult human patients with cutaneous/mucosal leishmaniasis (1 mg/kg body weight, not to exceed the accumulated dose of 1 g) (http://portal.saude.gov.br/portal/arquivos/pdf/manu_leishman.pdf). AMB is the second drug of choice in Brazil for antimony-resistant patients, because of the high cost of AMB liposomal formulations.6

The skin-healing effect we observed for topical GSNO application was comparable to that described in experimental treatment with paromomycin ointment.40 We observed significant reduction of the estimated parasite loads in the skin lesion site, but not in the draining LN. The persistence of parasites in healed lesions after topical therapy was observed by other authors, who stressed its importance in maintaining concomitant immunity to reinfection in a mouse model.41

Although GSNO was shown to be leishmanicidal to intracellular amastigotes in THP-1 cell cultures and also to reduce the parasite load in L. major lesions, it is possible that its skin-healing effects do not only result from the diminished parasite load. In fact, GSNO has known wound-healing properties and has been incorporated into hydrogels in order to accelerate skin repair and/or cicatrization.52,43 The size of LCL lesions in human patients correlates with higher tumour necrosis factor (TNF) secretion levels14 and local inhibition of cytokine synthesis pathways by NO or by nitrosation could be an additional mechanism of wound healing. In this context, recent work has focused on the importance of wound skin repair genes that influence the outcome and resistance to cutaneous leishmaniasis in mice.14,45

Because L. braziliensis is a common aetiology of LCL, we tested topical GSNO treatment on infected IFN-γ-KO mice and confirmed a marked reduction of the skin lesion. IFN-γ-KO mice were chosen as hosts for L. braziliensis because this parasite fails to cause significant ulcerative lesions in immunocompetent mice.31 IFN-γ-KO mice are extremely susceptible to infection with L. braziliensis, because of the major roles of IFN-γ as a co-stimulator of TH1-type immune responses and as a macrophage activator.15,31 IFN-γ-KO mice have reduced natural killer cell activity and the macrophages produce very low amounts of molecules that have microbicidal activities, such as NO and reactive oxygen species; inflammatory cytokines, such as TNF-α, interleukin-1 and interleukin-6, are also reduced.46 Topical application of an NO donor molecule to the lesion may partially compensate for the shortage of NO and promote healing, but it would not totally compensate for the other deficient microbicidal mechanisms of these immunodeficient hosts. However, although neither GSNO nor iv AMB completely healed the ulcer (as seen for L. major-infected BALB/c mice), there was significant improvement, suggesting that topical GSNO could be beneficial even in immunocompromised patients. It should be stressed that L. braziliensis skin infection of IFN-γ-KO mice has different characteristics from those observed in L. major-infected BALB/c mice. Namely, the lesion ulcerates

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**Figure 4.** Effect of topical treatment with GSNO of L. braziliensis-infected IFN-γ-KO C57BL/6 mice on ulcer size and parasitism of the draining lymph node. Mice were infected in the dermis of the low dorsum with $2 \times 10^6$ promastigotes of the RPL5 strain of L. braziliensis. Two independent experiments were performed. Topical treatment with GSNO and systemic treatment with amphotericin B were carried out as described in the legend to Figure 3. The data on lesion size are expressed by the variation (Δ) of ulcer size between the end (4 weeks) and the start of treatment. Parasite loads were estimated at the end of treatment by endpoint dilution, as described in the legend to Figure 3, only in the draining lymph node. In all graphs, the bars represent the mean and SEM of six to eight mice. *, $P < 0.05$ in comparison with the control PBS group.

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earlier and rapidly enlarges in L. braziliensis-infected IFN-γ-KO mice and is shallower and adherent to underlying tissues. For this reason, obtaining uniformly sized skin biopsies for parasite quantification became impossible. Nevertheless, the results indicate that topical treatment with GSNO may also have a beneficial effect in lesions caused by L. braziliensis, the most prevalent cause of LCL in South America.

In conclusion, our results on the successful topical therapy of cutaneous leishmaniasis with GSNO in the mouse model provide the experimental basis for a possible trial of this molecule in the treatment of LCL.

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Transparency declarations
None to declare.

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