VERA LÚCIA HIDEKO TATAKIHARA

# EFEITO DO BLOQUEIO DE COX-1 E DE COX-2 SOBRE A CARGA PARASITÁRIA, PRODUÇÃO DE ÓXIDO NÍTRICO E ESTRESSE OXIDATIVO DE ERITRÓCITOS DURANTE A FASE AGUDA DA INFECÇÃO EXPERIMENTAL POR *TRYPANOSOMA CRUZI*

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Orientador: Prof. Dr. Phileno Pinge Filho
Co-orientador: Prof. Dr. Rubens Cecchini

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Prof. Dr. Phileno Pinge Filho

Londrina, 27 de fevereiro de 2007.

**APOIO FINANCEIRO**

CAPES

FAEP/UEL

CNPq

A Deus, ao meu esposo e filhos, aos
meus amigos, companheiros de
todas as horas...

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## RESUMO

A infecção com o *T. cruzi* provocou aumento na porcentagem de reticulócitos no sangue periférico dos camundongos C57BL/6 em relação aos camundongos normais (12,5 ± 1,4 vs 7,6 ± 0,6). Os camundongos C57BL/6 infectados e tratados com celecoxibe aprsentaram no dia 12 pós infecção um aumento no número de reticulócitos (22 ± 2,2 vs 12,5 ± 1,4) em relação aos controles (infectados não tratados), o que não foi verificado com os outros inibidores de COX utilizados. A infecção também provocou aumento na porcentagem de reticulócitos no sangue periférico dos camundongos BALB/c em comparação aos animais normais (14,0 ± 1,5 vs 6,8 ± 2,9). O tratamento com indometacina aumentou cerca de 1,5 vezes a porcentagem de reticulócitos no sangue daqueles animais. Estes dados sugerem que produtos do metabolismo gerados via COX-2 estariam suprimindo a produção de reticulócitos durante a fase aguda da infecção experimental com o *T. cruzi*. Em adição, nossos dados mostram claramente que macrófagos obtidos da cavidade peritoneal de camundongos BALB/c infectados produzem espontaneamente muito menos óxido nítrico (NO) em relação aos macrófagos oriundos de camundongos C57BL/6 também infectados. O aumento da carga parasitária e morte precoce dos camundongos C57BL/6 e a tendência de queda da parasitemia e prolongamento da sobrevivência dos camundongos BALB/c, quando tratados com indometacina ou Asa podem ser mais bem compreendidos quando consideramos às diferenças na produção de NO mencionadas anteriormente e os efeitos dos diferentes inibidores utilizados sobre a produção de NO. O tratamento com indometacina não modificou a produção espontânea de NO por macrófagos obtidos de camundongos C57BL/6, mas provocou o aumento da produção de NO em macrófagos obtidos de BALB/c (que apresentaram tendência de diminuição da parasitemia no sangue e no aumento da sobrevida). Já o tratamento com Asa provocou diminuição da produção de NO por macrófagos de camundongos C57BL/6 (esses apresentaram 50% de morte no período do experimento). O tratamento de camundongos BALB/c infectados com Asa provocou aumento de NO o que poderia explicar a tendência observada no controle da parasitemia por parte destes animais Em adição, há evidências sugerindo que as miocardites chagásicas são devidas aos danos induzidos pelo estresse oxidativo, podendo contribuir para a evolução da doença de Chagas em humanos e animais de laboratório. Em doenças infecciosas, a formação de espécies reativas do oxigênio (ROS) é, principalmente, derivada de danos celulares mediados pela invasão e replicação do patógeno e por reações citotóxicas mediadas pelo sistema imunológico. No entanto, como as ROS são formadas e sua função no estresse oxidativo na cardiomiopatia chagásica (CCM) não estão completamente elucidadas. As evidências atuais dão ênfase para as anormalidades mitocondriais como a disfunção da cadeia de transporte de elétrons para explicar o aumento do estresse oxidativo na doença de Chagas. A análise do estresse oxidativo por quimiluminescência iniciada por *tert*-butil hidroperóxido, mostrou claramente que o estresse oxidativo em eritrócitos que se desenvolve no camundongo C57BL/6 infectado com *T. cruzi* é atenuado com o bloqueio farmacológico tanto de COX-1 como de COX-2. Em relação ao camundongo BALB/c a quimiluminescência mostrou que o tratamento com as NSAIds utilizadas não foi capaz de reduzir o estresse oxidativo em eritrócitos de camundongos infectados. Estes dados sugerem que o mecanismo gerador do estresse oxidativo que ocorre na fase aguda da infecção com *T. cruzi* depende da capacidade do hospedeiro em responder á infecção por meio da alta produção de NO e ROS. CONCLUSÕES: Nossos dados não deixam dúvidas de que na fase inicial da infecção com *T. cruzi* ocorre um aumento da

lipoperoxidação de eritrócitos. Mostramos que o bloqueio de COX atenua a lipoperoxidação de eritrócitos no início da infecção com *T cruzi* (12º dia pós-infecção).

**Palavras-chave**: *Tripanossoma cruzi*. Estresse oxidativo. Óxido nítrico.

TATAKIHARA, Vera Lúcia Hideko. **Efeito do bloqueio de COX-1 e de COX-2 sobre a carga parasitária, produção de óxido nítrico e estresse oxidativo de eritrócitos durante a fase aguda da infecção experimental por *Trypanosoma cruzi***. 2007. 92f. Dissertação (Mestrado em Patologia Experimental) – Universidade Estadual de Londrina, Londrina, 2007.

## ABSTRACT

The *T. cruzi* infection caused an increase in the percentage of reticulocytes in C57BL/6 mice's peripheral blood in relation to normal mice (12,5 ± 1,4 vs 7,6 ± 0,6). C57BL/6 mice that were infected and treated with celecoxib presented an increase in reticulocytes (22 ± 2,2 vs 12,5 ± 1,4) on day 12 post-infection, in relation to the controls (infected but not treated), which was not verified with others COX inhibitors used. The infection also caused an increase in the percentage of reticulocytes in BALB/c mice's peripheral blood in comparison to normal animals (14,0 ± 1,5 vs 6,8 ± 2,9). Treatment with indomethacin increased the percentage of reticulocytes in those animals by 1,5 times. This data suggests that metabolism products generated via COX-2 were suppressing the reticulocyte production during the acute phase of the *T. cruzi's* experimental infection. In addition, our data clearly show that macrophages obtained in the peritoneal cavity of infected BALB/c mice spontaneously produce much less nitric oxide (NO) in relation to macrophages originated in infected C57BL/6 mice. The increase in parasite load and premature death of C57BL/6 mice, the decrease in parasitemia pattern and the survival prolongation of BALB/c mice; when treated with indomethacin or Asa can better understood when we consider the differences in NO production previously mentioned and the effects of different inhibitors used over NO production. The indomethacin treatment did not change the spontaneous production of NO by macrophages obtained in C57BL/6 mice, but it caused an increase in NO production in macrophages obtained in BALB/c mice (which presented decrease in blood parasitemia and survival increase tendencies). The treatment with Asa caused a decrease in NO production by C57BL/6 mice macrophages (these presented 50% death rate during the experiment). The treatment of infected C57BL/6 mice with Asa caused an increase in NO, which could explain the pattern observed in parasitemia control in these animals. In addition, there is evidence suggesting that chagasic myocarditis occur due to damage induced by oxidative stress, allowing the evolution of Chagas disease in humans and lab animals. In infectious diseases, the formation of reactive oxygen species (ROS) is mainly derived from cell damage mediated by pathogen invasion and replication, and by cytotoxic reactions mediated by the immune system. Therefore, since ROS are formed and its role in oxidative stress in chagasic cardiomyopathy (CCM) is not completely clear. Actual evidence gives emphasis to mitochondrial abnormalities such as electron transport chain dysfunction in order to explain the increase in oxidative stress in Chagas disease. The analysis of oxidative stress via tert-butyl hydroperoxide-induced chemiluminescence clearly showed that the oxidative stress in erythrocytes that develops in *T. cruzi* infected C57BL/6 mice is weakened with either COX-1 or COX-2 pharmacological blockage. In regard to the BALB/c mice, the chemiluminescence showed that treatment with the NSAIds used were not able to reduce the oxidative stress in infected mice's erythrocytes. This data suggests that oxidative stress producing mechanism that occurs in *T. cruzi*'s infection acute phase depends on the host's capacity to respond to the infection through high production of NO and ROS. CONCLUSIONS: Our data shows that in *T. cruzi* infections' initial phase there is an increase in lipid peroxidation in erythrocytes. We show that blocking COX weakens lipid peroxidation in erythrocytes at the beginning of *T. cruzi* infections ($12^{th}$ day post-infection).

**Keywords**: *Trypanosoma cruzi*. Oxidative stress. Nitric oxide.

## LISTA DE ABREVIATURAS E SIGLAS

**BCG** – Bacilo de Calmette-Guérin

**CAT** – Catalase

**CCM** – Cardiomiopatia chagásica

**cDNA** – DNA complementar

**COX** – Ciclooxigenase

**COX-1** – Ciclooxigenase do tipo 1

**COX-2** – Ciclooxigenase do tipo 2

**DC** – Doença de Chagas

**DCH** – Doença de Chagas Humana

**DNA** – Ácido desoxirribonucléico

**GPI** – Glico-fosfatidil inositol

**GPx** – Glutationa peroxidase

**GSH** – Glutationa

**GSR** – Glutationa redutase

**IFN** – Interferon

**MDA** – Malondialdeído

**NK** – "Natural Killer"

**NO** – Nitric Oxide - Óxido Nítrico

**NOS** – Nitric Oxide Synthase - Óxido Nítrico Sintase

**NSAIDs** – Non-steroidal anti-inflammatory drugs - Drogas antiinflamatórias não-esteróides

**PAMPs** – Padrão de seqüências moleculares associadas ao patógeno

**PG** – Prostaglandins - Prostaglandina

**$PGE_1$** – Prostaglandina da classe $E_1$

**$PGE_2$** – Prostaglandina da classe $E_2$

**ROI** – Reactive intermediates from oxygen - Intermediários reativos de oxigênio

**ROS** – Reactive oxygen species - Espécies reativas de oxigênio

**SOD** – Superóxido dismutase

**TGF-β** – Fator transformador do crescimento-β

**Th1** – Linfócito T auxiliar produtor de citocinas do padrão 1

**TLR** – Toll like receptor - Receptor de toll-like

**TNF** – Tumor necrosis factor - Fator de Necrose Tumoral

**TS** – Trans-sialidase

**IL-1** – Interleucina do tipo 1

**MHC** – Complexo de histocompatibilidade principal

**QL** – Quimiluminescência

$\mathbf{T^{ind}}$ – Tempo de indução

**SUMÁRIO**

# 1 REVISÃO BIBLIOGRÁFICA

## 1.1 *Trypanosoma cruzi*: POSIÇÃO TAXONÔMICA, MORFOLOGIA E CICLO DE VIDA

*Trypanosoma cruzi* é um protozoário flagelado, segundo Levine *et al.* da Ordem *Kinetoplastida*, Família *Trypanosomatidae*, caracterizado pela existência de um único flagelo e do cinetoplasto, uma organela contendo DNA e localizada na mitocôndria (DE SOUZA, 1984; DE SOUZA 2000). Seu ciclo de vida envolve a passagem alternada em hospedeiros vertebrados e em invertebrados da Classe *Hemiptera*, Família *Reduviidae* e Sub-Família *Triatominae*.

O ciclo de vida de *T. cruzi* tem sido o foco de excelentes revisões (BRENER, 1973; DE SOUZA, 1984; VICKERMAN, 1985; BURLEIGH; ANDREWS, 1995; TYLER; ENGMAN, 2001). Em seu ciclo, *T. cruzi* apresenta três formas evolutivas, as quais são identificadas morfologicamente pela posição do cinetoplasto com relação ao núcleo da célula e à emergência do flagelo (BRENER, 1973; DE SOUZA, 1984). Na forma tripomastigota (estágio infectante do parasito) o cinetoplasto situa-se posterior ao núcleo, em posição terminal ou subterminal, e o flagelo emerge da chamada bolsa flagelar, de localização próxima ao cinetoplasto; nos epimastigotas (formas de multiplicação no inseto e em culturas axênicas) o cinetoplasto e a bolsa flagelar estão em posição anterior ao núcleo; por fim, os amastigotas (estágios evolutivos que se multiplicam dentro das células hospedeiras de mamíferos) são formas arredondadas que apresentam um flagelo curto, de difícil visualização (BRENER, 1973; DE SOUZA, 1984, BURLEIGH; ANDREWS, 1995; TYLER; ENGMAN, 2001).

Os transmissores de *T. cruzi* são insetos estritamente hematófagos. Ao ser ingerido pelo vetor, as formas tripomastigotas presentes no sangue de vertebrados infectados, passam por uma seqüência de diferenciação ao longo do tubo digestivo do inseto, transformando-se em formas epimastigotas; por fim, os epimastigotas atingem o reto, onde se diferenciam em tripomastigotas metacíclicos, que são eliminados com as fezes e urina do vetor e podem penetrar no hospedeiro vertebrado pelas mucosas (BRENER, 1973; PEREIRA, 1990; TYLER; ENGMAN, 2001).

**Figura 1** – Formas evolutivas do *T. cruzi*: tripomastigota, amastigotas e epimastigotas.

O ciclo evolutivo de *T. cruzi* no vetor é influenciado por vários fatores, tais como: a espécie do inseto, a cepa do parasita, número de parasitas ingeridos e fatores ambientais (BRENER, 1973). A contaminação das mucosas e do ferimento provocado pela "picada" do inseto com as fezes contendo as formas tripomastigotas metacíclicas leva à infecção. Recentemente, foi mostrado que a rota anatômica preferencial utilizada pelo *T. cruzi* após invasão da conjuntiva envolve a passagem dos parasitas através do ducto nasolacrimal para a cavidade nasal (GIDDINGS, *et al*., 2006).

Independente do mecanismo de transmissão de *T. cruzi* no hospedeiro vertebrado, as formas tripomastigotas devem necessariamente penetrar no interior de células, onde se diferenciam em amastigotas. Após um período de latência, ocorrem várias divisões binárias, e os amastigotas se transformam em tripomastigotas. Com o rompimento da célula parasitada, os tripomastigotas liberados podem infectar células vizinhas ou atingir órgãos e tecidos distantes, através da corrente circulatória, reiniciando o ciclo acima descrito (BURLEIGH; ANDREWS, 1995).

As formas tripomastigotas são as formas que invadem as células do hospedeiro por um mecanismo que envolve o acoplamento do parasita à membrana citoplasmática através de um padrão de seqüências moleculares associadas ao patógeno (PAMPs), como por exemplo âncoras de GPI que são reconhecidos por receptores "Toll-like" do complexo TLR2 – TLR6 e TLR4 (GAZZINELLI; DENKERS, 2006), recrutamento e fusão dos lisossomos com esse ponto da membrana (TARDIEUX *et al*.,1992; BURLEIGH; WOLLSLEY, 2002), permitindo formação de um vacúolo e a entrada do parasita (ANDRADE; ANDREWS, 2005). O pH ácido dentro desse vacúolo estimula a secreção, pelo parasita, de uma molécula chamada Tc-Tox, que permite a saída do parasita para o citoplasma da célula (ANDREWS, 1993).

*T. cruzi* não é uma população homogênea e está constituído por diferentes cepas que apresentam grande variabilidade bioquímica e comportamento biológico, isso tem

dificultado os estudos de caracterização taxonômica das diferentes cepas, ao nível molecular e a utilização de drogas no tratamento de pacientes chagásicos (BRENER; GAZZINELLI, 1997). Os diferentes isolados e clones de *T. cruzi* foram agrupados em duas linhagens filogenéticas principais, denominadas *T. cruzi* I e *T. cruzi* II, através de marcadores bioquímicos e moleculares (ANÔNIMO, 1999). As cepas CL e Y, do grupo *T. cruzi* II infectam quatro vezes mais as células humanas do que as cepas do grupo I (F, Tulahen, M226, Sylvio-X10, Dm28c), que estão freqüentemente associadas ao ciclo silvestre de transmissão. Mais recentemente, os clones e populações estudados têm sido agrupados, mediante estudos de perfil molecular e izoenzimático, em três grandes grupos ou linhagens, denominados GI e GIII (grupos basicamente de origem silvestre, naturalmente vinculado a marsupiais) e Z2 (encontrado na DCH – Doença de Chagas Humana - da América do Sul, naturalmente ligados a primatas) (ZINGALES *et al*., 1999; DE SOUZA, 2000).

Algumas cepas mostram preferências ou "tropismos" por diferentes tecidos, como foi mostrado em camundongos infectados com diferentes amostras de *T. cruzi* examinados na fase aguda da infecção (MOROCOIMA *et al*., 2006). Estudos revelaram que algumas cepas invadem principalmente células musculares (musculatura lisa e estriada), enquanto outras, como a cepa Y, mostram uma preferência por células macrofágicas de baço, fígado e medula óssea (MELO; BRENER, 1978).

## 1.2 DOENÇA DE CHAGAS HUMANA E EXPERIMENTAL

Em 1909 Carlos Chagas descreveu o primeiro caso humano da doença causada por *T. cruzi*, a tripanossomíase americana ou doença de Chagas (DC) (CHAGAS, 1909).

Embora a melhora nas condições de moradia e a iniciativa do Cone Sul no combate à doença de Chagas tenham contribuído para o declínio na transmissão da infecção nos países endêmicos (SCHOFIELD; DIAS, 1999), a DC continua sendo um grave problema de saúde pública e atualmente a Organização Mundial de Saúde estima que de 11 a 18 milhões de pessoas estão infectadas com *T. cruzi* em todo o mundo, especialmente na América Latina (WHO, 2002). Esta endemia encontra-se condicionada ao nível econômico e social da região e em particular a existência de vetores domiciliares. No entanto, dados conhecidos sobre a transmissão congênita e por transfusão de sangue, somados aos

movimentos migratórios das pessoas infectadas, sugerem a possibilidade de transformação da tripanossomíase americana, de endemia rural em enfermidade urbana (KIRCHHOFF, 1993; SCHMUNIS, 1999; GOLDBAUM *et al*., 2004).

A infecção caracteriza-se pela existência de uma fase aguda, na qual a parasitemia patente é comumente observada seguida por uma fase crônica, onde a carga parasitária é controlada sem que haja completa eliminação dos parasitos. No homem a fase aguda da infecção geralmente é pouco sintomática. Na fase crônica, 30% dos indivíduos infectados desenvolvem patologias que podem se manifestar por insuficiência cardíaca, distúrbios do ritmo e da condução cardíacos, ou dilatações do trato digestório (ANDRADE, 1999).

Como mencionado, o protozoário pode ser transmitido por via congênita, transplante de órgãos e transfusões sanguíneas (BRENER, 1973; DIAS, 1979; DIAS, 2006). Embora muitos países da América Latina adotem a triagem sorológica para doença de Chagas nas doações de sangue, estima-se que de 0,1% a 24,4% das infecções com *T. cruzi* ocorrem através de transfusão sanguínea (WHO, 2002).

A terapêutica da Doença de Chagas é limitada basicamente a dois fármacos (CANÇADO, 1997). É consenso geral que o nifurtimox (*Lampit*, Bayer) e o benzonidazol (*Rochagan*, Roche) são capazes de curar de 70 a 100% dos chagásicos na fase aguda. Na fase crônica em que se encontram em geral os pacientes, o quadro é diferente, e as taxas de cura comprovadas variam de 6 a 36,5% dos casos (CANÇADO,1997). Isso não quer dizer que tudo está resolvido. Há ainda sérias dificuldades a vencer, a começar pela alta toxicidade dos medicamentos ativos e pela incerteza ainda reinante na apreciação do grau de eficiência terapêutica, na fase crônica.

Não foi possível ainda produzir uma vacina contra *T. cruzi*, que pudesse ser utilizada em humanos. Embora antígenos do parasito ou de outros tripanossomatídeos, quando testados em camundongos, tenham sido considerados candidatos a compor essa vacina (BRENER; KRETTLI, 1990; BREGANO *et al*., 2003; PINGE-FILHO *et al*., 2005). No entanto, uma nova esperança surgiu pela possibilidade de se utilizar um plasmídeo contendo uma seqüência de DNA complementar (cDNA) que codifique uma proteína antigênica. Este, uma vez inoculado pode gerar uma resposta celular e humoral forte e de grande duração contra o organismo que expresse a referida proteína (vacina de DNA). Utilizando-se desta técnica, Costa e colaboradores (1998) e mais recentemente Machado e colaboradores (2006), mostraram que a imunização de camundongos com um plasmídeo, contendo seqüências gênicas codificadoras para o sítio catalítico da trans-sialidase e da proteína dois de formas

amastigotas (ASP-2) de *T. cruzi*, gerou uma resposta imunológica mediada por anticorpos e células T. Quando desafiados com formas tripomastigotas sanguícolas, os animais imunizados apresentaram parasitemia e mortalidade reduzida.

A fase aguda da infecção chagásica experimental realizada em camundongos apresenta características similares à fase aguda humana, com alta parasitemia e infiltrados inflamatórios em diversos tecidos, incluindo o coração e músculo esquelético. Alguns autores foram capazes de desenvolver modelos experimentais de fase crônica (LAGUENS *et al.*, 1980, GONÇALVES *et al.*, 1982).

Nos modelos murinos, o curso da infecção varia amplamente com a raça, idade, via de inoculação e sexo do hospedeiro (GOBLE, 1951; HAUSCHKA, 1974). TRISCHMAN e colaboradores (1978) trabalhando com diferentes linhagens de camundongos isogênicos, verificaram a existência de um padrão variável de susceptibilidade e de resistência à infecção chagásica. Dependendo da cepa podem ocorrer variações no curso da infecção (CORSINI *et al.*, 1980; ANDRADE *et al*., 1985). Camundongos C57BL/6, referidos como resistentes, quando infectados com a cepa Tulahuén de *T. cruzi*, mostram-se extremamente susceptíveis à infecção (SILVA *et al*., 1992). Quando se utiliza a cepa SylvioX10 os camundongos C3H e C57BL/6, descritos como susceptíveis e resistentes a essa cepa, respectivamente, apresentam respostas opostas a esse padrão de susceptibilidade e resistência (POSTAN *et al*.,1983). Portanto, como a base genética de susceptibilidade e resistência não é totalmente conhecida, é inapropriado referir-se a uma determinada linhagem de camundongo como susceptível ou resistente sem referência à cepa de *T.cruzi* utilizada para estabelecer a infecção (TARLETON, 1995).

Camundongos C57BL/6 e BALB/c são modelos para estudo de resistência e susceptibilidade, respectivamente, a várias doenças infecciosas. Em muitos casos, a resistência do C57BL/6 é devido ao efeito microbicida do óxido nítrico produzido por macrófagos em resposta ao IFN-γ e ao TNF (SANTOS *et al.*, 2006).

No modelo murino da doença de Chagas, a resistência ou susceptibilidade do hospedeiro à infecção é dependente tanto da imunidade inata quanto adquirida e está diretamente ligada ao padrão de produção de citocinas (DOS REIS, 1997; ABRAHAMSOHN, 1998; ROGGERO *et al*., 2002) o qual é crítico para a ativação de macrófagos e controle do parasitismo durante as primeiras semanas após a infecção com *T. cruzi*.

Nesse contexto a interleucina 12 (IL-12) é considerada essencial para o estabelecimento de uma imunidade mediada por células protetora durante os estágios iniciais

da infecção por *T. cruzi* (GAZZINELLI *et al*., 1998), pois essa citocina é capaz de estimular as células NK ("natural killer") e células T a secretar IFN-γ, que exerce papel fundamental na ativação dos macrófagos para a produção de TNF-α (MUÑOZ-FERNANDES *et al.*, 1992), que em conjunto com o IFN-γ estimula o macrófago a produzir óxido nítrico (NO), o principal efetor que controla a replicação intracelular do parasita (GAZZINELLI *et al.*, 1992; VESPA *et al.*, 1994; SILVA *et. al*., 1995).

A produção de outros mediadores inflamatórios, como por exemplo, IL-1 (TARLETON, 1988), IL-6 (CARDONI *et al*., 1997) e metabólitos decorrentes da via da ciclooxigenase (COX) está também aumentada (CARDONI; ANTÚNEZ, 2004).

Recentemente foi mostrado que o IFN do tipo I também co-estimula a síntese de NO na fase aguda da infecção com *T. cruzi*, contribuindo para o controle da parasitemia em camundongos resistentes (COSTA *et al*., 2006).

Por outro lado a interleucina 10 (IL-10) e o fator transformador do crescimento–β (TGF-β) possuem atividade reguladora negativa sobre aos mecanismos tripanocidas (SILVA *et al*., 1992). Possivelmente esse efeito inibitório da IL-10 sobre a produção de IFN-γ e conseqüentemente sobre a produção de TNF-α consista em um mecanismo endógeno de prevenção de choque endotoxêmico promovido por altas concentrações de TNF-α (ABRAHAMSOHN, 1998).

Após a fase aguda da infecção, a ativação de células T $CD_4^+$ e T $CD_8^+$ e conseqüente produção de anticorpos líticos específicos e citocinas (Th1), moduladoras da função dos macrófagos, determina o controle da parasitemia pelos componentes da imunidade adquirida específica (DOS REIS, 2000).

### 1.3 DESENVOLVIMENTO DA ANEMIA NA DOENÇA DE CHAGAS EXPERIMENTAL

Carlos Chagas, em seu trabalho pioneiro descrevendo a Tripanossomíase Americana, notou que crianças infectadas apresentavam profunda anemia. Camundongos experimentalmente inoculados com diferentes cepas de *T. cruzi* apresentam além da anemia, trombocitopenia (CARDOSO; BRENER, 1980) e leucopenia (MARCONDES *et al*., 2000). Essas alterações hematológicas estão associadas a altos níveis de parasitemia, revertendo a valores normais quando a parasitemia é controlada, por exemplo, pelo nifurtimox, como

mostrado por Marcondes e colaboradores (2000). Esses pesquisadores postularam que a anemia e a trombocitopenia durante a fase aguda da infecção pode ser conseqüência da supressão induzida por mediadores inflamatórios liberados por precursores mielóides.

Por meio da *trans*-sialidase (TS), *T. cruzi* transfere o ácido siálico das plaquetas para suas estruturas, tornando as plaquetas vulneráveis à captação e eliminação pelas células de Küpffer. Sabe-se que não apenas nas plaquetas, mas também nas hemácias a diminuição no conteúdo de ácido siálico resulta em redução no tempo de vida celular (STEINER; VANCURA, 1985). Recentemente Tribulatti e colaboradores (2005) mostraram que a TS é fator de virulência presente em *T. cruzi* que provoca trombocitopenia em camundongos infectados.

O fato de que a inoculação da cepa Y de *T. cruzi* em camundongos resistentes à infecção provoca, na fase aguda, anemia mais severa do que em animas susceptíveis (MALVEZI *et al*., 2004) e que as citocinas, como o TNF-α, podem provocar efeito supressivo sobre os precursores eritróides na medula (ROODMANN *et al.*, 1987; MALVEZI *et al*., 2004) durante a infecção experimental com *T. cruzi,* sugerem que as alterações hematológicas observadas podem ser conseqüência tanto da ação supressora dos mediadores produzidos por macrófagos durante a fase aguda (ABRAHAMSOHN; COFFMAN, 1995; PINGE-FILHO *et al*., 1999) quanto dos componentes da imunidade do animal infectado (MARCONDES *et al*., 2000).

A anemia observada durante a infecção aguda por *T.cruzi* é geralmente acompanhada por lipoperoxidação dos eritrócitos com a participação do NO (óxido nítrico) (MALVEZI *et al.*, 2004) indicando que os radicais livres resultantes do estresse oxidativo provocado tanto pelo parasita, quanto pela resposta imunológica antiparasitária pode ter papel importante na geração da lesão dos eritrócitos.

## 1.4 ESTRESSE OXIDATIVO NA DOENÇA DE CHAGAS EXPERIMENTAL

O radical livre é definido como uma molécula de existência independente que possui um ou mais elétrons não emparelhados em um orbital atômico ou molecular (HALLIWELL; GUTTERIDGE, 1989). Durante o processamento molecular do oxigênio pelas mitocôndrias, cerca de 1 a 5% dos elétrons escapam da cadeia respiratória e formam espécies reativas de oxigênio (ROS) (SCHANAIDER, 2000), os radicais livres. Esses radicais

reagem com moléculas transformando-as em moléculas reativas capazes de transferir elétrons para outras moléculas não radicais, tornando-as radicais.

Os principais radicais livres são: o ânion superóxido ($O_2^{\bullet -}$), peróxido de hidrogênio ($H_2O_2$), radical hidroxila ($OH^{\bullet}$), halaminas, oxigênio singleto, entre outros. As células possuem mecanismos antioxidantes como enzimas superóxido dismutase, catalase e glutationa peroxidase que às protegem da ação desses radicais (HALLIWELL; GUTTERIDGE, 1989). Quando a formação dos radicais livres excede a capacidade da célula de removê-los, os efeitos lesivos desses radicais tornam-se predominantes gerando estresse oxidativo. A origem desses radicais está representada nas equações abaixo (BABIOR, 1997).

A redução da molécula de oxigênio para duas moléculas de água é a maior fonte de energia nos sistemas biológicos aeróbicos. Essa redução necessita de quatro elétrons:

$$\mathbf{O_2 + 4e^- + 4H^+ \longrightarrow 2H_2O}$$

Mas se os elétrons são transferidos um a um para o oxigênio, uma série de produtos parcialmente reduzidos são gerados. O primeiro desses produtos é o superóxido ($O_2^{\bullet -}$):

$$\mathbf{O_2 + e^- \longrightarrow O_2^{\bullet -}}$$

A redução do $O_2^{\bullet -}$ pelo segundo elétron produz peróxido de hidrogênio:

$$\mathbf{O_2^{\bullet -} + e^- + 2H^+ \longrightarrow H_2O_2}$$

O radical hidroxila e a primeira molécula de água surgem quando o terceiro elétron é transferido para o peróxido de hidrogênio:

$$\mathbf{H_2O_2 + e^- + H^+ \longrightarrow OH^{\bullet} + H_2O}$$

Finalmente, o quarto elétron produz a segunda molécula de água a partir do radical hidroxila:

$$\mathbf{OH^{\bullet} + e^- + H^+ \longrightarrow H_2O}$$

Ao contrário da maioria das moléculas com elétrons não pareados, o ânion superóxido é surpreendentemente inerte e sua principal reação é com outra molécula de superóxido para formar água oxigenada:

$$\mathbf{2\ O_2^{\bullet -} + 2H^+ \longrightarrow H_2O_2 + O_2}$$

É também uma base fraca, sendo seu conjugado ácido, o radical hidroperoxil, muito mais reativo:

$$\mathbf{O_2^{\bullet -} + H^+ \longrightarrow HO_2^-}$$

Esses radicais formados nessas reações também participam de outras reações como a reação de Haber-Weiss:

$$O_2^{\bullet -} + Fe^{3+} \longrightarrow O_2 + Fe^{2+}$$

e a reação de Fenton:

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + OH^{\bullet}$$

O superóxido também reage com hidroxila para formar oxigênio singleto:

$$O_2^{\bullet -} + OH^{\bullet} \longrightarrow {}^1O_2 + OH^-$$

e com o óxido nítrico para formar peroxinitrito, um radical altamente oxidante:

$$O_2^{\bullet -} + NO^{\bullet} \longrightarrow OONO^- + H^+ \longrightarrow NO_2^+ + OH^-$$

O óxido nítrico participante da reação acima é gerado a partir da *L*-arginina pela ação da enzima óxido nítrico sintase (NOS) na presença de $O_2$ e NADPH (PALMER; MONCADA, 1989), principalmente por macrófagos ativados.

Em adição, o peróxido de hidrogênio formado por dismutação do superóxido é utilizado por fagócitos para formar ácidos hipohalosos (como HOCl), compostos altamente reativos que reagem com aminas para produzir halaminas (como $NH_2Cl$), que podem ser ainda mais reativas que os ácidos hipohalosos. O ácido hipohaloso também pode reagir com o peróxido de hidrogênio para formar oxigênio singleto:

$$OBr^- + H_2O_2 \longrightarrow {}^1O_2 + H_2O + Br$$

### 1.4.1 Enzimas Antioxidantes

A remoção dos radicais livres em células de mamíferos, é realizada principalmente por quatro enzimas que compreendem 3 sistemas :

### 1.4.1.1 Superóxido dismutase (SOD)

Essa enzima catalisa a conversão do superóxido ($O_2^{\bullet -}$) em oxigênio e água:

$$2O_2^{\bullet -} + 2H^+ \longrightarrow O_2 + H_2O$$

Embora a dismutação espontânea seja rápida, a SOD acelera bastante a reação (FRIDOVICH, 1995).

Duas formas de SOD estão presentes nos células eucarióticas, uma que contém $Cu^{2+}$ e $Zn^{2+}$ (CuZnSOD) encontrada no citosol, e outra que contém $Mn^{2+}$ (MnSOD) localizada na mitocôndria. Ao contrário da CuZnSOD que não é afetada pelo estresse oxidativo, a MnSOD é induzível e sua atividade aumenta com o estresse oxidativo (BABIOR, 1997).

### 1.4.1.2 Catalase (CAT)

O peróxido de hidrogênio é controlado por dois sistemas, catalase e sistema antioxidante dependente de glutationa que convertem o peróxido em água às custas de NADPH. A catalase catalisa a transformação de $H_2O_2$ em água e oxigênio

$$2H_2O_2 \xrightarrow{\text{Catalase}} O_2 + 2H_2O$$

Cerca da metade do $H_2O_2$ produzido é destruído pela catalase (BABIOR, 1997).

### 1.4.1.3 Sistema antioxidante dependente de glutationa

Esse sistema é composto de glutationa (GSH) mais duas enzimas, glutationa peroxidase (GPx) e glutationa redutase (GSR) e seu funcionamento está baseado no ciclo da GSH entre a forma oxidada e a forma reduzida:

$$2GSH + H_2O_2 \xrightarrow{\text{GSH Peroxidase}} GSSG + 2H_2O$$

$$GSSG + NADPH + H^+ \xrightarrow{\text{GSH Redutase}} GSH + NADP^+$$

#### 1.4.1.4 Avaliação do estresse oxidativo

Devida a sua alta reatividade, os radicais livres tem vida curta, o que torna difícil sua medição direta (HOLLEY; CHEESEMAN, 1996). Assim a avaliação dos radicais livres é baseada na pesquisa de produtos secundários da reação com radicais livres gerados ou consumidos durante o processo.

A membrana do eritrócito contém grande número de grupamentos tióis (R-SH) que podem ser convertidos em dissulfetos (R-SSG) pelos oxidantes, levando à desnaturação das proteínas de membrana (GILBERT; MC LEAN, 1990) com conseqüente lesão intracelular e oxidação da hemoglobina à metahemoglobina (RICE-EVANS; BAYSAL, 1987).

O componente lipídico da membrana do eritrócito também está sujeito ao estresse oxidativo. A lipoperoxidação, conseqüência do ataque de espécies reativas de oxigênio que retiram átomos de hidrogênio dos grupamentos metil dos lipídeos de membrana, pode ser medida como um indicador indireto da concentração de radicais livres.

A lipoperoxidação é uma reação em cadeia representada pelas etapas de iniciação, propagação e terminação (GARDÈS-ALBERT *et al*., 1991).

A reação começa com a remoção do átomo de hidrogênio do ácido graxo polinsaturado da membrana celular pelo oxidante. Essa remoção pode ser feita pelo $OH^{\bullet}$ ou $LO^{\bullet}$ (radical alcoxil), formando o $L^{\bullet}$ (radical lipídico). O $L^{\bullet}$ reage rapidamente com o $O_2$ formando $LOO^{\bullet}$ (radical peroxil) que remove novo hidrogênio do ácido graxo formando novamente o $L^{\bullet}$ e hidroperóxido lipídico (LOOH) que pode ser convertido em malondialdeído, um dos produtos finais da lipoperoxidação (HALLIWELL; GUTTERIDGE, 1989). A lipoperoxidação termina quando os radicais $L^{\bullet}$ e $LOO^{\bullet}$ destroem a si próprios.

A determinação dos níveis de glutationa, e enzimas antioxidantes (SOD, CAT, GPx e GSR) também pode ser uma indicador dos níveis de estresse oxidativo a que o tecido está sendo submetido (WEN *et al*., 2004).

### 1.4.2 Radicais livres e a infecção por *T. cruzi*

Evidências da participação dos macrófagos no controle da infecção por *T. cruzi* foram mostradas nos experimentos de Hoff (1975) e Nogueira e Cohn (1978) que observaram que macrófagos de animais imunizados com BCG (Bacilo de Calmette-Guérin) apresentam atividade tripanocida aumentada quando comparada com os macrófagos de animais não imunizados.

A ativação dos macrófagos pelo BCG está associada a um aumento na produção de intermediários reativos de oxigênio (ROI) como, por exemplo, $H_2O_2$ (NATHAN, 1979). Porém o tratamento dos macrófagos com enzimas antioxidantes (SOD, CAT) ou benzoato de sódio, removedores de radicais livres produzidos pela explosão respiratória não inibe a atividade tripanocida (MCCABE; MULLINS, 1990) desses macrófagos. Mais tarde foi mostrado que a atividade tripanocida dos macrófagos é dependente principalmente da produção de óxido nítrico (GAZZINELLI *et al*., 1992; VESPA *et al*., 1994).

A habilidade de *T. cruzi* para resistir aos radicais livres está relacionada com a presença de mecanismos como a tripanotiona e enzimas relacionadas que estão envolvidas na proteção contra dano causado por oxidantes, metais pesados e, possivelmente, xenobióticos (FAIRLAMB; CERAMI, 1992)

Embora a atividade no controle da multiplicação do parasita seja dependente de NO, os intermediários reativos de oxigênio, (ROI), têm papel importante nas lesões teciduais ocorridas durante a infecção. Modificações na estrutura do retículo endoplasmático liso das células do hospedeiro, provavelmente devido à produção de $O_2^{\bullet -}$ e $H_2O_2$ foram mostradas por Cecchini (1987).

O estresse oxidativo em tecidos como fígado e coração também foi constatado por Do Carmo (1995), que mostrou que os níveis de lipoperoxidação estão aumentados nesses órgãos durante a infecção experimental em camundongos.

Os experimentos de Wen e colaboradores (2004) mostram que alterações na cadeia respiratória mitocondrial são fontes de produção de ROI nos miócitos de camundongos C3H/HeN infectados com *T. cruzi* e que a severidade da miocardiopatia nesses animais está relacionada com o aumento no dano oxidativo, demonstrado pelo aumento dos níveis de malondialdeído (MDA) e proteína carbonílica e redução dos mecanismos antioxidantes das células (por exemplo a SOD).

Os ânions superóxido produzidos durante a infecção por *T. cruzi* também podem reagir com o NO para formar peroxinitrito ($ONOO^-$) ou, pela reação de Fenton, podem gerar $OH^{\bullet}$, dois radicais altamente reativos que podem estar associados ao dano oxidativo nos eritrócitos observado durante a infecção experimental em camundongos C57BL6 e Swiss (MALVEZI *et al*., 2004). Estes autores também sugerem, que nesse caso, o dano oxidativo no eritrócito pode ser dependente de um balanço entre os níveis de $O_2^{\bullet -}$ e $NO^{\bullet}$ produzidos pelo hospedeiro.

Em adição, há evidências que sugerem que as miocardites chagásicas são devidas aos danos induzidos pelo estresse oxidativo, podendo contribuir para a evolução da doença de Chagas (ZACKS *et al*., 2005). Em doenças infecciosas, a formação de espécies reativas do oxigênio (ROS) é, principalmente, derivada de danos celulares mediados pela invasão e replicação do patógeno e por reações citotóxicas mediadas pelo sistema imune.

No entanto, como as ROS são formadas e sua função no estresse oxidativo na cardiomiopatia chagásica (CCM) não estão completamente elucidadas. As evidências atuais dão ênfase para as anormalidades mitocondriais como a disfunção da cadeia de transporte de elétrons para explicar o aumento do estresse oxidativo na doença de Chagas (ZACKS *et al*., 2005).

## 1.5 PROSTAGLANDINAS E INFECÇÃO COM TRYPANOSOMA CRUZI

Prostaglandinas (PGs) sintetizadas pelos macrófagos, principalmente $PGE_2$, constituem uma classe de ácidos graxos poliinsaturados, de grande importância imunofarmacológica, produzidos pela ação da enzima ciclooxigenase (COX) da prostaglandina sintase sobre o ácido aracdônico liberado de fosfolípidios de membrana celular (PARK *et al.,* 2006). Sua presença foi constatada pela primeira vez em 1930, mas as primeiras estruturas só foram elucidadas em 1962 (STRYER, 1995).

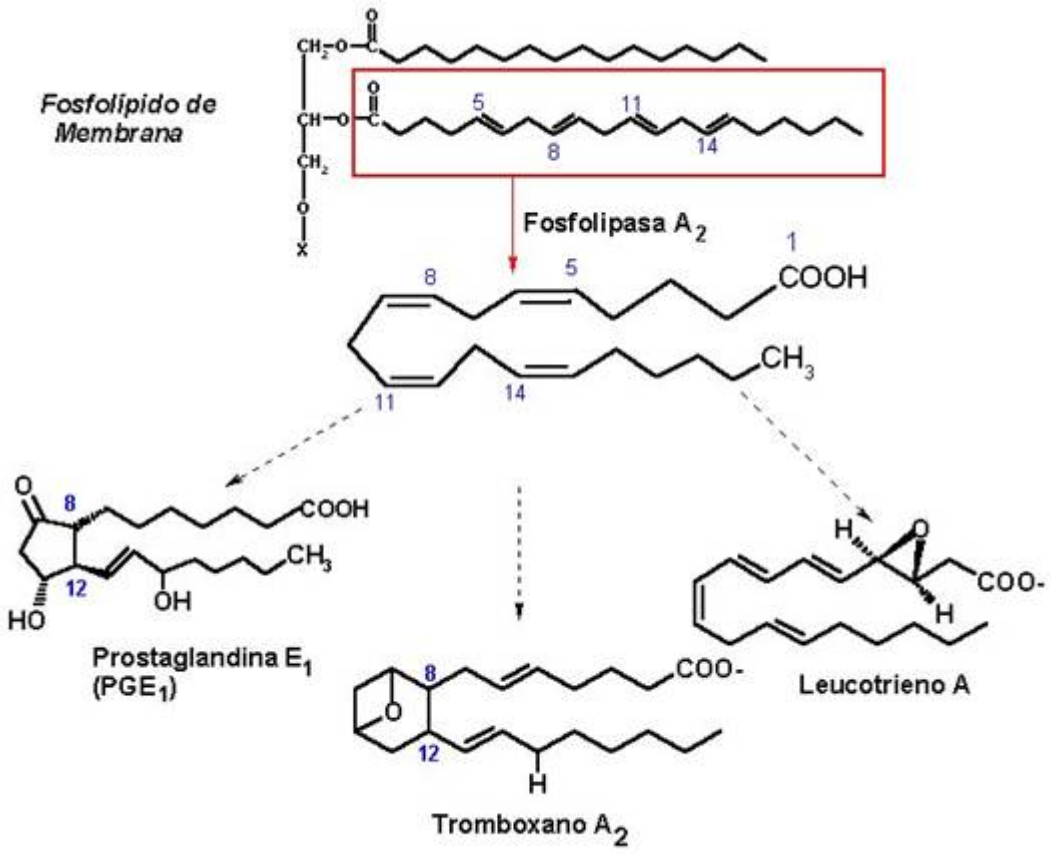


**Figura 2** – Origem das Prostaglandinas.

A COX pode apresentar-se em duas diferentes isoformas: COX-1 é a isoforma expressa de forma constitutiva, encontrada virtualmente em todos os tipos celulares dos mamíferos em um nível praticamente constante; COX-2 é praticamente indetectável na maioria dos tecidos, mas é rapidamente induzida em resposta a mitógenos, citocinas e endotoxinas (APLETON *et al*., 1995).

Resumidamente, a síntese de $PGE_2$, tem início a partir do araquidonato, que possui quatro carbonos. Um anel ciclopentano é formado e quatro átomos de oxigênio são introduzidos pela COX. Todos os átomos de oxigênio introduzidos na PG vêm do oxigênio molecular. O componente hidroxiperoxidase da COX, catalisa uma redação por $2e^-$ de um grupamento 15-hidroxiperoxi para um grupamento 15-hidroxila (descrito por STRYER, 1995).

Os macrófagos em particular, são capazes de produzir quantidades substanciais de imunomoduladores como as prostaglandinas da série E ($PGE_2$ e $PGE_1$),

especialmente após ativação por estímulos inflamatórios (KUNKEL *et al.*, 1986[a]). E sua síntese pode ser bloqueada por inibidores da ciclooxigenase, como drogas antiinflamatórios não-esteróides (NSAIDs) (MITCHELL *et al*., 1994). Estes inibidores podem exibir três tipos diferentes de mecanismos: competitiva reversível, irreversível e reversível não competitiva (LANDS, 1981; MITCHELL *et al*., 1994; HAWKEY, 1999;). Aspirina por exemplo, inativa irreversivelmente ambas COX-1 e COX-2 pela acetilação do grupo hidroxil de um resíduo de serina localizado no aminoácido 70 da região aminoterminal da enzima (ROTH *et al*., 1975). Outro inibidor bastante utilizado, a indometacina, causa uma inibição da COX-1 e da COX-2, reversível competitiva ou não (KURUMBAIL *et al*., 1996).

A $PGE_2$ tem múltiplos efeitos sobre o sistema imunológico, incluindo inibição da proliferação de células T murinas e de humanos (HASLER *et al*., 1983), inibição da atividade de células NK (GOTO *et al*., 1983), inibição da produção de IL-1, TNF-α (KUNKEL *et al*., 1986[b]). Foi mostrado também que $PGE_2$ inibe o desenvolvimento da resposta Th1 e secreção de citocinas Th1 específicas (IFN-γ, IL-2) (BETZ; FOX, 1991). $PGE_2$ também interfere nas interações *in vivo* entre monócitos e células T, gerando uma apresentação antigênica inadequada para células T e inibição da expressão de moléculas do MHC de classe II (STEPHAN *et al*., 1988). Em geral as prostaglandinas funcionam como agentes imunossupressores, então, a capacidade de um antígeno induzir a liberação de $PGE_2$ pelos macrófagos pode influenciar o desenvolvimento de uma resposta Th1 ou Th2 dominante e definir o estado de imunidade do hospedeiro (protetora ou não). Existem evidências de que a $PGE_2$ está aumentada em várias infecções parasitárias, tais como *Criptosporidium parvum* (ARGENZIO; RHOADS, 1997), *Toxoplasma gondii* (YONG *et al*., 1994), *Leishmania donovani* (REINER; MALEMUD, 1985), *Leishmania major* (CILLARI *et al*., 1986) e *T. cruzi* (CELENTANO *et al*., 1995; PINGE-FILHO *et al.,* 1999; CARDONI; ANTÚNEZ, 2004).

Prostaglandinas (PGs) juntamente com NO e TNF-α participam de um complexo circuito que controla a linfoproliferação e produção de citocinas durante a fase aguda da infecção com *T. cruzi* (PINGE-FILHO *et al*., 1999). O efeito supressor de $PGE_2$/TGF-β sobre a expressão de citocinas pró-inflamatórias (FADOK, 1998) poderia criar um ambiente favorável para o crescimento de *T. cruzi* dentro dos macrófagos.

Ainda que a ocorrência de imunossupressão induzida com *T. cruzi* esteja bem documentada, os papéis exercidos pelas PGs no estresse oxidativo associado à anemia não foram ainda investigados. Em adição, estudos que utilizaram diferentes NSAIDs para

determinar o papel das PGs no controle da carga parasitária e resistência à infecção com *T. cruzi* são discrepantes (CELENTANO *et al*., 1995; PINGE-FILHO *et al*., 1999; FREIRE-DE-LIMA *et al*., 2000; MICHELIN *et al.*, 2005).

Desta forma, estudos que buscam investigar o papel das PGs nos mecanismos de defesa e de geração de estresse oxidativo envolvidos na infecção por *T. cruzi* são essenciais para o melhor entendimento do desenvolvimento da doença de Chagas e podem indicar caminhos para o desenvolvimento de novas estratégias para o tratamento desta importante enfermidade.

# 2 OBJETIVOS

## 2.1 OBJETIVOS GERAIS

Investigar o efeito do bloqueio farmacológico de COX-1 e de COX-2 no curso da infecção experimental de animais na fase aguda da infecção com a cepa Y do *T. cruzi*, analisando parâmetros hematológicos, carga parasitária, produção de óxido nítrico e análise do estresse oxidativo em eritrócitos.

## 2.2 OBJETIVOS ESPECÍFICOS

**2.2.1** Avaliar o papel das prostaglandinas sobre o curso da infecção experimental com *T. cruzi* realizando o bloqueio farmacológico *in vivo* com inibidor específico de COX-2, celecoxibe, Asa inibidor de COX-1, e COX-2 e indometacina inibidor preferencial de COX-1, determinando os níveis de parasitemia no sangue e no coração e sobrevida de camundongos infectados com *T. cruzi*;

**2.2.2**. Correlacionar os níveis de parasitemia com o desenvolvimento da anemia;

**2.2.3** Avaliar o estresse oxidativo por meio da determinação do consumo de oxigênio, tempo de indução ($T^{ind}$) e quimiluminescência (QL) e ao mesmo tempo averiguar a participação das prostaglandinas como possíveis mediadores da lesão pré-hemolítica em eritrócitos provenientes de camundongos C57BL/6 (resistentes) e BALB/c (susceptíveis) à infecção com a cepa Y de T. cruzi;

**2.2.4** Analisar o efeito in vivo da inibição de COX-1 ou de COX-2 sobre a produção espontânea de óxido nítrico (NO) por macrófagos oriundos de camundongos infectados.com *T. cruzi.*

## 3 CONCLUSÕES

**3.1** A utilização de inibidores de COX-2 aumenta significativamente a parasitemia no sangue e no tecido cardíaco e diminuem a taxa de sobrevida de ambas as linhagens de camundongos utilizadas;

**3.2** O desenvolvimento da anemia e da hipoplasia medular observada em camundongos C57BL/6 e BALB/c durante a fase aguda da infecção por *T. cruzi* independe das PGs;

**3.3** Em camundongos C57BL/6 o tratamento com inibidor seletivo de COX-2 (celecoxibe), atenuou a trombocitopenia e a leucopenia e acentuou a reticulocitose;

**3.4** Em camundongos BALB/c o tratamento com inibidor preferencial de COX-1 (indometacina), acentuou a reticulocitose;

**3.5** Todos os três inibidores de ciclooxigenase utilizados atenuaram a lipoperoxidação de eritrócitos de camundongos C57BL/6 infectados, mas não atenuaram os de camundongos BALB/c;

**3.6** A partir desses resultados, nós sugerimos que a liberação de prostaglandinas através da cicloxigenase induzível é um dos fatores centrais para a proteção tecidual e para danos pré-hemolíticos de eritrócitos na doença de Chagas experimental.

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**5 MANUSCRITO**

# Differencial effects of cyclooxygenase inhibitors on parasite burden and oxidative stress in erythrocytes from mice susceptible and resistant to *Trypanosoma cruzi* infection

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***Abstract***

We investigated the effects of inhibition of COX-1 and COX-2 on control of parasite burden, and erythrocyte oxidative stressing during *Trypanosoma cruzi* infection in C57BL/6 and BALB/c mice. Mice were infected with 5 x $10^3$ trypomastigotes forms of *T. cruzi* (Y strain). After four hours, selected mice were treated or not with indomethacin (1.2mg/Kg/day) a preferential antagonist of COX-1, aspirin (50 mg/Kg/day) inhibitor of both COX and celecoxib (50mg/Kg/day) a COX-2 selective inhibitor. *Tert*-butyl hydroperoxide-initiated-chemiluminescence (CL) analysis carried out in non-lysed erythrocytes from *T. cruzi* infected-mice ($12^{th}$ day) showed a dramatic increase in CL indicating both reduced levels of antioxidants and increased erythrocyte membrane lipid peroxide. The COX-2 inhibitor employed, significantly increased the blood and cardiac tissue parasitemia and decreased the survival rate of both strains mice. All three cyclooxygenase inhibitors employed, reduced the lipid peroxidation in erythrocytes from C57BL/6 *T. cruzi*-infected-mice but not in BALB/c infected-mice. From these results, we propose that inducible cyclooxgenase-released prostaglandin is one of the pivotal factors to tissue-protective and pre-hemolytic damage of erythrocyte in the experimental Chagas' disease.

***Keywords:*** Trypanosoma cruzi*, Chagas' disease, cyclooxygenase, NSAIDs, anemia, oxidative stress, nitric oxide*

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## 1. Introduction

*Trypanosoma* (*Schizotrypanum*) *cruzi* is an intracellular parasite that causes American trypanosomiasis (Chagas'disease), characterized by a progressive inflammatory reaction mainly affecting the function of myocardial and skeletal muscles as well as the viscera of the digestive tract [1]. *T. cruzi* trypomastigotes can infect many different mammalian cell types in which they differentiate and multiply as amastigote forms. Macrophages can act both as host cells for the parasite and as effector cells in the anti-parasite immune responses [2,3].

The murine experimental infections usually show an acute stage with high parasitemia and prominent inflammatory infiltrates in several tissues, including the heart and skeletal muscles. At the moment, the mechanisms that induce this inflammatory reaction and its role in the resistance and/or the outcome of the disease are not fully understood. Several pro-inflammatory cytokines that tailor the protective immune response in the acute phase can play a role in its onset. In the early infection, host resistance depends on the T helper type 1(Th1) protective response, [4] triggered by IL-12 and IL-18 [5] that synergistically activate T cells and natural killer cells to produce interferon gamma (IFN-γ) [6]. In turn, IFN-γ and tumor necrosis factor alpha (TNF-α) stimulate phagocytic cells to destroy internalized parasites, mainly through nitric oxide (NO) generation [7], resulting in a raise of reactive intermediates from oxygen (ROS) [8] and reactive nitrogen species (RNS) [9]. The production of other inflammatory mediators, such as IL-1 [10] and IL-6 [11], and cyclooxygenase metabolites is also enhanced [12].

*T. cruzi* infection in mice is associated with severe hematological changes, including thrombocytopenia [13], neutropenia following by neutrophilia and eosinophilia [14], which may contribute to mortality. Similar hematological alterations have also been described in

experimental African trypanosomiasis [15] and are also common characteristic of human immunodeficiency virus infection [16] and malaria [17-19]. Marcondes and collaborators [20] showed that experimental acute *T. cruzi* infection is associated with anemia, thrombocytopenia, leukopenia, and bone marrow hypoplasia and that these alterations can be prevented by nifurtimox (an anti-trypanosomal drug) treatment.

The mechanisms responsible for these hematological alterations are not clearly understood, but some studies have provided a molecular explanation for the pathological findings. There are evidences indicating increased oxidative stress in the course of Chagas' disease [21-24]. Our previous results revealed that nitric oxide (NO) does not play direct role in development of anemia during *T. cruzi* infection, but contribute together with TNF-α to oxidative pre-hemolytic damage of erythrocytes in infected mice [25]. Tribulatti and collaborators [26] demonstrated that the trans-sialidase (TS) from *T. cruzi* deplete the sialic acid from platelets, increasing its clearance and leading afterwards to a thrombocytopenia observed during acute phase of infection. In addition, it was shown that IFN-inducible p47GTPase (LRG-47) influence *T. cruzi* control by simultaneously regulating macrophage-microbicidal activity and hemopoietic function [27].

The cyclooxygenase-released prostaglandin involvement in the oxidative stress associated with anemia in the early of *T. cruzi* infection is not known and date about its role in the control of parasitism and resistance to *T. cruzi* infection are discrepant [28-31].

Prostaglandins (PGs) are synthesized from arachidonic acid by enzymes known as cyclooxygenases (COX) that exist in at least two isoforms, type 1 (COX-1), which is constitutive and type 2 (COX-2), which is induced by inflammatory mediators, mainly cytokines [32]. Prostaglandin $E_2$ ($PGE_2$) is a principal mediator of inflammation in diseases such as rheumatoid, osteoarthritis and cancer [32,33].

$PGE_2$ has no effect on or enhances the production of Th2 cytokines, such as IL-4, IL-5 and IL-10, by Th2 cells, but inhibits drastically the production of Th1 cytokines, such as IL-2 and its receptor [34,35] and IFN-γ by Th 1 cells [36].

There is evidence that $PGE_2$ synthesis is increased in several parasitic infections such as *Cryptosporidium parvum* [37], *T. gondii* [38], *Leishmania donovani* [39], *Leishmania major* [34], and *T. cruzi* [28-31,12].

However, PG and RNS, such as $^{\bullet}NO$, along with reactive oxygen species (ROS) produced by activated macrophages have been implicated as mediators of tissue damage in numerous pathological conditions [40, 25], including regulation of lipid peroxidation [41].

The aim of this study was to compare the *in vivo* inhibition of cyclooxygenase-1 and cyclooxygenase-2 by non-steroidal anti-inflammatory drugs (NSAIDs) on the parasitism, resistance, anemia, erythrocytes oxidative damage and NO production in BALB/c and C57BL/6 mice infected with *T. cruzi* (Y strain). In the acute phase, we observed an increased NO production associated with anemia and oxidative stress in erythrocytes in both mouse strains. A parallelism was observed between the *in vivo* inhibition of COX and reduction of oxidative stress in erythrocytes with increased on parasite burden associate a decrease in the production of NO by macrophages.

## 2. Material And Methods

This study was reviewed and approved by the Internal Scientific Commission and the Ethics in Animal Experimentation Committee of the Londrina State University, Londrina, Brazil (Process nº 28568/05, CEEA 54/05).

*2.1 Mice and reagents*

Swiss mice were obtained from the breeding colonies of the animal facility of the Center for Biological Sciences at Londrina State University, Londrina, Brazil. C57BL/6 mice and BALB/c were obtained from the mouse breeding facilities of the Department of Immunology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil. We used 8 to 12 weeks old male mice in all experiments. Mice were maintained under standard conditions in animal facility within the Department of Pathological Sciences, Center for Biological Sciences, Londrina State University. Commercial rodent diet (Nuvilab-CR1, Nuvital, Campo Mourão, Brazil) and sterilized water were available *ad libitum*. All procedures with the animals were in accordance with the guidelines of the Brazilian Code for the Use of Laboratory Animals.

Indomethacin (1-[p-chlorobenzoyl]-5-methoxy-2-methylindole-3-acetic acid, $C_{19}H_{16}CINO_4$, Sigma, St Louis, USA) was dissolved in absolute ethanol and diluted in RPMI 1640 (GIBCO BRL, USA) without serum. Separate experiments indicated that 0.005% ethanol did not alter responses from normal mice. Aspirin (2-acetoxybenzoic Acid, $C_9H_8O_4$, Biotec, Brazil) and celecoxib ([4 - [5 - (4-methyllphenyl) – 3-(trifluoromethyl)-1H-pyrazol-1-yl] benzenesulfonamide, $C_{17}H_{14}F_3N_3O_2S$, Pfizer Pharmaceuticals, Porto Rico) were dissolved in phosphate-buffered saline (PBS, pH 7.2) [26,27]. All reagents were prepared fresh every day and aliquots were stored at 4ºC until used. Buffers and solutions were prepared in deionized water.

*2.2 Trypanosoma cruzi infection, parasitemia and survival rates determination*

The Y strain of *T. cruzi* [42] was kindly supplied by Dr. Paulo Maria Ferreira de Araújo (Institute of Biosciences, Campinas State University, Campinas, São Paulo, Brazil).

This strain was maintained by weekly intraperitoneally (i.p.) inoculation of Swiss mice with $2x10^5$ blood trypomastigotes forms. Infective blood trypomastigotes were obtained from *T. cruzi*-infected mice by drawing blood via cardiac puncture following anaesthetization. Motile blood forms were counted and the desired number of parasites ($5x10^3$) was injected intraperitoneally (i.p.) in C57BL/6 and BALB/c mice. Parasitemia was assessed by counting circulating parasites in 5 μL of blood obtained from a tail vein of infected mice. These data were expressed as the number of parasites per milliliter of blood. Parasitemia and survival rates were determined daily, beginning at the $5^{th}$ day post infection [43].

*2.3 Treatment of mice with cyclooxygenase inhibitors (NSAIDs)*

Indomethacin (INDO) (1.2 mg/kg), aspirin (ASA) (50 mg/Kg) and celecoxib (50 mg/Kg) were injected i.p. in mice at a daily dose. Groups of 6-10 mice were used. C57BL/6 and BALB/c mice received the first dose 4 h after infection and were treated until they died; parasitemia and mortality were evaluated in the same period. Untreated *T. cruzi*-infected mice were used as controls. In order to evaluate drug toxicity, normal mice were inoculated only with indomethacin, aspirin or celecoxib until 30 days, using the same treatment schedule. The dose of NSAIDs chosen for these experiments was based on previously published studies demonstrating its efficacy [28-30,44].

*2.4 Hematological methods*

Erythrocytes and leukocytes from normal and infected mice under ether anesthesia, were collected from cardiac puncture with heparinized needles and syringes and counted by standard methods [45]. Hemoglobin concentration was determined by the Drabkin method. Hematocrits were obtained by microcentrifugation of capillary tubes filled with heparinized

blood [46]. For enumerating reticulocytes, 40 µl of heparinized blood was incubated with 20 µl brilliant cresyl blue for 20 minutes at 37°C, and then thin blood smears were prepared on glass slides. After the blood smears air dried, reticulocytes were counted by light microscopy. All blood analysis and cell counts were performed 12 days post-infection. Platelets were counts from peripheral blood from normal and infected mice anaesthetized, in polypropylene tubes containing 3.8% (w/v) sodium citrate (citrate/blood ratio, 1:9) [26]. All the manipulations were carried out at room temperature. The number of platelets and blood cells were determined by manual counting with a Neubauer hemocytometer.

*2.5 Bone marrow cells harvest*

Bone marrow cells were harvested by flushing the femoral shafts with ice-cold PBS, as previously described [47]. The total number of cells collected was determined by manual hemocytometer count. For differential counts, cell suspensions from uninfected and day 12-infected mice were deposited on glass slide, stained with May Grünwald-Giemsa, then counted by light microscopy.

*2.6 Histopatological analysis*

On day 12 of infection, mice treated or not with NSAIDs were sacrificed. The heart was removed, fixed in 10% buffered formalin, were paraffin embedded, than sections were stained with hematoxylin/eosin (HE), and then analysed by light microscopy. The number of parasite nests was counted in 50 microscope fields (400x magnification) per tissue section. Three sections were counted and the results were expressed as the mean of them.

*2.7 Macrophages cultures and quantification of nitrite ($NO_2^-$)*

Peritoneal cells (PCs) were prepared from normal and infected (day 12 pi) mice treated or not with NSAIDs. Four days after i.p. injection of 1.5 mL of 5% thioglycollate to BALB/c and C57BL/6 mice, cells were harvested from the peritoneal cavity by injecting cold PBS, centrifuged at 500 x g for 10 min at 4ºC, resuspended in RPMI1640 medium supplemented with 10% heat inactivated fetal bovine serum (FBS, CRILON), 50 mg/mL gentamicin, 100 U/mL penicillin, 100 ug/mL streptomycin and 2 mM glutamine. The PCs suspension was dispensed into 48-well tissue culture plates ($10^6$ cells/ml) for 4 h to allow the cells to adhere to the plastic surface, followed by washing with warm PBS to remove nonadherent cells. For each experiment, PCs from three mice were pooled.

Macrophage monolayers from C57BL/6 and BALB/c mice were incubated in the presence of Asa (10 mM), Indo (10 μM) or celecoxib (10 mM) [48,49]. Nitrite ($NO_2^-$) accumulation in 24-h supernatants of cultured cells was used as an indicator of NO production and was determined by the Griess reaction with sodium nitrite as a standard, as previously describe (detection limit: 1:56 μM) [50]. Fifty microliters of supernatant were incubated for 10 min, in the dark, at room temperature, with an equal volume of Griess reagent (1% sulfanilamide, 0.1% N-1-naphthylethylenediamine dihydrochloride, 2.5% phosphoric acid). The absorbance was determined at 540 nm.

*2.8 Measurements of oxidative stress*

*2.8.1 Determination of oxygen uptake and induction time ($T^{ind}$) in erythrocytes*

Heparinized blood samples from uninfected and infected mice (day 12 post-infection) were used for erythrocyte oxidative stress determinations. After removal of plasma and white cells from whole blood, the remaining erythrocytes were washed twice with a 10 mM sodium

phosphate buffered saline (0.9% NaCl, pH 7.4), and then resuspended in the same buffer (1:99, v/v). Both *t*-butyl hydroperoxide 2 mM (*t*-BHT) induced oxygen uptake and induction time were measured with a Clark-type oxygen electrode at 37°C [51]. The induction time is directly related to the intracellular protective antioxidant capacity, while oxygen uptake is an indirect measure of the susceptibility of erythrocytes membranes to lipid peroxidation elicited by *t*-BHT [52].

*2.8.2 Tert-butyl hydroperoxide-induced chemiluminescence (CL) and protein concentration*

Mouse heparinized blood erythrocytes from different experimental groups were sedimented by centrifugation (800 *g*, 10 min) at 25ºC, and then washed twice with saline. Erythrocytes 1% suspension was prepared with 150 mM NaCl, 10 mM sodium phosphate at the moment of its use. The chemiluminescence reaction (CL) was initiated by the addition of 20 μL *tert*-butyl hydroperoxide at final concentration of 2 mM in 1 mL [53]. CL was measured in a Luminometer TD20/20 (Turner Biosystems, Sunnyvale CA, USA). The results were expressed in cpm/mg protein. The entire curve was used as an indicator of lipid peroxidation. The initial rate ($V_0$) was obtained in the ascending part of the CL curve [54]. Protein concentration was determination by the method of Lowry and collaborators [55] modified by Miller [56].

*2.9 Statistical analysis*

Arithmetic means (parasitaemia, amastigotes nests, and inflammatory foci) and standard deviations of means were calculated. The impact of infection and other treatments were determined by two-way analysis of variance (ANOVA). When significant main effects were noted, differences between individual groups were tested using Bonferroni multiple comparisons method. Survival curves were compared using the Mantel–Haenszel log rank

test. All statistical analysis was made using GraphPad Prism version 4.0 (GraphPad Software, San Diego, CA).

## 3. Results

### *3.1 Effects of the daily treatment with cyclooxygenase inhibitors on parasitemia, and survival in infected mice.*

To address the question of the involvement of prostaglandins in the protection during the acute phase of *T. cruzi* Y strain, two murine models of Chagas' disease were used. These vary with respect to the resistance (C57BL/6) and susceptibility (BALB/c) to the infection.

Fig. 1 and 2 shows the parasitemia (panel A) and the survival rate (panel B) of C57BL/6 and BALB/C mice infected with *T. cruzi*, treated or not with different NSAIDS, respectively. BALB/c infected with *T. cruzi* Y strain, were more susceptible than C57BL/6, reaching higher parasitemia and mortality. Parasitic load in the blood peaked 9 days post-infection was significantly higher in BALB/c compared to C57BL/6 mice and declined progressively thereafter. There were large and statistically significant differences ($P < 0.001$) in survival between all two strains of mice studied (Fig. 1 and Fig. 2, panel B). All of the C57BL/6 mice untreated survived the infection, while all of the BALB/c mice untreated died 26 days p.i. The treatment of infected C57BL/6 mice with ASA or celecoxib dramatically increased the blood parasites load starting from on the $7^{th}$ to $9^{th}$ day of infection, respectively (Fig 1, panel A, $P$ <0,05). ASA and celecoxib treatment showed no significant differences in parasitemia from the $17^{th}$ p.i. C57BL/6 treated with Indo, ASA and celecoxib die from the $7^{th}$ to $15^{th}$ and to $22^{th}$ day of infection, respectively. All C57BL/6 mice infected and treated with Indo died on day $12^{th}$ (Fig 1, panel B). The survival rate in C57BL/6 treated with Asa or celecoxib was half and fully that in C57BL/6 untreated mice, respectively (day 30 p.i) and the

mean survival time was little longer in the C57BL/6 mice treated with celecoxib (Fig 1, panel B, $P< 0.001$).

The treatment of infected BALB/c mice with celecoxib dramatically increased the blood parasites load starting from on the 13$^{th}$ day of infection (Fig 2, panel A, $P < 0.05$). Asa and Indo treatment showed no significant differences in parasitemia on days 7, 14 and 21 p.i. All BALB/c mice infected and treated with celecoxib died on day 18$^{th}$ (Fig 2, panel B). BALB/c treated with Asa or Indo die from the 20$^{th}$ to 22$^{th}$ and 22$^{th}$ day of infection, respectively. The mean survival time was little smaller in the BALB/c mice treated with celecoxib (Fig 2, panel B, $P< 0.001$).

In addition, we analyzed the effect of COX inhibition on parasitism in heart tissue taken from C57BL/6 and BALB/c mice treated with NSAIDs at 12 days after infection with *T. cruzi*.

On day 12 p.i, the heart tissue from C57BL/6 mice treated with the celecoxib had three-fold more parasites nests than those of untreated mice (Fig. 3A, $P < 0.05$) and those treated with Asa had two-fold more parasites nests than untreated. (Fig. 3A, $P < 0.05$). We observed an increase tendency in the number of parasites nests in the heart from infected mice treated with Indo, but the increase was not significant (Fig.3A, $P > 0.05$). In the same infection period, BALB/c treated with celecoxib, Asa and Indo had five and nine-fold more parasites nests than those of untreated mice, respectively (Fig. 3B, $P< 0.05$).

*3.2 Effects of COX-1 and COX-2 inhibition on anemia in* T. cruzi*-infected mice*

We conducted blood cells counts and measured several haematological values in uninfected and *T. cruzi*-infected treated or not with NSAIDs at 12 days post-infection. Based on significant decreases in hemoglobin, hematocrit and erythrocyte numbers, all two mice strains studied suffered from anemia on 12$^{th}$ day post-infection (Table I). The severity of the

anemia was greatest for C57BL/6 mice. Interestingly, the inhibition of endogenous PGs production did not impact on the extent of anemia that developed 12 days post-infection in the two mice strains (Table I). *T. cruzi* infection in BALB/c and C57BL/6 mice was associated with a significant decrease in total number of bone marrow cells (Table I). COX-1 and COX-2 inhibition had no effect on bone marrow hypoplasia in both strains of *T. cruzi*- infected mice (Table I).

*3.3 COX-2 inhibition with celecoxib treatment attenuates thrombocytopenia and leukopenia that develop in C57BL/6 infected with* T. cruzi

Because anemia, thrombocytopenia and leukopenia are associated with *T. cruzi* infection, we asked these changes occur in *T. cruzi*-infected mice and treated with inhibitors of PGs generation. As shown in Figures 4 and 5, platelet and white cell counts revealed thrombocytopenia and leukopenia in both infected strains mice. Interestingly, only the treatment of C57BL/6 infected mice with celecoxib (selective COX-2 inhibitor) was able to attenuate the thrombocytopenia (Fig. 4A, $P < 0.05$ when compared with infected group) and leukopenia (Fig. 4B, $P < 0.05$) observed on $12^{th}$ day of infection. None of the treatments with different NSAIDs had effect on the thrombocytopenia and leukopenia that occur in BALB/c mice infected with *T. cruzi* (Fig. 5A and Fig 5B, $P > 0.05$ when compared with infected group).

In addition, we noted a significant increase in reticulocytes present in both the C57BL/6 and BALB/c mice (Fig. 4 C and 5 C). Only the treatment of C57BL/6 mice with celecoxib increased the percentage of reticulocytes in *T. cruzi*-infected mice (day 12 p.i., Fig. 4 C, $P < 0.05$). Indomethacin but not celecoxib and Asa treatment of BALB/c mice significantly increased the reticulocytosis associated with *T. cruzi*-infection in this susceptible strain of mice (Fig. 5 C, $P < 0.05$).

*3.4 Effects of* in vivo *COX inhibition on differential production of NO in macrophages from* T.cruzi-*infected mice*

We determined the NO production in order to verify whether the *in vivo* treatment of infected C57BL/6 and BALB/c mice with NSAIDs could be alter the differential production of this compound by C57BL/6 (M-1) and BALB/c (M-2) macrophages during *T. cruzi* infection.

We observed that macrophages from BALB/c respond poorly to infection with *T. cruzi* for the spontaneous production of NO compared with macrophages from C57BL/6 (Fig 6A and Fig. 6B). In C57BL/6 mice on $12^{th}$ day of infection, the Asa treatment provoked decreased in the NO production by M-1 macrophages while that celecoxib treatment increased the NO production by these cells (Fig 6A, $P < 0.05$). The indo treatment had not effect on NO production by M-1 (Fig 6A, $P > 0.05$), when compared with the production of NO by M-1 from infected and untreated mice. In parallel, we verified that all NSAIDs treatment used increased NO production in M-2 macrophages when compared with infected untreated mice (Fig. 6B, $P < 0.05$).

*3.5 Erythrocyte oxidative stress*

Oxygen uptake by erythrocytes is directly associated with the susceptibility of the erythrocyte membrane to undergo lipid peroxidation elicited by *t*-BHP and is proportional to previous oxidative stress experienced by the erythrocyte *in vivo*. The oxygen uptake by erythrocytes from C57BL/6 and BALB/c mice was significantly increased 12 days following infection (Fig. 7A and Fig 7B; $P < 0.05$). The treatment with NSAIDs used significantly diminished erythrocyte oxygen uptake in both strains infected mice (Fig. 7A and Fig 7B; $P < 0.05$)

Induction time ($T^{ind}$) is directly related to the intracellular protective antioxidant capacity of the erythrocyte. *T. cruzi* infection in the C57BL/6 and BALB/c mice resulted in a significant reduction in $T^{ind}$ (Fig. 8; $P < 0.05$). Treatment of C57BL/6 mice with NSAIDs completely abrogated the infection-associated decline in $T^{ind}$ (Fig. 8A, $P < 0.05$). Prior to infection, $T^{ind}$ was significantly longer in erythrocytes isolated from BALB/c mice compared to C57BL/6 mice. Only the treatment with celecoxib protected against the reduction of antioxidant capacity of BALB/c erythrocytes on day 12 post-infection (Fig.8B; $p < 0.05$).

*Tert*-butyl hydroperoxide initiated CL was used to analyze the levels of lipoperoxides in erythrocytes from infected mice treated or not with NSAIDs. This test is based on the assumption that an increase in CL is related to the oxidative stress previously suffered by the erythrocyte during *T. cruzi* infection, inducing the consumption of antioxidant defenses and the formation of lipoperoxides resulting in an increase in photon emission ref.

The Figure 9 shown the time course of *tert* butyl hidroperoxide-initiated CL of mice from *T. cruzi* infection. The CL time course curves of erythrocytes from the C57BL/6 (Figure 9A) and BALB/c (Figure 9B) infected-mice (on day 12 after infection), showed a significant photon emission increase and shift of its maximum to the left. It means an increased lipid peroxides and reduced antioxidant defenses in the erythrocytes membrane from *T. cruzi* infected-mice (day 12 of infection). The NSAIDs treatment of C57BL/6 (Figures 9A) but not BALB/c mice (Figures 9B) was able to quench significantly the photon emission. These results indicate that PGs play a role in pre-hemolytic damage during experimental *T. cruzi* infection.

**4. Discussion**

C57BL/6 and BALB/c mice are prototype hosts for the study of resistance and susceptibility to several infectious diseases, including Chagas' disease [57]. In this study we

demonstrated that the experimental infection with *Trypanosoma cruzi* is characterized by exponential growth of parasites and oxidative stress associated with anemia in both resistant and susceptible strains of mice. In many cases, resistance of C57BL/6 is due to the microbicidal effect of nitric oxide (NO) produced by macrophages in response to interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α), mainly secreted by Th1 cells and macrophages, respectively. BALB/c, usually unable to give rise to Th1 lymphocytes, does not control certain infection [57].

Although it has been shown that prostaglandins (PGs), together with NO and TNF-α participate in a complex circuit that controls lymphoproliferative and cytokine responses in *T. cruzi* infection [30], the possible involvement of cyclooxygenase-released prostaglandin in the oxidative stress associated with anemia in the early of *T. cruzi* infection had not hitherto been investigate. In addition, studies that utilized inhibitors of cyclooxygenase to determine the role of PGs in the control of parasitism and resistance to *T. cruzi* infection are discrepants [28-31].

We tested three non-steroidal anti-inflammatory drugs (NSAIDs): aspirin, an inhibitor of both constitutive (COX-1) and inducible (COX-2) cyclooxygenases (COX); indomethacin a preferential antagonist of COX-1; and celecoxib, a COX-2 selective inhibitor [48]. We demonstrated that in the early of infection the treatment with Asa, indomethacin or celecoxib increased dramatically the parasitemia in the blood and declined the survival rate of *T. cruzi*-infected resistance C57BL/6 mice. The inhibition of COX-2 with celecoxib provoked the same in parasite-infected susceptible BALB/c mice. Although the blockade of COX-1 with Asa or with indomethacin has provoked a tendency of decrease of parasites in the blood from BALB/c, it was not capable to modify the survival rate of those mice.

In agreement with our results, Celentano and collaborators [28] observed that the treatment with cyclooxygenase inhibitors (aspirin or indomethacin) enhanced mortality rates

of C3H/HeN mice infected with *T. cruzi* (K98 strain). However, differently to ours results and those of Celentano and collaborators, others have found that the inhibition of the PGs synthesis abolish the parasitemia and delay the mortality of susceptible BALB/c *T. cruzi* infected-mice [29,31]. The answers to these questions are not easy to answer and the effects of NSAIDs on *T. cruzi* infection have been mysterious since the paper original of Celentano and collaborators [28].

Work in several laboratories has revealed that PGs is mainly produced by APCs (antigen-presenting cells) such as monocytes, macrophages, and dendritic cells, and the effects are almost suppressive on Th1-related immune responses, $PGE_2$ suppress IL-2 and IFN-γ production by Th1 clone, but no IL-4 and IL-5 production by Th2 clone [36,58]. The suppression of PGs by indomethacin prompted the development of a protective Th1 type response in BALB/c mice by a mechanism, which involves an enhancement of IL-12, IFN-γ and NO production [59].

In *T. cruzi* infection, BALB/c mice are susceptible and show higher IL-4 production and parasitemia than resistant C57BL/6 mice [60]. It is possible that the inhibition of PGs production accomplished in our studies do not induced alterations in the production of IL-4 in BALB/c infected with *T. cruzi*, but can might have inhibited the development of protective Th1 response in C57BL/6 mice. In fact, recent studies show that NSAIDs selectively inhibits interferon (IFN)-*γ* and tumor necrosis factor (TNF)-*α* production by spleen cells [31], natural killer (NK) and *γδ* T cells [61] with each NSAID displaying its own unique pattern of inhibition and these inhibitions were independent on cyclooxygenase inhibition. However, the high lethality after NSAIDs treatment could be related to a marked systemic increase of TNF-α production in a situation analogous to that observed for *T. cruzi*-infected C57BL/6 [30] and IL-$10^{-/-}$ mice [62]. In addition, there is a strong difference in ability to produce $PGE_2$ by

BALB/c and C57BL/6 mice [63]. All these data together may explain the discrepant results found by us and other authors.

Our additional experiments revealed that on day 12 of infection, celecoxib and Asa but not indomethacin treatment increased parasites nests than those infected-untreated C57BL/6 mice. Interestingly, the heart tissue from infected BALB/c (day 12 p.i) and treated with the antagonist of COX-1 (indomethacin), had eight-old more amastigotes nests than infected-untreated mice. The celecoxib or Asa increased the heart parasitism, but your action was smaller than mice infected-untreated.

So, our results suggest for the first time that cyclooxygenase-released prostaglandin plays a critical role in the heart protective immunity against *T. cruzi* in an experimental Chagas' disease model. More important, NSAIDs can be used clinically for fever control during *T. cruzi* infection. If we extrapolate our results to a clinical setting, treatment with NSAIDs may produce more severe disease and they serve as alert sign for those that defend the use of NSAIDs as potential drugs for therapy in the acute phase of Chagas´s disease [29].

It is clear that intraperitoneal *T. cruzi* infection leads to an early activation of the COX [12,30,31] and NOS system [30,64], besides it was of interest to determined whether NSAIDs treatment could affect the spontaneous production of NO by macrophages from peritoneal cavity of *T. cruzi*-infected mice.

Confirming previous reports [64], we have observed that macrophages from BALB/c *T. cruzi* infected-mice respond poorly for the production of NO compared with cells from C57BL/6 infected-mice. The inhibition of NO production by Asa in macrophages from C57BL/6 on day 12 p.i, could be explain the increased of parasitemia and dead these animals in the early of infection with *T. cruzi*. Interesting, the same treatment provoked an increased of NO production in BALB/c infected mice and this could explain the control of parasitemia in the early of infection for BALB/c Asa-treated infected-mice. The treatment with Indo had

not effect on NO production by macrophages from C57BL/6 and the treatment with celecoxib increased NO production by macrophages. The longer survival of C57BL/6 celecoxib treated infected-mice than Indo treated infected-mice can be explain for these differences in NO production. The little increase of NO production by macrophages from BALB/c infected and treated with celecoxib or Indo it was not enough to provoke a reduction in the parasitemia of those mice or to provoke increase in the survival rate.

In previous studies [25], we demonstrated that an acute infection of either C57BL/6 or Swiss mice with *T. cruzi* was generally associated with systemic oxidative stress evaluated by increased oxygen uptake and decreased induction time of erythrocytes. That aminoguanidine treatment of C57BL/6 mice hindered this oxidative lesion. That C57BL/6 $iNOS^{-/-}$ showed high oxidative stress, suggested that $^{\bullet}NO$ and possibly reactive oxygen species (ROS), played a role in peroxidative lesion of erythrocytes membrane in this disease.

In the present study, we evaluated the effect of cyclooxygenases inhibitors on the oxidative stress in erythrocytes of mice sensitive (BALB/c) and resistant (C57BL/6) to infection for *T. cruzi*. For this purpose, tert-butyl hydroperoxide-induced chemiluminescence (CL), a very sensitive and specific method, was employed to evaluate the integrity of non-enzymatic antioxidant defense mechanisms and quantify erythrocyte membrane lipid peroxide levels. The basis for this assay is the assumption that previous reactive oxygen and nitrogen species (i.e. $ONOO^{-}$ and $^{\bullet}OH$) attack generate membrane lipid peroxides and destroys the low molecular weight antioxidants in the tissue yielding a higher CL emission [54]. Our results reveals that the infection with *T. cruzi*, was associated with increased CL levels in erythrocytes of both C57BL/6 and BALB/c mice, nevertheless C57BL/6 mice erythrocytes had higher CL levels than BALB/c mice. This peroxidative lesion was confirmed by the increased oxygen uptake and decreased induction time in both strain mice. The data showed here and our previous results together [25] indicated that acute infection of mice with *T. cruzi*

leads to a oxidative damage to erythrocytes membrane and that •NO can partially be responsable for that process. Accordingly, in C57BL/6 mice, a good responder to iNOS induction gene, the nitrite levels in peritoneal macrophages arose about 5 times after 12 days of infection. Such increase was not seen in BALB/c mice namely, a poor responder to iNOS induction gene.

Oxidative stress in Chagas's disease has recently call attention as a possible pathogenetic mechanism. Erythrocytes GSH concentrations and GPx were reduced with the disease progression, suggesting systemic oxidative stress involvement in patients with Chagas cardiomyopathy [24,65]. Antioxidant such vitamin E and C administrated to these patients reverted the oxidative lesion [66]. Oxidative stress in heart but not in skeletal muscle of mice infected with *T. cruzi* evaluated by lipid peroxide and carbonylated proteins formation both in acute and cronic phases have been reported [21]. These authors propose a mitochondrial dysfunction as a causative of reactive oxygen species generation leading to progressive severity of chagasic cardiomyopathy. Extensive DNA damage in cardiac myocytes and in spleen cells have also been found in *T cruzi* infection [67]. Free radicals-mediated •NO generation was suggested as a mechanism of DNA breakage for the authors. Decreased antioxidant defense has also been related in human and experimental studies in Chagas's disease [22].

In our experimental model, that emphasize systemic oxidative injury, besides of oxidative stress contribution that takes place in important organs such heart, liver, spleen [24,67], others mechanism such circulating neutrophils [68], macrophages cells [69, 8], arachidonic acid cascade [12], endothelial cells [70, 71] generating ROS and RNS can play a more significant role in erythrocytes pre-hemolytic lesion. In fact, our results revealed that higher erythrocytes lipid peroxide levels observed in infected resistant C57BL/6 mice correlate with increased levels of •NO produced for peritoneal macrophages.

Increased of $^{\bullet}NO$ production has been observed in J77.4 murine macrophages [72] and in spleen cells after *T. cruzi* infection [69]. In susceptible BALB/c mice, however, was not observed any increase in $^{\bullet}NO$ after infection with *T. cruzi*. On contrary, ROS secreted by peritoneal macrophages during the chagasic infection of susceptible mice was higher than that observed in resistant one [73]. These data indicated that the C57BL/6 mice have high $^{\bullet}NO$ levels while the BALB/c mice shows higher $O_2^{\bullet}$ levels. Likewise the interpretation given to the comparision between C57BL/6 and Swiss mice [25], the erythrocytes oxidative injury found in this study might results of $^{\bullet}NO$ reaction with $O_2^{\bullet}$ yielding $ONOO^{-}$ or by the Fenton reaction producing $^{\bullet}OH$ near of outer leaflet of erythrocyte membrane both of which could lead to its oxidative lesion. In high $^{\bullet}NO$ levels the reaction between $^{\bullet}NO$ and $O_2^{\bullet}$ is favoured because of rate constant of this reaction to be higher than that between $O_2^{\bullet}$ and $H_2O_2$ [74]. This mechanism might be occurring in erythrocytes of C57BL/6 as suggested else were [25]. Moreover, since COX inhibitors efficiently inhibited the membrane lipid peroxide formation, the cyclooxygenase pathway may be involved in this process either increasing $^{\bullet}NO$ generation or directly producing additional reactive oxygen species [75]. Unfortunately in our results only Asa was able to diminished significantly the $^{\bullet}NO$ levels.

Involvement of cyclooxygenase-released prostaglandin in the oxidative stress induced by *T. cruzi* infection has not been investigated yet. All three cycloooxygenase inhibitors employed, reduced significantly the levels of CL, oxygen uptake and increased the induction time in C57BL/6 mice infected with *T. cruzi*. Such effect was not observed in BALB/c mice with exception to reduced oxygen uptake in celecoxib treatment. These results indicate that arachidonic acid metabolism via cyclooxygenase at last in part, play a significant role in oxidative damage in erythrocytes of C57BL/6 mice infected with *T. cruzi*. The oxidative stress verified for the BALB/c strain contrarely, do not depend on cyclooxygenase pathways. This profile was not reproduced in macrophages yielded $^{\bullet}NO$. Except for aspirin, the others

drugs did not inhibit the •NO increase in peritoneal macrophages of C57BL/6 infected., The •NO levels in BALB/c on contrary, were significantly increased. This is in accord with the finds showing *in vivo*, inhibition of protaglandin release by selective inhibition of COX-2 has only minimal effects on •NO production [76,77].

It could be suggested that the mechanism of oxidative stress in *T. cruzi* infection is dependent on strain. In C57BL/6 mice with high •NO generation, both •NO and ROS appears to be involved in CL emission curve observed in this strain. This statement is supported for the fact that aminoguanidine, a selective inhibitor of iNOS, reverted the erythrocytes lipid peroxidation in C57BL/6 and that C57BL/6 iNOS$^{-/-}$ mice revealed high erythrocytes membrane lipid peroxide in a similar experimental design (25). Additionally, the fact that inhibitor of cyclooxygenase pathway inhibited significantly the CL in erythrocytes of C57BL/6 mice infected means that arachidonic acid metabolism by the cyclooxigenase pathways might be involved in ROS generation. In BALB/c with low •NO levels production, the oxidative injury of erythrocyte depends almost exclusively of reactive oxygen species such •OH and singlet oxygen. Moreover, since our results showed that cyclooxygenases inhibitor were not able to reduce the oxidative stress, it can be suggested that in this strain the oxidative damage of erythrocytes do not depend on neither •NO nor cyclooxygenase-released prostaglandin.

In conclusion, our results indicated that the *T. cruzi* infection induced pre-hemolytic lesion in erythrocytes membrane through oxidative stress. This injury can be partially support the anemia observed in this disease. The mechanism by which the oxidative stress occur, depend on the strain. In susceptible mice with low •NO generation the oxidative injury happens almost exclusively in function of ROS production and do not depend of cyclooxygenase pathway activation. In resistant mice with high •NO yielding, the oxidative injury depend on •NO reaction with $O_2^{\bullet -}$ given $ONOO^-$.

*Speculative remark*: Higher oxidative lesion observed in erythrocytes of C57BL/6 mice was associated with anemia, lower parasitemia burden and higher survival when compared to BALB/c. The question to be answered is: In which extent the oxidative stress either brings benefits or damages to the host? It has been demonstrated that oxidative stress exerts two main actions. In high level, it can promote tissue damage [25,54,55]. In a moderate level it is involved in modulation of gene expression such proinflammatory cytokines through NFκB expression [78,79]. iNOS expression likely through NF-kappaB activation by *T. cruzi* infection, has been demonstrated recently [72]. Recent studies have shown that low oxidative stress levels modulates T cell polarization toward a Th2 phenotype that are related with the resistance to parasitic infection [80].

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**Disclosures**

The authors have no financial conflict of interest.

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**Table I –** Hematological values and bone marrow cells counts[▽] in *T. cruzi*-infected mice**.

| Group | Hemoglobin (g/dL) | Hematocrit (%) | Erythrocytes (x10 $^9$/mL) | Bone Marrow cells (x $10^3$/mL) |
|---|---|---|---|---|
| C57BL/6 | | | | |
| N | 14.4 ± 0.4 | 45.0 ± 0.6 | 7.1 ± 0.3 | 5200 ± 1110 |
| I | 10.2 ± 1.0* | 29.8 ± 2.2* | 5.6 ± 0.7* | 3560 ± 1370* |
| I + Celecoxib | 10.5 ± 2.0 | 34.0 ± 5.0 | 6.1 ± 0.3 | 3240 ± 1170 |
| I + Asa | 10.3 ± 1.0 | 32.8 ± 1.6 | 5.9 ± 0.2 | 2790 ± 790 |
| I + Indo | 10.3 ± 1.0 | 32.7 ± 3.2 | 5.8 ± 0.4 | 2630 ± 730 |
| BALB/c | | | | |
| N | 14.5 ± 1.4 | 44.6 ± 2.2 | 8.0 ± 0.1 | 4720 ± 830 |
| I | 12.0 ± 1.5* | 35.6 ± 2.0* | 5.5 ± 0.2* | 2660 ± 1370* |
| I + Celecoxib | 11.1 ± 0.5 | 34.7 ± 1.5 | 6.6 ± 0.1* | 2150 ± 660 |
| I + Asa | 10.0 ± 1.1 | 31.8 ± 2.9 | 5.3 ± 0.3 | 2740 ± 890 |
| I + Indo | 10.2 ± 1.6 | 33.4 ± 4.0 | 6.1 ± 0.4 | 2060 ± 210 |

[▽] Day 12 after *T. cruzi* infection. ** Groups of mice were infected (I) with 5x$10^3$ *T. cruzi* or infected and treated with celecoxib, Asa or Indo. N ormal mice (N). Values represent the mean ± SD and are representative of two independent experiments, using 5 to 12 mice per group * S ignificantly different ($p<0.05$) when compared with non-infected group.

**Figure Legends**

**Fig. 1.** A) Parasitemia and B) survival rates. Groups of ten C57BL/6 mice were infected with 5 x $10^3$ trypomastigote of *T. cruzi* (Y stain) and treated with indomethacin (Indo) (1.2 mg/kg$^{-1}$), aspirin (Asa) (50 mg/Kg$^{-1}$) and celecoxib (50 mg/Kg$^{-1}$). Mice received the first dose 4 h after infection and were treated until they died. Untreated *T. cruzi*-infected mice were used as controls. Results were analyzed by analysis of variance (ANOVA) and Bonferroni's test. **P* <0.05 was considered significantly.

**Fig. 2.** A) Parasitemia and B) survival rates. Groups of ten BALB/c mice were infected with 5 x $10^3$ trypomastigote of *T. cruzi* (Y stain) and treated with indomethacin (Indo) (1.2 mg/kg$^{-1}$), aspirin (Asa) (50 mg/Kg$^{-1}$) and celecoxib (50 mg/Kg$^{-1}$). Mice received the first dose 4 h after infection and were treated until they died. Untreated *T. cruzi*-infected mice were used as controls. Results were analyzed by analysis of variance (ANOVA) and Bonferroni's test. **P* <0.05 was considered significantly.

**Fig. 3.** Quantification of amastigotes nests from heart of C57BL/6 (A) and BALB/c (B), mice on day 12-pos infection. Groups of C57BL/6 mice were infected with 5 x $10^3$ trypomastigotes of *T. cruzi* (Y strain) and treated with indomethacin (Indo) (1.2 mg/$kg^{-1}$), Aspirin (Asa) (50 mg/$Kg^{-1}$) and celecoxib (50 mg/$Kg^{-1}$) during 12 days. Mice received the first dose 4 h after infection. Untreated *T. cruzi*-infected mice were used as controls. The number of amastigotes nests was counted in an area of 10 $mm^2$ per section. Three sections were counted for each animal and the result is expressed as the mean ± standard deviation of this three sections. Results were analyzed by analysis of variance (ANOVA) and Bonferroni's test. Means not sharing a letter differ with $P < 0.05$.

**Fig. 4** Platelet (A), leukocytes (B) and reticulocytes (C) were counts from peripheral blood from normal and infected mice. Groups of C57BL/6 mice were infected with 5 x $10^3$ blood trypomastigote *T. cruzi* (Y strain) and were treated with indomethacin (Indo) (1.2 mg/$kg^{-1}$), aspirin (Asa) (50 mg/$Kg^{-1}$) and celecoxib (50 mg/$Kg^{-1}$) during 12 days. Mice received the first dose 4 h after infection. Normal mice and untreated *T. cruzi*-infected mice were used as controls. Results were analyzed by analysis of variance (ANOVA) and Bonferroni's test.

**Fig. 5** Platelet (A), leukocytes (B) and reticulocytes (C) were counts from peripheral blood from normal and infected mice. Groups of BALB/c mice were infected with 5 x $10^3$ blood trypomastigote *T. cruzi* (Y strain) and treated or not with indomethacin (Indo) (1.2 mg/kg$^{-1}$), aspirin (Asa) (50 mg/Kg$^{-1}$) and celecoxib (50 mg/Kg$^{-1}$) during 12 days. Mice received the first dose 4 h after infection. Normal mice and untreated *T. cruzi*-infected mice were used as controls. Results were analyzed by analysis of variance (ANOVA) and Bonferroni's test.

**Fig. 6** $NO_2^-$ accumulation in cultures of macrophages from *T. cruzi* infected-mice (day 12 after infection). Macrophage monolayers (2x$10^5$ cells/ 200 μL) from C57BL/6 (A) and BALB/c (B), mice were incubated at 37ºC in 5% $CO_2$ in the presence or absence of indomethacin (Indo) (1.2 mg/kg$^{-1}$), aspirin (Asa) (50 mg/Kg$^{-1}$) or celecoxib (C) (50 mg/Kg$^{-1}$) during 12 days. After 24 h the spontaneous accumulated $NO_2^-$ was determined in the supernatant by the Griess reaction (see Material and Methods). Results are expressed as the mean ± standard deviation from 10 animals per group, and are representative of three independent experiments. Means not sharing a letter differ with $P < 0.05$.

**Fig. 7** Erythrocyte oxidative stress on day 12 after *T. cruzi* infection. Here we measured oxygen uptake induced by *tert*-butyl hydroperoxide in erythrocyte from C57BL/6 (A) and BALB/c (B) mice. Animals were infected with 5 x $10^3$ trypomastigotes of *T. cruzi* (Y strain). Four hours after the infection, mice received aspirin (Asa, 50 mg/kg$^{-1}$), indomethacin (Indo, 1.2 mg/kg$^{-1}$) or celecoxib (50 mg/kg$^{-1}$) by the i.p route. Normal mice and untreated *T. cruzi*-infected mice were used as controls. Results are expressed as the mean ± standard deviation from 5-10 animals per group, and are representative of three independent experiments. Controls received saline injections only. Values represent the mean ± standard deviation and are representative of five independent experiments, using 5 mice per group. Differences in oxygen uptake were analyzed by two-way analysis of variance (ANOVA) followed by Bonferroni's test. Means not sharing a letter differ, **P*<0.05.

**Fig. 8** Erythrocyte antioxidant capacity on day 12 after *T. cruzi* infection. Here we measured induction time induced by *tert*-butyl hydroperoxide in erythrocyte from C57BL/6 (A), and BALB/c (B) mice. Animals were infected with 5 x $10^3$ trypomastigotes of *T. cruzi* (Y strain). Four hours after the infection, mice received aspirin (Asa, 50 mg/kg$^{-1}$), indomethacin (Indo, 1.2 mg/kg$^{-1}$) or celecoxib (50 mg/kg$^{-1}$) by the i.p route. Normal mice and untreated *T. cruzi*-infected mice were used as controls. Results are expressed as the mean ± standard deviation from 5-10 animals per group, and are representative of three independent experiments. Controls received saline injections only. Values represent the mean ± standard deviation and are representative of five independent experiments, using 5 mice per group. Differences in induction time levels were analyzed by two-way analysis of variance (ANOVA) followed by Bonferroni's test. Means not sharing a letter differ, **P*<0.05.

**Fig. 9** Time course curve of *tert*-butyl hydroperoxide-initiated chemiluminescence (CL) in erytrocytes from C57BL/6 (A) and BALB/c (B) mice. Animals were infected with 5 x $10^3$ trypomastigotes of *T. cruzi* (Y strain). Four hours after the infection, mice received aspirin (Asa, 50 mg/kg$^{-1}$), indomethacin (Indo, 1.2 mg/kg$^{-1}$) or celecoxib (50 mg/kg$^{-1}$) by the i.p route. Normal mice and untreated *T. cruzi*-infected mice were used as controls. Data from the curves were compared by Turkey-Kramer multiple comparison procedure and Student's *t*-test.

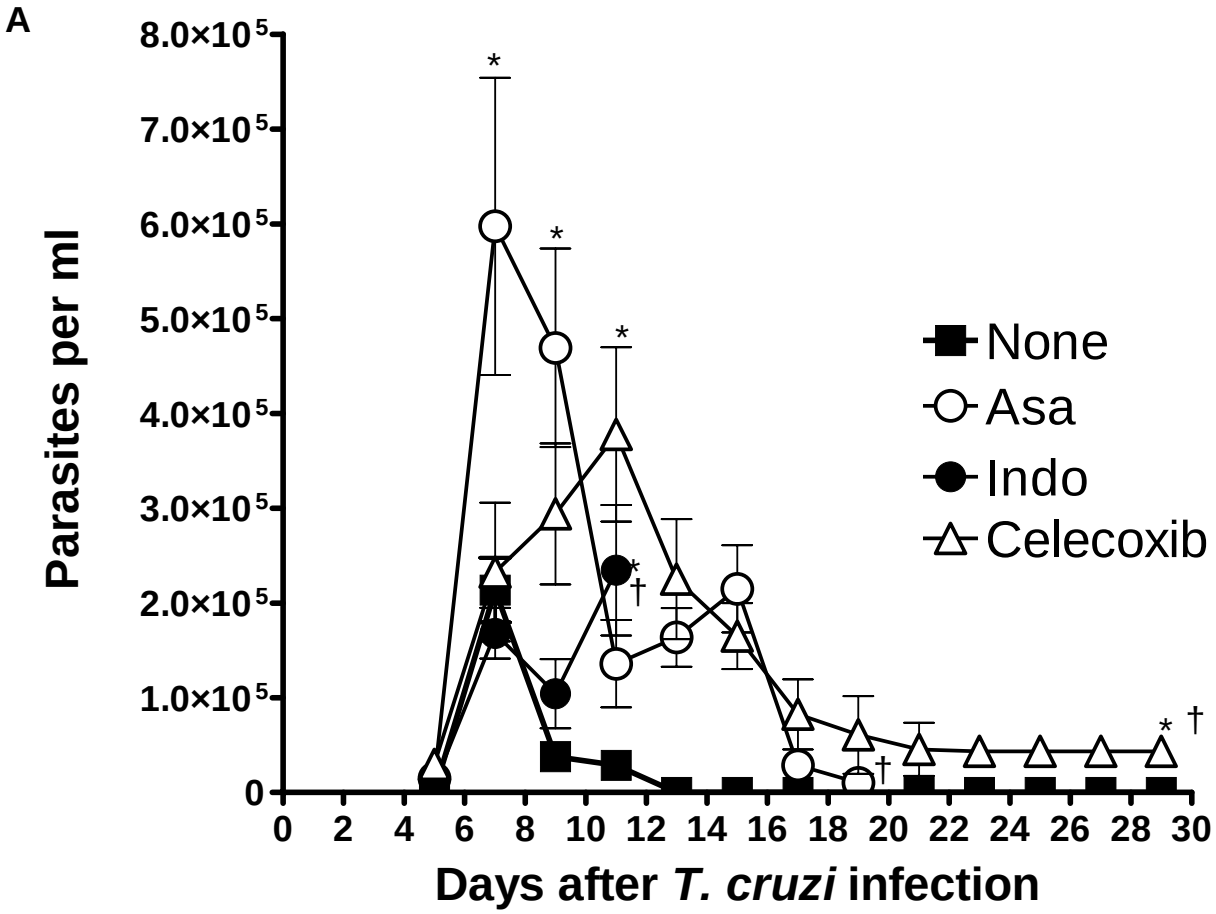
A
8.0×10⁵
7.0×10⁵
6.0×10⁵
5.0×10⁵
4.0×10⁵
3.0×10⁵
2.0×10⁵
1.0×10⁵
0
Parasites per ml
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
Days after T. cruzi infection
None
Asa
Indo
Celecoxib
*
†

Figure 1A

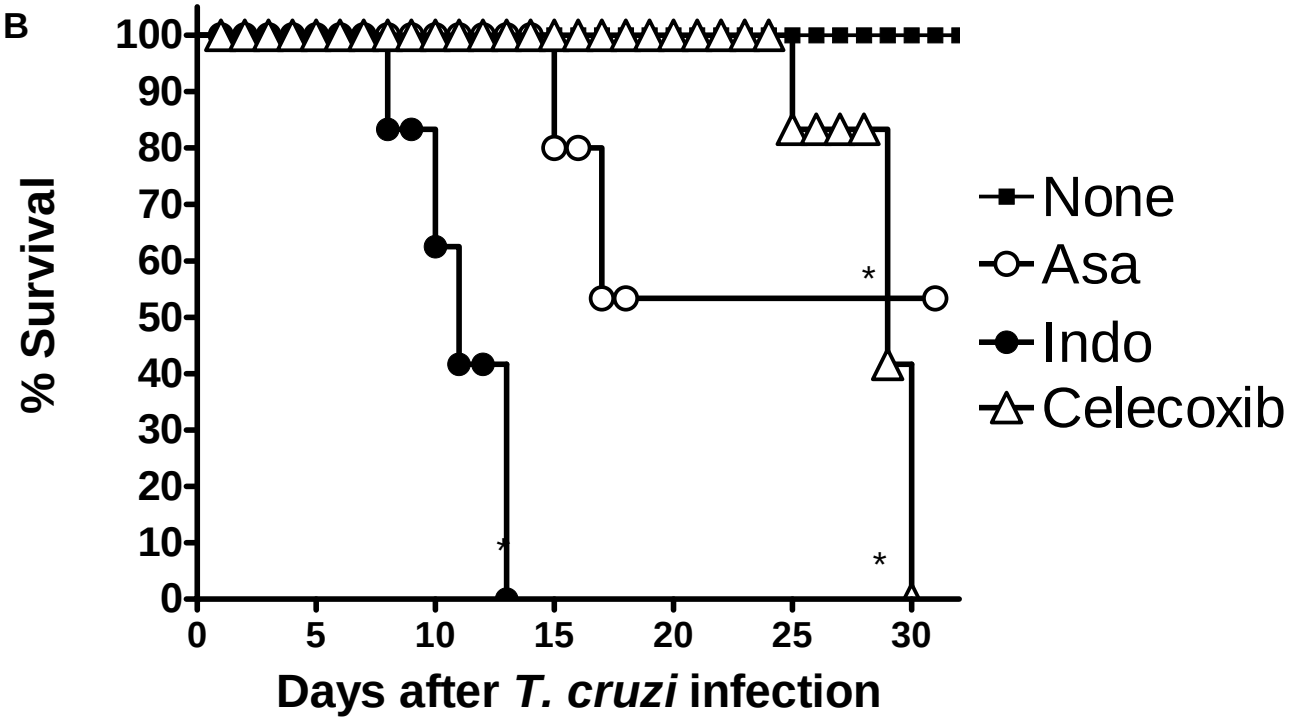
B
100
90
80
70
60
50
40
30
20
10
0
% Survival
0
5
10
15
20
25
30
Days after T. cruzi infection
None
Asa
Indo
Celecoxib
*
*
*

Figure 1B

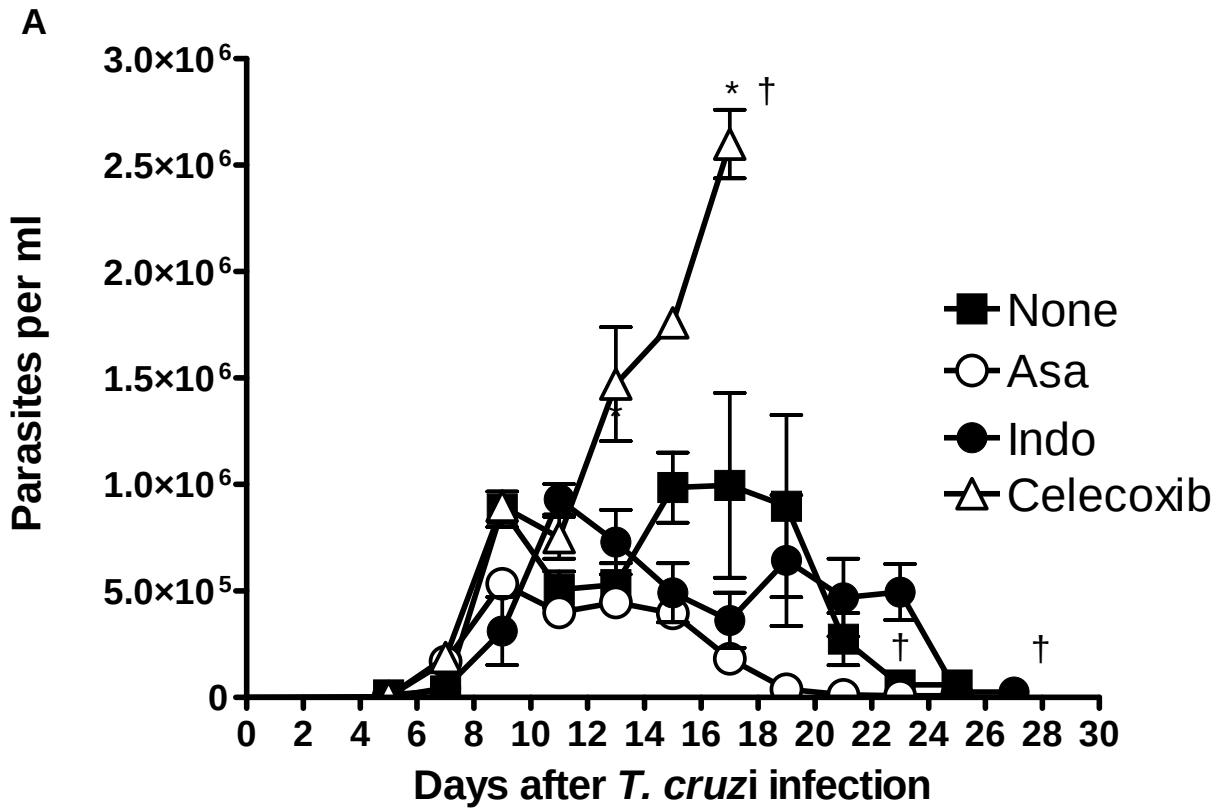
A
3.0×10⁶
2.5×10⁶
2.0×10⁶
1.5×10⁶
1.0×10⁶
5.0×10⁵
0
Parasites per ml
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
Days after T. cruzi infection
None
Asa
Indo
Celecoxib
*
†
†
†

Figure 2A

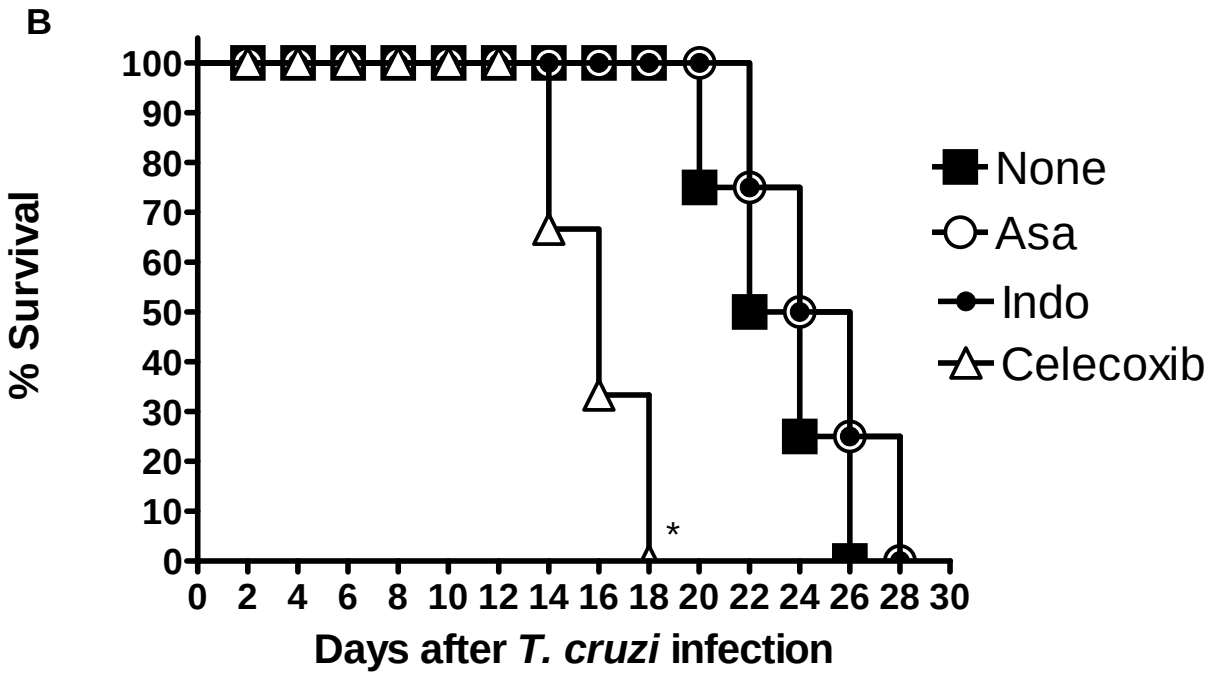
B
% Survival
100
90
80
70
60
50
40
30
20
10
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
Days after T. cruzi infection
*
None
Asa
Indo
Celecoxib

Figure 2B

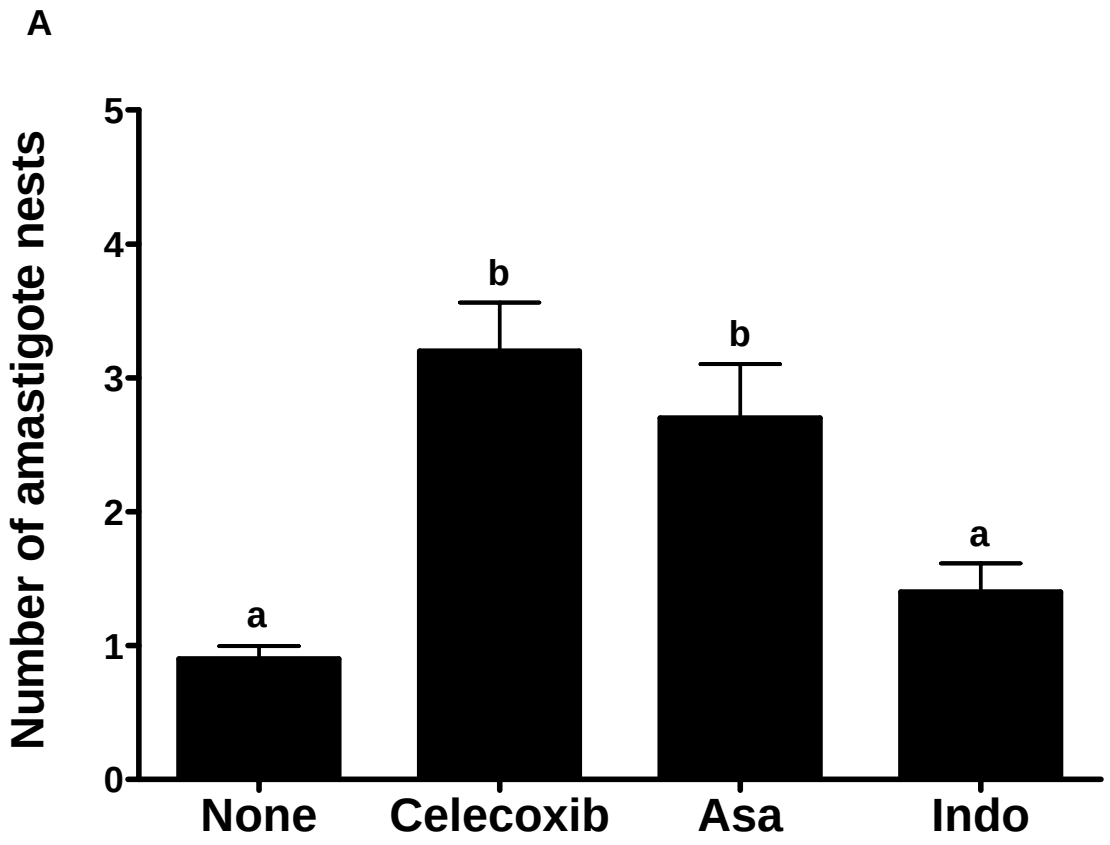
A
Number of amastigote nests
5
4
3
2
1
0
a
b
b
a
None
Celecoxib
Asa
Indo

Figure 3A

B

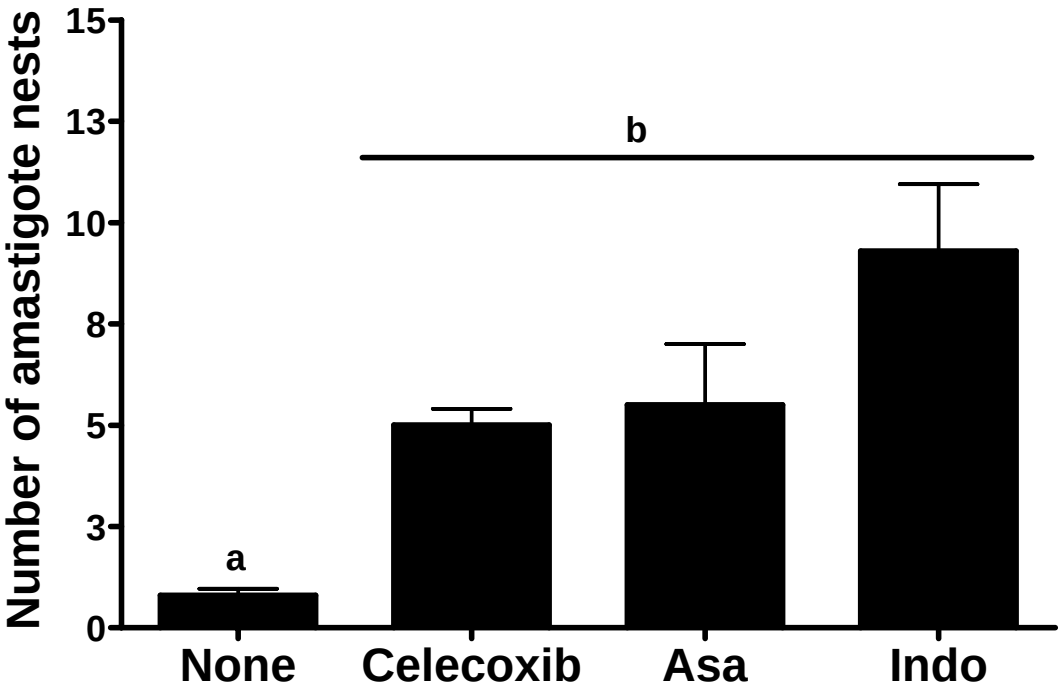
Number of amastigote nests
15
13
10
8
5
3
0
a
b
None
Celecoxib
Asa
Indo

Figure 3B

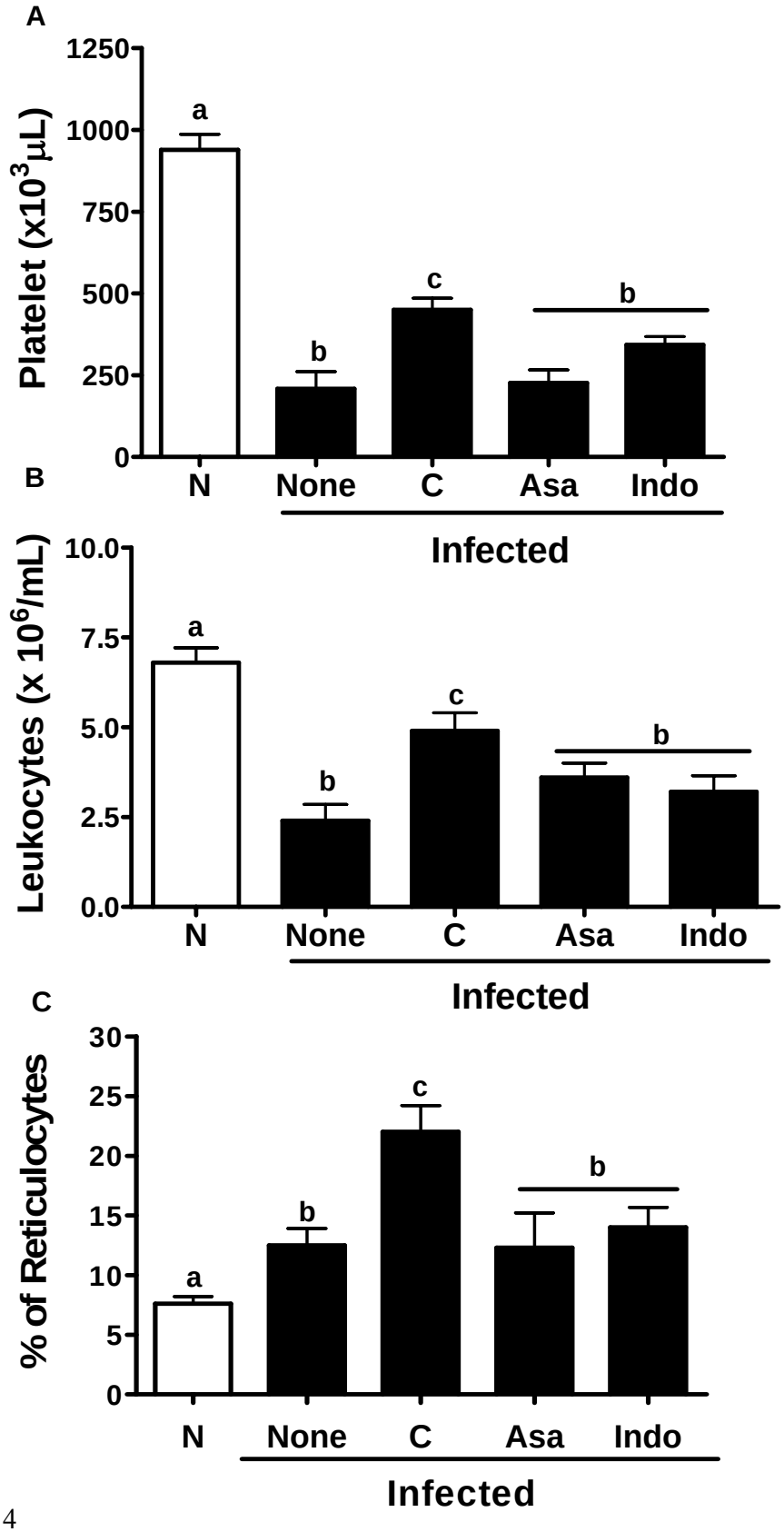
A
Platelet (x10$^3$µL)
1250
1000
750
500
250
0
a
b
c
b
N
None
C
Asa
Indo
Infected
B
Leukocytes (x 10$^6$/mL)
10.0
7.5
5.0
2.5
0.0
a
b
c
b
N
None
C
Asa
Indo
Infected
C
% of Reticulocytes
30
25
20
15
10
5
0
a
b
c
b
N
None
C
Asa
Indo
Infected

Figure 4

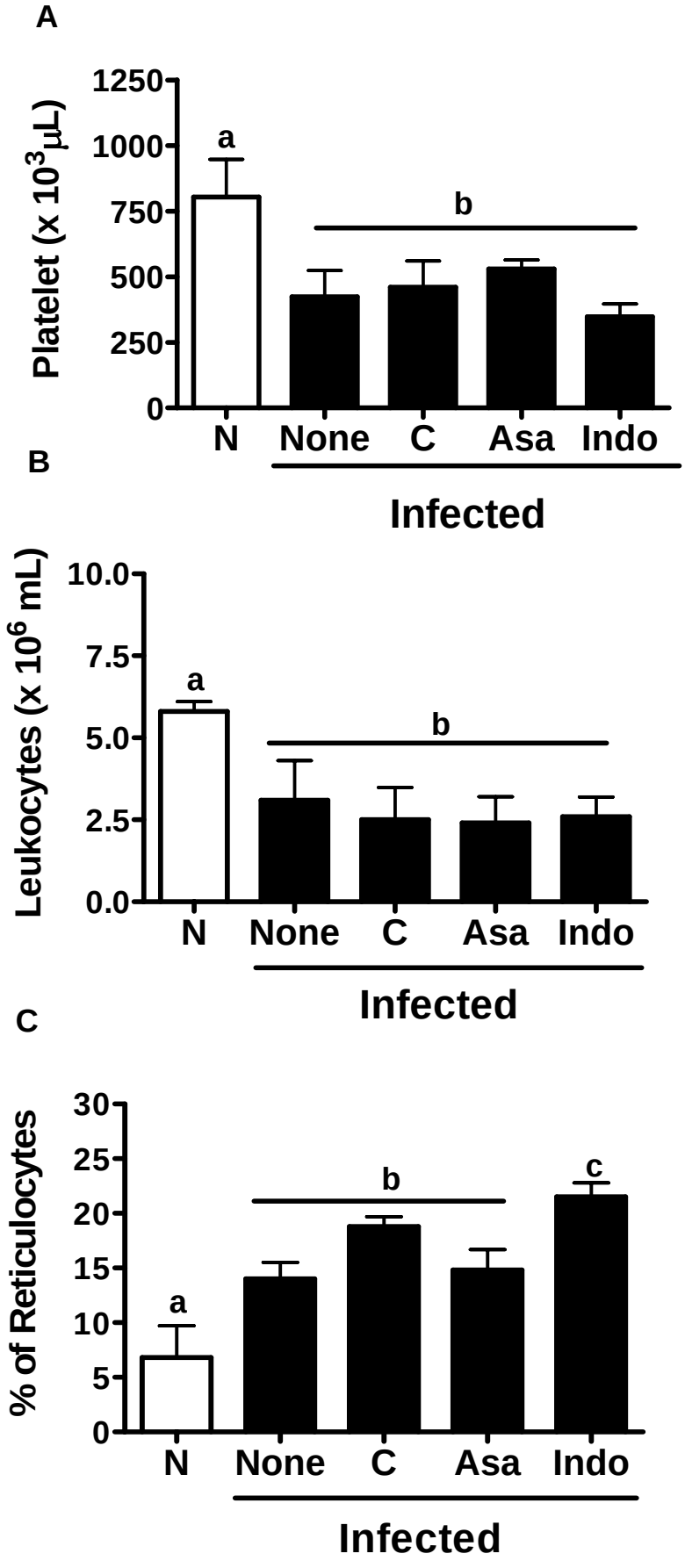
A
Platelet (x 10³µL)
1250
1000
750
500
250
0
a
b
N
None
C
Asa
Indo
Infected
B
Leukocytes (x 10⁶ mL)
10.0
7.5
5.0
2.5
0.0
a
b
N
None
C
Asa
Indo
Infected
C
% of Reticulocytes
30
25
20
15
10
5
0
a
b
c
N
None
C
Asa
Indo
Infected

Figure 5

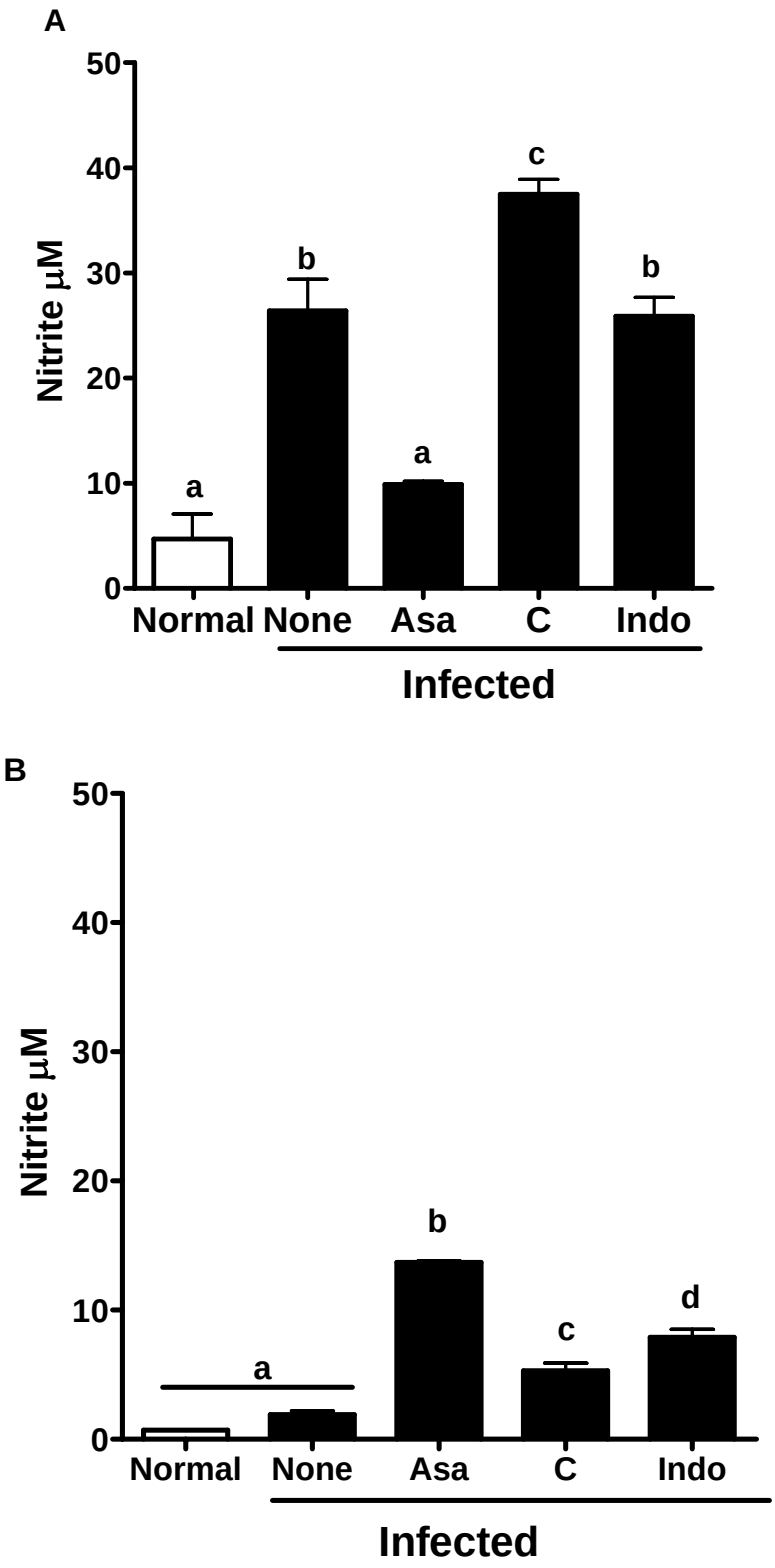
A
Nitrite µM
50
40
30
20
10
0
a
b
a
c
b
Normal
None
Asa
C
Indo
Infected
B
Nitrite µM
50
40
30
20
10
0
a
b
c
d
Normal
None
Asa
C
Indo
Infected

Figure 6

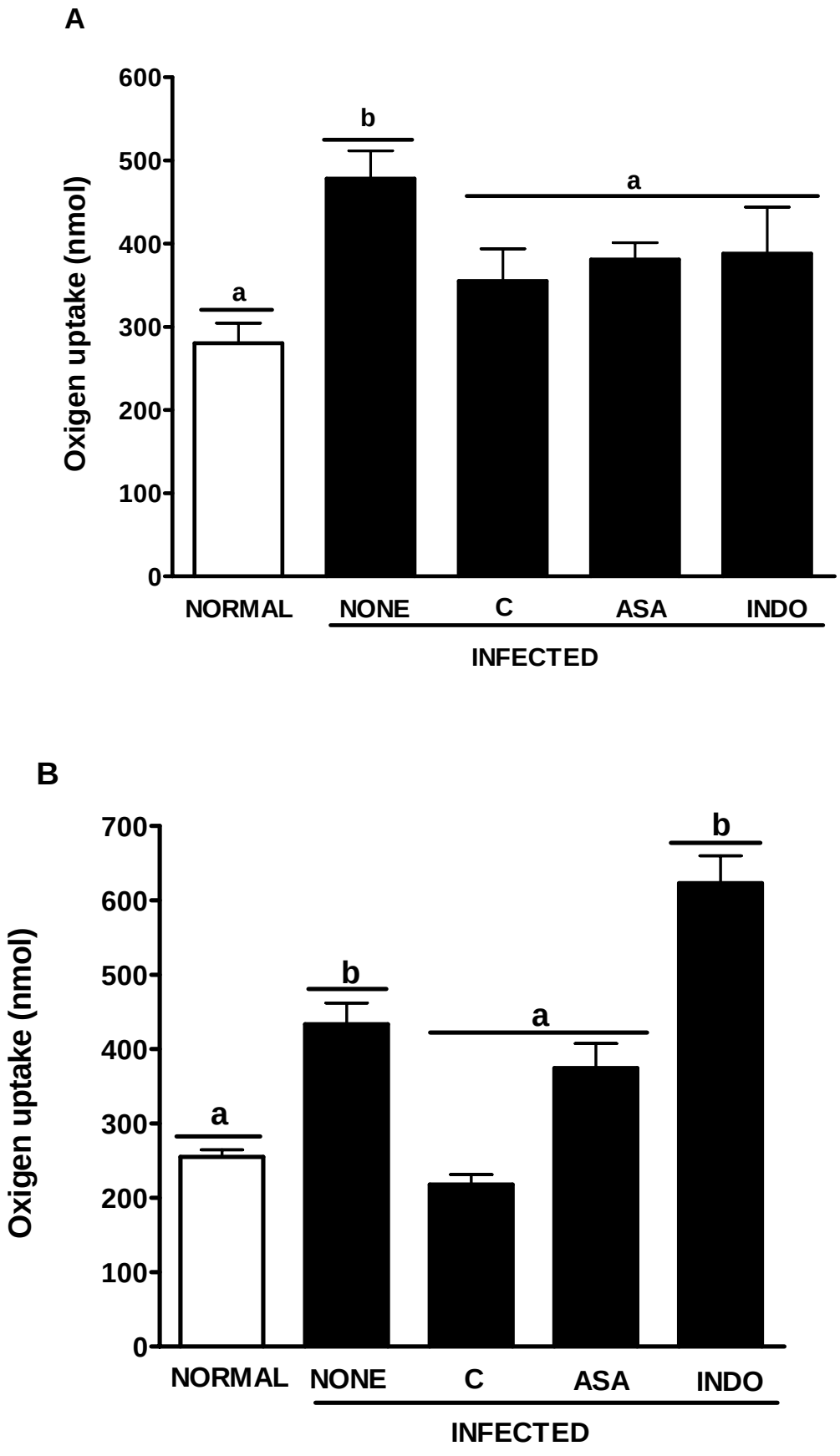
A
Oxigen uptake (nmol)
600
500
400
300
200
100
0
a
b
a
NORMAL
NONE
C
ASA
INDO
INFECTED
B
Oxigen uptake (nmol)
700
600
500
400
300
200
100
0
a
b
a
b
NORMAL
NONE
C
ASA
INDO
INFECTED

Figure 7

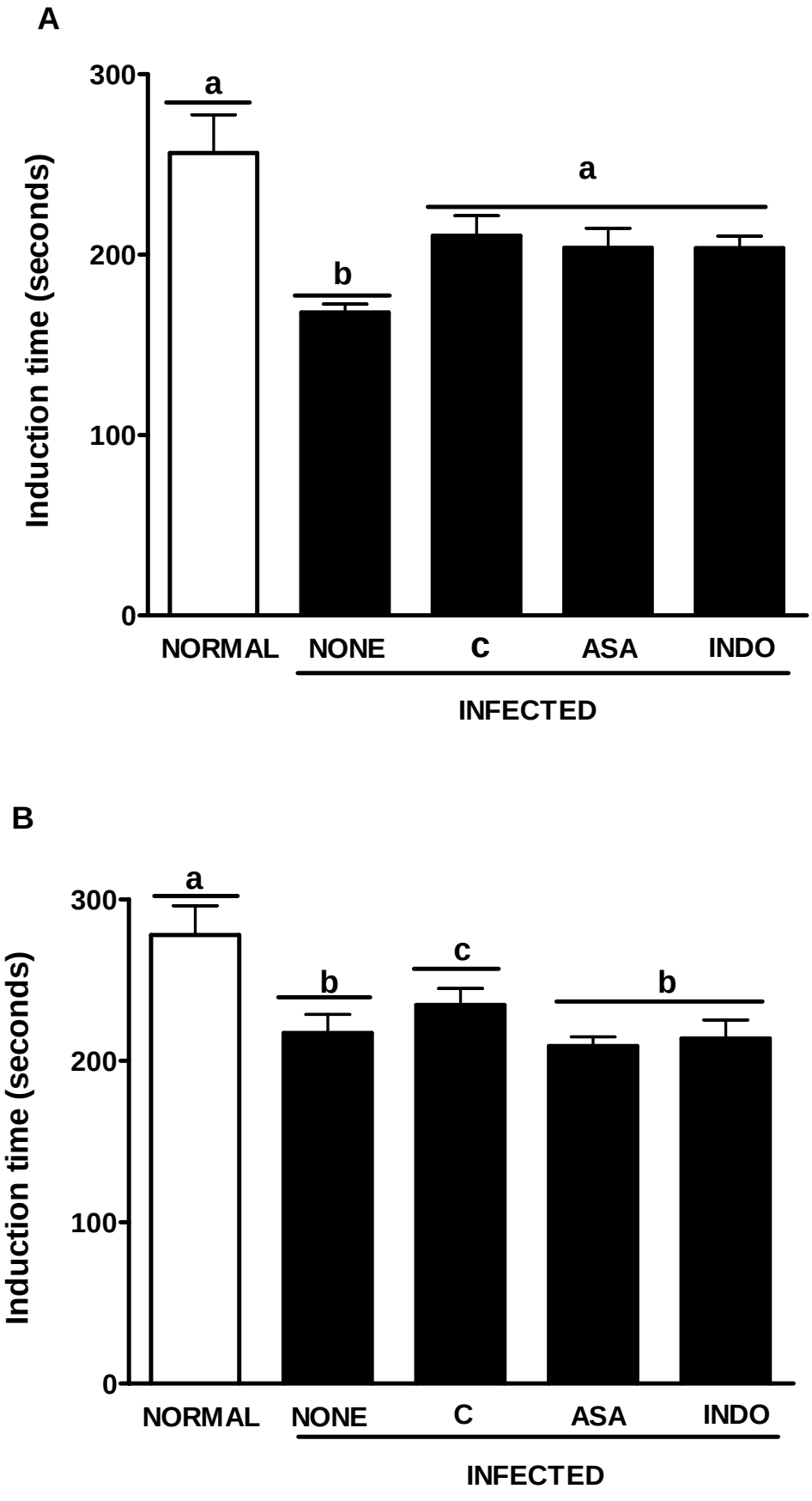
A
300
200
100
0
Induction time (seconds)
a
b
a
NORMAL
NONE
C
ASA
INDO
INFECTED
B
300
200
100
0
Induction time (seconds)
a
b
c
b
NORMAL
NONE
C
ASA
INDO
INFECTED

Figure 8

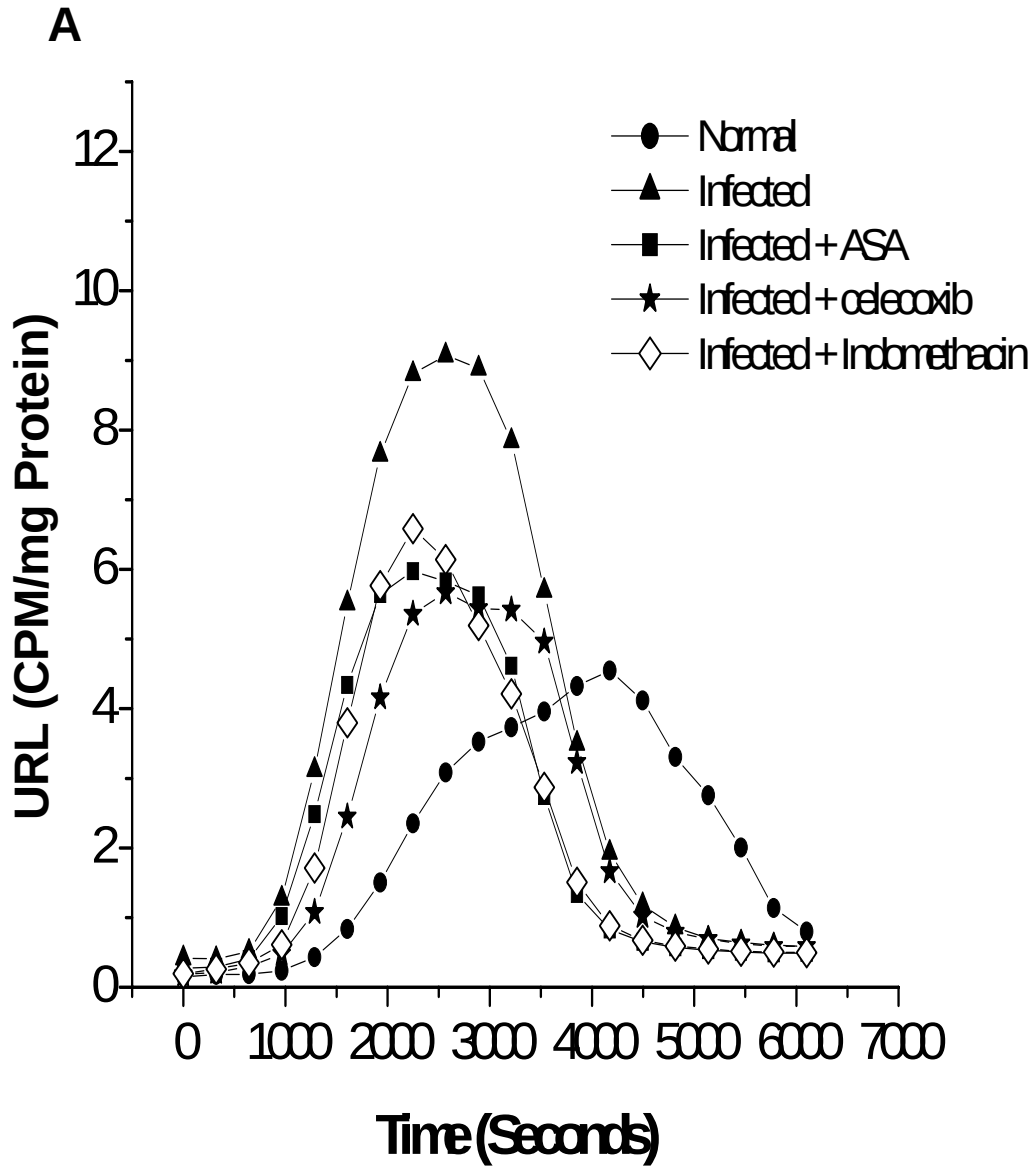
A
URL (CPM/mg Protein)
Time (Seconds)
Normal
Infected
Infected + ASA
Infected + celecoxib
Infected + Indomethacin
0
2
4
6
8
10
12
0
1000
2000
3000
4000
5000
6000
7000

Figure 9A

**B**

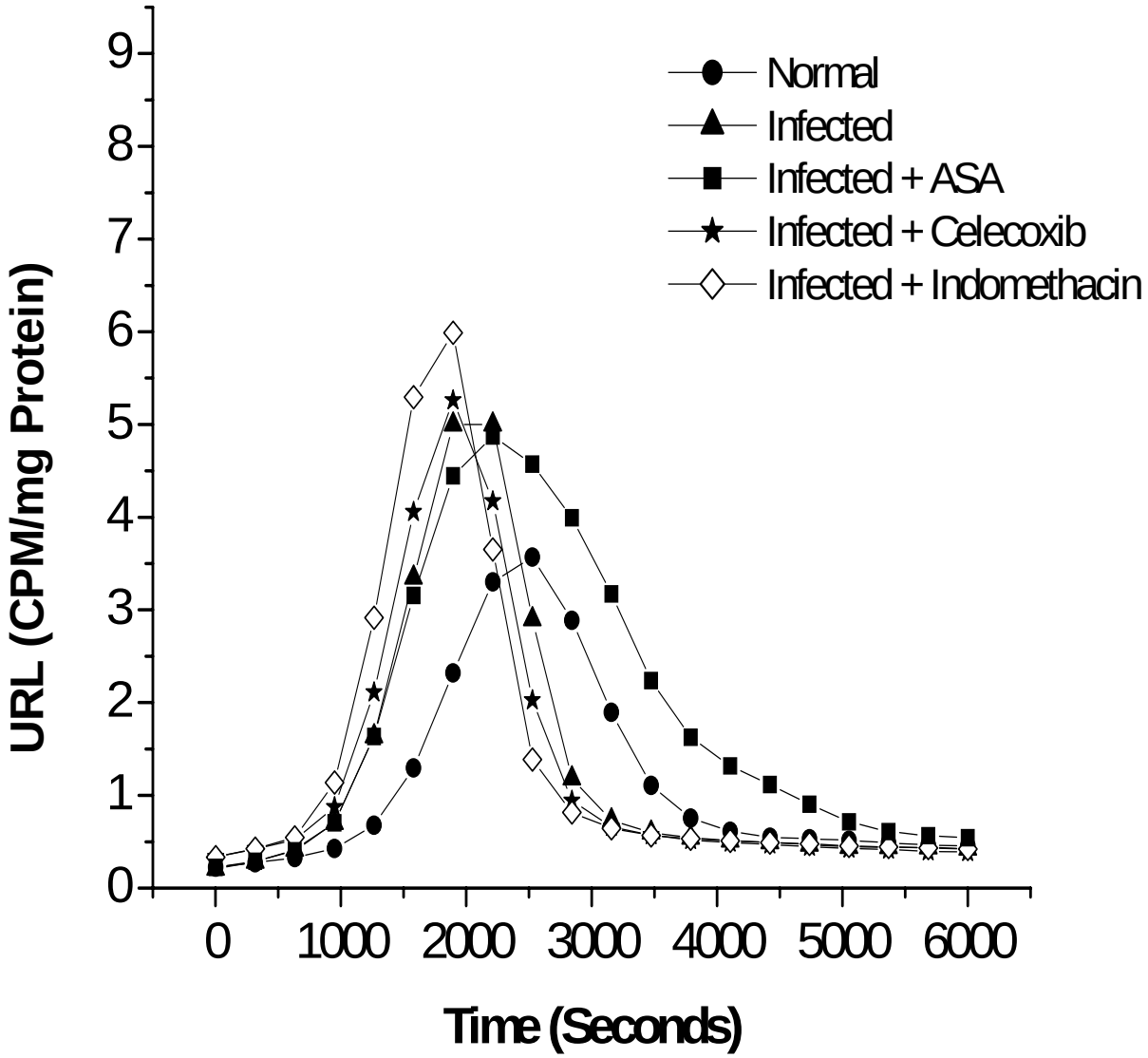
URL (CPM/mg Protein)
Time (Seconds)
0
1
2
3
4
5
6
7
8
9
0
1000
2000
3000
4000
5000
6000
Normal
Infected
Infected + ASA
Infected + Celecoxib
Infected + Indomethacin

Figure 9B