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O óxido nítrico e sua relação com diferentes microrganismos *in vitro* e durante a infecção aguda experimental por *Trypanosoma cruzi*

DANIELA LEITE FABRINO

Tese apresentada à Coordenação de Cursos de Pós-graduação do Instituto Oswaldo Cruz visando a obtenção do Título de Doutor em Ciências.
Área de concentração: Biologia Celular e Molecular

Orientadores:
Dr. Marcelo Genestra
Dra. Rossana Correa Netto de Melo

Rio de Janeiro
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DEDICATÓRIA

Dedico este trabalho a minha família, que sempre foi, e sempre será, minha maior motivação para vencer na vida; ao Johnny que me levantou todas as vezes que eu caí; aos meus Amigos que me seguraram todas as vezes em que quis sair correndo, e aos meus orientadores, que sempre me mostraram os melhores caminhos para seguir e nunca deixaram que eu me perdesse enquanto eu os seguia.

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“Saudade não é lembrança, nem mesmo recordação,

Saudade é a dor da ausência, maltratando o coração”

fica aqui registrada a minha saudade...

“Pensar é exercício de alegria
Entre veredas de erros
Cordilheiras de dúvidas
Oceanos de Perplexidade”

Carlos Drummond de Andrade

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LISTA DE ABREVIATURAS

A:T	Adenina/timina
ATP	Adenosina trifosfato
AINEs	Drogas antiinflamatórias não estereoidais
BH_4	6R-5,6,7,8-tetrahidrobiopterina
Ca^{2+}	Cálcio
CaM	Calmodulina
COXs	Ciclooxigenases
cNOS	NOS constitutiva
EBP-50	Esirina/radixina/moesina EMR 50
eNOS ou NOS III	NOS endotelial
FAD	Flavina adenina dinucleotídeo
Fe	Ferro
FMN	Flavina mononucleotídeo
G:C	Guanina-citosina
GAPDH	Gliceraldeído-3-fosfato desidrogenase
GCs	Guanilato ciclase solúvel
GMPc	Guanosina monofosfato cíclico
Heme	Ferro-protoporfirina IX
Hmp	Flavohemoglobina
H_2O_2	Peróxido de hidrogênio
Hsp 90	Proteína de choque térmico 90
IFN - γ	Interferon gama
IL	Interleucinas
iNOS ou NOS II	NOS induzida
L-NAME	N^G-nitro-L-arginina-metil-éster
L-NMMA	N^G-monometil-L-arginina
L-NNA	N^G-nitro-Larginina
LPG	Glicolipídio lipofosfoglicano
µL	Microlitro
µM	Micromolar
MET	Microscopia eletrônica de transmissão

N_2O	Óxido nitroso
NADPH	Nicotinamida adenina dinucleotídeo fosfato reduzida
NADPH-d	Nicotinamida adenina dinucleotídio fosfato diaforase
NFκB	Fator nuclear kappa beta
NMDA	N-metil-d-aspartato
nNOS ou NOS I	NOS neuronal
NO	Óxido nítrico
NO^-	Ânion nitroxil
NO_2^-	Nitrito
NO_3^-	Nitrato
nocNOS	Óxido nítrico sintase de *Nocardia sp.*
NOS	Óxido nítrico sintase
NOSs	Óxido nítrico sintases
O_2	Oxigênio
$ONOO^-$	Ânion peróxinitrito
PGE2	Prostaglandina E2
PKG	Proteína cinase G
RE	Retículo endoplasmático
REP	Ressonância eletrônica paramagnética
RIN	Radicais intermediários do nitrogênio
RYR	Receptor rianodino
S	Enxofre
SNP	Nitroprussiato de sódio
SERCA	Sarcoplasma/retículo endoplasma Ca^{2+} - ATPase
SOD	Superóxido dismutase
TcNOS	Óxido nítrico sintase de *Trypanosoma cruzi*
TGF-β	Fator de transformação do crescimento beta
TNF-α	Fator de necrose tumoral alfa

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O óxido nítrico e sua relação com diferentes microrganismos *in vitro* e durante a infecção aguda experimental por *Trypanosoma cruzi*

RESUMO

Daniela Leite Fabrino

O óxido nítrico (NO) é um radical livre gerado a partir da L-arginina, através da enzima oxido nítrico sintase (NOS). O NO é considerado um dos principais mediadores de citotoxicidade produzido por células da resposta imune, principalmente macrófagos (MØs), contra diversos microorganismos invasores. Embora, muitos trabalhos ressaltem a capacidade de diferentes parasitos em driblar os efeitos tóxicos desta molécula, ou mesmo de interferir em sua via de produção, os mecanismos envolvidos na habilidade desses microrganismos em manipular a via do NO permanecem pouco esclarecidos. Além disso, o papel do NO na resistência à infecções agudas ainda é controverso. Na presente tese, utilizamos o modelo rato (linhagens Wistar e Holtzman), para investigar a produção de NO no curso da infecção aguda experimental pelo *T. cruzi*, agente causal da Doença de Chagas. Demonstramos que houve uma queda significativa na produção de NO tanto de monócitos do sangue periférico quanto de macrófagos esplênicos. Entretanto, MØs dos animais infectados apresentaram importantes sinais de ativação, incluindo aumento do número e diâmetro de corpúsculos lipídicos, organelas envolvidas na síntese de mediadores inflamatórios, como a prostaglandina E2, um conhecido antagonista da via do NO. A via metabólica do NO também foi estudada no parasito. Nós demonstramos pela primeira vez a presença de uma TcNOS em formas tripomastigotas e amastigotas de *T. cruzi*. Entretanto, a atividade desta enzima se mostrou significativamente maior nas formas tripomastigotas, tal fato podendo estar correlacionado com sua intensa motilidade. Além disso, nós mostramos que tanto as formas tripomastigotas como as formas amastigotas do *T. cruzi* são capazes de produzir NO. A produção de NO pelo parasito também se revelou superior nas formas tripomastigotas, sugerindo que no *T. cruzi* a produção de NO é estágio-específica. Adicionalmente, usando um doador químico de NO, o nitroprussiato de sódio (SNP), nós demonstramos que as formas amastigotas tanto de *T. cruzi* como de *L. amazonensis* exibem maior perfil de resistência ao NO que as formas tripomastigotas e promastigotas, destes parasitos, respectivamente, sugerindo a participação da via metabólica do NO em diferentes aspectos relacionados ao ciclo biológico (diferenciação, multiplicação e/o manutenção da infecção) destes parasitos em seus hospedeiros vertebrados. Além disso, um triplo mutante para o gene das porinas de *Mycobacterium smegmatis*, também se mostrou resistente aos efeitos tóxicos do NO, sugerindo a participação destas proteínas de

superfície na captação deste mediador, interferindo no perfil de susceptibilidade de células hospedeiras a infecção por estas micobactérias. Nossos dados contribuem para o melhor entendimento da via do NO no curso de infecção de parasitas intracelulares obrigatórios assim como frente do potencial de regulação desta via metabólica a seu favor.

Palavras-chave: Óxido Nítrico. Óxido Nítrico Sintase. *Trypanosoma cruzi*. Doença de Chagas. *Leishmania amazonensis*. *Mycobacterium smegmatis*. Macrófagos.

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Nitric oxide effects on different microorganisms in vitro and its role during the experimental acute *Trypanosoma cruzi* infection in vivo

ABSTRACT

Daniela Leite Fabrino

Nitric oxide (NO) is a free radical generated from enzymatic deamination of L-arginine through the enzyme nitric oxide synthase. NO is claimed as a pivotal citotoxic molecule produced by activated macrophages during innate immune responses against invading microorganisms. It is also recognized that different microorganisms have developed a remarkable ability to survive to the toxic effects of this molecule, or even to modulate its metabolic pathway. However, little is known about the mechanisms involved in the NO manipulation by these microorganisms. Moreover, the role of NO in the resistance to the acute infections is still a matter of debate. In this work, we demonstrated through the use of two rat models (Wistar and Holtzman) that activated macrophages are able to kill the parasite *Trypanosoma cruzi*, the causal agent of Chagas'disease, in the absence of NO production. Activated macrophages were characterized by accentuated formation of cytoplasmic lipid bodies, organelles involved in the synthesis of inflammatory mediators, including prostaglandin E2, an antagonist of the NO pathway. The metabolic NO pathway was also studied in the parasite. We demonstrated the occurrence of a TcNOS in both trypomastigote and amastigote forms. However, the enzyme activity was significantly higher in the trypomastigotes, a fact that might be related to the intese motility of these forms. In addition, we showed, for the first time, that both evolutive stages of *T. cruzi* were able to produce NO. The synthesis of NO by the parasite might be important for the protozoan replication within the host cell contributing for the maintenance of parasite infection. Finally, using a NO donor – the sodiumnitroprusside (SNP), we demonstrated differential resistance of the parasite forms to NO with clear higher resistance of amastigotes forms from both, *T. cruzi* and *L. amazonensis* to the NO toxic effects compared to trypomastigotes and promastigotas forms, respectively. The triple mutant for the porin genes of *M. smegmatis*, also showed resistance to the NO effects. Our findings provide evidence for the existence of a consistent NO pathway in the parasite *T. cruzi* and suggest that this pathogen as well as the *L. amazonesis* can explore this pathway in its favor.

Key words: Nitric Oxide. Nitric oxide synthase. *Trypanosoma cruzi*. Chagas' disease. *Leishmania amazonensis*. *Mycobacterium smegmatis.* Macrophages.

1. INTRODUÇÃO

1.1 Óxido nítrico e Óxido nítrico sintase: aspectos gerais

A presença de radicais livres em sistemas biológicos foi descoberta há pouco mais de 50 anos. Naquela época foi hipotetizado que radicais oxigenados e nitrogenados eram formados a partir de reações enzimáticas, *in vivo*. Estas moléculas foram imediatamente relacionadas com processos prejudiciais ao organismo, tais como intenso dano celular, mutagênese e envelhecimento celular (revisão em Droge, 2002). Na década de 80 surgiram na literatura trabalhos demonstrando o papel benéfico dos radicais livres e sua importância nos processos de sinalização celular. Esses radicais foram reconhecidos como segundos mensageiros, ou mesmo como moléculas efetoras, em importantes processos fisiológicos tais como a resposta imune (revisão em Droge, 2002).

A evidência inicial da participação de óxidos de nitrogênio no metabolismo fisiológico surgiu de experimentos conduzidos no início da década de 80, que demonstraram a produção de nitratos em ratos *germ-free* (Green e cols., 1981b) e em humanos (Green e cols., 1981a), Em 1985, demonstrou-se que macrófagos ativados por lipopolissacarideos bacterianos eram capazes de induzir a produção de nitrito (NO_2^-) e nitrato (NO_3^-) (Stuehr & Marletta, 1985). Posteriormente, evidenciou-se que a L-arginina era o substrato de tal reação e a L-citrulina, também era formada como produto (Hobbs & Ignarro, 1996). Em 1998, identificou-se o óxido nítrico (NO) como o produto da reação de oxiredução da L-arginina (Marletta e cols., 1998).

O NO é um importante e versátil mediador gasoso nos sistemas biológicos, sendo relevante na regulação de uma ampla gama de processos fisiológicos e patológicos bem como na ação do sistema imune, atuando como um dos principais mediadores de citotoxicidade das células efetoras da resposta imune. (revisão em Martinez & Andriantsitohaina, 2008). Este radical livre é sintetizado a partir da L-arginina pela enzima óxido nítrico sintase (NOS), em uma reação de duas etapas de oxidação, sendo os produtos formados a L-citrulina e o NO, este último com uma vida extremamente curta cerca de 3 a 60

segundos, sendo rapidamente convertido em NO_2^- e NO_3^- nos sistemas biológicos **(Figura 1)** (revisões em Stamler e cols., 1992; Martinez & Andriantsitohaina, 2008).

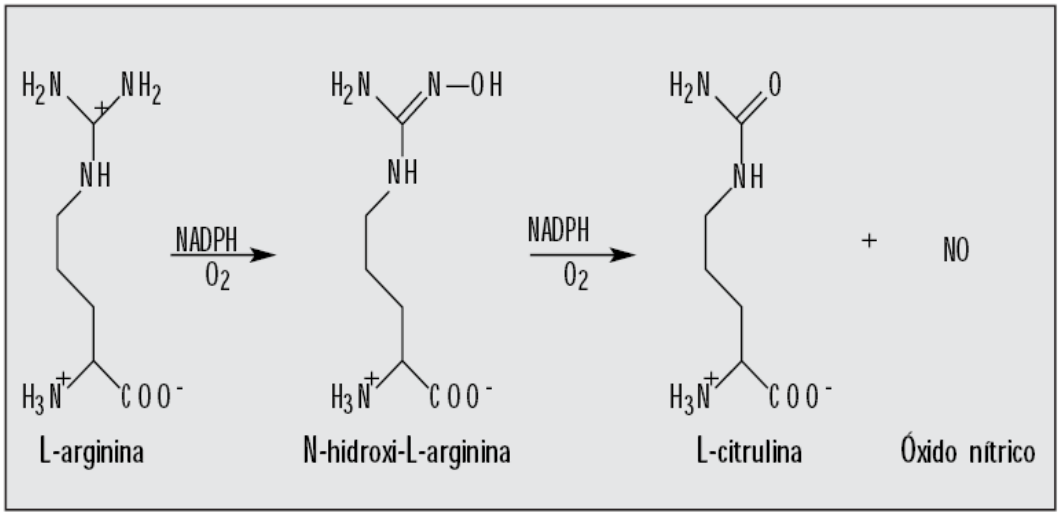

Figura 1 – Biossíntese de NO a partir da L-arginina (Dusse e cols., 2003)

Para que a reação enzimática protagonizada pela NOS ocorra, é necessária a participação de diferentes cofatores. A estrutura dimérica da NOS lhe confere dois domínios: um chamado de oxigenase N-terminal que contém sítios de ligação para o grupo Ferro-protoporfirina IX (heme) e para o cofator (6R)-5,6,7,8-tetrahidrobiopterina (BH_4); e o outro domínio é chamado redutase C-terminal, que contém locais de ligação para os cofatores flavina adenina dinucleotídeo (FAD), flavina mononucleotídeo (FMN) e nicotinamida adenina dinucleotídeo fosfato reduzido (NADPH). Os dois domínios são ligados entre si por um sítio de reconhecimento para calmodulina (CaM), sendo a presença de ambos fundamental para o funcionamento da enzima, visto que monômeros da enzima são inativos (revisões em Alderton e cols., 2001; Genestra, 2008) **(Figura 2).**

Cada cofator tem seu papel específico no funcionamento da NOS. FAD e FMN funcionam como aceptores de elétrons, que são doados pelo cofator NADPH, e também como repassadores desses elétrons para o grupo heme. O papel fundamental desses dois cofatores é permitir que o NADPH, que é capaz de doar dois elétrons, os doe para o grupo heme que é o aceptor final, que tem

capacidade para receber um elétron de cada vez. Quando passam de um cofator para outro, os elétrons são “armazenados” em intermediários estáveis, as semiquinonas (revisão em Alderton e cols., 2001).

Diversos foram os papéis propostos para a BH_4 no funcionamento da NOS. Atualmente este cofator é reconhecido como estritamente necessário ao funcionamento da NOS, servindo como um doador de elétrons para o grupo heme, contido no domínio oxigenase N-terminal. A razão para a alta demanda deste cofator pela NOS no papel de um transferidor de elétrons é promover uma transferência, rápida e acoplada, de prótons e elétrons, necessária para a ativação do oxigênio (O_2) requerido no passo final de produção do NO (revisão em Genestra, 2008)

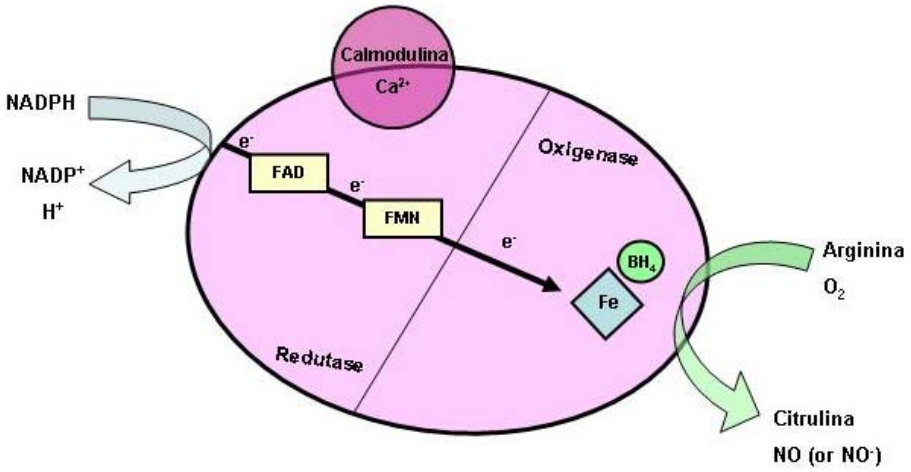

Figura 2 – Esquema de reações catalisadas pela NOS e seus cofatores. Modificado a partir da Figura 1 de (Alderton e cols., 2001)

A síntese enzimática de L-citrulina pode ser inibida por análogos da L-arginina tais como N^G-monometil-L-arginina (L-NMMA), N^G-nitro-Larginina (L-NNA) e N^G-nitro-L-arginina-metil-éster (L-NAME). Estes inibidores têm grande importância na pesquisa dos prováveis efeitos do NO em sistemas biológicos, uma vez que a substituição do substrato habitual pelos análogos inibe a produção de NO (Rees e cols., 1990; Snyder & Bredt, 1992).

A NOS apresenta 3 isoformas diferentes, as quais representam produtos gênicos distintos, e constituem uma família enzimática de NOSs (revisões em James, 1995; Alderton e cols., 2001). Duas de suas isoformas são NOS

constitutivas (cNOS) sendo (1) descrita em células neuronais (nNOS ou NOS I) e (2) descrita em endoteliais (eNOS ou NOS III).

As cNOS tem curtos períodos de ativação, controlados pelo aumento dos níveis intracitoplasmáticos do íon Ca^{2+}. Este íon se liga a CaM e este complexo (cálcio/CaM) é capaz de estimular a atividade da enzima (revisões em James, 1995; Fulton e cols., 2001; Genestra, 2007). Outra proteína que tem capacidade de estimular a cNOS é a proteína de choque térmico (*heat shock protein* 90) (hsp 90), tal proteína induz uma mudança conformacional na cNOS, resultando no aumento de sua atividade (Garcia-Cardena e cols. 1998). Além disso, Song e colaboradores mostraram que a hsp aumenta a afinidade da CaM pela nNOS (Song e cols., 2001).

Como proteínas que regulam as cNOS negativamente podemos citar as caveolinas, que são as principais proteínas estruturais de cavéolas e exibem capacidade de se ligar as cNOS e inibir diretamente sua função, tal interação é Ca^{2+}/CaM dependente (Felley-Bosco e cols., 2002) e revisões em (Alderton e cols. 2001, Martinez & Andriantsitohaina. 2008). Bem como a NOSIP, uma proteína com alta afinidade pela eNOS capaz de "seqüestrar" esta isoforma da enzima em compartimentos intracelulares, diminuindo sua disponibilidade citoplasmática (Dedio e cols. 2001).

Já a isoforma induzida (iNOS ou NOS II), foi descrita pela primeira vez em macrófagos (Xie e cols., 1992). Atualmente é sabido que os mais diferentes tipos celulares podem expressar iNOS (Nathan, 1997; Felley-Bosco e cols., 2002).

Para que a enzima iNOS alcance seu estado máximo de ativação, é preciso que além do estímulo antigênico; ocorra também um segundo estímulo fisiológico, mediado por citocinas como o fator de necrose tumoral alfa (TNF-α), interferon gama (IFN - γ) e interleucina (IL) 1β (revisões em Oswald e cols., 1994; James, 1995; MacMicking e cols., 1997; Brunet, 2001; Palmi & Meini, 2002). Tal indução leva a iNOS a produzir altos níveis (micromoles) de NO e embora seja necessário um tempo maior para sua ativação, em comparação com a NOSc, ela permanece ativa continuamente por horas ou mesmo dias (Green e cols., 1982) e revisões em (James, 1995; Alderton e cols., 2001). Para que ocorra a desativação da iNOS faz-se necessária a depleção total de seu substrato (Rivero, 2006) uma vez que a disponibilidade intracelular da L-

arginina, é fator limitante para a produção de NO (Mori & Gotoh, 2004). Diferentemente das cNOS a atividade da iNOS não é dependente das alterações dos níveis de Ca^{2+} (Nathan & Xie, 1994).

Outro mecanismo de desativação, ou supressão, da enzima é a ação de glicocorticóides que suprimem a iNOS em níveis transcricional e pós-transcricional, podemos citar ainda a ação de citocinas antiinflamatórias, que podem agir sinergicamente como, por exemplo, as IL - 4, IL - 10 e fator de transformação do crescimento beta (TGF-β) (revisões em Oswald e cols., 1994; MacMicking e cols., 1997; Brunet, 2001; Droge, 2002).

Devido ao fato das isoformas de NOS apresentarem diferentes vias de ativação, o controle das mesmas pode ocorrer de maneira distinta, até mesmo numa mesma célula. Entretanto, existem características bioquímicas importantes que são compartilhadas por estas enzimas e que podem ser resumidas, conforme abaixo:

a) as NOS apresentam esquema de catálise semelhante, que envolve a oxidação do nitrogênio guanido terminal da L-arginina para formar NO e L-citrulina, em uma complexa reação envolvendo flavina mononucleotídeo, flavina adenina dinucleotídeo, tetrahidrobiopterina e Ca^{2+}-calmodulina, produzindo N-hidroxi-L-arginina como produto intermediário, e consumindo 2 moles de O_2 e 1,5 moles de NADPH por mol de NO formado (Michel, 1999);

b) as enzimas cataliticamente ativas existem como homodímeros com HB_4 e heme, que facilitam a formação do dímero. Embora seja sempre citado na literatura que a NOS, em sua forma ativa, é uma proteína dimérica, é fato que todas as isoformas da NOS só atuam em presença de uma calmodulina (CaM) em cada um dos seus domínios, o que as torna portanto tetrâmeras. O domínio C-terminal é considerado homólogo entre as isoformas, existindo menor homologia no domínio N-terminal (Alderton e cols., 2001). Atualmente, apenas em humanos é conhecida a seqüência de DNA das três isoformas, existindo cerca de 55 a 60 % de identidade na seqüência de aminoácidos, com regiões fortemente conservadas e envolvidas na catálise, enquanto a homologia

de uma determinada isoforma pode ser de 85-92% entre espécies (Xie e cols., 1992; Genestra, 2008).

c) as três isoformas da enzima são reguladas pela presença da CaM, entretanto vale ressaltar que só as cNOS apresentam regulação aparente pelas alterações nas concentrações de Ca^{2+} (Nathan & Xie, 1994).

A dependência de íons Ca^{2+} para a ativação das NOS levou a classificação dessas enzimas como cálcio-dependentes (que são as isoformas I e III) e cálcio-independentes (isoforma II). Este íon é um importante sinalizador citoplasmático, atuando através de sua associação com receptores intracelulares específicos. Um desses receptores é a proteína citoplasmática calmodulina. Quando o Ca^{2+} se associa à calmodulina forma-se o complexo Ca^{2+}/calmodulina, que se constitui num elemento regulatório de várias atividades enzimáticas intracelulares, incluindo a ativação da NOS. No caso da cNOS, é necessária uma determinada concentração de Ca^{2+} intracelular para que ocorra a atividade enzimática, ocorrendo portanto, inativação da cNOS com a queda dos níveis de Ca^{2+} citoplasmático. Para a iNOS, não há inibição de atividade quando há queda dos níveis de Ca^{2+} intracelular (Nathan & Xie, 1994; Alderton e cols., 2001).

As NOSs também podem ser classificadas quanto à sua localização intracelular e a identificação destas enzimas pode ser feita de forma, (1) indireta: através de uma reação histoquímica que marca nicotinamida adenina dinucleotídio fosfato diaforase (NADPH-d), a partir da redução de sais de tetrazólio em um composto formazana (de coloração azul escura), com doação de elétrons provenientes do NADPH (Goldstein e cols., 2000); e (2) direta: através de técnica imunocitoquímica, atualmente mais utilizada, pois localiza a NOS através de anticorpos específicos (Johansson & Carlberg, 1995).

As NOSc permanecem ancoradas à membrana plasmática e a iNOS se localiza de maneira difusa no citoplasmática (Kroncke e cols., 1997; Shin e cols., 2004), podendo ser recrutada para um sítio citoplasmático específico, como por exemplo os fagossomos. Uma hipótese para o trânsito intracelular da NOS é a sua capacidade de ligação com a esirina/radixina/moesina (EMR) 50

(EBP-50), que é uma proteína de associação ao citoesqueleto, dessa maneira a iNOS se deslocaria no citoplasma celular através dos filamentos de actina (Davis e cols., 2007).

O fato do NO ser uma molécula efêmera e que, em alguns casos, como nos processos de sinalização celular, ser produzida em pequenas quantidades, pode tornar difícil sua mensuração. Entretanto, atualmente há diversas técnicas que permitem a detecção desta molécula, direta e indiretamente (Thatcher e cols., 2004).

O método de Griess foi descrito em 1879, e se baseia em uma reação colorimétrica, a diazotação (Griess, 1879). O NO é uma molécula instável que sofre rápida oxidação, podendo se transformar em NO_2^- e/ou NO_3^-. O NO_2^- tem capacidade de reação com aminas presentes no reagente de Griess e tal reação gera um produto de coloração púrpura cuja intensidade é medida por espectrofotometria (545nm) (Green e cols., 1982). Este método tem sido a técnica mais difundida para a mensuração de NO em sistemas biológicos, uma vez que apresenta bastante acuidade (aproximadamente 2,5µM) e permite o uso de pequenas quantidades de amostras (até 50µL), além de apresentar baixo custo (Green e cols., 1982; Bryan & Grisham, 2007)

É necessário ressaltar que o NO_3^- não tem capacidade de reação com o Griess, portanto, em alguns casos, é necessário o uso da enzima nitrato redutase que faz a transformação do NO_3^- em NO_2^-, antes da avaliação por espectrometria (Granger e cols., 1996).

A detecção de NO também pode ser feita por quimioluminescência, que consiste em reagir o NO com ozônio, produzindo o radical NO_2 no estado excitado (NO_2*). Quando retorna ao estado fundamental, cada NO_2* libera um foton que é medido por um foto-sensor. Este método consegue detectar de 1-5nM de NO (revisões em Tarpey e cols., 2004; Bryan & Grisham, 2007). Outra técnica usada é ressonância eletrônica paramagnética (REP), que detecta as propriedades magnéticas de elétrons não pareados. Entretanto, como tais elétrons provenientes de moléculas como o NO são encontrados em concentrações muito baixas, é necessário o uso adicional de compostos seqüestradores de elétrons não pareados (*spin traps*). As vantagens desta técnica são a sua alta sensibilidade, que chega aproximadamente a 10^{-9}M, e a

possibilidade de mensuração do NO *in vivo.* Entretanto é uma técnica extremamente dispendiosa (revisão em Tarpey e cols., 2004).

Outra técnica recentemente descrita é o uso de microeletrodos sensíveis a mudanças de potencial elétrico, o que permite a detecção direta de NO *in vivo*, pelo chamado método eletroquímico. Quando o NO passa pelo eletrodo, este tem seu potencial alterado e tal alteração é comparada com a de um eletrodo de referência (Thomson e cols., 2008). Entretanto, em muitos casos, como nas respostas imunológicas, o NO é produzido em altas concentrações, tornando-se, portanto, fácil a sua detecção pelo método de Griess (Rivero, 2006).

1.2 Participação do NO em processos fisiológicos e homeostáticos

Quando produzido em baixas concentrações (pico a nanomolares) o NO atua em diferentes funções homeostáticas, tais como o controle da pressão arterial, neurotransmissão (Felley-Bosco e cols., 2002) e revisão em (Martinez & Andriantsitohaina, 2008).

Um dos mecanismos de atuação do NO, no controle da fisiologia, é a sua capacidade de ativar proteínas fosforilativas, proporcionando assim a ativação ou desativação de diversas vias metabólicas intracelulares (revisão em Martinez & Andriantsitohaina, 2008). Como exemplo cita-se a capacidade do NO, produzido por células do endotélio vascular, em interferir no controle da pressão arterial. A isoforma eNOS, sensível a Ca^{2+}, é ativada por sinalização neuronal eletroquímica e produz pequenos pulsos de NO que se difunde para a vizinhança das células do músculo liso, ligando-se ao grupo heme da guanilato ciclase solúvel (GCs). Como resultado, ocorre a produção de guanosina monofosfato cíclico (GMPc), que, por sua vez, irá ativar as proteínas cinases do tipo G (PKG). Em sequência as PKGs vão ativar as fosfatases, proteínas responsáveis pela desfosforilação do complexo actina miosina, na célula muscular lisa e ocorrerá então o relaxamento muscular e provocando a diminuição da pressão arterial local (Hobbs & Ignarro, 1996).

A participação do NO em processos de neurotransmissão também se dá devido ao aumento dos níveis de GMPc, o glutamato age como molécula sinalizadora para receptores N-metil-d-aspartato (NMDA) e estes por sua vez

estimulam a nNOS que produz NO, deste modo há ativação de CGs e produção de GMPc (Dusse e cols., 2003).

Embora se mostre como excelente molécula sinalizadora por suas propriedades lipofílicas e baixo peso molecular, o NO é também bastante lábil e, como anteriormente citado, com “vida” ativa muito curta (Martinez & Andriantsitohaina, 2008). Por isso, acredita-se que a localização das NOSs no interior da célula pode influenciar a função biológica do NO produzido (Kelm, 1999; Davis e cols., 2007).

Alguns trabalhos destacam não só as propriedades fisiológicas do NO, mas também a sua capacidade citoprotetora. Em baixas concentrações micromolares (µMs), o NO impede a peroxidação de lipídios e também pode agir como uma molécula antioxidante, interrompendo a cadeia de reações oxidativas causadas por outros radicais livres (Kroncke e cols., 1997). Além disso, pode inibir apoptose através de inativação de proteínas da família das caspases, um estudo sobre injuria hepática, causada por isquemia/reperfusão, em modelos experimentais e humanos, mostra que a inibição deste processo de morte celular, pelo NO, pode ser benéfico (Abe e cols., 2008). Bem como há relatos de que em processo de isquemia cerebral, a indução da eNOS se mostra como importante ferramenta protetora (Endres e cols., 2004).

Quanto à capacidade tóxica do NO, diversos mecanismos de ação são descritos como, por exemplo, (a) a inativação de enzimas ferro-dependentes, pode ocorrer devido a associação do NO ao grupo prostético, ferro e enxofre (Fe-S), ocasionando nitrosilação do seu sítio ativo (Oswald e cols., 1994); (James, 1995; Martinez & Andriantsitohaina, 2008)); (b) a indução da quebra de dupla fita de DNA (Schapiro e cols. 2003); e (c) formação do ânion peroxinitrito ($ONOO^-$), numa reação entre o NO e o radical superóxido ($O_2^{\bullet-}$), que é tóxico para diversos tipos celulares (Denicola e cols., 1993; Peluffo e cols., 2004; Genestra, 2007).

Entretanto, ainda não está claro na literatura, exatamente qual concentração de NO é considerada tóxica para uma célula. Relata-se que uma concentração em torno de 4-5 µM de NO pode ser alcançada nas imediações de uma monocamada de células em cultura (Kroncke e cols., 1997). Por exemplo, na infecção aguda experimental por *Trypanosoma cruzi*, detectou-se em culturas de monócitos isolados a partir dos ratos do grupo controle (não

infectados) níveis de 8,6 ± 0,4 μM de NO. Estas células quando analisadas por microscopia eletrônica de transmissão (MET) não apresentaram qualquer sinal morfológico de injúria (Fabrino e cols., 2004). No entanto, sabe-se que, dependendo do tempo de contato e da situação sob a qual ele foi produzido, o NO pode se tornar uma molécula altamente tóxica (Kroncke e cols., 1997; Brunet, 2001; Martinez & Andriantsitohaina, 2008).

Portanto, todos os organismos que têm a capacidade de produzir NO possuem também o potencial para regular sua produção, bem como para desenvolver vias de detoxificação e resistência a esta molécula. Em mamíferos, o mais importante antioxidante intracelular talvez seja a glutationa, que reage com o NO gerando *S*- nitrosoglutationa, deste modo o NO perde seu potencial oxidativo. A depleção dessa molécula em culturas de células mostra um aumento na susceptibilidade aos danos causados pelo NO; sugerindo que *pools* de glutationa funcionem como "varredores" de NO (NO *scavenger*) (Kroncke e cols., 1997; Genestra e cols., 2008).

Em bactérias como a *Escherichia coli,* a presença de várias proteínas associadas ao estresse oxidativo, constitui um eficiente sistema de detoxificação, em relação ao NO. Um exemplo é a flavohemoglobina *Hmp*, codificada por um gene que é induzido, tanto em condições aeróbicas, quanto anaeróbicas, não só pela presença de NO_3^- mas, principalmente de NO_2^- e NO. Anaerobicamente, a *Hmp* detoxifica NO convertendo-o em óxido nitroso (N_2O) e ânion nitroxil (NO^-) (Corker & Poole, 2003). Outro exemplo é a presença de homocisteína em células bacterianas como a *Salmonella typhimurium*. Embora o mecanismo de ação desta molécula como antagonista do NO não esteja esclarecido, é fato que cepas de bactérias com mutações na via de síntese da homocisteína são altamente susceptíveis ao doador de NO S-nitrosoglutationa(Groote e cols., 1996; Cutruzzola, 1999; Spiro, 2007).

A *Micobacterium smegmatis*, uma micobactéria não patogênica, e supostamente susceptível às ações tóxicas do NO, também exibe capacidade de detoxificação e resistência a esse radical nitrogenado, por diferentes vias bioquímicas. Como exemplos, podemos citar a capacidade de redução do NO por meio de um tiol, análogo a glutationa, o chamado micotiol (MSH) (Miller e cols., 2007), ou presença de uma hemoglobina truncada, que apresenta capacidade de se ligar ao NO e inativá-lo (Lama e cols., 2006).

Em se tratando de protozoários, em especial os da ordem kinetoplastida, como a *Leishmania sp*. e o *T. cruzi*, ainda não foi feita a identificação de uma enzima estritamente ligada à neutralização de espécies reativas nitrogenadas; entretanto, esses protozoários conseguem interferir na via de produção do NO. Tais mecanismos serão abordados no capítulo 3 desta tese.

Deve-se citar também que o próprio NO participa de sua auto-regulação, exercendo "feed back" negativo (Stuehr, 1999; Brunet, 2001; Guzik e cols., 2003). Devido ao fato do NO formar complexos estáveis com o ferro, qualquer enzima que forme intermediários ferro-heme reduzidos, é potencialmente alvo de inibição por NO, e a família das NOSs não é exceção. Entretanto, parece que a iNOS é menos susceptível a tal inibição devido a facilidade do complexo Fe^{2+}–NO reagir com outros radicais livres e se dissociar. A cNOS, por sua vez, é muito mais passível de sofrer tal processo, pois reage com NO em seu sítio ativo, e parece formar um complexo mais estável, uma vez que a adição de NO *scavengers* não interfere no efeito inibitório nesta isoforma (Alderton e cols., 2001). Alguns autores propõem que a dissociação (catálise normal) do complexo Fe^{2+}–NO possa competir com sua formação (auto-inibição), e que devido ao sitio de ligação que o NO alcança na cNOS, seu sítio ativo, a dissociação do complexo formado com a mesma é muito mais lenta (revisão em Santolini e cols., 2001).

Em mamíferos, é necessário ainda citar outros mediadores fisiológicos, que também podem regular a produção de NO, como por exemplo, a prostaglandina E2 (James, 1995; Droge, 2002). Além disso, já foi relatada a interferência de hormônios femininos na modulação da produção tanto da cNOS, em células endoteliais, quanto da iNOS, em células da linhagem monocítica, como macrófagos peritoneais de ratos (Robert & Spitzer, 1997).

A expressão da iNOS é o resultado de uma resposta inflamatória localizada ou difusa oriunda de uma infecção ou dano tecidual. Os efeitos benéficos da expressão da iNOS podem ser vistos em infecções agudas tanto localizadas quanto sistêmicas. Por outro lado, em respostas inflamatórias crônicas localizadas, a síntese de NO dependente de iNOS, por macrófagos no espaço subendotelial, pode ter um impacto na patogênese dos vasos sangüíneos ateroscleróticos (Morris & Billiar, 1994; Guzik e cols., 2003). Os diferentes papéis do NO na homeostasia podem ser observados na **Figura 3.**

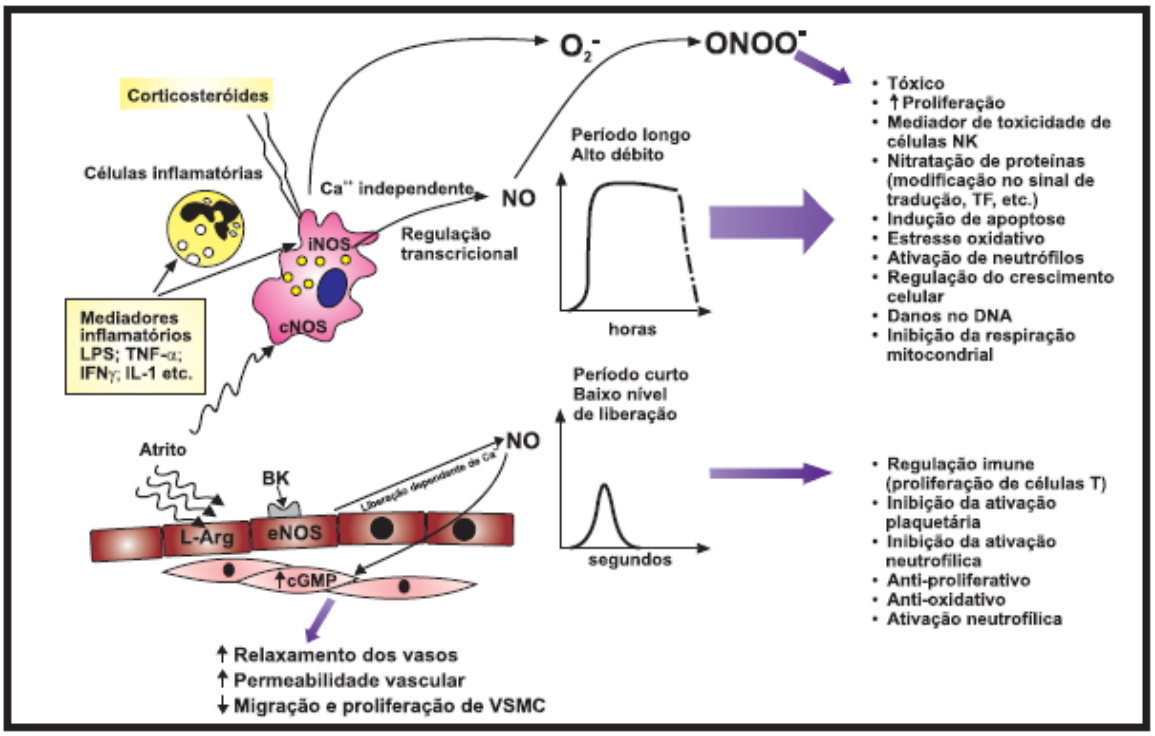

Figura 3 – Diferentes papeis do óxido nítrico na homeostasia.

Modificada a partir da Figura 2 de (Guzik e cols., 2003)

Ressalta-se que a síntese de NO, via NOS, não é restrita às células de mamíferos. Existem relatos na literatura de sua produção em plantas (Romero-Puertas e cols., 2004), insetos (Rivero, 2006), eucariotos inferiores como *T. cruzi* (formas epimastigotas) (Paveto e cols., 1995), *Leishmania* sp. (Basu e cols., 1997) (Geigel & Leon, 2003; Genestra e cols., 2006b) e fungos (Kanadia e cols., 1998) sempre através da NOS. Existem ainda relatos da produção de NO por procariotos (Corker & Poole, 2003; Zumft, 2005). Neste caso, a produção de NO pode ocorrer por uma via não enzimática, a partir de NO_2^- e NO_3^-, como ocorre nas bactérias denitrificantes (Corker & Poole, 2003).

A produção de NO, via NOS, já foi demonstrada em glândulas salivares de *Rhodnius prolixus*, um inseto hematófago, e neste inseto, o NO parece funcionar como vasodilatador e fator anti-plaquetário durante o repasto sanguíneo (Ribeiro e cols., 1993); a presença de NO neste invertebrado sugeriu um interessante exemplo de evolução convergente, uma vez que vertebrados utilizam a mesma molécula na regulação do tônus vascular e agregação plaquetária (Moncada e cols., 1991). A similaridade de sequências, e requerimentos de cofatores, entre a enzima de insetos, e a isoforma

constitutiva de vertebrados, indica alto grau de similaridade na estrutura e função dessas enzimas (Rivero, 2006).

O primeiro registro de produção de NO, via NOS, em procariotos, foi feito por Chen e Rosazza em *Nocardia sp.* (nocNOS) (Chen & Rosazza, 1994). *Nocardia sp.* são bactérias gram-positivas, que tem sua patogenicidade relacionada com infecções oportunistas, mas que podem causar quadro agudo de infecção A função da nocNOS não se encontra esclarecida, mas é sugerido que a presença de tal proteína nestas bactérias esteja relacionada com sua virulência, uma vez que *Nocardia sp* pode sobreviver por longo tempo dentro de macrófagos (Chen & Rosazza, 1994). A partir desse estudo, a NOS tem sido identificada nos mais diferentes tipos de bactérias, desde fermentadoras obrigatórias como o *Lactobacilus fermentum* (Morita e cols., 1997) até cepas virulentas como *Staphylococcus aureus* (Choi e cols., 1997; Hong e cols., 2003), mas o significado da atividade da NOS, e consequentemente do NO "per se" nestes microrganismos ainda está por ser esclarecido.

Em bactérias denitrificantes, o NO é um intermediário obrigatório no processo de redução do íon NO_3^- em di-nitrogênio (N2); entretanto vários representantes das enterobactérias, não denitrificantes, são capazes de produzir NO, embora em menor quantidade, nestas últimas mesmo o NO sendo, na maioria das vezes, um produto catabólico, é possível que tal molécula tenha uma atividade em processos de sinalização (Corker & Poole, 2003) e também há evidências que o NO exerça um papel específico na conservação da energia em diferentes bactérias denitrificantes (Zumft, 2005).

Após ser identificada em tripanosomatídeos, a via do NO foi relacionada com a motilidade flagelar (Pereira e cols., 1997) e regulação de processos de apoptose mediada por soro em *T. cruzi* (Piacenza e cols., 2001). Além disso, sugere-se que a capacidade do parasito em produzir NO possa interferir na resposta à infecção de macrófagos por *L. amazonensis* (Geigel & Leon, 2003; Genestra e cols., 2003b; Temporal e cols., 2005) e *T. cruzi* (Peluffo e cols. 2004).

Desta forma, destaca-se o papel do NO como uma importante molécula sinalizadora em eucariotos e fica claro que o campo de estudo sobre a fisiologia da mesma tanto em eucariotos como em procariotos ainda oferece muito a ser investigado.

1.3. Participação do NO em mecanismos de agressão e defesa

Embora o NO tenha grande importância nos processos de sinalização celular, e seja usado por diferentes organismos para tal finalidade, esta molécula vem recebendo grande destaque na literatura como relevante composto microbicida com papel reconhecido principalmente na imunidade inata (revisões em Gazzinelli e cols., 1992; Clark & Rockett, 1996; Bogdan, 1997; Bogdan e cols., 2000).

Em 1985, Sthuer e Marletta demonstraram pela primeira vez que macrófagos eram capazes de sintetizar NO_2 e NO_3 após serem estimuladas por lipopolissacarídeo (LPS) *in vitro* e durante o curso da infecção por *Escherichia coli*.(Stuehr & Marletta, 1985). Desde então, vários trabalhos vem enfocando a participação do NO em mecanismos de defesa. O NO é considerado como parte do arsenal de primeira defesa do organismo e com grande poder microbicida. Assim, já é bem descrita sua ação antibacteriana, antiparasítica e antiviral (Drapier e cols., 1988; Vincendeau & Daulouede, 1991; Gazzinelli e cols., 1992; Feng & Walker, 1993; Oswald e cols., 1994; Coleman, 2001; Sosroseno e cols., 2004).

A quantidade de NO produzida pela célula hospedeira e o seu local de produção são diferentes de acordo com o tipo de parasito que invade o hospedeiro (Brunet, 2001). Além disso, o limite de concentração de NO entre a não-toxicidade às células do hospedeiro e a toxicidade necessária para ação microbicida, é muito tênue (Nagy e cols., 2007). É importante lembrar que cada tipo celular/tecidual tem um limiar próprio de resistência ao NO (Kroncke e cols., 1997).

No caso de doenças autoimunes e situações de sobrecarga exagerada do organismo, o NO encontra-se em concentrações tóxicas para as células do próprio organismo (Nagy e cols., 2007). Deste modo, o balanço entre NO e citocinas inflamatórias, produzidas no curso da infecção, é crucial na determinação do potencial benéfico ou patológico dessa molécula para o hospedeiro (Brunet, 2001; Martinez & Andriantsitohaina, 2008).

O NO é produzido por células *natural killer* (NK), células dendríticas e principalmente por macrófagos, que são considerados como efetores da

imunidade inata, sendo uma de suas principais vias de ação mediada pela iNOS que, uma vez ativada, é capaz de produzir altas concentrações de NO por um período muito prolongado (Fang, 2004).

O equilíbrio da produção das citocinas, dos perfis Th1 e Th2, é fundamental para que ocorra um equilíbrio na produção de NO. A ativação da iNOS em macrófagos se dá através da estimulação de citocinas do perfil Th1, sendo o IFNγ o primeiro e mais importante sinal biológico, agindo sinergicamente ao fator TNFα e ao estímulo do patógeno. Por outro lado o perfil de citocinas Th2, composto por citocinas IL4, IL10 e TGFβ, diminui a capacidade de produção do NO pelo macrófago (Duffield, 2003). Um dos mecanismos propostos para tal ação é a ativação da enzima arginase, que compete com a NOS por seu substrato, a L-arginina, e tem como metabólitos poliaminas que podem ser usadas pelo parasito para multiplicação. (Duffield, 2003; Vincendeau e cols., 2003).

O papel do NO em diferentes processos infecciosos, incluindo a infecção por *T. cruzi*, é bastante controverso e nos capítulo 1 e 3 desta tese iremos abordar a produção e ação do NO durante a infecção experimental por *T. cruzi* em ratos e em ensaios *in vitro.*

Como já citado, são diversos os mecanismos de ação do NO, e alguns autores os classificam como diretos,por exemplo, a inativação de enzimas ferro-dependentes e a indução da quebra de dupla fita de DNA; e indireto através da formação de outro radical livre, o $ONOO^-$ (Miller & Britigan, 1997; Coleman, 2001). Entretanto, é difícil delimitar exatamente tais mecanismos; o mecanismo de dano mitocondrial envolve ações diretas e indiretas do NO. Em baixas concentrações, o NO inibe direta e reversívelmente, a citocromo C oxidase, competindo com o oxigênio; entretanto, quando o NO se liga à citocromo C oxidase ele induz à formação de $O_2^{\bullet-}$ e conseqüentemente de $ONOO^-$. Este radical danifica, irreversivelmente, os complexos I, II, IV e V da cadeia respiratória, bem como as enzimas aconitase, creatina quinase, superóxido dismutase (SOD) e o DNA mitocondrial (Coleman, 2001).

Além disso, o NO também inibe a gliceraldeído-3-fosfato desidrogenase (GAPDH) por *S*-nitrosilação inibindo assim a glicólise e a produção de ATP. A inibição pelo NO da via de respiração celular acarreta a depleção de fontes de

energia (ATP) e resulta em múltiplas conseqüências para a célula/tecido em questão (Coleman, 2001).

Em processos de sinalização celular o NO também pode causar grandes prejuízos com múltiplas conseqüências. Ele pode se ligar e nitrosilar a cisteína da proteína p21, que é uma molécula que controla proliferação, diferenciação e apoptose através de sua interação com proteínas, contra-reguladoras desses processos, como a proteína quinase RAF-1, fosfatidil-inositol 3-quinase e MAP-quinase, que em última instância controlam a ativação do NFκB (Coleman, 2001), um importante fator transcricional que está diretamente ligado com a regulação de processos inflamatórios e do sistema imune. O NO também pode induzir e estabilizar o inibidor do fator de transcrição nuclear-κβ (IκBα) que age diretamente no fator de transcrição nuclear-κβ (NFκβ) considerado uma molécula chave no controle da resposta imune (revisão em Kroncke e cols., 1997).

Em nível de núcleo celular, o NO pode causar uma série de danos na molécula de DNA, tais como, a indução de transições G:C (guanina-citosina) para A:T (adenina/timina) ou a quebra da dupla fita; esses dois processos seriam resultados da N-nitrosilação de desoxinucleotídeos, entretanto admite-se a possibilidade de que a produção dos radicais intermediários do nitrogênio (RIN) já citados anteriormente.também possam levar a tais processos. Além disso, a interação do NO com diversas proteínas reparadoras do processo de síntese, pode inativá-las o que causará dano na estrutura e função da molécula de DNA ou ainda atrasos no processo de síntese e consequentemente prejuízo de diferentes vias metabólicas celulares (revisão em Kroncke e cols., 1997).

O excesso de produção de NO pode ocorrer em diferentes processos patológicos tais como doenças auto-imunes, diabetes melitus, choque séptico e aneurisma cerebral, desta forma o NO acaba por aumentar as injúrias causadas por essas patogenias, podendo inclusive induzir processo de morte celular por apoptose (Gotoh & Mori, 1999).

O NO também pode induzir estresse funcional no retículo endoplasmático (RE), uma vez que a homeostasia de seus níveis intramembranares de Ca^{2+} é fundamental para conferir estrutura terciária a proteínas em processo de síntese. No interior do RE estão diversas chaperonas Ca^{2+} dependentes, que realizam os dobramentos das proteínas em

formação. O NO pode interferir nos níveis de Ca^{2+} do RE através de duas maneiras: (1) inibição da importação deste íon do citoplasma para o RE, aonde o NO se liga a Sarcoplasma/Retículo endoplasma Ca^{2+} - ATPase (SERCA) ou (2) pode se ligar ao receptor rianodino (RYR) e ativar a liberação descontrolada de Ca^{2+} do interior do retículo para o citoplasma da célula (Gotoh & Mori, 2006).

A capacidade de alterar o potencial de membrana das células durante sua difusão pelas membranas também foi atribuída ao NO. É documentado que em concentrações entre 0,1 e 1,0mM, bombas de Sódio/Potássio neuronais e bombas de prótons de vesículas sinápticas podem ser inibidas pela presença do NO (revisão em Kroncke e cols., 1997).

É importante ressaltar que o NO pode ter ação danosa ao próprio hospedeiro (Nagy e cols., 2007). Por exemplo, o NO produzido no endotélio dos vasos sanguíneos durante a infecção por *Plasmodium falciparum* (*P. falciparum*) interfere com a neurotransmissão local no cérebro e contribui para o desenvolvimento da malária cerebral (revisão em Bogdan, 1997). Na infecção chagásica já foi demonstrado que a produção exacerbada de NO acarreta lesões neuronais (Garcia e cols., 1999). Outro fato que se pode ressaltar é capacidade que o NO tem de exacerbar processos inflamatórios devido ao seu potencial de relaxamento dos vasos sanguíneos e por isso a facilitação do influxo de leucócitos para o sítio inflamado (Dedon & Tannenbaum, 2004).

Diante do exposto, conclui-se que o NO é uma molécula complexa e com atividade multifuncional que oferece muitos caminhos a serem explorados. A continuidade de estudos enfocando o NO, e suas vias de produção e atuação, são fundamentais para a elucidação de seu papel em processos fisiológicos e patológicos.

2. OBJETIVOS

2 – Objetivos

2.1 - Objetivo Geral

O presente trabalho teve por objetivo investigar a via metabólica do óxido nítrico frente a diferentes microorganismos, *in vitro*, e durante a infecção aguda experimental por *T. cruzi*.

2.2 - Objetivos Específicos

⇒ Avaliar o recrutamento, aspectos morfofuncionais e a produção de óxido nítrico de e por macrófagos na infecção aguda experimental por *Trypanosoma cruzi;*

⇒ Investigar a distribuição e o possível envolvimento de corpúsculos lipídicos de macrófagos durante a infecção aguda experimental por *T.cruzi* em ratas Holtzman*;*

⇒ Investigar a presença e atividade da enzima NOS, bem como a capacidade de produção de NO, pelo protozoário *T. cruzi*;

⇒ Avaliar *in vitro* a resistência de diferentes microorganismos (*T. cruzi, L. amazonensis e M. smegmatis*) a um doador de óxido nítrico;

3. RESULTADOS

3.1. CAPÍTULO 1

Recrutamento, aspectos morfofuncionais e avaliação da produção de óxido nítrico de macrófagos na infecção aguda experimental por *Trypanosoma cruzi*

A doença de Chagas ou tripanossomíase americana, cujo agente causal é o protozoário *Trypanosoma cruzi,* foi descoberta por Carlos Chagas, em 1909, e até hoje apresenta elevada prevalência e alto grau de morbi-mortalidade em países latino-americanos, onde existem cerca de 12-14 milhões de indivíduos infectados, constituindo importante problema social (revisões em Teixeira e cols., 1987; Tanowitz e cols., 1992; Brener & Gazzinelli, 1997; Barrett e cols., 2003; Stuart e cols., 2008).

O *T. cruzi* apresenta seu ciclo evolutivo em dois hospedeiros: um invertebrado, o inseto vetor triatomíneo, vulgarmente conhecido como "barbeiro"; e outro vertebrado (diferentes mamíferos incluindo o homem). A transmissão do protozoário ocorre no momento do repasto sangüíneo do triatomíneo, quando este elimina juntamente com suas fezes e urina as formas tripomastigotas metacíclicas, que penetram no hospedeiro através das mucosas, principalmente ocular, ou escoriações já existentes na pele ou provocadas ao coçar a região da picada. Após a invasão de células presentes nos sítios de inoculação, as formas metacíclicas diferenciam-se em formas amastigotas, multiplicando-se intensamente por divisão binária, constituindo os chamados "ninhos" de amastigotas. Posteriormente, ocorre um processo de diferenciação que leva ao aparecimento de inúmeras formas tripomastigotas sanguícolas, liberadas para a circulação sangüínea e linfática, pelo rompimento das células do hospedeiro. Estas formas parasitárias penetram em outras células nucleadas, tais como as do sistema muscular, reiniciando o processo de multiplicação ou podem ainda ser ingeridas pelo inseto vetor, encerrando o ciclo (Soares, 2007).

Além da transmissão vetorial a doença de chagas pode ainda ser transmitida por transfusão de sangue e órgãos infectados, congenitamente, ingestão de alimentos contaminados ou acidentes de laboratório (Prata, 2001).

Em termos gerais, a infecção chagásica manifesta-se sob 2 fases distintas: aguda e crônica.

A fase aguda se inicia logo após a infecção, em geral é assintomática e de curta duração (poucas semanas). Frente à resposta imune efetora (inata e específica), ocorre o controle tanto da parasitemia quanto do parasitismo tissular e a infecção entra na fase seguinte, denominada fase crônica. A maioria dos infectados permanece sem sintomatologia evidente, apresentandocontudo sorologia positiva, o que caracteriza a chamada, fase indeterminda. Porém, após anos (ou mesmo décadas) 30 a 40% passam a manifestar alterações cardíacas (mais freqüente) e/ou digestivas (megacolon e megaesôfago) (PIDC, 2009).

Um dos aspectos interessantes na infecção, humana e experimental por *T. cruzi,* e que tem sido objeto de numerosos estudos, é a intensa resposta imunológica iniciada na fase aguda e responsável pela resistência do hospedeiro ao parasito. Tal resistência tem importante participação da resposta mediada por células, envolvendo linfócitos T e macrófagos (Barrett e cols. 2003, Brener & Gazzinelli. 1997). Embora se reconheça o papel relevante dessa resposta celular, ainda não se encontram completamente esclarecidos quais os mecanismos envolvidos e a exata participação de cada uma das células efetoras no controle do parasito (Sun & Tarleton, 1993; Abrahamsohn & Coffman, 1996; Brener & Gazzinelli, 1997; Bogdan e cols., 2000).

Um grande número de estudos envolvendo ativação de macrófagos e seu papel na resistência à infecção por *T. cruzi,* vem sendo realizados (Nogueira & Cohn, 1978; Villalta & Kierszenbaun, 1984; Reed e cols., 1987; McCabe & Mullins, 1990; Silva e cols., 1995; Rodrigues e cols., 2000; Melo & Machado, 2001; Melo e cols., 2003a; Melo e cols., 2003b). Macrófagos ativados são capazes de matar o parasito, tanto *in vitro,* como *in vivo*, o que ressalta a importância dessas células na resistência à infecção (Villalta & Kierszenbaun, 1984; Reed e cols., 1987; Silva e cols., 1995; Melo & Machado, 1998; Rodrigues e cols., 2000; Melo & Machado, 2001; Fabrino, 2002; Fabrino e cols., 2004; Melo, 2008).

Utilizando-se o modelo rato, o nosso grupo tem demonstrado, *in vivo*, que o sistema monócito/macrófago apresenta papel crucial no controle da infecção aguda experimental por *T. cruzi* (revisão em Melo, 2008). Nesta fase

da doença, há uma intensa mobilização de monócitos do sangue periférico em paralelo ao intenso influxo de macrófagos no coração, órgão alvo da doença. Macrófagos infiltrados neste órgão encontram-se diretamente envolvidos na inibição da reprodução do parasito e apresentam morfologia de células ativadas, com muitas projeções citoplasmáticas, núcleo volumoso e eucromático e aumento de organelas (revisão em Melo, 2008).

Um aspecto morfológico interessante de macrófagos ativados é a formação aumentada de corpúsculos lipídicos (CL). CLs são inclusões citoplasmáticas ricas em lipídios presentes em virtualmente, todos os tipos celulares. Essas organelas caracterizam-se pelo fato de não apresentarem, dupla membrana trilaminar típica o que pode ser claramente identificado por microscopia eletrônica de transmissão (MET). CLs apresentam-se como organelas multi-funcionais. Leucócitos e macrófagos são capazes de sintetizar mediadores parácrinos, envolvidos na resposta inflamatória, tais como as prostaglandinas e leucotrienos (revisões em Bozza e cols. 2007, Melo, 2008).

O envolvimento de CLs em patogenias foi primeiramente observado em doenças inflamatórias crônicas, nas quais detectou-se que essas organelas aumentam em número e tamanho (Weller e cols., 1999). Recentemente, o envolvimento dessas organelas com mecanismos da resposta imune inata vem sendo estudado, principalmente em neutrófilos e macrófagos (revisão em Bozza e cols. 2007). O estímulo de leucócitos por LPS, *in vivo,* induz o aumento de sua capacidade para a produção de Leucotrieno B4 e prostaglandina 2 (PGE2), em paralelo com o aumento de número dos CL. Desta forma sugere-se que essas organelas possam funcionar como sítios distintos de produção de mediadores parácrinos da inflamação (Pacheco e cols. 2002).

Em acordo com os dados observados por Pacheco e colaboradores em 2002, no curso da infecção bacteriana; nosso grupo observou que na infecção aguda experimental por *T.cruzi,* macrófagos peritoneais e cardíacos de ratas, mostraram aumento do número de CLs no citoplasma, relacionado com maior síntese de mediadores da inflamação em especial a PGE (Melo e cols., 2003a).

Em 2006, nosso grupo demonstrou durante a infecção com *Mycobacterium bovis* BCG, a co-localização de PGE2 e da enzima

ciclooxigenase dentro de CLs em macrófagos ativados (D'Avila e cols., 2006). Além disso, tanto durante a infecção por micobactéria, como por *T. cruzi*, mostramos que essas organelas sofrem alterações ultra-estruturais de acordo com a intensidade da infecção e grau de inflamação (D'Avila e cols., 2006; Melo e cols., 2006). O primeiro artigo desta tese é focado em estudo ultra-estrutural detalhado de CLs em diferentes tipos de macrófagos durante a infecção aguda experimental por *T. cruzi*. Este artigo destaca os CLs como marcadores estruturais da inflamação, formados em resposta à infecção e aumentados em tamanho, em função do grau de parasitismo (Melo e cols., 2006).

A atividade funcional de macrófagos durante a infecção chagásica tem sido correlacionada com elevação da sua capacidade de produção, tanto *in vitro* como *in vivo*, de diversos compostos, como o peróxido de hidrogênio (H_2O_2) (Nathan, 1982; Villalta & Kierszenbaun, 1984; Melo e cols., 2003a; Fabrino e cols., 2004), NO (Gazzinelli e cols., 1992; Vespa e cols., 1994; Petray e cols., 1995; Silva e cols., 1995; Rodrigues e cols., 2000) e peroxinitrito (Alvarez e cols. 2004, Denicola e cols. 1993). Além disso, citocinas como IL-12, IFN-γ e TNF-α estão relacionadas com a atividade microbicida de macrófagos (Abrahamsohn & Coffman, 1996) e NO (Petray e cols., 1995; Rodrigues e cols., 2000).

A resposta de macrófagos à infecção por *T. cruzi* parece depender do tipo de macrófago, tipo de estímulo e microambiente em que se encontrar a célula em questão. Por exemplo, Melo e colaboradores (Melo e cols., 2003b) demonstraram que, *in vivo*, diferentes populações de macrófagos (baço e peritôneo) respondem diferentemente a infecção. Durante a fase aguda da infecção por *T. cruzi,* macrófagos esplênicos produzem altos níveis de H_2O_2, mas o mesmo não acontece com os macrófagos peritoneais (Melo e cols., 2003b).

O NO é considerado, por diversos autores como uma molécula chave no combate ao *T. cruzi* (Vespa e cols., 1994; Norris e cols., 1995; Petray e cols., 1995; Silva e cols., 1995; Aliberti e cols., 1996; Holscher e cols., 1998; Rodrigues e cols., 2000). Entretanto, ainda há muita controvérsia sobre o papel do NO no combate ao *T. cruzi*.

Diversos trabalhos, usando diferentes modelos experimentais, descreveram a ausência ou inibição da produção de NO, durante a fase aguda da doença de Chagas. Macrófagos de galinha (DaMatta e cols., 2000) não secretam níveis significativos de NO, quando cultivados na presença do parasito. Sabe-se que aves são refratárias ao *T. cruzi e* esta resistência está associada ao sistema complemento (Kierszenbaum e cols. 1981, Kierszenbaum e cols. 1976).

Por outro lado, demonstrou-se que o *T. cruzi* pode ser internalizado e destruído por macrófagos de galinha em mecanismo associado à produção de H_2O_2 e independente da produção de NO (Meirelles & De Souza, 1985). Da mesma forma, macrófagos do peritônio de *Calomys callosus* (Oliveira e cols., 1997) também foram incapazes de produzir NO ao mesmo tempo em que produziram níveis significativos de H_2O_2. A produção de H_2O_2 por monócitos do sangue e por macrófagos esplênicos também foi verificada durante a infecção aguda experimental no modelo rato. Neste trabalho, os autores apontam o H_2O_2 como uma importante molécula sinalizadora (Melo e cols., 2003b).

Saeftel e colaboradores (2001) questionaram o fato do NO ser indispensável na fase aguda da doença de Chagas ao observar que, em camundongos, ocorreu produção de NO, estimulada por IFN-γ, apenas nos primeiros dias de infecção. Tal produção passou a ser gradualmente substituída por mecanismos da imunidade adaptativa, na qual linfócitos $CD8^+$, $CD4^+$ e células B agiriam em conjunto para limitar a reprodução do parasito, por meio de mecanismo independente da produção de NO (Saeftel e cols., 2001).

Outros trabalhos realizados *in vitro,* revelaram diminuições significativas na produção do NO em macrófagos murinos, quando cultivados com diferentes patógenos. O *Toxoplasma gondii*, por exemplo, inibiu, parcialmente a produção de NO nessas células (Seabra e cols. 2002). Além disso, formas inativadas do *T. cruzi* também mostraram capacidade em bloquear a síntese de NO por macrófagos (Pakianathan & Kuhn, 1994). Também *in vitro*, foi demonstrado que o *T.cruzi* inibe a expressão da iNOS (Damatta e cols., 2007). *In vivo,* macrófagos peritoneais residentes de ratos *Sprague-Dawley* não produziram níveis altos de NO, enquanto macrófagos inflamatórios sofreram inibição da via

de produção do NO, por um mecanismo não esclarecido, após estimulação com esporos viáveis de *Microgemma caulleryi* (Leiro e cols., 2001).

Freire de Lima e colaboradores (2000) demonstraram que durante a infecção por *T.cruzi*, *in vitro*, macrófagos que entravam em contato com linfócitos apoptóticos exibiam produção aumentada de PGE_2 e TGF-β (Freire-de-Lima e cols., 2000). Nosso grupo descreveu, *in vivo*, aumento significativo dos níveis de PGE_2 em macrófagos peritoneais, durante a fase aguda da infecção experimental por *T. cruzi* em ratos Holtzman (Melo e cols., 2003a). Sugere-se, assim, que mecanismos envolvendo as prostaglandinas possam estar inibindo a síntese de NO em macrófagos inflamatórios, durante a fase aguda, neste modelo.

Entretanto, o papel das prostaglandinas na regulação da produção de NO durante a infecção chagásica, também é alvo de controvérsia. O fato de que a PGE_2 é capaz de estimular a NOS, consequentemente a produção de NO, foi demonstrado por Gaillard e colaboradores (Gaillard e cols., 1991; Gaillard e cols., 1992). Além disso, a inibição da produção de PGE_2 foi observada em paralelo a uma diminuição dos níveis de NO em culturas de células esplênicas de camundongos C57BL/6 (Pinge-Filho e cols., 1999). Contudo, alguns grupos ressaltam o papel deste mediador como um supressor da resposta imune inata ao *T. cruzi*, via inibição de produção de NO (Freire-de-Lima e cols. 2000, Michelin e cols. 2005).

O papel controverso das prostaglandinas na produção de NO, também foi demonstrado em um trabalho recente de Hideko de colaboradores (2008) no qual, macrófagos peritoneais de camundongos, C57BL/6 e BALB/c foram tratados com drogas antiinflamatórias não estereoidais (AINEs), tais como, a aspirina e o celecoxib. Essas drogas têm capacidade de inibição das enzimas responsáveis pela produção de prostaglandinas, as chamadas ciclooxigenases (COX).

Interessantemente os macrófagos peritoneais retirados de camundongos C57BL/6 e tratados com aspirina, demonstraram um decréscimo na produção de NO, aos 12 dias de infecção; enquanto que o tratamento com celecoxib causou aumento da produção de NO, por essas mesmas células. Já em camundongos BALB/c todos os AINEs usados causaram aumento da produção

de NO nos macrófagos dos animais infectados quando comparados aos controles (Hideko Tatakihara e cols., 2008).

No modelo rato nosso grupo observou que durante a infecção aguda experimental em ratos, monócitos de animais infectados mostram inibição na produção dos níveis de NO, em paralelo com aumento significativo dos níveis de PGE_2. Vale a pena ressaltar que mesmo com baixos níveis de NO produzidos por monócitos, os animais infectados foram capazes de controlar a infecção (Fabrino e cols., 2004).

Usando o mesmo modelo animal e as mesmas condições de estudo (inóculo e dias de observação), nosso grupo demonstrou que tipos diferentes de macrófagos (peritoneais e esplênicos), foram incapazes de produzir quantidades significativas de NO durante a fase aguda da infecção experimental por *T. cruzi,* ao contrário, estas células exibiram quedas nos níveis de NO em alguns momentos da infecção, sugerindo que processos de modulação da via do NO podem estar ocorrendo *in vivo*. Apesar da baixa produção de NO, os animais infectados foram capazes de controlar a infecção (Fabrino, 2002).

Cummings e colaboradores (2004) discutem o papel do NO no controle da infecção por *T. cruzi* ao usar 2 modelos de camundongos k*nockout* para iNOS. Esses autores demonstraram que mesmo não produzindo NO, ambos os modelos são tão resistentes quanto os animais selvagens de camundongo e que tal resistência se deve a uma possível compensação bioquímica por meio de produção de TNF-α, IL-1 e da proteína inflamatória de macrófagos 1-α (MIP-1-α) (Cummings & Tarleton, 2004). Esses dados corroboram o trabalho prévio de Saeftel e colaboradores (2001) que demonstraram em camundongos que a produção de NO, estimulada por IFN-γ, ocorre apenas nos primeiros dias de infecção. Os autores também demonstraram que tal produção passa a ser gradualmente substituída por mecanismos da imunidade adaptativa, na qual linfóctos $CD8^+$, $CD4^+$ e B agiriam em conjunto para limitar a reprodução do parasito, por meio de mecanismo independente da produção de NO (Saeftel e cols., 2001). Usando a cepa de baixa virulência Sylvio X10/4 de *T. cruzi*, outro grupo mostrou que camundongos infectados são capazes de controlar o parasitismo sem a produção aumentada de NO (Marinho e cols., 2007).

Além disso, existem evidências de que parasitos podem escapar da toxicidade do NO, através da depleção de L-arginina, fato que é mediado por um aumento da atividade da arginase. Esta enzima compete com a NOS pelo seu substrato e gera como produto final poliaminas que podem ser usadas na proliferação do parasito. Sugere-se que a expressão de arginase possa ser modulada pelo próprio patógeno invasor (revisão em Vincendeau e cols., 2003; Peluffo e cols., 2004) **(Figura 1.1)**.

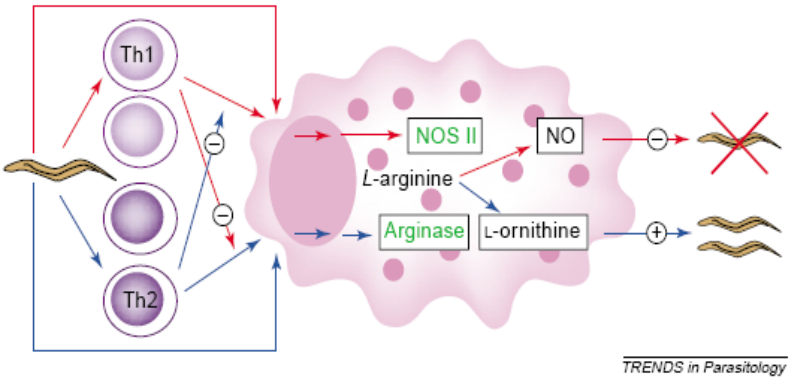

Figura 1.1 – Balanço entre as enzimas óxido nítrico sintase e arginase no macrófago e seu efeito em tripanosomatídeos (Vincendeau e cols. 2003).

Em 2004, demonstramos que, em paralelo a inibição da produção de NO por macrófagos esplênicos e monócitos do sangue periférico durante a infecção aguda em ratos, ocorre aumento na expressão da arginase por essas células. Entretanto, neste modelo, embora haja um aumento na expressão da arginase, parece que tal mecanismo não beneficia a manutenção do parasitismo uma vez que aos 20 dias de infecção o parasitismo nesses animais é quase ausente (Fabrino e cols. 2004).

O papel do NO durante a infecção aguda experimental por *T. cruzi* vem sendo investigado, principalmente em camundongos. Entretanto os dados apresentados por diferentes grupos ainda são contraditórios. Enquanto vários autores afirmam que o NO é fundamental no controle da infecção (Vespa e cols., 1994; Norris e cols., 1995; Petray e cols., 1995; Silva e cols., 1995; Aliberti e cols., 1996; Holscher e cols., 1998; Rodrigues e cols., 2000) outros questionam esse papel (Saeftel e cols., 2001; Cummings & Tarleton, 2004; Marinho e cols., 2007).

Os resultados contrastantes encontrados na literatura, sobre a produção de NO, no modelo camundongo, durante a infecção experimental por *T. cruzi* podem ser, em parte, explicados pela variedade de linhagens usadas, além da variedade de cepas do parasito e diferenças na intensidade dos inóculos usados nos diferentes trabalhos (Canto Cavalheiro & Leon, 1999).

Por outro lado o modelo rato também tem sido bastante usado para os estudos *in vivo* da infecção por *T. cruzi* (Machado e cols., 1987; Melo & Machado, 1998; Revelli e cols., 1998; Camargos e cols., 2000; Fichera e cols., 2004; Petersen e cols., 2006). Em ratos Wistar o controle do parasitismo parece relacionado à produção de NO (Fichera e cols., 2004).

Com o objetivo de comparar diferentes aspectos da infecção aguda e contribuir para o entendimento do papel do NO na resistência a infecção por *T. cruzi*, realizamos um estudo utilizando duas linhagens de ratos como modelos experimentais – Wistar e Holtzman. Além de avaliar a produção de NO, a infecção foi monitorada através da análise da parasitemia, histopatologia e ultra-estutura do coração, além da análise do recrutamento de monócitos do sangue periférico.

Nossos dados mostraram que na fase aguda os dois modelos apresentam patente parasitemia e importante parasitismo no coração. Entretanto nenhum dos dois modelos estudados produziu altos níveis de NO, quando comparados ao grupo controle (sem infecção). É interessante ressaltar que apenas o modelo Holtzman exibiu um relevante recrutamento de monócitos e macrófagos em paralelo a diminuição quase absoluta do parasitismo.

Desta forma, reiteramos a hipótese de que a participação de macrófagos no combate à infecção por *T. cruzi* aguda é crucial, mas que a produção de NO não parece ser o mecanismo central de ação dessas células conforme observado em (Fabrino, D. L. e cols., 2008) - submetido, inserido neste capítulo como o segundo artigo desta tese.

3.1.1 ARTIGO 1

Differential response to acute Trypanosoma cruzi infection in rat models is associated with high mobilization of monocytic lineage cells but not with nitric oxide production

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Differential response to acute *Trypanosoma cruzi* infection in rat models is associated with high mobilization of monocytic lineage cells but not with nitric oxide production

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Running title: Acute Chagas' disease and rat models

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Summary

The inflammatory response that follows the infection with *Trypanosoma cruzi*, the etiological agent of Chagas' disease, is essential for host resistance to infection. During the acute infection occurs a remarkable mobilization and activation of macrophages by parasite antigens, resulting in pro-inflammatory cytokine production, and consequent control of *T. cruzi* growth. Because *T. cruzi* infection has not been comparatively studied in different rat models, we evaluated the course of the acute infection in 2 strains of rats (Wistar and Holtzman). Host response was investigated by analyzing parasitemia, heart histopathology and ultrastructure, recruitment of monocytic lineage cells and nitric oxide (NO) production by these cells at different time points of infection. Acute infection in both rat strains was characterized by a marked parasitemia, but these animals showed a distinct behavior to manage the infection. While the early infection in Holtzman rats elicited a strong recruitment of monocytes/macrophages in parallel to a high parasitism in the myocardium, these aspects were not significant in Wistar rats. Moreover, the acute infection did not induce NO release by peripheral blood monocytes and splenic macrophages in both rat models, in spite of the consistent parasite clearance in blood and heart. Our findings demonstrate, for the first time, differential response of Holtzman and Wistar rats to the acute experimental *T. cruzi* infection and provide additional evidence that NO is not essential for the in vivo control of the parasite.

Key words: monocyte/macrophage system, rat models, nitric oxide, innate immunity, inflammation, *Trypanosoma cruzi*; heart; tissue pathology.

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INTRODUCTION

Parasitic protozoa infect hundreds of millions of people every year and are collectively some of the most important causes of human misery (Barrett*, et al.*, 2003). The flagellated protozoa *Trypanosoma cruzi* is the causal agent of Chagas' disease, which affects around 18 million people in Latin America. Unfortunately, there is no vaccine available to prevent this disease. In addition to humans, *T. cruzi* has the ability to infect several mammals. This parasite has an obligate intracellular, proliferative, non-flagellate form, called amastigote. After many division cycles, the amastigote forms convert into a flagellate form, the infective trypomastigote. Due to the high number of parasites into the host cell cytoplasm, the cell membrane disrupts and the infection spreads, affecting different organs, especially the heart (reviewed in Barrett, *et al*., 2003, Melo, 2008).

Acute Chagas'disease elicits a strong inflammatory response. In order to control parasite replication, cells of the monocytic lineage are recruited and have a pivotal role in the parasite clearance in both human beings and experimental models (reviewed in Melo, 2008).

In fact, during the acute phase of the infection, we have demonstrated an intense migration and extravasation of monocytes from the bloodstream into heart. Newly recruited monocyte-derived macrophages are strongly activated and actively involved in inhibiting parasite replication in the myocardium (Melo, 2008).

Activated macrophages are able to produce high levels of proinflammatory cytokines and microbicidal products such as hydrogen peroxide (H_2O_2) (Fabrino*, et al.*, 2004), peroxinitrite (Denicola*, et al.*, 1993) and nitric oxide (NO) (Rodrigues*, et al.*,

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2000). However, despite extensive experimental investigations, the mechanisms used by activated macrophages to kill *T. cruzi* are not fully understood. For instance, the production of high amounts of NO through the inducible nitric oxide synthase (iNOS) by macrophages has been claimed as one of the main biochemical pathways to kill the parasite during the murine infection (Brunet, 2001, Petray*, et al.*, 1995, Rodrigues, *et al*, 2000, Silva*, et al.*, 2003, Vespa*, et al.*, 1994). On the other hand, there are controversial data about the importance of this molecule in the control of parasite multiplication (de Oliveira*, et al.*, 1997, Fabrino, *et al,* 2004, Saeftel*, et al.*, 2001). In fact, it has also been demonstrated that neither NO production (Marinho*, et al.*, 2007) nor iNOS (Cummings and Tarleton, 2004) are essential for the in vivo control of *T. cruzi* in mice.

The contrasting results regarding the production of NO during the acute infection might be, in part, explained by the use of different animal models or mice lineages. Indeed, several studies have been undertaken in order to characterize murine models more effectively and their differential susceptibility to the acute *T. cruzi* infection, but the data in the literature are not fully in agreement. This is especially true for the two most widely used mouse model, BalbC and C57BL/6. Although it is often stated that BalbC is susceptible to *T. cruzi* while C57BL/6 is resistant with respect to the magnitute of parasitemia and/or mortality rate (Hideko Tatakihara*, et al.*, 2008, Meyer zum Buschenfelde*, et al.*, 1998, Silva*, et al.*, 1995), this pattern is not constantly observed (Andrade*, et al.*, 1985, Goncalves da Costa*, et al.*, 2002, Roggero*, et al.*, 2002).

On the other hand, rat models have also been used in experimental studies focused on the host resistance to the acute *T. cruzi* infection (Camargos*, et al.*, 2004, Machado*, et al.*, 1987, Melo and Machado, 1998, Petersen*, et al.*, 2006, Revelli*, et al.*,

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1998, Roffe*, et al.*, 2006). In Wistar rats, the parasite growth within cultured cardiac myocytes can be inhibited by cytokine-induced NO release (Fichera*, et al.*, 2004). However, in an in vivo study by our group using Holtzman rats, it is demonstrated absence of production of NO by blood monocytes (Fabrino, *et al*, 2004).

Because acute *T. cruzi* infection has not been comparatively studied in different rat models, we evaluate several aspects of the acute infection in these animals. We compare Holtzman and Wistar rats infected with the virulent Y strain of *T. cruzi* by the following parameters: (1) Parasitemia; (2) Mobilization/migration of monocytic cells lineage; (3) Parasite burden and pathological changes in the heart, and (4) Extent of inflammatory infiltrates in the myocardial tissue. In addition, because NO production during the acute in vivo infection is still controversial in the literature, we evaluate the ability of splenic macrophages and peripheral blood monocytes to produce NO. Remarkably, the acute in vivo infection did not induce NO release by monocytes/macrophages in both rat models, although they showed a distinct behavior to manage the infection. Inoculation of *T. cruzi* in both rat models led to an acute infection characterized by marked parasitemia. However, while the early infection in Holtzman rats elicited a strong recruitment of monocytes/macrophages in parallel to a high parasitism in the myocardium, these aspects were not significant in Wistar rats. In addition, inflammatory macrophages from Holtzman rats were able to control the parasite replication by the end of the acute infection. Our findings demonstrate, for the first time, differential response of Holtzman and Wistar rats to the experimental *T. cruzi* infection and provide additional evidence that NO is not essential for the control of the parasite.

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METHODS

Animals and Infection

Female Holtzman and Wistar rats aged 27-30 days comprised no infected and infected groups. Rats were inoculated intraperitoneally with a single inoculum of 3 X 10^5 trypomastigotes of *T. cruzi*, Y strain (Silva and V. Nussenzweig, 1953). Fresh blood samples taken from the tail showed living trypomastigotes in all animals at 6 or 12 days after inoculation. Animals were sacrificed in a CO_2 chamber at days 6, 12 or 20 of infection. Each group contained four to six animals, and survival was monitored daily. The Oswaldo Cruz Institute Ethical Committee approved the use of animals (CEUA: P-0069).

Parasitemia

Bloodstream forms of *T. cruzi* were assessed on sixteen animals (eight Holtzman/ eight Wistar) under standardized conditions, by direct microscopic observation of 5μL of fresh tail venous blood for twenty days post infection. Data were expressed as number of parasites x 10^4 mm^3.

Peripheral Blood Leukocyte Quantification

Samples of blood were taken from the tail of the controls and infected animals at 6, 12 and 20 days post-infection. The total number of leukocytes was estimated by hemocytometer counts (20 μL of blood diluted in 400 μL of a solution containing 2% acetic acid and 0.01% methylene blue). The percentage of each white blood cell in air-dried smears stained with May-Grünwald/Giemsa solution allowed an estimation of the

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total number of each leukocyte. The leukocyte percentage was determined after counting 200 cells per rat.

PBM and Macrophages Isolation and Viability

Peripheral blood was drawn by cardiac punction (3-5mL/animal). Monocytes were isolated from heparinized blood (100 units/mL blood) using Histopaque-1077 (Sigma, St. Louis, MO, USA) by density gradient centrifugation at 500g for 30 min, at room temperature. PBM were washed twice in PBS (280g for 10 min at 4ºC) and resuspended in RPMI 1640 medium. The cell viability was assessed using 0.4% Trypan blue dye. Trypan blue dye solution was added to the cell suspension at a ratio of 1:2. After mixing, the cells were observed under a microscope (Zeiss Axiolab, Carl Zeiss, Germany). The cell viability was higher than 95%.

Spleens were homogenized individually in 5 ml RPMI using a teflon coated tissue homogenizer (Gas-Col Apparatus Co. Terre Haute, IN, USA) and were washed twice; 280 g and 175 g/ for 10 min at 4ºC respectively and resuspended in 5 mL of the same culture cells medium. Viability of the cells was assessed by Trypan blue test as before (Fabrino, *et al.,* 2004). The concentration of all collected cells was adjusted to $2x10^6$ cells per ml with fresh medium RPMI 1640

NO/NO^-_2 measurement

NO generation by PBM and Splenic Macrophages was measured as accumulated supernatant nitrite (a stable breakdown product of NO) in controls and infected animals at 6, 12 and 20 days of infection by the Griess reaction (Green*, et al.*, 1982). The concentration of collected cells was adjusted to $2x10^6$ cell/ mL with fresh

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RPMI 1640 medium. Volumes (100μL) of cell suspensions were plated onto each well of 96-well flat-bottom tissue culture plates (Costar, Cambridge, MA, USA). Plates were incubated in humidified atmosphere at 37° C for 48h. Subsequently, 100 μL of the supernatant was collected and transferred to a new 96-well flat-botton tissue culture plates. The supernatants were incubated with 100 μL of Griess reagent (50 μL of 1% sulfanilamide (Sigma) plus 50μL of 0.1% of N-1- N-1-naphtylethylenediamine (Sigma) in in 5% phosphoric acid solution) at room temperature for 5 minutes. The absorbance was determined at 540 nm using a Spectra Max 190 spectrophotometer (Molecular Devices, USA). Conversion of absorbance to micromoles was deduced from a standard curve of sodium nitrite in medium. All determinations were performed in triplicate and expressed as micromoles of $NO^{-}_{2}/2x10^{6}$ cells.

Histological methods and Histoquantitative Analyses

Fragments of atria and ventricles from controls and infected animals (at 6, 12 and 20 days of infection) were fixed in 4% paraformaldehyde in buffered phosphate, pH 7.3, 0.1 M for 24 h, dehydrated and embedded in plastic resin - glycol metacrilate - (Leica, Heidelberger, Germany). Semi-serial 5-μm-thick sections were cut on a microtome (RM 2155, Leica) stained by haematoxylin and eosin or toluidin blue-basic fuchsin as previous works (Fabrino, *et al*., 2004) and examined for qualitative evaluation of the inflammatory and degenerative processes and quantification of parasitism. The number of amastigote nests in cardiomyocytes was evaluated at 70 μm intervals in order to avoid parasite recounting (Hanson and Roberson, 1974). For each group of animals

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were analyzed 1,600 fields (800 from atria and 800 from ventricles) in approximately 145 semi-serial sections at magnification of 400X.

The volumetric proportion of amastigote nests within cardiomyocytes and inflammatory processes were also examined in the heart at day 12 of infection, using a BX 51 Olympus microscope with the help of an integrating eyepiece with 100 squares at a final magnification of 400X (Melo and Machado, 1998). A total of 43,200 squares from both atria and ventricles were counted for each group.

Transmission Electron Microscopy (TEM)

Heart tissue fragments of controls and infected rats at 12 days post infection were fixed in phosphate-buffered 1% paraformaldehyde and 1% glutaraldehyde, pH 7.3, overnight at 4 ºC as before (Melo, 1999). After fixation, tissue fragments were washed in the same buffer and post-fixed in a mixture of 1% phosphate-buffered osmium tetroxide and 1.5% potassium ferrocyanide (final concentration) for 1 h prior to dehydration in graded ethanols and infiltration and embedding in a propylene oxide-Epon sequence (PolyBed 812, Polysciences, Warrington, PA, USA). Thin sections were cut using a diamond knife on an ultramicrotome (Sorvall MT2, Newton, MA, USA) and mounted on uncoated 200-mesh copper grids (Ted Pella, Redding, CA, USA) before staining with uranyl acetate and lead citrate and viewing with a transmission electron microscope (EM 10, Zeiss, Germany) at 60 KV. Electron micrographs were randomly taken at magnifications of x 12,000 and x 30,000. At least, 95 electron micrographs were analyzed.

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Statistical Analysis

Data from different groups were compared by the Mann Whitney "U" test ($P < 0.05$).

RESULTS

Holtzman and Wistar rats show similar parasitemia but distinct heart parasitism

Holtzman and Wistar rats showed a prominent parasitemia, with a similar pattern during the acute phase (Fig. 1A). Trypomastigotes, the invasive forms of the parasite, were firstly detected at day 5 and 6 for Wistar and Holtzman rats, respectively. At this time of infection, Wistar rats exhibit higher amount of parasites compared to Holtzman rats (Fig. 1A). The highest peak of circulating parasites for both rat models occurred at day 10 after infection (Fig. 1A), as previously described for Holtzman rats (Fabrino, *et al.*, 2004). Parasites were completely suppressed as early as 15-16 days after infection for respectively Wistar and Holtzman rats. Approximately 90-95% of both Holzman and Wistar rats survived beyond 90 days post-infection.

Heart parasitism was extensively studied at days 6, 12 and 20 of infection in both atrium and ventricle (Fig. 1B).To study heart histopathology, we used a histological approach that combines optimal fixation and processing with a plastic resin (glycol methacrylate) embedding. This allows increased tissue resolution and enables optimal parasite visualization and quantification (Fabrino, *et al*, 2004, Melo*, et al.*, 2003). Multiplying forms of the parasite (amastigotes) within cadiomyocytes were carefully

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examined and quantified by two different methods (number of nests/animal and volumetric proportion of nests/animal) in semi-serial sections.

Although both Wistar and Holtzman rats had comparable blood parasite levels, the 2 rat models showed a dramatic difference concerning the ability of the parasite to infect the heart. At day 6, the number of parasites was significantly higher in Holtzman compared to Wistar rats (mean ± SEM of 112.0 ± 28.6 and 4.0 ± 2.0 per animal for Holtzman and Wistar, respectively, $P < 0.05$) (Fig. 1B). The highest number of parasite nests was observed at day 12 of infection in both rat models. However, Holtzman rats had 7.5-fold higher amount of nests than Wistar rats at this time (mean ± SEM of 1987.8 ± 570.7 and 264.7 ± 151.5 per animal for respectively Holtzman and Wistar, $P < 0.05$) (Fig. 1B). Because the size of the amastigote nests apparently differed between the rat two models at day 12 of infection, we next evaluated the volumetric proportion occupied by these nests in the heart. Amastigote nests represented 2.2% of the heart in Holtzman rats while in Wistar rats this proportion was only 0.56%. Heart parasitism decreased significantly at day 20 in both rats, compared to day 12. This decrease was dramatically observed in Holtzman rats. At day 20 of infection, the number of parasite nests did not differ significantly between Wistar and Holtzman rats (mean ± SEM of 3.25 ± 2.0 and 1.16 ± 2.0, respectively) (Fig. 1B).

Of note, our histological approach enabled us to identify not only amastigote nests, but also breaching nests in the exact moment of liberation of trypomastigote forms on the way to reach nearby cardiomyocytes (Figure 2 C and 2Ci).

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Myocarditis is more intense in Holtzman than in Wistar rats

The rupture of amastigote nests during the acute phase of Chagas' disease provokes cell destruction and a potent inflammatory process, especially in the heart (Ropert*, et al.*, 2002). Because a high number of parasites have been observed within cardiomyocytes, we next evaluated the occurrence of inflammatory processes in both rat models. In addition to qualitative analysis, inflammation was quantitatively evaluated.

The presence of a diffuse mononuclear myocarditis was always observed in the heart of both rats, at all infection times, but the inflammation intensity varied greatly during the acute phase. At day 6, the inflammatory process was discrete in both rat models, but became very intense at day 12 only in Holtzman rats (Figure 1C and 2C). The Wistar ones had mainly a moderate inflammatory process at this time (Figure 1D). When we quantitated the area on sections occupied by the inflammatory process, Holtzman rats showed a significant higher proportion of inflammatory cells compared to Wistar rats ($P < 0.05$) (Fig. 2B and C).

Scattered, large macrophage-like cells were frequently observed in the myocardium of the infected animals, often invading amastigote nests at days 6 and 12 post-infection, especially in the heart of Holtzman rats (Fig. 2Ci, asterisks). At day 20, the extent of myocarditis consistently dropped in both rats, being virtually absent. At this time, cardiomyocytes had a normal appearance and nests were rarely observed. Degenerating cardiomyocytes showing vacuolated cytoplasm and condensed nucleus were also observed in the heart associated with the presence of edema. These alterations were more pronounced in Holtzman rats (Fig. 2C).

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Because a high number of infiltrating macrophage-like cells were observed in the heart of infected Holtzman rats at light microscopy, we next evaluated the heart of these animals by TEM (Fig. 2D and 2E). Our EM analyzes confirmed the presence of a high number of inflammatory macrophages exhibiting many phagocytic vacuoles in the cytoplasm. Intact or degenerating amastigotes were frequently observed within phagolysosomes (Fig. 2E) as previously demonstrated by our group (Fabrino, *et al.*, 2004, Melo, 1999, Melo*, et al.*, 2003, Melo*, et al.*, 2006) .

Mobilization/recruitment of monocytic lineage cells is high in Holtzman rats and deficient in Wistar rats

Because a prominent influx of macrophages is observed into the heart during the acute infection, we next compared the recruitment of peripheral blood monocytes (PBM) between the 2 rat models during different time points of the acute infection.

Acute infection induced prominent mobilization of cells from the monocytic lineage in Holtzman rats as previously observed (Fabrino, *et al.*, 2004, Melo and Machado, 2001). PBM numbers showed a significant increase at all time points of infection when compared to values from uninfected controls. In addition, PBM values significantly differed during the acute phase. At day 6 post-infection, PBM numbers increased 170% relative to the control group. At day 12, the highest mobilization of PBM was observed, being detected an increase of 578% and 990% compared to day 6 and controls, respectively. At day 20 post-infection, PBM numbers decreased significantly (59%) compared to day 12, but remained significantly higher (135%) than the value

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found at day 6. In sharp contrast to Holtzman rats, Wistar rats did not show a significant increase of PBM in infected rats compared with uninfected controls. This means that the acute *T. cruzi* infection did not induce significant PBM mobilization in Wistar rats (Fig 2A). Interestingly, at day 20 of infection the numbers of PBM were not significantly different between Holtzman and Wistar rats (Fig. 2A). Of note, the control values of PBM as well as total number of leukocytes did not differ between Wistar and Holtzman rats.

Acute Infection does not induce Nitric Oxide Release by Peripheral Blood Monocytes and Splenic Macrophages in both rat models

NO production was evaluated in both rat models. The acute infection with *T. cruzi* did not induce significant release of NO, measured as NO^{-}_{2} by PBM at 6, 12 and 20 days post-infection in Holtzman rats. On the contrary, as previously described by us in the same experimental model (Fabrino, *et al.* 2004), the levels of NO were significantly reduced at days 6 and 20 when compared to the control group. Interestingly, the acute infection did not trigger NO release by PBM in Wistar rats as well, at any time of infection. In controls, there is no significant difference between the basal levels of NO in PBM from Holtzman and Wistar rats (Fig. 3 A).

Splenic macrophages from both rat models did not to produce NO in response to the acute infection. As also demonstrated for PBM, splenic macrophages from infected Holtzman rats had the NO levels significantly reduced in all time points analyzed when compared to control group.

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DISCUSSION

The inflammatory response that follows the infection with *T. cruzi* is essential for host resistance to infection. During the acute infection occurs a remarkable recruitment and activation of macrophages by parasite antigens, resulting in pro-inflammatory cytokine production, and consequent control of *T. cruzi* growth (Melo, 2008). In this work, host response to the acute *T. cruzi* infection was investigated by evaluating the course of infection in 2 strains of rats. The infection was monitored by parasitemia, heart histopathology and ultrastructure, recruitment of monocytic lineage cells and NO production by these cells.

We showed that Wistar and Holtzman rats present a similar pattern of parasitemia, but greatly differ regarding tissue colonization pattern and inflammatory reaction. Parasitism in the heart, the main target organ of the Chagas' disease, was carefully analyzed and quantitated by 2 different methodologies. Heart parasitism was dramatically higher in Holtzman rats during different times of the acute phase of infection (Fig. 1B). In addition, a consistent parasitism was detected earlier in this model as observed at day 6 of infection. By this time, quantitative studies of both atrium and ventricle in semi-serial sections found a total of 112.0 ± 28.6 per animal in Holtzman rats compared to only 4.0 ± 2.0 nests per animal in Wistar rats. The infection in Holtzman, but not in Wistar rats, also elicited a strong recruitment of peripheral blood monocytes (Fig. 2A) in parallel to the increase of the parasite numbers in the heart (Fig. 1B). These data show that Holtzman rats are more susceptible to the acute infection compared to Wistar rats, but are able to respond to parasite with an accentuated

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mobilization and recruitment of monocytes/macrophages. Variation between inbred mice of susceptibility to experimental *T. cruzi* infection has frequently been described (Andrade, *et al.* 1985, Goncalves da Costa, *et al.*, 2002, Graefe*, et al.*, 2006, Hideko Tatakihara, *et al.*, 2008) but to our knowledge, this is the first work showing that variation in rat models.

As the acute phase progresses, a reduction of myocardial parasite burdens occurred in both rat strains. Interestingly, in spite of being more susceptible to the early infection, Holtzman rats did not show a retarded decrease of parasitism in comparison with Wistar rats. In fact, our analyses demonstrated that by day 20 of infection there is no significant difference between the numbers of parasites in the heart of these 2 models (Fig. 1B). This means that Holtzman rats had an efficient control of the acute infection, likely through the involvement of the monocyte/macrophage system remarkably mobilized in these animals. Indeed, activated macrophages exert critical activities in immunity to parasites and as evidenced here by EM, both mobilization of PBM and increase of the heart parasitism were paralleled by a consistent influx of macrophages in the myocardium. Heart inflammatory macrophages exhibited a morphological pattern of activation and were involved in the parasite phagocytosis (Fig. 2E) as previously demonstrated (Melo, 1999, Melo and Machado, 2001, Melo, *et al.*, 2003).

The immune control of many intracellular pathogens, including *T. cruzi*, is reported to be dependent on the production of NO by activated macrophages (Petray, *et al.*1995, Rodrigues, *et al.*, 2000, Silva, *et al.*, 2003, Vespa, *et al.*, 1994). Here we have performed a detailed in vivo study on the production of NO by PBM and splenic

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macrophages during the acute infection. We show that production of NO is not the decisive factor determining resistance or susceptibility to *T. cruzi*. Our data demonstrating consistent parasite clearance in both blood and heart in the absence of NO production support a recent study with a low virulent strain of the parasite and iNOS-KO mice (Marinho, *et al.*, 2007). By analyzing parasitemia and tissue pathology, the authors have shown that NO is not essential for the in vivo control of the parasite (Marinho, *et al.*, 2007). Accordingly, iNOS-deficient mouse strains, infected with the virulent Brazil or Tulahuen strains of *T. cruzi* had parasitemia, tissue parasite load, and survival rates similar to wild-type mice (Cummings and Tarleton, 2004). Our results, using another virulent strain of *T. cruzi* (Y strain) and a different experimental model strongly indicate that NO is not of primary relevance to the resistance of acute *T. cruzi* infection. Interestingly, NO levels in infected Holtzman but not in Wistar rats were significantly lower than the basal level found in control non-infected animals, as also observed in other experimental models of Chagas' disease (DaMatta*, et al.*, 2000, de Oliveira, *et al.*, 1997, Seabra*, et al.*, 2002). This may be related to the ability of the parasites to activate arginase, which in turn leads to a depletion of L-arginine, the iNOS substrate, resulting in lower levels of NO and increased production of polyamines. These substances are involved in parasite growth and differentiation (Vincendeau*, et al.*, 2003). Therefore, we can speculate that the high parasite burden in Holtzman rats might be responsible for the in vivo NO down-regulation found in these models. In fact, a previous study from our group has provided evidence for the occurrence of this pathway (Fabrino, *et al.*, 2004). On the other hand, production of other parasite-antigen-induced cytokines including interferon-gamma (INF-γ), tumor necrosis factor (TNF-α),

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and granulocyte-macrophage colony-stimulating factor (GM-CSF) may help compensate for the absence of NO (Cummings and Tarleton, 2004, Olivares Fontt*, et al.*, 1996).

The use of an adequate experimental model, especially for in vivo studies, is still an issue for Chagas' disease investigations. A good experimental model should mimic the human infection which includes supporting a subclinical parasitemia; developing a consistent cellular and/or humoral response; displaying lesions in a relatively short period of time and surviving to the acute phase of the infection (Canto-Cavalheiro and Leon, 1999). The present work sheds light on the use of rat models for in vivo studies of the acute *T. cruzi* infection. In contrast to mice models, both rat models present, as general feature, a high survival rate during the acute disease. This aspect is important because the human disease runs unnoticed in most of the infected persons and less than 5% of the patients with symptoms die during the disease's acute phase (Coura, 2007, Teixeira*, et al.*, 2006). However, Holtzman rats proved to be a more reliable model for *T. cruzi* infection studies than Wistar rats, at least, during the acute disease. Although both Wistar and Holtzman are able to develop a marked parasitemia, Holtzman rats mimic myocarditis and heart parasitism characteristic of the acute human disease in parallel to a consistent involvement of the innate immune response. These animals also are able to develop nerve lesions (Machado*, et al.*, 1994, Melo and Machado, 1998) classically identified in patients with the acute disease (reviewed in (Moncayo and Ortiz Yanine, 2006). Because recruitment and activation of monocytic lineage cells are a rapid and remarkable phenomenon during the acute infection with *T. cruzi*, our data particularly highlight the use of Holtzman rats as useful models to study

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different aspects of the monocyte/macrophage system. A better understanding of the functional capabilities of these cells, including a better characterization of monocyte and macrophage subsets may be instrumental for the development of appropriate therapeutic interventions and Chagas' disease control.

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FIGURE LEGENDS

Fig 1: Parasitemia (A), heart parasitism (B) and light micrographs of myocardium in infected Holtzman (C) and Wistar (D) rats at different time-points of infection with *Trypanosoma cruzi***.** (A) Both rats present a prominent parasitemia, with similar patterns during the acute phase. (B) However, the two rat models show a dramatic difference in the number of heart parasites at days 6 and 12 of infection. The highest number of parasite nests is observed at day 12 of infection in both Holtzman and Wistar rats. (C, D) At this time, amastigote nests (circles) are frequently seen in the myocardium, mainly in Holtzman rats (C). Numbers of parasites in the blood are expressed as sums/day of 8 animals per group. Tissue parasite numbers are expressed as mean ± SEM and are representative of three independent experiments. Four to six rats per group. Scale Bars 40 µm.

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Fig 2: Number of peripheral blood monocytes (PBM) (A), and myocarditis (B-D) during the acute infection with *Trypanosoma cruzi***.** (A) In Holtzman rats, PBM increased ($P < 0.05$) at day 6 compared to control values. At day 12, these numbers dramatically increased ($P < 0.05$) in comparison to numbers found at day 6 (*) while at day 20, they decreased ($P < 0.05$) compared to day 12 (*). In Wistar rats, there was no significant difference between PBM numbers in control and infected groups. Holtzman rats showed significant increase of PBM compared to Wistar rats at day 6 and 12 (**). At day 20, the numbers of PBM did not differ between Holtzman and Wistar rats. (B) Inflammatory processes in the heart were more intense in Holtzman compared to Wistar rats. (C) Histophatological analyses show myocarditis predominantly mononuclear and difuse, dissociated myocardial fibres (My) and edematous intersticial tissue. The boxed area in C is shown in higher magnification in Ci. Note the presence of macrophage-like cells (yellow asterisks) and trypomastigote forms (arrows) close to a disrupted nest. In (D) and (E) electron micrographs show a normal (D) and infected (E) myocadium. TEM reveals a large number of inflammatory macrophages infiltrated in the myocardium (My). Degenerating parasite amastigote forms (blue asterisks) are seen within these cells. Samples were processed at day 12 of infection with *T. cruzi*. N, nucleus; V, blood vessel. Data are expressed as mean ± SEM and are representative of three independent experiments. Four to six rats per group. Scale bars, 10μM (C and Ci), 1.5 μM (D), 2μM (E).

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Fig 3: **Nitric oxide (NO) production by peripheral blood monocytes (PBM) (A) and splenic macrophages (B) in response to the acute experimental** *Trypanosoma cruzi* **infection in rats (B).** In both Wistar and Holtzman rats the infection did not elicit NO production during the acute phase. On the contrary, PBM and splenic macrophages from infected Holtzman rats had the NO levels significantly reduced (*$P< 0.05$) during the acute infection when compared to control group. Data are expressed as mean ± SEM and are representative of three independent experiments. NO generation was measured as accumulated supernatant nitrite by the Griess reaction (Green, *et al*., 1982). Four to six rats per group.

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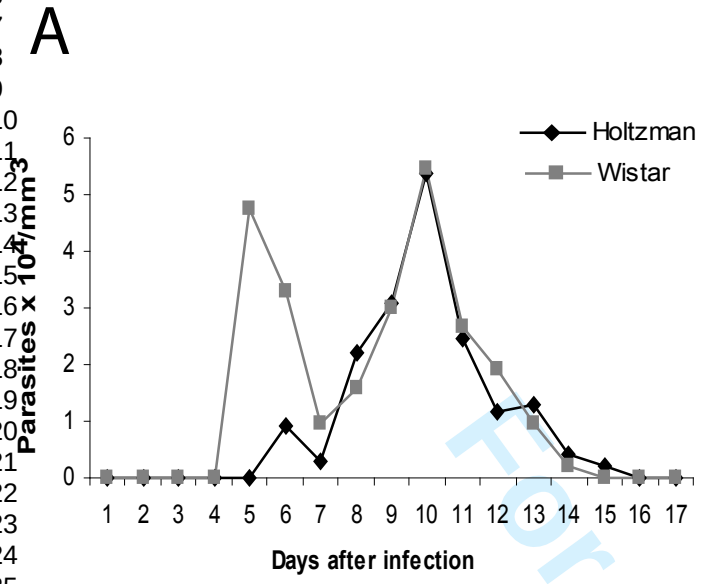
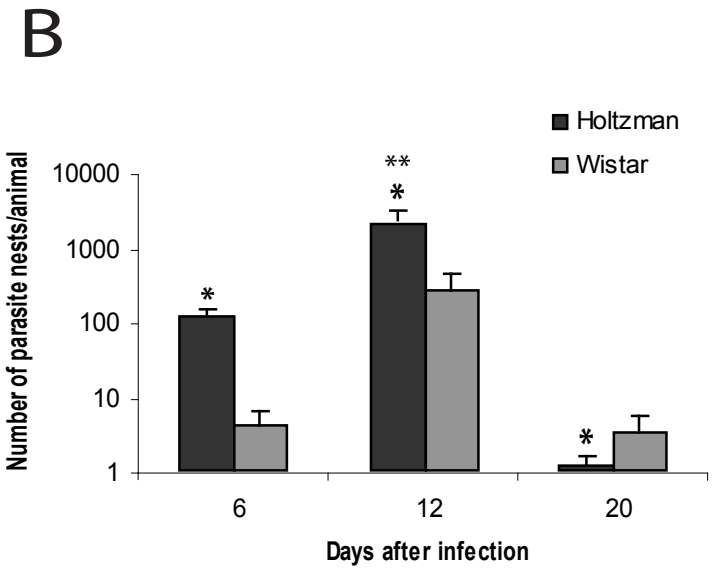
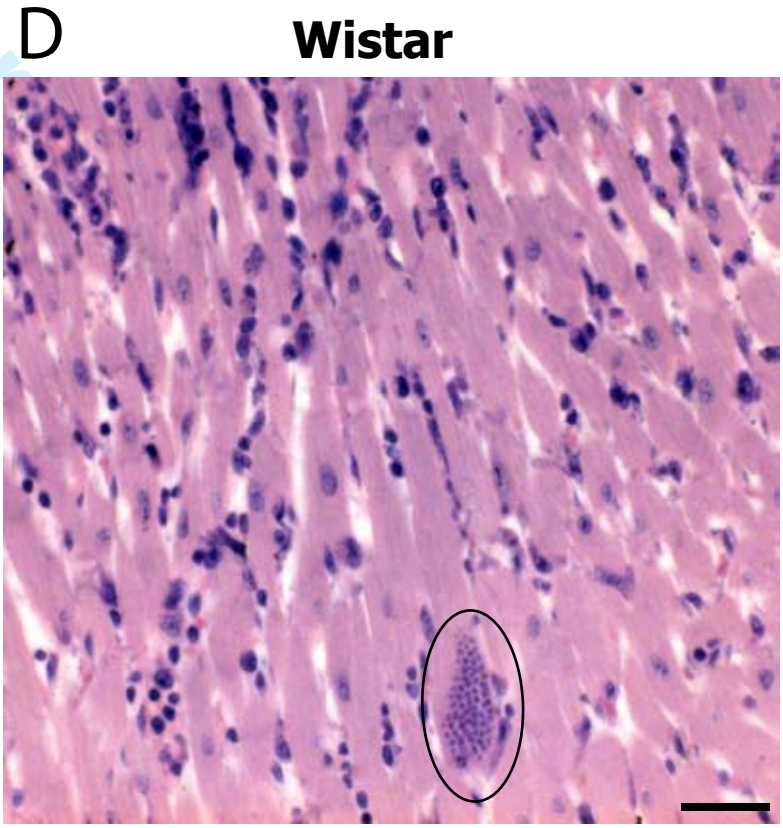

A
Holtzman
Wistar
Parasites x 10⁴/mm³
0
1
2
3
4
5
6
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Days after infection


B
Holtzman
Wistar
Number of parasite nests/animal
10000
1000
100
10
1
*
**
*
*
6
12
20
Days after infection

C
Holtzman


D
Wistar

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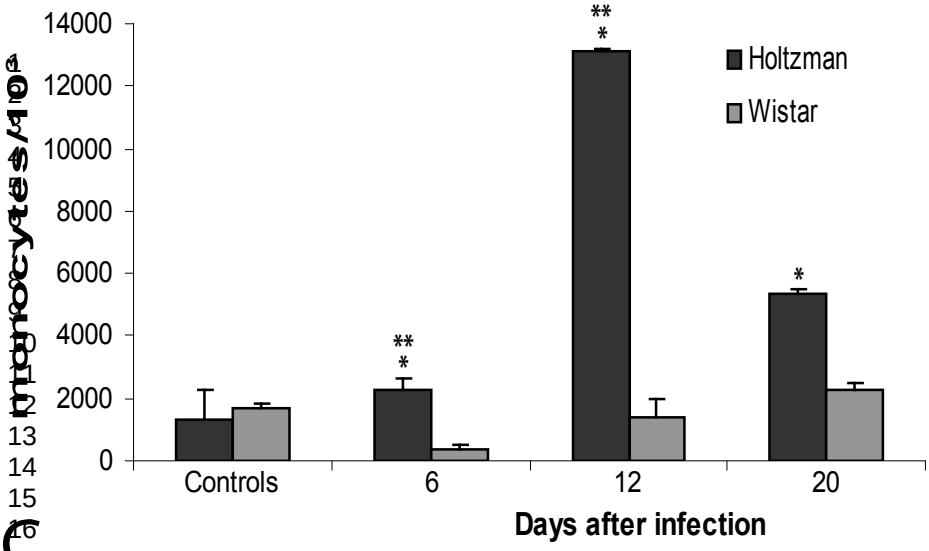
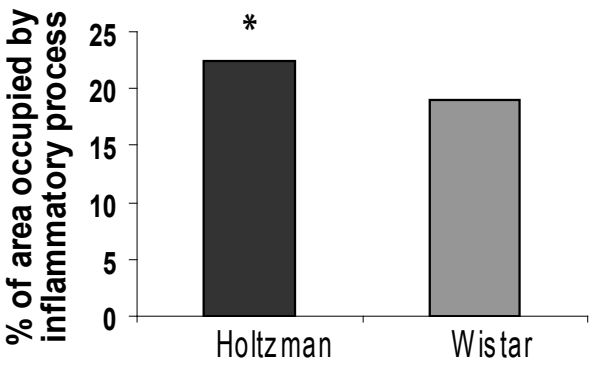
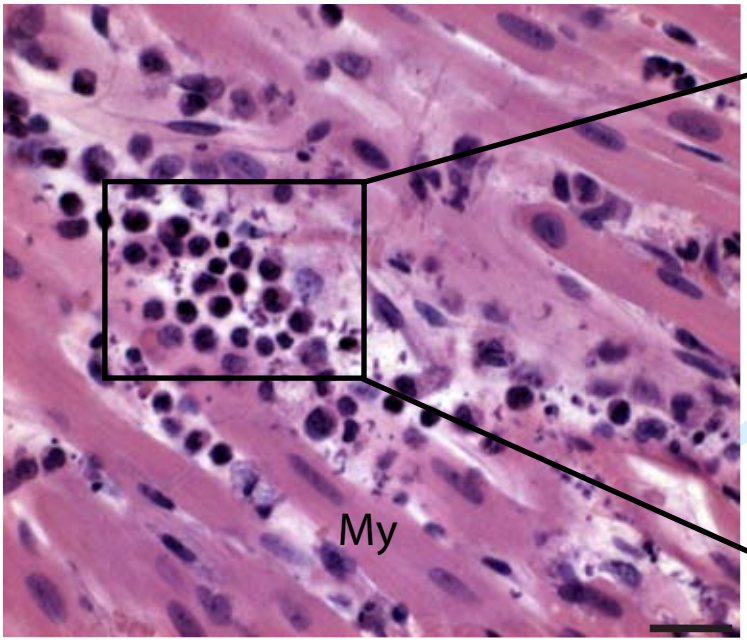
A


Mononucleocytes/µl
14000
12000
10000
8000
6000
4000
2000
0
Holtzman
Wistar
Controls
6
12
20
Days after infection

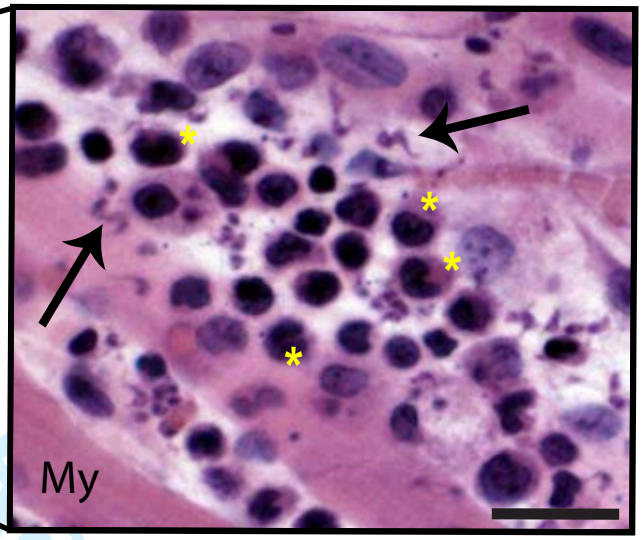
B


% of area occupied by inflammatory process
25
20
15
10
5
0
Holtzman
Wistar

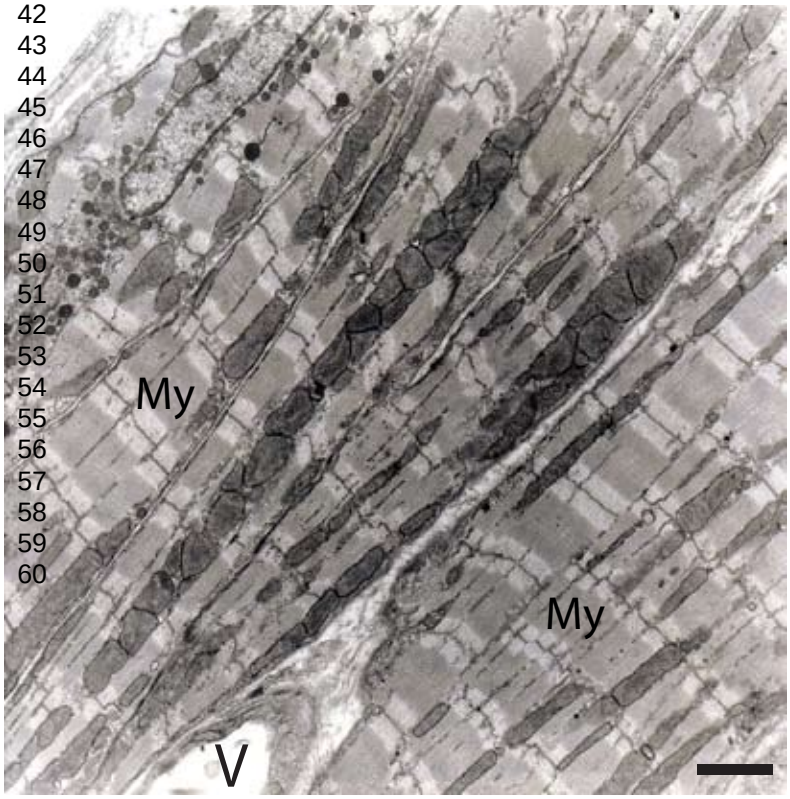
C


My

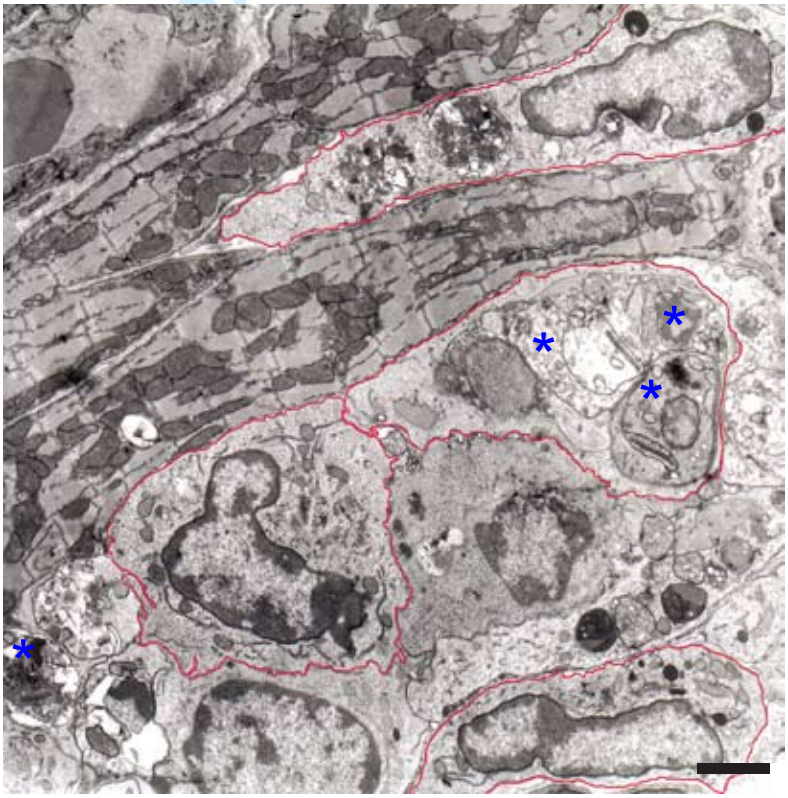
Ci


My

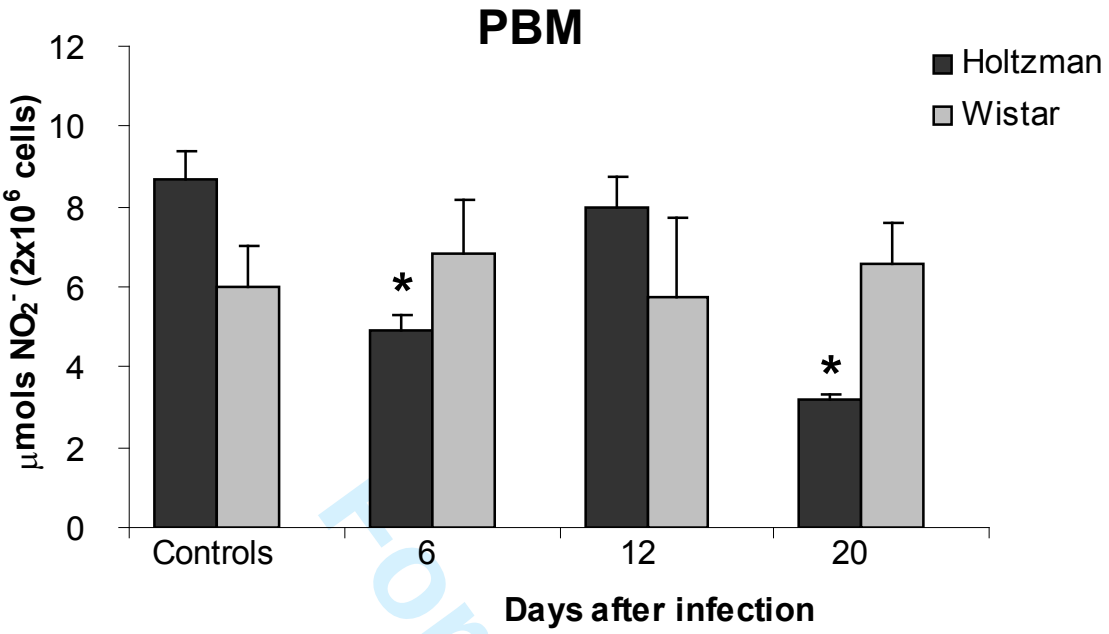
D


My
My
V

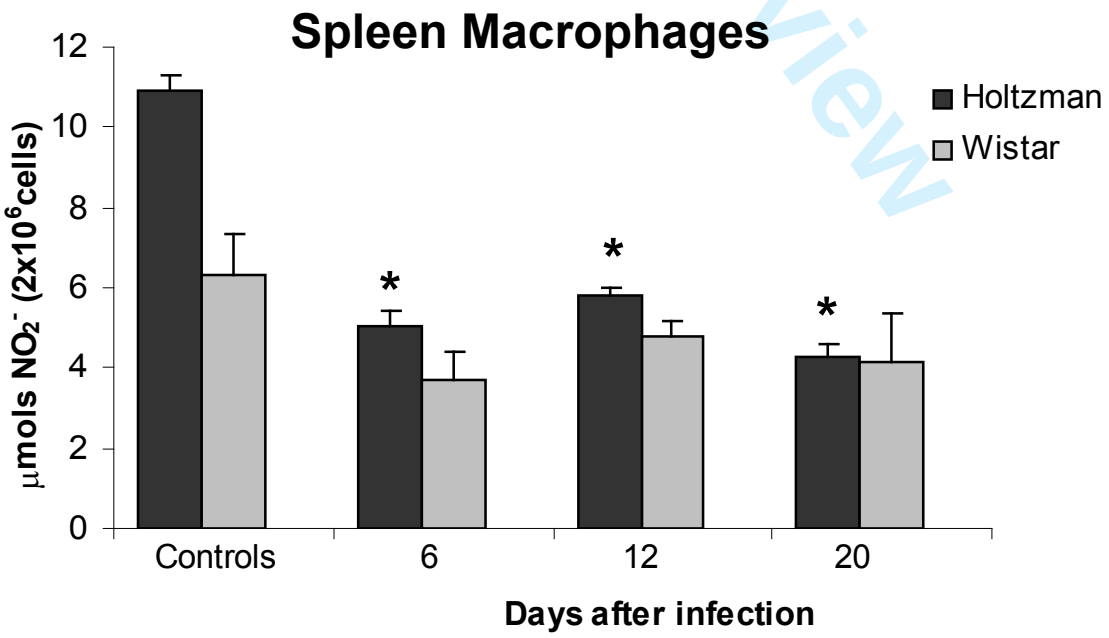
E



A



B



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3.1.2 ARTIGO 2

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Inflammation Research

Lipid bodies: structural markers of inflammatory macrophages in innate immunity

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Abstract. *Objective and design*: The correlation between innate immune responses and formation of cytoplasmic lipid bodies (LBs) was investigated *in vivo* in inflammatory macrophages from rats infected with *Trypanosoma cruzi*, the intracellular parasite which causes Chagas' disease.
Material and methods: We used an experimental model of high-dose irradiation prior to infection, which depletes the humoral and cellular immune responses except for the phagocytic activity of macrophages. Rats, irradiated or not, were infected with *T. cruzi* and macrophages from different origins (peritoneum, heart, uterus) were studied by transmission electron microscopy (TEM).
Results: As documented by quantitative TEM, innate immune responses induced prominent formation, structural changes and intracellular interactions of LBs. LBs significantly increased their size and changed their osmiophilia in response to both infection alone and when macrophages were challenged with irradiation-induced increased parasite load. Remarkably, a consistent LB-phagolysosome association was identified. LBs were surrounding, attached to or internalized by phagolysosomes.
Conclusions: We demonstrated that LBs are dynamic organelles notably involved in the host response to acute *T. cruzi* infection, an event that may be important for pathogen control during innate immunity. Our findings highlight LBs as structural markers of the innate immune responses in phagocytic cells.

Key words: Lipid bodies – Inflammation – Innate immunity – *Trypanosoma cruzi* – Electron microscopy – Macrophages – Phagocytosis – Chagas' disease

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Introduction

Innate immunity is the first line of defense to protect the host from invading microorganisms. Recruitment and activation of cells from the innate immune system has an important role in host resistance to early infection with the intracellular protozoan parasite *Trypanosoma cruzi*, the causative agent of Chagas' disease (reviewed in [1]). The acute infection, both in humans and experimental models, elicits cells from monocyte/macrophage lineage to produce high levels of cytokines and inflammatory mediators. The importance of these cells in the control of parasite replication has been pointed out by us and other groups during the *in vivo* acute infection [1–4]. At this time, we have demonstrated that a high mobilization of monocytes in the peripheral blood occurs in parallel to a prominent influx of macrophages into the heart, one of the main targets of the disease. In this organ, inflammatory macrophages are directly involved in inhibiting parasite multiplication [2] and are morphologically distinct from resident macrophages, exhibiting a patent increase in their organelle apparatus, nuclear changes and significant increase in size [3].

Another distinguishing feature of Chagas' disease-elicited macrophages is the presence of increased numbers of non-membrane bound cytoplasmic organelles termed lipid bodies (LBs) [5]. This increase was identified by us in both peritoneal and heart macrophages during different times of acute *T. cruzi* infection *in vivo* and it was positively correlated with augmented generation of prostaglandin E2 [5]. These findings indicated a role for LB in the heightened eicosanoid production observed during Chagas' disease [5]. In fact, LBs, which are present in limited numbers in the cytoplasm of most eukaryotic cells, have been recognized as cytoplasmic domains for synthesis of inflammatory mediators in leukocytes from natural and experimental inflammatory processes such as hypereosinophilic syndrome [6], acute respiratory distress syndrome [7] and Chron's disease [8]. A role for LB in eicosanoid production by macrophages and neutrophils

involved in innate immunity to bacterial infection has also been proposed by the demonstration that eicosanoid-forming enzymes, 5-lipooxygenase and cyclooxygenase-2, were immunolocalized within both experimentally induced (LPS in mice) or naturally occurring (septic patients) LBs [9]. In addition, our group has recently demonstrated that the *Mycobacterium bovis* BCG induces LB formation during the *in vivo* infection and that LBs were the predominant sites of prostaglandin E2 synthesis in activated macrophages [10].

The ultrastructure of LBs in cells involved in immune responses to infectious diseases is poorly characterized. The present study was, therefore, undertaken to investigate, by transmission electron microscopy (TEM), LB structure, formation and interactions within inflammatory macrophages actively involved in parasite phagocytosis during the acute Chagas' disease *in vivo*. Using quantitative TEM and an experimental model of high-dose irradiation prior to infection, which depletes the humoral and cellular immune responses except for the phagocytic activity of macrophages [11–13], leading to an dramatic increase of the parasitism [14, 15], we delineated new structural aspects of innate immunity-elicited LBs.

Materials and methods

Animals, Infection and Irradiation

Female Holtzman rats aged 27–30 days were irradiated with a single high dose (7 Gy) of gamma irradiation as described [14, 15]. The irradiation was performed prior to infection to avoid the increased radioresistance of the antigenically stimulated cells [11, 16]. Rats were inoculated intraperitoneally with a single inoculum of 3×10^5 trypomastigotes of *T. cruzi*, Y strain. Fresh blood samples taken from the tail showed living trypomastigotes in all infected animals. To monitor the irradiation-induced leukocyte depletion, blood analyses performed as previously described [15] at day 3 and 13 of irradiation showed a 95–97 % reduction of all kind of leukocytes, except monocytes (22 % reduction). Infected animals, irradiated or not, and non-infected controls (6 rats/group) were sacrificed in a CO_2 chamber at day 12 of infection.

Transmission Electron Microscopy (TEM)

Peritoneal cells and tissue fragments from heart and uterus were prepared for TEM. Peritoneal cells were obtained from peritoneal cavity washings with PBS (5 mL/animal). After centrifugation (80 g for 10 min at 4 °C), cells were washed in PBS and the cell viability was greater than 95 % as determined by the trypan blue test. Peritoneal cells in suspension and tissue fragments were fixed in phosphate-buffered 1 % paraformaldehyde and 1 % glutaraldehyde, 0.1 M, pH 7.3, overnight at 4 °C as before [5] and washed in the same buffer. Cell suspensions were centrifuged and the obtained pellets were embedded in molten 3 % agar (Merck, Darmstad, Germany) for further processing for TEM. After fixation, tissue fragments and agar pellets containing peritoneal cells were post-fixed in a mixture of 1 % phosphate-buffered osmium tetroxide and 1.5 % potassium ferrocyanide (final concentration) for 1 h prior to dehydration in graded ethanols and infiltration and embedding in a propylene oxide-Epon sequence (PolyBed 812, Polysciences, Warrington, PA, USA). After polymerization at 60 °C for 16 h, thin sections were cut using a diamond knife on an ultramicrotome (Sorvall MT2, Newton, MA, USA) and mounted on uncoated 200-mesh copper grids (Ted Pella, Redding, CA, USA) before staining with uranyl acetate and lead citrate and viewed with a transmission electron microscope (EM 10, Zeiss, Germany) at 60 KV.

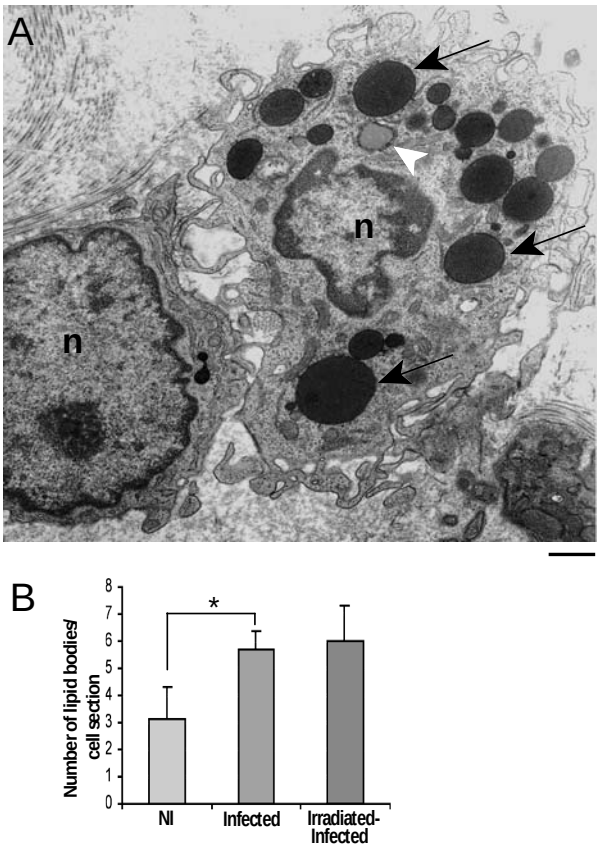

Fig. 1. Chagas' disease-induced lipid bodies (LBs) within inflammatory macrophages. (A) In an infected alone animal, a heart macrophage interacting with a lymphocyte shows several electron dense LBs (arrows) in the cytoplasm. A light dense LB is also indicated (arrowhead). (B) A significant increase of LB number is observed in response to infection alone (* $P < 0.05$). Irradiation followed by infection did not alter LB number. Over 100 electron micrographs taken from 3 different experiments were evaluated. Rats were irradiated or not with a high dose of gamma irradiation (7 Gy) and inoculated with Y strain of *Trypanosoma cruzi*. Samples from peritoneum and muscle tissues (heart and uterus) were processed by transmission electron microscopy at day 12 of infection. **n**, nucleus. **NI**, not infected. Scale bar, 0.9 µm.

EM quantitative analyses

Quantitative analyses were performed on electron micrographs randomly taken from macrophages and studied at final magnification of 20,000x. The following data were quantitated: i) numbers of LBs in non-infected and infected (irradiated or not) groups; ii) diameters of enumerated lipid bodies; iii) osmiophilia variation of LBs in each group; iv) numbers of phagolysosomes in infected groups; vi) numbers of LBs near or in contact with phagolysosomes. A LB was considered close to a phagolysosome when a mean distance of 0.5 µm or less was defined between individual LBs and phagolysosomes. A total of 105 electron micrographs were studied and a total of 611 LBs were counted and measured. Data from different groups were compared by the Mann Whitney "U" test ($P < 0.05$) and are presented as mean ±SEM.

Histological methods

Heart fragments were fixed in 25 % Bouin's fluid for 20 h, post-fixed in 10 % formalin for 4 h and embedded in Paraplast (Oxford®, Saint Louis,

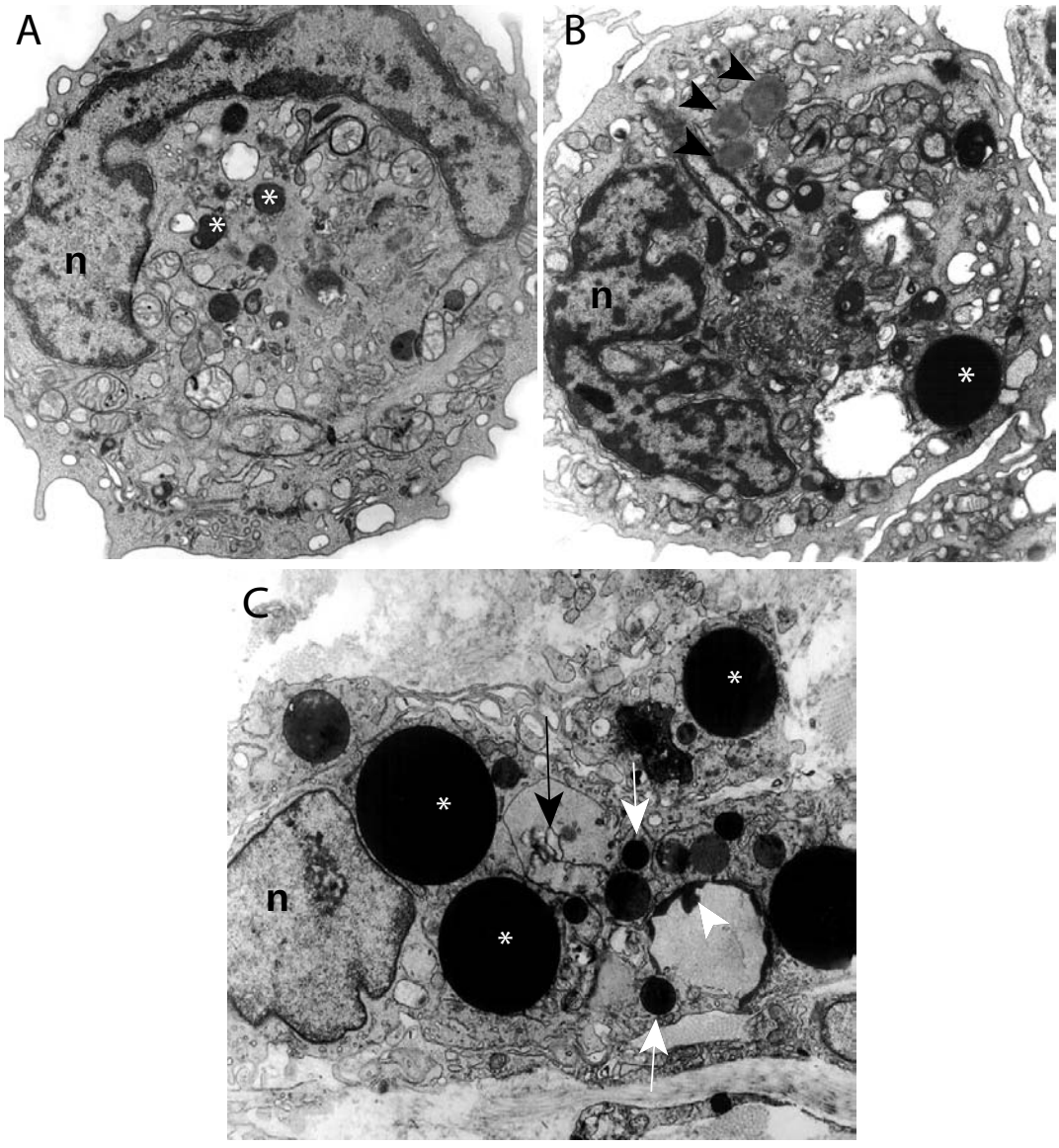

Fig. 2. Ultrastructural changes of lipid bodies (LBs) in response to infection. (A, B) LBs are observed in macrophages from a non-infected (A) and infected alone (B) animal. Small electron dense LBs are indicated in A (*). In B, a morphologically activated, organelle-rich macrophage exhibits a large, strongly electron-dense (*) and a cluster of light dense (arrowheads) lipid bodies. (C) Cytoplasmic, structurally diverse LBs within a macrophage from an infected-irradiated animal. In addition to giant strongly electron-dense (*) and small electron-dense (white arrows) LBs, a large lipid body showing an electron lucent center and an irregular and electron dense peripheral area is indicated (white arrowhead). The black arrow points to membranous structures within a light dense LB. Rats were irradiated or not and infected as in Figure 1. Samples from peritoneum (A, B) and uterus (C) were processed by transmission electron microscopy at day 12 of infection with *Trypanosoma cruzi*. Scale bar, 570 nm (A), 870 nm (B), 1.1 µm (C).

MO, USA) as previously described [15]. Semi-serial 7-µm-thick sections were stained with haematoxylin and eosin for a qualitative evaluation of inflammatory processes and quantification of myocardial parasitism. The number of amastigote nests in cardiomyocytes was evaluated at 70-µm intervals to avoid parasite recounting [17]. For each group (3 rats/group) were analyzed at least 1,400 fields (25 sections per animal) at a magnification of 400x.

Results

Lipid bodies within macrophages increase in size in response to increased parasite load

Exposure of rats to a single, high dose of gamma irradiation (7 Gy) 1 day before infection with *T. cruzi* induces dramatic increase of parasite burden [14, 15, 18]. We used TEM to delineate ultrastructural aspects of Chagas disease-elicited

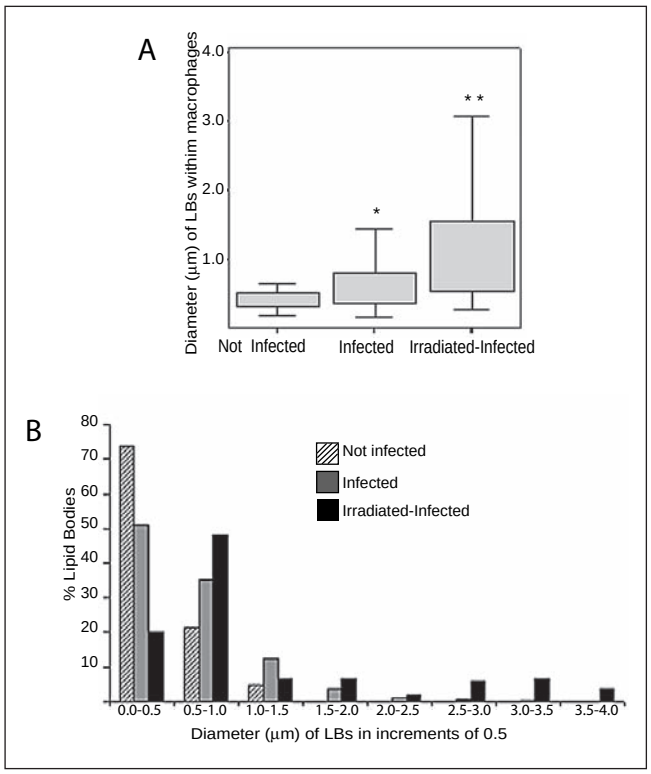

Fig. 3. Lipid bodies (LBs) increase in size in response to acute *Trypanosoma cruzi* infection and parasite load. (A) A significant increase of LB diameters occurred within macrophages in infected alone compared to non-infected (*$P < 0.05$) and in irradiated-infected compared to infected alone groups (**$P < 0.05$). Extreme values are not shown. (B) LB diameter variation in different groups. Irradiation induced-increased parasite load triggered formation of giant lipid bodies up to 4 μm. Evaluations were derived from 3 experiments. A total of 105 electron micrographs were studied and a total of 611 LBs were counted and measured. Rats were irradiated and infected as in Figure 1 and samples prepared at day 12 of infection.

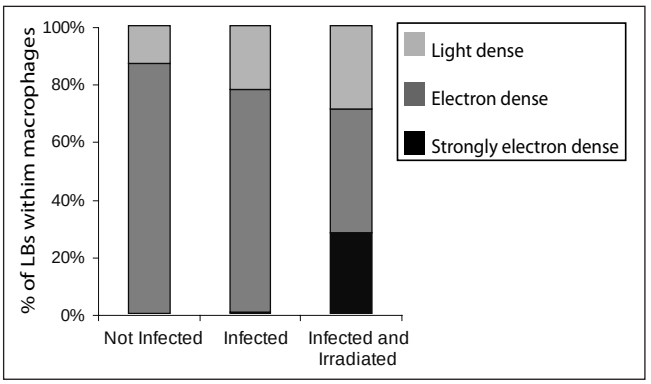

Fig. 4. Lipid bodies (LBs) change their osmiophilia within parasite-challenged macrophages. The number of light dense LBs increased in response to infection alone and when macrophages were challenged with higher parasite load. Strongly electron-dense LBs were prominently formed in response to higher parasitism. Evaluations were as in Figure 3. Rats were irradiated and infected as in Figure 1 and samples prepared at day 12 of infection with *Trypanosoma cruzi*.

LBs within radioresistant macrophages challenged *in vivo* with high parasitic load. Samples from peritoneum and muscle tissues from heart and uterus containing inflammatory macrophages were processed as before [19] for optimal membrane visualization with the reduced osmium potassium ferrocyanide method [20] so as to distinguish LBs from membranous organelles.

In non-infected and infected groups, macrophages from different origins showed LBs as distinct non-membrane bound cytoplasmic sites with different sizes. The infection alone induced a significant increase in number (Fig. 1A, B) and size (Fig. 2B) of LBs compared to non-infected controls (Fig. 2A). In parallel to these LB alterations, inflammatory macrophages showed other morphological features of activation such as increased number of vesicles and cell surface projections and a prominent Golgi complex (compare Fig. 2A with Fig. 2B). When macrophages were challenged with increased parasite load in irradiated-infected animals, LB numbers did not change compared to those induced by infection alone (Fig. 1B). However, there was a remarkable increase in LB sizes (Figs. 2C and 3A). For example, in scoring the diameters of LBs, in macrophages from non-infected animals, 74 % of LB had size lower than 0.5 μm whereas 54 % in infected and 80 % in irradiated-infected animals were greater than 0.5 μm (Fig. 3B). In addition, the irradiation followed by infection elicited 18.3 % of LBs with sizes greater than 2 μm whereas the infection alone triggered 2.4 % (Fig. 3B). Moreover, the occurrence of higher parasitism induced formation of gigantic LBs, with sizes up to 4 μm (Figs. 2C and 3B).

Infection-induced lipid bodies change in activated macrophages

LBs within macrophages showed variable osmiophilia, being classified as light, electron or strongly electron dense organelles. These 3 types of LBs were quantitated by TEM in macrophages from infected and non-infected groups. The infection alone induced a significant increase in the numbers of light dense LBs (Figs. 1A and 2B, arrowheads) compared to non-infected controls that showed LBs preferentially as electron dense organelles (Fig. 2A, asterisks). LBs changed consistently their osmiophilia in macrophages stimulated with higher parasite load in irradiated-infected animals compared to infection alone (Figs. 2C; 4 and 5B, C). There was a significant increase of both light dense and strongly electron dense LB numbers in response to increased parasitism and phagocytosis (Fig. 4). Moreover, LBs in activated macrophages were imaged as heterogeneous organelles. They displayed lucent areas, granular and/or membranous internal structures (Fig. 2C, black arrow) or showed surfaces with an irregular or thin intermittent electron dense line (Fig. 2C, arrowhead). Images suggesting budding (Fig. 5A, arrowhead), coalescence or fusion of LBs (Fig. 2B, arrowheads) were identified in inflammatory macrophages from both infected alone and infected-irradiated animals. In addition, Chagas' disease-induced LBs were also imaged as non-round, irregular structures (Fig. 5A, double asterisks).

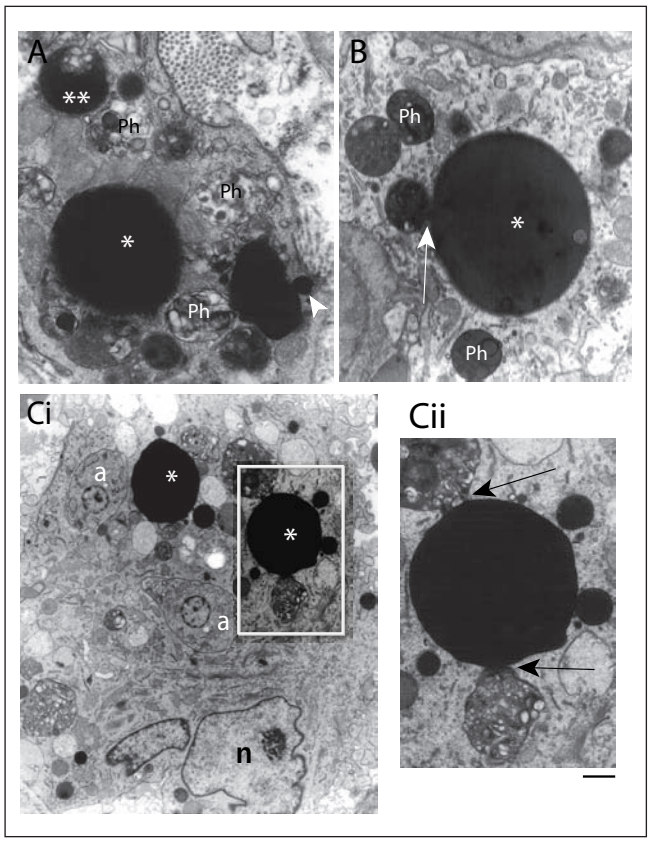

Fig. 5. Macrophage lipid bodies (LBs) interact with phagolysosomes. (A, B, C) Large, strongly electron-dense LBs (*) from an infected alone (A) and irradiated-infected (B, C) animals were formed in cytoplasmic areas showing many phagolysosomes (Ph). In A, an irregular LB (**) can also be visualized. A close association between phagolysosomes and LBs is indicated by arrows in B and Cii. Cii corresponds to the boxed area in Ci. Amastigotes (a), the intracellular form of the parasite, are seen within an activated macrophage (Ci). Rats were irradiated or not and inoculated with *Trypanosoma cruzi* as in Figure 1. Samples from peritoneum and muscle tissues (heart and uterus) were processed for macrophage evaluation by transmission electron microscopy at day 12 of infection. **n**, nucleus. Scale bar, 350 nm (A), 600 nm (B), 1.25 µm (Ci), 700 nm (Cii).

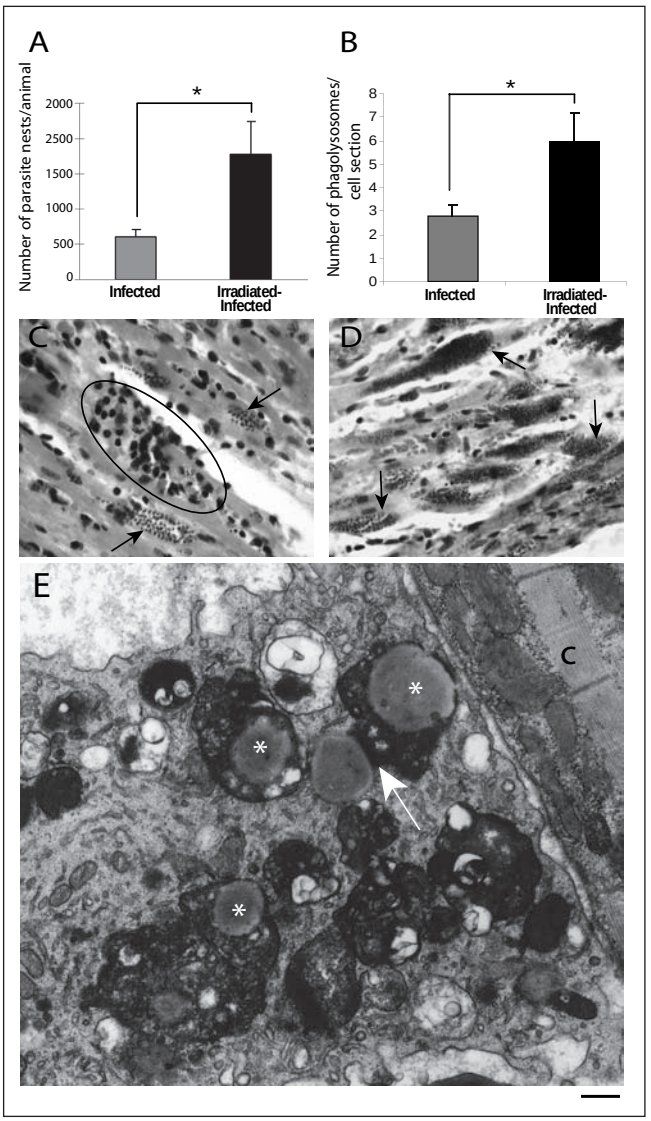

Fig. 6. Lipid bodies (LBs) are internalized by phagolysosomes. The number of heart parasites (A) and phagolysosomes within macrophages (B) significantly increased in irradiated-infected compared to infected alone groups (*$P < 0.05$). In C and D, myocardial amastigote nests (arrows) are indicated in infected alone and irradiated-infected animals, respectively. An area showing an inflammatory process characterized by predominance of mononuclear cells was outlined in C. (E) In a heart macrophage, LBs are seen within (*) or in contact (arrow) to phagolysosomes containing degenerating material. Note that the LB contacting a phagolysosome (arrow) exhibits the same osmiophilia (light dense) than internalized LBs (*). Samples from peritoneum and muscle tissues (heart and uterus) were processed for macrophage evaluation by light or transmission electron microscopy at day 12 of infection. **n**, nucleus; **c**, cardiomyocyte. Scale bar, 15 µm (C, D); 600 nm (E).

Lipid bodies interact with phagolysosomes

Macrophages from both infected and irradiated-infected animals showed a large number of cytoplasmic phagolysosomes (Ph) with degenerating material (Fig. 5A–C), including amastigotes, the intracellular form of the parasite. Our present histo-quantitative analyses showed that the numbers of myocardial parasites significantly increased in irradiated-infected animals compared to the infected group alone (Fig. 6A, C, D) as previously documented (14). Accordingly, TEM revealed that when macrophages were challenged with higher parasite load in irradiated-infected animals, the number of phagolysosomes significantly increased in the cytoplasm, an increase of 100 % [mean ±SEM of 5.9 ±1.3 per section for infected-irradiated versus 2.8 ± 0.5 for infected alone, n = 27 cells, $P < 0.05$] (Fig. 6B). Remarkably, large and strongly electron dense LBs frequently formed in cytoplasmic areas where phagolysosomes were concentrated (Fig. 5B, Ci, Cii).

A clear LB-phagolysosome association was identified. Quantitative TEM revealed that 47 % of LB in infected (Fig. 5A) and 39 % in irradiated-infected (Fig. 5B, C) rats were near or in contact with phagolysosomes. Notably, LBs were also present within phagolysosomes (Fig. 6B, asterisks). These LBs were preferentially light dense. Our results strongly suggest that LBs are able to interact with phagolysosomes during the *in vivo* acute disease.

Discussion

Macrophages exert critical activities in immunity to parasites. These cells play a pivotal role in the mechanism for halting the acute phase of Chagas' disease, one of the most prevalent infectious diseases in Central and South America [21]. We have previously reported that the *in vivo T. cruzi* infection in rats induces formation of LBs in activated macrophages [5]. We have now evaluated the ultrastructure of LBs formed within macrophages from different origins (peritoneum, heart and uterus) and exposed to variable parasite load *in vivo*. In addition, we have utilized quantitative analyses to delineate LB changes and intracellular interactions. The study of LBs by TEM is facilitated by the fact that these organelles constitute distinct cytoplasmic sites lacking a true delimiting membrane and, therefore, do not require a specific marker for their identification.

Inflammatory macrophages were studied at day 12 of infection with *T. cruzi* in rats. At this time point of infection is observed the most intense inflammatory process and parasitism in target organs of the disease compared to other points during the acute phase [2, 3]. In addition, different studies from our group have demonstrated a remarkable mobilization, migration and tissue infiltration of monocytes during this time [2–5]. Within inflammatory macrophages, we confirmed an increase in both LB number and size in infected compared to non-infected animals and further associated LB dynamics with macrophage response to higher parasite load induced by irradiation. Increased parasitism elicited gross alterations in newly formed LBs. Notably, while there was no increase in LB numbers compared with animals infected alone, a significant increase in size occurred in LB within inflammatory macrophages from irradiated-infected animals (Fig. 3). This structural change may represent an increased synthesis of inflammatory mediators within LBs. Indeed, LBs are recognized sites for localization of esterified arachidonic acid, the precursor for the synthesis of eicosanoids [6, 22] and eicosanoid-forming enzymes such as cyclooxygenase [23–25] and lipoxygenase [23, 26]. In addition, specific immunolocalization of newly formed leukotriene C4 demonstrated that LBs are sites of formation of this eicosanoid within infiltrating eosinophils during allergic inflammation [27].

Our results reveal that not only the number, but also the size of LBs represents an important structural indicative of the participation of these organelles in innate immune responses. Indeed, gigantic LBs occupied a large cytoplasmic area within inflammatory macrophages from irradiated-infected animals (Figs. 2C and 5C). These radioresistant macrophages were actively involved in phagocytosis, a crucial process in innate responses [28], showing an increased number of phagolysosomes compared to animals infected alone (Fig. 6B).

In general, LBs are intracellular distinct pools assembled as a neutral lipid core surrounded by a monolayer of polar lipids with a small amount of proteins embedded [29]. The differential fatty acid composition at the LB periphery may be visualized by TEM as an electron dense ring (Fig. 2). Within leukocytes, TEM reveals LBs as organelles with variable electron densities depending on the cell type [30]. In innate immunity-elicited inflammatory macrophages, our group has documented that the LB osmiophilia can consistently change depending on the time of infection with *Mycobacterium bovis BCG* [10]. During the acute infection with the parasite *T. cruzi*, newly formed LBs within macrophages can also exhibit distinct densities even in the same cell [5]. This under-appreciated, but important aspect of LB was evaluated in more detail in the present work. We wondered if the LB osmiophilia could represent a significant innate immune response-associated structural change of LBs. We demonstrated, for the first time by quantitative TEM, that the acute infection of *T. cruzi* induced a significant alteration of LB density. Moreover, when inflammatory macrophages were challenged with higher parasite load in irradiated-infected animals, LBs significantly changed their osmiophilia compared to LBs induced by the infection alone (Fig. 6). These LB alterations may be associated with different lipid composition, stages of formation of new LBs or fluctuations/mobilization of their arachidonate pools. Indeed, increases in LB size and number are accompanied by accumulation of neutral lipids in the hydrophobic core of these organelles [7]. Our results showing a variable osmiophilia within LBs highlight them as dynamic and not inert organelles, able to consistently change in concert with cell activation. In addition, we identify this morphological aspect of LB as an indicative of the cellular participation in inflammatory responses during innate immunity.

While the above findings provide evidence for LBs as a structural marker of inflammatory macrophages during innate immunity, mechanisms involved in pathogen control may also be active within LBs with the demonstration of a structural interaction between LBs and phagolysosomes. Our present results demonstrate that the acute infection triggers not only formation of LB but also a consistent distribution of these organelles close to phagolysosomes (Fig. 5). In addition, the number of phagolysosomes significantly increased in irradiated-infected animals. This demonstrates a high phagocytic activity of radioresistant inflammatory macrophages. Interestingly, *in vitro* stimulation of peritoneal macrophage phagocytosis with zymosan induced the same distribution of LBs [31]. Although these authors have suggested a possible discharge of LB contents into phagosomes, the occurrence of a LB-phagosome association and its functional role received little attention in the past. Recently, using high resolution, nonresonant confocal Raman microscopy, it was demonstrated that LBs rich in arachidonate associate with latex bead-containing phagosomes in neutrophilic granulocytes and an accumulation of LBs near ingested latex beads was also shown in macrophages by fluorescence microscopy [32]. Our EM quantitative studies show now a consistent interaction between LBs and phagolysosomes in inflammatory macrophages triggered in response to the *in vivo* infection with the parasite *T. cruzi*. Moreover, our data demonstrate that such interaction can result in LB internalization within phagosomes (Fig. 6B), a cellular event not discussed heretofore. Interestingly, a recent work from our group has also documented a LB-phagosome interaction during the *in vivo* infection with *Mycobacterium bovis* BCG in mice [10].

It has been demonstrated that arachidonic acid has important functions in the induction of apoptosis of mycobacterium-infected macrophages, and in actin recruitment to the phagosome, enabling phagosome maturation and increased mycobacterial killing [33, 34]. The LB-phagosome association as well as the internalization of LB by phagosomes

during the acute phase of *T. cruzi* infection may, therefore, represent different stages of phagosome maturation leading to killing of the parasite.

In summary, we have shown that LBs are dynamic organelles notably involved in the host response during the *in vivo* acute *T. cruzi* infection. Altogether, our results highlight LBs as structural markers of the innate immune response in phagocytic cells.

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3.2. CAPÍTULO 2

Estudo da via de produção de NO pelo protozoário *Trypanosoma cruzi*

O *Trypanosoma cruzi* é um parasito intracelular obrigatório, pertencente à ordem Kinetoplastidae, família Tripanosomatidae. Outros protozoários como *Leishmania sp.*, e outros tripanosomas também pertencem a esta mesma ordem. Embora todos esses protozoários sejam capazes de causar diferentes patogenias humanas, eles apresentam organização genômica e estrutural muito semelhante, e todos sofrem grandes transformações morfológicas durante seus ciclos de vida (Stuart e cols., 2008).

Muito da biologia destes protozoários (Kinetoplastidae) é similar. Todos eles apresentam um único flagelo e uma única e grande mitocôndria, a qual possui uma estrutura composta por milhares de pequenos e grandes DNAs circulares (essa região é denominada kinetoplasto). Outra característica desses protozoários é a presença de uma organela singular denominada glicossomo (um peroxissomo adaptado a realizar glicólise), além de uma densa rede de microtúbulos sub-peliculares (Donelson e cols. 1999, McConville e cols. 2002, Stuart e cols. 2008).

Tendo em vista que 6000 dos mais de 8000 genes desses protozoários são ortólogos, e que os kinetoplastidas estão entre os mais antigos eucariotos, é natural que eles apresentem várias vias bioquímicas semelhantes e é muito provável que eles compartilhem a semelhança de algumas vias com seus hospedeiros (Donelson e cols., 1999; Rivero, 2006; Stuart e cols., 2008).

Em se tratando da via metabólica do NO, tal hipótese já está sendo bem documentada na literatura. Como abordado na introdução desta tese, diferentes tipos celulares expressam ao menos duas vias diferentes de produção desta molécula, uma via induzida – com a participação da iNOS, e outra constitutiva – com a participação das cNOS. Cada via tem uma função metabólica, sendo que a via da cNOS está mais relacionada a processos homeostáticos e a via da iNOS está mais relacionada com mecanismos imunológicos (Alderton e cols., 2001). Entretanto, dependendo do patógeno em questão, nem sempre a ativação da via do NO ocorre, ou quando ocorre, ela

pode ser modulada por diferentes mecanismos induzidos ou protagonizados pelo patógeno como o consumo do substrato usado pela NOS ou liberação de alguma molécula antagonista (Brunet, 2001). O *T. cruzi,* por exemplo, age sobre a via metabólica da L-arginina, um aminoácido que tem posição crucial em duas vias metabólicas antagônicas na célula hospedeira (Peluffo e cols. 2004, Piacenza e cols. 2002, Vincendeau e cols. 2003).

Em protozoários, a primeira demonstração da via do NO em um eucariota unicelular foi feita em *T. cruzi* por Paveto e colaboradores em 1995 (Paveto e cols., 1995). Estes autores isolaram uma NOS específica de *T. cruzi* (TcNOS) e sua atividade foi descrita como muito semelhante as cNOS de mamíferos, incluindo sua Ca^{2+}-calmodulina dependência. No mesmo trabalho, foi sugerido que o *T. cruzi* possa ter receptores NMDA, especiais para o glutamato e que tais receptores seriam as principais unidades controladoras dos níveis citoplasmáticos de Ca^{2+} neste protozoário. Além disso, foi sugerido que a via do NO no *T. cruzi* seja controlada por esses receptores uma vez que sua estimulação leva ao aumento intracelular de guanosina monofosfato cíclico (GMPc) em formas epimastigotas do parasito (Paveto e cols., 1995). Entretanto, desde essa primeira descrição, ainda pouco foi estudado em relação à via do NO neste parasito.

Na tentativa de elucidar o significado biológico da ocorrência desta via em *T. cruzi*, os autores indicaram que a estimulação da atividade de GCs poderia ser responsável pelo aumento na motilidade de formas epimastigotas do parasito, (Johansson & Carlberg, 1995; Pereira e cols., 1997).

Outra possível função da via do NO em *T. cruzi* seria seu papel citoprotetor contra processos de apoptose neste protozoário. Usando soro fetal humano (10%) para indução de apoptose massiva em culturas de formas epimastigotas de *T. cruzi,* Pianceza e colaboradores em 2001 observaram que, quando incubados com L-arginina, substrato para vias de produção de NO e poliaminas, não foi observada a fragmentação de DNA e subseqüente incubação com L-NAME, um inibidor da NOS, levou a intensa fragmentação de DNA nestes parasitos, sugerindo o papel citoprotetor do NO. Por outro lado, a doação externa, por meio de incubação com doadores químicos de NO foi eficiente na recuperação dos níveis basais de replicação do *T.cruzi* (Piacenza e cols. 2001, Piacenza e cols. 2002).

Sabe-se que, em *T. cruzi*, a L-arginina pode atuar como precursor para a síntese de NO, fosfoargininas e possivelmente poliaminas. Entretanto, não há evidências para a síntese endógena de L-arginina neste protozoário, e sugere-se que ele adquira este aminoácido através de um transportador independente de alta afinidade por sódio (Na^+) (Pereira e cols., 1999). Deste modo, dados da literatura apontam para a possibilidade do parasito adquirir L-arginina da célula hospedeira, diminuindo a disponibilidade de substrato para a iNOS da célula hospedeira, o que levaria a uma diminuição dos níveis de NO produzidos por esta via. Como resultado, o parasito não só diminuiria os níveis nocivos de NO produzidos pela célula hospedeira, como se beneficiaria com o substrato (L-arginina) para produzir seu próprio NO, que poderia atuar não só como um modulador de morte celular (por apoptose) no parasito,, mas também como um gerador de GMPc (Peluffo e cols. 2004). Além disso, a produção de NO pelo *T. cruzi* pode estar associada também com sua capacidade de infecção e manutenção da multiplicação intracelular na célula hospedeira (Peluffo e cols. 2004). **(Figura 2.1)**

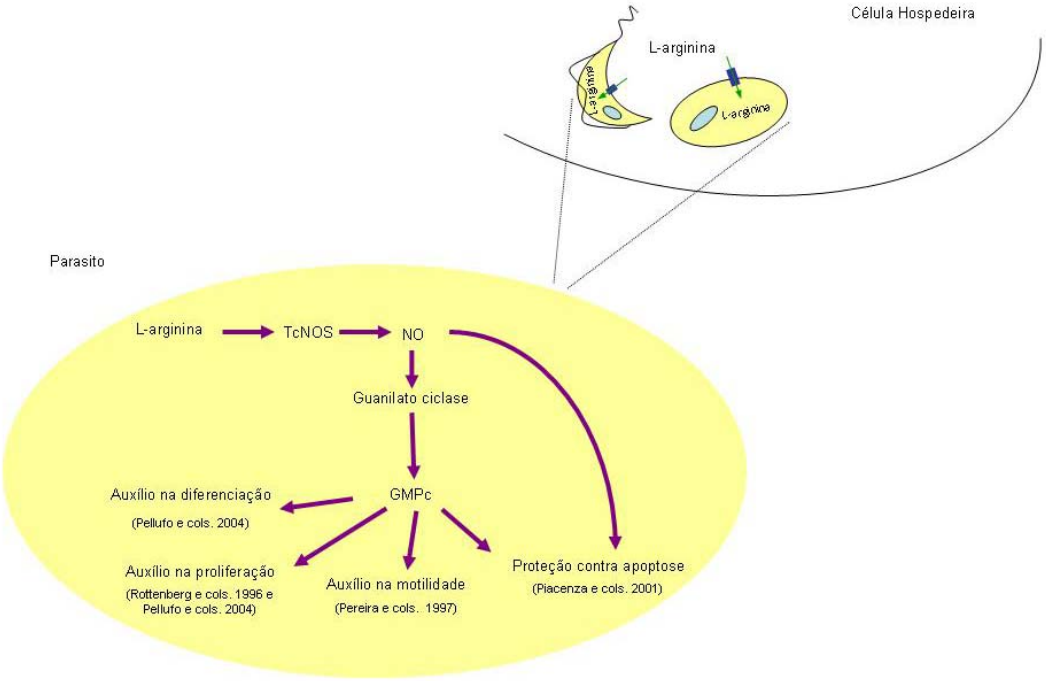

Figura 2.1 – Possíveis ações do NO na homeostasia do *T. cruzi*. Autoria Fabrino, D.L. 2009.

Neste capítulo apresentamos o terceiro artigo desta tese (Fabrino e cols., 2009b) – submetido; que teve como objetivo avaliar a capacidade e mecanismos de produção de NO por formas amastigotas e tripomastigotas do *T. cruzi* no sentido de contribuir para o melhor entendimento do papel do NO nesse parasito.

3.2.1 ARTIGO 3

ORIGINAL ARTICLE

Nitric Oxide Synthase (NOS) characterization in amastigote and trypomastigote forms of *Trypanosoma cruzi*

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Abstract

The nitric oxide (NO) synthesis has been observed in mammals and invertebrates, mostly through the nitric oxide synthase (NOS) enzyme. In Trypanosomatids, a TcNOS was previously identified in epimastigote forms of *T. cruzi,* but the ability of this parasite to express NO was not been defined until now. In this work , we demonstrate, for the first time, that both trypomastigote and amastigote forms are able to produce NO in a different rate. Remarkably, trypomastigote forms are able to produce higher levels of NO than the amastigote ones. We also isolated TcNOS from trypomastigote and amastigote forms. The enzyme activity was observed in both soluble fraction and in the purified soluble NOS. TcNOS from trypomastigotes showed higher activity compared to that from amastigote forms. The ability of NO synthesis by intracellular forms of the parasite might be important for the protozoan replication within the host cell, increasing its capacity of infection.

Key Words: *Trypanosoma cruzi,* Nitric oxide, Nitric oxide synthase, Trypomastigotes, Amastigotes; Chagas' disease.

Introduction

Nitric oxide (NO) is a highly reactive and unstable free radical that is produced by L-arginine oxidation to citrulline mediated by the enzyme nitric oxide synthase (NOS) (1).

In mammals, the NOS (EC 1.14.13.39) is recognized as responsible for NO production, and presents three isoforms (nNOS = neuronal NOS, eNOS = endothelial NOS and iNOS = inducible NOS). All the enzymes characterized so far are hemoprotein dimers comprised of subunits and require cofactors such as NADPH, tetrahydrobiopterin, FAD and FMN. The C-terminal domain is considered homologous among the isoforms, existing lesser homology in N-terminal domain. NOS is a modular enzyme in which each monomer consists of several discrete domains (2-5).

The DNA sequence of the NOS isoforms is known only in humans, existing 55–60% of identity in the amino acid sequence, with strongly conserved regions which are involved in catalysis, while the homology of one specific isoform can be of 85–92% among them (2). NOS isoforms are regulated by calmodulin, but only the constitutive isoform present apparent regulation according to alterations in Ca^{2+}concentrations (4).

NOS has been purified from a variety of mammalian tissues and has also been reported to be present in lower eukaryotic organisms (6-11).

The occurrence of NOS in epimastigotes of *T. cruzi*, the causal agent of Chagas' disease (12) was reported for the first time in 1995 by Paveto and collaborators. They showed not only the presence of a

Ca^{2+}/dependent NOS but also evidenced a NO signaling pathway in this protozoan. It was also demonstrated the existence of a nitroprusside-activated guanylyl cyclase and N-methyl-D-aspartate (NMDA) receptors in epimastigote forms of this parasite (13). However, the ability of *T. cruzi* forms present in mammalian host (trypomastigotes and amastigotes) to synthesize NO remains to be defined.

NO apparently affects a wide range of cellular functions in mammals such as cell-to-cell interactions, cellular proliferation, cytotoxicity and cytostasis, homeostatic and numerous other physiological mechanisms (14-16). Moreover, several works indicated NO as an important antimicrobial molecule (17-20). In invertebrates, however, little is known about the functional activity of this molecule. Regarding the protozoan *T. cruzi*, it was reported that NO can take part into the mechanism of the flagellum motility, through the guanylate cyclase enzymatic pathway (21) and that, the L-arginine metabolism by NO pathway can protect *T. cruzi* epimastigote forms against apoptotic death triggered by human fetal serum (21-23).

In the present work, we demonstrate, for the first time, that both amastigote and trypomastigote forms are able to produce NO through NOS. This enzyme was also demonstrated by us in both forms of the parasite. Remarkably, trypomastigote forms express higher amounts of both NO and NOS compared to amastigotes forms, which may be important for spread and sustaining the infection of the parasite.

Materials and Methods

Reagents

Benzamidine, trypsin inhibitor, penicillin G, KCl, leupeptin, *L*-glutamine, Schneider's Insect Medium, $MgCl_2$, phenylmethylsulfonyl fluoride (PMSF), N-1-naphtylethylenediamine, phosphoric acid, sulfanilamide, sucrose, Tris.HCl, dithiotreitol (DTT), aprotinin, L-arginine, NADH, NADP, NADPH, EGTA-AM (acetoxy methyl ethylene glycol-bis [β-aminoethyl ether]-N,N,N',N'-tetra acetic acid), L-NAME (N^{ω}-nitro-L-arginine methyl esther), (6R)-5,6,7,8-tetrahydrobiopterin (H_4B), 2′-5′-ADP agarose and rabbit IgG-anti nNOS (N7280/rat brain origin) were from Sigma Chemical Co. (St. Louis, MO). Glycerol, fluorescein isothiocyanate and formaldehyde were from Bio-Rad (Hercules, CA). Fetal bovine serum (FBS) was from Gibco BRL (Gaithersburg, MD).Polyethyleneglycol (Sigma, MA), Danvers, MA. Electrophoresis was performed using Phast-System (Pharmacia LKB Biotechnology Inc., Uppsala, Sweden).

Parasites

T. *cruzi* (Y strain) trypomastigote forms were maintained by *in vitro* infection of MK2 cell lineage. Semi-confluent MK2 cell cultures sustained in RPMI-1640 medium (Sigma, USA) supplemented with 10% FBS were infected (ratio of ten parasites per cell) and after 5–7 days, the cell culture

derived trypomastigotes forms were harvested from cell culture supernatants.

To obtain amastigote forms, the infected cell cultures were further maintained for 10 days at 37°C, replacing the culture medium every 48 hours. Then the supernatant of the infected cell culture was harvested and left at the same temperature for 1h, after this time the upper part was discarded and amastigotes collected from the bottom of the tube.

In addition, bloodstream forms were obtained by heart puncture from *T. cruzi*-infected Swiss mice at the parasitemia peak day and purified as previously described before (24).

NO/NO^-_2 measurement

NO generation by amastigote and trypomastigote forms was measured as accumulated supernatant nitrite (a stable breakdown product of NO) by the Griess reaction (25) The concentration of cultured cells was adjusted to $4x10^6$ cells and plated on 24-well flat-bottom tissue culture plates (Costar, Cambridge, MA, USA) with 600µL of RPMI 1640 (Sigma). Plates were incubated in a humidified atmosphere at 37° C for 24h. Subsequently, 100 µL of the supernatant was collected and transferred to a 96-well flat-bottom tissue culture plates. The supernatants were incubated with 100 µL of Griess reagent (50 µL of 1% sulfanilamide (Sigma) plus 50µL of 0.1% of N-1-naphythylethylenediamide (Sigma) in 5% phosphoric acid solution) at room temperature for 5 minutes. The absorbance was determined at 540 nm using a Spectra Max 190 spectrophotometer (Molecular Devices,

USA). Conversion of absorbance to micromoles was deduced from a standard curve of sodium nitrite in medium. All determinations were performed in triplicate and expressed as micromoles of NO^-_2/4x10^6 cells.

Isolation of NOS from Trypanosoma cruzi

Soluble fraction

Cell-free extracts were prepared by freeze-thawing the parasite suspensions (4 × 10^9 cells/mL for trypomastigotes and 5 × 10^9 cells/mL for amastigotes) three to five times, sonicated five times for 45 sec over ice in 0.25 mol/L sucrose containing 5 mmol/L KCl and put under pressure in a cavitation pump 1800 psi for 2 hours . The contents were centrifuged at 10,000 × g for 30 min and the supernatant was then centrifuged at 100,000 g for 60 minutes at 4°C. The supernatant which was considered the soluble fraction, was adjusted with antiproteolytic buffer containing 0.1 mmol/L PMSF (phenylmethylsulfonyl fluoride), 0.01% (w/v) leupeptin, 0.2 mg/mL trypsin inhibitor and 1 mmol/L benzamidine to a final volume of 5 mL.

Purified enzyme

The soluble fraction obtained as described above was applied to a column (8.5 × 1.5 cm) packet with 2′,5′- ADP agarose, equilibrated with 10 mmol/L Tris-HCl, pH 7.5 containing 1 mmol/L DTT (dithiotreitol), 1 mmol/L EDTA, 0.5 mmol/L PMSF, 25 units/ml aprotinin and 0.5 mmol/L L-arginine (buffer A). The column was then washed successively with 20 mL of buffer A, 20 mL of buffer A plus 0.5 mmol/L NADH, 20 mL of buffer

A plus 0.5 mmol/L NADP and 20 mL of buffer A. The enzyme was eluted with 10 mL of buffer A containing 10 mmol/L NADPH, 3 μmol/L H_4B and 10% (v/v) glycerol and concentrated to 2 mL using polyethyleneglycol. The protein concentration was estimated spectrophotometrically (260–280 nm) (26) and the sample was utilized to evaluate the pureness, through SDS-PAGE (6, 27).

SDS-PAGE

Polyacrilamide gel electrophoresis containing sodium dodecyl sulfate (SDS-PAGE) was performed using Phast-System using a 10% gel concentration, according to the manufacturer's directions (Bio-Rad), and a loaded with 45µg/line of protein. Gels were stained with silver nitrate using the kit Silver staining plus (Bio-Rad). Molecular weight standards (Bio-Rad) were myosin (200 kDa), β-galactosidase (116.2 kDa), rabbit muscle phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa) and egg white ovoalbumin (42.6 kDa).

Western-blot analysis

After transfer onto nitrocellulose membranes, non-specific binding sites were blocked with 5 % non-fat milk in Tris buffered saline-Tween (TBST; 50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 0.05 % Tween 20). Membranes were probed with the monoclonal anti-cNOS (Sigma) in TBST with 1 % non-fat dry milk. TcNOS was then identified by incubating the membrane with HRP-conjugated secondary antibodies in TBST,

followed by detection of antigen-antibody complexes by Supersignal Chemiluminescence (Pierce). The detection was done either by exposing the membrane to autoradiography film.

Determination of NOS Activity from T. cruzi trypomastigotes and amastigotes

The activity of the purified NOS from amastigote and trypomastigote forms was determined by measuring the decrease in the absorbance at 340 nm for 120 min continuously, as NADPH is consumed during the conversion of L-arginine to L-citrulline and NO by NOS (7)). Briefly, the complete enzyme reaction mixture contained 50 mmol/L potassium phosphate buffer (pH 7.4), 1 mmol/L $CaCl_2$, 0.1 mmol/L NADPH, 80 μmol/L H_4B, 10 μmol/L FAD, 10 μmol/L FMN, 0.1 mmol/L L-arginine, 0.2mmol/L DTT and 10µg of the pure soluble enzyme in a final volume of 300µL, at 25°C. Alternativelly, 5 mmol/L L-NAME and 40 mmol/L EGTA were used in the mixture reaction (27, 28).

Data Analysis

Three independent experiments were performed and the data obtained with different treatments were analyzed statistically by 1-way ANOVA (p <0.01 to 0.0001).

Results

Trypomastigote and amastigote forms of T. cruzi are able to release NO

The ability of NO production by the parasite was evaluated in trypomastigotes obtained from different sources (blood and from supernatant of MK2 cell cultures), and in amastigote forms obtained from MK2 cell cultures. Trypomastigotes are the extracelular infective forms while amastigotes represent the intracellular replicative forms present in mammalian hosts (12). Our data show that both forms were able to release NO, in a different rate, depending on its stage of life cycle.

Remarkably, bloodstream trypomastigotes were able to produce significantly higher levels of NO measured by nitrite formation (mean ± SEM of 5.3 ± 1.1 µmol/L) ($p < 0.001$) when compared to amastigote forms (mean ± SEM 2.5 ± 0.3 µmol/L), representing an increase of up to 47% (Fig 1).

Cell culture derived trypomastigote forms collected from MK2 infected cell cultures were also evaluated. Again the data showed a significant higher ($p < 0.01$) amount of NO (45% more) in these forms (mean ± SEM 4.6 ± 0.5 µmol/L) as compared to amastigotes (Fig 1). When the ability to produce NO was compared between bloodstream trypomastigote forms and those obtained from MK2 cell culture, there was no significant difference (Fig 1).

NOS isolated from trypomastigote and amastigote forms of T. cruzi show different levels of activity

Once the NO levels released by trypomastigotes obtained from blood sources did not show statistical significant differences from those levels released by cell culture derived trypomastigotes, we next used trypomastigotes and amastigotes obtained from the supernatant of MK2 infected cultures to evaluate TcNOS activity. Tests were performed by measuring consumption of NADPH in a spectrophotometer as described before (8, 13).

First, TcNOS activity was evaluated in the soluble fraction from crude extracts obtained from trypomastigote and amastigote forms. Both evolutive forms clearly showed TcNOS activity and this activity was significantly inhibited by L-NAME ($p < 0.001$), a specific and non reversible NOS inhibitor (2) (Fig 2A and B). Interestingly, the enzyme activity found in trypomastigotes was significantly higher compared to that observed in amastigote forms ($p < 0.01$). Moreover, NOS activity was increased earlier in trypomastigotes (after just 20 minutes of incubation) compared to amastigote forms (Fig 2C). When both extracts were boiled, the trypomastigote extracts lost part of its activity in a significant rate, but the same was not true for the amastigote ones. The amastigotes exhibited only a slight impairment in its activity but was less sensitive to the heating process that the enzyme from trypomastigotes (Fig 2A and B).

Since TcNOS activity was consistently detected in soluble fraction from crude extracts, we next isolated the enzymes from these fractions to

test their activity. Purified proteins were incubated with all its essential co-factors (see material and methods) and the activity was again measured by NADPH consumption in a spectrophotometer. The activity of the purified enzymes of both, trypomastigotes and amastigotes, could be observed only after one hour of incubation with its essential co-factor. NOS isolated from trypomastigote forms was more active than the enzyme from amastigote forms, as previously noted in the soluble fraction assay. This difference, however, was significantly observed only at 120 min of reaction ($p < 0.01$) (Fig 3A).

When incubated with L- NAME (5mmol/L), the specific non reversible, NOS inhibitor (2), the activity of the purified NOS from trypomastigote was significantly and time-dependent inhibited ($p< 0.01$ to 0.0001), reaching its highest level at 120 min (Fig 3B). NOS purified from the amastigote forms, showed significant inhibition by L-NAME at 60min of reaction ($p< 0.001$), remaining at the same rate until 120min (Fig 3C).

To get insights into the enzyme isoform, we next incubated them with EGTA, a calcium chelator (2). NOS from both trypomastigote and amastigote forms had its activity significantly impaired by 60 min of reaction (Fig. 3B and C). This means that both enzymes seems to represent the constitutive form since they showed Ca^{2+} dependence (2). Of note, EGTA-induced inhibition of the enzyme isolated from the trypomastigote forms was higher ($p< 0.0001$) than that detected upon the enzymes from amastigote forms ($p<0.01$ and 0.001 at 60 and 120 minutes respectively) (Fig 3 B and C).

To confirm the presence of NOS in *T.cruzi* forms a SDS-PAGE/ western blot was performed using the same amount of proteins (45 µg/line) for both trypomastigotes and amastigotes. The molecular weight of the protein was around 116 kDa and the enzyme from both evolutive forms appeared as a double band protein (Fig 4A and B).

Discussion

NO is an intriguing and potent mediator molecule with multiple functional activities as extensively demonstrated in mammalian cells (revision in (16)). The occurrence of this molecule in protozoan parasites, however, has received much less attention. To date, it has been described just in members of the genus *Leishmania* (6, 8, 29) and in epimastigote forms of *T. cruzi* (13).

In this work, we show, for the first time, that both trypomastigote (from blood and cell culture) and amastigote forms are able to release NO. Moreover, we demonstrate that trypomastigotes release significantly higher amounts of NO than amastigote forms. In parallel, we also demonstrated, using different approaches that the enzyme TcNOS was differentially expressed in these two forms of the parasite, being its activity prominent in trypomastigotes compared to amastigote forms.

Providing more evidence for the isolation of a TcNOS, the purified enzymes showed high NADPH consumption, and the enzyme activity was inhibited by the specific inhibitor L-NAME, as also observed in bacterial-NOS from *Nocardia* species (7). *T. cruzi* epimastigotes (13) and in *L. amazonensis* promastigotes and axenic amastigotes (8). In addition, our results demonstrated that NOS activities, from both forms, are impaired by EGTA incubation, reinforcing the hypothesis that NOS of *T cruzi* is also a constitutive NOS isoform as previously described (13) and observed by our group in *L. amazonensis* (28).

The fact of the enzyme from *T. cruzi* trypomastigotes exhibits more

activity than the corresponding one from amastigote, either in soluble fraction or in the purified enzyme, corroborate those data concerning the higher release of NO by this stage of the parasite.

It is matter of note that in the assay with the purified soluble enzyme, a longer time was necessary to detected the enzyme function than in the soluble fractions assays. We speculate that this might be due to the fact that in the soluble fractions the enzyme was already in direct contact with all necessary activation cofactors. However, we can not discard the possibility of some molecular damage over the purification process.

The effect of L-NAME in the trypomastigote purified enzyme was stronger than in the amastigote one. The inhibitory effect on NOS activity was observed since the first moment of incubation ($p< 0.01$-0.0001) reaching its highest level at 120 min. Whereas on the purified soluble enzyme, from amastigote forms, the inhibition happened to start significantly ($p< 0.001$) only at 60min of reaction and remains at the same rate till 120min (Fig 2c).

When incubated with EGTA, an intracellular calcium chelator, the NOS activity from both, trypomastigote and amastigote, was significantly impaired from 60 min and up. Curiously, the inhibition by EGTA upon the enzyme from the trypomastigote form was higher ($p< 0.0001$) than upon the enzyme from the amastigote form ($p<0.01$ and 0.001 at 60 and 120 minutes, respectively)

All these data together allow us to think about some functional differences between the TcNOS from trypomastigote and amastigote

forms. The enzyme from trypomastigote forms is clearly more sensible to heat inactivation, lack of available Ca^{2+} (provoked by EGTA action) and inhibition by L-arginine analogues. It seems that the amastigote form present a more stable (robust) enzyme than the trypomastigote forms. It is tempting to think that once the amastigote form of *T. cruzi* is the multiplicative form, it has to keep all its metabolic pathways in optimal condition, to be able to multiplicate and maintain the infection.

The biological significance of a NO/NOS pathway in *T. cruzi* is not clear. It was suggested that the stimulation of the soluble guanylate cyclase (GCs) activity by NO, would be responsible for the increase of the motility of the flagellum of *T. cruzi* epimastigotes (21). We can think about different purposes of this pathway in the parasite cell: (1) as proposed to the epimastigote forms, the greater activity of NOS and higher levels of released NO by trypomastigotes, might also be related to the high motility of these forms.

Trypomastigotes are considered the main infective forms of *T. cruzi*, they reach the host cell through the blood stream and are able to infect the host cells; so it is reasonable to think that the higher level of NO produced by trypomastigotes is linked to the flagellum movements, (2) once trypomastigotes are the parasite forms which get in contact with the harsh environment of the fagosome, it might be possible that a negative feedback can be driven by the NO produced by the parasite on the iNOS from the host cell. This mechanism has also been suggested in the literature when high levels of NO is produced by iNOS from host cell (30) and (3) the parasite can be using its produced NO to protect itself from

apoptosis process. The presence of a caspase-like activity was shown in *T. cruzi* epimastigotes, suggesting a possible enzymatic step that could be down regulated by NO through a S-nitrosylation (rewied in (22, 23)).

Parasites present various strategies to escape the host defense systems, taking advantages of different host biochemical factors. Murine infection with *L. major* , for example, is a well-studied model for evaluating resistance or susceptibility to infection. In macrophages, the first line of host defense, a Th1 response, is mainly through the production of gamma interferon (IFN-γ), that leads to NO synthesis by the host and parasite killing, whereas Th2 cytokines, mainly interleukin IL- 4 and IL-10 inhibit NO synthesis and favour parasite growth (31).

How can *T. cruzi* manipulate the host cell signaling pathway, specifically the host NO pathway? It has been suggested that the parasite can acquire L- arginine from the host cell sources and use it to produce putrescine and other poliamines to “build” new molecules for its own growth (32). Here, by the demonstration that the parasite is also able to synthesize NO, we speculate that the parasite NO can somehow interfere with the host NO production by negative feedback.

The lower levels of NO and NOS activity found in amastigotes can reflect the lack of substrate for it. Muscle cells, one of the favorite cell types for the maintenance of infection, and also macrophages, are differentiated cells, thus they have lower rates of arginine to offer than proliferating cells (32). The available substrate is a rate limiting factor for the NOS (2), if we consider that the macrophage spends a great amount of L-arginine in the first hours of infection, in the process called oxidative

burst (33) and that the trypomastigotes forms are also requiring the L-arginine in order to produce its own NO, as suggested by Peluffo and collaborators (2004) (32); it is possible to hypothesize that the amastigotes not only are living in a poorer L- arginine environment but are also driving the major part of the obtained L-arginine for multiplication.

Besides, the literature suggest that trypomastigotes are able to elicit asynchronous and repetitive intracellular Ca^{2+} pulses in the host cell, such changes regulate parasite invasion by promoting transient actin rearengements and lysosome-plasma membrane fusion and subsequently the level of this ion declines at later times of infection (34, 35). This calcium kinectics can be interfering in the trypomastigotes and amastigotes ability to produce NO once they exhibit a constitutive Ca^{2+} dependent TcNOS.

Further studies are necessary to investigate if the NO produced by the parasite can modulate the production of Th1/Th2 cytokines during the infection. Although macrophages trigger their defense mechanism to neutralize the parasite, our results show that the *T cruzi* NO pathway is a consistent system differentially expressed in the parasite forms and that, like in *Leishmania,* might modulate host NO/NOS production, infection range or other intracellular signaling pathways (21, 36, 37).

In fact, the recognition of NOS sites provides multiple ways to regulate NO levels and cross-talk between second messenger systems (14). Future studies are therefore important to understand the complexity of the mechanisms involved in the interaction of the parasite with the host NO signaling system and the significance of its intrinsic NO/NOS activity.

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Figures Legends

Figure 1. NO production by trypomastigote (from blood and MK2 cell culture) and amastigote forms of *T. cruzi* evaluated after 24 hours of culture by Griess reaction. Parasites (4×10^6) were cultured in RPMI medium. Values are expressed as mean ± SEM and are representative of three independent experiments. Statistical analysis by 1-way ANOVA (p <0.01 to 0.0001).

Figure 2. NOS activity *per se*, after heat shock or with L-NAME incubation. The assays were done with the soluble crude extract of (A) trypomastigotes (from MK2 cell culture) and (B) amastigote forms evaluated by consumption of NADPH at 340nm. The comparison between NOS activity from trypomastigotes (from MK2 cell culture) and amastigote forms is seen in (C). Values are expressed as mean ± SEM and are representative of three independent experiments. Statistical analysis by 1-way ANOVA (p <0.01 to 0.0001).

Figure 3. The comparison between NOS activity from trypomastigotes (from MK2 cell culture) and amastigote forms is seen in (A). NOS activity *per se*, after incubation with L-NAME or EGTA. The assays were done with the soluble fraction of (B) trypomastigotes (from MK2 cell culture) and (C) amastigote forms evaluated by consumption of NAPH at 340nm. (C). Values are expressed as mean ± SEM and are representative of

three independent experiments. Statistical analysis by 1-way ANOVA (p <0.01 to 0.0001).

Figure 4. Western blot of NOS molecule purified from *T. cruzi* cell cultured derived trypomastigotes and amastigotes (A) and silver staining (B). For both electrophoresis was performed using Phast-System on a 10% gel according to manufacturers directions. Proteins samples (45µg/mL) were used in two independent experiments.

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Figure - 1

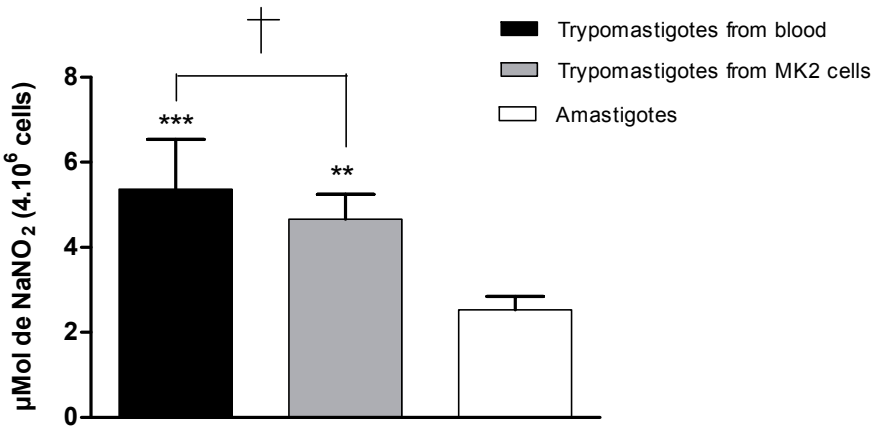

Trypomastigotes from blood
Trypomastigotes from MK2 cells
Amastigotes
µMol de NaNO2 (4.10^6 cells)
8
6
4
2
0
***
**
†

Figure - 2

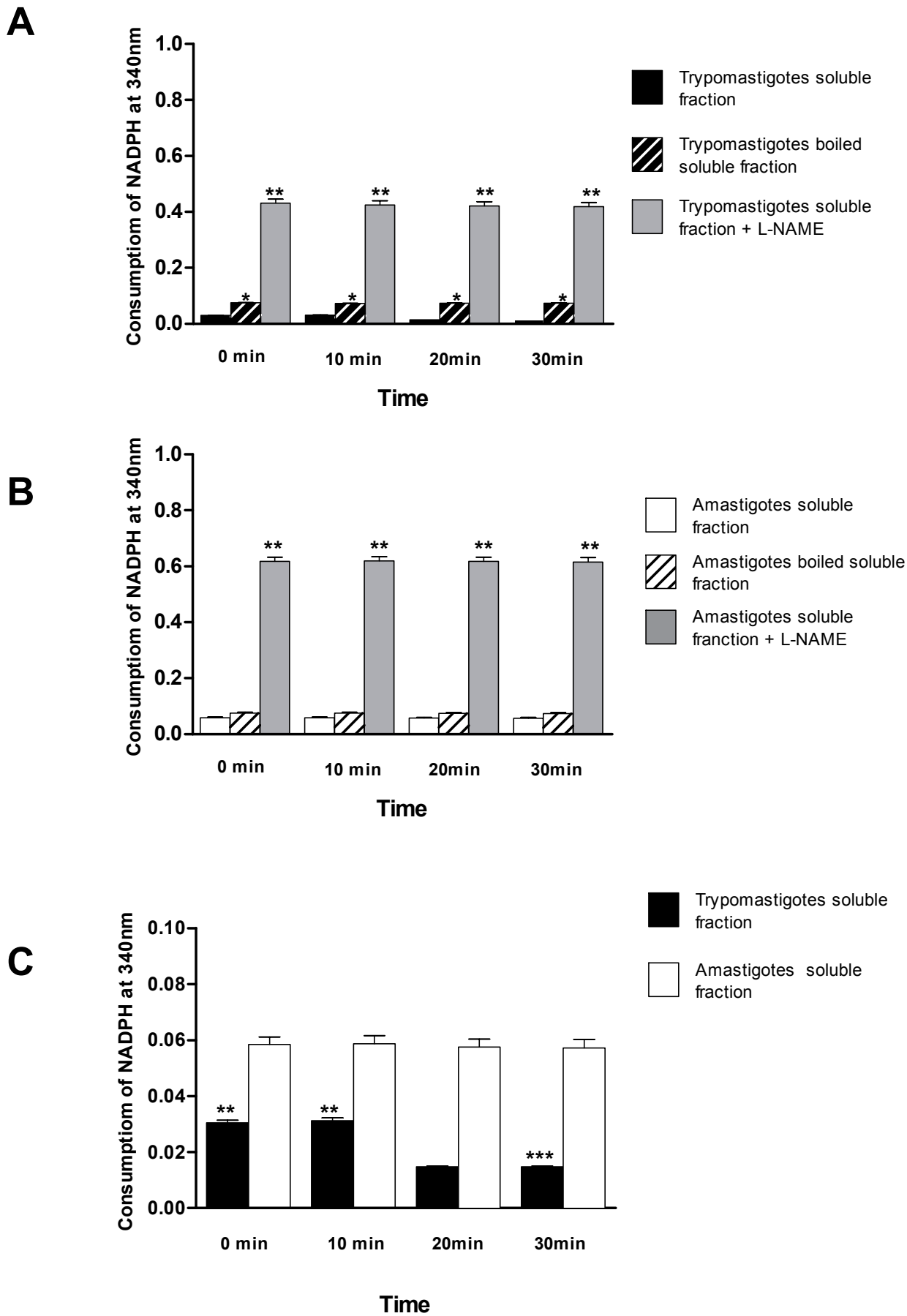
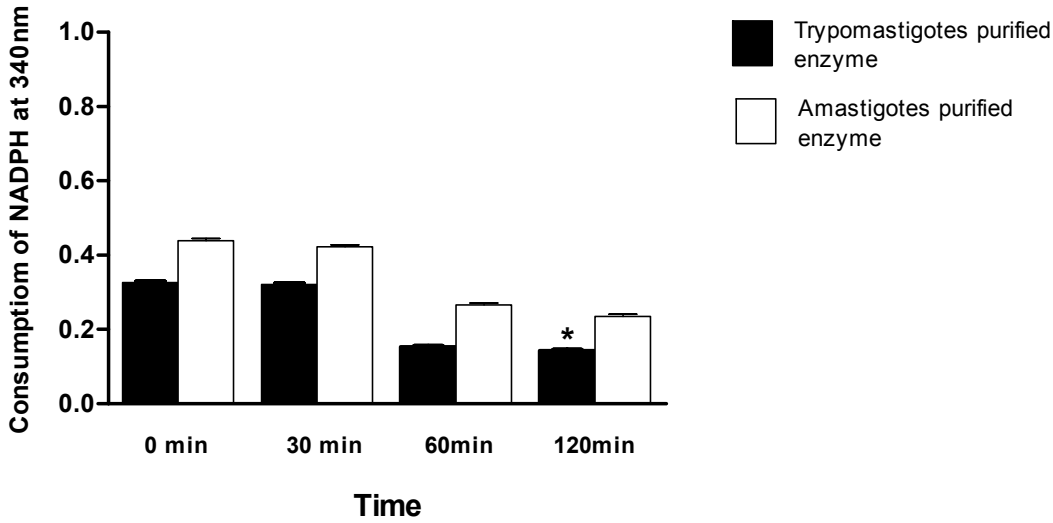
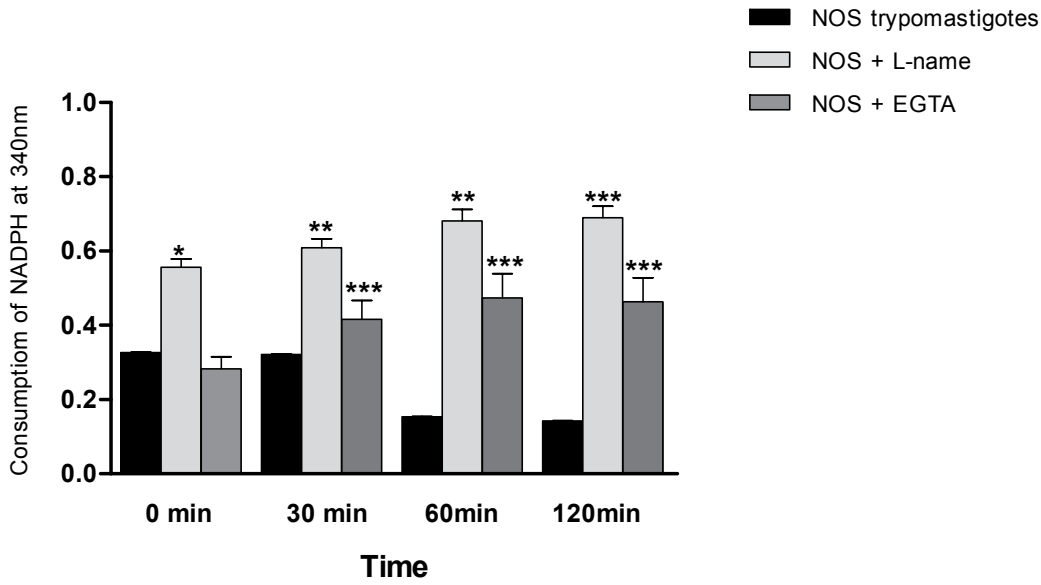

A
Consumptiom of NADPH at 340nm
1.0
0.8
0.6
0.4
0.2
0.0
**
*
0 min
10 min
20min
30min
Time
Trypomastigotes soluble fraction
Trypomastigotes boiled soluble fraction
Trypomastigotes soluble fraction + L-NAME
B
Consumptiom of NADPH at 340nm
1.0
0.8
0.6
0.4
0.2
0.0
**
0 min
10 min
20min
30min
Time
Amastigotes soluble fraction
Amastigotes boiled soluble fraction
Amastigotes soluble franction + L-NAME
C
Consumptiom of NADPH at 340nm
0.10
0.08
0.06
0.04
0.02
0.00
**
***
0 min
10 min
20min
30min
Time
Trypomastigotes soluble fraction
Amastigotes soluble fraction

Figure - 3

A


Consumptiom of NADPH at 340nm
1.0
0.8
0.6
0.4
0.2
0.0
*
0 min
30 min
60min
120min
Time
Trypomastigotes purified enzyme
Amastigotes purified enzyme

B


NOS trypomastigotes
NOS + L-name
NOS + EGTA
Consumptiom of NADPH at 340nm
1.0
0.8
0.6
0.4
0.2
0.0
*
**
***
**
***
***
***
0 min
30 min
60min
120min
Time

C

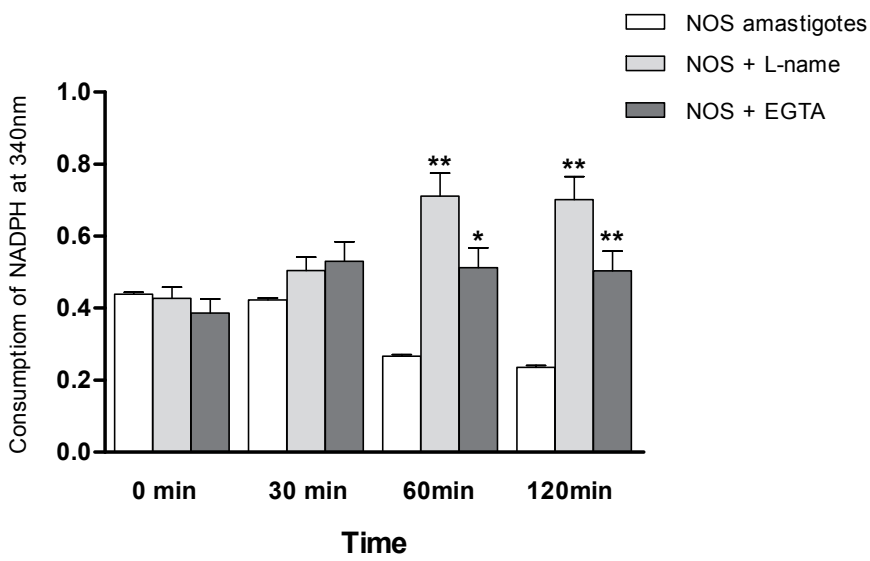
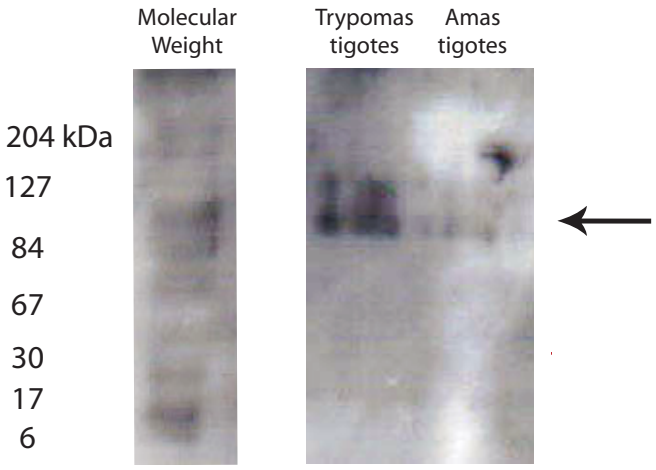
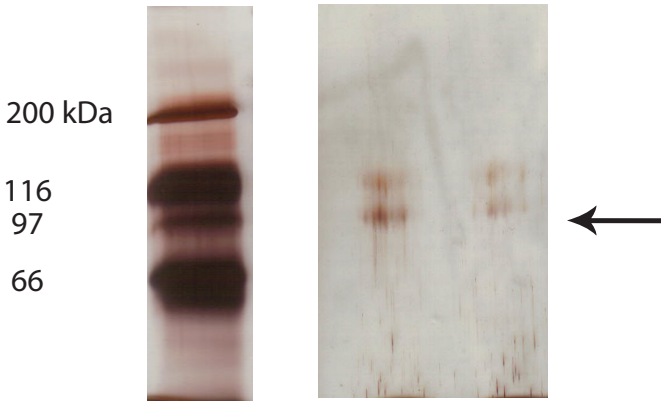

NOS amastigotes
NOS + L-name
NOS + EGTA
Consumptiom of NADPH at 340nm
1.0
0.8
0.6
0.4
0.2
0.0
**
*
**
**
0 min
30 min
60min
120min
Time

Figure - 4

A


Molecular Weight
Trypomas tigotes
Amas tigotes
204 kDa
127
84
67
30
17
6

B


200 kDa
116
97
66

3.3. CAPÍTULO 3

Susceptibilidade de diferentes microrganismos ao Nitroprussiato de Sódio, um doador de Óxido Nítrico

Protozoários parasitos infectam centenas de milhões de pessoas todo ano e, em conjunto, são uma das principais causas das maiores misérias humanas. Em especial, os protozoários da ordem Kinetoplastida, família Tripanosomatidae, como *T. cruzi* e *Leishmania sp.,* constituem importante problema de saúde pública na América Latina (revisões em Barrett e cols. 2003, Stuart e cols. 2008). Embora compartilhem diversas características biológicas e bioquímicas; estes parasitos exibem profundas diferenças quanto à sua relação com o hospedeiro, culminando numa diversidade patológica que produz variedade de sintomas e injúria, no homem e em diferentes modelos animais (revisões em Barrett e cols., 2003; Ouaissi & Ouaissi, 2005; Stuart e cols., 2008). Por isso, o entendimento das diferenças, e congruências entre esses dois patógenos causadores de doenças humanas em nível genético, molecular e de biologia celular pode prover novas abordagens para o desenvolvimento de diagnósticos, fármacos ou vacinas (Ouaissi & Ouaissi. 2005; Stuart e cols. 2008).

De fato, as doenças causadas por esses protozoários apresentam ainda sérias limitações quanto ao diagnóstico e tratamento uma vez que nem sempre a infecção manifesta sintomas clínicos evidentes, podendo ser silenciosa por vários anos. Em indivíduos infectados por *T. cruzi*, 95% dos casos são assintomáticos na fase aguda (Coura, 2007) e 30-40% desenvolvem sintomas clínicos da fase crônica (PIDC, 2009); dados semelhantes podem ser observados em pacientes infectados por diferentes espécies de *Leismania sp.* Muitos pacientes apresentam uma forma não aparente ou oligossintomática da doença, e o número de casos graves ou com sintomatologia evidente é relativamente pequeno em relação ao número total de infectados (WHO/CID/Leish., 2008).

Outro grande problema de saúde publica na America Latina, e no mundo, que apresenta a mesma particularidade quanto à manifestação clínica (assintomatologia), é a tuberculose, causada pelo *Mycobacterium tuberculosis* (*M. tuberculosis*). Transmitida facilmente por aerossóis, esta bactéria causa infecção das vias respiratórias. Estima-se que 1/3 da população mundial esteja infectada por *M. tuberculosis*; entretanto apenas 10% da população infectada desenvolve a doença (Russell, 2001). Na maioria dos casos, a bactéria e o hospedeiro estabelecem um equilíbrio que pode perdurar por vários anos, e alguns indivíduos podem permanecer assintomáticos a vida toda (Sundaramurthy & Pieters, 2007).

Diante do exposto, nota-se que as três patogenias citadas acima têm em comum o fato de que os parasitos que as causam conseguem enfrentar o ambiente hostil do compartimento endossomal e permanecer, ainda que de modo sub-patente e mesmo temporariamente (no caso do *T. cruzi*) de modo silencioso em número diminuto, silenciosamente e viáveis por muito tempo (anos) nas células de seus hospedeiros vertebrados (Zambrano-Villa e cols., 2002; Barrett e cols., 2003; Sundaramurthy & Pieters, 2007).

Os fármacos usados no combate desses patógenos apresentam sérios efeitos colaterais, e em todas as três patogenias, o controle da severidade das formas clínicas desenvolvidas pelos pacientes é alcançado mais efetivamente quando o diagnóstico é efetuado na fase inicial da infecção e o paciente não sofre de nenhum outro estresse imunológico (Barrett e cols., 2003; Pieters, 2008; WHO/CID/Leish., 2008). Faz-se, portanto, necessário o entendimento dos eventos iniciais da interação parasito-hospedeiro, para o desenvolvimento de melhores estratégias no combate a tais microrganismos. Neste contexto o entendimento dos mecanismos de ação do NO, uma molécula associada, na grande maioria das vezes ás respostas imune inata e inflamatória, que são os eventos mais precoces de uma infecção, merece atenção especial.

Os mais diversos patógenos incluindo protozoários (Stafford e cols., 2002), bactérias (Weinberg, 2000) e fungos (Gonzalez e cols., 2000) de modo geral, iniciam sua interação com o hospedeiro vertebrado através dos macrófagos, células componentes do sistema imune inato, que exibem diversos mecanismos de defesa, entre eles o NO. Tais células exercem muitas vezes um duplo papel na batalha imunológica travada pelos hospedeiros contra

infecções. Por um lado, são consideradas importantes células efetoras no controle e morte de microrganismos através de mecanismos oxidativos e não oxidativos. Entretanto, podem também servir de local de hospedagem e multiplicação para os mesmos, protegendo-os de substâncias adversas do meio extracelular ou oferecendo condições microambientais favoráveis para sua sobrevivência como no caso dos protozoários *Leishmania sp.*, *T. cruzi* e *T. gondii* (Bogdan & Rollinghoff, 1999) e da bactéria *M. tuberculosis* (Anes e cols., 2006; Sundaramurthy & Pieters, 2007). Todos os organismos citados acima são capazes de disparar respostas imunológicas intensas em seus hospedeiros, ativando vias de oxidação distintas em macrófagos. Entretanto, muitas vezes conseguem evadir os mecanismos de defesa e estabelecer e manter a infecção (Brunet, 2001; Stafford e cols., 2002; Zambrano-Villa e cols., 2002; Pieters, 2008).

A infecção murina por *Leishmania major* é um modelo bem estudado no que concerne ao estabelecimento de resistência ou susceptibilidade do hospedeiro à infecção, especialmente em macrófagos. Nestas células, fica claro que a estimulação por citocinas componentes do perfil Th1, principalmente o IFNγ leva à síntese de NO, o que viria a ser tóxico para o parasito. Entretanto, o perfil de citocinas Th2 é capaz de inibir a produção de NO e estimular a via bioquímica da arginase, que usa o mesmo substrato usado na produção de NO. Deste modo, a síntese do NO é regulada negativamente e paralelamente a arginina produzirá como metabólitos finais, poliaminas que são usadas para multiplicação e crescimento do parasito (Vincendeau e cols., 2003); ver Figura 1.1 no capítulo 1.

Em *T. cruzi,* um mecanismo semelhante pode estar acontecendo, uma vez que, durante a infecção aguda experimental em ratas, há uma inibição na produção de NO por macrófagos esplênicos e monócitos do sangue periférico (Fabrino, D. L. e cols., 2008); artigo número 2 desta tese. Já foi demonstrado no mesmo modelo que os monócitos exibem altos níveis de arginase, aos 20 dias de infecção (Fabrino e cols., 2004). Além disso, embora a produção de L-arginina, molécula precursora do NO não tenha sido detectada em *T. cruzi*, dados revelam que o parasito é capaz de sintetizar poliaminas, mas o mecanismo exato pelo qual ocorre essa produção ainda não está totalmente esclarecido (Peluffo e cols. 2004).

Sugere-se que o *T. cruzi* capte a L-arginina do citoplasma da célula hospedeira para sua produção de poliaminas através de um canal transportador independente de sódio. Desta forma, o parasito faz uso do substrato da iNOS ativada na célula hospedeira, regulando negativamente a produção de NO por estas células (Peluffo e cols., 2004).

Outra observação interessante é que a ativação de macrófagos pode ser modulada pela fagocitose de células apoptóticas. A fagocitose de células T apoptóticas, por meio de receptores para vitronectina, o que leva a síntese de citocinas antiinflamatórias, favorecendo a replicação do parasito dentro do macrófago tanto *in vivo*, quanto *in vitro* (Freire-de-Lima e cols. 2000). A infecção por *T. cruzi* induz a expressão desse receptor em macrófagos, o que conseqüentemente leva à secreção de TGF-β, que por sua vez suprime NO e estimula arginase no macrófago (Freire-de-Lima e cols., 2000; Peluffo e cols., 2004; Freire-de-Lima e cols., 2006). A síntese de PGE_2 e TGF-β é também observada quando neutrófilos apoptóticos e infectados com *Mycobacterium bovis BCG* são fagocitados por macrófagos; tal síntese é acompanhada pela formação de corpúsculos lipídicos no citoplasma do macrófago (D'Avila e cols. 2008).

O *M. tuberculosis*, por sua vez, interfere nas vias de tráfego intracelular de vesículas, modulando os eventos de maturação dos compartimentos endossomal/fagossomal. Nesse caso, ocorre formação de um fagossomo que contém bactérias viáveis; entretanto a fusão/maturação do mesmo com/para os lisossomos não ocorre (Sundaramurthy & Pieters, 2007). Acredita-se que a capacidade que as micobactérias patogênicas tem de manipular essa via de tráfego de vesículas seja o evento chave na sobrevivência deste microorganismo dentro da célula hospedeira. Por outro lado, micobactérias não patogênicas como a *M. smegmatis* não são capazes de modular tais eventos e são, na maioria das vezes, eliminadas eficientemente por macrófagos (Anes e cols., 2006; Sundaramurthy & Pieters, 2007).

Além disso, há evidencias de que o *M. tuberculosis* possa afetar o acesso do NO aos compartimentos fagocíticos, impedindo o recrutamento da iNOS para a proximidade destes compartimentos. O mecanismo exato deste processo ainda precisa ser esclarecido, mas sugere-se que o *M. tuberculosis* interfira na funcionalidade da EBP50, uma proteína de associação do

citoesqueleto que está envolvida na migração de moléculas pelos filamentos de actina na célula (Davis e cols., 2007).

Vale ressaltar que a capacidade de interferir no processo de fusão de vesículas do compartimento endossomal também já foi evidenciada em *L. donovani*. Usando uma cepa selvagem e um mutante para o glicolipídio lipofosfoglicano (LPG), Desjardin e Descoteaux, demostraram que o LPG tem importante papel no impedimento da fusão dos vacúolos parasitóforos com vesículas do sistema endossomal; tal inibição foi sugerida pelos autores como uma estratégia de sobrevivência utilizada pelos promastigotas durante sua transformação em amastigotas (Desjardins & Descoteaux, 1997).

As interações entre organismos intracelulares e a via bioquímica do NO podem ser investigadas, através de estudos envolvendo: (1) a produção de NO em presença de diferentes quantidades de substrato; (2) a produção de NO combinada com adição de um análogo inerte da L-arginina como o L- NAME, que compete com o substrato pela ligação do sitio ativo da enzima; e (3) pela regulação da produção de NO por meio do uso de RNA de interferência (RNAi) que pode, dessa forma, ser silenciada em nível transcricional (Rivero, 2006). Outra possibilidade para avaliar a via do NO é testando-se a susceptibilidade de diversos patógenos *in vitro* a partir do uso de compostos doadores deste radical livre, como por exemplo, o S-nitroso-N-acetilpenicilamina (SNAP) e o pentacianonitrosilferrato de sódio, conhecido como nitroprussiato de sódio (SNP) (Geigel, 2000; Genestra e cols., 2008)

Quando se estuda a via do NO por meio de doadores químicos do mesmo é necessário ficar atento para o fato de que o NO produzido naturalmente num sistema biológico é produzido nas imediações das células alvo. O uso de doadores químicos de NO vai liberar este radical em todo o sobrenadante da cultura, portanto, a maioria do NO gerado é provavelmente auto-oxidado antes do mesmo alcançar seu alvo. Devido a este fato, concentrações de no mínimo 0.1-1.0 μM são necessárias para alcançar o mesmo efeito, causado por micromoles de NO em um sistema natural de geração desta molécula (Kroncke e cols., 1997).

Um doador de NO bastante usado em ensaios *in vivo*, e *in vitro*, é o SNP ($Na_2[Fe(CN)_5(NO)]$) que vem sendo utilizado há mais de 40 anos como vasodilatador, para o controle da pressão sanguínea em tratamentos pós-

operatórios e no tratamento de hipertensão crônica (Ross e cols. 1994). Atualmente, sabe-se que a ação do SNP é baseada na produção e liberação de NO, de forma semelhante à ação dos nitratos orgânicos. A reação de liberação de NO pelo SNP requer sua interação com um agente redutor, por exemplo, um tiol, e a presença de luz. Estas observações têm aumentado o interesse nas reações do SNP com outros agentes redutores como hemoproteínas, pois acredita-se que estes agentes redutores podem ser os responsáveis pelo transporte e liberação do NO *in vivo* (Bates e cols., 1991; Yamamoto & Bing, 2000).

Diante da complexidade dos mecanismos de ação da via bioquímica do NO, o uso de modelos experimentais que permitam um estudo mais detalhado se faz necessário, para (1) uma melhor compreensão dos mecanismos de interação entre parasitos e hospedeiros, (2) capacidade de estabelecimento e manutenção de um processo patológico e (3) funcionalidade do NO em sistemas biológicos de procariotos e eucariotos inferiores.

Desse modo, nosso grupo utilizou três organismos diferentes, uma bactéria e dois protozoários, submetendo-os um tratamento com um mesmo doador de NO, o SNP, e avaliando o impacto de diferentes doses deste composto químico, na fisiologia e capacidade de interação desses organismos com células hospedeiras.

No primeiro trabalho apresentado neste capítulo, o quarto trabalho desta tese, foram usados os 2 estágios do ciclo evolutivo de *L. amazonensis*; as formas promastigotas, que são flageladas, extracelulares e consideradas as formas infectantes ao hospedeiro; e as formas amastigotas axênicas, com flagelo curto e interiorizado, e capazes de multiplicação por fissão binária. Para este estudo, o SNP foi usado, *in vitro*, em diferentes concentrações e foi verificado que embora o SNP exerça uma toxicidade dose dependente nestes parasitos, as formas amastigotas axênicas, responsáveis pela manutenção da infecção do hospedeiro, são significativamente mais resistentes ao NO do que as promastigotas (Genestra e cols., 2008).

Posteriormente, foi realizado o quinto trabalho desta tese (Fabrino e cols., 2009a) usando-se a mesma abordagem com o *T. cruzi* no intuito de avaliar se também, as diferentes formas dos diferentes estágios do ciclo de vida deste protozoário exibiriam diferente resistência ao SNP. Em acordo com

os achados envolvendo *L. amazonensis*, verificou-se em *T. cruzi,* que as formas amastigotas, com flagelo curto e interiorizado, intracelulares e capazes de multiplicação por fissão binária e, portanto, responsáveis pela manutenção da infecção, são mais resistentes que as formas tripomastigotas deste protozoário. Curiosamente foi verificado que tripomastigotas, quando tratados com SNP e observados à microscopia de luz parecem se diferenciar, numa maneira dose dependente, em formas amastigotas (Fabrino e cols., 2009a) (dados de microscopia eletrônica estão sendo presentemente processados para melhor avaliar este processo de diferenciação).

E finalmente, usando uma bactéria não patogênica, a *M. smegmatis*, numa abordagem mais complexa, foi observado não só a reação do microorganismo ao SNP, mas também durante sua interação com a célula hospedeira, macrófagos da linhagem J774. A *M. smegmatis* vem sendo muito usada para o melhor entendimento da evolução da patogênese causada pelo *M. tuberculosis*, agente causal da tuberculose, pois por serem do mesmo gênero, essas duas bactérias compartilham muitas vias bioquímicas (Anes e cols., 2006).

Entretanto, após ser fagocitada por macrófagos, a *M. smegmatis* é eliminada dentro de fagossomos por uma combinação de fatores entre eles os intermediários reativos nitrogenados (RNIs), enzimas lisossomais, e outros fatores ainda não identificados. Por outro lado, o mesmo não acontece com o *M. tuberculosis*, pois esta bactéria consegue modular a via endocítica de macrófagos criando, nestas células, um ambiente propício à sua multiplicação (Jordao e cols. 2008).

Dessa forma, usando-se uma bactéria que mimetiza os eventos patológicos do *M. tuberculosis* é possível estudar os mecanismos citotóxicos e de interação com a célula hospedeira. .Além disso, a *M. tuberculosis* é uma bactéria de crescimento lento e de grande risco à saúde humana, o que limita o seu uso para investigações. A utilização de uma bactéria não patogênica e de crescimento rápido que, no entanto, compartilha muitas características biológicas e bioquímicas de uma bactéria patogênica, se mostra uma alternativa bastante viável (Anes e cols., 2006; Gutierrez e cols., 2008).

Portanto, no sexto e último trabalho, desenvolvido e apresentado nesta tese (Fabrino, D. L. e cols., 2008), foi usada uma cepa mutante de *M.*

smegmatis para estudo da ação do SNP. Esta cepa apresenta deleção para 3 porinas, proteínas que facilitam a entrada de nutrientes e de moléculas tóxicas, para o espaço intracitoplasmático de micobactérias. Nossos resultados demonstram que a *M. smegmatis*, *in vivo*, não consegue impedir a fusão/maturação do compartimento endossomal/lisossomal, corroborando dados anteriores (Anes e cols., 2006; Jordao e cols., 2008). Entretanto, os mutantes que exibem a falta das porinas em sua parede celular curiosamente se apresentam mais resistentes aos efeitos do SNP *in vitro.* Esses dados sugerem um papel relevante das porinas na susceptibilidade das micobacterias, possivelmente facilitando o transporte de NO pela membrana das mesmas.

3.3.1 ARTIGO 4

CELL BIOCHEMISTRY AND FUNCTION
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In vitro sodium nitroprusside-mediated toxicity towards *Leishmania amazonensis* promastigotes and axenic amastigotes

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Leishmania parasites survive despite exposure to the toxic nitrosative oxidants during phagocytosis by the host cell. In this work, the authors investigated comparatively the resistance of *Leishmania amazonensis* promastigotes and axenic amastigotes to a relatively strong nitrosating agent that acts as a nitric oxide (NO) donor, sodium nitroprusside (SNP). Results demonstrate that SNP is able to decrease, *in vitro*, the number of *L. amazonensis* promastigotes and axenic amastigotes in a dose-dependent maner. Promastigotes, cultured in the presence of 0.25, 0.5, and 1 mmol L^{-1} SNP for 24 h showed about 75% growth inhibition, and 97–100% when the cultures were treated with >2 mmol L^{-1} SNP. In contrast, when axenic amastigotes were growing in the presence of 0.25–8 mM SNP added to the culture medium, 50% was the maximum of growth inhibition observed. Treated promastigotes presented reduced motility and became round in shape further confirming the leishmanicidal activity of SNP. On the other hand, axenic amastigotes, besides being much more resistant to SNP-mediated cytotoxicity, did not show marked morphological alteration when incubated for 24 h, until 8 mM concentrations of this nitrosating agent were used. The cytotoxicity toward *L. amazonensis* was attenuated by reduced glutathione (GSH), supporting the view that SNP-mediated toxicity triggered multiple oxidative mechanisms, including oxidation of thiols groups and metal-independent oxidation of biomolecules to free radical intermediates.
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KEY WORDS — *Leishmania amazonensis*; nitric oxide; sodium nitroprusside; promastigotes; axenic amastigotes

INTRODUCTION

Leishmania parasites cause a disease which has been associated with different clinical forms, including cutaneous, hyperergic mucocutaneous, anergic diffuse cutaneous, and visceral Leishmaniasis. The disease is endemic in some geographical areas of world, where it constitutes a serious public health problem.[1,2] *Leishmania amazonensis* has been isolated from patients with all the different clinical forms of the disease.[3–5] The extracellular promastigote stage of this protozoan parasite is introduced into subcutaneous tissue in the human host during the bite of an infected sandfly vector. It is phagocytosed by a mononuclear phagocyte, after which it converts into the obligate intracellular amastigote form.[6] When cultivated in liquid medium *L. amazonesis* promastigotes transform from a less infectious form during log-phase growth to a highly infectious metacyclic promastigote when they reach stationary phase. The development is accompanied by an increased ability to survive intracellularly in macrophages. The amount and/or physicochemical structure of several promastigote molecules has been found to change during development to stationary phase in *Leishmania* sp. studies.[6,7]

Immune control of leishmaniasis involves a dominant Th1 response, leading to macrophage

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activation and elimination of intracellular amastigotes through the induction of nitric oxide synthase (NOS II) and nitric oxide (NO) synthesis from L-arginine. Given the necessity for avoiding the toxic effects of macrophage-generated oxidant agents in order to cause infection, several hyphotheses may account for the differential survival of the different forms of *Leishmania*.[8] The increased virulence that occurs during promastigote stationary phase and amastigote may be triggered in part by enviromental stimuli encountered by the promastigote during its life cycle, similar to the starvation-induced protection against both heat and oxidative killing observed in some bacteria.[9,10] *In vitro* models have documented that promastigotes attach to several receptors on the surface of the macrophage before phagocytosis.[11–13] This receptor-mediated ingestion is accompanied by an oxidative burst of the phagocyte, during which oxidants such superoxide (O_2^-) and NO are formed.[14–16]

NO donors are pharmacolocically active substances that, *in vivo* and *in vitro*, release NO.[17] All of them have nitrate functionality within the molecule, and a nitroso functional group is present in all of these compounds. In sodium nitroprusside (SNP) ($Na_2[Fe(CN)_5NO].2H_2O$) a molecule of NO is coordinated to iron metal forming a square bipyramidal complex (Figure 1). This nitroprusside dianion is a complex of ferrous ion with five cyanide anions (CN^-) and a nitrosonium in (NO^+). Interaction of SNP with a reducing agent such as a thiol leads to the formation of NO.[17–20] Being small and hydrophobic, NO can pass easily through membranes and as it persists *in vivo* for a few seconds, it can diffuse several cell diameters from its site of production. The transcellular diffusion of NO is more rapid than the rate of its intracellular reactions, consequently the steady-state NO concentration experienced by a cell is determined by the number of NO-producing cells nearby or by NO-releasing compounds.[21–23]

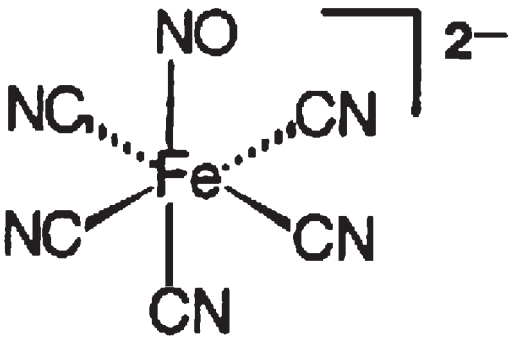

Figure 1. Sodium nitroprusside molecule structure

Low levels of NO have been implicated in cellular proliferation.[24] However, nitrosating agents have been shown to be highly microbicidal by killing *Escherichia coli*,[25–27] *Trypanosoma cruzi*,[28,29] and *L. amazonensis*.[30,31] Investigations on the mechanisms of *Leishmania* killing by mouse macrophages *in vitro* and *in vivo* have clearly identified NO and/or its many congeners (NO_2^-, NO_3^-, N_2O_3, N_2O_4, S-nitrosothiols, nitrosyl-metal complexes) as the cytotoxic molecule(s) most likely responsible for intracellular parasite destruction in experimental murine leishmaniasis.[31] The complexity of NO chemistry in oxygenated aqueous solution and studies of NO toxicity in the context of macrophage activation are rendered even more complex by the fact that NO can combine with other molecular released by phagocytes to generate further molecular species endowed with distinct toxic activities.[32–34] Therefore, the experiments described in this report were aimed at investigating comparatively, the resistance of metacyclic promastigotes and axenic amastigotes to SNP *in vitro*.

MATERIALS AND METHODS

Reagents

Benzamidine, trypsin inhibitor, penicillin G, KCl, leupeptin, L-glutamine, Schneider's Insect Medium, $MnCl_2$, H_2SO_4, α-isonitrosopropienophenone, sodium nitrite, Triton X-100, phenylmethylsulfonyl fluoride (PMSF), N-1-naphthylethylenediamine, phosphoric acid, sulfanilamide, sucrose, Tris HCl, SNP, and urea, were from Sigma Chemical Co., St. Louis, MO (USA). L-arginine was from Nutritional Biochemicals Corporation (USA). Ethanol was from Merck S.A. Indústrias Químicas (Brazil). Fetal calf serum (FCS) was from Gibco BRL (USA).

Parasites

L. amazonensis (MHOM/BR/77/LTB0016 strain), infective promastigotes, and axenic amastigotes were studied comparatively. Infective promastigotes (3rd passage) had a high percentage (~73%) of metacyclic forms, evaluated through a complement lysis test. Promastigotes were maintained in Schneider's Insect Medium supplemented with 10% of heat inactivated FCS at 26°C and pH 7.2. Axenic amastigotes were also cultivated in the same medium, but with 20% FCS at 32°C and pH 5.5.[35]

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Assessement of SNP-mediated effect on parasite survival

Baseline viability for promastigotes was quantified microscopically according to flagellar motility by grading a minimum of 200 promastigotes as motile (viable) or non-motile (nonviable). Usually over 98% of promastigotes were viable prior to SNP exposure. Experiments with <95% baseline viability were discounted. Axenically grown axenic amastigotes appeared homogeneous, round to ovoid in shape, without apparent flagella and non-motilic. The parasite cultures in 24-well flat-bottom plates (4×10^6 cells/well) were treated with SNP (300 μl final volume). Promastigotes and axenic amastigotes were incubated in 0.25–8.0 mmol L^{-1} SNP for 24 h. During some viability assays, parasites were incubated with SNP plus 8 mmol L^{-1} glutathione (GSH) or only GSH. The final pH of the incubations was routinely checked and did not exceed 7.0 for promastigotes and 5.5 for axenic amastigotes cultures unless stated.

NO/nitrite measurement

After 24 h to SNP exposure, parasites were counted in a Neubauer's chamber at 40× magnification after suitable dilution in medium, centrifuged at 1500*g* for 10 min, and the supernatants were collected. The pellet was added to 0.5 ml of 0.1% Triton X-100 in an antiproteolytic buffer containing 0.1 mmol L^{-1} PMSF, 0.01% w/v leupeptin, 0.2 mg ml^{-1} trypsin inhibitor and 1 mM benzamidine in 0.25 mol L^{-1} sucrose and 5 mmol L^{-1} KCl for arginase activity assay.

Nitrite, a byproduct of NO, was measured spectrophotometricaly by adding Griess reagent (0.1% N-1-naphtylethylenediamine in 5% phosphoric acid and 1% sulfanilamide) to the same volume of parasite (promastigotes or axenic amastigotes) culture supernatants. After 10–15 min at room temperature, the absorbance of the chromophore was measured at 540 nm (μ-Quant Spectrophotometer, BIO-TEK Instruments Inc., Vermont, USA). Nitrite concentrations were estimated by comparison with a standard curve prepared with sodium nitrite in Schneider's medium.[36–40]

Arginase activity from parasites

Arginase activity from *L. amazonensis* promastigotes and axenic amastigotes was verified as previously described,[41] with some modifications.[42] Briefly, pellets (100 mg ml^{-1}) previously washed with a solution of 0.25 mol L^{-1} sucrose and 5 mmol L^{-1} KCl were added to 0.5 ml of 0.1% Triton X-100 in an antiproteolytic buffer containing 0.1 mmol L^{-1} PMSF, 0.01% w/v leupeptin, 0.2 mg ml^{-1} trypsin inhibitor and 1 mmol L^{-1} benzamidine in 0.25 mol L^{-1} sucrose and 5 mmol L^{-1} KCl. The mixture was then stirred for 30 min at room temperature. After the cells were lysed, 0.5 ml of 25 mmol L^{-1} Tris-HCl plus 5 mmol L^{-1} of $MnCl_2$, pH 7.4 were added and the enzyme was activated for 10 min at 56°C. Arginine hydrolysis was initiated by the addition of 25 μl of 0.5 mol L^{-1} L-arginine, pH 9.7 to a 25 μl aliquot of the previously activated lysate. Incubation was performed at 37°C for 60 min and the reaction was stopped by the addition of 400 μl of an acid mixture containing H_2SO_4, H_3PO_4, and H_2O (1:3:7). The urea formed was colorimetrically quantified at 540 nm after addition of 25 μl of 9% α-isonitrosopropienophenone (dissolved in 100% ethanol) and heating at 100°C for 45 min. After 10 min in the dark, the optical absorbance was determined in a spectrophotometer (μ-Quant Spectrophotometer, BIO-TEK Instruments Inc., Vermont, USA) using 200 μl aliquots in a non-sterile micro-culture plate. A calibration curve was prepared with increasing amounts of urea between 1.5 and 30 μg ml^{-1}.

Light microscopy

After 24 h incubation of parasites, motility or viability were reassessed microscopically. Pictures were taken at ×40 magnification under a Zeiss Axiolab microscope (Carl Zeiss, Inc.) in a Trypan Blue exclusion test. The values are presented as percentage of parasites that remained viable after incubation with SNP and/or GSH. The imaging system used to detect alterations of parasite morphology consisted of a digital DSC-W30 Sony camera fitted with a zoom microscope-magnifying lens producing enhanced black and white images yielding image tiles of 6.0 megapixels, zoom 4.4.

Data analysis

Three independent experiments in duplicate were performed and the data obtained with different treatments were analyzed statistically by one-way ANOVA and Fisher's exact test ($p < 0.05$). The figures show representative experiments demonstrating consistently repeatable patterns of the toxic effects of SNP on parasite viability.

RESULTS

Considerable variation in susceptibility of parasite number to SNP-mediated injury was noted over the

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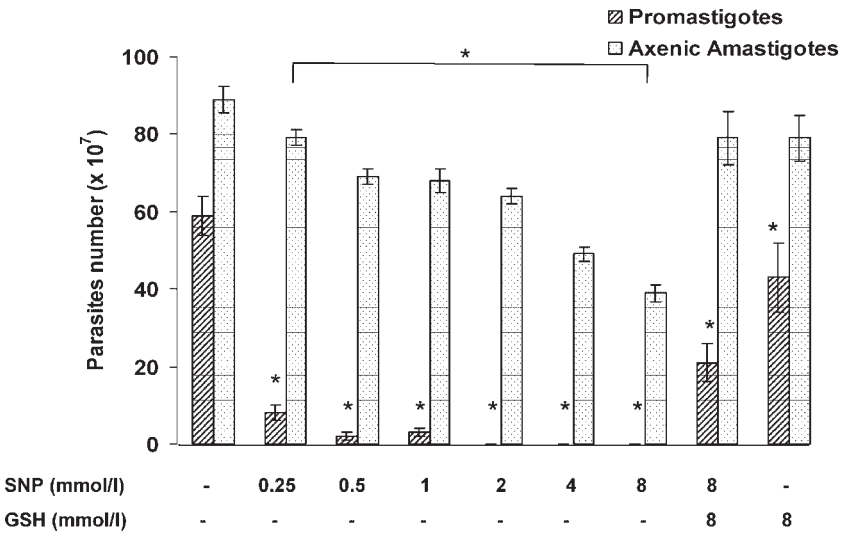

Figure 2. Effect of SNP on the death of *L. amazonensis* infective promastigotes (empty symbols) and axenic amastigotes (filled symbols) demonstrating relative sensitivities to SNP-induced toxicicity. Suspensions of parasites in Schneider's medium were exposed or not to SNP at 26°C 7.2 (for promastigotes) and pH 5.5 (for axenic amastigotes) for 24 h; after which time the cells were counted in a hemocytometer and the viability was reassessed microscopically by Trypan Blue exclusion. Results are expressed as the mean and standard deviation of three independent experiments made in duplicate

course of our experiments, as reflected in Figure 2. This variability was probably due to a number of factors, including the enriched medium, time, and cultivation *in vitro*. Examining the *in vitro* toxicity of SNP towards *L. amazonensis* promastigotes and axenic amastigotes in Schneider's medium revealed a dose-dependent decrease of parasite number. Treatment of *L. amazonensis* promastigotes with SNP, a potent NO donor, led to a dramatic loss of parasite viability with regard to morphological changes, as well as a concentration-dependent decrease of parasite number by 98% (4 mmol L^{-1}) and 99% (8 mmol L^{-1}). The sensitivity of axenic amastigotes was significantly less to SNP at all the concentrations tested ($p = 0.0001$). About 50% of axenic amastigotes exposed to 4.0 or 8.0 mmol L^{-1} SNP were killed within 24 h of incubation. Figure 3 shows *in situ* analysis of control *L. amazonensis* infective promastigotes and axenic amastigotes and morphological changes observed after 24 h incubation of parasites with SNP in different concentrations. The addition of GSH at 8.0 mmol L^{-1} restore promastigotes and axenic amastigotes viability, and striking morphological change was observed under the microscope (Figures 3p and q). Plots represent parasites that remained viable after SNP exposure during a representative experiment.

Figure 4 shows nitrite measurements on supernatant of parasites culture after 24 h incubation. Late log-phase promastigotes and axenic amastigotes of *L. amazonensis* were incubated at the indicated concentrations of SNP or/and GSH. The effect of different concentrations of SNP on arginase activity of promastigotes and axenic amastigotes is shown in Figure 5. No apparent difference in arginase activity among the tested groups was observed, except for 8 mmol L^{-1} SNP ($p = 0.003$).

DISCUSSION

Several factors beyond those addressed in this study contribute to the parasite's ability to survive macrophage-derived oxidant-mediated toxicity, especially related to the resistance of axenic amastigotes to SNP. *Leishmania*-infected murine macrophages have diminished capacity to undergo an oxidative response,[43] suggesting that parasites may partially inhibit their oxidative capacities, involving negative feedback of the NO pathway between *Leishmania* and host cell.[39,44–46]

Oxidative stress is the major cause of damage associated with elevated NO.[47,48] NO and its products react with a large number of biological molecules[49] contributing to the breadth of its signaling and

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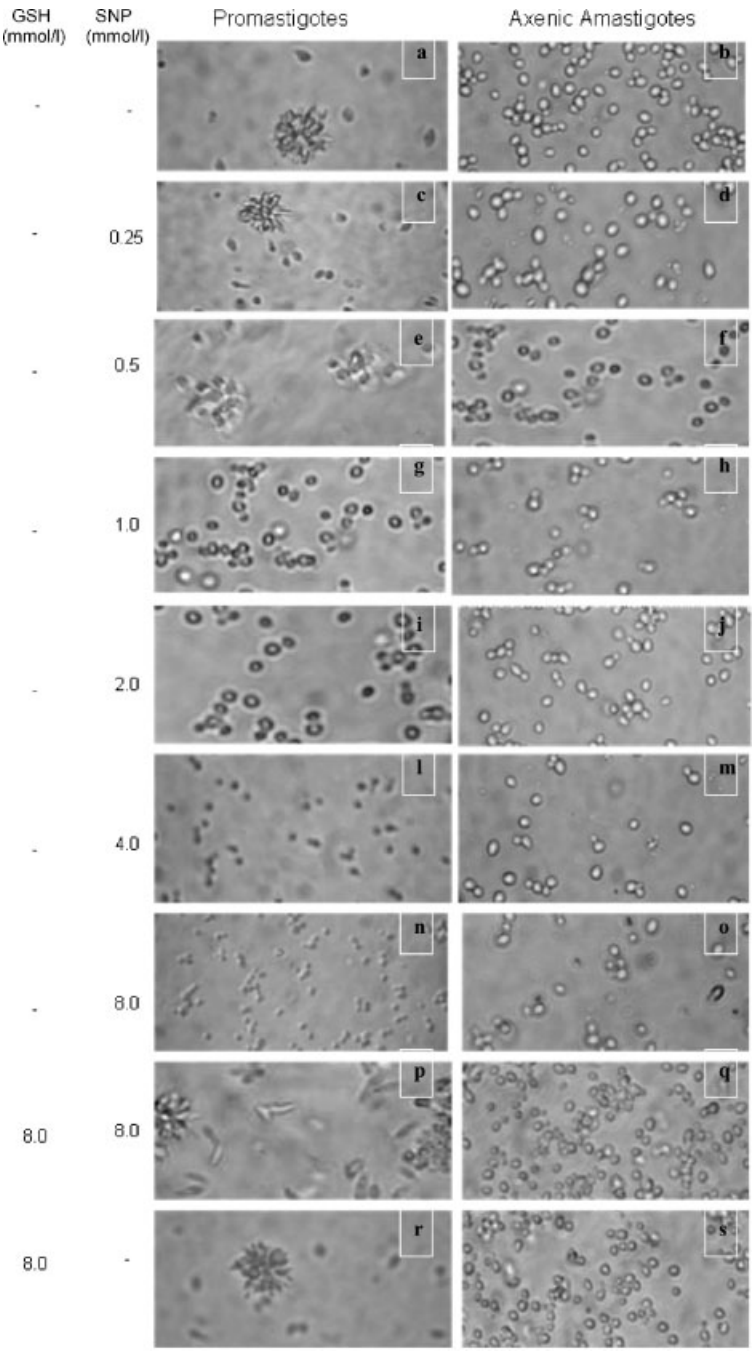

Figure 3. *In situ* analysis of control *L. amazonensis* infective promastigotes and axenic amastigotes and morphological changes observed after 24 h incubation of parasites with SNP in different concentrations. A light microscopy view of: (a) untreated *L. amazonensis* promastigotes and (b) axenic amastigotes, as a negative control; (c,e,g,i,l,n) *L. amazonensis* promastigotes treated with 0.25, 0.5, 1.0, 2.0, 4.0 and 8.0 mmol L^{-1} SNP respectively. Letters d,f,h,j,m,o indicate *L. amazonensis* axenic amastigotes treated with the same concentrations above cited of SNP; (p) cytotoxic effect of 8.0 mmol L^{-1} SNP attenuated by 8.0 mmol L^{-1} GSH on promastigotes and (q) axenic amastigotes; (r) promastigotes and (s) axenic amastigotes treated 24 h only with 8.0 mmol L^{-1} GSH as a positive control. Plots represent parasites that remained viable after SNP exposure during a representative experiment. Magnification 40 x.

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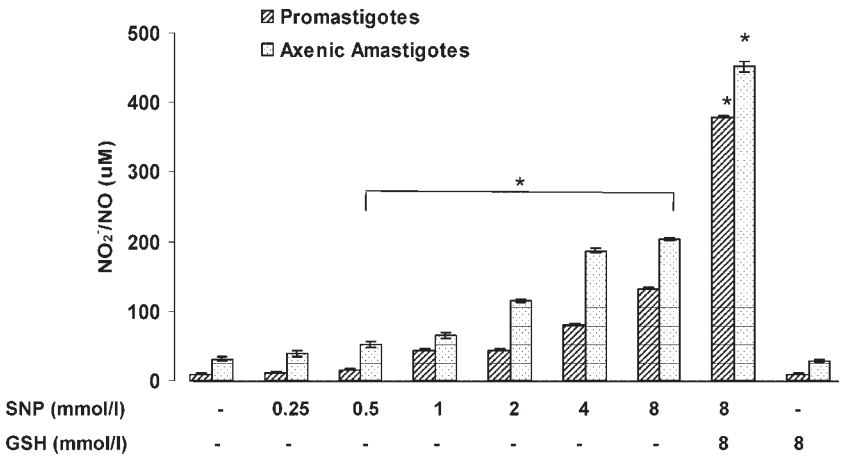

Figure 4. Nitrite measurement on supernatant of parasite cultures after 24 h incubation. Late log-phase promastigotes (empty symbols) and axenic amastigotes (filled symbols) of *L. amazonensis* were incubated in indicated concentrations of SNP or/and GSH. The results represent the average of triplicate cultures + SEM

deleterius effects. Reaction of O_2^- with NO forms the short-lived peroxynitrite ($ONOO^-$, half-life <1 s) which is far more reactive and damaging than its precursors.[50] Peroxynitrite oxidatively damages a wide range of biological molecules, including proteins, lipids, and nucleic acids, and also oxidizes thiols, nitrates protein tyrosine residues and damages mitochondria.[48,51] NO excess also causes disturbances in energy metabolism and in calcium homeostasis, and all of these disturbances can lead to cell death by apoptosis or necrosis, depending on the context and severity of the damage.[52] Some studies found that several targets may be affected by NO toxicity in eukaryotic cells. These targets include metabolic enzymes such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), aconitase,[53,54] and cysteine proteinase.[55]

In our experiments, exposure of *Leishmania* promastigotes or axenic amastigotes to a strong NO donor—SNP—in the millimolar range led to loss of viability of the microorganisms, as determined from inhibition of motility and the capacity to multiply,

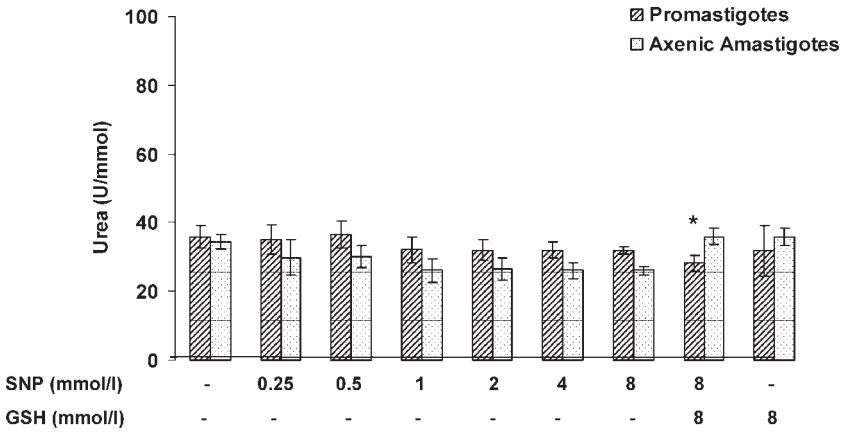

Figure 5. Effect of different concentrations of SNP on arginase activity of promastigotes (empty symbols) and axenic amastigotes (filled symbols). After SNP 24 h incubation, parasite cultures were centrifuged $10.000 \times g$, lysed with an antiproteolytic buffer and then submitted to measurement of arginase activity as described by Corraliza and collaborators (1994),[41] with some adaptations. No apparent difference on arginase activity among the tested groups was observed, except in 8 mmol L^{-1} SNP ($p = 0.003$)

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respectively. The cytotoxic properties of SNP towards *L. amazonensis* promastigotes were evident from examination of the parasites by light microscopy (Figure 3). Untreated cells (Figure 3a) remained highly mobile and spindle shaped over 24 h incubation time, whereas treated cells became non-mobile and round in shape (Figures 3c, e, g, i, l, and n). Similar parasite alterations have been described for *L. amazonensis* promastigotes treated with lytic sera[56] and *T. cruzi* epimastigotes treated with peroxynitrite.[57] Similarly, promastigotes or amastigotes of *L. enriettii* demonstrated a very rapid drop in oxygen uptake, reaching significant levels after 5 min of exposure to NO_2^- at pH 5.0 and falling to less than 20% of the starting oxygen consumption after 30 min.[58]

In this study, we showed that the leishmanicidal effect of a NO donor seemed to be the consequence of the induction of apoptosis, with more evident morphological changes in promastigotes. However, NO donors can also induce a pattern of DNA fragmentation typical of an apoptotic process in axenic amastigotes of *L. amazonensis*.[59] Holzmuller's group investigated the genomic DNA status of NO-treated *L. amazonensis* amastigotes and verified a strong correlation between nuclear DNA fragmentation, increased NO_2^- levels, and antileishmanial activity. We verified that the leishmanicidal effect of SNP was attenuated (34% for promastigotes and 97% for axenic amastigotes) by 8 mmol L^{-1} GSH. This effect was observed also at a mophological level, especially in *L. amazonensis* promastigotes (Figure 3p). GSH is the major intracellular antioxidant thiol protecting mammalian cells against oxidative stress induced by oxygen- and nitrogen-derived reactive species. In Trypanosomes and Leishmanias, trypanothione plays a central role in parasite protection against mammalian host defence systems by recycling trypanothione disulfide by the enzyme trypanothione reductase (TR).[60] Although Kinetoplastida parasites lack GSH reductase, they maintain significant levels of GSH. We also verified the higher resistance of axenic amastigotes to SNP (Figure 3) than promastigotes, corroborating with recent results of our Laboratory, that showed higher TR activity in axenic amastigotes than in promastigotes.[60] The hypothesis that SPN-mediated oxidation of thiol groups critical for parasite survival is a major mechanism accounting for its trypanocidal activity is supported by Gatti's work[30] and our results, demonstrating a protective effect of GSH against leishmanicidal effects of oxidants.

Arginase is an enzyme involved in the urea cycle, responsible for the conversion of arginine to urea and ornithine. Promastigotes of *Leishmania* have been previously shown to exhibit arginase activity.[61] However, our results demonstrated that promastigotes and axenic amastigotes SNP treated lacked a direct correlation between nitrosative effect and arginase activity. Only at 8 mmol L^{-1} GSH arginase did activity of promastigotes and axenic amastigotes demonstrate a significant difference, that was restored when 8.0 mmol L^{-1} GSH plus 8.0 mmol L^{-1} SNP were present in culture medium for both developing stages of parasite. However, this phenomenon could be related from the known reactivity of the oxidant with amino acid (e.g., L-arginine)[62] and sera constituents.[63] On the other hand, in this study we showed that the leishmanicidal effect of SPN is likely the consequence of the induction of a programmed cell death-like process in *L. amazonensis* verified by Holzmuller's group,[59] without correlation with L-arginine metabolism by arginase from the parasite. An apoptosis-like cell death pathway could represent an important and highly regulated mechanism used for the clearance of *Leishmania* within infected macrophages when stimulated to produce NO endogenously or during treatments with NO-releasing drugs. Based on the data presented here we hypothesize that increased resistance of axenic amastigotes to the toxic effects of SNP contributes to the *in vitro* development of virulence in *L. amazonensis*. The investigation of an apoptotic process in parasites and the possible feedback with the NO pathway within the parasite from NO-treated *Leishmania* sp. might merit further research.

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3.3.2 ARTIGO 5

Amastigote forms of the parasite *Trypanosoma cruzi* are more resistant to nitric oxide than trypomastigote ones. An in vitro assay with sodium nitroprusside.

Short title: *T.cruzi* and resistance to sodium nitroprusside

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Abstract

Despite the myriad of molecules produced by the immune cells against *Trypanosoma cruzi*, including NO, this protozoan generally by pass the adversities and is able to establish the infection. Studies on the evasion mechanisms in different protozoans can supply important tracks to better understand the interaction of parasites with their hosts. Several hypotheses have been done on the ability of the *T. cruzi* to modulate the host cell response, however, this is the first time that the resistance of trypomastigote (both bloodstream and MK2 cell culture derived) and amastigote (MK2 cell culture derived) forms of *T. cruzi* to NO toxic effects were directly tested *in vitro*. Using a chemical NO donor, the sodium nitroprusside (SNP), we evaluated the effect of NO on *T. cruzi*, and demonstrated that NO is not such a straight trypanocidal molecule, at least not for the amastigote forms. At high dosages of released NO by SNP amastigotes were able to survive to the toxic effects of NO, showing that these multiplicative forms are far more resistant than trypomastigote ones. Additionally the trypomastigote forms seem to undergo into a differentiation process, assuming a round shape and losing its apparent flagellum. Our findings points to amastigotes as the more resistant life stage of *T.cruzi* regarding to NO, and also suggest that the oxidative stress might be related to the differentiation process of this protozoan parasite.

Keywords: *Trypanososma cruzi*; nitric oxide; sodium nitroprusside; trypomastigotes; amastigotes.

Introduction

Trypanosoma cruzi is an obligate intracellular parasite with a complex life cycle involving two different hosts, one invertebrate, the Reduviidae bugs generally called barber bug; and the vertebrate, which can be several different mammals, including humans. When the infection is established in humans it will cause the pathology named Chagas disease, considered one of the most important public health problems in Latin America, where several million people are infected (1).

The parasite life cycle involves an extracellular, proliferative stage (epimastigote) that resides in the insect vector, and two forms that occur in the mammalian host, a nonproliferating, infective form (trypomastigote) and an intracellular, proliferative form (amastigote) (2).

Unlike many other pathogens that adeptly avoid fusion with host cell lysosomes, transient residence within an acidic lysosomal compartment is a pre-requisite for *T. cruzi* survival. Exposure of trypomastigotes to the acidic environment of the lysosomal vacuole is thought to be an important trigger for trypomastigote to amastigote differentiation, a process that is initiated in the vacuole and completed in the cytoplasm (3).

Immune control of *T. cruzi* has been linked to a dominant Th1 response, leading to macrophage activation and elimination of intracellular amastigotes through the induction of nitric oxide synthase (NOS II) and nitric oxide (NO) synthesis from L-arginine (4-7). However, studies *in vivo* have demonstrated that the parasite is able to manipulate the NO pathway of the host cell and resist to the NO attack (8-10). Given the necessity to avoid the toxic effects of

macrophage-generated NO, different hypotheses might account for the differential survival of the different forms of *T. cruzi* (2, 7, 11).

For instance, it has been proposed that the parasite is able to induce the expression of arginase in the host cell, an enzyme which competes with nitric oxide synthase (NOS) by its substrate, the L-arginine. The induction of arginase would lead to lower levels of NO and, in parallel, higher production of polyamines, the final metabolite of arginase. Polyamines are used for the parasite growth and differentiation (9, 12).

Another possible mechanism involved in the decrease of NO levels released by the host cell is the modulation of its Th1/Th2 cytokines profile, by the parasite. During the infection of macrophages with *T. cruzi*, the engulfment of apoptotic T cells leads to production of TGFβ, a known down regulator of NO production (8). However, how the parasite survives to the NO hostile response is not well understood.

The use of chemical NO donors such as the sodium nitroprusside (SNP) is helpful to evaluate both the resistance of microorganisms to this molecule and the amount of NO needed to cause toxicity (13-15). NO donors are pharmacologically active substances, with a functionality active nitrate and nitroso group within the molecule. These compounds are able to release NO both, *in vivo* and *in vitro*, (16). SNP ($Na_2[Fe(CN)_5NO].2H_2O$), is a complex of ferrous ion with five cyanide anions (CN^-) and a nitrosonium (NO^+). Interaction of SNP with a reducing agent such as a thiol leads to the formation of NO (16-19).

Using the SNP against *Leishmania amazonensis*, *in vitro*, our group has recently demonstrated that although SNP induced loss of viability in both

promastigotas and axenic amastigotes the latter parasite forms were statistically more resistant to the effects of NO than the promastigotes (13).

In the present work, we evaluated for the first time, by the use of the SNP, the effects of different doses of NO towards directly the trypomastigote and amastigote forms of *T. cruzi*, *in vitro.* We demonstrate differential resistance of the parasite forms to NO with clear higher resistance of amastigotes as compared to trypomastigotes and suggest the possible participation of NO in the differentiation process of this protozoan.

Materials e Methods

Reagents

Benzamidine, trypsin inhibitor, penicillin G, KCl, leupeptin, *L*-glutamine, RPMI Medium, $MnCl_2$, H_2SO_4, sodium nitrite, Triton X-100, phenylmethylsulfonyl fluoride (PMSF), N-1-naphtylethylenediamine, phosphoric acid, sulfanilamide, sucrose, Tris.HCl and SNP, were from Sigma Chemical Co., St. Louis, MO (USA). L-arginine was from Nutritional Biochemicals Corporation (USA). Ethanol was from Merck S.A. Indústrias Químicas (Brazil). Fetal bovine serum (FBS) was from Gibco BRL (USA).

Parasites

T. cruzi (Y strain) trypomastigote forms were maintained in vitro by infection of MK2 cell lineage. Semi-confluent MK2 cell cultures maintained in RPMI-1640 medium (Sigma, USA) supplemented with 10% FBS were infected (ten parasites per cell) and after 5–7 days the differentiated trypomastigotes or cell culture supernatants were harvested.

To obtain amastigote forms, the infected cell cultures were left for 10 days at 37°C , replacing with new medium every 48h. The supernatant was left at the same temperature for 1h, after this time the upper part was discarded and amastigotes were collected from the bottom of the tube.

Evaluation of SNP-mediated effect on parasite survival

Trypomastigotes and amastigotes were incubated with 0.25 to 8.0 mmol/L SNP for 24 hours. The final pH of the incubations was routinely checked and did not exceed 7.0. Parasite cultures in 24 well flat-bottom plates (4 x 10^6 cells/well) were treated with 300 μL of SNP (final volume). Baseline viability for parasites was quantified under light microscopy using a Neubauer chamber. Usually over 98% of parasites were viable prior to SNP exposure as determined by the trypan blue test. Trypomastigotes were evaluated according to flagellar motility; by grading a minimum of 100 trypomastigotes as motile (viable) or nonmotile (nonviable). Amastigotes appeared homogeneous, round to ovoid, without apparent flagellum and presenting Brownian movements. Experiments with <95% baseline viability were discarded.

NO/Nitrite measurement

After 24 h to SNP exposure, parasites were counted in a Neubauer chamber at a 40 x magnification after adequate dilution of the parasites in medium, then they were centrifuged at 1.500 g for 10 minutes and the supernatants collected.

Nitrite, a byproduct of NO, was measured spectrophotometricaly by adding Griess reagent (0.1 % N-1-naphtylethylenediamine in 5% phosphoric acid and 1 % sulfanilamide) to the same volume of culture supernatants were taken from controls and treated parasite cultures (trypomastigotes and amastigotes). After 10 -15 minutes at room temperature, the absorbance of the

chromophore was measured at 540 nm (μ-Quant Spectrophotometer, BIO-TEK Instruments Inc., Vermont, USA). Nitrite concentrations were estimated by comparison with a standard curve prepared with sodium nitrite in RPMI medium (20)

Data analysis

Three independent experiments in duplicate were performed. The data obtained with different treatments were analyzed statistically by 1-way ANOVA and Fisher's exact test ($p < 0.01 – 0.001$). Figures contain representative experiments demonstrating consistently repeatable patterns of the toxic effects of SNP on parasite viability.

Results

To evaluate the effect of NO upon *T. cruzi*, trypomastigote and amastigote forms were incubated for 24hours with different concentrations of SNP (0.25mmol/L; 4.0mmol/L; 8.0mmol/L).

Trypomastigotes forms were highly sensitive to the effects of the nitric oxide released by SNP, in a dose dependent manner. After 24h of incubation with a low dose of the NO donor (0.25mmol/L), we observed a significant rate of death. There was a reduction of 48% and 42% in the number of viable parasites derived from blood and cell cultures, respectively (Fig 1). The death rate increased up to 75.2% and 74.6%, respectively, at the concentration of 4.0mmol/L of SNP (Fig 1). At the concentration of 8.0mmol/L, trypomastigotes could barely handle the toxicity of the NO, and only 14.1% (blood derived) and 22.9% (MK2 derived) of the parasites showed viability (Fig 1). The total numbers of viable trypomastigotes obtained from the blood and cell cultures were not significantly different at any given concentration.

We next evaluated the morphology of the cells which survived to the SNP treatment compared to controls. By light microscopy, we observed that most cells collected after incubation with different doses of SNP exhibited consistent morphological alterations. These changes were characterized mainly by loss of flagellum and acquirement of round shape. Of note, these rounded parasites were provided of incredible moving force (data not shown).

However, when toxicity of SNP was evaluated on amastigote forms, we observed that there was no significant death with the dosages of 0.25mmol/L and 4.0mmol/L. Non-viable amastigotes were observed only when the higher

dosage of SNP (8.0mmol/L) was used. At this SNP concentration, the percentage of non viable parasites rate was 54.5%, being statistically different from the controls, but significantly higher as compared to those found on trypomastigote forms (Fig 1). Also, in these forms we could observe a remarkable increase in motility (data not shown). When compared with trypomastigotes obtained from blood, amastigotes exhibited a significant higher survival rate (30%) under 8.0μmmol/L of SNP treatment (Fig 1).

Functionality of SNP was assessed by the evaluation of the NO levels in all cultures. The production of NO was SNP-dose dependent, increasing in all doses (Fig 2). Moreover, NO production was differentially observed when untreated trypomastigotes and amastigotes were compared. As is shown in the figure 3, trypomastigotes obtained from blood were able to produce significantly higher levels of NO (mean ± SEM of 4.9 ± 1.3 mmol/L) ($p < 0.001$) when compared to amastigote forms (mean ± SEM 2.3 ± 0.6 mmol/L). Trypomastigote forms from the MK2 cell culture were also evaluated, and also showed a significant higher amount of NO in comparison with amastigotes (mean ± SEM 5.0 ± 0.4 mmol/L) ($p < 0.01$) (Fig 3). When the ability to produce NO was compared between trypomastigote forms from the blood and those obtained from MK2 cells, there was no significant difference (Fig 3).

Discussion

The literature provides a bulk of data showing that *T. cruzi* can somehow interfere with the NO pathway of the host cells by down regulating the NO metabolic pathway (9, 12, 21). However, despite the myriad of molecules produced by the immune cells against this protozoan, including NO, generally it by pass the adversities and is able to establish the infection (7, 22, 23).

The acute phase of experimental *T. cruzi* infection is characterized by increased formation of eicosanoids as documented by several authors (24-27). Our group has previously demonstrated that, during this phase of *T. cruzi* infection, peritoneal macrophages from infected rats are able to produce prostaglandin E_2 (28). Further studies from our group showed that, monocytes isolated from infected rats during the acute phase of *T. cruzi* infection do not produce high levels of NO, on the contrary, these cells present high levels of arginase, a enzyme which competes with NOS by its substrate, the amino acid L-arginine (21).

It has been also proposed that a common mechanism where different parasites, including trypanosomes, escape from NO toxicity is the activation of arginase which leads to a depletion of L-arginine, resulting in lower levels of NO and increased production of polyamines, which are molecules involved in parasite growth and differentiation (8, 9, 12). It has also been shown that heat-treated or glutaraldehyde-fixed trypomastigotes of *T. cruzi* significantly decrease NO production by interferon-γ activated macrophages (29) and the possibility that NO would down-regulate the NOS by itself has also been proposed (30).

However, this is the first time that the resistance of trypomastigote (both

from blood and MK2 cell culture derived) and amastigote (MK2 cell culture derived) forms of *T. cruzi* to NO toxic effects directly assayed *in vitro*.

Using different doses of SNP to evaluate the effect of NO on *T. cruzi*, we demonstrate that NO is not such a straight trypanocidal molecule, at least not for the amastigote forms. Once even at high concentrations of NO released by SNP, 53.5% of the amastigotes were still viable, being far more resistant than trypomastigote ones.

Our observation that *T. cruzi* amastigotes are quite more resistant to NO than trypomastigotes is in agreement with our previous work with *L. amazonensis,* in which we point to amastigotes as the more resistant life stage of this intracellular pathogen. When treated with the same doses, of the same NO donor used in this work, promastigotas of *L. amazonenzis* showed dramatic loss of viability (99% at 8.0μM of SNP) while on amastigotes forms the loss of viability was about 50%. Also as observed on promastigotas of *L. amazonesis* in that work, herein the evaluated trypomastigotes from *T. cruzi* exhibited the same dramatic morphological changes, such as loss of the apparent flagellum and changing in its shape (from elongated to rounded) (13).

Even though NO is considered a pivotal killing molecule against parasites (31-33)), including *T. cruzi* (4-6, 34-37), there are data in the literature proposing its homeostatic, and even a protective, role in mammals, for example, in cellular proliferation (38), molecular signaling through the PKG pathway (39) and protection against apoptosis (40). The physiological role of NO was also reported in lower eukaryotes such as in *Leishmania sp.*, in which the NO could play a role in its infectivity (41) and *T. cruzi*, in which the NO has been linked with its flagellum motility (42) and protection against apoptosis (10).

In the present study we observed, under light microscopy, that the trypomastigote forms seem to undergo into an amastigote-like form after being in contact with NO. These changes in morphology seems to be dose dependent, once at higher dosages we found higher number of rounded shape forms lacking an apparent flagellum (data not shown). These observations added to the ability of amastigotes into survive at high doses of NO, allow us to speculate a possible role for NO in the differentiation process of this parasite.

Surely a more detailed study is necessary to confirm a role of the NO in the differentiation process and maintenance of chronic infection ability of *T. cruzi*. Electron microscopy assays are being performed by our group to add new information in this field.

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Figure Legends :

Figure 1: Effect of SNP on the viability of *T. cruzi* trypomastigotes (from blood sources and MK2 cell culture derived) and amastigotes (MK2 cell culture derived) demonstrating relative sensitivities to SNP-induced toxicicity. Values are expressed as mean ± SEM and are representative of three independent experiments. Statistical analysis by 1-way ANOVA (p <0.01 to 0.0001).

Figure 2: Nitrite measurement on supernatant of *T. cruzi* trypomastigotes (from blood sources and MK2 cell culture derived) and amastigotes (MK2 cell culture derived) after 24 h incubation with SNP (0.25 to 8.0 μM). Values are expressed as mean ± SEM and are representative of three independent experiments. Statistical analysis by 1-way ANOVA (p <0.01 to 0.0001).

Figure 3: Nitrite measurement on supernatant of *T. cruzi* trypomastigotes (from blood sources and MK2 cell culture derived) and amastigotes (MK2 cell culture derived) after 24 h incubation only in RPMI medium at 37 ^{o}C. Values are expressed as mean ± SEM and are representative of three independent experiments. Statistical analysis by 1-way ANOVA (p <0.01 to 0.0001).

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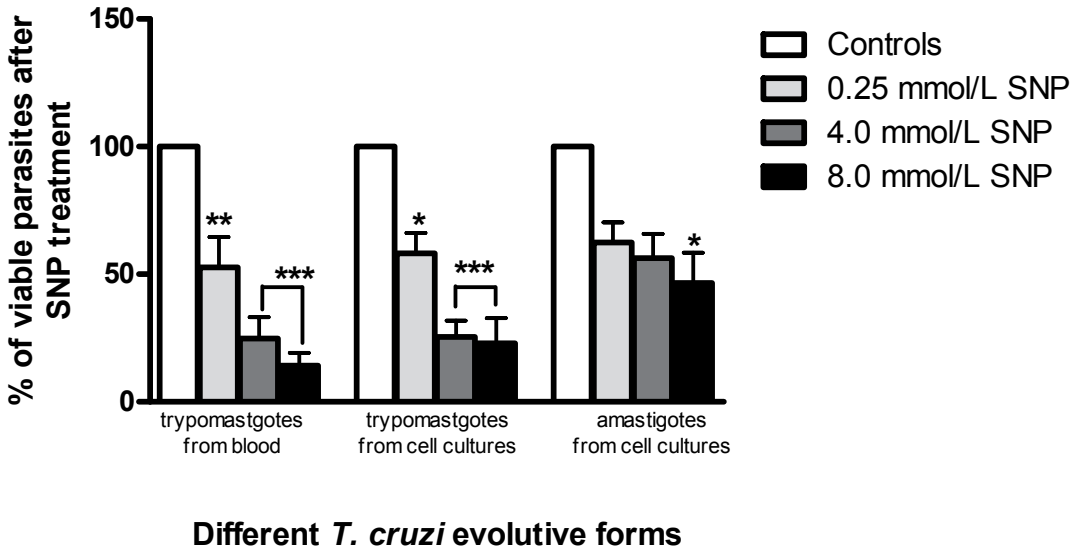
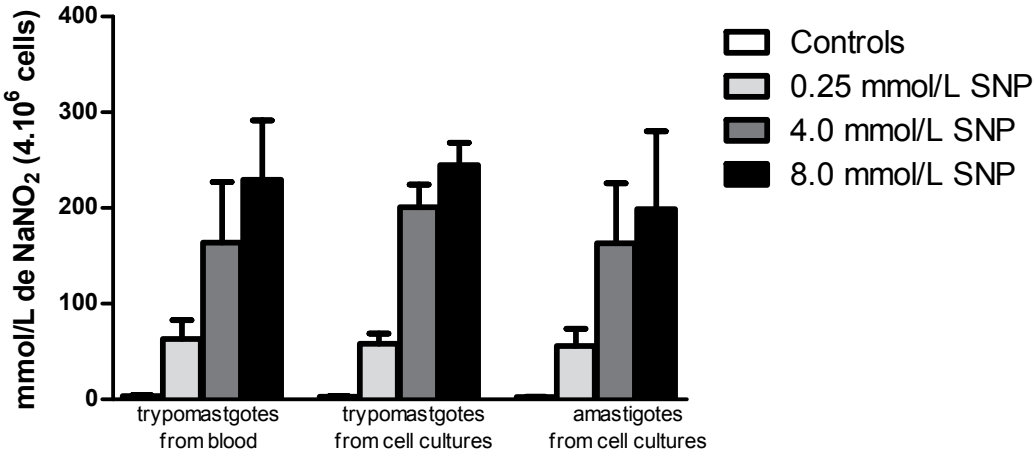
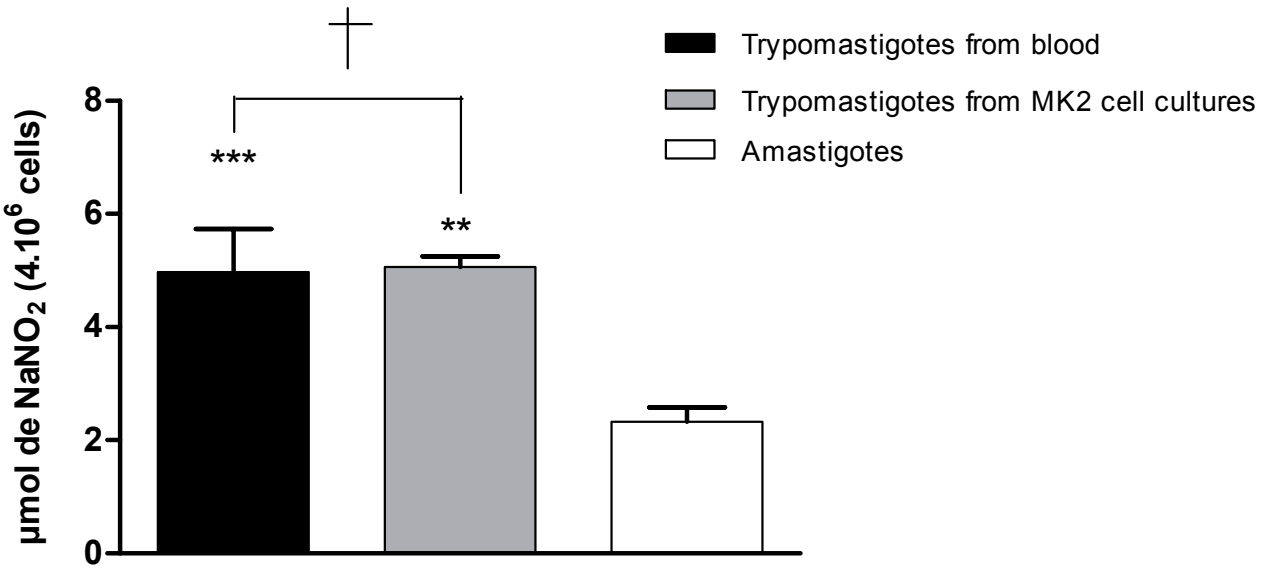

% of viable parasites after SNP treatment
150
100
50
0
**
***
*
***
*
trypomastgotes from blood
trypomastgotes from cell cultures
amastigotes from cell cultures
Controls
0.25 mmol/L SNP
4.0 mmol/L SNP
8.0 mmol/L SNP
Different T. cruzi evolutive forms



Different *T. cruzi* evolutive forms

Figure - 3


Trypomastigotes from blood
Trypomastigotes from MK2 cell cultures
Amastigotes
µmol de NaNO2 (4.10^6 cells)
8
6
4
2
0
***
**
†

3.3.3 ARTIGO 6

Porins facilitate nitric oxide-mediated killing of mycobacteria

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Summary

Non-pathogenic mycobacteria such us *Mycobacterium smegmatis* reside in macrophages within phagosomes that fuse with late endocytic/lysosomal compartments. This sequential fusion process is required for the killing of non-pathogenic mycobacteria by macrophages. Porins are proteins that allow the influx of hydrophilic molecules across the mycobacterial outer membrane. Deletion of the porins MspA, MspC and MspD significantly increased survival of *M. smegmatis* in J774 macrophages. However, the mechanism underlying this observation is unknown. Internalization of wild-type *M. smegmatis* (SMR5) and the porin triple mutant (ML16) by macrophages was identical indicating that the viability of the porin mutant *in vivo* was enhanced. This was not due to effects on phagosome trafficking since fusion of phagosomes containing the mutant with late endocytic compartments was unaffected. Moreover, in ML16-infected macrophages, the generation of nitric oxide (NO) was similar to the wild type-infected cells. However, ML16 was significantly more resistant to the effects of NO *in vitro* compared to SMR5. Our data provide evidence that porins render mycobacteria vulnerable to killing by reactive nitrogen intermediates within phagosomes probably by facilitating uptake of NO across the mycobacterial outer membrane.

Keywords: mycobacterium, phagosome, porins

1. Introduction

M. smegmatis is a non-pathogenic mycobacteria that is phagocytosed by macrophages and killed within phagosomes by a combination of factors delivered from lysosomes such as reactive nitrogen intermediates (RNI), and likely other still unidentified components [1, 2]. As *M. smegmatis* is killed, these bacteria are a useful model to study the mechanisms that macrophages employ to eliminate intracellular mycobacteria [1, 3].

Once in the phagosome, *M. smegmatis* is exposed to the antimicrobials present in the intra-phagosomal environment and subsequently killed [1, 4]. One mechanism of intra-phagosomal killing of mycobacteria is the delivery of lysosomal enzymes [1, 4]. The acquisition of hydrolases by phagosomes is thought to be dependent on phagosomal fusion with late endosomes and lysosomes [1, 4]. One of the anti-mycobacterial components present in the phagosome is nitric oxide (NO) and other reactive nitrogen intermediates (RNI) to which NO is converted [5]. These compounds are generated in macrophages mainly by the inducible nitric oxide synthase (iNOS) [5]. The production of RNI at the acidic phagosomal pH generates a “nitroxidative” stress that is considered a very potent antimicrobial mechanism [6].

It is unknown how NO and the other toxic RNI enter into mycobacterial cells. It was recently demonstrated by cryo-electron microscopy that mycobacteria have an outer membrane [7, 8]. The outer membrane of mycobacteria is an unusual asymmetric supported lipid bilayer and presents an effective barrier against hydrophilic and hydrophobic solutes [9, 10]. In mycobacteria small hydrophilic compounds diffuse through water-filled channels called porins. Porins are non-specific channels present in the mycobacterial outer membrane that facilitate the influx of hydrophilic solutes [10, 11]. The loss of porins in mycobacteria leads to deficient nutrient uptake and consequently bacterial growth rates are significantly reduced

in mutants lacking porins [12]. Mycobacterial porins also provide the major pathway for the influx into the cytoplasm of hydrophilic drugs such as antibiotics [13-15].

An interesting feature to consider in the biology of mycobacterial porins is the intracellular lifestyle of mycobacteria within phagosomes. In contrast to the extracellular bacteria, intracellular mycobacteria are exposed to the phagosomal milieu. There, some of the accumulated toxic factors, including RNI, need to pass through the relatively impermeable cell wall of mycobacteria.

It has been reported that the deletion of some porins of mycobacteria result in improved survival in eukaryotic cells, demonstrating that the intracellular killing of *M. smegmatis* depends upon the permeability through the outer membrane [16]. However, the mechanism that leads to an improved intracellular survival of *M. smegmatis* porin mutants is still elusive.

Our hypothesis was that porins might enable the entry of one or several cytotoxic solutes inside the mycobacterial cell. In this report, we analyzed the intracellular fate of a porin triple mutant of *M. smegmatis* lacking MspA, MspC and MspD [12]. The number of Msp porins in this mutant is at least 15-fold lower that the *M. smegmatis* wild type strain SMR5 [12]. Our results indicate that the *M. smegmatis* porin mutant demonstrates an increased survival in J774 macrophages. On the other hand, we observed that the anti-mycobacterial capacity involving either phagosome-lysosome fusion or NO production in macrophages is not affected during intracellular infection with this mutant. Further, *in vitro* experiments that mimic the phagosomal environment provide direct evidence that *M. smegmatis* with less porins are more resistant to the microbicidal effects of NO. These findings indicate that porins are required for the bactericidal effect of NO and point to a role for porins in the susceptibility of mycobacteria to bactericidal components present in the phagosome.

2. Material and Methods

2.1 Reagents

All chemicals were from Sigma (St Louis, USA). Rat monoclonal anti-LAMP-2 was purchased from the Iowa Hybridoma Bank. Mouse monoclonal anti iNOS was from BD Biosciences (Heidelberg, Germany). Secondary antibody anti-rat was conjugated with Alexafluor 555 (Invitrogen, Germany).

2.2 Cell lines and bacterial culture conditions

The mouse macrophage cell line J774A.1 was cultured as previously described [1]. *M. smegmatis* SMR5 is a derivate of *M. smegmatis* mc^2155 and is resistant to Streptomycin due to a mutation in ribosomal protein S12 [17]. *M. smegmatis* ML16 (ΔmspA, ΔmspC ΔmspD) was previously described and derives from the SMR5 strain [12, 18]. Both strains were grown under agitation, at 37°C in Middlebrook 7H9 liquid medium (Difco Laboratories, USA) supplemented with 0.2% glycerol, 0.05% Tween 80 and 5% of glucose or on Middlebrook 7H10 plates (Difco Laboratories, USA) supplemented with 0.2% glycerol and 5% of glucose. For both strains Streptomycin (15µg/ml) was added to the medium.

2.3 Macrophage infection

Bacterial cultures in exponential growth phase were pelleted, washed in PBS and re-suspended in D-MEM medium to a reach a multiplicity of infection (MOI) of 100. Clumps of bacteria were removed by ultrasonic treatment of bacteria suspensions in an ultrasonic water bath for 15 min followed by a low speed centrifugation for 2 min. Macrophages were seeded onto 24 well tissue culture plates at 70% confluence. In each experiment, after 1 h of infection, cells were washed and medium plus Gentamicin (10 µg/ ml) was added to kill extracellular bacteria.

2.4 Colony forming Unit (CFU) assay

Macrophages were plated in a 24 wells plate and infected with *M. smegmatis* SMR5 or ML16. At the indicated time points, cells were washed with PBS and lysed with sterilized water. Quantitative cultures for *M. smegmatis* SMR5 and ML16 were performed by 10-fold

serial dilutions inoculated on 7H10 agar plates. 5 µl were plated by triplicate and the number of colonies was counted after 48 h and referred as number of colonies (CFU)/ml.

2.5 Indirect Immunofluorescence

Cells were fixed with 3% paraformaldehyde solution in PBS for 10 min and quenched by incubating with PBS 50mM NH_4Cl. Subsequently, cells were permeabilized with 0.05% saponin in PBS containing 0.2% BSA, and then incubated with the primary and secondary antibodies. Cells were mounted with Dako mounting media (Dako, Denmark) and analyzed by confocal microscopy in a Leica SP2 microscope (Leica Microsystems, Germany). Internalization of mycobacteria was tested using SMR5 or ML16 rhodamine-labeled bacteria. After 1 h infection, cells were fixed and extracellular bacteria were detected by immunofluorescence by incubation of non-permeabilized cells with a rabbit anti-mycobacteria polyclonal antibody (a gift from Dr. Pascale Salek, Institute de Pharmacologie et de Biologie Structurale, Toulouse, France) followed by a Alexa 488-conjugated secondary antibody (Invitrogen, Germany). Number of intracellular bacteria was counted in at least 100 cells from 3 different experiments.

2.6 Assessment of phago-lysosomal fusion by electron microscopy

J774.A1 macrophages were prepared as described before and incubated with medium containing 1% of (Fetal Calf Serum) FCS (PAA, Germany) for 16 h. Then, 5nm gold (OD_{520}: 5.0) was internalized by 1 h pulse and 4 h chase. Infection with SMR5 and ML16 was performed as described above. 1 and 4 h after infection, cells were fixed in 2.5 % glutaraldehyde (50 mM sodium cacodylate, pH 7.2, 50 mM KCl, 2.5 mM CaCl2) for 30 min at room temperature. After several rinses in cold buffer (50 mM sodium cacodylate, pH 7.2), cells were post fixed in 2 % osmium tetroxide (50 mM sodium cacodylate, pH 7.2) for 90 min on ice, subsequently rinsed in water, and stained overnight at 4°C in aqueous 0.5 % uranyl acetate. Samples were then dehydrated in graded ethanol series and propylene oxide,

followed by embedding in epoxy resin. Ultrathin sections were obtained on a Leica Ultracut UCT or Ultracut S microtome (Leica Microsystems, Germany), counter-stained with uranyl acetate and lead citrate, and examined with a Philips CM120 TEM (Biotwin, 120 kV) electron microscope.

2.7 Measurement of Nitric Oxide production

NO generation by J774.A1 macrophages was measured as accumulated supernatant nitrite (a stable breakdown product of NO), of the controls and infected cells at 1, 4 and 24 h after infection by the Griess reaction. Briefly, $1x10^5$ cells were seeded onto 6 well plates and left for 48 h in a 5% CO_2 incubator at 37° C prior the infection. The infection was performed using an MOI 100 for the *M. smegmatis* strains ($O.D_{600} \cong 0.1$). After 1 h infection Gentamicin (10µg/ml) was added to all wells. 200 µl of the supernatants were collected at the indicated time points and centifugated at 2000 g. 100µl of the supernatants were incubated with 100 µl of Griess reagent (50 µL of 1% sulfanilamide plus 50µL of 0.1% of N-1-naphyethylethylenediamine in 5% phosphoric acid solution at room temperature for 10 min. The absorbance was determined at 545 nm using a Safire II (TECAN, Austria). Conversion of absorbance to micromoles was deduced from a standard curve. All determinations were performed in triplicate and expressed as micromoles of NO/ml.

2.8 Western-blot

Macrophages under the different conditions were washed with PBS, scraped in lysis buffer and processed as described before [3]. Briefly, 50 µg of protein extracts were subjected to electrophoresis in 10% SDS-PAGE gels, transferred to a nitrocellulose membrane and blocked with 0.1% Tween 20, 5% of milk TBS. The nitrocellulose membrane was then incubated with primary antibodies, washed and incubated with secondary HRP-conjugated antibodies. The bands were visualized with a chemioluminiscent reagent (Amersham Biosciences, UK).

2.9 Analysis of bacterial susceptibility *in vitro*

SMR5 and ML16 strains were grown in 7H9 liquid medium as described above for 12 h. From this cultures, a 1% inoculum was prepared in fresh medium for control and in fresh 7H9 medium pH 5.5 containing 2mM, 4mM or 8mM of Sodium nitroferricyanide (III) dehydrate (SNP) as described previously by Genestra and collaborators [19] or a mixture of purified lysosomal "dense pool" fraction from a homogenate of human placental tissue using Percoll gradient centrifugation [20]. After 24h incubation at 37°C, 100µl of each sample was collected and serial dilutions in PBS Tween 0.05% were plate on 7H10 plates. CFU were counted as described above.

2.9 Statistical analysis

Data are presented as means ± SEM of at least three independent experiments; p values (ANOVA, two ways) are from comparison between SMR5 and ML16 strains.

3. Results

3.1 A mutant of *M. smegmatis* lacking the MspA, MspC and MspD porins is more resistant to killing by J774 macrophages

The presence of porins in the outer membrane of mycobacteria facilitates the entrance into the bacteria of hydrophilic compounds including antibiotics [21]. We rationalized that a mutant of *M. smegmatis* with reduced number of porins could exhibit a stronger resistance to the macrophage antimicrobial compounds within the phagosome. To analyze whether the absence of porins has any impact, we used the *M. smegmatis* mutant strain ML16 which lacks the three main porins of *M. smegmatis*, namely MspA, MspC and MspD [12]. This porin mutant grew more slowly on Middlebrook 7H9 agar plates (data not shown) in agreement with previous observations [12].

We infected J774 macrophages with the wild type strain SMR5 and the porin mutant ML16. To ensure that the number of bacteria used for the infections was similar for both SMR5 and ML16, the colony forming units (CFU) of serial dilutions of fresh cultures were determined (data not show). After infection with the *M. smegmatis* strains macrophages were lysed and CFU determined. As shown in **Fig.1A**, the mutant of *M. smegmatis* ML16 showed a significantly higher survival compared to the SMR5 strain. This increase in survival was observed at both 4 h ($p \leq 0.01$) and 24 h ($p \leq 0.001$) after infection. Strikingly, the effect was particularly significant at 24 h of infection, where the number of bacteria that survived was almost double for ML16 compared to SMR5 (**Fig.1A**). One explanation for these observations could be that ML16 is more efficiently internalized by macrophages. To examine this, we analyzed internalization using immunofluorescence to reveal extra and intra-cellular bacteria (see material and methods). We observed that the number of intracellular bacteria per macrophage after 1h infection was the same for both strains (**Fig.1B**). Therefore, this observed increase in the number of viable bacteria was not due to an improved internalization of the mutant and likely the bacteria were able to multiply during the first 24 h.

Taken together, our data indicated that the viability of the ML16 mutant strain in macrophages was higher. Our data also suggest that the ML16 increased survival is most likely due to enhanced resistance of the mutant to macrophage bactericidal mechanisms.

3.2 Phagosomes containing ML16 and SMR5 fuse with late endosomes and lysosomes

We next sought to identify the mechanism which enables the increased survival of the *M. smegmatis* porin mutant in macrophages. Macrophages kill non-pathogenic mycobacteria within phagosomes by a combination of factors delivered from the lysosome to the phagosome [1, 3]. First, we tested whether the survival of ML16 was related to impaired phago-lysosomal fusion. Since the killing of non-pathogenic mycobacteria is an early event

occurring mainly during the first four hours after infection [1], we focused on these first time points. For that, we analyzed by confocal microscopy the acquisition of the glycoprotein LAMP-2. LAMP-2 acquisition is a marker of late endosomes/lysosomes and it has been shown that this marker is acquired by phagosomes containing non-pathogenic mycobacteria [3]. As shown in **Fig.2A**, both SMR5 and ML16 acquired similar amounts of LAMP-2 after infection. The number of phagosomes positive for LAMP-2 was similar for both strains one and four hours after infection (**Fig.2B**). Additionally, we analyzed the number of phagosomes positive for the lysosomal enzyme cathepsin D. In agreement with the LAMP-2 co-localization data, differences between SMR5 and ML16 were not observed (data not shown).

To provide an independent assessment of the intracellular trafficking of SMR5 and ML16, we also performed electron microscopy in J774 macrophages pre-loaded with BSA-coated colloidal gold to label the late endosomal/lysosomal population. As shown in **Fig.3**, most of the phagosomes containing SMR5 or ML16 contained the pre-loaded gold. The fusion with gold pre-loaded compartments was observed already at 1 h and remained at four hours after infection. These observations indicated that ML16 fused with late endosomal/lysosomal compartments indistinguishable from the wild-type strain SMR5. Therefore, the increased survival observed for the porins mutant ML16 is not due impaired lysosomal fusion within the host cell.

3.3 Infection with either SMR5 or ML16 induced NO production by J774 macrophages

Since the phagosomes containing ML16 allows fusion with late endosomes/lysosomes, we next studied whether infected macrophages were indeed producing NO, another well-known killing factor for mycobacteria [22]. It is known that porins from other Gram-negative bacteria are able to enhance the production of NO by macrophages [23]. Therefore, the improved intracellular survival of ML16 could be due to this mutant inducing a lesser production of NO. Consequently, this diminished production of NO could lead to the

increased survival of the mutant ML16. To test this hypothesis, we measured the NO production of macrophages infected with either SMR5 or ML16. As shown in **Fig.4**, infection with both SMR5 and ML16 strains clearly induced NO production by J774 macrophages. The levels of NO production were similar to the ones obtained with LPS and reached higher values at 24h after infection. Importantly, SMR5 and ML16 elicited the same NO response in infected macrophages. In addition, iNOS was clearly induced in both SMR5 and ML16 after 4 h of infection compared to the controls (**Fig.4**). This clearly indicates that the increased survival of the ML16 mutant is not due to a reduced production of NO in J774 macrophages.

3.4 ML16 is more resistant *in vitro* to NO

We observed that the ML16 mutant survived better in macrophages, indicating that this mutant is more resistant to the bactericidal activity of macrophages. We have shown that phago-lysosomal fusion and NO production remained unaffected in macrophages infected with either SMR5 or ML16. Based on these observations we hypothesized that the intrinsic susceptibility of the *M. smegmatis* porin mutant to toxic agents generated within the phagosome is different. To examine this assumption, we tested *in vitro* conditions that mimic the phagosomal milieu. As NO is one of the main mechanisms of killing of mycobacteria [24], we analyzed the susceptibility of both SMR5 and ML16 to NO *in vitro*. As the minimal condition that mimics the phagosomal milieu is the combination of weak pH and RNI [6], we used Middlebrook 7H9 at pH 5.5 combined with the NO donor sodium nitroprusside (SNP). As shown in **Fig.5**, treatment of both strains with different concentrations of sodium nitroprusside (SNP) (see material and methods) led to a dramatic reduction in the number of colonies recovered after treatment. Strikingly, in the case of ML16 the number of bacteria recovered after treatment with NO-donor SNP was significantly higher compared to the wild type strain SMR5. Even at the highest concentration of the nitric oxide donor tested (8 mM), we recovered porin mutants, but not wild-type *M. smegmatis*. We also tested the ability of

SMR5 and ML16 to resist lysosomal enzymes from a homogenate of human tissue [20]. We observed that both SMR5 and ML16 were highly susceptible to treatment with lysosomal enzymes under oxidizing conditions. However, no differences were noticed between both strains after treatment ($p \geq 0.05$). Thus, ML16 is more resistant to the bactericidal action of NO *in vitro* (Fig.5).

4. Discussion

The outer membrane of mycobacteria serves as a protective barrier against the external environment but is rendered selectively permeable to nutrients and small compounds by proteins called porins [10]. Here, we show that porins render mycobacteria susceptible to toxic molecules such us RNI, present in the intra-phagosomal compartment. This augmented susceptibility to NO is most likely related to the increased survival profile observed for the mutant ML16 in J774 macrophages. Interestingly, only acidic pH (pH 5) had no significant effect in the survival of ML16 compared to SMR5 (data not shown).

It has been shown that in *Escherichia coli*, the treatment with NO activates several genes controlled by the redox-sensitive transcriptional regulator *soxR*. One of those *soxRS*-regulated genes encodes an antisense RNA that down-regulates OmpF, a major outer membrane porin of *E. coli* [25]. The reduction of OmpF levels contributes to resistance by slowing down the entry of toxic solutes into the cell [11] and might be similarly protective against some toxic compounds generated by macrophages. Interestingly, strains of enteric bacteria bearing mutations in a system involved in the control of OmpF proteins expression showed elevated resistance not only to many antibiotics but also to certain oxidants [25]. Similarly, *M. smegmatis* also protects itself by reducing the outer membrane permeability in response to toxic solutes via down-regulation of porin gene expression [26]. In the light of our results, it would be interesting to address how the expression of porins is regulated in mycobacteria during intracellular infection.

It has been reported that the porin PorB from *Neisseria gohorreae* is associated with the plasma membrane and latex bead phagosomes and modulates phagosome maturation [27]. Here, we observed that the lack of MspA, MspC and MspD in *M. smegmatis* does not influence phagosome maturation. This is most likely due to differences between porins from the soil dweller *M. smegmatis* and the pathogenic bacteria such as *Neisseria*. For example, PorB inserts into host membranes including mitochondrial membranes [28].

The bacterial proteasome has been shown to protect mycobacteria against oxidative or nitrosative stress. One of the postulated mechanisms of protection involved the degradation of proteins that are irreversibly nitrosated [29]. Based on our data, this could be facilitated by the presence of porins in the mycobacterial outer membrane. NO is soluble in water and lipids [22]. Although diffusion of NO is fast within cells, numerous components may interact with NO, limiting its biologically effective radius. These molecules include cellular antioxidants, multivalent metal centers, proteins, and lipids [30]. In this context, within the phagosome RNI need to pass through the permeability barrier established by the mycobacterial outer membrane to produce its toxic effect. Our data suggest that the entrance of RNI into the bacteria through a water-filled outer membrane channel is important for the toxic properties of NO.

The finding that low levels of porins in the outer membrane of *M. smegmatis* led to increased resistance to NO suggest that a similar mechanism could be operating for *M. tuberculosis*. These pathogenic mycobacteria could regulate porin expression to shield itself from toxic compounds such as RNI. Survival in phagosomes might be facilitated by the fact that *M. tuberculosis* does not express MspA-like proteins [10, 21]. It is tempting to speculate that in order to avoid stress in the phagosomal environment, mycobacteria down-regulate specific membrane proteins in response to environmental signals minimizing the entry of toxic molecules into the bacteria. However, Bacillus Calmette-Guérin (BCG) expressing porins

from *M. smegmatis* demonstrated improved growth in macrophages [16]. These differences observed here may be related to the fact that BCG inhabits a phagosome that fails to acidify whereas *M. smegmatis* phagosomes fully acidify [1].

Our results also show that the triple porin mutant survives better than wild-type *M. smegmatis* in macrophages, although it grows more slowly *in vitro* [12]. This suggests a potential link between growth rate and pathogenesis. A low number of less efficient porins might cause *M. tuberculosis* to grow slowly due to the slow uptake of nutrients, but also increased resistance to the effect of toxic molecules and intracellular persistence. This hypothesis was raised earlier [12] and needs to be tested experimentally.

In summary, our data indicate for first time that the presence of porins in *M. smegmatis* led to an increased susceptibility to NO *in vitro*. Our data also suggest a new potential mechanism of resistance to the harsh environment of the phagosome. Further studies in *M. tuberculosis* will provide insights into the role of porins during infection with this pathogen.

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7. **Figure Legends**

Figure-1 Survival of SMR5 versus ML16 in J774 macrophages A. J774 macrophages were infected with *M. smegmatis* SMR5 and ML16 strains and CFU determined at the indicated time points. Data is represented as the mean of the percentage of survival ± SEM of three independent experiments. (*), $p \leq 0.01$; (**), $p \leq 0.001$. B. Quantitative analysis of the number of intracellular bacteria after 1h of infection, at least 100 cells were counted in three independent experiments †, $p \geq 0.05$. Right panels: representative pictures of the quantitations. The image shows two cell profiles with a few internalized bacteria (red, black arrow), whereas the extracellular bacteria were double labeled presenting both red and green fluorescence (white arrow).

Figure-2 Fusion of phagosomes containing either SMR5 or ML16 with late endosomes and lysosomes A. J774 macrophages were infected with SMR5 or ML16 stained with rhodamine-NHS. After 1h and 4h of infection cells were fixed and LAMP-2 localization determined by confocal microscopy (green). Insets show a representative phagosome for each condition. Bars: 10 µm B. Quantitative analysis of the percentage of colocalization of LAMP-2 with SMR5 and ML16 phagosomes. Results are shown as means ± SEM of three independent experiments. †, $p \geq 0.05$ n= number of phagosomes that were counted in total.

Figure-3 Ultrastructural analysis of the fusion between phagosomes containing either SMR5 or ML16 and lysosomes. J774 macrophages were pre-loaded with gold-albumin for 1 and 4h pulse-chase and subsequently were infected with SMR5 or ML16. After 1h and 4h of infection cells were fixed and processed for electron microscopy. Pictures show representative images. Both SMR5 and ML16 were found inside phagosomal compartments that fused with gold-preloaded lysosomes. Arrows: 5nm gold; PM, plasma membrane; Nu, nucleus

Figure-4 NO production in macrophages infected with SMR5 or ML16. A. J774 macrophages were infected with SMR5 or ML16 and after the indicated time points supernatants were collected and NO measured by Griess. LPS (1ug/ml) was used as a positive control. Results are shown as means ± SEM of three independent experiments.†, $p \geq 0.05$. B. J774 macrophages were left untreated, treated with LPS or infected with SMR5 or ML16. After 4 h, cell lysates were obtained and processed for western blotting to detect iNOS. Tubulin was used as a loading control.

Figure-5-Effect of NO and lysosomal enzymes treatment *in vitro* on SMR5 and ML16 survival.

An overnight bacterial culture was incubated in fresh medium pH for 24 h in presence of different NO donor SNP or a purified lysosomal fraction (100 µg/ml). Subsequently, treated bacteria were plated and CFU number counted. Results are shown as means ± SEM of three independent experiments. (**), $p \leq 0.001$; †, $p \geq 0.05$ CTRL; non-treated bacteria; SNP: Sodium nitroferricyanide (III) dehydrate; Lys: purified lysosomal fraction.

Figure 1-Fabrino et al

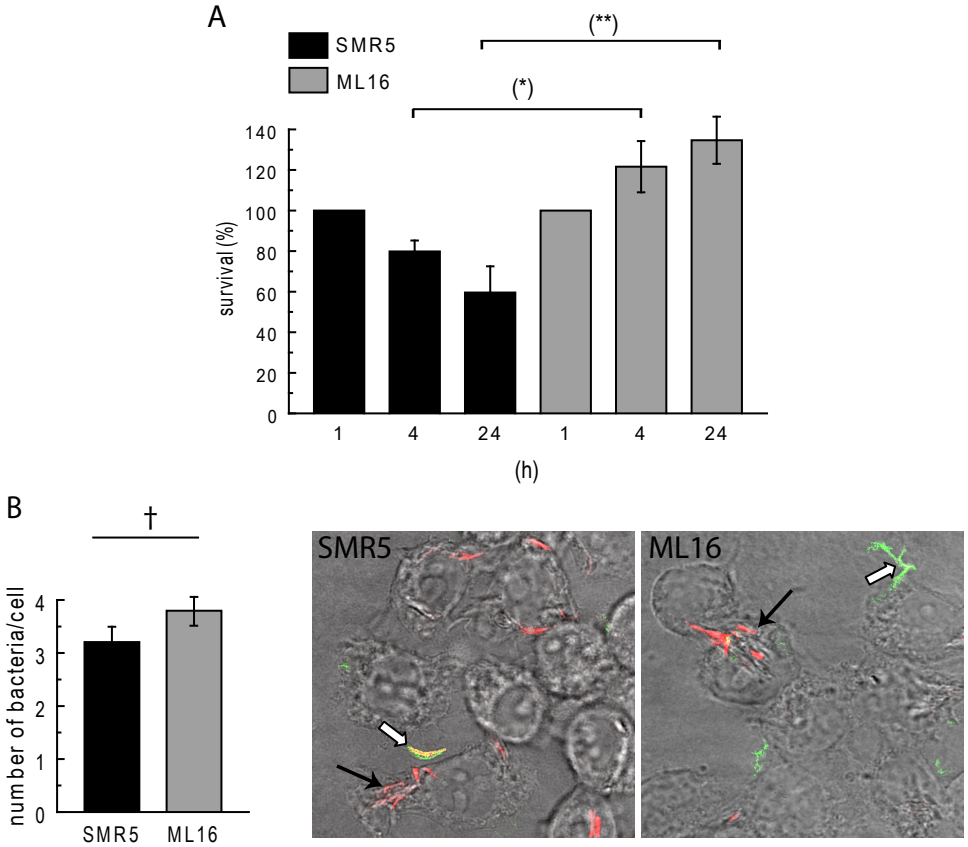
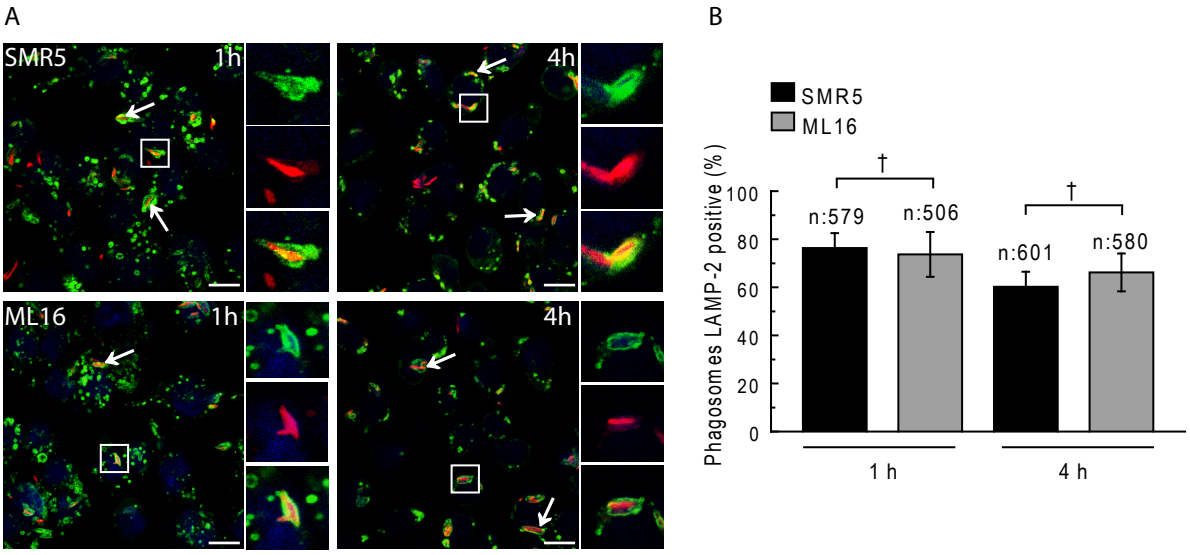


Figure 2-Fabrino et al



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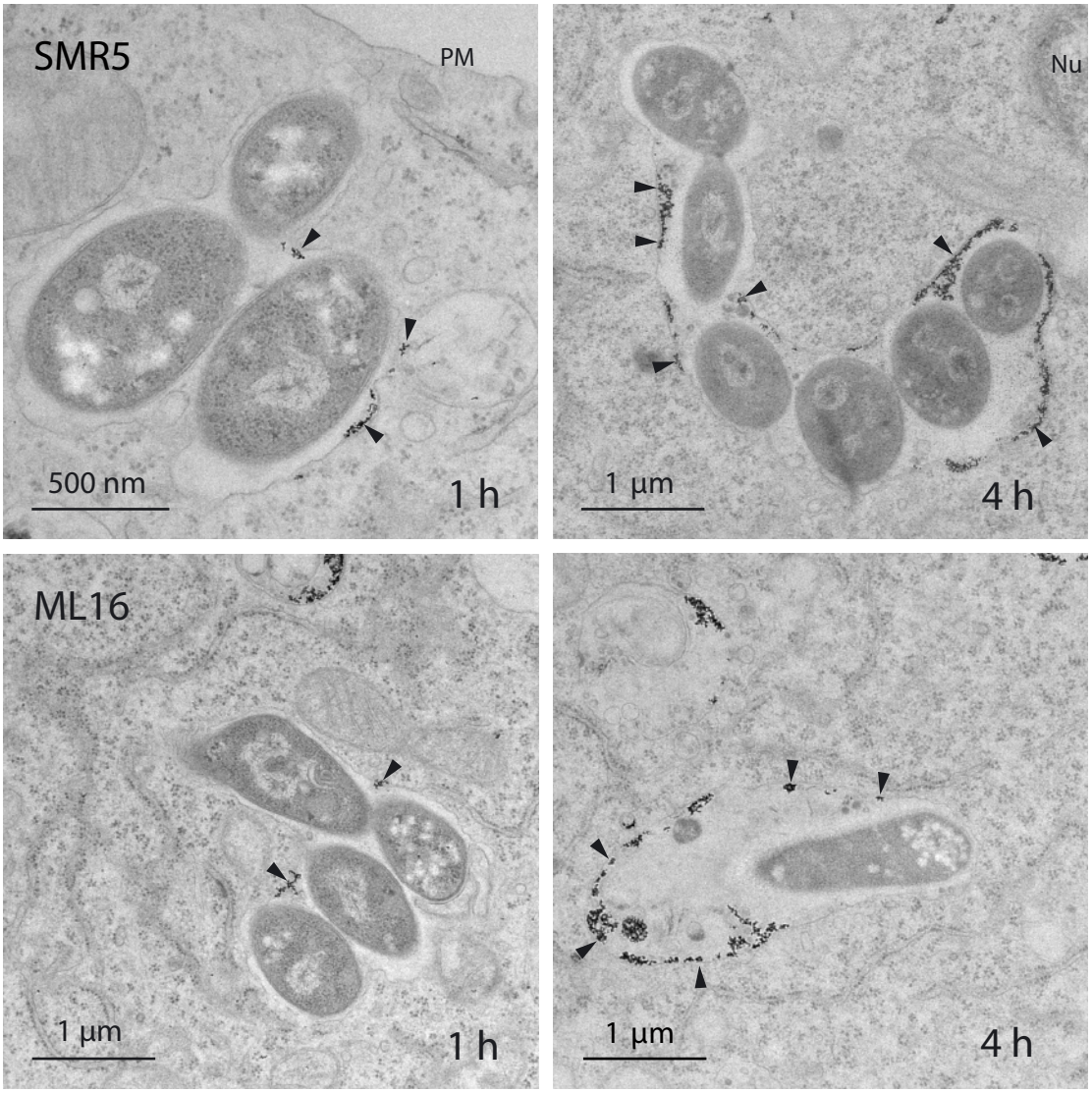

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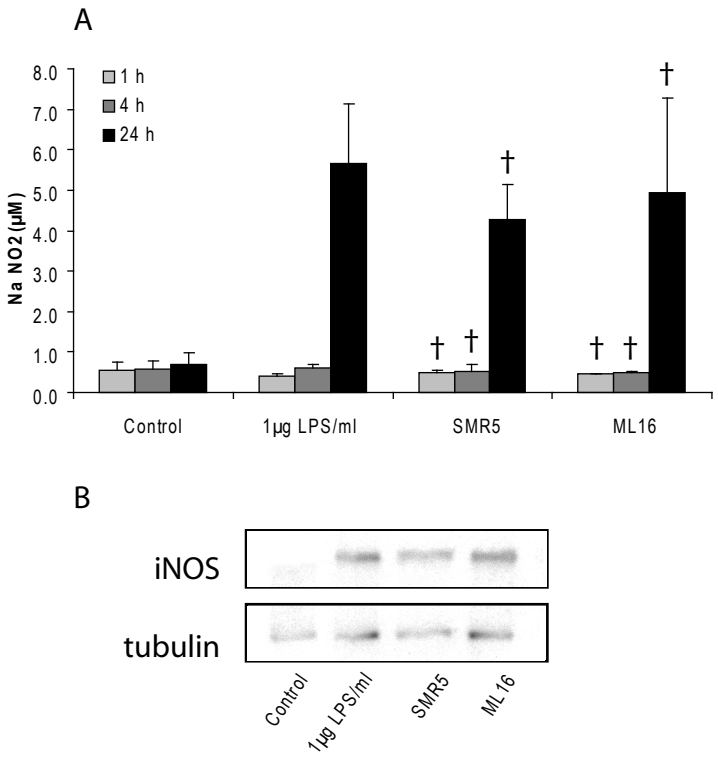

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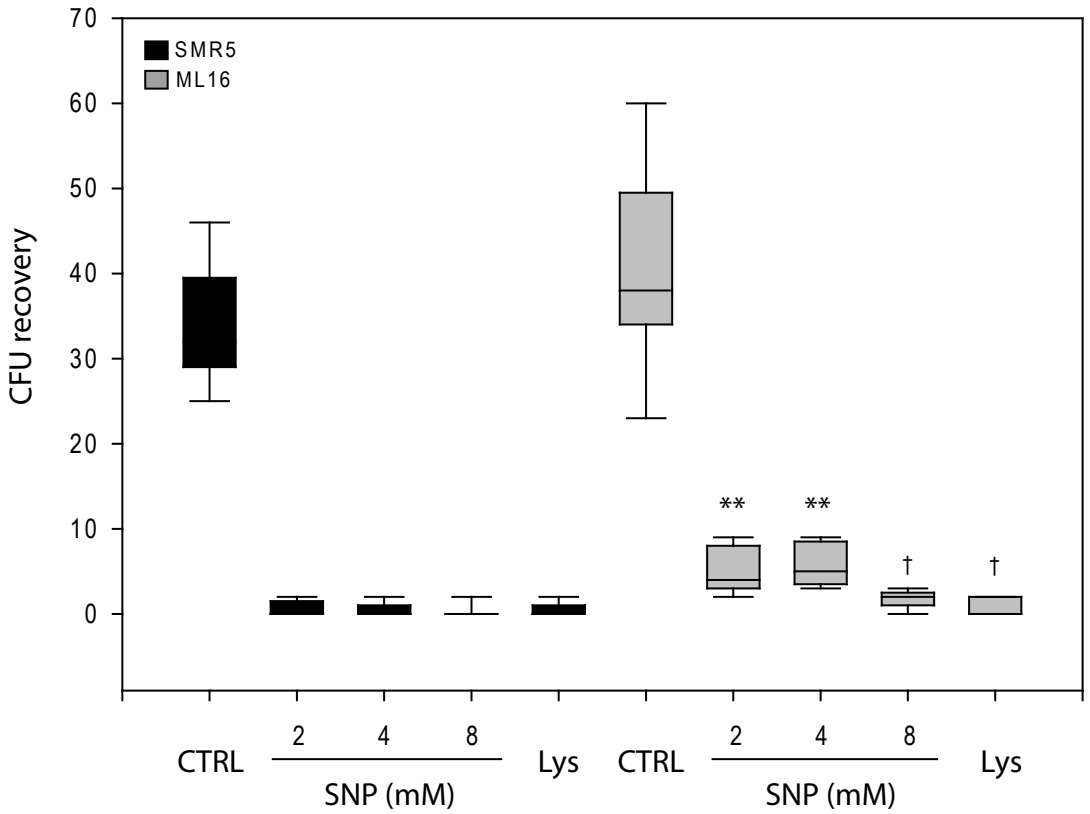

4. CONSIDERAÇÕES FINAIS

Muitos trabalhos têm sido elaborados no sentido de esclarecer o papel do óxido nítrico durante diversos processos infecciosos, tais como a doença de Chagas, leishmaniose e tuberculose. Tais estudos demonstram que o NO é uma molécula com grande complexidade em termos de atividade funcional e que sua via bioquímica pode ser influenciada por inúmeros fatores inerentes tanto a célula hospedeira como ao patógeno Os dados relatados na literatura até o momento, com relação à produção e papel desse composto, ainda não se encontram completamente esclarecidos (revisão em Mauel, 1996; Bogdan e cols., 2000; Russell, 2001).

O desenvolvimento de diversos mecanismos de escape ocorreu em diversos parasitos devido à sua longa coexistencia com células hospedeiras, e até mesmo a capacidade de aproveitar os fatores de crescimento presentes nessas células para sua própria multiplicação e crescimento (Rivero, 2006). Uma vez que a resposta imune dos hospedeiros produz diferentes moléculas tóxicas, ela exibe diversos mecanismos regulatórios que são passíveis de interferência pelo próprio parasito. Em se tratando da via metabólica do NO o parasito pode estar interferindo em etapas distintas da mesma, resultando na diminuição da produção dessa molécula ou mesmo usando-a a seu favor (James, 1995; Clark & Rockett, 1996; Bogdan e cols., 2000; Brunet, 2001; Vincendeau e cols., 2003; Peluffo e cols., 2004). O entendimento desses mecanismos é crucial para o estabelecimento de estratégias para o controle de diferentes doenças infecciosas.

Durante a infecção aguda experimental por *T. cruzi,* a produção de NO vem sendo estudada principalmente *in vitro* no modelo murino (Vespa e cols., 1994; Norris e cols., 1995; Petray e cols., 1995; Silva e cols., 1995; Holscher e cols., 1998). Esses autores descreveram aumento dos níveis de NO, produzidos por macrófagos estimulados com lipopolissacarídeo (LPS), IFN-γ, TNF-α, ou com o próprio antígeno parasitário, e o correlacionaram à resistência do hospedeiro ao parasito.

Por outro lado, trabalhos recentes questionam a importância do NO na infecção chagásica. Em 2004, Cummings e colaboradores, discutem o papel do NO no controle da infecção chagásica, ao usar 2 modelos de camundongos

k*nockout* para iNOS. Os autores demonstraram que mesmo na ausência da produção de NO, tais camundongos são tão resistentes à infecção quanto o modelo selvagem de camundongo. Sugere-se que tal resistência se deva a uma compensação bioquímica por meio de produção de TNF-α, IL-1 e da proteína inflamatória de macrófagos 1-α (MIP-1-α) (Cummings & Tarleton, 2004).

Os dados acima corroboram o trabalho prévio de Saeftel e colaboradores (2001) que demonstraram em camundongos que a produção de NO, estimulada por IFN-γ, ocorre apenas nos primeiros dias de infecção e passa a ser gradualmente substituída por mecanismos da imunidade adaptativa, na qual linfócitos CD8, CD4 e B agiriam em conjunto para limitar a reprodução do parasito, por meio de mecanismo independente da produção de NO (Saeftel e cols., 2001). Usando a cepa de baixa virulência Sylvio X10/4 de *T. cruzi*, outro grupo mostrou que camundongos infectados são capazes de controlar o parasitismo sem a produção aumentada de NO (Marinho e cols., 2007).

Os resultados contrastantes encontrados na literatura sobre a produção de NO podem se dar por diferentes razões. No modelo cão a procedência da forma infectiva usada (tripomastigotas metacíclicos provenientes de ninfas do inseto vetor ou do sangue de camundongos infectados) causaram diferença nos níveis séricos de NO (Vieira e cols., 2009). No modelo camundongo, durante a infecção experimental por *T. cruzi* podem, em parte, ser explicados pela variedade de linhagens usadas, além da variedade de cepas do parasito e intensidade do inóculo usado nos diferentes trabalhos (Canto Cavalheiro & Leon, 1999; Fabrino, D. L. e cols., 2008).

No modelo rato, existem poucos trabalhos investigando o papel do NO na infecção chagásica, apesar desse modelo ser bastante usado para o estudo de diferentes aspectos dessa infecção (Machado e cols., 1987; Melo & Machado, 1998; Revelli e cols., 1998; Camargos e cols., 2000; Fichera e cols., 2004; Petersen e cols., 2006). Em culturas de cardiomiócitos de ratos Wistar infectados com tripomastigotas da cepa Sylvio-X10/4, o controle do parasitismo foi relacionado com produção de NO (Fichera e cols., 2004). Entretanto, nosso grupo demonstrou que, *in vivo*, no modelo rato (Holtzman), o NO não parece

exercer papel fundamental no combate a infecção aguda experimental por *T. cruzi* (Fabrino e cols., 2004).

Em 2003, mostramos que macrófagos do peritôneo e coração, de ratos infectados, exibem formação aumentada de CLs, organelas intimamente relacionadas com o metabolismo do ácido araquidônico e síntese de mediadores da inflamação. Tal aumento foi correlacionado com a produção de PGE2, um derivado daquela via e conhecido antagonista do NO, (Melo e cols., 2003a).

Além disso, demonstramos que macrófagos esplênicos e monócitos do sangue periférico não produzem NO durante a infecção aguda em ratos Holtzman (Fabrino e cols., 2004). Essas células exibem altos níveis de arginase, uma enzima que compete com a NOS pelo seu substrato, a L-arginina, e tem como produto final poliaminas que podem ser usadas pelo parasito para sua multiplicação. Entretanto, neste modelo, embora haja um aumento na expressão da arginase, parece que tal mecanismo não beneficia a manutenção do parasitismo prolongadamente uma vez que, aos 20 dias de infecção o parasitismo nesses animais é quase ausente (Fabrino e cols., 2004).

O nosso grupo tem demonstrado, *in vivo*, que o sistema monócito/macrófago apresenta papel crucial no controle da infecção chagásica aguda (Melo, 2008). Nesta fase da doença, ocorre intensa mobilização de monócitos do sangue periférico em paralelo ao intenso influxo de macrófagos no coração, órgão alvo da doença. Macrófagos infiltrados neste órgão encontram-se diretamente envolvidos na inibição da reprodução do parasito e apresentam morfologia de células ativadas (Melo, 2008).

Com base nos dados expostos acima, utilizamos dois modelos de ratos (Wistar e Holtzman) com o objetivo de contribuir para o entendimento do papel do NO na resistência a infecção aguda e em um desses modelos (Holtzman), avançamos no estudo dos corpúsculos lipídicos em animais submetidos a maior carga parasitária. Essas investigações geraram dois artigos. No primeiro artigo, focado no estudo de corpúsculos lipídicos em macrófagos, reiteramos a importância dessas células no combate a infecção aguda por *T. cruzi* e ressaltamos os CLs como marcadoras estruturais e funcionais da inflamação (Melo e cols., 2006).

Este estudo revela que diferentes populações de macrófagos exibem uma característica bioquímica/morfológica em comum, a formação de grande número de CLs em resposta a infecção. O aumento da carga parasitária, resultante da irradiação dos animais, induz significativa variação da eletro-densidade e do tamanho dos CLs que podem alcançar até 4 µm de diâmetro. Neste estudo, foi também realizada uma criteriosa análise ultra-estrutural das interações entre CLs e outras organelas. Demonstramos que a infecção por *T. cruzi* induz modificação na distribuição citoplasmática dos CLs que passam a se localizar preferencialmente ao redor de fagolisossomos. Além disso, os CLs interagem nitidamente com essas organelas, apresentando pontos de fusão com as mesmas e podem até ser observados dentro dos fagolisossomos (Melo e cols., 2003a). A interação CL-fagolisossomo vem despertando grande interesse, uma vez que CLs parecem exercer papel importante no destino do parasito, favorecendo sua manutenção ou morte. A variação em elétron-densidade dos CLs, em macrófagos inflamatórios, por outro lado, denota um estado bioquímico de intenso metabolismo nestas organelas (revisão em Bozza e cols., 2007). Tal metabolismo está centrado numa via antagônica à produção de NO, uma vez que mediadores inflamatórios como a PGE2 são geralmente observados atuando como inibidores da síntese de NO (Meerpohl & Bauknecht, 1986; Freire-de-Lima e cols., 2000; Fabrino e cols., 2004).

Já no segundo trabalho apresentado nesta tese, discutimos o uso de diferentes modelos experimentais para o estudo da infecção chagásica, destacando o rato Holtzman como modelo extremamente útil para o estudo do sistema monócito/macrófago na fase aguda dessa infecção. Além disso, nossos estudos demonstraram que o NO não parece constituir uma molécula central na resistência a este parasito *in vivo*. Usando tripomastigotas sanguícolas da cepa Y em dois modelos de ratos, Wistar e Holtzman, mostramos que ambos não exibem altos níveis de NO produzidos por monócitos e nem por macrófagos esplênicos; entretanto, esses animais são capazes de combater o parasito e sobreviver à infecção ((Fabrino, D. L. e cols., 2008) – submetido).

Uma vez que a produção de NO se mostrou nitidamente diminuída na infecção aguda, e que moléculas antagonistas da via do NO como a PGE2 e

arginase, por sua vez, encontram-se em níveis aumentados na mesma fase da infecção (Melo e cols., 2003a; Fabrino e cols., 2004; Fabrino, D. L. e cols., 2008), perguntamos se o parasito poderia estar, de alguma forma, modulando a via metabólica do NO em seu favor.

No terceiro trabalho desta tese, investigamos, pela primeira vez, a capacidade do parasito em produzir NO. Verificamos não só a existência da TcNOS em formas amastigotas e tripomastigotas do *T. cruzi*, bem como a sua atividade diferenciada nestes dois estágios evolutivos do parasito. Observamos que a TcNOS das formas tripomastigotas é mais ativa e corroboramos esse dado através da demonstração da maior capacidade de produção de NO por essas formas.

Sabe-se que o *T. cruzi* possui um transportador independente, de alta afinidade por sódio (Na^+) para a L-arginina (Pereira e cols., 1999). Dados da literatura sugerem que, dessa forma, o parasito possa adquirir L-arginina da célula hospedeira, diminuindo a quantidade de substrato para a iNOS da célula hospedeira, o que levaria a uma diminuição dos níveis de NO produzidos pela via induzida da NOS. Como resultado, o parasito não só diminuiria os níveis nocivos de NO produzidos por esta via, como se beneficiaria com o substrato para produzir seu próprio NO, mas também como um gerador de guanosina monofosfato cíclico (*GMPc) (Peluffo e cols., 2004).

A estimulação da atividade de GCs, por meio do NO, poderia ser responsável pelo aumento na motilidade de formas epimastigotas do parasito (Johansson & Carlberg, 1995; Pereira e cols., 1997). Demonstramos no presente estudo que o NO é produzido em maior quantidade pelas formas tripomastigotas deste parasito, formas dotadas de motilidade e infectivas. Portanto, sugerimos que NO possa atuar como uma molécula sinalizadora no *T. cruzi* (Fabrino e cols., 2009b) – submetido.

Outra possível função da via do NO em *T. cruzi* é seu papel citoprotetor contra processos de apoptose neste protozoário. Quando incubados com L-arginina, substrato para vias de produção de NO e poliaminas, não foi observada a fragmentação de DNA em culturas de formas epimastigotas de *T. cruzi*. Neste foi observado que a incubação subseqüente das culturas com N-nitro-L-arginina-metil-ester (L-NAME), um inibidor da NOS, causou intensa

fragmentação de DNA nestes parasitos, indicando o papel citoprotetor do NO; por outro lado, a doação externa, por meio de incubação com doadores químicos de NO foi eficiente na recuperação dos níveis basais de replicação do *T. cruzi* (Piacenza e cols., 2001).

A produção de NO pelo *T. cruzi* pode estar associada também com a capacidade de infecção e manutenção da multiplicação do mesmo dentro da célula hospedeira (Peluffo e cols., 2004), conforme sugerido para a *Leishmania sp.* Além da demonstração da presença de NO em *Leishmania* (Basu e cols., 1997; Geigel & Leon, 2003; Genestra e cols., 2003b; Genestra e cols., 2006a), tal ocorrência tem sido relacionada à capacidade de infecção, visto que o tratamento com L-NAME, afeta intensamente a capacidade infectiva deste parasito (Genestra e cols., 2003a; Genestra e cols., 2006b).

A presença de uma mesma enzima, a NOS, em dois protozoários que compartilham diversas rotas bioquímicas, além de características biológicas e morfológicas, nos faz pensar sobre a função desta rota bioquímica nestes protozoários, e desperta o interesse sobre a toxicidade do NO nos mesmos. Embora a literatura pontue o NO como uma molécula tóxica para ambos (James, 1995; Mauel, 1996; Ouaissi & Ouaissi, 2005), há ainda discussão acerca deste tema (Piacenza e cols., 2001; Peluffo e cols., 2004; Genestra e cols., 2006b; Genestra e cols., 2008).

Vários trabalhos apontam mecanismos de escape dos mais diferentes microorganismos em relação à via do NO. Um exemplo é a micobacteria *M. smegmatis*, uma bactéria não patogênica, que exibe capacidade de detoxificação e resistência a esse radical nitrogenado, por diferentes vias bioquímicas. Como exemplos, podemos citar a capacidade de redução do NO por meio de um tiol, análogo a glutationa, o chamado micotiol (MSH) (Miller e cols., 2007), ou presença de uma hemoglobina truncada, que apresenta capacidade de se ligar ao NO e inativá-lo (Lama e cols., 2006).

Em se tratando de protozoários, em especial os da ordem kinetoplastida, como a *Leishmania sp*. e o *T. cruzi*, ainda não foi feita a identificação de uma enzima estritamente ligada à neutralização de espécies reativas nitrogenadas. No entanto, esses parasitos conseguem driblar a resposta imune do hospedeiro e estabelecer processos infecciosos.

Deste modo, fazendo uso do nitroprussiato de sódio (SNP), um doador químico de NO, testamos a toxicidade do NO nos três microorganismos acima citados e demonstramos, nos últimos 3 trabalhos desta tese, que as formas amastigotas tanto *L. amazonesis* quanto *T. cruzi* e uma cepa de *M. Smegmatis,* triplo mutante para porinas, são capazes de sobreviver a altas doses de NO *in vitro*.

O trabalho realizado com as formas, promastigota e amastigota axênica (amastigotas cultivadas sem a presença de célula), de *L. amazonensis* mostrou que a forma amastigota, que é a que enfrenta o ambiente hostil fagossomal dentro da célula hospedeira, é 50% mais resistente, ao SNP do que a forma promastigota (Genestra e cols., 2008). Corroborando esses dados, nosso outro trabalho com *T. cruzi*, mostra que usando o mesmo doador (SNP) e nas mesmas concentrações, que formas amastigotas resistem significativamente mais do que as formas tripomastigotas deste parasito. Além disso, à microscopia de luz, parece que os tripomastigotas quando expostos ao estresse oxidativo sofrem uma transformação morfológica, perdem seu flagelo aparente e se diferenciam em formas amastigotas-like (Fabrino e cols., 2009a) – submetido. Tais dados necessitam ser confirmados por microscopia eletrônica (material em processamento).

Entretanto, os dados desses dois trabalhos juntos apontam para uma alta capacidade de resistência da forma amastigota, que é intracelular e que tem de enfrentar, face a face, o ambiente hostil e oxidativo do fagossomo.

Vale ressaltar que na infecção de macrófagos da linhagem J774, com a *M. smegmatis*, os macrófagos são capazes de debelar a infecção dentro de 48 horas (Jordao e cols., 2008). Entretanto, usando um mutante para porinas, proteínas que servem como um canal de influxo, de moléculas do meio externo para o citoplasma dessas bactérias, nota-se que o mutante é mais resistente à ação do SNP do que a cepa selvagem. O *M. tuberculosis*, que é altamente patogênico e consegue estabelecer infecções crônicas em seus hospedeiros, também exibe um número reduzido de porinas (Sharbati-Tehrani e cols., 2005; Stephan e cols., 2005). Embora o *M. tuberculosis* exiba outros mecanismos de resistência aos “ataques” da célula hospedeira, não se pode descartar o possível papel do número reduzido de porinas (Niederweis, 2003; Fabrino, D. L. e cols., 2008).

A partir de nossos estudos, destacamos a importância de investigações enfocando a via metabólica do NO não apenas em células hospedeiras, mas também em microorganismos unicelulares como protozoários e bactérias. A capacidade desses microorganismos de driblar a ação tóxica do NO e, potencialmente usá-la a seu favor constitui uma vasta área a ser estudada.

5. CONCLUSÕES

A partir dos resultados apresentados nos trabalhos desta tese concluímos que:

- ✓ O NO não se revelou uma molécula chave na resistência a infecção aguda experimental por *T. cruzi* em ratos;
  - Dois modelos de ratos (Holtzman e Wistar) estudados numa cinética de infecção diferentes da fase aguda (6, 12 ou 20 dias após infecção) mostraram que os mesmos são capazes de combater o parasitismo apesar de um declínio na de NO.

- ✓ O recrutamento e ativação de células do sistema monócito/macrófago durante a infecção aguda é mais consistente em ratos da linhagem Holtzman em comparação com ratos da linhagem Wistar.

- ✓ A formação de corpúsculos lipídicos, bem como sua atividade metabólica, está relacionada com a carga parasitária aplicada no modelo animal. Tendo em vista que estas organelas sintetizam PGE2, um antagonista do NO, sugere-se que os corpúsculos lipídicos possam estar interferindo na via metabólica do NO.

- ✓ O protozoário *T. cruzi* exibe uma TcNOS e é capaz de produzir NO de acordo com o estágio evolutivo em que o parasito se encontra;
  - A produção de NO e a atividade enzimática da TcNOS são mais elevadas nas formas tripomastigotas, o que pode estar relacionado com a motilidade flagelar e ser relevante para o processo de infecção.

- ✓ As formas amastigotas de *T. cruzi* e *L. amazonensis* são capazes de resistir a ação tóxica do NO, o que pode estar relacionada com a manutenção da infecção causada por esse protozoários.

- ✓ Um triplo mutante para porinas, da bactéria não patogênica *M. smegmatis,* mostrou que pode suportar altos níveis de NO *in vitro*. Tal

capacidade de resistência pode ter papel relevante na sobrevivência da mesma durante infecções intracelulares e além disso, o mecanismo de resistência parece envolver a ação de porinas.

Diante do exposto, fica clara a complexidade da via metabólica do NO em células hospedeiras e de parasitos, e possivelmente há uma grande interface nas vias metabólicas desta molécula nessas duas células.

Considerada uma molécula fundamental em ações microbicidas da resposta imune inata, na década de 80 e começo de 90, nossos resultados ressaltam outras possíveis atividades funcionais do NO como fazer parte do metabolismo do parasito ou mesmo contribuir para mecanismos de sobrevivência do mesmo em células do hospedeiro.

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