JULIANO YASUO ODA

TRATAMENTO COM BAIXAS DOSES DE ASPIRINA NAS FASES AGUDA E CRÔNICA DA INFECÇÃO MURINA POR *Trypanosoma cruzi* PROMOVE NEUROPROTEÇÃO ENTÉRICA

Londrina
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Londrina, _____de _____de _____.

Dedico este trabalho a minha família em especial minha esposa Thais Tatiana Pfau e meus filhos Luiz Eduardo Y. Oda e Miguel Augusto S. Oda.

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Combati o bom combate,
Acabei a carreira,
Guardei a fé

(II Timóteo 4:7)

ODA, JULIANO YASUO. **TRATAMENTO COM BAIXAS DOSES DE ASPIRINA NAS FASES AGUDA E CRÔNICA DA INFECÇÃO MURINA POR *Trypanosoma cruzi* PROMOVE NEUROPROTEÇÃO ENTÉRICA.** 2016. 90P. Tese – Universidade Estadual de Londrina, Londrina, 2016.

RESUMO

A doença de Chagas (DC) representa um importante problema de saúde pública no Brasil, devido ao surgimento de novos casos e também por ainda não possuir cura. A DC acomete o controle nervoso do trato gastrointestinal, resultando em severos distúrbios de motilidade. Essas alterações de motilidade devem-se, principalmente, à perda de neurônios do plexo mientérico, o qual é um dos plexos ganglionados do sistema nervoso entérico. Neste estudo, objetivou-se avaliar se o uso de baixas doses de aspirina (ASA) durante a fase aguda (20 mg/kg) ou crônica (50 mg/kg) da infecção experimental por *T. cruzi* seria capaz de proteger neurônios mientéricos do cólon de camundongos. Todos os procedimentos foram aprovados pela Comissão de Ética no Uso de Animais da Universidade Estadual de Londrina (UEL). Camundongos Swiss foram distribuídos em grupos que receberam PBS ou foram tratados com baixas doses de aspirina durante a fase aguda (20 mg/Kg, ASA_{Early}) ou crônica (50 mg/Kg, $ASA_{Delayed}$) da infecção causada pela cepa Y de *T. cruzi*. Após 75 dias de infecção, coletou-se o cólon para quantificar focos inflamatórios em cortes histológicos e neurônios mientéricos gerais, nitrérgicos, VIPérgicos e colinérgicos evidenciados por imunofluorescência. Além disso, avaliou-se o trânsito gastrointestinal. Contou-se o número total de corpos celulares de neurônios gerais, nitrérgicos e VIPérgicos presentes em 24,06 mm^2 de cada animal, valor que projetado para 1 cm^2. O número de neurônios colinérgicos foi estimado pela diferença entre o número de neurônios gerais e o número de neurônios nitrérgicos. Foi também realizada a mensuração da área do corpo celular de 300 neurônios evidenciados em cada marcação de cada animal. Foram mensuradas 700 varicosidades nervosas mientéricas VIPérgicas e contendo Substância P. O tempo de trânsito intestinal foi avaliado com a administração de um marcador não absorvível, via gavagem (tempo zero), até a eliminação do primeiro pelete de fezes na cor vermelha. Os dados foram expressos por média ± desvio padrão, e os grupos foram comparados utilizando ANOVA, seguido pelo pós-teste de Tukey, considerando significante $p<0,05$. O tratamento com ASA na fase aguda provocou uma significativa redução no pico de parasitemia ($p<0,05$). Com relação a

quantidade de foco inflamatório, ambos tratamentos foram eficientes para reduzi-los. Os camundongos infectados do grupo PBS apresentaram intensa perda neuronal mientérica, tanto na população geral (60,67%), como também nas subpopulações nitrérgica (49,0%), colinérgica (67%) e VIPérgica (38,0%) ($p<0,05$). Essa morte de neurônios foi significativamente reduzida pelo tratamento com ASA tanto na fase aguda (ASA_{Early}) como na fase crônica ($ASA_{Delayed}$) da infecção ($p<0,05$). A intensa morte de neurônios colinérgicos nos animais do PBS provocou significativo retardo do trânsito do tubo digestório ($p<0,05$). O tratamento com ASA contribuiu para que o tempo de trânsito gastrointestinal dos animais infectados permanecesse semelhantes aos animais saudáveis. A infecção por *T. cruzi*, provocou uma hipertrofia no corpo celular dos neurônios remanescentes. O tratamento com ASA na fase aguda da infecção contribuiu de forma mais expressiva para manutenção da área do corpo celular dos neurônios mientéricos remanescentes. Camundongos infectados com *T. cruzi* não tratados com ASA apresentaram varicosidades nervosas mientéricas VIPérgicas atrofiadas e as contendo substância P estavam hipertrofiadas. Além disso, as fibras nervosas apresentaram maior intensidade de brilho para SP. Conclui-se que a administração de ASA apresentou efeito neuroprotetor para os neurônios do plexo mientérico do cólon de camundongos infectados por *T. cruzi*.

Palavras-Chave: Doença de Chagas, Neuropatia Entérica, Aspirina, Neurônios mientéricos.

ODA, JULIANO YASUO. **TREATMENT WITH LOW DOSES OF ASPIRIN IN ACUTE AND CHRONIC PHASES OF MURINE INFECTION BY *Trypanosoma cruzi* PROMOTES ENTERIC NEUROPROTECTION.** 2016. 90P. Thesis – State University of Londrina, Londrina, 2016.

Abstract

Chagas disease (DC) represents an important public health problem in Brazil, due to the emergence of new cases and still has no cure. The DC affects the nervous control of the gastrointestinal tract, resulting in severe disorders of motility. These alterations of motility are due, mainly, to the loss of neurons of the myenteric Plexus, which is one of the ganglionated Plexus of the enteric nervous system. In this study, the objective to assess whether the use of low-dose aspirin (ASA) during the acute phase (20 mg/kg) or chronic (50 mg/kg) of the experimental infection by *t. cruzi* would be able to protect myenteric neurons of the mice colon. All procedures were approved by the Ethics Committee on use of Animals at the State University of Londrina (UEL). Swiss mice were divided into groups that received PBS or were treated with low doses of aspirin during the acute phase (20 mg/Kg, ASA_{Early}) or chronic (50 mg/Kg, $ASA_{Delayed}$) of the infection caused by the Y strain of *t. cruzi*. After 75 days of infection, collected the colon to quantify inflammatory focus in histological sections and general myenteric neurons, nitrergic, VIPergic and cholinergic evidenced by immunofluorescence. In addition, assessed the gastrointestinal transit. The total number of cell bodies of general neurons, nitrergic and VIPergic present in 24.06 mm^2 of each animal was couted, which designed for 1 cm^2. The number of cholinergic neurons was estimated by the difference between the number of general neurons and the number of nitrergic neurons. The measurement was performed in the area of the cell body of neurons identified 300 in each marking of each animal. Were measured 700 VIPergic myenteric nerve varicosities and containing Substance P. The intestinal transit time was evaluated with the administration of a nonabsorbable marker, gavage pathway (zero time), until the removal of the first pellet of stool in red color. The data were expressed by mean ± standard deviation, and the groups were compared using ANOVA, followed by the Tukey post-test, considering significant $p < 0.05$. The ASA during the acute phase treatment caused a significant reduction in peak parasitaemia ($p < 0.05$). Regarding the amount of inflammatory focus, both treatments were efficient to reduce them. The mice infected

PBS group showed intense myenteric neuronal loss, both in the general population as well as in subpopulations (60.67%), nitrergic (49.0%), cholinergic (67%) and VIPergic (38.0%) ($p < 0.05$). This death of neurons was significantly reduced by treatment with ASA both in the acute phase (ASA_{Early}) as in the chronic phase ($ASA_{Delayed}$) of the infection ($p < 0.05$). The intense death of cholinergic neurons in animals of the PBS caused significant traffic delay of the digestive tube ($p < 0.05$). Treatment with ASA contributed to the gastrointestinal transit time of infected animals remain similar to healthy animals. Infection with *t. cruzi*, provoked a hypertrophy in the cell body of neurons. Treatment with ASA in the acute phase of the infection contributed more expressive for maintenance of the cell body of myenteric neurons remaining. Mice infected with *t. cruzi* treated with ASA presented VIPergic myenteric nerve varicosities atrophied and the containing substance P were hypertrophied. In addition, the nerve fibers showed greater intensity of brightness to SP. Concluded that the wing administration presented a neuroprotective effect to neurons of the myenteric plexus of the mice colon infected by *t. cruzi*.

Keywords: Chagas disease, Enteric Neuropathy, Aspirin, Myenteric Neurons.

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LISTA DE ABREVIATURAS E SIGLAS

AINES – Anti-inflamatórios não Esteroidais

ASA - Aspirina

BSA – Albumina Bovina Sérica

°C – Graus Celcius

CEUA/UEL – Comissão de Ética no Uso de Animais da Universidade Estadual de Londrina

COX-1 – Cicloxigenase 1

COX-2 – Cicloxigenase 2

DATASUS – Departamento de Informática do Sistema Único de Saúde

DC – Doença de Chagas

DPI – Dias Pós-Infecção

HE – Hematoxilina e Eosina

iNOS – Oxido Nítrico Sintase Induzida

MHC – Complexo Principal de Histocompatibilidade

µL – Microlitro

µm – Micrômetro

MS – Ministério da Saúde

nNOS – Óxido Nítrico Sintase Neuronal

NO – Óxido Nítrico

PBS – Tampão Fosfato-salino

PCR - Reação de cadeia da polimerase

PGE2 – Prostaglandina E2

pH – Potencial hidrogeniônico

SNC – Sistema Nervoso Central

SNE – Sistema Nervoso Entérico

SIM – Sistema Informatizado de Mortalidade

SP – Substância P

SUS – Sistema Único de saúde

T. cruzi – Trypanosoma cruzi

VIP – Peptídeo Vasoativo Intestinal

SUMÁRIO

1. INTRODUÇÃO

1.1 Doença de Chagas

A descoberta da tripanossomíase humana por Carlos Chagas (DC) em 1909 foi um dos trabalhos mais completos e bem-sucedidos na história da medicina tropical. Carlos Chagas não apenas descobriu uma doença nova, mas primeiramente descreveu com riqueza de detalhes não apenas um novo parasito, mas seu ciclo de transmissão, seu vetor e reservatório mamífero intermediário, bem como as manifestações clínicas agudas do primeiro caso a acometer humanos (COURA e BORGES-PEREIRA, 2010; RASSI-Jr, RASSI e MARIN-NETO, 2010).

A história natural da DC começou há milhões de anos como uma doença enzoótica entre os animais selvagens, e isso ainda persiste em áreas enzoóticas, tais como a região amazônica. Devido ao extenso desmatamento para agricultura e criação de gado ao longo dos últimos 200-300 anos na América Latina, triatomíneos que ficaram sem suas fontes de alimentos devido à remoção de animais selvagens começaram a colonizar áreas circundantes habitações humanas, e as próprias habitações. Eles se adaptaram a este novo nicho, alimentando-se do sangue de humanos e animais domésticos (COURA e BORGES-PEREIRA, 2010; RASSI Jr, RASSI e REZENDE, 2012).

A DC é a manifestação clínica causada pelo protozoário *Trypanosoma cruzi* e a transmissão pode ocorrer de forma vetorial, congênita, oral, por transfusões sanguíneas e transplantes de órgãos. Atualmente é reconhecida pela Organização Mundial da Saúde como sendo a 13ª doença tropical mais negligenciada e representa um importante problema social e econômico na América Latina, haja vista

que ainda não há um tratamento que promova a cura dessa enfermidade (COURA; DIAS, 2009; RASSI-Jr, RASSI e MARIN-NETO, 2010; BRASIL, 2010).

Essa doença é amplamente dispersa, principalmente, na América Latina, onde se estima que existam 7 milhões de pessoas infectadas e a cada ano são relatados cerca de 50 mil novos casos, sendo que aproximadamente 75 a 90 milhões de pessoas estejam sob risco potencial de se tornarem infectadas (COURA; DIAS, 2009; MAYA, et al., 2010; MUKHERJEE, et al., 2011; MOREIRA, et al., 2011; WHO 2014).

No Brasil, em virtude da implementação de medidas de controle na transmissão vetorial e transfusional, ocorreu um decréscimo significativo no surgimento de novos casos de DC. No entanto, dados epidemiológicos dos últimos dez anos apontam para a continuidade dos números de casos de DC. Nos anos de 2000 a 2010 foram registrados 1.093 casos de DC aguda no Brasil, sendo que a maior parte (71%) ocorreu por transmissão oral devido à ingestão de alimentos contaminados (caldo de cana, açaí, entre outros). Mas, no Brasil predominam os casos crônicos de DC, decorrentes de infecções adquiridas no passado, com aproximadamente três milhões de indivíduos infectados representando um alto custo para o serviço de saúde, tendo em vista a sua característica de uma longa cronicidade (MEDEI et al., 2008; COURA; DIAS, 2009; BRASIL, 2011; MARTINS-MELO, et al., 2012).

Essa doença representa a quarta causa de morte entre as doenças infecto-parasitárias, considerando a faixa etária acima de 45 anos. Martins-Melo et al. (2012), analisando os óbitos registrados no período entre 1979 a 2009 cadastrados no Sistema de Informação de Mortalidade SIM/MS/DATASUS, verificaram que de um total de 27 milhões de óbitos registrados no Brasil a DC foi mencionada como a

causa de morte em mais de 172 mil (0,62%) registros (MEDEI et al., 2008; ANDREOLLO; MALAFAIA, 2009; MAYA, et al., 2010; BRASIL, 2010).

A DC está emergindo na América do Norte (Figura 1), provavelmente a partir da migração de indivíduos infectados (COURA e VIÑA, 2010; EPTING, COATES e ENGMAN, 2010). Dessa forma, o Centro de controle de doenças e prevenção estima que mais de 300 mil pessoas estejam infectadas com *T. cruzi* e que um total de 30 a 45 mil pessoas provavelmente serão diagnosticadas com cardiopatia chagásica grave e, aproximadamente 3 a 5 mil desenvolverão a forma digestiva da DC. Assim, são necessárias medidas como aprimorar os métodos de diagnóstico e alternativas terapêuticas para possibilitar uma melhor assistência ao paciente, bem como investimento para prevenir novas infecções (BERN e MONTGOMERY, 2009; NUNES et al., 2013; MONTGOMERY, et al., 2014).

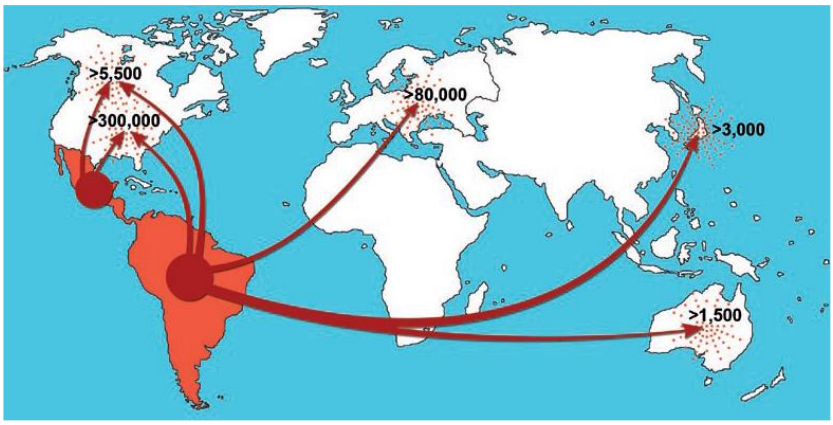

Figura 1: Rotas de migração da América Latina e estimativa do número total de infectados em países não-endêmicos (COURA e VIÑA, 2010).

1.2 Interação Parasito X Hospedeiro

O ciclo de vida do *T. cruzi* é complexo, com diferentes estágios de desenvolvimento no inseto vetor e no hospedeiro mamífero (Figura 2). As formas típicas no hospedeiro mamífero são a tripomastigota não-replicativa e a amastigota (intracelular replicativa), enquanto as formas epimastigota e tripomastigota

metacíclica (infectante) são encontradas no hospedeiro invertebrado (BRENER, 1971; RASSI-Jr, RASSI e MARIN-NETO, 2010).

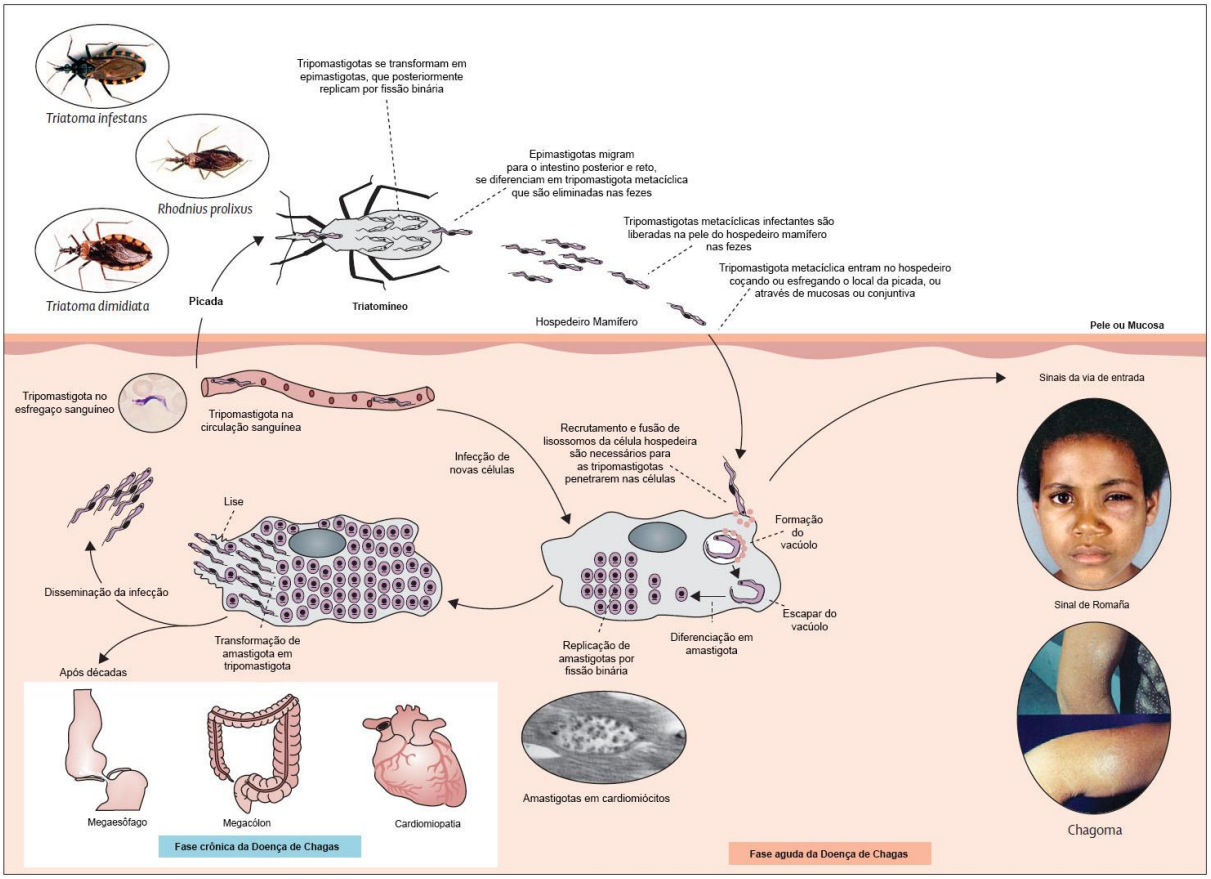

Figura 2: Transmissão vetorial e ciclo de vida do *T. cruzi*. (Adaptado de RASSI Jr, RASSI e MARIN-NETO, 2010).

Como parte de seu ciclo evolutivo, o *T. cruzi* um parasito intracelular obrigatório, apresenta-se sob três formas evolutivas diferentes: tripomastigota, epimastigota e amastigota. A diferenciação ocorre como forma adaptativa necessária para o desenvolvimento nos dois hospedeiros: o invertebrado (triatomíneo hematófago) e o vertebrado (mamíferos, incluindo o homem) (COURA e BORGES-PEREIRA, 2010; RASSI-Jr, RASSI e MARIN-NETO, 2010; SOUZA, CARVALHO e BARRIAS, 2010; BARRIAS, CARVALHO e SOUZA, 2013).

Portanto, durante a fase aguda, as formas infectantes do *T. cruzi* (amastigotas e tripomastigotas) são hábeis para infectar todas as células nucleadas do

hospedeiro mamífero devido a um complexo mecanismo de invasão celular. A forma tripomastigota metacíclica invade principalmente macrófagos, fibroblastos e outros tecidos mesenquimais no sítio primário da infecção. Após a transformação na forma tripomastigota sanguínea, o parasito precisa resistir a resposta imune humoral mediada pelo complemento e para iniciar o novo ciclo de vida, o parasito precisa invadir novas células (SOUZA, CARVALHO e BARRIAS, 2010; TEIXEIRA et al., 2011; OSORIO et al., 2012).

O fato do *T. cruzi* possuir a capacidade de infectar célula nucleada *in vitro* e infectam músculo estriado, músculo cardíaco e neurônios entéricos, leva os pesquisadores a inferir um possível e intrínseco tropismo celular. Demonstra ainda perfil geográfico restrito, levando a hipótese de que existe uma relação entre determinada cepa de *T. cruzi* e seu tropismo tecidual e clones de cepas distintas podem ser isoladas de pacientes com a forma cardíaca ou gastrintestinal da doença (MELO e BRENER, 1978; EPTING, et al., 2010).

Após a invasão, as tripomastigotas devem lançar mão de mecanismos de evasão do sistema imune para sobreviver ao ambiente altamente oxidado no interior dos macrófagos, com a finalidade de estabelecer a infecção. Para isso, o *T. cruzi* possui uma complexa rede de enzimas antioxidantes localizadas em diferentes compartimentos subcelulares que defendem o parasito contra um ambiente oxidadado. Depois de se multiplicar e se transformar na forma tripomastigota sanguínea, os parasitos devem resistir a resposta imune humoral (De MORAES et al., 2015).

Mediante o reconhecimento entre o parasito e a célula do hospedeiro vertebrado, guiados pelo processo de sinalização celular, eles iniciam seu ciclo intracelular e são internalizados em um processo que envolve a formação de um

vacúolo endocítico o vacúolo parasitóforo, seguindo vários ciclos de divisão celular que culminam com a disseminação do parasito para os tecidos (SOUZA, CARVALHO e BARRIAS, 2010; TEIXEIRA et al., 2011; BARRIAS, CARVALHO e SOUZA, 2013).

Epting et al. (2010) e Moraes et al. (2015) relatam que o *T. cruzi* possui uma vasta diversidade de moléculas de superfície e secretadas que estão envolvidas direta ou indiretamente na adesão e invasão da célula hospedeira. Osorio et al. (2012) complementam que, as estruturas, estratégias ou moléculas produzidas por um agente patogênico no intuito de invadir e estabelecer relação de parasitismo no hospedeiro, provocando doença e evadindo das defesas do hospedeiro, define-se como fatores de virulência, que são listados na tabela 1.

Tabela 1. Fatores de virulência expressados pelo *T. cruzi* durante sua interação com a célula hospedeira.

Fatores de Virulência envolvidos na resistência do *T. cruzi* ao estresse oxidativo	**Referência(s)**
Peroxidases (detoxificação de hidroperóxidos)	
Glutationa Peroxidase-I (TcGPXI)	Alvarez et al. (2004), Alvarez et al. (2011)
Glutationa Peroxidase-II (TcGPXII)	Alvarez et al. (2004), Alvarez et al. (2011)
Triparedoxina Peroxidase Citosólica (TcCPx)	Piacenza et al. (2008), Piacenza et al. (2009)
Triparedoxina Peroxidase Mitocondrial (TcMPx)	Piacenza et al. (2008), Piacenza et al. (2009)
Superóxido Dismutases	
Ferro superóxido dismutase (Fe-SOD)	Mateo et al. (2008)
Fatores de Virulência envolvidos na resistência do *T. cruzi* ao Sistema Imune da Célula Hospedeira e Evasão Imune	**Referência(s)**
Moléculas envolvidas na Resistência ao Sistema Complemento	
Fator de Aceleração de Decaimento do *T. cruzi* (T-DAF)	Norris et al. (1991), Tambourgi et al. (1993)
Proteína reguladora do complemento (CRP)	Norris et al. (1991), Norris et al. (1998), Beucher

	(2008)
Proteína trispanning inibidora do receptor de C2 (CRIT)	Cestari et al. (2008), Cestari et al. (2009), Blom et al. (2009)
Calreticulina (CRT)	Valck et al. (2010), Ramirez et al. (2011)
Prolina racemase (PR)	Chamond et al. (2005), Coutinho et al. (2009)
Tc52	Ouaissi et al. (1998), Ouaissi et al. (2002)
Evasão Imune mediada pela indução de microvesículas derivadas da célula hospedeira	Cestari et al. (2012)
Fatores de Virulência Envolvidos na Adesão e Invasão da Célula Hospedeira por Tripomastigotas	**Referência(s)**
gp82 e gp35/50	Atayde et al. (2004), Staquicini et al. (2010)
Mucinas	Bugliala et al. (2006), Alcaide et al. (2004)
Cruzipaína	Berasain et al. (2003), Alvarez et al. (2012)
Oligopeptidase B (OpB)	Burleigh et al. (1998), Coetzer et al. (2008)
gp85/Família Trans-sialidase (TS)	Magdesian et al. (2007), Tonelli et al. (2011)
Superfamília das TS	Lieke et al. (2011), Rubin e Schenkman (2012)
Calcineurina	Araya et al. (2009), Naderer et al. (2011)
Peptidil-prolil cis-trans Isomerase (TcMIP)	Moro et al. (1995)
Fosfolipase A1 (PLA1)	Belaunzaran et al. (2011)
Gp 63	Yao (2010)
Fatores de Virulência envolvidos no escape do *T. cruzi* do Fagolissomo	**Referência(s)**
Tc-tox	Rubin-de-Celis et al. (2006)
LYT1	Zago et al. (2008)
Fatores de Virulência envolvidos na diferenciação ou proliferação do *T. cruzi*	**Referência(s)**
Proteasoma	de Diego et al. (2001)
Fosfatidilinositol Fosfolipase C (TcPI-PLC)	Vde et al. (2010)
Proteína Fosfatase 2A (TcPP2A)	Lauwaet et al. (2007),

	Madeira da Silva et al. (2010)
Calpaínas	Sangenito et al. (2009), Ennes-Vidal et al. (2010)

Assim, a invasão da célula hospedeira por tripomastigotas é um processo complexo, compreendendo diferentes etapas, envolvendo moléculas de adesão, eventos de sinalização e atividades proteolíticas.

No entanto, a patogenia da DC ainda permanece controversa. Diversas teorias tentam elucidar os mecanismos que provocam lesões teciduais e provocam intensas e severas disfunções, entre elas (1) persistência do parasito, (2) a proposta neurogênica e (3) a autoimunidade (TEIXEIRA, et al., 2011).

A teoria da persistência do parasito baseia-se na detecção precoce de ninhos de amastigotas nos tecidos musculares durante a fase aguda da infecção (TORRES, 1960). No entanto, a ausência de parasitismo nas lesões encontradas na fase crônica coloca em dúvida sua credibilidade, uma vez que aproximadamente 90% dos pacientes que morrem em decorrência da DC não apresentam parasitos nos tecidos (TORRES, et al., 2004; TEIXEIRA, et al., 2009).

A detecção de perdas significativas de neurônios em gânglios autonômicos e entéricos na ausência de *T. cruzi in situ*, é a base para a hipótese da liberação de uma neurotoxina do ninho do parasito escondido em alguma parte do corpo do hospedeiro (KOEBERLE, 1970). No entanto, não há relatos que demonstrem a estrutura da neurotoxina ou qualquer substância neurotóxica liberada pelo parasito (CILLARI, et al., 1995; TEIXEIRA, et al., 2011).

Já a teoria da autoimunidade é baseada na demonstração de uma interação citotóxica acelerada entre linfócitos T reativos ao *T. cruzi* com células alogênicas não parasitadas. Esses linfócitos aderem às miofibras e lisam as fibras musculares livres

de parasitos. Além disso, são capazes de destruir neurônios dos plexos entéricos (TEIXEIRA, NASCIMENTO e STURM, 2006).

As respostas imunes contra antígenos próprios na DC humana e experimental foram demonstrados em vários estudos, entre os quais, anticorpos contra antígenos expressos em células cardíacas (MCCORNICK e ROWLAND 1989, CUNHA-NETO et al., 1995), células nervosas (RIBEIRO-dos-SANTOS et al., 1979, VAN VOORHIS e EISEN, 1989), entre outros, foram detectados durante a infecção pelo *T. cruzi*. No entanto, os autoanticorpos são comumente encontrados após a infecção com patógenos diferentes, sem qualquer implicação sobre uma patologia autoimune (ARGOV et al., 1989, DANIEL-RIBEIRO e ZANINI, 2000; SOARES, PONTES-de-CARVALHO e RIBEIRO-dos-SANTOS, 2001).

Dessa forma, Teixeira et al. (2011) concluem que a teoria da autoimunidade da DC continua sendo indefinida, uma vez que o mecanismo direto que reconhece o próprio como não-próprio e resulta na ativação de linfócitos inflamatórios efetores ainda é desconhecido.

1.3 Manifestações Clínicas da Doença de Chagas

A infecção que causa a DC apresenta duas fases bem definidas: a fase aguda e a fase crônica. A fase aguda, com duração de aproximadamente dois a três meses, com a ocorrência de sintomas não específicos, comumente encontrados em outras doenças que dificultam seu diagnóstico e caracterizada por uma parasitemia, febre, mal-estar, linfocitose e astenia. Poucos indivíduos desenvolvem síndromes clínicas severas nesta fase, porém cerca de 10% dos acometidos podem vir a óbito como resultado de severa miocardite ou meningoencefalite (RASSI-Jr, RASSI e MARIN-NETO, 2010; MONTGOMERY et al., 2014).

Segundo Montgomery et al. (2014), a fase aguda é diagnosticada pela identificação do parasito na circulação sanguínea com avaliação microscópica ou hemocultura do sangue periférico. De acordo com Hofflin et al. (1987) e Grauert et al. (1993) a parasitemia desenvolve-se em uma fase indetectável microscopicamente (período pré-patente), outra detectável e crescente e uma terceira, detectável e decrescente.

Segundo os estudos de Correa Oliveira et al. (1999), as manifestações clínicas observadas na DC são em parte devido à resposta imune dirigida ao parasito. O sistema imune, portanto, estaria envolvido tanto na redução da carga parasitária quanto nas lesões teciduais verificadas na fase crônica da doença (CUNHA- NETO, 2014).

As manifestações da fase aguda da DC, geralmente, se resolvem espontaneamente em 90% dos indivíduos infectados, mesmo se a infecção não for tratada com drogas tripanocidas e aproximadamente 60% destes não manifestarão as formas clínicas cardíaca, neurológica, mista ou digestiva. Esses indivíduos possuem a forma indeterminada da DC que é caracterizada por ausência de sintomatologia clínica, sorologia positiva para *T. cruzi*, e eletrocardiograma e radiografias de tórax e abdome normais (DIAS, 1995; RASSI-Jr, RASSI e MARIN-NETO, 2010).

De acordo com Barrias, Carvalho e Souza (2013), 30 a 40% dos pacientes podem desenvolver uma forma crônica e sintomática da doença, que se desenvolve 10-20 anos após a infecção inicial, provocando lesões irreversíveis no coração, esôfago e intestino grosso com alteração na condução nervosa desses órgãos, caracterizando as formas clínicas: cardíaca, digestiva e neurológica. Durante a fase aguda da infecção experimental por *T. cruzi* observa-se uma curva de parasitemia,

que por consequência leva a um intenso processo inflamatório com lesões secundárias em diversos tecidos do hospedeiro (COURA e BORGES-PEREIRA, 2010). Normalmente, o parasito não circula no sangue do hospedeiro durante a fase crônica (Da SILVEIRA, et al., 2007a; CAMPOS, et al., 2016). Por isso, assume-se que a maior parte das lesões teciduais observadas na DC ocorra na fase aguda da infecção (CAMPOS, et al., 2016).

A progressão direta da fase aguda para as formas clínicas da DC acomete uma pequena parcela dos pacientes (5 a 10%) (BRAZ, AMATO-NETO e OKAY, 2008). A reagudização da doença pode acontecer em indivíduos imunossuprimidos ou que fazem uso de medicamentes imunossupressores.

A morbimortalidade da DC ocorre principalmente na fase crônica, em decorrência de lesões teciduais que ocorrem ainda na fase aguda (RASSI e MARIN-NETO, 2010; MARTINS-MELO et al., 2012). Estudos epidemiológicos realizados em países da América Latina demonstram que 70% dos acometidos por DC são assintomáticos, e 30% podem desenvolver cardiopatia severa ou lesões digestivas ou distúrbios neurológicos. É importante ressaltar que a cada ano aproximadamente 2 a 3% dos indivíduos assintomáticos passam a manifestar alterações cardíacas, digestivas ou neurológicas (TEIXEIRA, NASCIMENTO e STURM, 2006; MAYA et al., 2010; ZINGALES, 2011; POVEDA, et al., 2014).

Segundo Verani et al. (2009) e Afonso, Ebell e Tarleton (2012), na fase crônica da DC o parasito pode ser encontrado esparsamente distribuído intracelularmente em tecidos por todo o corpo e raramente no sangue. O parasitismo não pode ser detectado por microscopia, mas apenas por mecanismos mais sensíveis como a PCR (Reação de cadeia da polimerase).

Como as manifestações gastrointestinais da DC provocam baixas taxas de mortalidade em comparação com as manifestações cardíacas, elas acabam recebendo pouca atenção por parte dos pesquisadores e até mesmo políticas públicas de saúde, no entanto os pacientes que manifestam a forma digestiva da DC possuem altos índices de morbidade, o que resulta em uma qualidade de vida severamente prejudicada (PINAZO, et al., 2014).

A forma digestiva da DC é caracterizada por alterações na função motora, secretória e absortiva do trato gastrointestinal (RASSI, et al., 2010). Ela é encontrada quase que exclusivamente em países ao sul da bacia amazônica (Brasil, Chile, Argentina e Bolívia) e raramente em países da América Central e do Norte. Essa distribuição geográfica ocorre devido a diferenças nas cepas do parasito (RASSI, et al., 2010; RASSI-Jr, RASSI e MARIN-NETO, 2010).

As alterações nos órgãos do trato gastrointestinal que se manifestam durante a fase crônica da DC são atribuídas a lesões no plexo mientérico, resultando em movimentos peristálticos incoordenados, hipertrofia muscular e dilatação de órgãos como esôfago e intestinos (ADAD, et al., 2001; CAMPOS, et al., 2016). Em geral os sintomas digestivos são inespecíficos e diversos fatores, incluindo outras infecções comuns acabam gerando dificuldade no diagnóstico.

O megaesôfago e o megacólon são as maiores causas de morbidade na forma clínica digestiva da DC crônica (da SILVEIRA, et al., 2007ab; MATSUDA, MILLER e EVORA, 2009), sendo que para o desenvolvimento do megaesôfago é necessária uma redução de aproximadamente 85% do número de neurônios, e no megacólon, uma perda de pelo menos 50% do número de neurônios (KOEBERLE, 1970; TAFURI, MARIA e LOPES, 1970).

Ainda são escassos os estudos que visam a elucidar as alterações no sistema nervoso entérico após infecção por *T. cruzi*, bem como o papel das células inflamatórias no desenvolvimento das manifestações gastrointestinais. É possível que ocorra uma inflamação crônica ao redor dos gânglios entéricos nos indivíduos infectados que desenvolvem os sintomas gastrointestinais (Da SILVEIRA, et al., 2007b). Acredita-se que a causa da morte neuronal durante a fase aguda da DC seja, em partes, provocada pela presença do parasito em altas concentrações nos tecidos (ANDRADE, 1983; Da SILVEIRA, et al., 2005), em contraste durante a fase crônica a carga parasitária é muito baixa nas lesões, assim a destruição dos neurônios mientéricos pode ser uma consequência da resposta imune que segue a infecção (VAGO, et al., 1996; Da SILVEIRA, et al., 2007a). Da Silveira, et al. (2007a) postulam que o processo inflamatório e a redução das células gliais encontradas em pacientes chagásicos pode perturbar o funcionamento do SNE, contribuindo para o desenvolvimento das manifestações gastrointestinais.

1.4 Neuropatia Entérica Chagásica

O trato gastrointestinal difere de todos os demais órgãos periféricos, pois é dotado de um extenso sistema nervoso intrínseco, denominado de sistema nervoso entérico (SNE), que pode controlar suas funções intestinais mesmo quando totalmente isolado do sistema nervoso central (Bayliss e Starling, 1899 APUD FURNESS, 2012).

O SNE é a maior e mais complexa divisão do sistema nervoso autônomo em vertebrados. Distribuído por todo o trato gastrointestinal (TGI), vesícula biliar e o pâncreas, é organizado como uma rede interconecta de neurônios e células da glia

que são agrupados no interior de gânglios, localizados nos dois maiores plexos: o mientérico e o plexo submucoso, demonstrados na figura 3 (FURNESS, 2006).

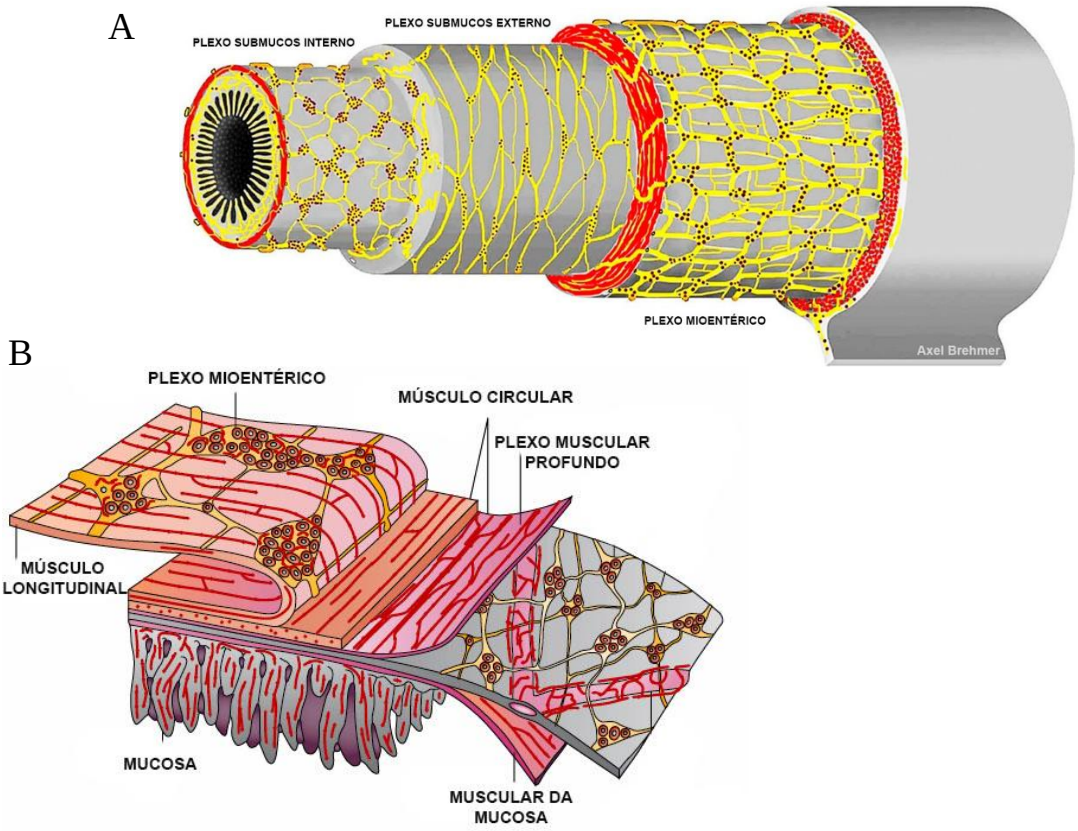

Figura 3. (A) Desenho esquemático dos plexos ganglionados do sistema nervoso entérico (Adaptado de Brehmer, 2006). (B) Organização do plexo mioentérico de humanos e mamíferos médios e grandes (Adaptado de Furness, 2006).

Os componentes do SNE formam um circuito integrado que desempenha diversas funções como o controle dos padrões de movimento do TGI, troca de fluídos por meio da superfície da mucosa, alterações no fluxo sanguíneo e interação com os sistemas imunológico e endócrino do intestino. Embora o TGI receba influência autonômica via sistema nervoso simpático e parassimpático, os circuitos neuronais intrínsecos do SNE são capazes de gerar atividade contrátil do intestino independente de intervenção do SNC (FURNESS, 2012; SASSELI, PACHNIS e BURNS, 2012).

Furness (2012), ressalta que o SNE, no entanto, não é autônomo, pois o controle neural da função gastrointestinal é um sistema integrado que envolve interações entre reflexos entéricos locais, reflexos que passam por gânglios simpáticos e reflexos que passam pelo intestino e volta ao SNC.

Ele contém vários tipos diferentes de neurônios comparáveis em número ao da medula espinal (80-100 milhões) e uma série de neurotransmissores e neuromoduladores semelhantes aos encontrados no SNC. Com base nas propriedades eletrofisiológicas e histoquímicas podem ser classificados em subpopulações funcionalmente distintas, incluindo neurônios aferentes primários intrínsecos, interneurônios, neurônios motores, neurônios intestinofugais, neurônios secretores e vasomotores (GIORGIO e CAMILLERI, 2004; BREHMER, 2006; FURNESS, 2006).

A maior parte dos neurônios mientéricos humanos são colinérgicos ou nitrérgicos (MURPHY, et al., 2007; JABARI, et al., 2014). Como em outras espécies os neurônios nitrérgicos humanos parecem ser interneurônios descendentes ou neurônios motores inibitórios (BREHMER, 2006). Em contraste, os neurônios mientéricos colinérgicos são neurônios motores excitatórios (FURNESS, 2006).

O VIP (peptídeo vasoativo intestinal) é um neuropeptídeo não-adrenérgico e não-colinérgico encontrado em neurônios secretomotores do intestino. Inervam diretamente o epitélio e regulam a concentração de íons e a secreção de fluidos (KEITA, et al., 2013). Em diferentes estudos VIP é demonstrado como regulador da resposta inflamatória (DELGADO e GANEA, 2001; CONLIN, et al., 2009). VIP é principalmente produzido por neurônios entéricos dos plexos mientérico e submucoso. Esse neuropeptídeo apresenta um potente efeito anti-inflamatório, afetando as respostas imunes inata e adaptativa (Di GIOVANGIULIO, et al., 2015).

Já a Substância P, um neuropeptídeo expresso em diversas regiões, incluindo o TGI é liberada principalmente por neurônios do plexo mientérico e submucoso, bem como neurônios sensitivos intrínsecos e extrínsecos (GOODE, et al., 2000). Vários autores (STURIALE, et al., 1999; PELAYO, et al., 2014) sugerem um efeito pró-inflamatório para a SP na inflamação intestinal.

Os distúrbios de motilidade são comuns em pacientes com a forma digestiva da DC (da SILVEIRA, et al., 2007b; MATSUDA, MILLER e EVORA, 2009), os quais são decorrentes de lesões no plexo mientérico (MAIFRINO et al., 1999; ADAD et al., 2001; MEDEIROS, et al., 2010).

Estudos experimentais (MOREIRA, et al., 2011; MOREIRA et al., 2014; NOGUEIRA-PAIVA, et al., 2014) e em pacientes (da SILVEIRA, et al., 2007b; JABARI, et al., 2011; 2012; 2014) revelam morte de neurônios mientéricos durante a infecção chagásica. No entanto, Jabari et al. (2011) relatam que os neurônios nitrérgicos são mais resistentes aos fatores patológicos que levam à morte neuronal quando comparados com os neurônios colinérgicos.

A desnervação leva à perda de coordenação motora e alteração no funcionamento dos esfíncteres, e a musculatura lisa do segmento permanece em estado de contração (JABARI, et al., 2014), prejudicando o esvaziamento de material semissólido, provocando assim a dilatação, sendo este o mecanismo fisiopatológico subjacente ao megaesôfago e megacólon (RASSI, REZENDE e LUQUETTI, 2010).

No megacólon, os distúrbios da motilidade são relacionados com a dilatação do colo e a constipação. O reto e o colo sigmoide são os segmentos mais comprometidos (da SILVEIRA et al., 2007ab; JABARI et al., 2014). Assim, a dificuldade em defecar contribui para a dilatação do colo provocando dor e desconforto (TEIXEIRA et al., 2011).

O mecanismo patofisiológico que leva a lesão e morte neuronal observada na DC ainda é muito discutido no meio científico. Embora a maioria dos danos aos neurônios do plexo mientérico e suas fibras nervosas ocorram durante a fase aguda da infecção devido à ação direta do parasito (JABARI, et al., 2014), grande perda neuronal ocorre lentamente ao longo da fase crônica da doença. A desnervação ocorre em graus variáveis, é irregular e provavelmente esteja relacionada com fatores próprios do hospedeiro e sua interação com o parasito (RASSI Jr, RASSI e REZENDE, 2012), reação de autoimunidade (DUTRA, et al., 2009), ou persistência do parasito no tecido do hospedeiro (da SILVEIRA, et al., 2005; CLAYTON, 2010).

No entanto, as lesões inflamatórias que acometem as fibras musculares e afetam os neurônios mientéricos (ADAD, et al., 2001; TEIXEIRA, NASCIMENTO e STURM, 2006; da SILVEIRA, et al., 2007a), estão fortemente associadas com a morte desses neurônios provocada pela lise mediada pelos linfócitos (TEIXEIRA, et al., 2011). Da Silveira et al. (2007b) complementam que o desenvolvimento do megacólon, após a infecção aguda por *T. cruzi* está associado com a invasão permanente dos gânglios entéricos por células T citotóxicas, levando a perda da inervação do músculo liso da parede do colo. Jabari et al. (2012) sugerem que o predomínio de fibras nervosas inibitórias intramusculares pode ser um fator importante no desenvolvimento do megacólon.

1.5 Tratamento Farmacológico da Doença de Chagas

O tratamento da DC constitui-se basicamente em controlar a replicação do parasito, particularmente nos casos agudos, congênitos, acidentes de laboratório, em casos crônicos de baixa idade e ocorrência de reinfecção e reagudização, além do tratamento sintomatológico durante o curso da infecção (MAYA, et al., 2010).

Existem no mercado dois fármacos para o tratamento da DC, o Benznidazol (Rochagan®, Roche Pharmaceutical, patente doada ao Ministério da Saúde, Brasil) e o Nifurtimox (Lampit®, Bayer Healthcare).

Nifurtimox e benznidazol atuam através da geração de radicais livres e/ou metabólitos eletrofílicos. O grupo nitro de ambos os fármacos é reduzido a um grupo amina pela ação das nitroredutases, formando intermediários de radicais livres e metabólitos eletrofílicos (MAYA, et al., 2007).

É provável que os metabólitos reduzidos do benznidazol estejam envolvidos nos efeitos tripanocidas por ligação colalente a macromoléculas (MAYA, et al., 2004). O benznidazol melhora a fagocitose e aumenta a morte de tripanossomas por intermédio do IFN-γ (ROMANHA, et al., 2002), promove ainda inibição da enzima NADH-fumarato redutase, que está presente no T. cruzi e catalisa a redução do fumarato gerando succinato. A atividade da enzima é importante na geração de energia do parasito (TURRENS, et al., 1996).

Diversos estudos relatam sobre os efeitos adverso com o uso do benznidazol, entre eles: dermatite alérgica, erupções cutâneas, edema generalizado, linfoadenopatia, artralgia, prurido, distúrbios gastrointestinais (náusea, anorexia, vômito, diarréia, cólica intestinal), anorexia, perda de peso, cefaléia, neuropatia periférica (parestesia e hipoestesia astenia ou leve tremor das mãos). A depressão da medula óssea (púrpura trombocitopênica, agranulocitose e neutropenia) é um efeito adverso raro, sendo a neutropenia a manifestação mais frequente (STOPPANI, 1999; PONTES, et al., 2010; PINAZZO, et al., 2013).

O tratamento ainda não é efetivo para as duas fases clínicas da doença, sendo eficazes para o tratamento da doença quando administrados na fase aguda, alcançando índice de cura que varia de 30-70% (GONTIJO, GALVÃO e ELOI-

SANTOS, 1999). Entretanto, em virtude da DC na fase aguda se manifestar com sintomatologia pouco específica, grande parte dos indivíduos infectados não procuram o tratamento em decorrência da dificuldade de diagnóstico e tornam-se portadores crônicos da DC (RASSI, et al., 1999); momento em que os tratamentos são pouco eficazes (URBINA, 2010). No Brasil, o SUS (Sistema Único de Saúde) disponibiliza o medicamento benznidazol após prescrição médica, seja em casos agudos ou crônicos (FIOCRUZ, 2013).

De acordo com Abdalla et al. (2008), durante a fase aguda da DC, há um estado de imunossupressão evidenciado por uma baixa resposta humoral sobre antígenos parasitários específicos e não específicos, pela inibição da proliferação e apoptose de células T (FREIRE-DE-LIMA, et al., 2006), além de liberação de mediadores inflamatórios como a prostaglandina (PINGE-FILHO, et al., 1999), possibilitando que o *T. cruzi* possa evadir-se do sistema imune. Esse estado é mediado pela prostaglandina E2 (PGE2, ABDALLA, et al., 2008). As prostaglandinas são sintetizadas a partir do ácido araquidônico por meio da ação da enzima ciclo-oxigenase (COX). A PGE2 é um potente imunomodulador, com efeitos tanto estimuladores quanto inibidores (MAYA et al., 2010).

Experimentos realizados *in vitro* demonstraram que as PGE2 induzem a imunossupressão por interferir no processo de apresentação de antígeno, inibindo a expressão de molécula de MHC de classe II, provocando inibição da produção de IL-2 e redução da ativação de linfócitos T (HARRIS, et al., 2002; DURAND et al., 2009; GANZINELLI et al., 2009).

Abdalla et al., (2008) verificaram que níveis aumentados de PGE2 estavam relacionados a maior lesão tecidual no parênquima cardíaco de animais infectados com a cepa Y do *T. cruzi* e que o tratamento com drogas inibidoras seletivas para

COX-2 foi capaz de diminuir essas lesões e contribuir no aumento da síntese de óxido nítrico.

Freire-de-Lima et al. (2000) demonstraram que a prostaglandina E2 pode estar envolvida no processo de reprodução do parasito e que, portanto, inibidores de ciclooxigenases podem afetar este processo. Tatakihara et al. (2008) demonstraram que o uso de aspirina provocou aumento de ninhos amastigotas de *T. cruzi* no músculo cardíaco de camundongos C57BL/6, uma linhagem susceptível que apresenta progressiva imunossupressão que é correlacionada com o agravo da DC (PINGE-FILHO, 1999).

Os efeitos do tratamento com anti-inflamatórios não esteroidais (AINEs) inibidores das ciclooxigenases (COX) dentre eles a aspirina (ASA), ainda não estão totalmente esclarecidos na infecção chagásica. Os efeitos da ASA demonstram ser dose-dependente, uma vez que doses baixas (25 a 50mg/kg) exercem efeito protetor para o hospedeiro e doses acima de 50mg/kg aumentam a parasitemia e a taxa de mortalidade do hospedeiro (TATAKIHARA, et al., 2008; MUKHERJEE et al., 2011; MOLINA-BERRÍOS, et al., 2013a; MOLINA-BERRÍOS, et al., 2013b). Freire de Lima et al. (2000) encontraram redução da parasitemia, enquanto outros trabalhos mostram acréscimo da mortalidade e do parasitismo, especialmente no tecido cardíaco (TATAKIHARA et al., 2008). Não há relatos sobre efeitos do tratamento com ASA sobre o plexo mientérico do intestino durante a instalação da neuropatia entérica na infecção por *T. cruzi*.

O papel dos lipídios bioativos na DC ainda é muito inexplorado e complicado pelo fato de que o hospedeiro e o parasito são as principais fontes de síntese (MUKHERJEE et al., 2011). Estudos recentes destacam avanços no conhecimento dos mecanismos de resolução inflamatória. Nesses, sugerem que as lipoxinas são

promissoras ferramentas terapêuticas para o tratamento de doenças inflamatórias (MEDEIROS, et al., 2013) e que a inibição da COX-2 e a consequente redução na produção de PGE2 com um shift metabólico para derivados de lipoxinas tem a capacidade de diminuir a parasitemia e melhorar a mortalidade de camundongos infectados com *T. cruzi* (MOLINA-BERRÍOS, et al., 2013a).

No entanto, ainda são escassos os estudos que relacionam os efeitos do tratamento com ASA para reduzir os efeitos prejudiciais provenientes da infecção por *T. cruzi* na neuropatia entérica Chagásica.

2. OBJETIVO

2.1 OBJETIVO GERAL

Avaliar o efeito de baixas doses de aspirina (ASA) sobre neurônios mientéricos do cólon durante a fase aguda (20 mg/kg) ou crônica (50 mg/kg) durante a infecção murina por *T. cruzi*.

2.1.1 OBJETIVOS ESPECÍFICOS

- Verificar se o tratamento com ASA na fase aguda ou na fase crônica da infecção provocada por *T. cruzi* é capaz de mudar o curso da infecção e proteger da desnervação a população neural mientérica;

- Avaliar se o tratamento com ASA interfere na plasticidade neuronal mientérica;

- Verificar se o protocolo experimental proposto provoca alteração na densidade celular e na morfometria das subpopulações neuronais produtoras de óxido nítrico, VIP e substância P, do plexo mientérico de camundongos infectados com *T. cruzi*.

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Running Title: Aspirin treatment and infection with *T. cruzi*

MYENTERIC NEUROPROTECTIVE ROLE OF ASPIRIN IN ACUTE AND CHRONIC EXPERIMENTAL INFECTIONS WITH *Trypanosoma cruzi*

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ABSTRACT

Background Million individuals have Chagas disease and new cases are diagnosed every year in Latin America, North America, and Europe. Experimental and clinical studies have shown that myenteric neuron cell death during infection with *Trypanosoma cruzi* mainly occurs in the esophagus and colon, resulting in megaesophagus and megacolon, respectively**.** Evidences suggest that the cyclooxygenase enzyme (COX) is involved in the *T. cruzi* invasion process. The use of low-dose aspirin (ASA), a COX-1/COX-2 inhibitor, have been shown capable to reduce infection with *T. cruzi*. Therefore, in this study, we evaluated the effects of treatment with low-dose ASA on myenteric colonic neurons during murine infection with *T. cruzi*. ***Methods*** Swiss mice were assigned into groups treated with either phosphate-buffered saline or low doses of ASA during the acute phase (20 mg/kg) and chronic phase (50 mg/kg) of infection with the Y strain of *T. cruzi*. Seventy-five days after infection, colon samples were collected to quantify inflammatory foci in histological sections and also general (myosin-V^{+}), nitrergic, and VIPergic myenteric neurons in whole mounts. Gastrointestinal transit was also measured. ***Key results*** ASA treatment during the acute phase of infection reduced parasitemia ($p < 0.05$). ASA treatment reduced the intensity of inflammatory foci in the colon, protected myenteric neurons from cell death and plastic changes, and recovered the gastrointestinal transit of mice infected with *T. cruzi* ($p < 0.05$). ***Conclusion & Inferences*** Thus, treatment with low-dose ASA can reduce the morphofunctional damage of colonic myenteric neurons caused by murine *T. cruzi* infection.

Keywords: American trypanosomiasis, Chagas disease, enteric denervation, enteric nervous system, nonsteroidal anti-inflammatory drugs.

KEY POINTS

1) Colonic motility disorders occur because of myenteric neuron death, which is caused by inflammatory processes in the digestive tract.

2) During the acute and chronic phases of experimental infection with *Trypanosoma cruzi*, administration of low doses of ASA reduced the intensity of colonic inflammatory foci, protected myenteric neurons against cell death, and recovered the gastrointestinal transit in mice.

3) The use of low doses of ASA may represent a therapeutic alternative for Chagas disease and should be evaluated in combined treatment with benznidazole.

INTRODUCTION

Approximately 7–8 million individuals have Chagas disease (American trypanosomiasis) and 50,000 new cases are diagnosed every year in Latin America, North America, and Europe[1,2]. It is estimated that more than 90 million individuals are currently at risk of infection with the Chagas disease etiologic agent (*Trypanosoma cruzi)*[2,3]. Furthermore, slightly invasive or non-invasive strains of *Trypanosoma cruzi* (CL-14 and G strains) are capable of infecting mice with gastric lesions[4], indicating that the number of oral infection cases has potential to increase.

The Chagas disease-associated morbidity and mortality occur mainly during the chronic phase, as a result of tissue damage during the acute phase[5,6]. Although many infected individuals remain asymptomatic during their lives[5,7], 30–40% develop cardiac[8], neurologic[1,5], digestive[3,8,9], or mixed complications.

Motility disorders commonly occur in patients with digestive form of Chagas disease[10,11] as a result of myenteric plexus lesions. Experimental[12,13,14] and clinical studies[10,15,16,17] have shown that myenteric neuron cell death during infection with *T. cruzi* mainly occurs in the esophagus and colon, resulting in megaesophagus and megacolon, respectively[10,17]. However, there is evidence of selective and partial

survival of nitrergic and VIPergic neurons[15,16]. There is no agreement regarding the cause of myenteric neuron death; however, the most accepted hypothesis is that it is associated with an inflammatory process induced by the presence of the parasite[3,10,18,19].

Evidence suggests that the cyclooxygenase enzyme is involved in the *T. cruzi* invasion process[20]. Thus, the use of aspirin (ASA) and celecoxib, respective COX-1/COX-2 and COX-2 inhibitors, can inhibit infection with *T. cruzi*[20,21]. The effects of ASA have been shown to be dose-dependent, as low doses (20–50 mg/kg) have a protective effect and doses above 50 mg/kg increase host parasitemia and mortality rates in mice[22,23,24,25].

The objective of this study was to evaluate the effects of low doses of ASA on colonic myenteric neurons during the acute phase (20 mg/kg) or chronic phase (50 mg/kg) of murine infection with *T. cruzi*.

MATERIALS AND METHODS

All performed procedures were approved by the Animal Ethics Committee of the State University of Londrina (Reference number: 156/2012).

Experimental groups

Sixty-day-old male Swiss mice (*Mus musculus*) weighing 25–30 g were sourced from the animal house at State University of Londrina (Brazil). Mice were kept in polypropylene boxes in groups of five individuals under standardized conditions: 21–23°C and 12-h light/dark cycle. Rodent chow (Nuvilab CR1 from Nutrient Nuvil Ltda.®, Curitiba, Brazil) and water were provided *ad libitum*.

Mice were randomly assigned into six groups (Fig. 1A). Two groups were treated 0.1 M phosphate-buffered saline (PBS, pH 7.4); the remaining four groups were treated with ASA (A2093 from Sigma-Aldrich, St. Louis, MO, USA). One PBS-treated-group and two ASA-treated-groups were infected with 1,300 blood trypomastigotes of *T. cruzi* (Y strain) via i.p.. The trypomastigotes were sourced from the Parasitology Laboratory of the Department of Basic Health Science at State University of Maringá (Brazil). To induce the chronic phase of *T. cruzi* infection, 100 mg/kg doses of benznidazole (Rochagan® from Roche Pharmaceuticals, Mannheim, Germany, patent donated to the Brazilian Ministry of Health) were administered by gavage on days 11, 13, 15, 25, 29, and 48 post infection[12] (dpi). ASA treatment was performed during the acute phase (Fig. 1B) or chronic phase (Fig. 1C) of infection with *T. cruzi*. The doses and duration of the ASA treatment in this experiment were defined for an exploratory study.

Course of experimental infection

Host parasitemia was evaluated using Brener's Method (1962)[26], counting the parasite number in 5 µL of blood extracted from the mice tail each day on days 4–15 dpi. The parasitemia curve was plotted using the average count of parasites in the infected mice.

Euthanasia and colon collection

All mice were euthanized using an euthanasia chamber with halothane vapor saturation (Tanohalo®) at 135 days of age (75 dpi). Colon extraction was performed and its length and width were measured in order to calculate total area.

Inflammatory infiltrate analysis

One centimeter distal segment of each colon was used to histological processing in order to obtain four semi-serial histological sections stained with hematoxylin-eosin. All sections were 5-µm thick and 20 µm away from each other.

For inflammatory foci evaluation, 10 microscopic field magnified by 400X were analyzed in each section (1.9 mm^2 total area). Foci were classified as absent (0–9 inflammatory cells), discrete (10–24 inflammatory cells), moderate (25–50 inflammatory cells, and intense (50+ inflammatory cells), as suggested by Michailowsky et. al.[27]. Final results were obtained by summing the number of inflammatory cells observed in each histological section.

Myenteric plexus evaluation

Immunofluorescence

Colons were washed with 0.1 M PBS (pH 7.4) and fixed using Zamboni's fixative solution for 18 h at 4°C. After washing with different concentrations of ethanol, the colons were stored in 0.1 M PBS, pH 7.4, containing 0.08% sodium azide at 4°C for microdissection using a stereomicroscope. Whole mounts containing the external muscular layer and myenteric plexus were processed according to standard immunofluorescence technique using the antibodies shown in Table 1.

Quantitative analysis

The total number of myenteric neurons immunoreactive (IR) to myosin-V (a general marker), neuronal nitric oxide synthase (nNOS), and vasoactive intestinal

polypeptide (VIP) was counted using 35 images captured with a high-definition camera (AxioCam, Carl Zeiss, Jena, Germany) coupled to a fluorescence microscope (Axioskop Plus light microscope, Carl Zeiss) using a 20X objective lens. The difference between the number of myosin-V-IR neurons and nNOS-IR neurons was used as the estimated number of cholinergic neurons as suggested by Philips et al[28]. The counting result was extrapolated to 1 cm^2.

Morphometric analysis

Three hundred myenteric cell body area (μm^2) immunoreactive to myosin-V, nNOS, and VIP of each mouse was measured using Image Pro-Plus 4.5v software (Media Cybernetics, Rockville, MD, USA). Furthermore, 700 VIPergic myenteric varicosities and 700 varicosities containing substance P (SP) of each mouse were measured using the same software.

Bright field analysis

Bright field intensity was evaluated in 60 images captured with a high-definition camera (AxioCam, Carl Zeiss) using a 40X objective lens. The image capture settings (exposure, gain, and offset) were standardized during the experiment in order to avoid external influences on image brightness. The bright field intensity was analyzed using ImageJ 1.48v software (NIH, Bethesda, MD, USA). For this purpose, the area containing the myenteric ganglion was selected. Areas of different images and the bright field intensity of each mouse were summed. The values found in each mouse were extrapolated to 1 cm^2.

Gastrointestinal transit time evaluation

The gastrointestinal transit time was evaluated by administering a non-absorbable marker (3% carmine red and 0.5% ethylcellulose) by gavage[29]. The mice were assigned into individual boxes containing food and water. The time required for elimination of the first evacuated red pellet was considered as the gastrointestinal transit time.

Statistical analysis

All data were expressed as the mean ± standard error of the mean. Data were analyzed to determine the distribution using the Shapiro-Wilk test. The Mann-Whitney test was performed to analyze parasitological parameters. For the other parameters, significant statistical differences were identified using the one-way ANOVA test, followed by Tukey's test. For this purpose, the GraphPad Prism6 software (GraphPad Software Inc. San Diego, USA) was used. The significance level was set to 5%.

RESULTS

Effects of ASA in the course of infection by *T. cruzi*

The parasitemia curve of the three groups infected with *T. cruzi* (PBS, ASA_{Early} and $ASA_{Delayed}$) showed the characteristic profile of the Y strain. ASA treatment during the acute phase (ASA_{Early}) reduced the peak parasitemia by 46% by day 8 ($p < 0.01$), 65% by day 9 ($p < 0.01$), and 75% by day 11 ($p < 0.01$) after infection, thus contributing to the reduction in total parasitemia ($p < 0.01$) (Fig. 1D).

ASA treatment reduces the number of inflammatory foci

Even after 75 dpi with *T. cruzi*, the infected mice treated with PBS showed intense ($p < 0.01$) inflammatory foci in the colonic wall. ASA treatment during the

acute and chronic phases of the infection was capable to reduce the number of inflammatory foci in the colonic wall ($p < 0.01$; Fig. 2).

Neuroprotective effect of ASA treatment during the infection with *T. cruzi*

PBS-treated-mice infected with *T. cruzi* presented intense myenteric neuron loss in the general population (60.7%; Fig. 3A–3D) and nitrergic (49.0%; Fig. 4A–4D), VIP (38.0%; Fig. 5A–5D), and cholinergic (67.0%; Fig. 6A) subpopulations. Neuronal death was dramatically reduced by ASA treatment in both the acute and chronic phases of infection ($p < 0.01$; Fig. 3A, 3E–3F, 4A, 4E–4F, 5A, 5E–5F). However, more intense death of the cholinergic population was observed (Fig. 6A). As there were no modifications in the colonic area ($p > 0.05$), the neuronal population density showed myenteric denervation in groups infected with *T. cruzi*. The high cholinergic neuronal death rate in the infected mice from the PBS group caused a significant delay in gastrointestinal transit ($p < 0.01$; Fig. 6B). ASA treatment contributed to gastrointestinal transit of the infected mice had no difference compared with the uninfected mice ($p > 0.01$; Fig. 6B). Notably, ASA treatment of uninfected mice led to a discrete (15.0%) neuronal death (Fig. 3A), mainly for the cholinergic population (Fig. 6A).

The cell body of the remaining neurons of the PBS-treated-mice infected with *T. cruzi* became hypertrophic. ASA treatment during the acute phase of infection contributed most significantly to maintaining the cell body area of the remaining myenteric neurons ($p < 0.05$; Fig. 3B, 4B, and 5B). Additionally, ASA treatment of uninfected mice led to cell body atrophy of myenteric neurons ($p < 0.05$; Fig. 3B and 5B). PBS-treated-mice infected with *T. cruzi* showed atrophied VIPergic myenteric varicosities and hypertrophied myenteric varicosities containing substance P ($p <$

0.05; Fig. 6C and 6D). Furthermore, myenteric nerve fibers showed higher bright field intensity when marked for SP in PBS-treated-mice infected with *T. cruzi* ($p < 0.05$; Fig. 6E). None of the ASA treatments contributed to restoring the balance of VIP and substance P ($p > 0.05$; Fig. 6C–6E).

DISCUSSION

This is the first study to investigate the effects of ASA treatment for colonic neuropathy caused by *T. cruzi*. Low-dose ASA treatments reduced the lesions resulting from murine infection with *T. cruzi*. It was observed lowered parasitemia, decreased inflammatory foci in the colonic wall, protection of myenteric neurons, and reestablishment of gastrointestinal transit.

The parasitemia curve observed during the acute phase of the experimental infection with *T. cruzi* leds to an intense inflammatory process with secondary lesions in several tissues[30]. Typically, the parasite does not circulate in the host's blood during the chronic phase[10,19]. Therefore, it is assumed that most tissue lesions observed in Chagas disease occur during the acute phase of infection[19]. Lower parasitemia was observed in BALB/c mice treated with 5–50 mg/kg of ASA during the acute phase of infection with *T. cruzi*[24]. However, Mukherjee et al.[23] reported an increase in parasitemia and the mortality of C57BL/6 and CH3/HeJ mice treated with 20 and 50 mg/kg of ASA during the acute phase of infection with *T. cruzi*. The results of experimental studies evaluating the treatment with nonsteroidal anti-inflammatories during the infection with *T. cruzi* are controversial. In general, these results vary according to the administrated drug[20,21,31] and its doses[23,24,25], as well as also according to the species and strains of the host[32]. Furthermore, the *T. cruzi* strain[33] may interfere with the results.

Low-dose ASA treatments for *T. cruzi*-infected mice have shown a protective effect against lesions observed during the course of infection[34]. This effect may be related to NF-κB translocation to the nucleus[35,36,37], alternative production of 15-epi-lipoxin-A_4[36], and increase in anti-inflammatory cytokines[36,38]. As a result, inflammatory mediators are reduced, which can explain the reduction of inflammatory foci found in the colonic wall of ASA_{Early}- and $ASA_{Delayed}$-infected mice groups. These findings were also observed in the heart of BALB/c mice treated with 25–50 mg/kg of ASA[24]. This inflammatory process control can contribute to a better survival rate of mice infected by *T. cruzi*[18]. Therefore, the use of low doses of ASA may represent a therapeutic alternative against the evolution of Chagas disease[20] and should be evaluated in combined treatment with benznidazole, the only trypanocidal drug available in Brazil for humans[39].

Several studies have detected intense denervation in the myenteric plexus during infection with *T. cruzi*[10,12,40,41,42], which was also observed in the infected mice colon samples used in this study. However, in a parallel study using the same mice, myenteric denervation was not found in nitrergic neurons of the esophagus[34]. Myenteric neuronal losses are consistent with clinical findings of the digestive form of Chagas disease[10,17,42]. It has been suggested that this loss is related to the inflammatory process caused by *T. cruzi*[19,41], and thus is related to parasitemia[3]. ASA_{Early} and $ASA_{Delayed}$ treatment protected the colonic myenteric neurons. Because the parasitemia curve did not change for mice treated with ASA during the chronic phase of infection, the mechanisms involved in preserving myenteric neurons with low-dose treatment with ASA may be related to reducing the inflammatory process during infection with *T. cruzi* rather to control the parasitemia.

Our results revealed higher denervation of cholinergic myenteric neurons in PBS-treated-mice infected with *T. cruzi*. These results differ from those of Da Silveira et al.[42] in chagasic humans, which showed a higher denervation of nitrergic and VIPergic myenteric neurons. However, Jabari et al.[15,16,17] observed a selective survival of nitrergic and VIPergic myenteric neurons in chagasic humans, suggesting that these types of neurons are more resistant to neuronal death provoked by Chagas disease. Nitrergic myenteric neuronal death was not observed in the esophagus of the PBS-treated-mice infected with *T. cruzi*[34]. Some authors have suggested that the survival imbalance between excitatory and inhibitory motor neurons is strongly associated to the chagasic megacolon patogeny[10,43].

Regarding the death and survival of neurons, we observed a discrete reduction in the number of neurons in uninfected mice treated with ASA. Additional studies are required to investigate whether the use of low-doses of ASA can cause myenteric neuronal death.

Studies have indicated that the relationship between reduction in myenteric neurons and modifications of contractility can determine digestive symptoms of the Chagas disease[10,41,44]. Campos et al.[19] suggested that myenteric denervation and the formation of fibrotic areas, resulting from intense inflammatory processes, may modify the smooth muscle architecture and promote modifications in the intermuscular nerve fibers, thus resulting in symptoms such as constipation. In this study, a higher cholinergic myenteric neuron loss was observed compared to that for nitrergic myenteric neurons in the PBS-treated-mice infected with *T. cruzi*, resulting in delayed gastrointestinal transit. ASA_{Early} and $ASA_{Delayed}$ treatment partially preserved myenteric neurons from cell death and thus was efficient for maintaining the gastrointestinal transit.

Regarding the myenteric neuronal morphometric, we observed that the infection with *T. cruzi* led to hypertrophy of the remaining neurons in the PBS-treated-mice. This result was similar to those of previous studies, which analyzed the myenteric plexus of humans[41] and mice[12,45] infected with *T. cruzi*. However, atrophied nitrergic myenteric neurons were found in the esophagus of the PBS-treated-mice[34]. The increase in the neuronal cell body size may be related to a morphological adaptation (neuronal plasticity) as a compensatory mechanism related to higher physiological activity and resulting in greater neurotransmitter release, such as nitric oxide (NO) and the VIP. An increased level of NO promotes over relaxation of the muscle layer and peristaltic disorders; both of these characteristics are common in Chagas disease[15,46]. VIP has been shown to have neuroprotective[47,48,49] and neuroeffector[50] roles in Chagas disease. Jabari et al.[16] suggested that VIP can protect neurons from death, despite the colonic motility being irreversibly disrupted. Only ASA_{Early} treatment was able to control the hypertrophy of the all evaluated myenteric neuronal populations in infected mice. The $ASA_{Delayed}$ treatment was not able to control the hypertrophy of the nitrergic myenteric neuronal population in infected mice. This indicates that although inflammatory process control was established by the $ASA_{Delayed}$ treatment, it was unable to restore the nitrergic myenteric neuronal area. ASA treatment minimized atrophy of the nitrergic myenteric neurons in the esophagus of PBS-treated-mice infected with *T. cruzi*[34].

Dutra et al.[51] reported that increased VIP levels and decreased SP levels induce regulatory cytokines, resulting in the indeterminate form of Chagas disease. However, a microenvironment with decreased VIP levels and increased SP levels promotes an increasing of pro-inflammatory cytokines, resulting in the various clinical forms of Chagas disease. Accordingly, larger varicosities containing SP and smaller

varicosities containing VIP were found in PBS-treated-mice infected with *T. cruzi*. Furthermore, SP-producing nerve fibers were brighter in these mice, indicating a larger amount of this neuropeptide. This suggests that PBS-treated-mice infected with *T. cruzi* showed an imbalance contributing to the development of clinical forms of Chagas disease. The VIPergic and SP varicosities behaved in opposite manners based on the results for the ASA_{Early} and $ASA_{Delayed}$ treatment. However, ASA treatment did not show a significant difference from PBS-treated-mice infected with *T. cruzi*. On the other hand, both ASA_{Early} and $ASA_{Delayed}$ treatments reduced the number of inflammatory foci in the colonic wall. Considering the role of VIP and SP in inflammatory process[49,51], the strong influence of these two neuropeptides over colonic motility[43] and their contribution to the physiopathology of Chagas disease[10,16,17,47,48,49], we suggest that ASA treatment positively influenced the balance of these two neuropeptides. This benefic effect occurred regardless of whether ASA treatment was performed during the acute or chronic phase, as treatment in both phases appeared to be effective for reducing inflammatory foci and recovering the gastrointestinal transit.

We conclude that only the ASA_{Early} treatment was capable of reducing parasitemia. Both ASA_{Early} and $ASA_{Delayed}$ treatments were capable of reducing the intensity of inflammatory foci in the colonic wall, protecting myenteric neurons against cell death and plastic changes, and recovering the gastrointestinal transit in mice infected with the Y strain of *T. cruzi*. Therefore, low-dose ASA treatment reduces myenteric neuron loss in the colon of mice infected with *T. cruzi*.

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AUTHORS CONTRIBUTIONS
JYO acquisition of data, statistical analysis, interpretation of data and drafting of the manuscript; MOB, TMC, RG, NMM & CLM acquisition of data and technical support; SMA *Trypanosoma cruzi* providing and critical revision of the manuscript for important intellectual content; DMGS study design and critical revision of the manuscript for important intellectual content; NCB antibody against mysion-V providing and critical revision of the manuscript for important intellectual content; PPF study design, critical revision of the manuscript for important intellectual content and study supervision (second supervisor); EJAA Senior author, study design, obtained funding from Araucária Foundation for Scientific and Technological Development (Brazil), critical revision of the manuscript for important intellectual content, study supervision (first supervisor); All authors approved of the final version submitted.

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Table 1. Primary and secondary antibodies used for the immunofluorescence technique.

ANTIBODY	SPECIES	DILUTION	MANUFACTURER	CODE
nNOS	Rabbit	1:1000	Santa Cruz	SC8309
VIP	Rabbit	1:200	ABCAM	AB8556
SP	Rabbit	1:1000	Millipore	AB1566
MYOSIN-V	Rabbit	1:1000	Buttow, et al.[52]	------
ALEXA FLUOR 568 ANTI-RABBIT	Donkey	1:500	Invitogen	A100042

nNOS (neuronal nitric oxide synthase); VIP (vasoactive intestinal peptide); SP (substance P).

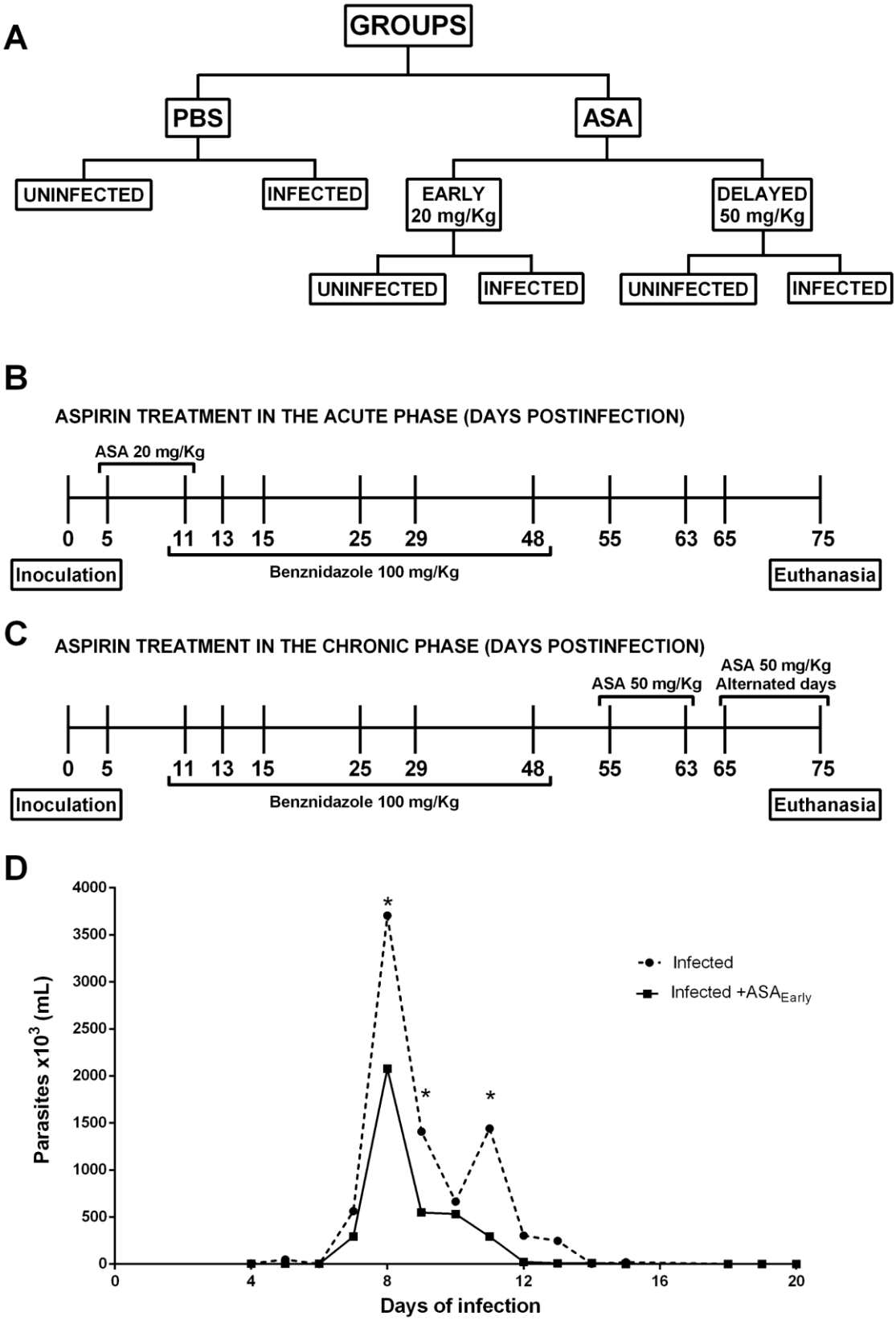

Figure 1. Flowchart showing the method used in the study. (A) Experimental groups, (B) Aspirin (ASA) treatment protocol performed during the acute phase (ASA_{Early}) of infection with *Trypanosoma cruzi*, (C) ASA treatment protocol performed during the chronic phase ($ASA_{Delayed}$) of infection with *T. cruzi*, and (D) Parasitemia curve for male Swiss mice infected with *T. cruzi* (Y strain) and treated with ASA during the acute phase (ASA_{Early}) of the infection. *$p < 0.05$ compared to the infected group (Mann-Whitney's test).

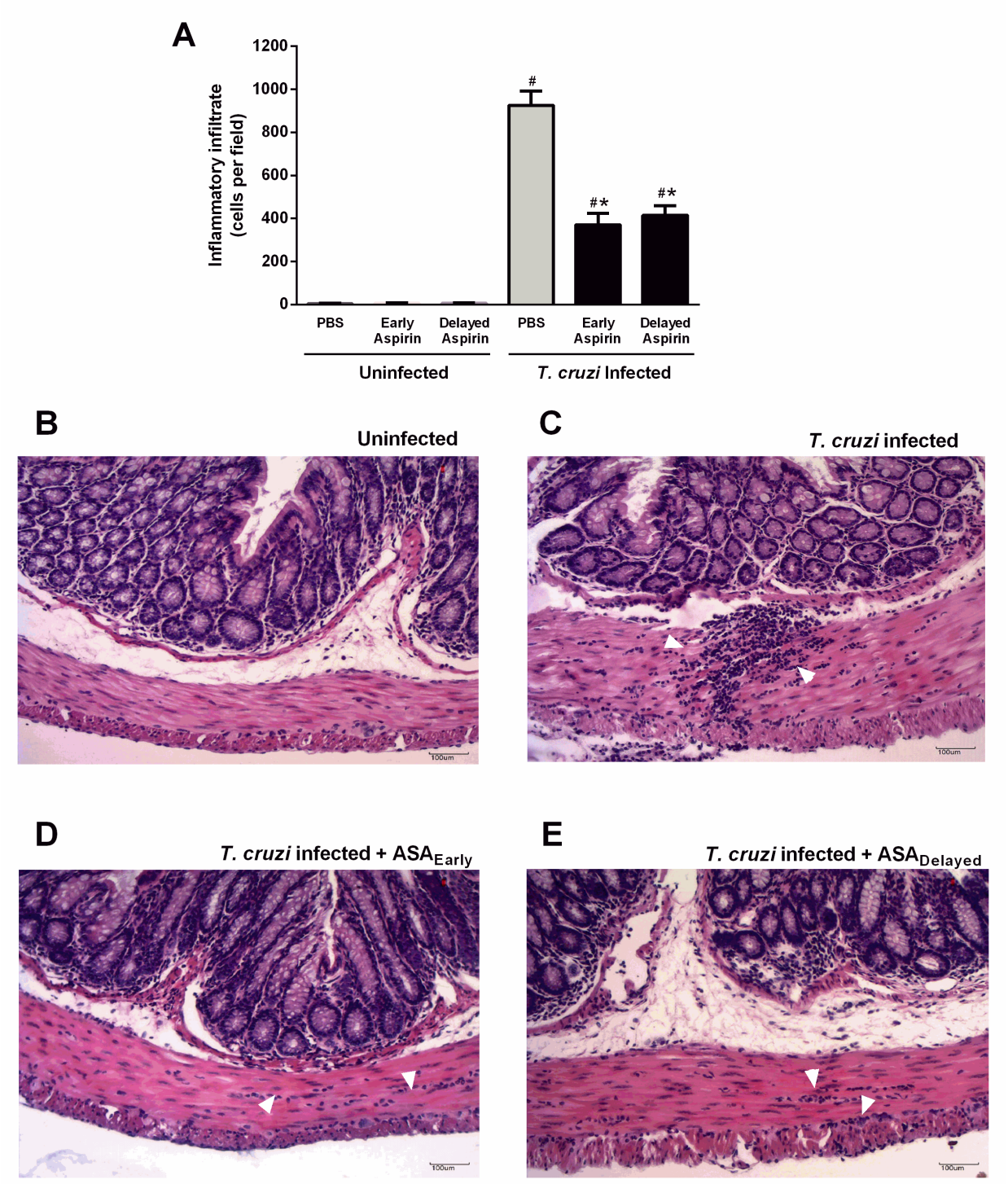

Figure 2. Evaluation of inflammatory infiltrate in uninfected mice, mice infected with *Trypanosoma cruzi* (Y strain) and treated with aspirin (ASA). (A) Quantification of inflammatory infiltrate present on the colonic wall after histopathological analysis, (B) Photomicrography showing absence of inflammatory foci in uninfected mice, (C) Intense inflammatory foci presence (see arrow head) in PBS-treated-mice infected with *T. cruzi*, (D) Reduction of inflammatory foci (see arrow head) ASA_{Early}-treated-mice infected with *T. cruzi*, (E) Reduction of inflammatory foci (see arrow head) in $ASA_{Delayed}$-treated-mice infected with *T. cruzi*. # $p < 0.05$ compared to uninfected PBS-treated-group. *$p < 0.05$ compared to infected PBS-treated-group. ANOVA one-way, followed by Tukey's post-test.

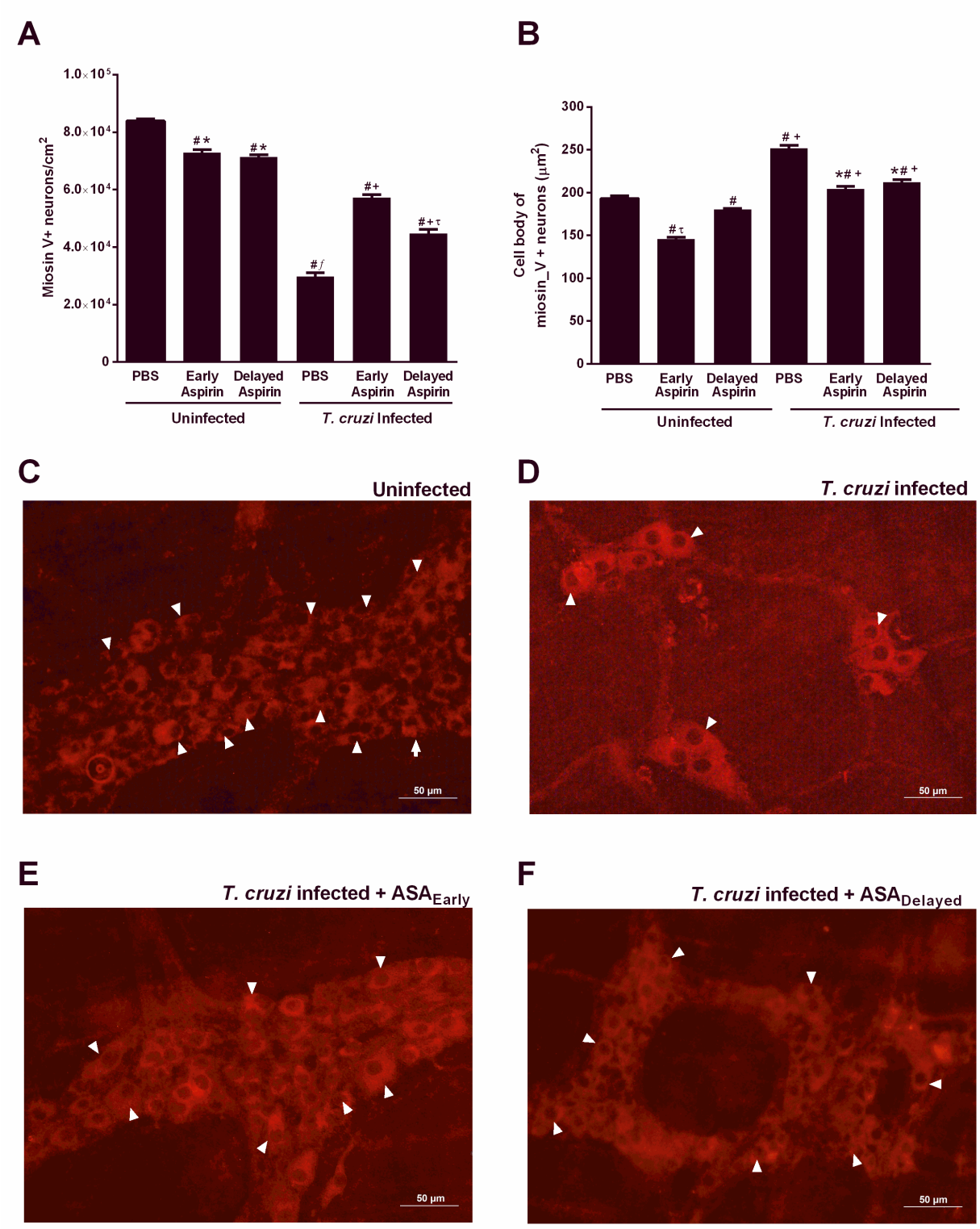

Figure 3. Morphoquantitative and morphometric evaluation of myosin-V$^+$ myenteric neurons of uninfected mice, mice infected with *Trypanosoma cruzi* (Y strain) and mice treated with aspirin (ASA). (A) General myenteric neuronal population density (myosin- V$^+$), (B) Cell body area of the general myenteric neuronal population, (C) to (F) Immunofluorescence photomicrographies showing myenteric neurons (see arrow heads) in the colon of uninfected mice, infected with *T. cruzi* (Y strain) and treated with ASA during the acute (ASA_{Early}) or chronic ($ASA_{Delayed}$) phases of the infection. $^{\#}p < 0.05$ compared to the uninfected PBS-treated-group. *p < 0.05 compared to the infected PBS-treated-group. $^{+}p < 0.05$ compared to uninfected ASA-treated-groups. $^{f}p < 0.05$ compared to infected ASA-treated-groups. $^{\tau}p < 0.05$ comparation between the two ASA-treated-groups (uninfected or infected). ANOVA one-way followed by Tukey's post-test.

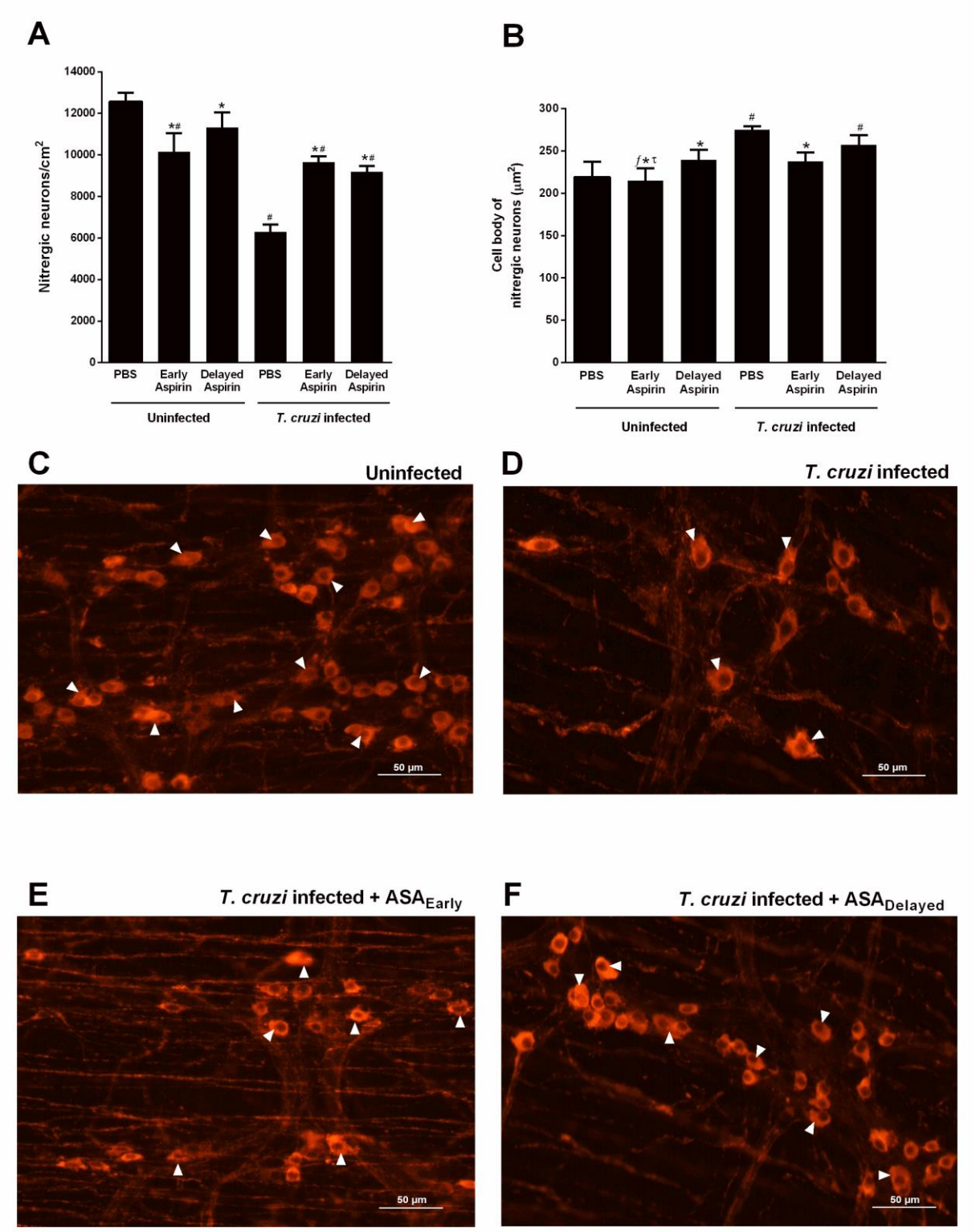

Figure 4. Morphoquantitative and morphometric evaluation of nitrergic myenteric neurons of uninfected mice, mice infected with *Trypanosoma cruzi* (Y strain) and mice treated with aspirin (ASA). (A) Nitrergic myenteric neuronal population density, (B) Cell body area of the nitrergic myenteric neuronal population, (C) to (F) Immunofluorescence photomicrographies showing myenteric neurons (see arrow heads) in the colon of uninfected mice, infected with *T. cruzi* (Y strain) and treated with ASA during the acute (ASA_{Early}) or chronic ($ASA_{Delayed}$) phases of the infection. $^{\#}p < 0.05$ compared to the uninfected PBS-treated-group. *$p < 0.05$ compared to the infected PBS-treated-group. $^{+}p < 0.05$ compared to uninfected ASA-treated-groups. $^{\int}p < 0.05$ compared to infected ASA-treated-groups. $^{\tau}p < 0.05$ comparation between the two ASA-treated-groups (uninfected or infected). ANOVA one-way followed by Tukey's post-test.

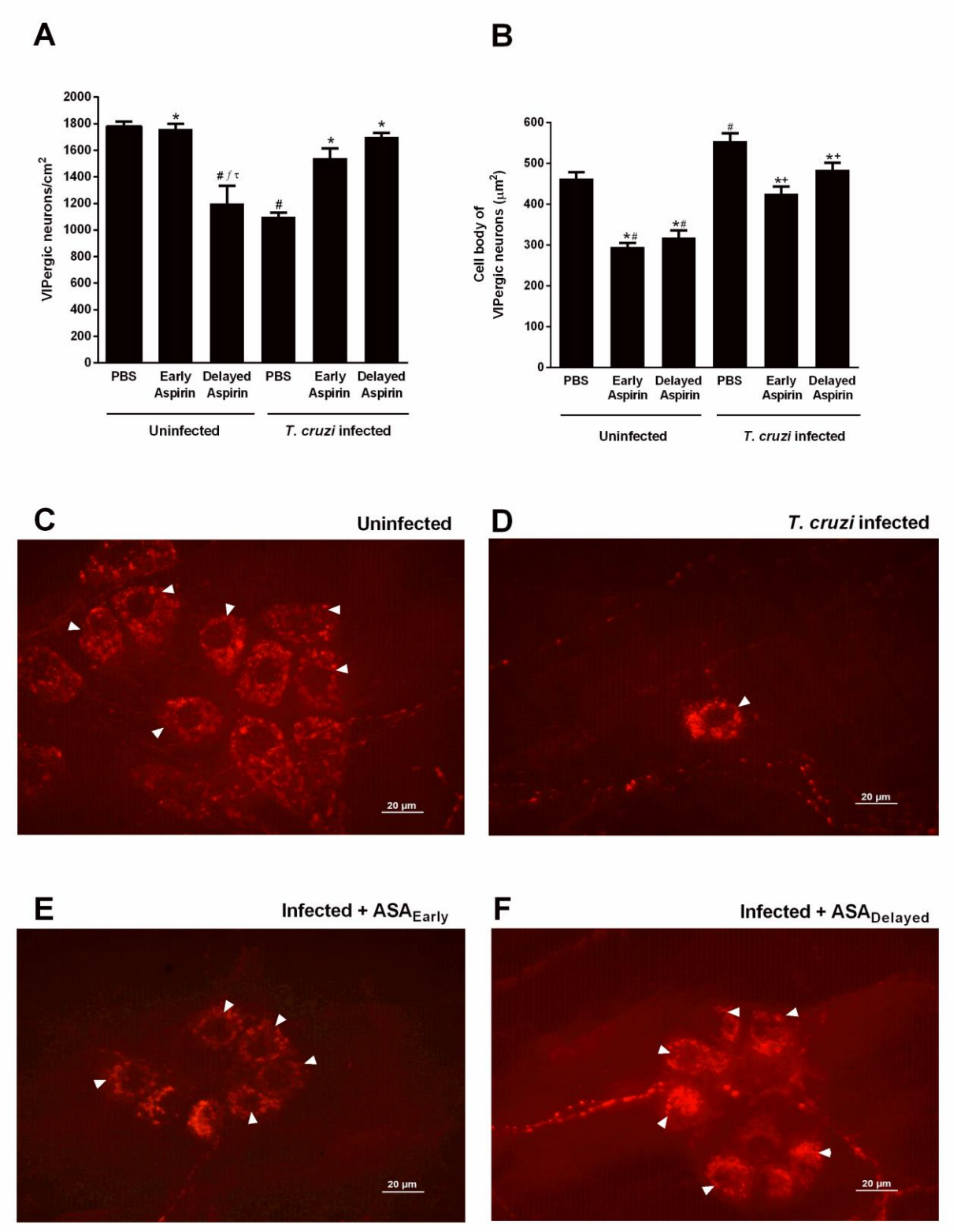

Figure 5. Morphoquantitative and morphometric evaluation of VIPergic myenteric neurons of uninfected mice, mice infected with *Trypanosoma cruzi* (Y strain) and mice treated with aspirin (ASA). (A) VIPergic myenteric neuronal population density, (B) Cell body area of the VIPergic myenteric neuronal population, (C) to (F) Immunofluorescence photomicrographies showing myenteric neurons (see arrow heads) in the colon of uninfected mice, infected with *T. cruzi* (Y strain) and treated with ASA during the acute (ASA_{Early}) or chronic ($ASA_{Delayed}$) phases of the infection. $^{\#}p < 0.05$ compared to the uninfected PBS-treated-group. *$p < 0.05$ compared to the infected PBS-treated-group. $^{+}p < 0.05$ compared to uninfected ASA-treated-groups. $^{\int}p < 0.05$ compared to infected ASA-treated-groups. $^{\intercal}p < 0.05$ comparation between the two ASA-treated-groups (uninfected or infected). ANOVA one-way followed by Tukey's post-test.

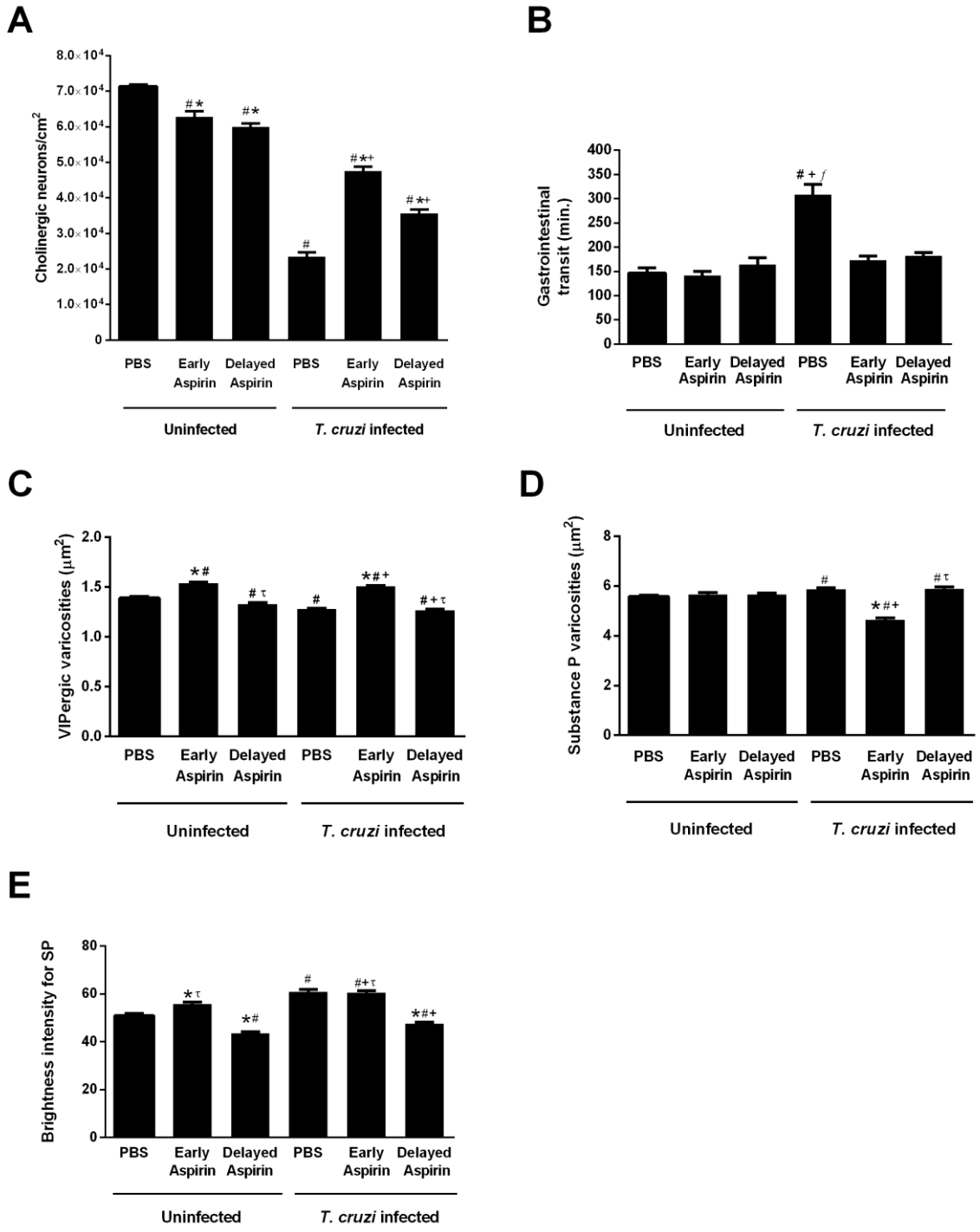

Figure 6. Morphoquantitative, morphometric, and physiological evaluation of myenteric neurons of uninfected mice, mice infected with *Trypanosoma cruzi* (Y strain) and mice treated with aspirin (ASA). (A) Cholinergic myenteric neuron population density, (B) Gastrointestinal transit evaluation, (C) to (D) Varicosities area of VIP and substance P (SP) producing nerve fibers, (E) Bright field intensity of varicosities containing SP. $^{\#}p < 0.05$ compared to the uninfected PBS-treated-group. $^{*}p < 0.05$ compared to the infected PBS-treated-group. $^{+}p < 0.05$ compared to uninfected ASA-treated-groups. $^{f}p < 0.05$ compared to infected ASA-treated-groups. $^{\tau}p < 0.05$ comparation between the two ASA-treated-groups (uninfected or infected). ANOVA one-way followed by Tukey's post-test.

CONCLUSÃO

Baseados na hipótese de que o tratamento com aspirina em baixas doses seria eficiente para controle da neuropatia colônica induzida pela infecção com *T. cruzi*, em camundongos, e que os efeitos terapêuticos seriam benéficos para os neurônios do plexo mientérico tornou-se um guia para a realização deste trabalho experimental. Conclui-se, portanto, que a hipótese inicial era verdadeira.

O tratamento com ASA na dose de 20 mg/kg durante a fase aguda da infecção por *T. cruzi* foi capaz de reduzir significativamente a parasitemia, fato este não observado no grupo $ASA_{Delayed}$. Devido a esse efeito antiparasitário demonstrado no grupo ASA_{Early}, foi possível verificar uma relação entre a carga parasitária e as lesões que se manifestam na fase crônica da infecção: redução na quantidade de focos inflamatórios; preservação estrutural e quantitativa dos neurônios do plexo mientérico colônico e restabelecimento da função intestinal. Como os camundongos do grupo $ASA_{Delayed}$ não tiveram alteração nos parâmetros parasitológicos, pode-se inferir que os benefícios terapêuticos oriundos da administração de ASA, possivelmente podem estar relacionados com a redução do processo inflamatório.

Desse modo, o tratamento com ASA mostrou-se eficaz para reduzir as alterações teciduais e celulares que acompanham as manifestações clínicas da DC. Entretanto, benefícios maiores são adquiridos quando o tratamento ocorre precocemente, nos períodos iniciais do processo infeccioso. Portanto, sugere-se a realização de trabalhos que possuam um maior tempo de tratamento com ASA, iniciando na fase aguda da infecção, bem como a inclusão de métodos fisiológicos como a manometria que possam avaliar as alterações da motilidade do cólon.

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ANEXOS

A
Aprovação da Comissão de Ética no Uso de Animais da Universidade Estadual de Londrina (CEUA/UEL) protocolo 156/2012 e processo 7371.2012.56.

Universidade
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COMISSÃO DE ÉTICA NO USO DE ANIMAIS

OF. CIRC. CEUA Nº 156/12 **Londrina, 25 de Junho de 2012**

Prezada Pesquisadora,

A CEUA/UEL, reunida em 19 de Junho de 2012, reavaliou o projeto de pesquisa intitulado "*Análise dos efeitos do tratamento com aspirina sobre o desenvolvimento de neuropatia entérica de camundongos infectados com Trypanosoma Cruzi*", processo registrado na CEUA sob nº 7371.2012.56, pesquisa do Centro de Ciências Biológicas, desenvolvido sob sua responsabilidade. Esclarecidos os aspectos metodológicos solicitados, o projeto está aprovado para execução entendendo-se que os princípios éticos postulados pelo Conselho Nacional de Controle da Experimentação Animal estão respeitados.

Serão utilizados 192 camundongos da raça Suíços com idade de 60 dias, machos, com procedência do Biotério Central CCB-UEL. Neste projeto será avaliado se o uso de aspirina, uma droga antiinflamatória não esteroidal (AINES) convencional, durante a fase aguda da infecção experimental causada por *Trypanosoma cruzy* (cepa y) interfere no desenvolvimento da neuropatia entérica chagásica observada na fase crônica da infecção. Para isto camundongos que não serão infectados serão tratado com PBS sem aspirina (G1), camundongos não infectados será tratado com a Aspirina (G2), camundongos infectados serão tratados com PBS (G3) e camundongos infectados serão tratados com aspirina (G4). Os camundongos receberão *T. cruzi* para indução de uma fase crônica e serão tratados com Benznidazol nos dias 11, 16 e 18 após a infecção. O tratamento com a aspirina será iniciado no 20º dia após a infecção, sendo o tratamento diário e 75 dia após a infecção os camundongos serão eutanasiados. Os colóns dos animais serão coletados e analisados quanto a quantidade de neurônios mioentéricos, também será realizada a análise morfométrica dos neurônios e dosagem de citocinas. O projeto está previsto para ser desenvolvido em 24 meses

Cumpre orientar que caso pretendam-se quaisquer alterações no protocolo experimental aprovado, deve-se submeter o novo protocolo à apreciação da CEUA/UEL anteriormente à execução das modificações. Sem mais para o momento, subscrevo-me. Cordialmente,

Waldiceu Cp.° Verri Junior
Prof. Dr. Waldiceu Aparecido Verri Junior
Coordenador da CEUA/UEL

Ilmo. Sr.
Prof. Dr. Eduardo José de Almeida Araújo
Coordenadora do Projeto
Departamento de Histologia
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Com cópia para Srª Égle Maria de Sousa (Chefe da DCA/PROPPG) e Prof. Luiz Carlos Juliani (Diretor do Biotério Central da UEL).

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