Nitric Oxide and Brazilian Propolis Combined Accelerates Tissue Repair by Modulating Cell Migration, Cytokine Production and Collagen Deposition in Experimental Leishmaniasis

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Abstract

The fact that drugs currently used in the treatment of Leishmania are highly toxic and associated with acquired resistance has promoted the search for new therapies for treating American tegumentary leishmaniasis (ATL). In this study, BALB/c mice were injected in the hind paw with Leishmania (Leishmania) amazonensis and subsequently treated with a combination of nitric oxide (NO) donor (cis-[Ru(bpy)2imN(NO)](PF6)3) (Ru-NO), given by intraperitoneal injection, and oral Brazilian propolis for 30 days. Ru-NO reached the center of the lesion and increased the NO level in the injured hind paw without lesion exacerbation. Histological and immunological parameters of chronic inflammation showed that this combined treatment increased the efficacy of macrophages, determined by the decrease in the number of parasitized cells, leading to reduced expression of proinflammatory and tissue damage markers. In addition, these drugs in combination fostered wound healing, enhanced the number of fibroblasts, pro-healing cytokines and induced collagen synthesis at the lesion site. Overall, our findings suggest that the combination of the NO donor Ru-NO and Brazilian propolis alleviates experimental ATL lesions, highlighting a new therapeutic option that can be considered for further in vivo investigations as a candidate for the treatment of cutaneous leishmaniasis.
Introduction

*Leishmania (Leishmania) amazonensis* is an obligatory intracellular parasite of mammalian cells and one of the causative agents of American tegumentary leishmaniasis (ATL). Infection occurs from the bite of an infected phlebotomine sand fly, and depending on the host immune response, various clinical manifestations may result, ranging from a single granulomatous skin lesion at the site of the bite to diffuse lesions, where it may or may not affect the mucous membranes or even progress to visceral disease [1].

The first choice of antiparasitic agent for treating this disease in Brazil since the 1940s has been Glucantime (N-methyl glucamine antimoniate); however, despite being used against all forms of leishmaniasis, it has serious limitations in clinical practice, due to its extensive toxicity and limited efficacy [2].

Experimental models have shown that the outcome of *Leishmania* infection is critically dependent on the activation of CD4 T cell subsets [3]. Host susceptibility is ascribed to Th2 immune response, which leads to the development and multiplication of the parasite [4]. On the other hand, resistance is established by preferential activation of a Th1 subpopulation of lymphocytes, which produce IFN-γ and TNF-α, leading to macrophage activation and increased activity of inducible nitric oxide synthase (iNOS) and NADPH oxidase, with consequent increase in the production of nitric oxide (NO) and reactive oxygen species (ROS), respectively [4–6]. Among the microbialicidal mechanisms exhibited by phagocytic cells, NO production has been shown to be one of the most important for eliminating *Leishmania* spp.[7, 8].

Nevertheless, *Leishmania* has numerous escape mechanisms [9]. For instance, it can reduce NO production in macrophages by increasing the expression of arginase, which catalyzed the cleavage of L-arginine [10–12]. Therefore, the parasite can multiply and escape from the microbialicidal actions of the host, and in attempt to eliminate it and control the infection, there is an intense activation of many defense mechanisms, resulting in a strong inflammatory response, increasing tissue damage and exacerbation of the injury [13, 14].

Synthetic or natural substances with leishmanicidal and even anti-inflammatory capacity have emerged as an alternative to conventional treatment. In this context, the use of exogenous compounds that can donate microbialicidal molecules, such as NO, can be an important strategy for the effective treatment of leishmaniasis. Some *in vitro* studies had already reported that NO donors can inhibit mitochondrial respiration in amastigote and promastigote forms of *Leishmania* spp., killing the parasite [15, 16]. Moreover, NO plays an important role in wound healing and collagen deposition [17, 18]. Accordingly, ruthenium-NO-donor complexes have emerged as a potential therapeutic approach for disease models that require NO as the effector microbialicidal molecule. The advantages of such drugs include controlled release of NO, low toxicity and stability in aqueous media. NO molecules are released from such complexes by reducing agents commonly present in biological media [19, 20].

Another compound of interest that has been widely investigated in parasitic infections is propolis extract. Propolis has been widely employed in several disease models, showing great potential in protective immune response against leishmaniasis [21–23]. The major components of this bee product are phenolic compounds (flavonoids, aromatic acids and benzopyranes), di- and triterpenes and essential oils [24], which have already been described as having anti-inflammatory [25, 26], antioxidant [27], immunomodulatory [28–31] properties and being able to promote wound healing and reepithelization process [32, 33]. Furthermore, it has been shown that propolis is able to increase the generation of hydrogen peroxide, suggesting that this product modulates the activation of macrophages and acts against intracellular parasites [34, 35].
Since NO and propolis can play an important role in the control of various parasite diseases, the objective of this study was to evaluate the effect of combined therapy using the NO donor cis-[Ru(bpy)$_2$imN(NO)](PF$_6$)$_3$ (Ru-NO), where bpy = 2,2'-bipyridine and imN = imidazole, and Brazilian propolis against infection with *L. amazonensis*.

**Material and Methods**

**Parasite**

*L. amazonensis* (MHOM/BR/1989/166MJO) was used as promastigote forms in the stationary growth phase for the infection. The parasites were obtained from popliteal lymph nodes of *L. amazonensis*-infected mice and maintained in 199 culture medium (GIBCO-Invitrogen) supplemented with 10% fetal bovine serum (FBS; GIBCO-Invitrogen), 1 M Hepes, 0.1% human urine, 0.1% L-glutamine, 10 U/mL penicillin and 10 μg/mL streptomycin (GIBCO-Invitrogen) and 10% sodium bicarbonate. Cells were maintained in 25-cm$^2$ flasks in a BOD-type incubator at 25°C.

**Chemicals, drugs, and reagents**

Propolis was collected in the Beekeeping Section of the Lageado Farm, São Paulo State University (UNESP), Botucatu, SP, Brazil, from *Apis mellifera* L. colonies. Propolis was ground and a 30% extract in 70% ethanol was prepared. Its chemical composition was analyzed using gas-chromatography (GC), gas chromatography-mass spectrometry (GC-MS) and thin layer chromatography (TLC) [24]. The final concentration of the solvent, ethanol, in the experiments did not exceed 0.1%. NO donor compound cis-[Ru(bpy)$_2$imN(NO)](PF$_6$)$_3$ (Ru-NO) was synthesized and characterized as described previously by Silva *et al.* [36]. Glucantime (Sanofi-Aventis, Brazil; 300 mg/ml, 81 mg/ml Sb$^V$) was used as standard drug for treatment of ACL[15].

**Animals and infection**

Female BALB/c mice weighing approximately 25–30 g, aged 6–8 weeks old, were obtained from the Institute Carlos Chagas-Fiocruz, Curitiba-PR, Brazil. They were infected in the right hind paw with 1x10$^5$ *L. amazonensis* promastigote forms. Mice were kept under pathogen-free conditions and used according to protocols approved by the Institutional Animal Care and Use Committee. This study was approved by Londrina State University Ethics Committee for Animal Experimentation (No. 56/2012).

**Experimental procedures**

Groups of five *L. amazonensis*-infected BALB/c were treated for 30 consecutive days, starting after lesion appearance in all mice, which occurred 8 weeks post-inoculation (p.i.). The treatment groups were as follows: Glucantime (33 μmol.kg$^{-1}$ day$^{-1}$) by intraperitoneal (i.p.) injection; propolis diluted in PBS, administered orally (p.o.) (5 mg.kg$^{-1}$ day$^{-1}$) [35]; Ru-NO diluted in PBS (0.385 μmol.kg$^{-1}$ day$^{-1}$, i.p.) [37]; and Ru-NO (0.385 μmol.kg$^{-1}$ day$^{-1}$, i.p.) plus propolis (5 mg.kg$^{-1}$ day$^{-1}$, p.o.). Uninfected and infected mice were used as control groups and received only PBS vehicle (p.o. and i.p.). The lesion size (in mm) was measured weekly during the treatment using a digital caliper (Starrett 799). At the end of therapy (86 days p.i.), animals were euthanized. Plasma was collected and the injured hind paw of each animal was excised, which was divided into four fragments for analysis.
AST and ALT levels

Five BALB/c mice were treated for 30 consecutive days with Ru-NO (0.385 μmol.kg⁻¹ day⁻¹, i.p.) plus propolis (5 mg.kg⁻¹ day⁻¹, p.o.). Plasma was collected for measurement of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), markers of hepatocellular damage, using a colorimetric assay with a commercial kit from Labtest Diagnóstica (Lagoa Santa, MG, Brazil).

Ruthenium detection by energy dispersive spectroscopy (EDS)

Injured paw fragments were immersed in 8% paraformaldehyde and 0.2 M cacodylate buffer fixing solution for 48 h at room temperature. The paws were then dehydrated in an increasing graded ethanol series (70, 80, 90 and 100% GL) and critical-point dried in CO₂ (Bal-Tec CPD 030). The samples were placed on stubs and coated with carbon using a sputter coater (Bal-Tec SCD 050). For the detection of ruthenium, samples were analyzed by spectroscopy energy dispersive (EDS, Oxford), INCA software, coupled to a scanning electron microscope (SEM) FEI Quanta 200.

Real-time detection of NO/peroxynitrite by high sensitivity chemiluminescence

NO production was evaluated employing a highly sensitive NO detection system described by Kikuchi et al. (1993) [38], with some modifications. In this method, NO/peroxynitrite reacts with hydrogen peroxide, which in the presence of luminol produces triplet oxygen, which decays to singlet oxygen and emits photons, detected by a luminometer system coupled to a computer. One of the hind paw fragments was mechanically homogenized (Tissue-tearor, BioSpec), and the supernatants (100 mg/ml) were diluted 1:1 in fresh sterile 2 mM Na₂CO₃ buffer, pH 8.5, previously degassed with bubbling N₂ for 20 min to eliminate the presence of molecular oxygen and oxidation of NO to nitrite/nitrate. The final reaction volume was 500 μl of macerated paw plus 500 μl of Na₂CO₃ buffer. The starting reagent was prepared by mixing equal volumes of luminol solution (4.39 μM dissolved in 1 M KOH) diluted 1:10 in 36.58 μM desferrioxamine and 2.44 μM H₂O₂, with 3 parts degassed Na₂CO₃ buffer. This mixture was vortexed for 5 min before use. All solutions were sterile and kept at 25°C in covered tubes, protected from light. Finally, the luminometer chamber was injected with 50 μl of starting reagent and the reaction was performed in a Glomax luminometer (Promega), with automatic reagent injector, employing a kinetic protocol which allowed following the reaction at 10 readings per second. All chemicals were purchased from Sigma.

DNA extraction and parasite quantification by real-time PCR

Real-time quantitative PCR (RT-qPCR) was performed to determine the tissue parasite load in each group. A hind paw fragment was weighed, washed in PBS, and homogenized in lysis buffer (50 mM Tris-HCl [pH 7.6], 10 mM EDTA, 0.5% SDS, and 0.2 mg/ml proteinase K (Invitrogen, Carlsbad, CA), followed by phenol-chloroform extraction of DNA. Briefly, samples were mechanically homogenized (Tissue-tearor, BioSpec), incubated at 55°C for 12 h, and extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1). Two volumes of cold ethanol (Merck) were added to the aqueous phase, and samples were stored at -20°C for 12 h. Samples were then centrifuged for 30 min at 10,000 g, washed with 70% ethanol, dried at room temperature, and resuspended in 10 mM Tris HCl (pH 8.5) [39]. Real-time PCR was performed by using Platinum SYBR Green qPCR SuperMix UDG with ROX reagent (Invitrogen Corporation, New York, NY) with 100 ng total genomic DNA (gDNA). Parasite quantification was
performed using JW11 (forward, 5’-CCTATTTTACACCAACCCCCAGT-3’) and JW12 (reverse, 5’-GGTTAGGGGCGTTCTGCGAAA-3’) Leishmania-specific primers [40]. The samples were amplified with a Corbett Rotor-Gene thermal cycler under the following PCR conditions: an initial step of 2 min at 50°C, a second step of 10 min at 95°C, and 40 cycles of 30 s at 95°C, 30 s at 57°C, 30 s at 72°C, and 15 s at 82°C, followed by a dissociation step. The results were based on a standard curve constructed with DNA from culture samples of *L. amazonensis* promastigote forms.

**Histological analysis**

Hind paw fragments collected were fixed in Bouin’s solution, decalcified for 45 days in 5% EDTA, processed for paraffin embedding, sectioned (4 μm) and stained with hematoxylin-eosin (H&E) or sirius red by the picrosirius technique to assess the presence of collagen. Cellular profile was scored by counting the different cell types (macrophages, vacuolated macrophages, fibroblasts and lymphocytes) in H&E-stained paw sections, analyzed with a photomicroscope (Olympus, Miami, FL, USA) at a final magnification of 200x. Five images from each mouse were captured using Motic Images Plus v.2.0 (Motic China Group Co. Ltd., Xiamen, China). These images were divided into four quadrants, two of which were randomly selected for cell quantification with ImageJ 1.45 s software (NIH, USA—2011). Collagen quantification of the lesion site was determined in sirius red-stained paw sections under polarized light using a photomicroscope (Nikon Eclipse 80i) with a camera (Nikon DSFi1C) coupled to a computer using Nis Element software (Shinjuku, Japan), at a final magnification of 200x. Four images of four sections from each mouse were considered for the study and analyzed by Image Pro Plus (version 4.5). The results were expressed as percentage of area with the presence of collagen compared to the total measured area.

**Immunohistochemistry**

The paw sections were also analyzed by immunohistochemistry [41] to identify CD4+ and CD8+ T lymphocytes, iNOS and nitrotyrosine (3NT) by the labeled streptavidin—biotin method using an LSAB kit (DAKO Japan, Kyoto, Japan). The paraffin-embedded sections were deparaffinized and rehydrated, treated for 40 min with 2% BSA and incubated overnight at 4°C with primary antibody (anti- CD4+, anti- CD8+, anti-iNOS and anti-3NT rabbit polyclonal antibody diluted 1:500, Sigma). Horseradish peroxidase activity was visualized by treatment with H₂O₂ and 3, 30–diaminobenzidine (DAB) for 5 min. In the last step, the sections were weakly counterstained with Harry’s hematoxylin (Merck). For each assay, negative controls were prepared on serial sections. Intensity and localization of immunoreactivity with all primary antibodies used were examined on all sections using a light microscope (Olympus BX41, Olympus Optical Co., Ltd., Tokyo, Japan). As a negative control, the primary antibody was omitted. For the image analysis study, color photomicrographs of representative areas (magnification of 400x) were digitally acquired. For semi-quantitative scoring, images were evaluated by using the color deconvolution tool in Image J software (NIH, USA). Pixels were categorized as previously described by Chatterjee et al. [42] as strong positive (3+), positive (2+), weak positive (1+) and negative (0).

**Determination of cytokine profile by Western blotting**

The supernatants from hind paw fragments (100 mg/ml) was used to measure the cytokines and transcription factor expressed in the lesion by Western blotting [43]. Supernatant samples were first centrifuged and total protein determined by Lowry’s method modified by Miller [44]. Supernatant samples were diluted 1:2000 in 0.9% NaCl and incubated with 300 μl of cupric
reagent for 10 min. Afterwards, the mixture was vortexed and added to 900 μL of Folin-Ciocalteu's reagent and kept at 50°C in a water bath for 10 min. Absorbance of the samples was read at 660 nm, and the protein concentration was obtained from the standard curve of bovine serum albumin. A volume of sample corresponding to 20 μg protein was subjected to 10% acrylamide gel electrophoresis followed by transfer to a nitrocellulose membrane [43]. Membranes were blocked in 5% milk and incubated overnight with antibodies to NF-κB (p65), STAT3, TNF-α, IL-10 and TGF-β1 diluted 1:1000 (Santa Cruz Biotechnology, USA). Subsequently, the membranes were washed in PBS-0.1% Tween for 30 min and incubated with secondary antibody specific for each primary antibody for 2 h at room temperature (diluted 1:2000). The detection of the bands of respective antibodies was performed using the ECL Plus kit (GE Healthcare, United Kingdom). The images of the bands were scanned (Labscan Software, EG Healthcare) and quantified in Image J software (NIH, USA).

Statistical analysis
Results were expressed as the mean ± standard error of the mean (SEM) and analyzed with the Prism 5.0 statistical program (GraphPad Software, San Diego, CA), using the Student t-test, comparing with infected control. Differences were considered significant when \( P < 0.05 \).

Results
Ru-NO by intraperitoneal injection was able to reach the site of Leishmania-induced lesion
Initially, we checked if the route of drug administration delivered Ru-NO to the injured paw. Energy dispersive spectroscopy (EDS) analysis of the infected control revealed the presence of many elements including carbon, oxygen, iron, sodium, chlorine, phosphorus, magnesium and sulfur in the paw section (Fig 1A). Ru-NO treated mice showed the same elements plus the presence of ruthenium, proving that the intraperitoneal injection of Ru-NO donor was sufficient to ensure the delivery of this compound to the lesion site (Fig 1B). The presence of silicon and calcium are from mouse hair and bone, respectively.

Intraperitoneal injection of Ru-NO increased NO levels at the lesion site
Experimental interest in the use of NO-donors in ATL is based on the fact that Leishmania parasites are able to deplete NO levels by several mechanisms, reducing the bioavailability of NO for microbicidal action [9].

The complex cis-[Ru(bpy)₂imN(NO)](PF₆)₃ releases NO through electrochemical, photochemical and chemical processes [36]. The reaction of this compound with antioxidants could lead to the availability of free NO [37]. Since we demonstrated that the drug reached the lesion, we wondered if NO was released at the site of injury, employing a high-sensitivity chemiluminescence method based on the detection of NO/nitrosative stress [38].

According to our results, the infected group showed a marked depletion of NO levels when compared to uninfected control (Fig 2). Infected BALB/c mice treated with Ru-NO, i.p., showed increased NO levels at the lesion site, indicating its local release by the Ru-NO donor (Fig 2), which demonstrated that systemic administration of Ru-NO donor could represent a useful strategy for NO release at the lesion site, overcoming the NO depleting mechanisms of Leishmania.
Treatments delayed the development of the cutaneous lesion and significantly reduced the parasite load

Several studies have reported that Brazilian propolis exerts important immunomodulatory effects [22, 23], which could improve the effects of the Ru-NO donor against the *Leishmania* pathogenic mechanisms. Therefore, we aimed to investigate the immunomodulatory effect of combined propolis and Ru-NO in experimental leishmaniasis. BALB/c mice were infected with *L. amazonensis* and the lesion was monitored for 30 days, with or without treatments.

The lesions of the infected control group increased progressively, and the ulcer size ranged from 9.7 to 12.5 mm at day 30 of treatment (Fig 3A). In contrast, the lesions in all treated groups showed a significantly slower development compared to infected control. At day 30, mean lesion size was 8.75±1.32 mm in the propolis-treated group, 8.20±1.13 mm with RuNO, 8.91±1.03 mm with RuNO plus propolis and 8.10±1.01 mm with Glucantime (Fig 3A).

Concerning the efficacy of treatments on parasite load, we determined parasite DNA levels at the lesion site by real-time PCR. A marked reduction in *L. amazonensis* number at the lesion site was found in all treated groups with no difference between them (Fig 3B). Interestingly, treatment with Ru-NO, propolis or Ru-NO plus propolis produced similar results as the
Glucantime group. Furthermore, cutaneous lesions and parasite load in all treated animals were smaller than those found in the untreated infected control, showing the establishment of an inflammatory process with reduced tissue parasitism and severity of lesions.

It was important to assess the possible toxicity of combined treatment, and we found that Ru-NO plus propolis treatment daily for 30 days did not foster hepatic lesions since the hepatic enzymes ALT and AST remained unchanged (S1 Fig).

Ru-NO plus propolis treatment increased the infiltrating macrophage population and modulated the expression of inflammatory markers

The complex interactions between Leishmania and immune cells have fundamental effects on the outcome of the disease. The success or failure of infection depends on the development of adaptive immune response. To investigate which immune cells migrate to the site of lesion and their role in the modulation on the cytokines synthesis after the treatment with Ru-NO and propolis, we performed histopathological analysis of the cellular infiltrate by light microscopy, immunohistochemical analyses for inflammatory markers and Western blotting to determine the expression of cytokines and transcription factor.

Regarding the cell profile of the lesion, the number of infiltrating macrophages was significantly increased at the lesion site in all treated groups when compared with infected control (Fig 4A). T cells were similar in the infected control and Glucantime-treated group. However, mice treated with propolis, Ru-NO or Ru-NO plus propolis exhibited a reduced T cell population (Fig 4B).

Immunohistochemical studies using specific monoclonal antibodies for CD4+ and CD8+ T labeling revealed that treatment with propolis and Glucantime increased the number of CD4+ and CD8+ T cells, respectively (Fig 4C and 4D). Nevertheless, Ru-NO only and the combined treatment Ru-NO plus propolis did not induce CD4 and CD8 recruitment to the lesion site (Fig 4C and 4D).
Fig 4. Effect of Ru-NO plus propolis on inflammatory process at lesion site. BALB/c mice were infected with $1 \times 10^5$ promastigotes of *L. amazonensis* in the right hind paw and treated with Ru-NO (0.385 μmol.kg$^{-1}$ day$^{-1}$, i.p.), propolis (5 mg.kg$^{-1}$ day$^{-1}$, p.o.) or Glucantime (33 μmol.kg$^{-1}$ day$^{-1}$, i.p.) for 4 weeks post-lesion appearance. The paw sections were analyzed for number of A) macrophages (H&E), B) lymphocytes (H&E), C) CD4$^+$ T (IHC), and D) CD8$^+$ T (IHC) at a final magnification of 200x. The paw supernatants were assayed for expression of E) NF-κB(p65), F) TNF-α, G) IL-10 and H) STAT3 by Western blotting. The dotted line (—) represents the uninfected control. Bars represent the mean ± SEM (n = 5). Significant difference relative to the infected control * $P<0.05$, ** $P<0.01$ and *** $P<0.0001$, unpaired t-test.

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We evaluated the signaling pathways during the infection by measuring the expression of NF-κB (p65) (Fig 4E), TNF-α (Fig 4F), IL-10 (Fig 4G) and STAT3 (Fig 4H) by Western blot analysis of hind paw supernatant of each group. The expression of all markers was significantly altered during infection.

Glucantime treatment caused an increase in NF-κB and IL-10 expression compared to the infected control group. The animals treated with propolis showed a proinflammatory profile, with significant increase in expression of NF-κB, TNF-α and STAT3, associated with a reduced expression of IL-10, compared to infected control. The Ru-NO-treated group showed increased expression of NF-κB and STAT3 with concomitant reduction in the expression of TNF-α and IL-10 when compared to the infected control. Treatment with Ru-NO plus propolis caused a reduction in the expression of NF-κB, TNF-α and IL-10 and increased expression of STAT3, compared to the infected control. According to these findings, the combined use of Ru-NO and propolis seems to primarily impact the innate immune response, by improving the recruitment of effective macrophages to the lesion site, resulting in parasite killing besides the attenuation of the inflammatory process without any impact on the number of infiltrating T cells.

Ru-NO plus propolis treatment did not promote the formation of NO-derived nitrotyrosine residues, favoring the control of lesion exacerbation

NO has been shown to be a crucial and versatile molecule in the control of a variety of intracellular organisms. In leishmaniasis, this compound is mainly produced by macrophages through the activation of inducible NO synthase (iNOS), which catalyzes the production of a huge amount of NO during infectious processes. Nevertheless, excess NO can yield peroxynitrite, which causes protein oxidation by nitration of tyrosine residues, resulting in nitrotyrosine formation and consequently increased tissue damage [45, 46]

To determine iNOS activity in treated groups and whether the administration of exogenous NO exacerbates tissue damage, we performed immunohistochemistry analysis. The results showed that after 30 days of treatment, the groups treated with Glucantime and with Ru-NO plus propolis showed lower iNOS expression when compared with the infected control group, returning to levels found in animals without infection (Fig 5A).

Concerning nitrotyrosine expression, the results were complementary and although all treated groups tended to show a decline in labeling, Glucantime and Ru-NO plus propolis groups showed similar nitrotyrosine levels as baseline levels also found in uninfected control (Fig 5B). Thus, the results indicated a reduction in the inflammatory process, demonstrating that exogenous NO supply along with the protective effects of propolis was beneficial to the host, favoring the control of the inflammatory response.

Ru-NO plus propolis treatment induced tissue repair

Nitric oxide and propolis are also significant regulators of wound healing, and during healing of Leishmania lesions, there is a production of collagen and metalloproteinases [47]. We investigated the presence of tissue repair markers in the injured area.

Only the animals treated with Ru-NO plus propolis showed a considerable increase in fibroblasts at the lesion site, accompanied by the upregulation of TGF-β1 synthesis (Fig 6A and 6B). The groups that received propolis or Glucantime showed a decrease in fibroblasts in the lesions, and the Ru-NO group did not show any change in this cell type (Fig 6A). TGF-β1 levels were also altered with a reduction in the propolis group and an increase in the group treated with Ru-NO only (Fig 6B).
Concerning the synthesis of collagen, picrosirius staining showed significantly greater levels of collagen deposition at the lesion site in the Ru-NO plus propolis group, indicating enhanced wound healing (Fig 6C and 6D). The group that received Glucantime also showed a slight increase in the amount of collagen fibers. However, in all other groups analyzed, collagen fibers were only weakly visualized at the injury site.

Discussion

The inflammatory response developed during Leishmaniasis is essential for controlling the parasite burden, and the clinical outcome of ATL is a result of the immune capacity of the host in building an effective adaptive response against the parasite. In most cases, this host-parasite fight results in chronic disease and development of severe cutaneous lesions, where uncontrolled or continued inflammation is correlated with extended collateral damage and may constitute a crucial factor for the development of features of chronic disease (e.g., deforming ulcers) and subsequent morbidity [13, 14, 48].
Fig 6. Effect of Ru-NO + propolis on healing process at the lesion site. The paw sections were analyzed for A) number of fibroblasts (H&E), B) TGF-β1 by Western blotting. C) Photomicrographs of paw sections stained with picrosirius technique D) and the quantification of collagen deposition. The paw sections were analyzed at a final magnification of 200x. The data represent the means±SEM of five animals per group. The dotted line (—) represents the uninfected control. Significant difference relative to infected control ** P<0.01, ***P<0.0001, unpaired t-test. Scale bars = 50 μm.

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In this context, the present study provided new perspectives for the treatment of experimental cutaneous leishmaniasis, especially regarding the use of combined therapy with the microbiidal molecule Ru-NO and the immunomodulatory propolis extract. Our data demonstrated that this combined therapy was able to increase the infiltration of competent macrophages at the site of leishmanial infection, decreasing the parasite load, modulating the inflammatory response and accelerating the healing of the lesion.

The analysis of the lesion development in infected footpad at 86 days p.i. showed that all treatments tested resulted in the control of the lesion, due to the enhanced recruitment of competent macrophages, consequently controlling the parasitic burden.

Several studies have shown that NO is involved in the death of *Leishmania* amastigotes during macrophage burst [7, 8, 49]. Recently, Olekhnovitch et al.[8] showed a new perspective of NO action in an experimental model of *Leishmania*. These authors proved that the collective production and subsequent diffusion of NO creates an antimicrobial environment that permits parasite killing in cells, demonstrating a cooperative mechanism at the tissue level to control *L. major* amastigotes, reinforcing our choice of an exogenous nitric oxide donor to control this disease.

Some molecules of a group commonly known as NO donors have been tested against *Leishmania* spp., showing a considerable effect against cutaneous leishmaniasis. The leishmanicidal effects of trans-[Ru(NO)(NH3)4L](X)3 have been demonstrated in *vitro* and *in vivo* against *L. major* [15]. *L. amazonensis*, when co-cultured with sodium nitroprusside *in vitro*, also showed a decrease in the number of promastigotes and axenic amastigotes in a concentration-dependent manner [50], and S-nitrosoglutathione (GSNO) was found to be cytotoxic to amastigotes and to promote the healing of topically treated *L. major* or *L. braziliensis* skin lesions [51].

Nevertheless, topical treatment of cutaneous leishmaniasis with NO donors, potassium nitrite and transdermal patches for continuous NO delivery, did not perform well against the disease [52, 53]. These variable results could possibly be related to the instability of NO release.

Our results demonstrated for the first time that the i.p. inoculation route of Ru-NO was effective in delivering NO to the lesion site, and consequently, the noted effect was due to the presence of NO and its reaction with compounds at the injury site and not to a systemic effect of this compound.

Regarding Brazilian propolis, our earlier study demonstrated that this propolis had antileishmanial and immunomodulatory effects against *L. braziliensis* in and *in vitro* experimental infection, increasing macrophage internalization and further killing of parasites [23]. The propolis tested contains mainly phenolic compounds, including flavonoids, di- and triterpenes, and essential oils [24]. In experimental leishmaniasis, propolis components such as prenylated compounds and benzophenones [54] have already been associated with the inhibition of amastigote proliferation by macrophage activation or by direct effect on promastigote forms (caffeic acid, p-coumaric acid, aromadendrine-4’-methyl ether, 3-prenyl-p-coumaric and 3,5-diprenyl-p-coumaric) [55]. Besides the leishmanicidal effects, peritoneal macrophages were activated by Brazilian green propolis, increasing the phagocytic ability [56].

When evaluating the combination of Ru-NO and propolis only with regard to amastigote number, we did not observe a greater reduction in parasite load; however, unlike with the other treatments, we found a considerable synergistic effect of these compounds in reducing the inflammatory process at the lesion site.

The effect of Ru-NO plus propolis on cell profile found in the lesions revealed a moderate inflammatory infiltrate, composed mainly of macrophage cells, and the quantification of CD4+ and CD8+ T lymphocytes showed a significant decrease in mice treated with Ru-NO plus propolis. It is possible that this attenuated inflammatory response had been, in part, due to reduced
parasite load, suggesting that the combined drugs are crucial to promote parasite clearance and modulation of the local inflammatory response.

This immunosuppressive activity of NO has already been reported in *in vitro* and *in vivo* models, including microfilariae [57], mycobacterial [58] and *Toxoplasma gondii* [59] infections. NO donors are able to decrease the rolling and adhesion of leukocytes to endothelial cells, reducing transmigration to inflammatory sites [60–62].

Proving the alleviation of inflammation, we also observed reduced expression of TNF-α levels and transcription nuclear factor NF-κB, which induces several genes that are responsible for the coding of various proinflammatory mediators [63, 64], in mice treated with Ru-NO plus propolis. Propolis and Ru-NO, when administered alone, caused an increase in NF-xb transcription, and TNF-α level were also higher in the group treated with propolis.

Pavanelli *et al.* [65] also observed that treatment with the NO donor cis-[Ru(bpy)2(NO)SO3](PF6) decreased TNF-α production and consequently inflammation in an *paracoccidioidomycosis* infection model. The reduced production of TNF-α, IL-10 and INF-γ was also associated with reduced myocarditis in mice treated with the ruthenium NO donor trans-[RuCl([15]aneN4)NO]2+ in a Chaga’s infection model [66].

When evaluating the presence of protein tyrosine nitration, another marker of inflammation associated with the upregulation of iNOS [67], we observed that the expression of iNOS and nitrotyrosine levels were significantly diminished only in the groups treated with Ru-NO plus propolis and Glucantime, similar to that observed in the group without infection. Indeed, NO can control its own production by inhibiting iNOS activity, an important feedback control mechanism [68]. Interestingly, this result allows us to infer that while iNOS expression was low in the combined treatment group, NO delivered to the injury site did not aggravate the process of protein nitration, suggesting that this molecule may be consumed in another process. Taken together, the data suggest that the drug combination is crucial for parasite elimination and to modulate the local inflammatory response.

Another important property of both NO and propolis is the ability to promote tissue repair. In fact, in recent years, NO has emerged as a critical molecule in wound healing, where it increases collagen content in experimental wounds [69–71]. It should be noted that propolis extract is already used for wound healing, burns, external ulcers, shortening healing time, increasing wound contraction and accelerating tissue repair [72].

Soneja *et al.* [18] support the strategy of accelerating the wound healing process with antioxidants, leading to decreased oxidative stress and delivery of NO to the lesion site. The Ru-NO plus propolis treatment would be ideal for this approach, since various compounds in propolis have been described as powerful antioxidants, capable of scavenging free radicals, making the donated NO available for the healing process [73, 74].

Our results showed that only Ru-NO plus propolis treatment was able to induce the healing process, because it increased fibroblasts, a cell type that is a central player in tissue repair [17, 18], TGF-β and STAT3 levels and collagen deposition. IL-10 production was not altered by combined treatment, indicating that repair of tissue damage was not dependent on this cytokine.

TGF-β is a cytokine involved in orchestrating wound repair and participates in all phases of the healing process [75]. This cytokine helps in the chemotaxis of fibroblasts to the site of injury and stimulates collagen deposition [76, 77]. Nakamura *et al.* [78] and Frank *et al.* [17] showed that NO was able to convert latent TGF-β1 to the active form in fibroblasts, regulating collagen synthesis, where it is predominantly expressed during the repair process [79]. Transcription factor STAT3 when activated by various cytokines and growth factors plays a key role in wound healing [80]. Studies have shown that tissue-specific STAT3 gene deletion leads to impaired tissue remodeling [80–82].
Propolis, NO and TGF-β1 play central roles in collagen synthesis and can cross-regulate each other [33, 83, 84]. The synergistic signals of Ru-NO and propolis at the lesion site, provide a rapid deposition of remodeling tissue at the lesion site. Our data are in line with the findings of Baldwin et al. [85] in an ear model of Leishmania infection, which showed that the collagen deposition was more prominent in areas where re-epithelialization had occurred, when parasites had been cleared and inflammation controlled.

The data clearly showed that combined therapy resulted in alleviation of the inflammatory response, protected against tissue damage, and significantly accelerated the healing process when compared to the other groups.

It is important to emphasize that our therapy showed very similar effects as Glucantime conventional treatment, in relation to the control of lesion size and reduced amount of parasites. However, when considering the inflammatory response, which can be so aggravating in this parasitosis, and the induction of the healing process, there is no doubt that the effect of Ru-NO plus propolis was superior to that of Glucantime, also highlighting the lack of hepatotoxic damage in this treatment period.

In summary, the data obtained strongly suggest that the combination of cis-[Ru(NO)(bpy)2imN] (PF6)3 and Brazilian propolis is effective against L. amazonensis in vivo, allowing us to infer that this combined therapy can be an alternative for the treatment of leishmaniasis.

Supporting Information

S1 Fig. Effect of treatment with Ru-NO + propolis or saline on ALT and AST levels. BALB/c mice were treated with Ru-NO (0.385 μmol.kg⁻¹ day⁻¹, i.p.) and propolis (5 mg.kg⁻¹, p.o.) for 30 consecutive days. ALT and AST were measured 24 h after the last treatment day (n = 5). Results are expressed as mean ± SEM * indicates P < 0.05 versus control, unpaired t-test. (TIF)

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Author Contributions

Conceived and designed the experiments: MMM CP SSS ICC WRP JMS JJNS LGFL. Performed the experiments: MMM CP AHDC NYK SSS ATM LMY CGTJA. Analyzed the data: MMM CP AHDC NYK SSS ATM LMY CGTJA RC JMS ICC WRJ LGFL. Contributed reagents/materials/analysis tools: LGFL JJNS JMS RC CGTJA LMY CP. Wrote the paper: MMM CP AHDC NYK SSS LGFL ATM LMY JJNS RC WRJ CGTJA.

References

1. Barral A, Pedral-Sampaio D, Grimaldi G, Momen H, McMahon-Pratt D, Ribeiro de Jesus A, et al. (1991) Leishmaniasis in Bahia, Brazil: Evidence that Leishmania amazonensis produces a wide spectrum of clinical disease. Am J Trop Med Hyg 44: 536–546. PMID: 2063957
2. Alvar J, Croft S, Olliaro P (2006) Chemotherapy in the treatment and control of leishmaniasis. Adv Parasitol 61: 223–274. doi: 10.1016/s0065-308x(05)61006-8 PMID: 16735166
3. Reiner SL, Locksley RM (1995) The regulation of immunity to Leishmania major. Annu Rev Immunol 13: 151–177. doi: 10.1146/annurev.iy.13.040195.001055 PMID: 7612219
4. Sacks DN-T, Nancy (2002) The immunology of susceptibility and resistance to Leishmania major in mice. Nat Rev Immunol 2: 845–858 doi: 10.1038/nri933 PMID: 12415308
1. Lemesre JL, Sereno D, Dauloué W, Wanasen N, Soong L (2008) L-arginine metabolism and its impact on host immunity against Leishmania amazonensis. J. Clin Invest. 124: 1711–1722. doi: 10.1172/JCI72058 PMID: 24614106

2. Babior BM (2000) Phagocytes and oxidative stress. Am J Med. 109: 33–44. doi: 10.1016/S0002-9343(00)00481-2 PMID: 10936476

3. Van Assche T, Deschacht M, da Luz RAI, Maes L, Cos P (2011) Leishmania–macrophage interactions: Insights into the redox biology. Free Rad Biol Med. 51: 337–351. doi: 10.1016/j.freeradbiomed.2011.05.011 PMID: 21620959

4. Olekhnovitch R, Ryffel B, xFc, iler AJ, Bousso P (2014) Collective nitric oxide production provides tissue-wide immunity during Leishmania infection. J. Clin Invest. 124: 3293–3295. doi: 10.1172/JCI72058 PMID: 24614106

5. Olivier M, Gregory DJ, Forget G (2005) Subversion mechanisms by which Leishmania parasites can escape the host immune response: a signaling point of view. Clin Microbiol Rev. 18: 293–305. doi: 10.1128/cmrl.18.2.293-305.2005 PMID: 15831826

6. Bogdan C, Röllinghoff M (1999) How do protozoan parasites survive inside macrophages? Parasitol. Today. 15: 22–28. doi: 10.1016/S0169-4758(98)01362-3 PMID: 10234174

7. Lira R, Doherty M, Modi G, Sacks D (2000) Evolution of lesion formation, parasitic load, immune response, and reservoir potential in C57BL/6 mice following high- and low-dose challenge with Leishmania major. Infect Immun. 68: 5176–5182. doi: 10.1128/IAI.68.9.5176-5182.2000 PMID: 10948141

8. Oliveira WN, Ribeiro LE, Schrieffer A, Machado P, Carvalho EM, Bacellar O (2014) The role of inflammatory and anti-inflammatory cytokines in the pathogenesis of human tegumentary leishmaniasis. Cytokine. 66:127–32. doi: 10.1016/j.cyto.2013.12.016 PMID: 24485388

9. Toledo JC, Lima Neto BS, Franco DW (2005) Mutual effects in the chemical properties of the ruthenium metal center and ancillary ligands upon coordination. Coord Chem Rev. 249: 419–431. doi: 10.1016/j.ccr.2004.09.016 PMID: 120598778

10. Van Assche T, Deschacht M, da Luz RAI, Maes L, Cos P (2011) Leishmania–macrophage interactions: Insights into the redox biology. Free Rad Biol Med. 51: 337–351. doi: 10.1016/j.freeradbiomed.2011.05.011 PMID: 21620959

11. Lemesre JL, Sereno D, Dauloué W, Wanasen N, Soong L (2008) L-arginine metabolism and its impact on host immunity against Leishmania amazonensis. J. Clin Invest. 124: 1711–1722. doi: 10.1172/JCI72058 PMID: 24614106

12. Babior BM (2000) Phagocytes and oxidative stress. Am J Med. 109: 33–44. doi: 10.1016/S0002-9343(00)00481-2 PMID: 10936476

13. Cunningham AC (2002) Parasitic adaptive mechanisms in infection by Leishmania. Exp Mol Pathol. 72: 132–141. doi: 10.1006/expm.2002.2418 PMID: 11890722

14. Melo Pereira JC, Carregaro V, Costa DL, Santana da Silva J, Cunha FQ, Franco DW (2010) Antileishmanial activity of ruthenium([I]tetraamine nitrosyl complexes. Eur J Med Chem. 45: 4180–4187. doi: 10.1016/j.ejmech.2010.06.010 PMID: 20598778

15. Toledo JC, Lima Neto BS, Franco DW (2005) Mutual effects in the chemical properties of the ruthenium metal center and ancillary ligands upon coordination. Coord Chem Rev. 249: 419–431. doi: 10.1016/j.ccr.2004.09.016 PMID: 120598778

16. Lemesre JL, Sereno D, Dauloué W, Veyret B, Brajon N, Vincendeau P (1997) Leishmania spp.: nitric oxide-mediated metabolic inhibition of promastigote and axenically grown amastigote forms. Exp Parasitol. 86: 58–68. doi: 10.1006/expar.1997.4151 PMID: 9149241

17. Toledo JC, Lima Neto BS, Franco DW (2005) Mutual effects in the chemical properties of the ruthenium metal center and ancillary ligands upon coordination. Coord Chem Rev. 249: 419–431. doi: 10.1016/j.ccr.2004.09.016 PMID: 120598778

18. Toledo JC, Lima Neto BS, Franco DW (2005) Mutual effects in the chemical properties of the ruthenium metal center and ancillary ligands upon coordination. Coord Chem Rev. 249: 419–431. doi: 10.1016/j.ccr.2004.09.016 PMID: 120598778

19. Toledo JC, Lima Neto BS, Franco DW (2005) Mutual effects in the chemical properties of the ruthenium metal center and ancillary ligands upon coordination. Coord Chem Rev. 249: 419–431. doi: 10.1016/j.ccr.2004.09.016 PMID: 120598778

20. Silva FON, Cândido MCL, Holanda AKM, Diógenes ICN, Sousa EHS, Lopes LGF (2011) Mechanism and biological implications of the NO release of cis-[Ru(bpy)2L(NO)]n+ complexes: A key role of physiological thiols. J Inorg Biochem. 105: 624–629. doi: 10.1016/j.jinorgbio.2011.02.004 PMID: 21443852

21. Sforzin JM (2009) Própolis e imunidade—Comprovações científicas. São Paulo. p.67

22. Sforzin JM, Bankova V (2011) Propolis: Is there a potential for the development of new drugs? J Ethnopharmacol. 133: 253–260. doi: 10.1016/j.jep.2010.10.032 PMID: 20970490

23. da Silva SS, Thomé M, Cataneo A, Miranda MM, Felipe I, Andrade CGTJ, et al. (2013) Brazilian propolis antileishmanial and immunomodulatory effects. Evid Based Complement Alternat Med 2013:673058. doi: 10.1155/2013/673058 PMID: 23762152

24. Sforzin JM (2007) Propolis and the immune system: a review. J Ethnopharmacol. 113: 1–14. doi: 10.1016/j.jep.2007.05.012 PMID: 17580109

25. Hu F, Hepburn HR, Li Y, Chen M, Radloff SE, Daya S (2005) Effects of ethanol and water extracts of propolis (bee glue) on acute inflammatory animal models. J Ethnopharmacol. 100: 276–283. doi: 10.1016/j.jep.2005.02.044 PMID: 15899563

26. Amarante MK, Watanabe MAE, Conchon-Costa I, Fiori LL, Oda JMM, Bufolo MC, et al. (2012) The effect of propolis on CCL5 and IFN-γ expression by peripheral blood mononuclear cells from leishmaniasis patients. J Pharm Pharmacol. 64: 154–160. doi: 10.1111/j.2042-7158.2011.01385.x PMID: 22150683
27. Daleprane JB, Abdalla DS (2013) Emerging roles of propolis: antioxidant, cardioprotective, and antiangiogenic actions. Evid Based Complement Alternat Med 2013: 175135. doi:10.1155/2013/175135 PMID: 23662115

28. Orsatti CL, Missima F, Pagliarone AC, Bachiega TF, Búfalo MC, Araújo JP, et al. (2010) Propolis immunomodulatory action in vivo on Toll-like receptors 2 and 4 expression and on pro-inflammatory cytokines production in mice. Phytother Res 24: 1141–1146. doi:10.1002/ptr.3086 PMID: 20041423

29. Orsatti CL, Missima F, Pagliarone AC, Sforcin JM (2010) Th1/Th2 cytokines’ expression and production by propolis-treated mice. J Ethnopharmacol 129: 314–318. doi:10.1016/j.jep.2010.03.030 PMID: 20392428

30. Sá-Nunes A, Faccioli LH, Sforcin JM (2003) Propolis: lymphocyte proliferation and IFN-γ production. J Ethnopharmacol 87: 93–97. doi:10.1016/S0378-8741(03)00121-1 PMID: 12787960

31. Sforcin JM, Orsi RO, Bankova V (2005) Effect of propolis, some isolated compounds and its source plant on antibody production. J Ethnopharmacol 98: 301–305. doi:10.1016/j.jep.2005.01.042 PMID: 15814263

32. Olczyk P, Komosinska-Vassev K, Winsz-Szczotka K, Stojko J, Klimek K, Kozma EM (2013) Propolis induces chondroitin/dermatan sulphate and hyaluronic acid accumulation in the skin of burned wound. Evid Based Complement Alternat Med 2013:290675. doi:10.1155/2013/290675 PMID: 23533471

33. Olczyk P, Wisowski G, Komosinska-Vassev K, Stojko J, Klimek K, Olczyk M, et al. (2013) Propolis modifies collagen types I and III accumulation in the matrix of burnt tissue. Evid Based Complement Alternat Med 2013: 423809. doi:10.1155/2013/423809 PMID: 23781260

34. Missima F, Sforcin JM (2008) Green brazilian propolis action on macrophages and lymphoid organs of chronically stressed mice. Evid Based Complement Alternat Med 5: 71–75. doi:10.1093/ecam/nel112 PMID: 18317551

35. Orsi RO, Funari SRC, Soares AMVC, Calvi SA, Oliveira SL, Sforcin JM, et al. (2000) Immunomodulatory action of propolis on macrophage activation. J Venom Anim Toxins 6: 205–219. doi:10.1590/S0104-79302000000200006

36. Silva FON, Araújo SXB, Holanda AKM, Meyer E, Sales FAM, Diógenes ICN, et al. (2006) Synthesis, characterization, and NO release study of the cis- and trans-[Ru(Bpy)2(SO3)(NO)]+ complexes. Eur J Inorg Chem 2006: 2020–2026. doi:10.1002/ejic.200500871

37. da Silva JJN, Guedes PMM, Zottis A, Balliano TL, Nascimento Silva FO, Lopes LGF, et al. (2010) Novel ruthenium complexes as potential drugs for Chagas's disease: enzyme inhibition and in vitro/in vivo trypanocidal activity. Br J Pharmacol 160: 260–269. doi:10.1111/j.1476-5381.2009.00524.x PMID: 20105182

38. Kikuchi K, Nagano T, Hayakawa H, Hirata Y, Hirobe M (1993) Real time measurement of nitric oxide produced ex vivo by luminol-H2O2 chemiluminescence method. J Biol Chem 268: 23106–23110. PMID: 8226828

39. Valdez RH, Tonin LTD, Ueda-Nakamura T, Silva SO, Dias Filho BP, Kaneshima EM, et al. (2012) In vitro and In vivo trypanocidal synergistic activity of n-butyl-1-(4-dimethylamino)phenyl-1,2,3,4-tetrahydro-β-carboline-3-carboxamide associated with benznidazole. Antimicrob Agents Chemother 56: 507–512. doi:10.1128/AAC.05575-11 PMID: 22037851

40. Nicolas L, Prina E, Lang T, Milton G (2002) Real-Time PCR for detection and quantitation of Leishmania in mouse tissues. J Clin Microbiol 40: 1666–1669. doi:10.1128/jcm.40.5.1666-1669.2002 PMID: 11980939

41. Chatterjee S, Malhotra R, Varghese F, Bukhari AB, Patil A, Budrukkar A, et al. (2011) 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 127: 58–65. doi:10.1016/j.exppara.2010.06.030 PMID: 20599987

42. Pizzatti L, Panis C, Lemos G, Rocha M, Cecchini R, Souza GH, et al. (2012) Label-free MSE proteomic analysis of chronic myeloid leukemia bone marrow plasma: disclosing new insights from therapy resistance. Proteomics 12: 2618–2631. doi:10.1002/pmic.201200066 PMID: 22761178

43. Miller GL (1959) Protein determination for large numbers of samples. Anal Chem 31: 964. doi: 10.1021/ac60149a611

44. Giorgio S, Linares E, Capurro MdL, de Bianchi AG, Augusto O (1996) Formation of nitrosyl hemoglobin and nitrotyrosine during murine leishmaniasis. Photochem Photobiol 63:750–754. doi:10.1111/j.1751-1097.1996.tb09626.x PMID: 8992499
46. Pacher P, Beckman JS, Liaudet L (2007) Nitric oxide and peroxynitrite in health and disease. Physiol Rev 87:315–424. PMID: 17237348

47. Sakhthianandeswaren A, Elso CM, Simpson K, Curtis JM, Kumar B, Speed TP, et al. (2005) The wound repair response controls outcome to cutaneous leishmaniasis. Proc Natl Acad Sci USA 102:15551–15556. PMID: 16223880

48. Oliveira F, Bafica A, Rosato AB, Favali CBF, Costa JM, Cafe V, et al. (2011) Lesion Size Correlates with Leishmania Antigen-Stimulated TNF-Levles in Human Cutaneous Leishmania. Am J Trop Med Hyg 85:70–73. doi: 10.4269/ajtmh.2011.10-0680 PMID: 21734128

49. Stuart LM, Ezekowitz RAB (2005) Phagocytosis: elegant complexity. Immunity 22: 539

50. Genestra M, Soares-Bezerra RJ, Gomes-Silva L, Fabrino DL, Bellato-Santos T, Castro-Pinto DB, et al. (2014) S-nitrosoglutathione (GSNO) is cytotoxic to intracellular amastigotes and promotes healing of topically treated Leishmania major or Leishmania braziliensis skin lesions. J Antimicrob Chemother 68: 2561–2568. doi: 10.1093/jac/dku223

51. López-Jaramillo P, Rincón MY, García RG, Silva SY, Smith E, Kampeerapappum P, et al. (2010) A bactericidal synergy of a Brazilian green propolis extract and axenic amastigotes. Cell Biochem Funct 26: 709–717. doi: 10.1002/cbf.1496 PMID: 18720423

52. Ayres DC, Marcucci MC, Giorgio S (2007) Effects of Brazilian propolis on Leishmania amazonensis. Mem Inst Oswaldo Cruz 102: 215–220. doi: 10.1590/S0071-72652007005000020 PMID: 17426888

53.珍珠母K，da Silva Filho A，Santos F，Silva M，Cunha W，Nanayakkara NP，et al. (2008) In vitro and in vivo antileishmanial activities of a Brazilian green propolis extract. Parasitol Res 103: 487–492. doi: 10.1007/s00436-008-0970-z PMID: 18491139

54. Gao W, Wu J, Wei J, Pu L, Guo C, Yang J, et al. (2014) Brazilian green propolis improves immune function in aged mice. J Clin Biochem Nutr 55:7–10. doi: 10.3164/jcbn.2013.10-070 PMID: 24078064.

55. o J, Hunter CA (2002) NF-κB activation pathways and their role in innate and adaptive immune functions. Clin Microbiol Rev 15: 414–429. doi: 10.1128/CMR.15.3.414-429 PMID: 12097249

56. Hickey MJ (2001) Role of inducible nitric oxide synthase in the regulation of leucocyte recruitment. Clin Sci 100: 1–12. doi: 10.1042/cs20000135 PMID: 11154111

57. Spiecker M, Darius H, Kaboth K, Hübner F, Liao JK (1998) Differential regulation of endothelial cell adhesion molecule expression by nitric oxide donors and antioxidants. J Leukoc Biol 63: 732–739. PMID: 9620666

58. dal Secco D, Paron JA, de Oliveira SHP, Ferreira SH, Silva JS, Cunha FdeQ (2003) Neutrophil migration in inflammation: nitric oxide inhibits rolling, adhesion and induces apoptosis. Nitric Oxide 9: 153–164. doi: 10.1016/j.niox.2003.11.001 PMID: 14732339

59. Caamaño J, Hunter CA (2002) NF-κB family of transcription factors: central regulators of innate and adaptive immune functions. Clin Microbiol Rev 15: 414–429. doi: 10.1128/CMR.15.3.414-429 PMID: 12097249

60. Bonizzi G, Karin M (2004) The two NF-κB activation pathways and their role in innate and adaptive immunity. Trends Immunol 25: 280–288. doi: 10.1016/j.it.2004.03.008 PMID: 15145317

61. Pavaneli WR, da Silva JJ, Panis C, Cunha TM, Conchon-Costa I, de Menezes MC, et al. (2011) Experimental chemotherapy in paracoccidioidomycosis using ruthenium NO donor. Mycopathologia 172: 95–107. doi: 10.1007/s11046-011-9416-8 PMID: 21437728

62. Guedes PMM, Oliveira FS, Gutiérrez FRS, da Silva GK, Rodrigues GJ, Bendhack LM, et al. (2010) Nitric oxide donor trans-[RuCl([15]aneN4)NO]2+ as a possible therapeutic approach for Chagas’ disease. Br J Pharmacol 160: 270–282. doi: 10.1111/j.1476-5381.2009.00576.x PMID: 20128813
67. Ahsan H (2013) 3-Nitrotyrosine: A biomarker of nitrogen free radical species modified proteins in systemic autoimmunogenic conditions. Hum Immunol 74: 1392–1399. doi: 10.1016/j.humimm.2013.06.009 PMID: 23777924

68. Liew FY (1994) Regulation of nitric oxide synthesis in infectious and autoimmune diseases. Immunol Lett 43: 95–98. doi: 10.1016/0165-2478(94)00157-X PMID: 7737695

69. Thornton FJ, Schäffer MR, Witte MB, Moldawer LL, MacKay SLD, Abouhamze A, et al. (1998) Enhanced collagen accumulation following direct transfection of the inducible nitric oxidesynthase gene in cutaneous wounds. Biochem Biophys Res Commun 246: 654–659. doi: 10.1006/bbrc.1998.6861 PMID: 9618268

70. Muscará MN, McKnight W, Asfaha S, Wallace JL (2000) Wound collagen deposition in rats: effects of an NO-NSAID and a selective COX-2 inhibitor. Br J Pharmacol 129: 681–686. doi: 10.1038/sj.bjp.0703112 PMID: 10683192

71. Shi HP, Efron DT, Most D, Tantry US, Barbul A (2000) Supplemental dietary arginine enhances wound healing in normal but not inducible nitric oxide synthase knockout mice. Surgery 128: 374–378. doi: 10.1067/msy.2000.107372 PMID: 10923019

72. Ramos AFN, Miranda JL (2007) Propolis: a review of its anti-inflammatory and healing actions. J Venom Anim Toxins inclu Trop Dis 13: 697–710. doi: 10.1590/S1678-91992007000400002

73. Hoşnuter M, Gürel A, Babucçu O, Armutcu F, Kargi E, Iskdemir A (2004) The effect of CAPE on lipid peroxidation and nitric oxide levels in the plasma of rats following thermal injury. Burns 30: 121–125. doi: 10.1016/j.burns.2003.09.022 PMID: 15019118

74. Kolankaya D, Selmanoğlu G, Sorkun K, Salih B (2002) Protective effects of Turkish propolis on alcohol-induced serum lipid changes and liver injury in male rats. Food Chem 78: 213–217. doi: 10.1016/S0308-8146(01)00400-9

75. Faler BJ, Macsata RA, Plummer D, Mishra L, Sidaway AN (2006) Transforming growth factor-β and wound healing. Perspec Vasc Surg Endovasc Ther 18: 55–62. doi: 10.1177/153100350601800123 PMID: 16628336

76. Kim WH, Gittes G, Longaker M (1998) Signal transduction in wound pharmacology. Arch Pharm Res 21: 487–495. doi: 10.1007/BF02975363 PMID: 9875483

77. Crowe MJ, Doetschman T, Greenhalgh DG (2000) Delayed wound healing in immunodeficient TGF-β1 knockout mice. J Invest Dermatol 115: 3–11. doi: 10.1046/j.1523-1747.2000.00010.x PMID: 10886500

78. Nakamura H, Herzenberg LA, Bai J, Araya S, Kondo N, Nishinaka Y, et al. (2001) Circulating thioredoxin suppresses lipopolysaccharide-induced neutrophil chemotaxis. Proc Nat Acad Sci U S A 98: 15143–15148. doi: 10.1073/pnas.191498798 PMID: 11742067

79. Sano S, Itami S, Takeda K, Tarutani M, Yamaguchi Y, Miura H, et al. (1999) Keratinocyte-specific ablation of Stat3 exhibits impaired skin remodeling, but does not affect skin morphogenesis. EMBO J 18: 4657–4668. doi: 10.1093/emboj/18.17.4657 PMID: 10466945

80. Gao H, Guo RF, Speyer CL, Reuben J, Neff TA, Hoesel LM, et al. (2004) Stat3 activation in acute lung injury. J Immunol 172: 7703–7712. doi: 10.4049/jimmunol.172.12.7703 PMID: 15187153

81. Baldin T, Saksikanandeswaren A, Curtis JM, Kumar B, Smyth GK, Foote SJ, et al. (2007) Wound healing response is a major contributor to the severity of cutaneous leishmaniasis in the ear model of infection. Parasite Immunol 29: 501–513. doi: 10.1111/j.1365-3024.2007.00969.x PMID: 17883453

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Nitric Oxide and Propolis Combined in Experimental Leishmaniasis