

Nitric Oxide Donors with Therapeutic Strategic in Experimental *Schistosomiasis Mansoni*

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Abstract: Schistosomiasis, an immune disease, remains a major public health problem in endemic area. To determine the influence of Nitric Oxide (NO) on this disease, we tested two compounds (Trans-[Ru(bpy)₂(NO)SO₃](PF₆)-PF₆ and Na₂[Fe(CN)₅(NO)]-SNP, which releases NO when activated by biological reducing agents, in BALB/c mice infected subcutaneously by *Schistosoma* BH strains. The parasitic activity of NO-donors was evaluated in this model by measuring the immune cellular response in liver with: Cytokines levels; histopathological characteristics and the number of the granulomatous lesions; and NO levels. We found that NO-donors treated mice were more resistant to infection, since they exhibited higher survival. Furthermore, we observed in histopathological analysis a decreased influx of inflammatory cells in the hepatic tissue of mice treated with both donors. The parasite counting (estimated as eggs and worms number) was also minor in treated mice. Moreover, decreased levels of IL-10 were detected in the liver of infected mice treated with SNP. The animals treated with PF₆ showed high plasmatic NO levels at 45 days after infection. Altogether, these data suggest that NO is a pivotal factor of resistance during schistosomiasis by controlling parasites proliferation, influencing cytokine production and consequently modulating the development of inflammatory response.

Keywords: NO Donors, PF₆, SNP, Nitric Oxide, Immune Response, Schistosomiasis

Introduction

Schistosoma mansoni is an important helminth parasite. During its course of infection, different stages of *S. mansoni* cause markedly varied patterns of inflammatory responses in hosts. The etiopathogenesis associated with schistosomiasis is largely attributed to the intense granulomatous inflammatory response and subsequent fibrosis induced by parasite eggs that become trapped in host organs such as the liver and intestine.

Granuloma formation occurs in an environment that is initially proinflammatory that rapidly polarizes to the Th2 immune pattern (Davies *et al.*, 2004). The relative influence of Th1 and Th2 cytokines on the pathogenesis of schistosomiasis remains controversial in both mice and humans experimental models. Nevertheless, decreased fibrosis in the murine model is associated with diminished production of Th2 cytokines and increased production of Th1 cytokines (Cheever *et al.*, 2002). Therefore, modulation of both Th1 and Th2 responses

could down regulate the granulomatous inflammation and consequently reduce the parthenogenesis of schistosomiasis (Schramm *et al.*, 2010).

The interventional control of liver granuloma formation and fibrosis becomes another key therapeutic strategy after efficacious killing of the parasite by schistosomicides. Associated this factor, the resistance to praziquantel, which is actually the main treatment for schistosomiasis, has been reported (Ross *et al.*, 2002; Burke *et al.*, 2009).

There is a wide range of direct and indirect evidences implicating that Nitric Oxide (NO) can act as an antischistosomal and more broadly, antiparasitic molecule (Rivero, 2006; Brunet, 2001). NO produced by human white cells has been shown to kill larval schistosome parasites (James and Glaven, 1989). In addition, NO produced through inducible NO synthase activation is a core component of the immune response and there is direct evidences of NO being a primary aspect of host defense against other parasites as *Echinococcus granulosus* and *Trypanosoma congolense* (Amri *et al.*, 2007; Magez *et al.*, 2007). Winberg *et al.* (2007) demonstrated that NO production diminished *Leishmania donovani* promastigotes ability to inhibit periphagosomal F-actin breakdown, allowing normal phagosomal maturation and parasite killing. Beyond innate activation of the NO synthase, exogenous NO from small molecule NO donors has shown promise in several arenas. A topical formulation of S-nitroso-N-Acetylpenicillamine (SNAP) was reported as an effective treatment option for the control of cutaneous leishmaniasis (López-Jaramillo *et al.*, 1998). A class of bicyclic nitroimidazoles was recently reported as a deazaflavin-dependent nitroreductase prodrug that releases oxidized nitrogen molecules that are converted to NO and are capable of anaerobic killing of *Mycobacterium tuberculosis* (Singh *et al.*, 2008). Further, the mainstays of Chagas disease chemotherapy are benzimidazole and nifurtimox (agents with heteroaromatic nitro moieties) (Mady *et al.*, 2008). These compounds are activated to release NO by reducing agents present in the biological milieu (Bogdan, 2001). Hence, the features shown by these types of compounds are quite promising and motivate development of novel NO-based metallopharmaceuticals. This is particularly interesting, to combat infectious diseases where the NO concentration has to be high enough to prevent the development of the microorganisms but not so high as to cause immunosuppression, inhibition of respiratory complexes and acotinase, DNA modifications or apoptosis in the host cells (Zanichelli *et al.*, 2006).

In fact, previous studies (Sánchez-Delgado *et al.*, 1998; Navarro *et al.*, 2000) have shown that javascript: Void (0); ruthenium complexes are able to inhibit 70% of the proliferation of epimastigote forms of *T. cruzi*

Sánchez-Delgado *et al.*, 1998). Following this approach, the $[\text{Ru}(\text{NH}_3)_4\text{L}]^{n+}$ moiety has been successfully tested as a NO carrier *in vitro* and *in vivo* (Torsoni *et al.*, 2002), because the trypanocidal effect of activated macrophages has been ascribed to NO production (Silva *et al.*, 2007). In addition, these authors observed that trans- $[\text{Ru}(\text{NO})(\text{NH}_3)_4\text{L}]^{n+}$, exhibited not only low cytotoxicity but also anti-*T. cruzi* activity due to NO action (Silva *et al.*, 2007). Guedes *et al.* (2010) verified that the NO-donor trans- $[\text{RuCl}(\text{[15]aneN(4)NO}]\text{(2+)}$ in concentration of 0.1 mM induced 100% of trypanocidal activity (trypomastigotes forms) in assay *in vitro*, promote suppression of parasitaemia and 100% survival in a murine model of acute Chagas' disease.

We have previously demonstrated that mice treated with Cis- $[\text{Ru}(\text{bpy})_2(\text{NO})\text{SO}_3](\text{PF}_6)$ are more resistant to Paracoccidioidomycosis (PCM) infection, thus, presenting prolonged survival with reduced leukocyte recruitment and TNF- α production in the lung and liver as well as increased production of the anti-inflammatory cytokine IL-10 (Pavanelli *et al.*, 2011). This compound has exhibited interesting biological behavior with promising therapeutical potential such as in brain ischemia/reperfusion (Campelo *et al.*, 2012) and cardiovascular agent (Campelo *et al.*, 2011). Recently, it was showed this ruthenium-based NO-donor can efficiently release nitric oxide upon reaction with thiols, such as glutathione (Silva *et al.*, 2011). This property might be strategically used for treatment of parasitic infection, particularly for Schistosomiasis which is known due to the production of biological thiols, including glutathione, as protective tools against oxidative stress. On the other hand, Nitroprusside (SNP) has been already in clinical use for many decades and is one of the oldest NO-donors described. A similar version of these two NO-donors employed in this study were prepared on silica particles, which could be eventually be used impregnated in bandages or gels for topical (Silva *et al.*, 2010).

There is evidence that inflammatory cytokines and NO play an important role in the genesis and control of schistosomiasis. Therefore, NO donor, (Trans- $[\text{Ru}(\text{bpy})_2(\text{NO})\text{SO}_3](\text{PF}_6)\text{-PF}_6$ and $\text{Na}_2[\text{Fe}(\text{CN})_5(\text{NO})]\text{-SNP}$, were chosen as a good model for assessing parasitic activity *in vivo*. Thus, in the present study we proposed to determine the influence of the ruthenium NO-donor on the inflammatory response induced against *S. mansoni*.

Materials and Methods

Animals

Female BALB/c mice, aged 6 to 8 weeks, were bred and maintained in microisolator cages in the animal housing facility of the Department of

Pathology Science, CCB, State University of Londrina-UEL, in accordance with the local protocols of ethics in animal care. The procedures involving animals and their care were conducted in agreement with national and international policies.

Parasites and Infection

Mice were infected by percutaneous exposure of tail skin for 40 min in water containing 70 cercariae of BH strain. For survival determination, the animals were followed for up to 60 days of infection.

Eggs and Adults Worms Quantification

At 45 and 90 days post-infection, all the mice were sacrificed and the livers were excised for worm and egg recovery. The livers were rinsed with chilled saline solution and homogenized in ice-cold Tris-HCL buffer (10 mM, pH 7.4) containing Triton X-100 and sucrose (0.25 M). An aliquot of the liver homogenate was subjected to egg counting with a light microscope. All treated and control groups of mice were killed in 45 and 90 days post-infectious by bloodletting and schistosomes were collected from hepatic and portomesenteric veins using a perfusion technique (Yolles *et al.*, 1947). Schistosomes obtained from each animal were sexed and counted.

AST and ALT Levels

The levels of aspartate Aminotransferase (AST) and alanine Aminotransferase (ALT) markers of hepatocellular damage, in serum were determined at 45 and 90 days p.i., by a colorimetric assay by using a commercial kit from CHIRON Diagnostics Corporation (East Walpole, Mass).

Chemicals, Drugs and Reagents

Ruthenium trichloride from Aldrich Chemical Company (ACC) was the starting material for the synthesis of the ruthenium complex described here. All solvents were purified following known procedures (Armarego and Chai, 2012) and double-distilled water was used throughout. The synthesis and all manipulations were carried out under argon atmosphere (Shriver, 1986).

Synthesis and Instrumentation

The ruthenium NO-donor Trans-[Ru(bpy)₂(NO)SO₃](PF₆)-PF₆ and Na₂[Fe(CN)₅(NO)]-SNP were synthesized following published procedures (Silva *et al.*, 2006; Cerecetto and Gonzalez, 2002). Elemental analysis of hydrogen, carbon and nitrogen was carried out using an EA 1110 CHNS-O CE instrument. Analysis of ruthenium was performed as described elsewhere (Clarke, 1978), using a Polarized Zeeman atomic absorption spectrophotometer, Hitachi (model Z-

8100), with a Hitachi Hollow Cathode Lamp, 12 mA and $\lambda = 349.9$ nm. UV-visible measurements were performed in a 1.0 cm quartz cell in a Hewlett-Packard diode array model 8452A spectrophotometer. IR spectra were recorded with a Bomem FTIR, model MB-102, spectrophotometer in the 400-4000 cm⁻¹ range, with the sample supported in potassium bromide pellets. A polarographic analyzer/stripping voltammeter model 264A from Princeton Applied Research attached to a microcomputer and employing Microquímica Electrochemical software was used for the electrochemical measurements. The electrochemical cell used was a conventional three-electrode type with an aqueous saturated calomel electrode as a reference electrode and a glassy-carbon and platinum wire as working and auxiliary electrodes, respectively.

Histological and Fibrosis Analysis

Groups of 5 mice were euthanized after 45 and 90 days of infection with *S. mansoni*. The liver were fixed in 10% formaldehyde in PBS, embedded in paraffin, sectioned, stained with hematoxylin-eosin and examined by light microscopy. The number of granulomatous lesions in the liver was quantified by histocytometry using an image analyzer (BioScan/OPTIMAS; Media Cybernetics, Silver Spring, MD). Values were expressed as the mean \pm SEM of triplicate sections. For the collagen detection (fibrosis) sections were washed and stained with the picro-sirius red solution. To study the matrix of fibrillar collagen, 5 μ m sections were immersed in 0.2% phosphomolybdic acid for 1-5 min before staining for 90 min in a 0.1% solution of Sirius red F₃BA dissolved in saturated aqueous picric acid at pH 2.0. The sections treated with picrosirius red, collagen is readily identified as red staining fibers with direct light or as green thin fibers with polarization microscopy. A method for the quantitative examination of the liver tissue was carried out at medium power light microscopic fields ($\times 250$): A 100-point ocular Integration eyepiece II (Carl Zeiss) was used to estimate the volume fraction (%) of fibrosis in picrosirius-red-stained sections. Twenty fields of hepatic tissue were analyzed for each mouse, as described (Hoffmann *et al.*, 2000; Rossi, 1998).

Cytokine Assays

ELISA measured concentrations of cytokines in tissue homogenates from liver. IL-10 (OpTEIA, BD Bioscience, San Diego-CA, USA) and IL-4 (Duoset R and D Systems, Minneapolis-MN, USA), were assayed following the manufacturer's instructions. The reaction was revealed with peroxidase-conjugated streptavidin (Vector Laboratories, Burlingame-CA, USA) followed by the substrate containing TMB (Promega, Madison-WI, USA) as a chromogen. Optical Densities (O.D.) of samples were then read at 450 nm and the concentrations

of cytokines were determined by extrapolation from a standard curve of each recombinant cytokine.

Immunohistochemical Staining of Vascular Endothelial Growth Factor (VEGF)

Immunohistochemistry for VEGF was performed on 3 μm -thick paraffin-embedded sections from liver in each group by the labeled streptavidin-biotin method using a LSAB kit (DAKO Japan, Kyoto, Japan) with microwave antigen retrieval. The paraffin-embedded sections were heated for 30 min at 65°C, deparaffinized in xylene and rehydrated through a graded ethanol series at room temperature. Incubations were performed in a humidified chamber. Sections were treated for 40 min at room temperature with 2% BSA and incubated overnight at 4°C with primary antibody (anti-VEGF rabbit polyclonal antibody diluted 1:50, Sigma). Horseradish peroxidase activity was visualized by treatment with H_2O_2 and 3,3'-Diaminobenzidine (DAB) for 5 min. In the last step, the sections were weakly counterstained with Harry's hematoxylin (Merck). For each case, negative controls were performed on serial sections. On the control sections, incubation with the primary antibodies was omitted. Intensity and localization of immunoreactivities against all primary antibodies used were examined on all sections using a photomicroscope (Leica DM 2500) and the score determined.

Determination of Nitrite Levels

Sample nitrite was determined according to (Panis *et al.*, 2011). Briefly, plasma aliquots were deproteinized by adding 50 μL of 75 mM ZnSO_4 and 70 μL of NaOH, shaken and centrifuged at 10,000 rpm for 5 min, 25°C. The clear supernatant was recovered and diluted in glycine buffer (45 g L^{-1} , pH 9.7). Cadmium granules were rinsed in sterile distilled water for 5 min and added to a 5 mM CuSO_4 solution in glycine-NaOH buffer (15 g L^{-1} , pH 9.7) and the copper-coated cadmium granules were used within 10 min. The activated granules were added to glycine buffer diluted supernatant and stirred for 10 min. Aliquots of 200 μL were recovered in appropriate tubes for nitrite determination and the same volume of Griess reagent was added. After an incubation of 10 min at room temperature, the tubes were centrifuged at 10,000 rpm, for 2 min at 25°C and the resuspended pellet added to 96-well microplates in triplicate. A calibration curve was prepared by dilution of NaNO_2 and the absorbance was determined at 505 nm in a microplate reader.

Statistical Analysis

Results were expressed as means and standard errors of the means \pm SEM of two independent

experiments. Student's t test was used to analyze the statistical significance of the observed differences. The Kaplan-Meier method was used to compare survival rates of the groups studied. Values of $p \leq 0.05$ were considered significant.

Results

Influence of Treatment with Ruthenium NO-Donors on the Survival of S. Mansoni-Infected Mice

To determine the effect of ruthenium (PF_6) and iron (SNP) NO-donors on mortality during the experimental schistosomiasis, mice were treated i.p., for 20 days with 100 μM of PF_6 , SNP or PBS solution (control group). We observed that 80% of infected mice treated with the donors survived up to 60 days p.i., the mortality of the infected mice treated with vehicle (control) was only of 40%, with initiation on day 25 p.i., (Fig. 1).

Parasiticidal Activity of Trans-[Ru(bpy)₂(NO)SO₃](PF₆) and Na₂[Fe(CN)₅(NO)] in Schistosomiasis

With the knowledge of the NO effects as anti-parasitidal demonstrated by several works, it was carried out experiments to verify the parasitic ability of these inflammatory mediators on eggs and worms of *S. mansoni*. We observed that at 45 days post-infection, mice treated with both NO-donors showed a reduced number of eggs (Fig. 2A) and worms (Fig. 2B) of *S. mansoni* in liver, without spreading to the spleen (data not shown) and after 90 days, only SNP presented reduction of this morphological form when compared with control. This result demonstrated that treatment with the NO-donor was able to eliminate both morphological forms of parasite during the infection.

The Influence of NO-Donors Administration on Inflammatory Response in Liver of S. Mansoni-Infected Mice

Several studies also suggest that NO is implicate in mediating the inflammatory or anti-inflammatory actions. Therefore, we evaluated the inflammatory response in liver of *S. mansoni*-infected mice. The animals treated with both NO-donors showed decreased and focal inflammation in liver in both periods (Fig. 3A), when compared to mice treated with vehicle (control). In fact, the quantitative analysis clearly showed that the treatment promoted a reduction of the number of granulomas (Fig. 3B). Accordingly, this difference was most evident at 90 days after infection. These results indicated that NO released in the liver of *S. mansoni*-infected mice promoted a control of inflammatory response.

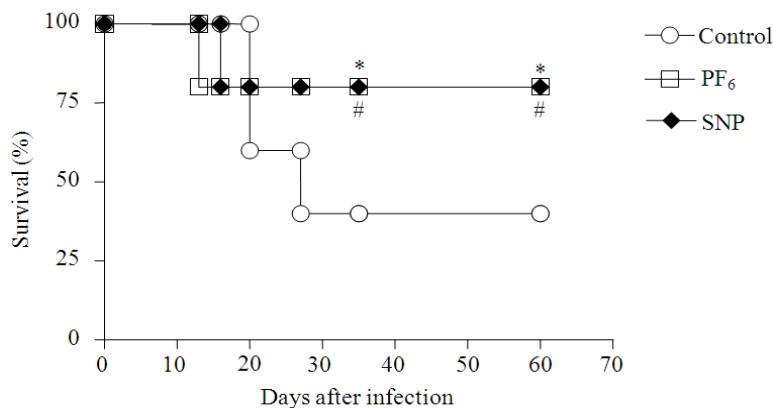


Fig. 1. Survival curves of BALB/c mice treated with Trans-[Ru(bpy)₂(NO)SO₃](PF₆)-PF₆ and Na₂[Fe(CN)₅(NO)]-SNP or control. BALB/c mice were infected by percutaneous exposure of tail skin with 70 cercariae of BH strain and treated with Trans-[Ru(bpy)₂(NO)SO₃](PF₆)-PF₆ and Na₂[Fe(CN)₅(NO)]-SNP or control 20 days. The mortality of these mice was also evaluated. Data are representative of two independent experiments with 5 mice per group. The data shown represent the mean ± SEM of the results obtained. *p<0.05 for PF₆ and #p<0.05 for SNP treated mice versus control mice

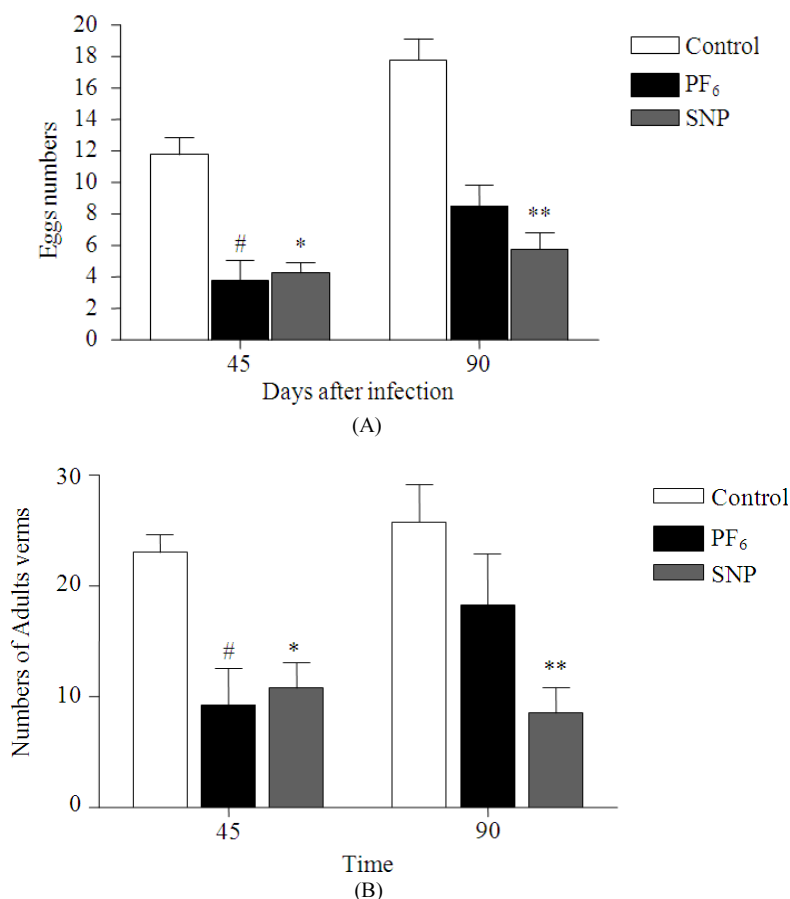
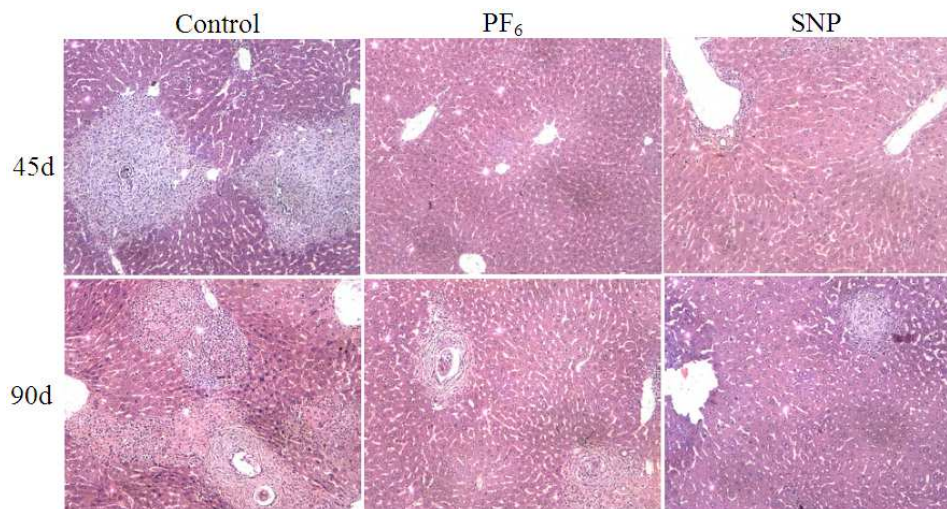
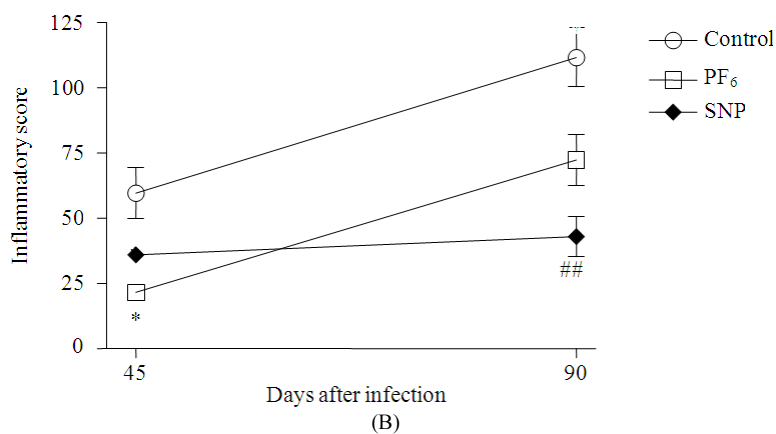


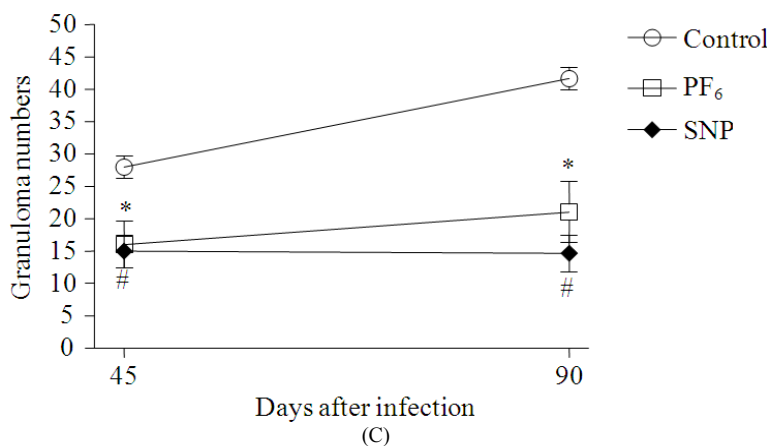
Fig. 2. Antischistosomal activity of Trans-[Ru(bpy)₂(NO)SO₃](PF₆)-PF₆ and Na₂[Fe(CN)₅(NO)]-SNP in BALB/c mice with *S. mansoni*. BALB/c mice were infected by percutaneous exposure of tail skin with 70 cercariae of BH strain and treated with Trans-[Ru(bpy)₂(NO)SO₃](PF₆)-PF₆ and Na₂[Fe(CN)₅(NO)]-SNP or control 20 days; All treated and control groups of mice were killed in 45 and 90 days pos-infectious by bloodletting and schistosomes were collected from hepatic and portomesenteric veins using a perfusion technique. Figure 2A and B showed the numbers of eggs and worms in liver, respectively. Data are representative of two independent experiments with 5 mice per group. The data shown represent the mean ± SEM of the results obtained. *p<0.05 and **p<0.01 for PF₆; #p<0.05 for SNP treated mice versus control mice



(A)



(B)



(C)

Fig 3. NO release attenuated histological alteration in the liver of mice infected with *S. mansoni*. BALB/c mice were infected by percutaneous exposure of tail skin with 70 cercariae of BH strain and treated with Trans-[Ru(bpy)₂(NO)SO₃](PF₆)-PF₆ and Na₂[Fe(CN)₅(NO)]-SNP or vehicle by 20 days and the liver removed, fixed and examined using photomicrography. Data are representative of two independent experiments with 5 mice per group. The data shown represent the mean ± SEM of the results obtained. *p<0.05 for PF₆; ###p<0.01 for SNP treated mice versus control mice. Original magnification for all microphotographs ×200. Scale bars = 150 μm. Fine arrow/egg and Wide arrow/inflammatory response. (A) Representative photomicrography of liver histopathology, (B) Quantification of inflammatory score and (C) Granuloma numbers

Effect of NO-Donor Administration on Hepatic Lesion

In addition, the present study investigated if the NO-donor is associated with hepatic lesions and accordingly transaminases alteration. For such, it was measured the AST and ALT activity in serum and observed that the AST (Fig. 4A) and ALT (Fig. 4B) levels were significantly higher in mice treated only with vehicle when compared with NO-donors treated mice in all periods evaluated. The examination of AST and ALT levels demonstrated that NO-donors treatment protect the liver of injury.

Effect of NO-Donor Administration on Cytokine Production in *S. Mansoni*-Infected Mice

Several works showed the importance of a different profile of cytokines during the schistosomiasis. Therefore, we determined the kinetics of cytokine production (IL-10 and IL-4) in the liver of infected mice treated with ruthenium and iron NO-donors or vehicle.

The SNP treatment caused a significant decrease in IL-10 levels in the liver by 45 and 90 days p.i., compared to control mice (Fig. 5A). However, the production of IL-4 was not significantly different between NO-treated mice and control group (Fig. 5B). Therefore, these results demonstrate that NO delivery directly induced an anti-inflammatory condition.

Effect of NO-Donor Administration on Nitric Oxide Production in Infected Mice

NO is one of the most important mediators involved in microbicidal mechanisms during *parasite* infection. Therefore, we measured NO levels in the serum of mice treated with PF₆, SNP or PBS solution. After 45 days of infection, only the animals treated with PF₆ showed higher levels of NO when compared to control mice (Fig. 6). This finding suggests that the elevated production of NO in mice treated with PF₆ could be responsible for the elimination of *S. mansoni*, mainly in the liver, as observed in histopathological analysis.

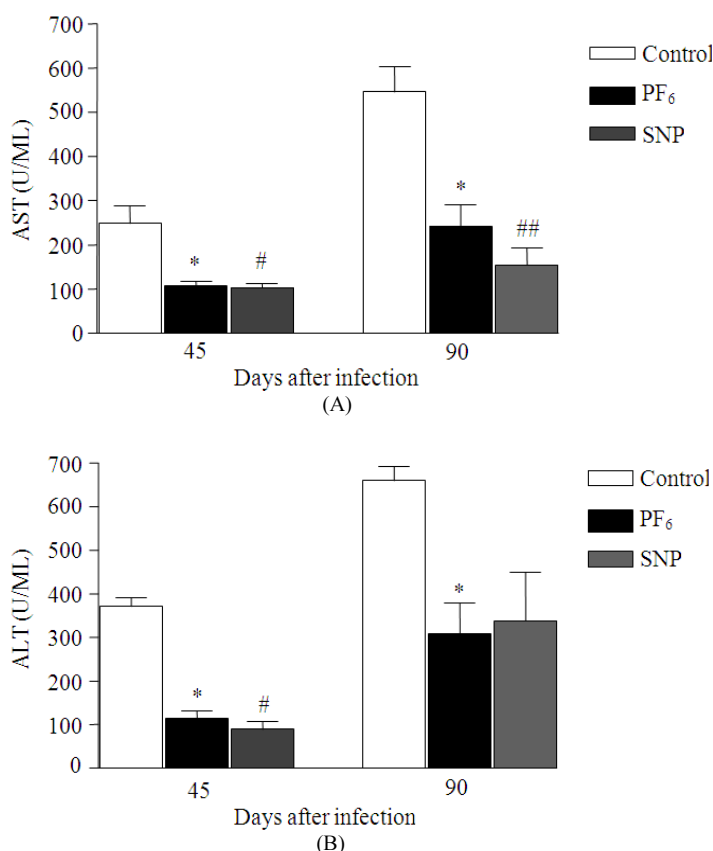


Fig. 4. NO-donors treatment reduces transaminase levels mice infected with *S. mansoni*. PF₆ (black bar) and SNP (gray bar) treated mice were infected by percutaneous exposure of tail skin with 70 cercariae of BH strain. At the showed time-points the mice were sacrificed and serum collected by analyze of AST (A) and ALT (B), as described in materials and methods. Data are representative from two independent experiments of five mice per group are shown. The data shown represent the mean \pm SEM of the results obtained. * $p < 0.05$ for PF₆; # $p < 0.05$ and ## $p < 0.01$ for SNP treated mice versus control mice

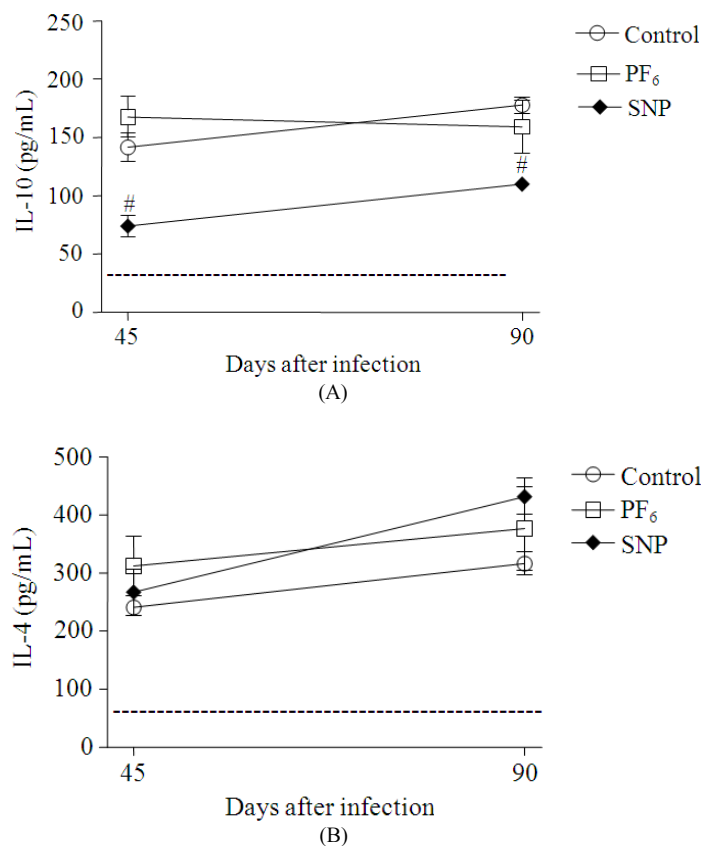


Fig. 5. Kinetics of cytokine production during Schistosomiasis infection. BALB/c mice were infected by percutaneous exposure of tail skin with 70 cercariae of BH strain and treated with Trans-[Ru(bpy)₂(NO)SO₃](PF₆)-PF₆ and Na₂[Fe(CN)₅(NO)]-SNP or vehicle by 20 days; the animals were euthanized at different time points after infection. The concentrations of IL-10 (5A) and IL-4 (5B) were determined in liver homogenate by ELISA. Data are representative of two independent experiments with 5 mice per group. The data shown represent the mean \pm SEM of the results obtained. #p<0.05 for SNP treated mice versus control mice. The dashed line represents values obtained on day zero for mice not infected and treated

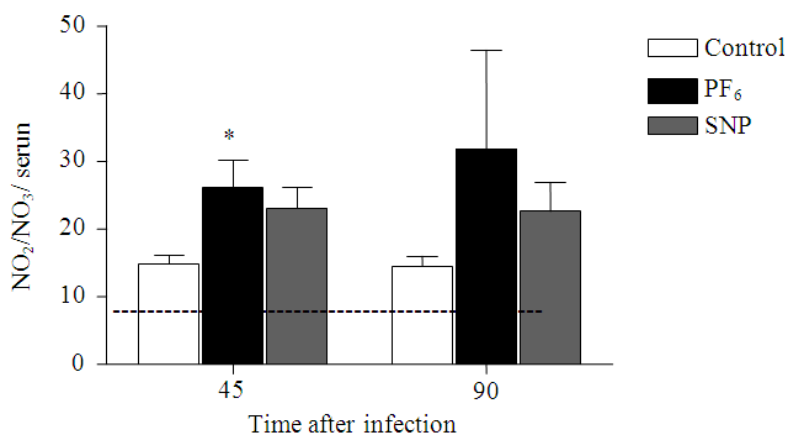


Fig. 6. Kinetics of nitric oxide production in serum of BALB/c mice, treated with NO-donor and control. BALB/c mice were infected by percutaneous exposure of tail skin with 70 cercariae of BH strain and treated with Trans-[Ru(bpy)₂(NO)SO₃](PF₆)-PF₆ and Na₂[Fe(CN)₅(NO)]-SNP or vehicle by 20 days. Serum levels of nitrite/nitrate are shown for BALB/c mice treated with NO-donors or vehicle at days 45 and 90 p.i. Data are representative of two independent experiments with 5 mice per group. The data shown represent the mean \pm SEM of the results obtained. *p<0.05 for PF₆ treated mice versus control mice. The dashed line represents values obtained on day zero for mice not infected and treated

Detection of Vascular Endothelial Growth Factor (VEGF) Positive Cells in Liver of *S. Mansoni*-Infected mice Treated with Trans-[Ru(bpy)₂(NO)SO₃](PF₆) and Na₂[Fe(CN)₅(NO)]

VEGF is widely regarded as a potent stimulator of angiogenesis, edema, inflammation and vascular remodeling (Furuta *et al.*, 2010). Immunohistochemistry was performed to detect VEGF cells, with positive immunostaining found present in live sections from mice treated with NO donors or vehicle. The results demonstrated that the treatment with NO-donors promotes a decrease in the expression of VEGF-positive cells at the two times examined, when compared with the control group (Fig. 7A). In fact, quantitative analysis revealed that SNP induces reduction of VEGF-positive cells at the two times examined (Fig. 7B).

Fibrosis is Attenuated in NO-Donors Treated Mice

Some Extracellular Components Matrix (ECM) deposition is necessary for wound healing to provide strength and temporary structure to damaged tissues; however, if not limited, it can be pathologic. For such, further to evaluate the damage in the hepatic tissue, provoked by exacerbated inflammatory response, we quantified fibrosis during the course of infection. Analysis of picosirius red-stained sections by conventional microscopy revealed mild interstitial fibrosis in both, PF₆ and SNP treated mice in 45 and 90 days p.i., (Fig. 8A) In fact, at day 90th p.i., control mice exhibited a marked diffuse interstitial hepatic fibrosis in comparison with the more discrete fibrosis in the liver from NO-donors treated mice.

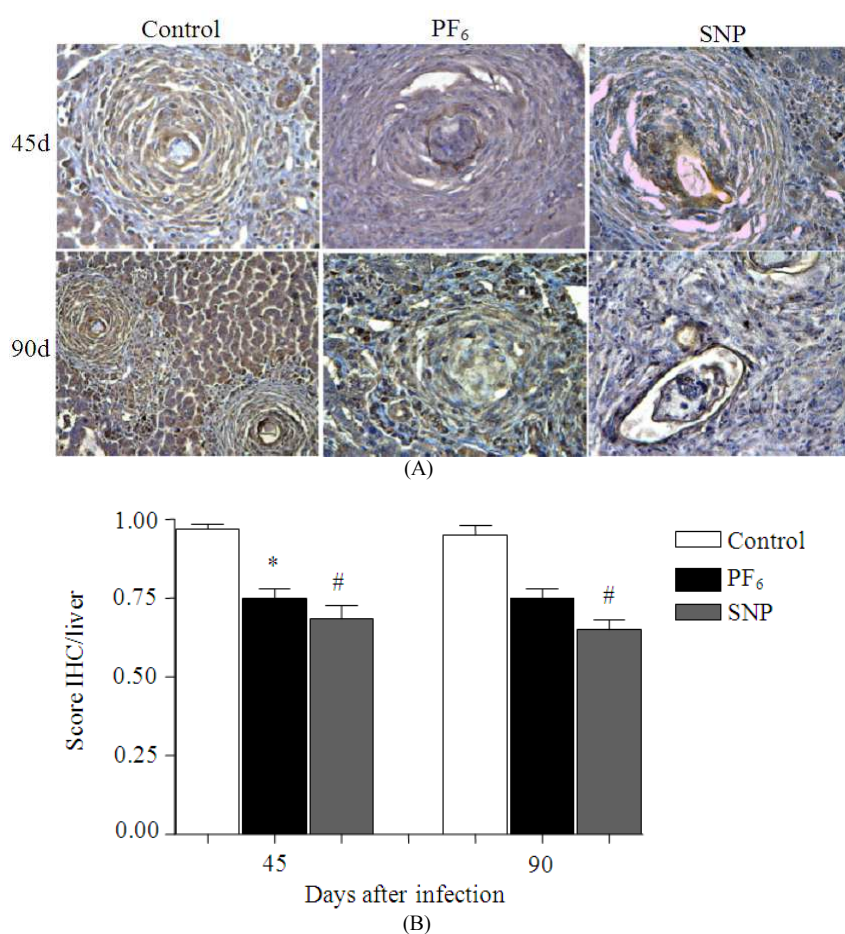


Fig. 7. Vascular Endothelial Growth Factor (VEGF) immunoreactivity in hepatic tissue of BALB/c mice, treated with NO-donor and control. BALB/c mice were infected by percutaneous exposure of tail skin with 70 cercariae of BH strain and treated with Trans-[Ru(bpy)₂(NO)SO₃](PF₆)-PF₆ and Na₂[Fe(CN)₅(NO)]-SNP or vehicle by 20 days and the liver removed, fixed and examined using photomicrography. (A) Representative photomicrography of VEGF presence in liver of BALB/c mice treated with NO-donor or vehicle at days 45 and 90 days p.i. (B) Percentage of VEGF per area in liver. Data are representative of two independent experiments with 5 mice per group. The data shown represent the mean ± SEM of the results obtained. *p<0.05 for PF₆ and #p<0.05 for SNP treated mice versus control mice. Original magnification for all microphotographs ×400. Scale bars = 150 μm

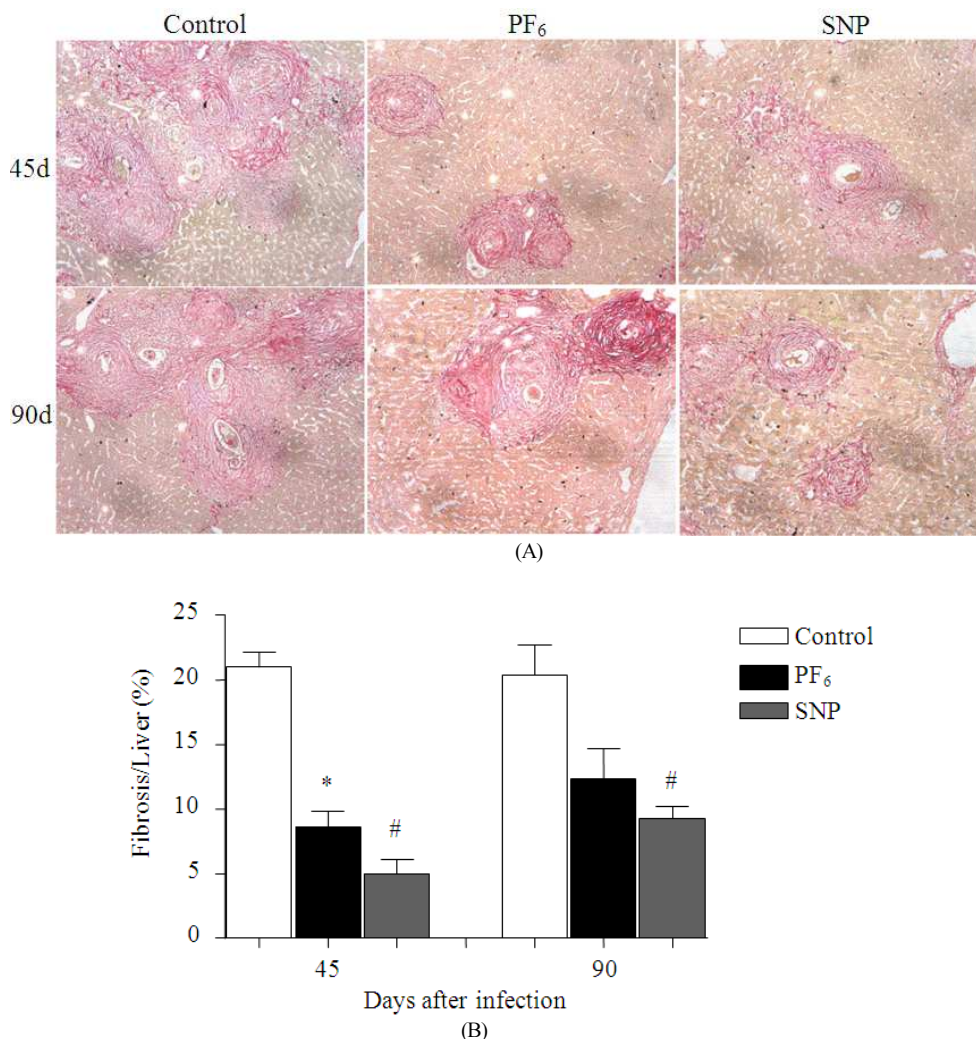


Fig. 8. NO-donors treatment reduces fibrosis in the liver mice infected with *S. mansoni*. PF₆ (black bar) and SNP (gray bar) treated mice were infected by percutaneous exposure of tail skin with 70 cercariae of BH strain. At the showed time-points the mice were sacrificed in at 45 and 90 days after infection and the liver was processed of collagen content as described in materials and methods. Photomicrographies are representative of both periods after infection from two independent experiments with five mice per group. (A) and the quantification of collagen deposition (B) are showed. The data shown represent the mean \pm SEM of the results obtained. * $p < 0.05$ for PF₆ and # $p < 0.05$ for SNP treated mice versus control mice. Original magnification for all microphotographs $\times 200$. Scale bars = 150 μ m. Fine Arrow/egg and Wide Arrow/collagen deposition

The intensity fraction of control groups increased approximately 2.5 and 4.5 times as compared with the values found 45 days after infection while the intensity of fibrosis in PF₆ and SNP treated mice, respectively. Already, in 90 days p.i., the amount of hepatic tissue fibrosis in control mice was of 2 and 2.75 times higher than the volume fraction of fibrosis in the myocardium from PF₆ and SNP treated mice, correspondingly (Fig. 8B).

Discussion

The immunopathological mechanisms involved in the pathogenesis of schistosomiasis are not completely elucidated. Although the immune response presents an

inflammatory profile to control the infection, an exacerbated inflammatory stimulation may also cause damage to the host. The murine model of schistosomiasis has been used extensively to study the mechanism of granuloma formation and its immune regulation. In both murine and human schistosomiasis, findings suggest that Nitric Oxide (NO) plays an essential role in host defense against this parasite.

In fact, in evaluating the influence of treatment with NO-donors in experimental schistosomiasis, we found that NO release induced host resistance against infection, since 80% of treated mice survived for at least 60 days (Fig. 1).

In recent studies from our laboratory it has showed that treatment with Cis-[Ru(bpy)₂(NO)SO₃](PF₆)

increases the survival in 100% of mice in a model of *Paracoccidioides brasiliensis* infection (Pavanelli *et al.*, 2011). In another study using a model of *T. cruzi* infection, the treatment of infected animals with an NO-donor, similar to that used in this study, markedly increased survival (to 60%) (Silva *et al.*, 2007). These results suggest that NO is involved in the induction of resistance on several types of infection. One of the possible mechanisms of NO resistance is the capacity of this mediator to promote the death of the pathogen.

Other studies have demonstrated that NO reacts with reactive oxygen species, resulting in substances capable of killing microorganisms and inactivating proteins through nitration and nitrosilation (Boscá *et al.*, 2005; Eu *et al.*, 2000). Rodrigues *et al.* (2012), characterized that the NO release, from a nitrosyl ruthenium complex and its Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) mediated actions on mitochondria, emphasizing the involvement of the mitochondrial NADH oxidation and promoting the opening of Permeability Transition Pores (PTPs). This PTP opening involves the oxidation of mitochondrial membrane protein-thiols by ROS/RNS and is implicated in the release of cytochrome *c* from mitochondria, which is, in turn, associated with cell death induction. Although these direct cellular effects of NO over the pathogen biology are not yet fully understood, the strong oxidative capacity of NO and its sub-products appears to act in synergism with other lethal reactive oxidant species, accounting for most of the host's microbial toxicity.

We found that the treatment with the NO-donors (PF₆ and SNP) reduced the eggs and worms adult numbers in liver analysed (Fig. 2A and B) on 90th p.i., showing that circulating NO is able to kill and hence control parasite multiplication. In fact, killing of pathogens mediated by NO was described in several infectious and parasitic diseases including those caused by *Mycobacterium tuberculosis* (MacMicking *et al.*, 1995), *Leishmania major* (Liew *et al.*, 1990) and *Histoplasma capsulatum* (Lane *et al.*, 1994), including of *Schistosoma mansoni* (James and Glaven, 1989). Bocca *et al.* (1998) observed that treating the infected animals daily with *N*ω-nitro-L-arginine (nitro-Arg) blocked NO production and caused a significant increase in the lung fungal burden by day 60 of infection and the time to death of the Nitro-Arg-treated animals was sorted compared with untreated animals. Pavanelli *et al.* (2011) also demonstrated that the microbicidal effect of NO released by Cis-[Ru(bpy)₂(NO)SO₃](PF₆), results in reduced microbial counts and consequently inflammation reduction. These results might be related to the microbicidal effect of NO released by NO-donors, which results in reduced parasite counts and reduced inflammation.

It is known that one of the most prominent functions of NO in the immune system is its

participation in protective immunity, which may directly and indirectly modulate the inflammatory response. In attempt to evaluate the participation of NO-donors in the hepatic inflammation, we initially verified that the microscopic examination revealed a discrete interstitial inflammatory infiltrate composed mainly of mononuclear cells presented a remarkably severe tissue destruction with large and disseminated necrotic loci in liver of control group than NO donors treated mice (Fig. 3A). At day 90 p.i., the liver from control group presented a markedly severe hepatitis and disruption associated with an inflammatory infiltrate composed of leukocytes cells. In contrast, the hepatic tissue from SNP treated mice showed a moderate inflammation characterized by mononuclear cells (Fig. 3A). In fact, the quantification (inflammatory score) of inflammatory cells in the liver showed a significant decrease in the numbers of leukocytes in the PF₆ (45 day p.i.) and SNP (90 day p.i.), compared to control (Fig. 3B).

The immunosuppressive activity of NO has been reported *in vitro* and in several *in vivo* models of infection, including *Toxoplasma gondii* (Candolfi *et al.*, 1994); *Listeria monocytogenes* (Gregory *et al.*, 1993) and *Plasmodium vinckei* (Rockett *et al.*, 1994).

In several works (Hickey, 2001; Spiecker *et al.*, 1998; Dal Secco *et al.*, 2003) it was observed that NO donors decrease leukocytes rolling and adhesion to endothelial cells, as well as neutrophil transmigration to inflammatory sites. Moretti *et al.* (2012) verified that NO deficiency results in a prolonged inflammatory reaction in model the mesh implant.

It is possible that this attenuated inflammatory response found in the liver of animals treated with NO-donors was due in part to the reduced burden parasites found in hepatic tissue. Together these results suggest that NO is crucial in modulating the local inflammatory response, exerting both regulatory and effectors roles during the parasite elimination.

Another possibility is that this attenuated inflammatory response and reduced hepatic lesion, found in the liver of animals treated with NO-donors is due in part to reduced expression of Vascular Endothelial Growth Factor (VEGF) in cells (Fig. 7A), which act on leukocyte migration. Possibly, this reduced expression of VEGF is partly due to the reduced amount of egg granuloma, since (Loeffler *et al.*, 2002) related that studies *in vitro* with Soluble Egg Antigens (SEA) induce up-regulate of VEGF.

Fibrosis is the process of excessive deposition of collagen and other Extracellular Components Matrix (ECM). Extracellular matrix formation and degradation are balanced processes dependent on the same cell types and products secreted. Some ECM deposition is necessary for wound healing to provide strength and temporary

structure to damaged tissues; however, if not limited, it can be pathologic. Analysis of picosirius red-stained sections by conventional or polarization microscopy revealed mild interstitial fibrosis in PF₆ (in 45 day after infection) and SNP (45 and 90 day after infection) treated mice. The fibrosis was manifested by a discrete increase in the amount of perimysial and endomysial fibrosis as compared to control livers from infected mice and not treated (Fig. 8A and B). The quantification of myocardial fibrosis in liver sections of both groups of mice revealed that the fibrosis is significantly higher on day 45 p.i., in infected control group mice compared with NO-donors treated mice. Therefore, the treatment with NO abrogated the inflammatory response and consequently the hepatic fibrosis.

Another possibility is that this attenuated inflammatory response and reduced hepatic lesion (fibrosis), found in the liver of animals treated with a SNP is due in part to increased production of IL-10 (Fig. 5A), which has immunomodulatory activity. A known function of IL-10 is its ability to modulate the production of several proinflammatory mediators, including cytokines and chemokines, thus reducing the severity of the inflammatory response to *S. mansoni* infection. IL-10 plays an anti-pathological role during many infections, including schistosomiasis and the distinction between IL-12 versus IL-10 secretion underlies the concept of macrophage polarization (Mosser and Edwards, 2008; Martinez *et al.*, 2008; Biswas and Mantovani, 2010). In fact, it was observed that egg of *S. mansoni* induced Th2 differentiation depends on IL-10, but IL-10 reduces both Th1 and Th2-mediated damage (Hoffmann *et al.*, 2000). Wynn *et al.* (1998) conclude that while IL-10 plays an important role in controlling acute granulomatous inflammation, it plays no essential role in the process of immune down modulation of the chronic schistosome infection. Although the production of IL-4 was no present it was noticed difference between NO-donors treated mice and the control group (Fig. 5B). Therefore, these results demonstrate that the NO deliverance directs induced anti-inflammatory condition, but not Th2 profile.

Our data showed a correlation between the activity of NO in to promote the development of minor inflammation hepatic and the production of IL-10 immunomodulatory cytokine, suggesting that this molecule might also play a significant role in schistosomiasis disease hepatic fibrosis.

In addition, the present study verified that NO-donors would be associated with hepatic lesions and accordingly transaminases alteration. For such, was measured the AST and ALT activity in serum and observed that the AST (Fig. 4A) and ALT (Fig. 4B) levels were significantly smaller in PF₆ and SNP when compared with control mice in all periods evaluated. The examination the AST and

ALT level is correlated with histological analysis, demonstrating association between hepatic lesions and transaminase level smaller.

Our results clearly show that the treatment with NO-donor resulted in the killing of the parasite, amelioration of the inflammatory response in the liver, protected the tissue damage and significantly promoted an overall increase in the survival these mice.

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Disclosures

The authors have no financial conflict of interest.

Author's Contributions

Wander Rogério Pavanelli: Conducted the BALB/c infection and treatment; participated in the design of the study, statistical analyzing, writing and discussion of the manuscript.

Jean Jerley Nogueira da Silva: Conducted and analyzed the ruthenium complex synthesis; participated in the design of the study, writing and discussion of the manuscript.

Carolina Panis: Conducted and analyzed the immunohistochemical assay and nitrite measurement; participated in the design of the study, writing and discussion of the manuscript.

Thiago Mattar Cunha: Assisted in the histological and fibrosis analysis.

Francisco José de Abreu Oliveira: Performed the eggs and adults worms quantification and data analysis.

Maria Claudia Noronha Dutra de Menezes: Performed the eggs and adults worms quantification and data analysis.

Ivete Conchon Costa: Conducted and analyzed the experiments for cytokine determination by ELISA.

Graciele da Silva Thomé: Conducted and analyzed the ALT/AST assay.

Francisco Ordelei Nascimento da Silva: Conducted and analyzed the ruthenium complex synthesis.

Eduardo Henrique Silva de Sousa: Conducted the histological and fibrosis analysis.

Luiz Gonzaga de França Lopes: Conducted and analyzed the ruthenium complex synthesis.

Rubens Cecchini: Contributed to the conception of the study, financial support and data discussion.

Fernando de Queiroz Cunha: Contributed to financial support, data analysis, discussion and writing of the manuscript.

Eiko Nakagawa Itano: Contributed to financial support, data analysis, discussion and writing of the manuscript.

Maria Angélica EharaWatanabe: Coordinator of the study, contributed to the design of the study, financial support, data analysis and the writing and discussion of the manuscript.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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