Major Article

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Sodium nitroprusside has leishmanicidal activity independent of iNOS

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ABSTRACT

Introduction: Leishmaniasis is a zoonotic disease caused by protozoa of the genus *Leishmania*. Cutaneous leishmaniasis is the most common form, with millions of new cases worldwide each year. Treatments are ineffective due to the toxicity of existing drugs and the resistance acquired by certain strains of the parasite. **Methods:** We evaluated the activity of sodium nitroprusside in macrophages infected with *Leishmania (Leishmania) amazonensis*. Phagocytic and microbicidal activity were evaluated by phagocytosis assay and promastigote recovery, respectively, while cytokine production and nitrite levels were determined by ELISA and by the Griess method. Levels of iNOS and 3-nitrotyrosine were measured by immunocytochemistry. **Results:** Sodium nitroprusside exhibited *in vitro* antileishmanial activity at both concentrations tested, reducing the number of amastigotes and recovered promastigotes in macrophages infected with *L. amazonensis*. At 1.5µg/mL, sodium nitroprusside stimulated levels of TNF-α and nitric oxide, but not IFN-γ. The compound also increased levels of 3-nitrotyrosine, but not expression of iNOS, suggesting that the drug acts as an exogenous source of nitric oxide. **Conclusions:** Sodium nitroprusside enhances microbicidal activity in *Leishmania*-infected macrophages by boosting nitric oxide and 3-nitrotyrosine.

Keywords: Leishmaniasis. Nitric oxide. 3-nitrotyrosine. *Leishmania amazonensis*. Sodium nitroprusside.

INTRODUCTION

Leishmaniasis, a group of infectious diseases found worldwide, is caused by protozoa of the genus *Leishmania*. Approximately 20 to 40 thousand deaths each year are attributed to this disease, which can manifest in various forms with different symptoms depending on the infecting species and the host immune response. The main forms of the disease are cutaneous, mucocutaneous, and visceral, of which the cutaneous form is the most common, with 0.7 to 1.2 million new cases each year in 98 countries(1). Pentavalent antimonials are the standard treatments, of which meglumine antimoniate and sodium stibogluconate are the most frequently used(2). However, these drugs require long treatment regimens and parenteral or intralesional administration(3), and cause numerous side effects, including pancreatitis, hepatitis, and cardiotoxicity(3) (4). In addition, some strains of the parasite have acquired resistance to these drugs(5).

Macrophages mount various mechanisms to combat parasites, including oxidative burst, acidification of vesicles, and expression of inducible nitric oxide synthase (iNOS)(6). iNOS synthesizes nitric oxide, a highly reactive, membrane-diffusible molecule used to control various pathogens(7). Nitric oxide reacts with reactive oxygen species to generate reactive nitrogen species such as peroxynitrite(8), which damage DNA, inhibit enzymes, and peroxidize lipids(9).

Leishmania spp. has evolved several mechanisms to evade macrophage activity. For instance, parasites suppress nitric oxide production by taking up L-arginine, a required substrate for iNOS, as well as by inhibiting the release of IFN-γ and TNF-α, proinflammatory cytokines that stimulate expression of this enzyme(10). These observations suggest that exogenous sources of nitric oxide may potentially be used to control leishmaniasis.

Indeed, studies have shown that such sources are active against *Leishmania in vitro*(11) and *in vivo*(12), as well as other microorganisms, including *Strongyloides venezuelensis*(13), *Paracoccidioides brasiliensis*(14), and *Trypanosoma cruzi*(15). One such source is sodium nitroprusside ($Na_2[Fe(CN)_5NO]•2H_2O$),

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an inorganic compound[16] active against promastigotes and axenic amastigotes of *Leishmania (Leishmania) amazonensis*[17]. In this report, we demonstrate that sodium nitroprusside enhances the microbicidal activity of *Leishmania*-infected macrophages by enhancing production of nitric oxide and 3-nitrotyrosine.

METHODS

Leishmania (Leishmania) amazonensis

Promastigotes of *L. amazonensis* (MHOM/BR/1989/166MJO) were maintained in 199 medium pH 7.18-7.22 (Invitrogen-GIBCO) supplemented with 10% fetal bovine serum (Invitrogen-GIBCO), 10mM HEPES, 0.1% human urine, 0.1% L-glutamine, 10U/mL penicillin, 10µg/mL streptomycin (Invitrogen-GIBCO), and 10% sodium bicarbonate. Cultures were grown in 25cm^2 flasks at 24°C.

Culture of peritoneal macrophages and phagocytosis assay

Macrophages were obtained from the peritoneal cavity of BALB/c mice, re-suspended in RPMI 1640 medium pH 7.2 (Gibco BRL), and incubated for 2h at 37ºC and 5% CO_2 in 24-well plates (5×10^5 cells per well) with 13mm glass coverslips with 200µL RPMI 1640 medium. Adherent cells were infected for 2h with *L. amazonensis* promastigotes at a ratio of 1:5, washed with phosphate-buffered saline to remove non-phagocytized parasites, and treated for 24h at 37ºC and 5% CO_2 with RPMI 1640 (control) or with 0.5 and 1.5µg/mL sodium nitroprusside. Subsequently, cells were stained with Giemsa, and 200 cells per sample were imaged at 1,000× under a CX31RBSFA light microscope (Olympus Optical Co. Ltd., Tokyo, Japan) to quantify the number of infected macrophages and the average number of amastigotes per macrophage.

Promastigote recovery

Promastigote recovery was performed as previously described[18]. Briefly, peritoneal macrophages (5×10^5 cells) were infected with *L. amazonensis*, treated for 24h at 37ºC and 5 % CO_2 with 0.5 and 1.5µg/mL sodium nitroprusside, washed with phosphate-buffered saline, and incubated at 24°C with 199 medium to induce differentiation of intracellular amastigotes into promastigotes. Promastigotes recovered were counted daily in a Neubauer chamber for three days after infection.

Cytokine levels

Supernatants were collected from cultures of infected macrophages 24h after treatment with sodium nitroprusside, centrifuged at 460 ×*g* for 7 min at 4°C, and stored at -80°C until analysis. TNF-α, IFN-γ, and IL-12-p70 were determined by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (eBiosciences®, USA). Plates were read at 450nm using a plate reader (Thermo-TP-Reader).

Nitrite levels

Nitrite was also determined in the supernatant of cultures of infected macrophages treated with sodium nitroprusside. Nitric oxide was measured with the Griess reagent according to published methods[19]. Briefly, aliquots were diluted in 45g/L glycine pH 9.7, and treated for 10 min with cadmium granules previously activated with 5mM $CuSO_4$. Subsequently, 200µL of this mixture reacted for 10 min at room temperature with an equal volume of Griess reagent. Tubes were then centrifuged at 10,845 ×*g* for 2 min at 25°C, and transferred to 96-well microplates in triplicate. Absorbance at 550nm was determined in a microplate reader. A calibration curve was constructed using dilutions of $NaNO_2$.

Immunocytochemistry for iNOS and 3-nitrotyrosine

Slides with adherent macrophages were prepared in triplicate as described for the phagocytic assay, and labeled by the streptavidin-biotin method (Universal Dako LSAB®+ System-HRP Kit, DAKO Japan, Kyoto, Japan) without microwave pretreatment. Slides were then treated with 10% Triton-X for 15 min, washed with phosphate-buffered saline, and incubated in 1% fetal bovine serum for 30 min. Subsequently, slides were probed overnight at 4 ºC with 1:300 dilutions of rabbit polyclonal primary antibodies against iNOS and 3-nitrotyrosine (Santa Cruz Biotechnology), and then with biotinylated anti-rabbit, anti-mouse, and anti-goat IgG (LSAB+ System-HRP, DAKO, Japan, Kyoto, Japan) for 2h at room temperature. Negative controls were performed omitting the primary antibodies. Horseradish peroxidase activity was visualized with H_2O_2 and 3,3'-diaminobenzidine for 5 min, and cells were counterstained with Harris hematoxylin (Merck). Finally, slides were digitally imaged in color at 400× using a BX41 photomicroscope (Olympus Optical Co. Ltd., Tokyo, Japan). Representative fields of view from 10 images of each replicate were scored semi-quantitatively using color deconvolution in ImageJ (NIH, USA). Pixels with intensity 0-255 were categorized as strongly positive (3+, intensity 0-60), positive (2+, intensity 61-120), weakly positive (1+, intensity 121-170), and negative (0, intensity 171-230), as previously described[20]. Slides that were not probed with primary antibody were used as negative control.

Statistical analysis

Data are reported as mean ± standard error of the mean. Duplicate datasets from three independent experiments with three animals per experiment were analyzed in Prism GraphPad 5.00 (GraphPad Software, Inc., USA). Data were found to be normally distributed by Kolmogorov-Smirnov test, and variances were found to be homogeneous by F test. Treatments were compared by Student's t-test or analysis of variance followed by Tukey's test for multiple comparisons. Differences were considered statistically significant when $p < 0.05$.

Ethics statement

Female BALB/c mice weighing approximately 25-30g and aged 6-8 weeks were housed in pathogen-free conditions according to protocols approved by the Institutional Animal Care and Use Committee at Londrina State University. This study was approved by the Londrina State University Ethics Committee for Animal Experimentation (33064.2012.42).

RESULTS

Sodium nitroprusside alters phagocytic capacity and increases microbicidal activity

To characterize the impact of sodium nitroprusside on phagocytic and microbicidal activity, macrophages were treated with different concentrations of the compound for 24h after infection. We found that exposure to sodium nitroprusside for 24h did not significantly affect the number of infected macrophages. However, the number of amastigotes per macrophage was significantly reduced in cells treated with 0.5µg/mL ($p = 0.0188$) and 1.5µg/mL ($p = 0.0409$) sodium nitroprusside (**Figure 1A** and **1B**). Accordingly, sodium nitroprusside also reduced the number of promastigotes recovered, with $p < 0.0001$ for both concentrations 72h after exposure (**Figure 1C**). The data indicate that treatment with sodium nitroprusside for 24h enhanced leishmanicidal activity in macrophages.

Sodium nitroprusside increases nitric oxide and favors formation of 3-nitrotyrosine

Exposure to 1.5µg/mL sodium nitroprusside for 24h significantly increased nitric oxide ($p = 0.0304$, **Figure 2A**)

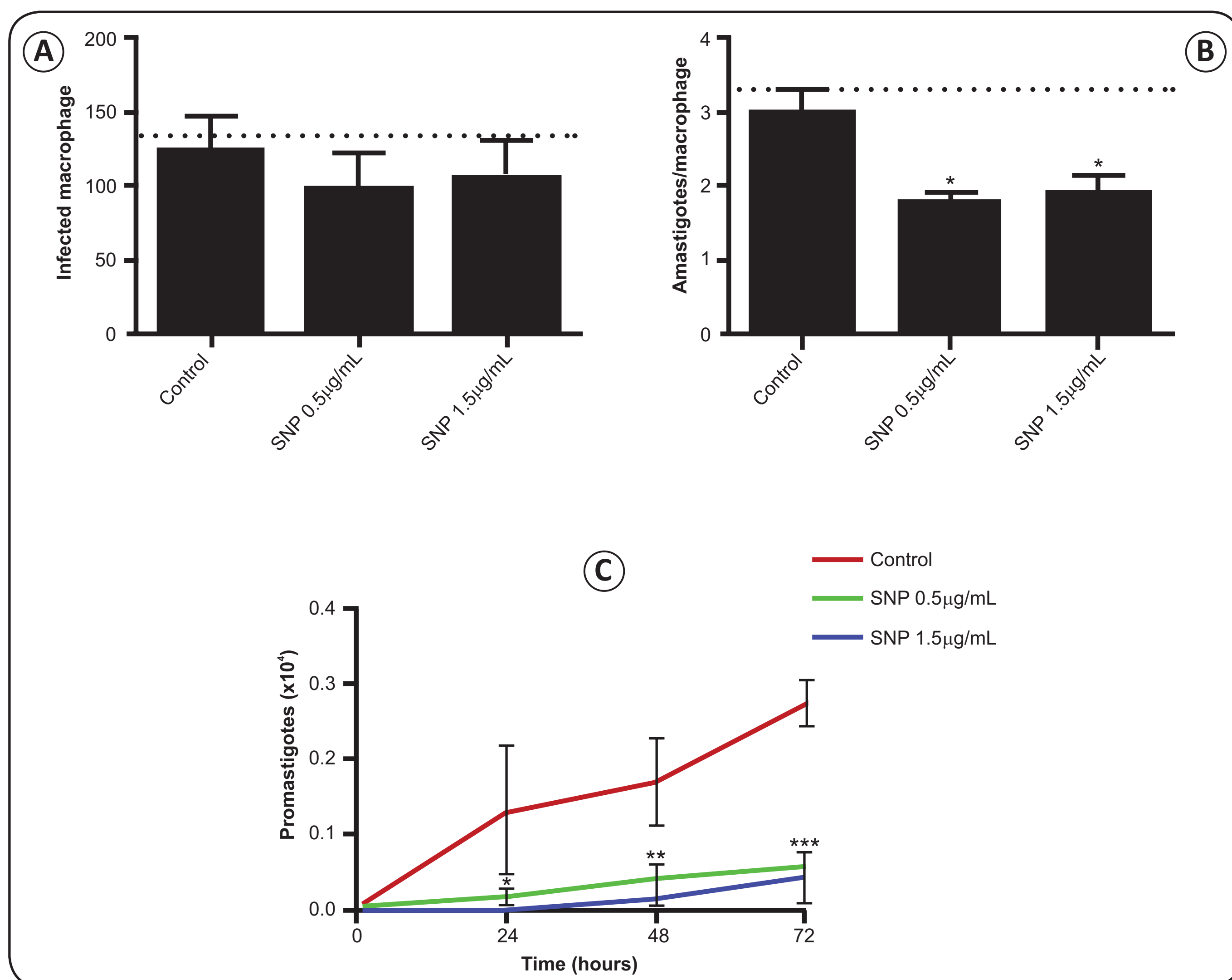

FIGURE 1 – Peritoneal macrophages from BALB/c mice were infected *in vitro* with *Leishmania amazonensis* and treated for 24h with 0.5 and 1.5µg/mL sodium nitroprusside (SNP). A) Number of infected macrophages. B) Number of amastigotes per macrophage. Dashed line indicates the number of (A) infected macrophages and (B) the amount of internalized parasites after infection period (2 h). C) *Leishmania amazonensis* promastigotes recovered over three days after infection. *p < 0.05; **p < 0.01; ***p < 0.001 vs. control, by one-way ANOVA followed by Tukey test.

in macrophages infected with *L. amazonensis*. Accordingly, there was increased immunostaining for 3-nitrotyrosine ($p = 0.0467$, **Figure 2B**), implying that sodium nitroprusside acts as an exogenous source of nitric oxide. Notably, 3-nitrotyrosine colocalized with the parasite in some cells.

Sodium nitroprusside does not induce iNOS expression

Semi-quantitative immunocytochemistry demonstrated that sodium nitroprusside exposure was not associated with elevated expression of iNOS (**Figure 2B**), indicating that the increase in nitric oxide was exogenous.

Sodium nitroprusside increases TNF-α

Treatment of infected macrophages for 24h with 1.5µg/mL sodium nitroprusside increased TNF-α ($p = 0.0065$, **Figure 3A**), but not IFN-γ and IL-12 (**Figure 3B** and **3C**).

DISCUSSION

Nitric oxide is well known to be a key effector in clearing *Leishmania*[21], although the parasite is capable of suppressing nitric oxide production via several mechanisms[22] [23]. Thus, drugs that release nitric oxide, including sodium nitroprusside[24], may enhance leishmanicidal activity in macrophages. The pharmacological characteristics of these drugs were established in 1955[25], and clinical application has since widened[26] [27]. Indeed, sodium nitroprusside remains in use due to its effectiveness and rapid action[26] [28], despite reports of cyanide toxicity.

A previous study *in vitro* demonstrated that sodium nitroprusside decreased the number of *L. amazonensis* promastigotes and axenic amastigotes in a dose-dependent manner[17]. In accordance with this result, we observed that the drug reduced the number of intracellular amastigotes, and, consequently, the number of promastigotes recovered (**Figure 1B** and **1C**). Thus, we investigated the mechanism by which sodium nitroprusside enhanced leishmanicidal activity. We found that nitric oxide levels increased in the supernatant of cultured macrophages exposed to 1.5µg/mL sodium nitroprusside (**Figure 2A**). Consequently, 3-nitrotyrosine was generated, indicating enhanced formation of reactive nitrogen radicals[29]. Notably, 3-nitrotyrosine and nitrated proteins accumulated in phagosomes and in intracellular parasites (**Figure 2B**), reinforcing the idea that parasite clearance depends on reactive nitrogen species. Indeed, 3-nitrotyrosine peaks early in infection in leishmaniasis-resistant C57BL6 mice, presaging a subsequent decline in parasitosis. In contrast, 3-nitrotyrosine peaks late in leishmaniasis-susceptible BALB/c mice[30], implying that 3-nitrotyrosine formation is a relevant indicator of antiparasitic activity.

In addition to 3-nitrotyrosine, the outcome of *Leishmania* infection is critically dependent on the host immune response. In mice, protective immunity depends on the ability of IL-12 to trigger Th1 activity and release of IFN-γ[22] [31] [32]. Indeed, macrophages stimulated with IL-12 secrete IFN-γ[33], although

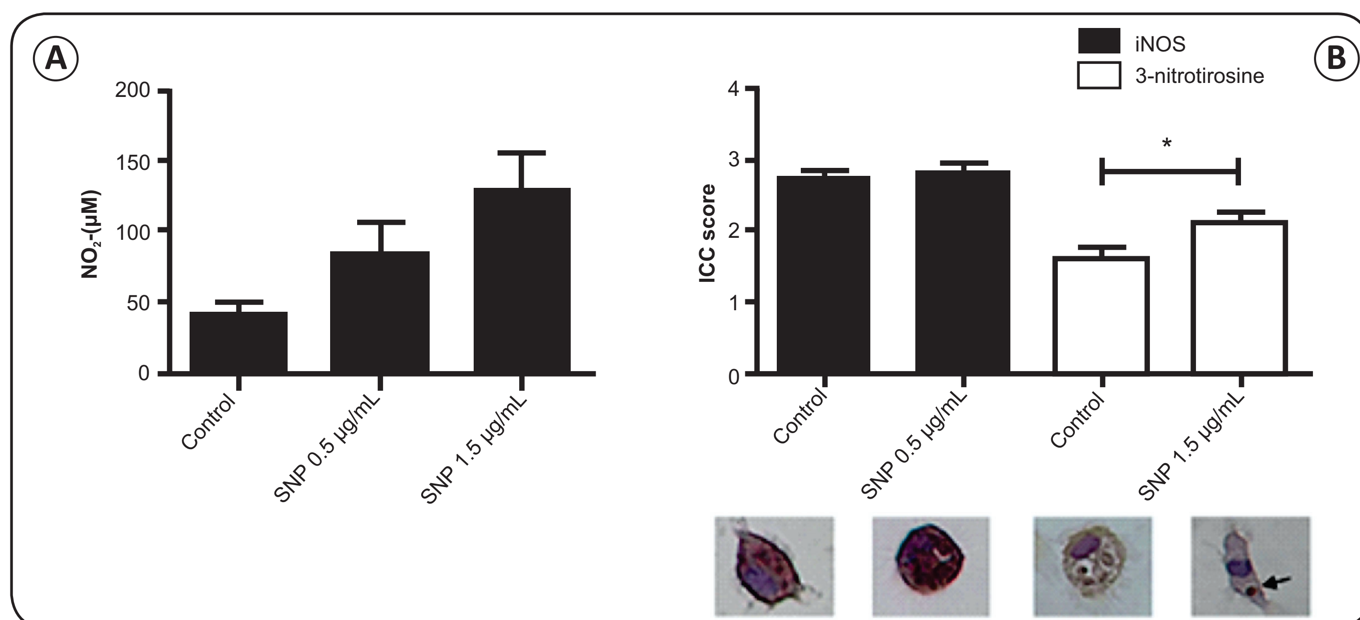

FIGURE 2 - A) Nitrite levels in peritoneal macrophages isolated from BALB/c mice, infected *in vitro* with *Leishmania amazonensis*, and treated for 24h with 0.5 and 1.5µg/mL sodium nitroprusside (SNP). B) iNOs and 3-nitrotyorsine, as measured by immunocytochemistry, in BALB/c peritoneal macrophages infected with *L. amazonensis*, and treated with 1.5µg/mL sodium nitroprusside for 24h. Pixel intensities (ICC score) were scored as strongly positive (3+), positive (2+), weakly positive (1+), and negative (0). Sodium nitroprusside did not affect expression of iNOS, but increased 3-nitrotyrosine, which colocalized with the parasite (arrow). *, $p < 0.05$ vs. control, by t test or one-way ANOVA followed by Tukey test.

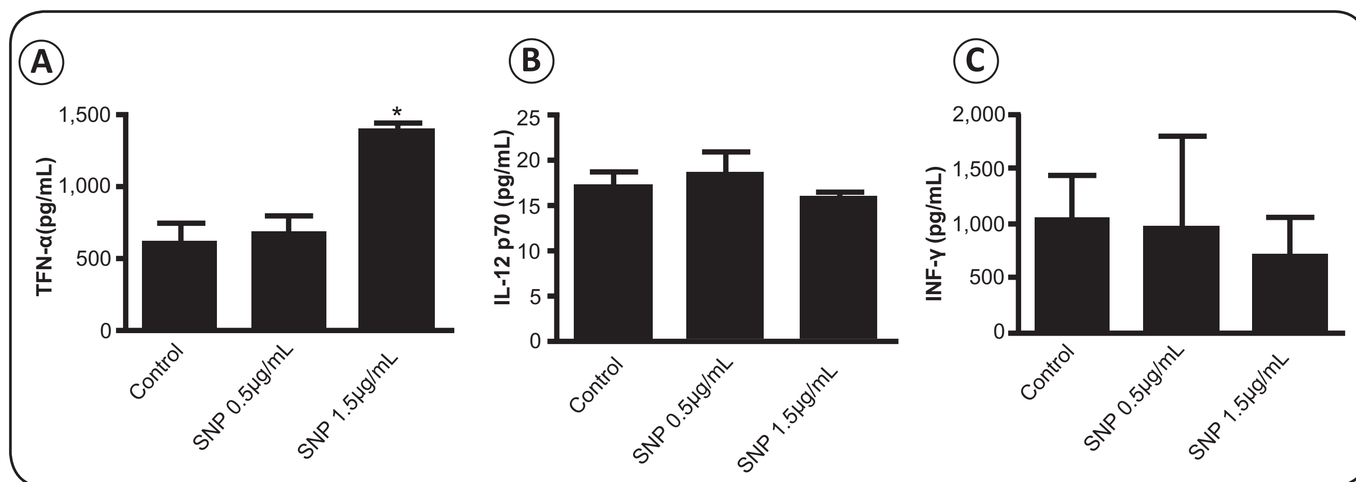

FIGURE 3 - TNF-α (A), IL-12 (B), and IFN-γ (C), as measured by ELISA, in BALB/c peritoneal macrophages infected *in vitro* with *Leishmania amazonensis* and treated with 0.5 and 1.5μg/mL sodium nitroprusside (SNP) for 24h. *p < 0.01 vs. control, by one-way ANOVA followed by Tukey test.

this cytokine is primarily produced by natural killer cells, $CD4^+$, and $CD8^+$ T lymphocytes[34]. We note that *Leishmania* is capable of suppressing IL-12 expression[35] [36]. Importantly, we found that IL-12 production (**Figure 3B**) and IFN-γ secretion were not affected by sodium nitroprusside (**Figure 3C**).

In contrast, sodium nitroprusside stimulated levels of TNF-α (**Figure 3A**), a key cytokine involved in macrophage expression of iNOS[37]. However, the increase in TNF-α did not stimulate iNOS expression (**Figure 2B**), in line with published data demonstrating that exogenous sources of nitric oxide suppress expression of this enzyme[38] [39]. Thus, we conclude that the antileishmanial activity of sodium nitroprusside depends on its properties as a source of nitric oxide[40] [41].

In summary, we have demonstrated *in vitro* that sodium nitroprusside enhances leishmanicidal activity in macrophages infected with *L. amazonensis* via release of nitric oxide. Even though the drug has some toxicity and is challenging to administer, the results provide a rationale for further studies *in vivo*, in light of the serious limitations of current therapies, which have limited efficacy and significant toxicity, and require long treatment regimens.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

REFERENCES

1. Alvar J, Vélez ID, Bern C, Herrero M, Desjeux P, Cano J, et al. WHO Leishmaniasis Contro Team. Leishmaniasis worldwide and global estimates of its incidence. PLoS One 2012; 7:e35671.
2. Palumbo E. Treatment stra tegies for mucocutaneous leishmaniasis. J Glob Infect Dis 2010; 2:147-150.
3. Frézard F, Demicheli C. New delivery strategies for the old pentavalent antimonial drugs. Expert Opin Drug Deliv 2010; 7:1343-1358.
4. Sundar S, Chakravarty J. Leishmaniasis: an update of current pharmacotherapy. Expert Opin Pharmacother 2013; 14:53-63.
5. Kaur G, Rajput B. Comparative analysis of the omics technologies used to study antimonial, amphotericin B, and pentamidine resistance in *leishmania*. J Parasitol Res 2014; 2014:726328.
6. Cunningham AC. Parasitic adaptive mechanisms in infection by leishmania. Exp Mol Pathol 2002; 72:132-141.
7. Qadoumi M, Becker I, Donhauser N, Röllinghoff M, Bogdan C. Expression of inducible nitric oxide synthase in skin lesions of patients with american cutaneous leishmaniasis. Infect Immun 2002; 70:4638-4642.
8. Rosselli M, Keller PJ, Dubey RK. Role of nitric oxide in the biology, physiology and pathophysiology of reproduction. Hum Reprod Update 1998; 4:3-24.
9. James SL. Role of nitric oxide in parasitic infections. Microbiol Rev 1995; 59:533-547.
10. Perrella-Balestieri FM, Queiroz AR, Scavone C, Costa VM, Barral-Netto M, Abrahamsohn IA. *Leishmania (L.) amazonensis*-induced inhibition of nitric oxide synthesis in host macrophages. Microbes Infect 2002; 4:23-29.

11. Tfouni E, Truzzi DR, Tavares A, Gomes AJ, Figueiredo LE, Franco DW. Biological activity of ruthenium nitrosyl complexes. Nitric Oxide 2012; 26:38-53.

12. Miranda MM, Panis C, Cataneo AH, da Silva SS, Kawakami NY, Lopes LG, et al. Nitric oxide and Brazilian propolis combined accelerates tissue repair by modulating cell migration, cytokine production and collagen deposition in experimental leishmaniasis. PLoS One 2015; 10:e0125101.

13. Ruano AL, López-Abán J, Fernández-Soto P, de Melo AL, Muro A. Treatment with nitric oxide donors diminishes hyperinfection by *Strongyloides venezuelensis* in mice treated with dexamethasone. Acta Trop 2015; 152:90-95.

14. Pavanelli W, da Silva JJ, Panis C, Cunha TM, Costa IC, de Menezes MC, et al. Experimental Chemotherapy in Paracoccidioidomycosis Using Ruthenium NO Donor. Mycopathol 2011; 172:95-107.

15. Silva JJN, Guedes PMM, Zottis A, Balliano TL, Nascimento-Silva FO, França Lopes LG, et al. Novel ruthenium complexes as potential drugs for Chagas's disease: enzyme inhibition and *in vitro*/*in vivo* trypanocidal activity. Br J Pharmacol 2010; 160:260-269.

16. Bates JN, Baker MT, Guerra Jr R, Harrison DG. Nitric oxide generation from nitroprusside by vascular tissue. Evidence that reduction of the nitroprusside anion and cyanide loss are required. Biochem Pharmacol 1991; 42:S157-S165.

17. Genestra M, Soares-Bezerra RJ, Gomes-Silva L, Fabrino DL, Bellato-Santos T, Castro-Pinto DB, et al. *In vitro* sodium nitroprusside-mediated toxicity towards *Leishmania amazonensis* promastigotes and axenic amastigotes. Cell Biochem Funct 2008; 26:709-717.

18. Da Silva SS, Thomé GS, Cataneo AHD, Miranda MM, Felipe I, Guadalupe C, et al. Brazilian propolis antileishmanial and immunomodulatory effects. Evid Based Complement Alternat Med 2013; 2013:673058.

19. Panis C, Mazzuco TL, Costa CZ, Victorino VJ, Tatakihara VL, Yamauchi LM, et al. *Trypanosoma cruzi*: Effect of the absence of 5-lipoxygenase (5-LO)-derived leukotrienes on levels of cytokines, nitric oxide and iNOS expression in cardiac tissue in the acute phase of infection in mice. Exp Parasitol 2011; 127:58-65.

20. Chatterjee S, Malhotra R, Varghese F, Bukhari AB, Patil A, Budrukkar A, et al. Quantitative Immunohistochemical Analysis Reveals Association between Sodium Iodide Symporter and Estrogen Receptor Expression in Breast Cancer. PLoS ONE 2013; 8:e54055.

21. Horta MF, Mendes BP, Roma EH, Noronha FSM, Macêdo JP, Oliveira LS, et al. Reactive oxygen species and nitric oxide in cutaneous leishmaniasis. J Parasitol Res 2012; 2012:203818.

22. Isnard A, Shio MT, Olivier M. Impact of *Leishmania* metalloprotease GP63 on macrophage signaling. Front Cell Infect Microbiol 2012; 2:72.

23. Shio MT, Hassani K, Isnard A, Ralph B, Contreras I, Gomez MA, et al. Host cell signalling and *leishmania* mechanisms of evasion. J Trop Med 2012; 2012:819512.

24. Lockwood A, Patka J, Raninovich M, Wyatt K, Abraham P. Sodium nitroprusside-associated cyanide toxicity in adult patients - fact or fiction? A critical review of the evidence and clinical relevance. J Clin Trials - Dovepress 2010; 2:133-148.

25. Page IH, Corcoran AC, Dustan HP, Koppanyi T. Cardiovascular actions of sodium nitroprusside in animals and hypertensive patients. Circulation 1955; 11:188-198.

26. Hottinger DG, Beebe DS, Kozhimannil T, Prielipp RC, Belani KG. Sodium nitroprusside in 2014: A clinical concepts review. J Anaesthesiol Clin Pharmacol 2014; 30:462-471.

27. Taylor TH, Styles M, Lamming AJ. Sodium nitroprusside as a hypotensive agent in general anaesthesia. Br J Anaesth 1970; 42:859-864.

28. Friederich JA, Butterworth JF. Sodium nitroprusside: twenty years and counting. Anesth Analg 1995; 81:152-162.

29. Schopfer FJ, Baker PR, Freeman BA. NO-dependent protein nitration: a cell signaling event or an oxidative inflammatory response? Trends Biochem Sci 2003; 28:646-654.

30. Linares E, Giorgio S, Mortara RA, Santos CX, Yamada AT, Augusto O. Role of peroxynitrite in macrophage microbicidal mechanisms *in vivo* revealed by protein nitration and hydroxylation. Free Radic Biol Med 2001; 30:1234-1242.

31. Heinzel FP, Rerko RM, Hatam F, Locksley RM. IL-2 is necessary for the progression of leishmaniasis in susceptible murine hosts. J Immunol 1993; 150:3924-3931.

32. Sypek JP, Chung CL, Mayor SE, Subramanyam JM, Goldman SJ, Sieburth DS, et al. Resolution of cutaneous leishmaniasis: interleukin 12 initiates a protective T helper type 1 immune response. J Exp Med 1993; 177:1797-1802.

33. Gessani S, BelardellI F. IFN-gamma expression in macrophages and its possible biological significance. Cytokine Growth Factor Rev 1998; 9:117-123.

34. Farrar MA, Schreiber RD. The molecular cell biology of interferon-gamma and its receptor. Annu Rev Immunol 1993; 11:571-611.

35. Lapara NJ, Kelly BL. Suppression of LPS-induced inflammatory responses in macrophages infected with *Leishmania*. J Inflamm (Lond) 2010; 7:8.

36. Murphy TL, Cleveland MG, Kulesza P, Magram J, Murphy KM. Regulation of interleukin 12 p40 expression through an NF-kappa B half-site. Mol Cell Biol 1995; 15:5258-5267.

37. Fonseca SG, Romão PR, Figueiredo F, Morais RH, Lima HC, Ferreira SH, et al. TNF-alpha mediates the induction of nitric oxide synthase in macrophages but not in neutrophils in experimental cutaneous leishmaniasis. Eur J Immunol 2003; 33:2297-3306.

38. Chang K, Lee SJ, Cheong I, Billiar TR, Chung HT, Han JA, et al. Nitric oxide suppresses inducible nitric oxide synthase expression by inhibiting post-translational modification of IkappaB. Exp Mol Med 2004; 36:311-324.

39. Colasanti M, Persichini T, Menegazzi M, Mariotto S, Giordano E, Calderena CM, et al. Induction of nitric oxide synthase mRNA expression. Suppression by exogenous nitric oxide. J Biol Chem 1995; 270:26731-26733.

40. Emre A, Bayram O, Salman B, Ercan S, Anadol Z, Akin O. Sodium Nitroprusside as a Nitric Oxide Donor in a Rat Intestinal Ischemia-Reperfusion Model. Clinics 2008; 63:91-96.

41. William M, Vien J, Hamilton E, Garcia A, Bundgaard H, Clarke RJ, et al. The nitric oxide donor sodium nitroprusside stimulates the Na+–K+ pump in isolated rabbit cardiac myocytes. Journal Physiol 2005; 565:815-825.