Sodium nitroprusside has leishmanicidal activity independent of iNOS

Natália Yoshie Kawakami[1], Fernanda Tomiotto-Pellissier[1], Allan Henrique Depieri Cataneo[1], Tatiane Marcusso Orsini[1], Ana Paula Fortes Dos Santos Thomazelli[1], Carolina Panis[2], Ivete Conchon-Costa[1] and Wander Rogério Pavanelli[1]


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[3].

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, 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].

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Indeed, studies have shown that such sources are active against *Leishmania in vitro*[10] 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)$_5$NO]•2H$_2$O),
an inorganic compound\textsuperscript{(16)} active against promastigotes and axenic amastigotes of \emph{Leishmania (Leishmania) amazonensis}\textsuperscript{(17)}. In this report, we demonstrate that sodium nitroprusside enhances the microbicidal activity of \emph{Leishmania}-infected macrophages by enhancing production of nitric oxide and 3-nitrotyrosine.

\section*{METHODS}

\textbf{Leishmania (Leishmania) amazonensis}

Promastigotes of \emph{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\textsuperscript{2} flasks at 24°C.

\textbf{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 37°C and 5\% CO\textsubscript{2} in 24-well plates (5 × 10\textsuperscript{5} cells per well) with 13mm glass coverslips with 200µL RPMI 1640 medium. Adherent cells were infected for 2h with \emph{L. amazonensis} promastigotes at a ratio of 1:5, washed with phosphate-buffered saline to remove non-phagocytized parasites, and treated for 24h at 37°C and 5\% CO\textsubscript{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.

\textbf{Promastigote recovery}

Promastigote recovery was performed as previously described\textsuperscript{(18)}. Briefly, peritoneal macrophages (5 × 10\textsuperscript{5} cells) were infected with \emph{L. amazonensis}, treated for 24h at 37°C and 5\% CO\textsubscript{2}, 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.

\textbf{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-\alpha, IFN-\gamma, and IL-12-p70 were determined by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s instructions (eBioscences®, USA). Plates were read at 450nm using a plate reader (Thermo-TP-Reader).

\textbf{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\textsuperscript{(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\textsubscript{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\textsubscript{2}.

\textbf{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\textsuperscript{+} 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°C 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\textsuperscript{+} 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\textsubscript{2}O\textsubscript{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\textsuperscript{(19)}. Slides that were not probed with primary antibody were used as negative control.

\textbf{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...
this cytokine is primarily produced by natural killer cells, CD4⁺, and CD8⁺ T lymphocytes. We note that *Leishmania* is capable of suppressing IL-12 expression. 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. 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. Thus, we conclude that the antileishmanial activity of sodium nitroprusside depends on its properties as a source of nitric oxide.

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**