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BENZNIDAZOL LIVRE E NANOESTRUTURADO NA TERAPIA DA DOENÇA DE CHAGAS EXPERIMENTAL E SEUS EFEITOS SOBRE BIOMARCADORES DO SISTEMA COLINÉRGICO E ESTRESSE OXIDATIVO

Santa Maria, RS
2022

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Tese apresentada ao Curso de Doutorado em Ciências Biológicas: Bioquímica Toxicológica (PPGBTox), Área de Ciências Naturais e Exatas, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do título de **Doutora em Ciências Biológicas: Bioquímica Toxicológica.**

Orientador: Prof. Dr. Aleksandro Schafer da Silva
Coorientadora: Profª. Drª. Maria Rosa Chitolina

Santa Maria, RS
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Aniélen Dutra da Silva

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“O educador se eterniza em cada ser que ele educa.”

Paulo Freire

RESUMO

EFEITOS DO BENZNIDAZOL LIVRE E NANOESTRUTURADO SOBRE O SISTEMA COLINÉRGICO E ESTRESSE OXIDATIVO EM UM MODELO EXPERIMENTAL DE DOENÇA DE CHAGAS

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A doença de Chagas (DC) apresenta ampla distribuição na América Latina, sendo ocasionada pelo *Trypanosoma cruzi*. A infecção promove um processo inflamatório, que desencadeia diversos fatores da resposta imunológica. O sistema colinérgico atua na modulação da resposta inflamatória, através da via colinérgica anti-inflamatória. No Brasil, o tratamento da DC é feito com o fármaco benznidazol (BNZ), o qual apresenta efeitos adversos e alta toxicidade. A utilização da biotecnologia é uma aliada na busca por estratégias no desenvolvimento de formas de administração de medicamentos com maior eficácia e menos prejuízos para o organismo. O objetivo desse estudo foi compreender a participação do sistema colinérgico na patogenia da doença e trazer novas possibilidades no tratamento, bem como interações entre tratamentos e sinalização colinérgica na regulação imune. Foram realizados três protocolos experimentais, através dos quais analisou-se a atuação do sistema colinérgico durante a DC, testes de eficácia do fármaco livre (BNZ) e nanoestruturado (NBNZ) em diferentes doses, além de avaliar os tratamentos na terapia DC e seus efeitos sobre parâmetros hematológicos, bioquímicos, de estresse oxidativo, patológico e no sistema colinérgico. Foi possível demonstrar que o sistema colinérgico pode estar envolvido na imunomodulação durante a infecção, visto que houve uma redução na atividade da acetilcolinesterase no sistema nervoso central, bem como um aumento dos níveis de acetilcolina, caracterizando uma resposta anti-inflamatória. Demonstrou-se a eficiência do processo de nanoencapsulação do benznidazol na terapia de DC. A dose 20 mg/kg NBNZ apresentou uma redução significativa da parasitemia, similar ao fármaco livre. Além disso, baixa citotoxicidade, redução de danos teciduais e aumento da taxa de sobrevivência em comparação as demais doses de nanocápsulas (5mg/kg, 10mg/kg, 15mg/kg). Sendo assim, a dose 20 mg/kg NBNZ demonstrou melhor desempenho, nas análises realizadas. Ainda foi possível observar que o processo de nanoencapsulação do BNZ, apesar de promover redução da parasitemia, não demonstrou eficácia maior que o tratamento com BNZ. A infecção por *T. cruzi* ocasionou um quadro de anemia, leucopenia e trombocitopenia nos animais, porém a tratamento com BNZ foi capaz de evitar essa alteração. Por outro lado, todos os grupos de tratamento infectados apresentaram alterações nos marcadores bioquímicos de dano hepático. A avaliação da via colinérgica anti-inflamatória mostrou-se em um perfil pró-inflamatório através da avaliação da atividade da AChE, expressão da AChE, e dos receptores M1 e M2 mAChR em linfócitos, esse perfil foi observado no grupo infectado e tratado com BNZ, demonstrando que o fármaco atua estimulando essa via de sinalização pró-inflamatória. Ao avaliar o status oxidante/antioxidante, observou-se um padrão pró-oxidante nos tecidos avaliados, principalmente nos níveis de EROs e NOx dos grupos tratados, moléculas importantes no controle de proliferação do parasito na fase aguda da doença. De modo geral, concluímos que a nanoencapsulação não potencializou a eficácia terapêutica do benznidazol, mas minimizou alterações patológicas causadas pela infecção por *T. cruzi* e que o sistema colinérgico apresenta-

se como um alvo farmacológico potencial para o controle do processo inflamatório e da evolução dos danos ocasionados pela infecção.

Palavras-chave: Doença de Chagas, acetilcolinesterase, estresse oxidativo, nanotecnologia, benznidazol.

ABSTRACT

EFFECTS OF FREE AND NANOSTRUCTURED BENZNIDAZOLE ON THE CHOLINERGIC SYSTEM AND OXIDATIVE STRESS IN AN EXPERIMENTAL MODEL OF CHAGAS DISEASE

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Chagas disease (CD) is widely distributed in Latin America, caused by *Trypanosoma cruzi*. The infection promotes an inflammatory process, which triggers several factors of the immune response. The cholinergic system acts in the modulation of the inflammatory response, through the anti-inflammatory cholinergic pathway. In Brazil, the treatment of CD is done with the drug benznidazole (BNZ), which has adverse effects and high toxicity. The use of biotechnology is an ally in the search for strategies in the development of forms of drug administration with greater efficiency and less damage to the body. The objective of this study was to understand the participation of the cholinergic system in the pathogenesis of the disease and bring new possibilities in the treatment, as well as interactions between treatments and cholinergic signaling in immune regulation. Three experimental protocols were carried out, through which the performance of the cholinergic system during CD, efficacy tests of free drug (BNZ) and nanostructured (NBNZ) at different doses were analyzed, in addition to evaluating treatments in CD therapy and their effects. on hematological, biochemical, oxidative and pathological stress parameters and on the cholinergic system. It was possible to demonstrate that the cholinergic system may be involved in immunomodulation during infection, as there was a reduction in acetylcholinesterase activity in the central nervous system, as well as an increase in acetylcholine levels, characterizing an anti-inflammatory response. The efficiency of the benznidazole nanoencapsulation process in CD therapy was demonstrated. The 20 mg/kg NBNZ dose showed a significant reduction in parasitemia, similar to the free drug. In addition, low cytotoxicity, reduced tissue damage and increased survival rate compared to other doses of nanocapsules (5mg/kg, 10mg/kg, 15mg/kg). Thus, the 20 mg/kg NBNZ dose showed better performance in the analyzes performed. It was also possible to observe that the BNZ nanoencapsulation process, despite promoting a reduction in parasitemia, did not demonstrate greater efficacy than the treatment with BNZ. *T. cruzi* infection caused anemia, leukopenia and thrombocytopenia in the animals, but treatment with BNZ was able to prevent this change. On the other hand, all infected treatment groups showed changes in biochemical markers of liver damage. The evaluation of the anti-inflammatory cholinergic pathway showed a pro-inflammatory profile through the evaluation of AChE activity, AChE expression, and M1 and M2 mAChR receptors in lymphocytes, this profile was observed in the infected group treated with BNZ, demonstrating that the drug acts by stimulating this pro-inflammatory signaling pathway. When evaluating the oxidant/antioxidant status, a pro-oxidant pattern was observed in the evaluated tissues, mainly in the levels of ROS and NOx of the treated groups, important molecules in the control of parasite proliferation in the acute phase of the disease. In general, we concluded that nanoencapsulation did not potentiate the therapeutic efficacy of benznidazole, but minimized pathological changes caused by *T. cruzi*

infection and that the cholinergic system presents itself as a potential pharmacological target for the control of the inflammatory process and the evolution of damage caused by the infection.

Keywords: Chagas disease, acetylcholinesterase, oxidative stress, nanotechnology, benznidazole.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

Acetilcoenzima A	Acetil-CoA
Acetilcolina	ACh
Acetilcolinesterase	AChE
Benznidazol	BNZ
Catalase	CAT
Colina acetiltransferase	ChAT
Doença de Chagas	DC
Espécies reativas de nitrogênio	ERNs
Espécies reativas de oxigênio	EROs
Eudragit L100	EL-100
Fator de necrose tumoral α	TNF-α
Glutationa peroxidase	GPx
Glutationa redutase	GSR
Interferon γ	IFN-γ
Interleucina	IL
Nanocápsula de benznidazol	NBNZ
Organização Mundial da Saúde	OMS
Receptores acopladas a proteína G	GPCRs
Receptores muscarínicos	mAChR
Receptores nicotínicos	nAChR
Receptores *Toll-like*	TLRs
Sistema Nervoso Central	SNC
Superóxido dismutase	SOD

SUMÁRIO

1. INTRODUÇÃO

A doença de Chagas (DC) é uma antropozoonose de ampla distribuição na América Latina. Cerca de 7 milhões de pessoas estão infectadas pelo *Trypanosoma cruzi* em regiões endêmicas, apresentando risco de desenvolver a forma crônica da doença (WHO, 2012). Em algumas regiões do Brasil, estudos epidemiológicos buscam esclarecer o perfil de infectados por *T. cruzi*, os principais meios de infecção e a possível relação com a presença do inseto vetor da DC. As principais características dos grupos acometidos pela doença foram relacionadas, por uma análise de soro prevalência de DC na região nordeste do país, com baixa renda, baixa escolaridade, condições de trabalho precárias, as quais propiciam a ocorrência de infecções através do vetor transmissor. Grande parte dos participantes da pesquisa foram capazes de reconhecer facilmente o triatomíneo vetor da infecção. Além disso, alguns indivíduos com sorologia positiva para DC relatam ter realizado doações sanguíneas recentes, porém sem conhecimento da destinação dessas doações ou descarte das mesmas (FREITAS *et al.*, 2017).

O *T. cruzi* é um patógeno intracelular obrigatório (LOPES *et al.*, 2015). Possui um ciclo evolutivo complexo que compreende diferentes estágios de desenvolvimento. Entre suas formas estão a forma epimastigota e tripomastigota metacíclica infectante, presentes no inseto vetor e as formas amastigotas e tripomastigota sanguícola, presentes no hospedeiro vertebrado (TANOWITZ *et al.*, 1992). A forma de amastigota pode ser encontrada em células musculares (TEIXEIRA *et al.*, 2011), podendo permanecer na forma dormente por muito tempo, sem causar danos para o hospedeiro (TEIXEIRA *et al.*, 2006; 2011). A forma tripomastigota do *T. cruzi* infecta principalmente macrófagos, fibroblastos e tecido muscular (TEIXEIRA *et al.*, 2011). Ao invadir as células, a forma tripomastigota é englobada em um vacúolo lisossomal, porém é capaz de sair do ambiente ácido desse e originar a forma amastigota no citoplasma, onde irá replicar-se (TOMLINSON *et al.*, 1995; ANDRADE, ANDREWS, 2005). A ocorrência desse processo pode se dar durante a fase S do ciclo celular, na qual há um consumo maior de glicose e energia, desencadeando vias de sinalização das MAPK, o que resulta no crescimento e diferenciação celular (TEIXEIRA *et al.*, 2011).

A infecção pelo parasito pode ocorrer através do contato com as excretas do triatomídeo contaminado por *T. cruzi*, por acidentes laboratoriais, transmissões congênitas, transfusões sanguíneas, transplantes de órgãos e contaminação oral através da ingestão do parasito (FERREIRA *et al.*, 2011). Os processos de doações de sangue podem ser considerados um meio

de infecção pelo parasito, principalmente quando os testes para a detecção do mesmo não são sensíveis o bastante para identificar a infecção, mesmo em condições de baixa ou nenhuma detecção de parasitemia sanguínea (CARDOSO *et al.*, 2018). Recentemente, alguns processos de infecção oral foram documentados no Brasil, no Rio Grande do Norte, os quais ocorreram através da ingestão de caldo de cana-de-açúcar contaminado por *T. cruzi*. Na fazenda onde ocorria o processo de moagem da cana foram encontrados exemplares de *Triatoma brasiliensis*, compreendendo a maioria dos triatomídeos encontrados, com alto índice de infecção natural pelo parasito (VARGAS *et al.*, 2018). O estado do Pará é uma região com alto risco de contaminação oral, já se demonstrou o potencial da infecção oral, nessa região, através da polpa do açaí. A contaminação pode ocorrer durante o processamento da fruta sem higienização adequada, entre outros fatores (BARBOSA *et al.*, 2018).

Durante a infecção intracelular pelo parasito, ocorre o desencadeamento de uma resposta imune inata, com a liberação de citocinas pró inflamatórias, as quais poderão induzir danos em neurônios periféricos entéricos e cardíacos, por exemplo (CHUENKOVA, PERRIN, 2011). Esse dano ao sistema nervoso periférico está relacionado a altas concentrações de óxido nítrico (NOO) produzidas, na tentativa de combater o parasito, levando a danos nas células (KNOTT, BOSSY-WETZEL, 2009; ALIBERTI *et al.*, 2001; GU *et al.*, 2010). Durante a fase aguda é possível detectar a presença do parasito flagelado no sangue ou fluído cerebrospinal. (DIAZ-GRANADOS *et al.*, 2009). Alguns pacientes apresentam sintomas durante a fase aguda da doença, entre 10 a 60 dias após a infecção. Durante essa fase há a ocorrência de febre, edema ocular e facial, conjuntivite, entre outros (PITTELLA, 1993). Alguns pacientes imunossuprimidos, como portadores do vírus da imunodeficiência adquirida humana (HIV), podem apresentar abcessos granulomatosos cerebrais (MORETTA *et al.*, 2009).

O início rápido do tratamento é muito importante, pois grande parte dos pacientes apresentam a parasitemia negativa após o quinto dia de tratamento por via oral, tendo uma continuidade desse por 60 a 90 dias (CORDOVA *et al.*, 2010). Muitos pacientes não apresentam sintomas, mesmo sem tratamento. Porém, a realização do mesmo representa a principal chance de cura do paciente na fase aguda e de evitar transição para a fase crônica. Pacientes na fase crônica apresentam sorologia positiva, mas a doença clínica pode não aparecer em até 30 anos pós-infecção, isto é, o surgimento dos primeiros sintomas cardíacos ou digestivos nos pacientes só ocorrem com o envelhecimento do indivíduo (WHO, 2011).

A infecção por *T. cruzi* leva a um aumento na formação de espécies reativas de oxigênio (EROs), isso ocorre em função do estímulo de mediadores inflamatórios, como as citocinas e quimiocinas, o que resulta no estresse oxidativo em células fagocitárias (GUPTA *et al.*, 2009). Proteínas, lipídios e DNA podem sofrer a ação de potentes agentes oxidantes, o que leva a um dano estrutural e até a morte das células (WEN *et al.*, 2004). Na DC o estresse oxidativo é considerado um mecanismo de defesa em relação ao parasito na fase aguda da infecção, mas que contribui para o consequente desenvolvimento de danos teciduais (WEN *et al.*, 2004; ZACKS *et al.*, 2005).

O estresse oxidativo e a inflamação ocasionam sérias lesões ao organismo do hospedeiro. O sistema colinérgico está, notavelmente, envolvido em reações anti-inflamatórias (BOROVIKOVA *et al.*, 2000). A células imunológicas possuem um sistema colinérgico completo, composto de acetilcolina (ACh), colina acetiltransferase (ChAT), receptores nicotínicos e muscarínicos (nAChR e mAChR) e acetilcolinesterase (AChE) (KAWASHIMA, FUJII, 2003; TAYEBATI *et al.*, 2002). De acordo com literatura, a via colinérgica anti-inflamatória pode mediar interações neuro-inflamatórias, visto que o sistema nervoso é o maior produtor de ACh, podendo ainda atuar como um regulador interno da resposta imune (BOROVIKOVA *et al.*, 2000)

A ACh atua como um neurotransmissor, tanto no sistema nervoso central como no sistema nervoso periférico (TUCEK, 1988). Além disso, essa molécula está envolvida na regulação de funções imunológicas (KAWASHIMA, FUJII, 2003), apresentando propriedades anti-inflamatórias (SOREQ, SEIDMAN, 2001; DAS, 2007). As enzimas colinesterases estão presentes em tecidos colinérgicos e não colinérgicos. A Acetilcolinesterase (AChE) é uma enzima ligada à membrana, encontrada no cérebro, músculos, eritrócitos, linfócitos e neurônios colinérgicos (SCHETINGER *et al.*, 2000; KAWASHIMA, FUJII, 2003). Por outro lado, a enzima butirilcolinesterase (BChE: EC 3.1.1.8) está presente no intestino, fígado, rins, coração, pulmões e soro (ECOBICON, CORNEAU, 1973). Apesar de hidrolisar ésteres como butirilcolina, a BChE pode atuar na degradação da ACh quando houver uma situação de inibição ou ausência da AChE (TAYLOR, BROWN, 1999; LI *et al.*, 2000). Trabalhos na literatura já demonstram a ocorrência de alterações na atividade das enzimas acetiltransferase e AChE relacionadas a tripanossomíases, ocasionadas por *T. cruzi* e *Trypanosoma evansi* (RODRIGUES *et al.*, 2005; DA SILVA *et al.*, 2010).

O estudo da fase aguda da infecção induzida por *T. cruzi* pode ser de grande importância, considerando que diferentes autores sugerem que muitas lesões tardias são influenciadas pelo curso da doença durante fase aguda. A DC pode ser tratada com benznidazol e nifurtimox (BRASIL, 2009), e ambos os medicamentos são quase 100% eficazes na cura da doença (FERREIRA, 1988; OMS, 2015), se administrados no início da fase aguda. No entanto, na fase crônica as chances de cura são de aproximadamente 8% (FERREIRA, 1990).

O benznidazol consiste no mesmo fármaco de escolha desde 1976, tendo apenas novas dosagens de apresentações dos comprimidos, bem como ajustes de doses administradas. Esse fármaco é bem tolerado pelos pacientes infectados quando comparando ao nifurtimox, mas ambos causam efeitos adversos com o uso prolongado. O fármaco é administrado na fase aguda da doença, sendo rapidamente absorvido no trato gastrointestinal e metabolizado no fígado pelo sistema citocromo P450 (MAYA *et al.*, 2007). O benznidazol apresenta a seguinte recomendação de dosagem para pacientes com idade superior a 12 anos de 5,0 a 7,0 mg/kg de peso corporal/dia; para pacientes menores de 12 anos 5 a 7,5 mg/kg de peso corporal/dia. A administração deve ser subdividida em duas a três doses diárias, durante 60 dias (COURA, 2009). Essa recomendação é para minimizar efeitos de toxicidade desse fármaco, tais como alterações neurológicas, gástricas, dermatológicas e hematológicas (MAYA *et al.*, 2007; LIMA *et al.*, 2011; WHO, 2015)

Nesse sentido, a utilização de nanoestruturas, visando um melhoramento na disponibilidade do fármaco, bem como uma redução de concentração utilizada, pode trazer benefícios ao tratamento. As nanopartículas têm sido utilizadas em diversos estudos com parasitoses, com resultados promissores tanto para redução da concentração de fármacos administrados, como para associações de tratamentos. Além disso, diferentes formulações permitem um ajuste de doses administradas (BOTTARI *et al.*, 2015; PALMEIRO-ROLDÁN *et al.*, 2014; SALOMON, 2011; ROMERO, MORILLA, 2010). Palmeiro-Roldán e colaboradores (2014) demonstraram uma melhora na eficácia do tratamento com benznidazol, quando o mesmo foi disponibilizado em dispersão sólida, reduzindo a parasitemia e apresentando uma liberação mais lenta do mesmo. Considerando a necessidade contínua de se elucidar novos mecanismos relacionados a patogenia e controle imunológico da DC, bem como a busca por formulações mais eficientes do medicamento e menos tóxicas, é de extrema importância a realização de estudos com esse enfoque, promovendo ampliação de conhecimentos acerca de mecanismos envolvidos na doença e a contínua melhora nas opções de tratamento da DC.

1.1 Revisão da Literatura

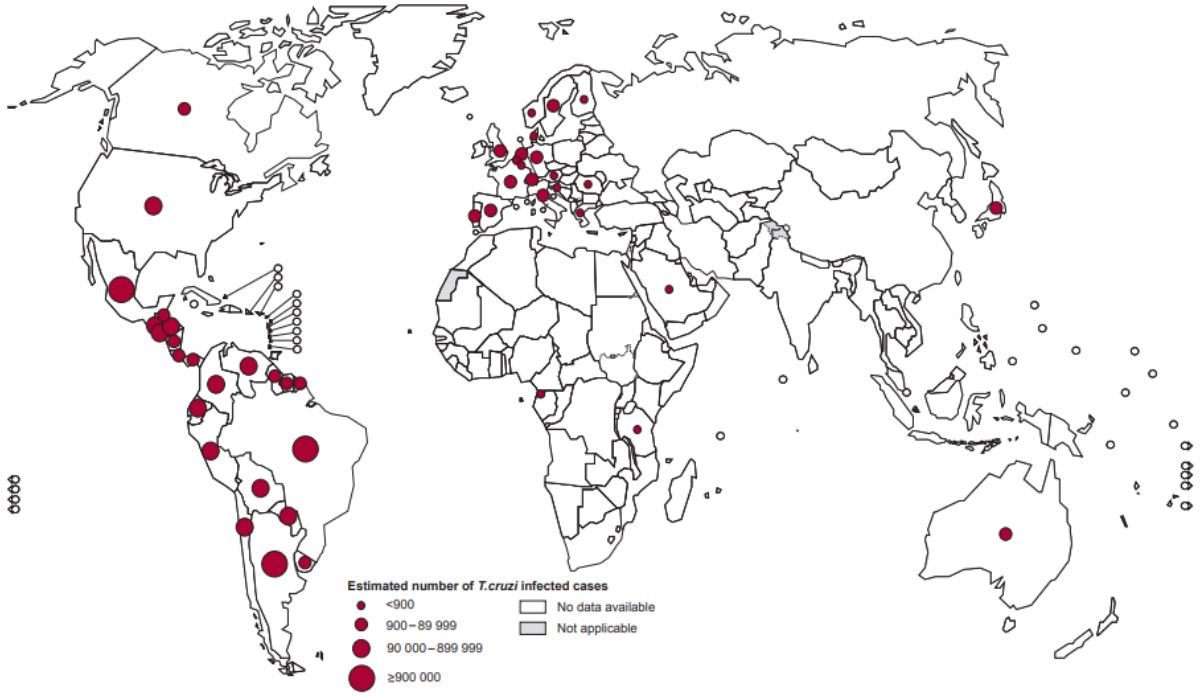
1.1.1 Doença de chagas e sua incidência

A DC, também conhecida como tripanossomíase americana, foi detalhadamente descrita por Carlos Ribeiro Justiniano Chagas, no ano de 1909. Porém, pesquisas paleoparasitológicas demonstraram a presença da tripanossomíase americana datando mais de 9.000 anos atrás na américa latina e 7.000 anos no Brasil. A doença estava presente antes mesmo da domesticação de animais, sendo o contato prévio com triatomídeos, vetores transmissores, já relatado por Charles Darwin. No entanto, Carlos Chagas foi o primeiro a descrever a doença, envolvendo todas as características referentes ao parasito causador da doença, vetor transmissor e características clínicas da mesma, passando a ser conhecida como DC (ARAÚJO-JORGE *et al.*, 2017). Atualmente a DC é considerada uma doença tropical negligenciada, presente principalmente em áreas endêmicas da américa latina, porém os processos migratórios facilitaram a disseminação da doença pelo mundo, tornando-a um problema de saúde pública mundial (WHO, 2010; WHO, 2021).

Os infectados pela DC estão distribuídos por diversos locais do mundo, somando aproximadamente de 6 a 7 milhões de infectados (WHO, 2021). Dados baseados em estimativas demonstram a distribuição e incidência de infectados em diferentes países (Figura 1). Através dessas estimativas observa-se um número mais significativo de infectados na região da América Latina, na qual algumas regiões concentram cerca de 900.000 infectados (WHO, 2018). Os índices de infectados na América latina estão relacionados a infecções principalmente por vetores e via oral (Pérez-Molina & Molina, 2018). Além disso, há uma ampla distribuição de infectados na Europa e em algumas regiões da América do Norte, os quais estão mais relacionados a processos de migração, infecções congênitas, por doações de sangue e transplante de órgãos (WHO, 2015; PÉREZ-MOLINA & MOLINA, 2017).

As taxas de incidência da DC no Brasil apresentaram uma queda significativa entre 2020 e 2021. O estado do Pará concentra o maior número de registro de novos casos, bem como um maior índice de mortalidade, quando comparado a outras regiões do país. O predomínio de infecções ocorre em pessoas pardas, do sexo masculino e na faixa etária de 20 a 59 anos, no entanto, ainda é registrada a incidência da doença em gestantes (MINISTÉRIOS DA SAÚDE, 2021).

Figura 1. Distribuição global de casos de doença de chagas, baseado em estimativas, 2018.



Fonte: WHO, 2018

1.1.2 Agente etiológico da tripanossomíase americana

O *Trypanosoma cruzi* é o parasito causador da DC, através de alguns trabalhos de infecção experimental, Carlos Chagas descreveu detalhadamente o mesmo, demonstrando com excelência suas formas morfológicas. Além disso, elucidou as manifestações patológicas do mesmo na infecção em humanos, encontrando-o no sangue da primeira paciente descrita com a DC, identificou o protozoário como *Trypanosoma cruzi*, em homenagem a seu mestre Oswaldo Cruz (CHAGAS, 1909; ARAÚJO-JORGE *et al.*, 2017). Esse parasito apresenta diferentes subpopulações capazes de causar infecção, tendo uma grande diversidade de hospedeiros. Alguns estudos demonstram diferentes hipóteses para o processo evolutivo do *T. cruzi*, sua dispersão e adaptação a diferentes espécies. Diversas ordens de mamíferos podem ser hospedeiras do *T. cruzi*, funcionando como reservatórios silvestres ou domésticos do parasito, apresentando significativa importância na disseminação do mesmo. Apesar da identificação da doença ocorrer em conjunto com a identificação de um vetor hematófago, alguns estudos demonstram e reforçam a teoria de que a disseminação do parasito, entre diferentes grupos que compõem a classe dos

mamíferos, ocorreu inicialmente por transmissão oral (SCHOFIELD, 2000; JANSEN *et al.* 2017).

O parasito apresenta-se em 4 formas morfológicas principais, as quais são identificadas como: amastigota, epimastigota, tripomastigota metacíclica e tripomastigota sanguínea. Alguns hospedeiros podem apresentar o desenvolvimento de todas as formas morfológicas, porém, nos humanos encontram-se as formas de amastigota e de tripomastigota sanguícola. Enquanto no vetor transmissor as formas presentes são de epimastigota e tripomastigota metacíclica (TEIXEIRA *et al.*, 2011; LANA & MACHADO, 2017). A forma de epimastigota está presente no trato digestivo do vetor e pode ser encontrada em alguns hospedeiros, como nas glândulas odoríferas de *Dídelphis* spp, essa forma apresenta reprodução assexuada, sem necessidade de adesão a tecidos ou células (TEIXEIRA *et al.*, 2011; JANSEN *et al.*, 2017; LANA & MACHADO, 2017). A forma de tripomastigota metaciclica é a forma infectante do parasito, presente na porção final do tubo digestivo do inseto vetor, onde é eliminada juntamente com as excretas do mesmo. Essa forma não apresenta processo reprodutivo ativo, tendo capacidade de movimentação com o auxílio de um flagelo e membrana ondulante. Além disso, é a forma morfológica que realiza a invasão inicial de células no processo de infecção (TEIXEIRA *et al.*, 2011; LANA & MACHADO, 2017). A forma de amastigota está presente nos tecidos e células dos hospedeiros. Não apresenta estruturas celulares anexas para movimentação, realiza o processo de reprodução de forma assexuada e pode permanecer nos tecidos por anos, ou ainda ser reativada se diferenciando na forma tripomastigota (TEIXEIRA *et al.*, 2011; LANA & MACHADO, 2017).

1.1.3 Formas de infecção na doença de chagas

No que tange as formas de infecção pelo *T. cruzi* são elucidadas algumas rotas de infecção dentre as quais são: vetorial, materno-fetal, acidental, oral e através de transplante de órgãos. De forma negativa, ainda há um *déficit* de 15% no que diz respeito aos registros específicos relacionados a via de infecção, comprometendo a geração de informações epidemiológicas e o planejamento de estratégias de controle de infecções (MINISTÉRIOS DA SAÚDE, 2021).

A forma mais conhecida é a vetorial, que, dar-se-á pelas fezes eliminadas durante o repasto sanguíneo de hemípteras infectados pelo protozoário. No Brasil, três gêneros são o de maior importância na infecção *Triatoma*, *Rhodnius* e *Panstrogylus* (MONTEIRO, 2017).

Anteriormente, a DC tinha como área endêmica apenas os países da América Latina, todavia, a migração de pessoas para fora do continente, fizeram com que a moléstia passasse a ter uma abrangência global através da migração dos indivíduos infectados (MÉNDES *et al.*, 2015; MONCAYO e SILVEIRA, 2009).

Sabe-se que todos os triatomíneos são potenciais fontes de infecção do parasito, entretanto, apenas aqueles que possuem o hábito alimentar do tipo hematófago é que oferecem risco, em especial, os que possuem hábitos peridomiciliares, porque são os responsáveis em fechar o ciclo silvestre-domiciliar do parasito, sendo assim, apresentam maior importância (FERREIRA, 2020).

Como descrito anteriormente a distribuição do vetor dar-se-á em todo o Brasil, sendo as espécies de maior importância na disseminação da DC *Panstrongylus megistus*, *Triatoma brasiliensis* e *T. pseudomaculata*. Ao que se sabe, a espécie de maior importância no Rio Grande do Sul que se tem registro é *T. tubrovaria*. Todavia, sabe-se que a espécie *T. infestans* é a espécie vetorial de maior importância, porque além de ter hábitos domiciliares é a de maior incidência em toda a América do Sul (FERREIRA, 2020).

Pesquisas demonstram que é possível ter uma infecção vertical, ou seja, uma vez estabelecida a infecção em uma gestante, a mãe pode transmitir a doença para o feto. Um estudo conduzido por Fracasso e col. (2019) evidenciou através de PCR a transmissão materno-fetal em um modelo de camundongos e DC. Na transmissão materno-fetal os fatores determinantes para a efetividade da transmissão estão envolvidos em torno da carga parasitaria além da reinfecção em mulheres gestas (ANDRADE *et al.*, 2006). Na maioria dos casos de transmissão materno-fetal, lesões placentárias acabam sendo a porta de entrada para o parasito infectar o embrião, que uma vez infectado, as formas tripomastigotas infectam qualquer tipo celular embrionário (ANDRADE *et al.*, 2006; LANA e TAFURI, 2000; REY, 1991).

Apesar da forma vetorial ser a mais elucidada, em 2005 o estado de Santa Catarina registrou um surto de DC (INOUE E GUEDES, 2005), a partir do consumo do caldo de cana, uma vez que os cuidados sanitários não foram tomados, alguns hemípteras foram moídos juntos, e os mesmos continham formas infectantes as quais foram ingeridas junto ao caldo. A infecção oral dar-se-á no hospedeiro através da gp82 descrito por Neira *et al.* (2003), uma glicoproteína de superfície que, ao que se sabe, desempenha papel chave para que se estabeleça a invasão celular através da mucosa gástrica. A gp82 tem ligação direta com a mobilização de Ca^{2+} intracelular,

componente essencial para que as formas infectantes de *T. cruzi* possam realizar o processo de internalização/invasão celular e por consequência a multiplicação e instalação da doença no hospedeiro (RUIZ *et al.*, 1998; TARDEUX, NATHANSON & ANDREWS 1994; MORENO *et al.*, 1994; DORTA *et al.*, 1995; YOSHIDA, 2008).

A forma acidental de infecção acontece através de materiais perfuro cortantes contendo secreções (sangue) oriundas de animais ou pessoas infectadas por *T. cruzi* e os manipuladores acabam se acidentando e infectando-se (PINTO e AMATO NETO, 2011). Nos últimos 15 anos, evidenciou-se um maior índice de infecções ocorrendo por via oral e por interação acidental com o agente etiológico da doença, durante seu ciclo silvestre (MINISTÉRIO DA SAÚDE, 2021).

A primeira infecção através da transfusão de sangue foi descrita em 1936 na Argentina conforme Gómez (GUTIERREZ E PEÑUELA, 2019) e nos anos posteriores (1949 e 1952) de acordo com Pedreira de Freitas (1952) ocorreram os primeiros casos observados no Brasil, haja vista que anteriormente nenhum tipo de diagnóstico era utilizado para a doação de sangue e/ou órgãos. A infecção por transfusão de sangue depende de muitos fatores, incluindo a quantidade de sangue transfundido, a carga da parasitemia no momento da doação de sangue, a disponibilidade de testes de triagem e a taxa de infecção na população de doadores de sangue (SCHMUNIS & CRUZ 2005; CARDOSO *et al.*, 2018). Com o conhecimento a cerca da biologia do parasito, bem como a implementação da triagem de sangue e órgãos, é que foi possível a diminuição de infecção a partir de transfusões e transplantes.

1.1.4 Ciclo biológico do *T. cruzi*

A partir do conhecimento da biologia da espécie e através de estudos que foram conduzidos por Carlos Chagas a partir de pessoas infectadas, bem como xenodiagnóstico é que foi possível a elucidação do ciclo da doença no vetor, hospedeiro e conhecimento das espécies reservatório da DC. Sendo assim, o ciclo da doença (Figura 2) se dá inicialmente pelo repasto sanguíneo dos invertebrados em um hospedeiro infectado. No invertebrado, ao que se sabe o desenvolvimento acontece no tubo digestivo.

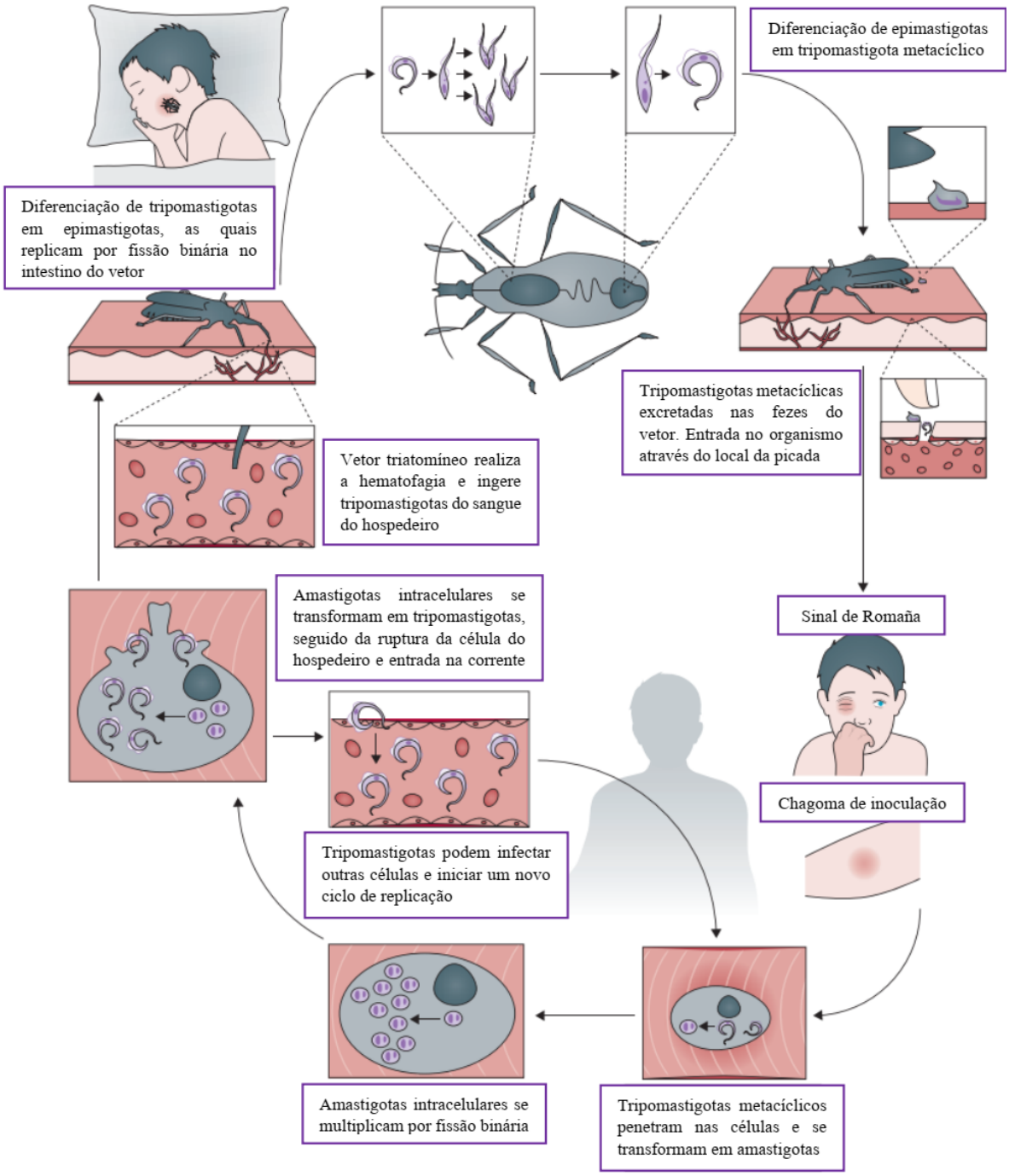
Ao chegarem no intestino do hemíptero, as formas tripomastigotas evoluem para a forma de epimastigotas intestinais, essas que mantem nessa forma evolutiva enquanto durar a vida do inseto. Uma vez que essas formas evolutivas migram, mais especificamente para o terço final do intestino (reto), sofrem diferenciação e evoluem para forma tripomastigota metaciclica (REY,

2009). Nessa fase, que apresenta durabilidade aproximada de sete dias (FERREIRA, 2020), o parasito é incapaz de reproduzir, pois a síntese de DNA é suspensa. Ou seja, a sua reprodução depende exclusivamente de um hospedeiro vertebrado para que o ciclo de vida possa ser concluído. Ainda nessa fase, essas formas evolutivas supracitadas, sessam a aderência no tecido intestinal e migram para a porção terminal do tubo digestório.

O invertebrado outrora infectado, ao realizar o repasto sanguíneo, ou até mesmo pouco tempo após o repasto, realiza a defecação. As fezes contendo formas infectantes de *T. cruzi* são eliminadas sobre a derme do mamífero. As formas infectantes, podem entrar no organismo do hospedeiro por duas vias: através de escarificações, ou seja, o ato de coçar, carrega as fezes com formas infectantes para dentro do local da picada do inseto, ou então, através de mucosas (BARRIAS *et al.,* 2013). Após esse processo de infeção, será iniciado o processo de invasão celular, que possui duas vias: via dependente de fagocitose e via independente de fagocitose, as quais despendem energia do parasito (GUARNERI & LORENZO 2017).

O processo de invasão celular é finito, ou seja, a depender do pH as formas tripomastigotas iniciam a sua evolução no sentido da diferenciação em amastigotas. Uma vez que as formas amastigotas estejam livres no citosol, dar-se-á a multiplicação por fissão binária. Em alguns casos, as amastigotas entram em um estado de dormência (SÁNCHES-VALDÉZ *et al.,* 2018). Depois disso, dois caminhos podem ser tomados: reiniciação do ciclo formando amastigotas, ou então, diferenciar-se em tripomastigotas infectantes. Essas últimas, através do rompimento das células hospedeiras, podem atingir tanto a corrente sanguínea quanto adentrar em células vizinhas e assim migrarem para tecidos e órgãos aos quais possuem tropismo (BONFIM-MELO *et al.,* 2018).

Figura 2. Ciclo biológico entre vetor e hospedeiro humano.



Fonte: Adaptado de Pérez-Molina & Molina, 2018.

1.1.5 Evolução da doença de chagas

Uma vez infectado, pela forma vetorial, o indivíduo encontra-se na fase aguda da doença, estima-se que essa fase dure de 6 a 12 semanas após a infecção (CAROD-ARTAL & GASCON, 2010; CHAGAS, 1909; MAGUIRE; 2004; BUCKNER, 2017). Geralmente observa-se a presença de edema no local da inoculação do parasito, que é conhecido como chagoma de inoculação. Outro sinal característico acontece, quando, a porta de entrada é via ocular, neste caso, observa-se, celulite perioftálmica unilateral clássica e edema palpebral conhecido como sinal de Romaña. Nessa fase, é possível observar formas tripomastigotas na circulação do hospedeiro, além de febre, erupções cutâneas, mal-estar, anorexia, vômitos além de mialgia, todavia são sintomas inespecíficos e acabam sendo uma barreira no diagnóstico precoce da parasitose, bem como a intervenção medicamentosa que possui maior eficiência curativa nessa fase. Por não apresentar nenhum sinal patognomônico, na maioria dos casos a fase aguda passa despercebida, sendo diagnosticada entre 1 a 2% dos casos. Grande parte dos casos agudos da DC a evolução (RASSI *et al.*, 2007; RASSI *et al.*, 2010; VILLAR *et al.*, 2014; BUCKNER, 2017) é benigna, dependendo principalmente da competência imune do hospedeiro, alguns casos requerem hospitalização onde comumente os indivíduos apresentam hepatoesplenomegalia, miocardite aguda grave, linfadenopatia além de meningoencefalite. Passado o período da fase aguda, os pacientes podem apresentar dois estados distintos, podendo evoluir para a fase indeterminada ou para a fase crônica. A fase indeterminada é assim denominada, porque não é sabido se o paciente desenvolverá sintomas ou não. Essa fase afeta entre 50 e 80% dos indivíduos, podendo ser detectada a sorologia positiva, embora a radiografia bem como o eletrocardiograma encontrem-se normais. A ausência de sintomas faz com que alguns indivíduos se tornem reservatórios, sem ter conhecimento de que estão infectados pelo *T. cruzi* (CAROD-ARTAL & GASCON, 2010; BUCKNER, 2017).

A evolução crônica sintomática da DC afeta principalmente o coração, causando cardiomiopatia chagásica. No trato intestinal é possível observar a formação do megacolon e, recentemente, veem sendo observadas desordens de ordem neurológica. A gravidade dos acometimentos nos órgãos supracitados está diretamente relacionada ao processo inflamatório causado pelo parasito, ao status imune bem como a persistência parasitária nos tecidos (CAROD-ARTAL & GASCON, 2010). Nos pacientes que são acometidos pela forma cardíaca os sintomas comumente encontrados são: palpitações, dispneia, edema e dor no peito. A cardiopatia chagásica

é caracterizada por uma variedade de arritmias, com frequência são registrados bloqueios sinoatriais. O bloqueio de ramo direito com hemibloqueio anterior esquerdo é a anormalidade de condução clássica e mais comum (CAROD-ARTAL & GASCON, 2010), também são observados sopros sistólicos. Em análises histopatológicas são comuns achados de pancardite, ou seja, inflamação do pericárdio, miocárdio bem como das válvulas cardíacas. Além do mais, comumente a morte súbita está atrelada a fibrilação ventricular. Os danos gerados no tecido cardíaco estão relacionados a dois processos durante a infecção. Primeiramente, deve-se a formação de ninhos de formas amastigotas no tecido cardíaco. Além disso, devido a esse parasitismo nas fibras musculares cardíacas, há um processo inflamatório intermitente, promovendo a agressão do tecido cardíaco advinda da resposta imune que continuamente é induzida, visto que é atrelada ao parasitismo persistente nesse tecido, levando a apresentação de antígeno continuada (SIMÕES *et al.,* 2018).

Embora os dois processos supracitados, ainda há embasamento cientifico, que sustenta outros dois processos: Anormalidades na microcirculação coronária, que é resultado da resposta imune e que desencadeia processos de origem trombocitária, agregação plaquetária e disfunção do tecido endotelial, esses processos culminam em distúrbios de ordem microvascular, levando a isquemia e lesão das fibras musculares cardíacas e por consequência a insuficiência cardíaca (SIMÕES *et al.,* 2018; MARIN-NETO *et al.,* 2007). Já as anormalidades de ordem nervosa autônoma cardíaca são desencadeadas pela resposta imune, que leva a lesão nas fibras miocárdicas e, por consequência, causa a desenervação autonômica. O que leva a sobrecarga hemodinâmica além da perda da regulação de ordem homeométrica evoluindo para a dilatação e insuficiência cardíaca (SIMÕES *et al.,* 2018; MARIN-NETO *et al.,* 2007; RASSI *et al.,* 2010).

Ademais, o trato gastrointestinal também sofre as consequências causadas pelo parasito. Neste, as lesões inflamatórias são encontradas numa região específica, ou no plexo de Auerbach, que é responsável pela coordenação dos movimentos peristálticos. Sendo assim, comumente, os segmentos mais afetados são: esôfago e o cólon retossigmóide. Inicialmente é observada a constipação, que é ocasionada pela dismotilidade do colón. Também é observada a ocorrência de volvo, além de megacólon dilatado, fecaloma e dor intensa na região abdominal. Em alguns casos é possível visualizar radiograficamente anormalidades de contração e estreitamento esofágico, evidenciando a lesão de megaesôgafo.

A degeneração do plexo ou pós-desnervação do gânglio parassimpático endógeno leva à hipertrofia muscular e hiperplasia da mucosa, as quais resultam no aumento visceral na fase crônica da infecção por *T. cruzi* (megaesôfago e megacólon) (GARCIA *et al.*, 1996). Sendo assim, a ação do parasito nesse tecido, pode ser de maneira direta, ou seja, lesão tissular em células-alvo, ou então de maneira indireta, através do antígeno F1-160, o antígeno do parasito mimetiza uma proteína expressa por neurônios de mamíferos (VAN VOORHIS *et al.*, 1991). Isso resulta em uma reação cruzada de imunorreatividade, promovendo o recrutamento de células imunes, levando a uma ganglionite mioentérica aguda. Esse processo ocorre porque as células imunes são recrutadas para dentro dos gânglios. Por fim, leva a uma contração desordenada ou mesmo a ausência de contração da musculatura (GULLO *et al.*, 2012; IANTORNO *et al.*, 2007).

Embora o *T. cruzi* não tenha tropismo pelo encéfalo, estudos vem demonstrando que paciente chagásico, com insuficiência cardíaca crônica, entre 18 a 20% tiveram infartos cerebrais (ARAS *et al.*, 2003). Além disso, é de conhecimento que a DC pode causar acidente vascular cerebral (AVC) (PITELLA, 1984). Existem alguns fatores que favorecem o AVC chagásico como aneurisma apical cardíaco, arritmias, trombo mural e disfunção ventricular esquerda (NUNES *et al.*, 2009). Embora não seja frequente a parasitemia em neurônios, os principais tipos celulares parasitados no SNC são as células gliais e as de Schwann (DA MATA *et al.*, 2000). Além do mais, na fase aguda, o parasito pode atravessar a barreira hematoencefálica e na fase crônica pode ser encontrado no líquor. Bem como, a longo prazo pacientes com neuro-infecção, causada pelo *T. cruzi*, pode desenvolver casos de *déficit* cognitivo e/ou síndromes cerebelares, que são sintomas característicos da fase crônica (BRENER & ANDRADE, 1979).

1.1.6 Resposta imune e sistema colinérgico na infecção aguda por *T. cruzi*

A resposta imune inicial do hospedeiro se dá através de componentes da resposta inata. Uma vez que o parasito invade o organismo do hospedeiro, encontrará diversos componentes solúveis que constituem o sistema complemento, anticorpos naturais, entre outros componentes (CESTARI *et al.*, 2013). Além disso, células envolvidas nessa resposta inicial atuam para o controle do processo de infecção. Células natural killer e neutrófilos são componentes da resposta imune inata que atuam na produção de citocinas que realizem o processo inicial de sinalização imunológica e controlem a multiplicação do parasito, como o IFN-ɤ (TRUYENS & CARLIE, 2017).

Os macrófagos desempenham um papel muito importante na infecção por *T. cruzi*, visto que são, simultaneamente, a célula hospedeira do parasito, além de atuar como apresentadora de antígeno. Moléculas presentes no próprio parasito são capazes de promover a ativação dos macrófagos, levando a produção interleucinas, TNF, óxido nítrico e espécies reativas de oxigênio. Além disso, as células dendríticas desempenham um papel extremamente importante no desenvolvimento da resposta imune adaptativa, secretando citocinas que estimulam o processo de ativação e diferenciação celular (TRUYENS & CARLIE, 2017).

Na DC a principal resposta imunológica caracterizada é a do tipo Th1, sendo essencial para o controle e eliminação do parasito. Porém a DC traz a necessidade de um processo de regulação das respostas imunológicas do organismo. Visto que as mesmas podem causar danos significativos aos tecidos (BORGES *et al.*, 2013). A resposta imunológica mediada pela ação das células T apresenta um perfil misto de Th1/Th2/Th17 (TRUYENS & CARLIE, 2017). Através desse equilíbrio entre os tipos de resposta imunológica, o hospedeiro consegue combater o parasito, enquanto regula a resposta para reduzir os danos dessa ação. Porém as respostas regulatórias que visam a proteção do hospedeiro, beneficiam a permanência do parasito no organismo uma vez que podem levar a inibição na produção de óxido nítrico e INF-ɤ, por exemplo (BORGES *et al.*, 2013).

A fase aguda da DC é caracterizada por um intenso processo inflamatório nos tecidos alvo do parasito, com infiltrados inflamatórios significativos, em função do aumento na produção de citocinas pró-inflamatória que realizam o processo de sinalização na resposta imunológica. No entanto, esse tipo de resposta leva destruição de cardiomiócitos funcionas, induz necrose a apoptose no tecido, promovendo a quimiotaxia de células inflamatórias para o tecido (TEIXEIRA *et al.*, 2011). Durante a DC, é relata uma degeneração do sistema nervoso autônomo, relacionado as alterações patológicas que ocorrem no tecido cardíaco, bem como no trato gastrointestinal. Essas alterações consistem na degradação das conexões do sistema nervoso simpático e parassimpático nos órgãos em questão, comprometendo a estimulação nervosa e, consequentemente, a contração desses tecidos musculares (MACHADO *et al.*, 2012).

Nesse sentido, algumas pesquisas utilizam o sistema colinérgico como um alvo farmacológico, buscando, através do processo de inibição/estimulação de alguns componentes desse sistema reduzir os processos inflamatórios que resultam no comprometimento das terminações nervosas locais. A partir desses estudos foi possível ressaltar a importância do

sistema colinérgico, não só na patogenia da doença, como na regulação e controle da resposta imunológica na DC (KAWASHIMA 2004; KAWASHIMA *et al.*, 2012; MACHADO *et al.*, 2012; FUJII *et al.*, 2017a; FUJJI *et al.*, 2017b). O sistema colinérgico é expresso em diversas células imunológicas, sendo composto por colina acetiltransferase (ChAT), acetilcolina (ACh), receptores muscarínicos (mAChR) e nicotínicos (nAChR), e acetilcolinesterase (AChE). Através da atividade desses componentes a resposta imunológica pode ser regulada (KAWASHIMA 2004; KAWASHIMA *et al.*, 2012; FUJII *et al.*, 2017a).

A ACh consiste em um neurotransmissor presente tanto no sistema nervoso central, quanto no sistema nervoso periférico. Além disso, é uma molécula que atua nos processos de regulação das funções imunológicas. A ACh apresenta uma ação anti-inflamatória, influenciando nos processos de sinalização celular através da interação com receptores ou no comunicação célula-célula (KAWASHIMA 2004; KAWASHIMA *et al.*, 2012; FUJII *et al.*, 2017a; FUJJI *et al.*, 2017b; LU & WU, 2021). A síntese da ACh nas células imunes é realizada pela ChAT. Essa enzima tem sua expressão influenciada por diversos fatores, na sinalização imunológica, que podem resultar em um aumento ou redução na produção de ACh nessas células. A estimulação de TLR leva a um aumento na produção de ACh, evidenciando uma relação da ACh na regulação da resposta imune inata. Além disso, alguns dados na literatura demonstram que a ativação de linfócitos T e B, além da adesão linfocitária, podem resultar em um aumento na síntese e liberação de ACh, podendo atuar, também, na regulação da resposta imune adaptativa (KAWASHIMA 2004; KAWASHIMA *et al.*, 2012; FUJII *et al.*, 2017a; FUJJI *et al.*, 2017b; LU & WU, 2021).

Os receptores muscarínicos e nicotínicos são expressos nas células imunológicas, atuando de diferentes formas na regulação imune. A acetilcolina produzida nessas células pode atuar de forma autócrina e/ou parácrina nos receptores, A maior parte das pesquisas envolvendo a via colinérgica anti-inflamatória tem seu foco nos receptores muscarínicos M1 e M5 e nos receptores niconíticos α7. A ativação desses receptores leva a alterações, via segundos mensageiros, em fatores de transcrição da célula, promovendo ou inibindo a produção de moléculas como citocinas, as quais atuam na regulação da resposta imune e inflamatória (KAWASHIMA 2004; PAVLOV & TRACEY, 2006; KAWASHIMA *et al.*, 2012; FUJII *et al.*, 2017a; FUJJI *et al.*, 2017b; LU & WU, 2021). Através da avaliação de dados encontrados na literatura, é possível observar que os receptores muscarínicos e nicotínicos parecem atuar de forma antagônica, em

relação a regulação da sinalização imunológica. A estimulação de receptores muscarínicos, nas células imunes, é mais associada a uma resposta pró-inflamatória, enquanto a estimulação de receptores nicotínicos é relacionada a uma modulação anti-inflamatória. A estimulação desses receptores parece estar relacionada com a produção de citocinas como, por exemplo, TNF-α, IFN-ɤ, IL-6 e IL-2, atuando na regulação de ativação e proliferação celular, modulando a resposta imunológica (KAWASHIMA 2004; PAVLOV & TRACEY, 2006; KAWASHIMA *et al.*, 2012; FUJII *et al.*, 2017a; FUJJI *et al.*, 2017b; LU & WU, 2021).

As enzimas colinesterases podem ser encontradas em tecidos colinérgicos e não colinérgicos. A Acetilcolinesterase (AChE) consiste em uma enzima ligada à membrana, encontrada no cérebro, músculos, eritrócitos, linfócitos e neurônios colinérgicos (SCHETINGER *et al.*, 2000; KAWASHIMA, FUJII, 2003). A enzima butirilcolinesterase (BChE: EC 3.1.1.8), por sua vez, está presente no intestino, fígado, rins, coração, pulmões e soro (ECOBICON, CORNEAU, 1973). Ambas podem atuar na degradação da ACh, sendo a BuChE atuante principalmente quando houver uma situação de inibição ou ausência da AChE (TAYLOR, BROWN, 1999; LI *et al.*, 2000). Trabalhos na literatura já demonstram a ocorrência de alterações na atividade das enzimas acetiltransferase e AChE relacionadas a tripanossomíases, ocasionadas por *T. cruzi* e *Trypanosoma evansi* (RODRIGUES *et al.*, 2005; DA SILVA *et al.*, 2010). Além disso, através da ação da AChE, principalmente, os níveis de acetilcolina podem ser regulados no meio extracelular. Por consequência, essa ação interfere diretamente nos processos de sinalização exercidas pela ACh. Dessa forma as alterações na expressão e atividade da AChE também integram as vias de imunomodulação, tendo em vista sua ação catalítica (FUJII *et al.*, 2017a; FUJJI *et al.*, 2017b). Essa via de regulação imunológica vem sendo avaliada na DC, algumas pesquisas já tem demonstrado a íntima relação entre o intenso processo inflamatório, presente na fase aguda da doença, com componentes do sistema colinérgico. Esses componentes desempenham papel imunomodulatório, demonstrando estar relacionado na manutenção do equilíbrio entre a resposta pró e anti-inflamatória (MACHADO *et al.*, 2012, SILVA *et al.*, 2017).

1.1.7 Estresse oxidativo

Os processos bioquímicos da célula, como a formação de ATP via cadeia respiratória na mitocôndria, levam naturalmente a formação de espécies reativas (ER) (ANDERSON, 1996). Entretanto, em algumas condições há, uma formação exacerbada de ER, como por exemplo em

resposta a algum agente patogênico (parasitas, fungos, bactérias, vírus) e palotogias que provoquem processos inflamatórios persistentes (doenças autoimunes, síndrome metabólica). Nessas situações, ocorre um desiquilibro na formação/remoção de ER, levando assim, o organismo a um perfil pro-oxidante. Ademais, a formação de ER em processos inflamatórios tem como objetivo a produção de mediadores, que atuam de maneira pro-inflamatória, como o NF-κB, p53, TNF-α entre outros (CHATTERJEE, 2016).

Para equilibrar a formação/remoção de ER, o organismo lança mão de dois mecanismos conhecidos como: mecanismos enzimáticos e não enzimáticos (Sies, 1993). As enzimas que integram o aporte enzimáticos são inúmeras, podemos destacar o papel da enzima superóxido dismutase (SOD) que dismuta o radical superóxido (O_2•-) em peroxido de hidrogênio (H_2O_2) (HALLIWEL, 1987; HALLIWEL, 1990). Já a catalase (CAT), que tem por função a decomposição do peroxido de hidrogênio (H_2O_2) em água e oxigênio (ANSCHAU, 2011). Por outro lado, mecanismos não enzimáticos utilizados são exógenos, sendo assim, há necessidade de serem ingeridos via alimentação. Dentre os quais encontram-se as vitaminas e outros compostos naturais, como o resveratrol, a cúrcuma, β-caroteno entre outros (STAVRIC, 1994).

Estudos recentes vêm demonstrando a efetividade do parasito no sentido pro-oxidante. Um estudo conduzido por Fracasso *et al.* 2021, ilustraram a capacidade do parasito em aumentar as ER no fígado de animais infectados experimentalmente por *T. cruzi*. Bem como, o aumento da atividade das enzimas SOD e CAT, demonstrando um aumento da produção de ER que, embora possam levar a danos relacionados ao DNA, o organismo tenta utilizar ER no sentido de rompimento do patógeno. Em uma infecção experimental por *Toxoplasma gondii* (Machado *et al.* 2016), pesquisadores evidenciaram um aumento na peroxidação lipídica (TBARS) em cérebro, esse aumento pode estar intimamente ligado com a interação do agente patogênico e o hospedeiro, o que, por consequência, pode estar ligado inclusive com o aumento de ER (BARBOSA *et al.,* 2014). Kumar *et al.* 2018 conduziram um estudo com *Plasmodium falsiparum* e *Plasmodium vivax* com humanos e puderam observar que em ambas as infecções parasitárias, a medida que os níveis de ROS se mostravam aumentados, a capacidade antioxidante total (TAC) era diminuída nos pacientes infectados com ambas espécies de *Plasmodium,* podendo esse aumento de ROS estar ligado a tentativa do organismo em romper o agente patogênico.

Por outro lado, muitos fármacos utilizados no tratamento das doenças supracitadas, estimulam a produção de ER, que por sua vez induz um estresse oxidativo e que tem como

consequência a morte dos parasitos. Como é o caso do fármaco benznidazol (BNZ), atualmente o único que é utilizado para o tratamento da DC no Brasil. Um estudo conduzido por Fracasso *et al.* (2021) demonstrou que o BNZ é capaz de aumentar os níveis de ER em córtex de camundongos infectados experimentalmente com *T. cruzi* quando comparado ao grupo sadio e tratado com BNZ, ou seja, essa produção de ER visa o controle da carga parasitária, aumentando os níveis de agentes pro-oxidativos.

1.1.8 Tratamento doença de Chagas no Brasil

De acordo com o Protocolo Clínico e Diretrizes Terapêuticas DC do Ministério da Saúde (2018), no Brasil para o tratamento da DC, em qualquer fase, há dois medicamentos principais: benznidazol e nifurtimox, entretanto o benznidazol é mais utilizado. Esses medicamentos, quando utilizados na fase aguda da doença, possuem boa eficácia, visto a taxa de cura pode chegar a 60% entre crianças e adultos (SOSA *et al.*, 1998; PINAZO *et al.*, 2010). Porém, a terapia medicamentosa diminui eficácia com a duração da infecção, como no caso da fase crônica (MARIN-NETO *et al.*, 2008; PINAZO *et al.*, 2010). O tratamento em estágios crônicos é controverso e a verdadeira eficácia nestes estágios é desconhecida, apesar de ser preconizado pelo Ministério da Saúde em qualquer fase da doença (GARCIA *et al.*, 2005; DAVANÇO *et al.*, 2016; PERIN *et al.*, 2017). O objetivo da terapia farmacológica é a redução da parasitemia e impedir a reativação da doença, além de melhora dos sintomas clínicos (com consequente aumento da expectativa de vida) e redução de complicações clínicas. Porém o uso do benznidazol e nifurtimox estão associados a alta frequência de efeitos adversos (53 e 85% respectivamente) (PÉREZ-MOLINA, MOLINA, 2018; PAVAN *et al.*, 2018; SALES JUNIOR *et al.*, 2017).

Em relação aos efeitos adversos mais observados com o uso do benznidazol, destacam-se parestesias, artralgias, intolerância gastrointestinal, alopecia, dermatites e rash cutâneo. Também podem ocorrer complicações mais graves, como depressão da medula óssea com neutropenia (MINISTÉRIO DA SAÚDE 2018; PAVAN *et al.*, 2018; SALES JUNIOR *et al.*, 2017). O benznidazol é um fármaco derivado do nitroimidazol, de baixa solubilidade em água (PALMEIRO-ROLDÁN *et al.*, 2014), tem boa biodistribuição (92%) em órgãos como: baço, fígado, pulmões, rins, coração e cérebro (órgãos mais afetados pela infecção pelo *T. cruzi*), porém possui baixa absorção durante o metabolismo de primeira passagem no fígado (DAVANÇO *et al.*, 2016; PERIN *et al.*, 2017). Embora haja menor resposta terapêutica após fase crônica,

estudos apontam menor mudança no padrão eletrocardiográfico bem como retardo do aparecimento de complicações clínicas entre pacientes tratados com benznidazol. É um pró-fármaco ativado por nitrorredutase do tipo I, expressa pelo tripanossoma, produzindo um composto citotóxico e mutagênico (DAVANÇO *et al.*, 2016; RITTER *et al.*, 2020).

Devido ao efeito do benznidazol sobre os parasitos circulantes, a eficácia envolvendo esse fármaco tem sido relatada durante a fase aguda da infecção, podendo levar, inclusive, à cura parasitológica. Isso reflete o efeito tripanocida pela ação das espécies reativas de oxigênio (EROs) e nitrogênio (ERNs) produzidos pelo benznidazol, além daqueles provenientes do metabolismo aeróbio do parasita e por meio da resposta imunológica do hospedeiro. Esses metabólitos interagem com a membrana celular, levando à inativação das principais enzimas e morte do parasito (BATISTA *et al.*, 2020; GOMEZ, 2017; SALES JUNIOR *et al.*, 2017). Este medicamento é comercializado sob a forma de comprimidos de 100 mg. Sua administração é feita por via oral, sendo rápida a absorção com concentrações máximas após 2 a 4 horas. Tem meia-vida de cerca de 12 horas e metabolização conduzida pelo fígado e eliminação pela urina; uma pequena parcela não absorvida é eliminada nas fezes (BATISTA *et al.*, 2020; RITTER *et al.*, 2020).

Apesar da toxicidade sistêmica e da baixa tolerabilidade orgânica associada aos efeitos colaterais marcantes com o uso de benznidazol, estudos clínicos relataram que não há medicamentos disponíveis com eficiência terapêutica superior à do benznidazol, o que torna esse fármaco o único para o tratamento clínico da DC no Brasil (BATISTA *et al.*, 2020; RITTER *et al.*, 2020). Nos casos em que houver intolerância ao benznidazol ou falha, poderá ser indicado o nifurtimox. Este composto nitroaromático é reduzido por enzimas citosólicas do tripanossoma, promovendo estresse oxidativo e morte do parasita por excessiva produção de EROs. Efeitos adversos limitam seu uso crônico e incluem sintomas gastrointestinais e neurológicos, sendo que tais efeitos adversos são mais importantes que aqueles observados pelo benznidazol (GOMEZ, 2017). Já existem relatos na literatura, entretanto de resistência cruzada do *T. cruzi* aos dois medicamentos (BATISTA *et al.*, 2020; RITTER *et al.*, 2020).

Considerando a toxicidade do benznidazol, vários alvos terapêuticos ou novas formulações têm sido investigados *in vitro* e *in vivo* (MINISTÉRIO DA SAÚDE 2018; PAVAN *et al.*, 2018; SALES JUNIOR *et al.*, 2017). Rycker *et al*. (2016) estudaram alguns fármacos com alvo na protease cruzipaina e na enzima citocromo p450, envolvidas na formação do ergosterol, que é um

dos esteróis de membrana produzidos pelo *T. cruzi*. Alguns dos fármacos testados neste estudo se mostraram promissores, porém resultaram em morte lenta dos parasitos e muitas células permaneceram infectadas. Outro alvo promissor foi com inibidores da enzima N-miristoil transferase (NMT), facilitadora de ligação com proteínas de membrana do parasito vs hospedeiro. Porém se observou muitos efeitos danosos as células do hospedeiro, necessitando ajustar a estrutura química do fármaco para possibilitar uma maior especificidade para NMT (HERRERA *et al.*, 2016). Outros estudos têm apontado a relevância da combinação de fármacos como a suramina associada a diferentes doses do benznidazol (SANTOS *et al.*, 2015). A curcumina, composto bioativo extraído de rizomas de *C. longa*, também foi estudada em associação a diferentes doses de benznidazol (NOVAES *et al.*, 2016). Foi observada uma atividade antiparasitária limitada, porém uma redução da inflamação cardíaca e diminuição da toxicidade hepática produzida pelo benznidazol. Ainda que promissores, são resultados restritos a fase aguda da doença. Apesar das tentativas descritas para controle da doença, ela permanece negligenciada e continua sendo um grave problema de saúde pública.

1.1.9 Nanotecnologia e a doença de chagas

O uso de nanocápsulas pode reduzir a toxidade do fármaco, aumentar biodisponibilidade e prolongar efeito devido a liberação lenta, tudo isso pode resultar em um maior sucesso e eficácia no tratamento da doença. Essas hipóteses são baseadas em diversos estudo com nanotecnologia realizados em nosso grupo de pesquisas, que têm constatado potencialização terapêutica de componentes fornecido nas mais diferentes formulações nanoestruturadas, como por exemplo as nanocápsulas e nanoemulsões contra parasitos. Bottari *et al.* (2015, 2016) utilizaram complexos de inclusão, com resveratrol, no tratamento coadjuvante da toxoplasmose experimentalmente induzida. Os resultados demonstraram uma redução do número de cistos no cérebro dos animais no grupo tratado com o resveratrol livre e em complexo de inclusão. Além disso, demonstraram o efeito protetor do resveratrol na doença, pois os grupos tratados com o composto apresentaram uma redução do estresse oxidativo e um aumento de agentes antioxidantes.

A DC ainda ocasiona muitos danos à população, representando um problema de saúde pública, visto que apresenta diversas formas de contaminação. Além disso, sua distribuição ocorre em diferentes regiões do planeta, pelos processos de migrações populacionais. Mesmo com a presença de muitos casos de infectados por *T. cruzi*, não se tem total compreensão dos

processos bioquímicos e imunológicos envolvidos no curso da doença, e ainda não há um tratamento que seja eficaz nas diferentes fases do desenvolvimento da doença. Assim, torna-se muito importante o desenvolvimento de pesquisas para a melhor compreensão dos mecanismos pró e anti-inflamatórios envolvidos na DC. A elucidação desses processos pode permitir o encontro de novos alvos farmacológicos. Além disso, a combinação de fármacos ou o uso de novas tecnologias disponíveis podem ser uma solução para um tratamento mais eficiente e com menor impacto ao organismo.

2. OBJETIVOS

2.1 Objetivo Geral

Avaliar os efeitos do benznidazol nanoencapsulado e livre na terapia da fase aguda da DC, assim como os efeitos da infecção por *T. cruzi* e do tratamento sob a via colinérgica e reações oxidativas.

2.2 Objetivos Específicos

- Desenvolver e produzir nanocápsulas a base de Eudragit L-100 com benznidazol e avaliar suas propriedades.
- Em camundongos experimentalmente infectados com a cepa Y de *T. cruzi*:
    - Avaliar a eficácia do tratamento à base de nanocápsulas de benznidazol
    - Verificar a toxicidade das nanocápsulas de benznidazol, bem como do fármaco livre através de ensaio cometa.
    - Avaliar se a terapia usada no tratamento da DC afeta o sistema colinérgico em linfócitos e córtex, através da avaliação de enzimas e receptores.
    - Verificar se a terapia minimiza os danos patológicos causados pela doença em coração, fígado, rim e córtex através de análise histológicas.
    - Investigar a relação entre status oxidativo/antioxidante e patogenia da DC em fígado, rim e córtex através da avaliação de enzimas e moléculas pró e antioxidantes.

3. EXPERIMENTOS

3.1 Desenhos experimentais

Os protocolos experimentais foram aprovados pela Comissão de Ética no Uso de Animais (CEUA) da Universidade Federal de Santa Maria (protocolo número 2842070618).

3.2 Experimento I – Avaliação da ação da acetilcolinesterase na Doença de Chagas

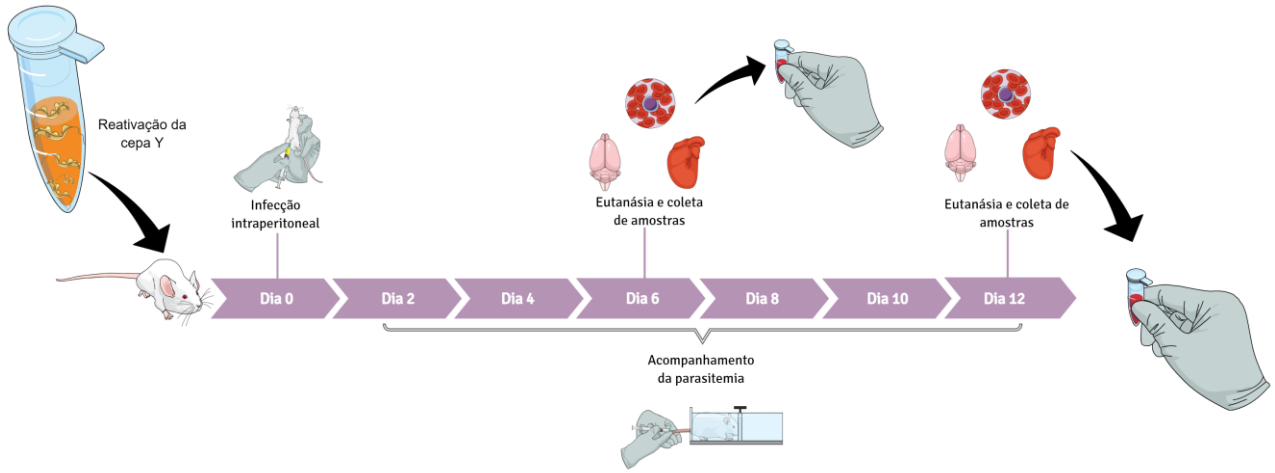
3.2.1 Modelo Animal e desenho experimental

Para a realização do protocolo experimental I (fig.1), foram utilizados 24 camundongos *Swiss* fêmeas (com 45 dias de idade e peso corporal aproximado de 20 ± 3 g). Os animais foram obtidos do Biotério Central da Universidade Federal de Santa Maria (UFSM) e mantidos em condições controladas em temperatura constante (23 ± 1 ºC, umidade relativa de 70 %), com ciclo claro/escuro de 12h, água e alimentação *ad libitum*. Os mesmos foram distribuídos em dois grupos: grupo não infectado (controle, n=12) e grupo infectado (n=12). A infecção foi realizada via intraperitoneal, usando 0,2 mL de sangue, contendo 1,05 x 10^4 tripomastigotas de *T. cruzi* (cepa Y), de camundongos previamente infectado para reativação e multiplicação da cepa.

3.2.2 Cepa Y:

Tripomastigotas de *T. cruzi* (cepa Y) (SILVA E NUSSENZWEIG, 1953) criopreservadas em nitrogênio líquido foram utilizadas para a infecção do modelo experimental. Inicialmente um camundongo foi infectado, para a reativação da cepa e para obter parasitos suficientes para a infecção.

Figura 3 - Desenho experimental I – os procedimentos experimentais iniciam-se com a reativação da cepa em animais, via intraperitoneal. Após a reativação o sangue, com formas tripomastigotas, será inoculado via intraperitoneal nos grupos experimentais para realizar a infecção. O acompanhamento da parasitemia é realizado ao longo de todo o período experimental. Em momentos específicos, a eutanásia é realizada para a coleta de amostras para as análises.



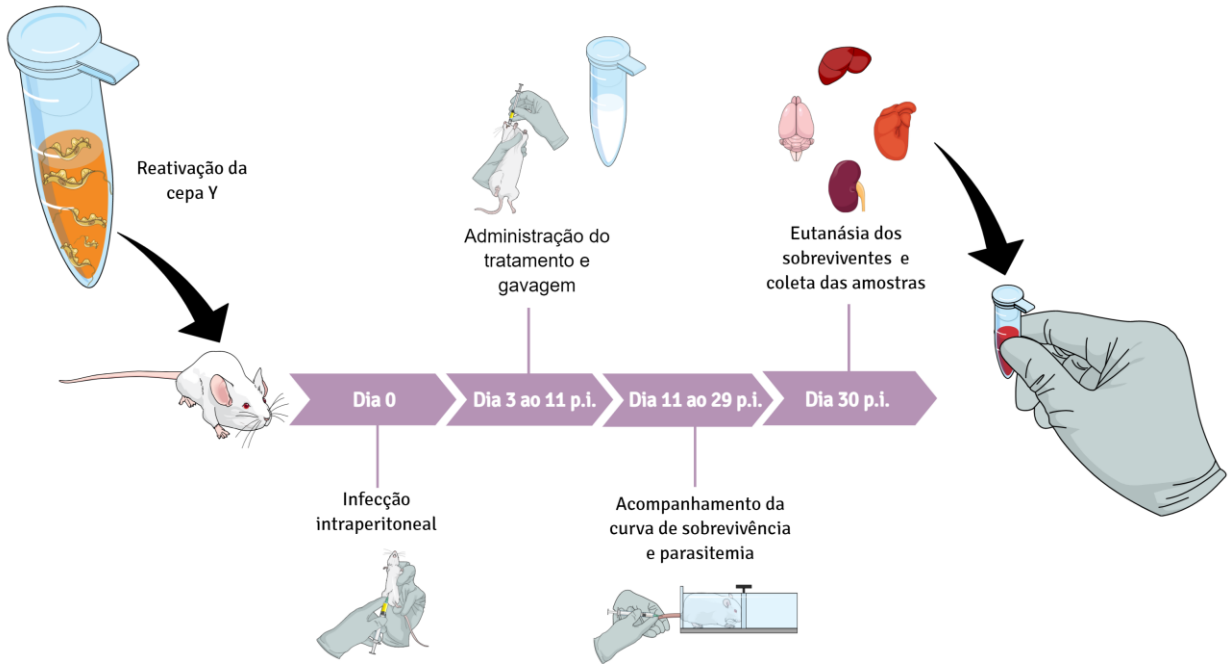
Fonte: Autora.

3.3 Experimento II – Efeitos de diferentes doses de benznidazol nanoencapsulado no tratamento da infecção experimental com *T. cruzi*

3.3.1 Modelo Animal e desenho experimental

Para a realização do experimento de curva de doses de NBNZ (fig. 2), foram utilizados 40 camundongos *Swiss* fêmeas, com 60 dias de vida (25 – 30g), obtidos do Biotério Central da Universidade Federal de Santa Maria (Santa Maria, RS, Brasil). Os animais foram mantidos em um ambiente com temperatura controlada (23°C ± 1) e com um ciclo de 12 horas claro/escuro. As dietas, tanto sólida quanto hídrica, foram fornecidas *ad libitum*. Antes do início do experimento, os animais passaram por um período de adaptação de 7 dias.

Figura 4 - Desenho experimental II - os procedimentos experimentais iniciam-se com a reativação da cepa em animais, via intraperitoneal. Após a reativação o sangue, com formas tripomastigotas, será inoculado via intraperitoneal nos grupos experimentais para realizar a infecção. O acompanhamento da parasitemia é realizado ao longo de todo o período experimental. Os tratamentos foram administrados via gavagem. Acompanhou-se a sobrevivência dos animais ao longo de 30 dias e ao final do período os animais que permaneciam vivos foram submetidos a eutanásia.



Fonte: Autora.

3.3.2 Delineamento Experimental

Para a realização do protocolo experimental, os animais foram divididos em sete grupos, de forma aleatória, além disso, 5 animais foram destinados ao processo de reativação da cepa. Os sete grupos contendo um total de 5 animais por grupo, foram identificados da seguinte forma:

Grupo 1: Controle negativo – animais não infectados e não tratados

Grupo 2: Controle positivo – animais infectados e não tratados

Grupo 3: NBNZ 5mg/Kg – animais infectados e tratados com a dose de 5mg/Kg de benznidazol nanoencapsulado

Grupo 4: NBNZ 10mg/Kg – animais infectados e tratados com a dose de 10mg/kg de benznidazol nanoencapsulado

Grupo 5: NBNZ 15mg/Kg – animais infectados e tratados com a dose de 15mg/kg de benznidazol nanoencapsulado

Grupo 6: NBNZ 20mg/Kg – animais infectados e tratados com a dose de 20mg/kg de benznidazol nanoencapsulado

Grupo 7: BNZ – animais infectados e tratados com benznidazol livre na dose de 100mg/Kg

Os tratamentos foram iniciados após a confirmação da infecção, realizados via oral, por gavagem, ao longo de 8 dias. Após o período do tratamento, acompanhou-se a sobrevivência dos animais, de todos os grupos, durante os 30 dias subsequentes.

3.4 Experimento III: Efeitos do benznidazol nanoestruturado no tratamento da Doença de Chagas.

3.4.1 Modelo Animal

Foram utilizados 55 camundongos fêmeas com 60 dias de vida (25 – 30g), obtidos do Biotério Central da Universidade Federal de Santa Maria (Santa Maria, RS, Brasil). Os animais foram mantidos em um ambiente com temperatura controlada (23°C ± 1) e com um ciclo de 12 horas claro/escuro. As dietas, tanto sólida quanto hídrica, foram fornecidas *ad libitum*. Antes do início do experimento, os animais passaram por um período de adaptação de 7 dias.

3.4.2 Delineamento experimental

Para o tratamento com benznidazol, na forma livre e de nanocápsula (fig. 3), foram utilizados 55 animais divididos em grupos não infectados e infectados com *T. cruzi*, sendo que 7 animais foram utilizados em processos de reativação das cepas. Os animais foram divididos então de forma aleatória em oito grupos, com 6 camundongos em cada grupo, como descrito abaixo:

Grupo 1: Controle negativo- animais sadios sem tratamento

Grupo 2: Controle positivo- animais infectados com cepa Y, sem nenhum tratamento

Grupo 3: Controle negativo veículo - animais sadios e tratados com veículo da nano (nano branca) via oral por 8 dias consecutivos, em intervalos de 24h;

Grupo 4: Controle positivo veículo- animais infectados com a cepa Y e tratados com veículo da nano (nano branca) via oral por 8 dias consecutivos, em intervalos de 24h;

Grupo 5: Animais não- infectados e tratados com 100 mg/kg/dia de benznidazol via oral por 8 dias consecutivos, em intervalos de 24h;

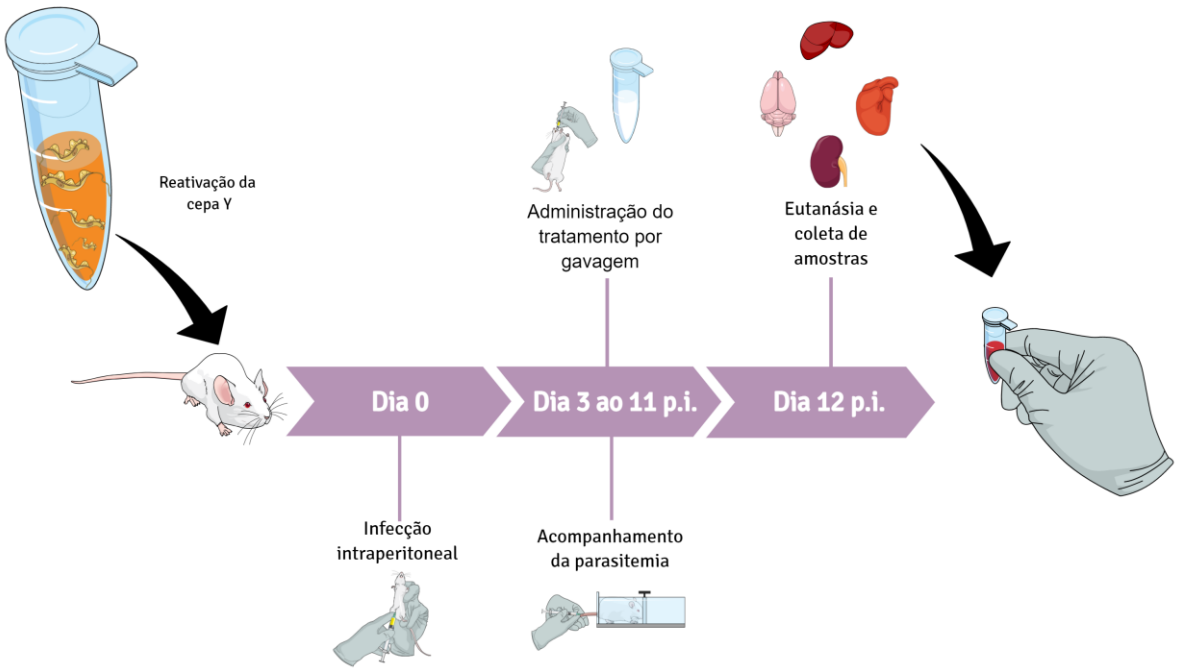
Grupo 6: Animais infectados com a cepa Y e tratados com 100 mg/kg/dia de benznidazol via oral por 8 dias consecutivos, em intervalos de 24h;

Grupo 7: Animais não infectados com a cepa Y e tratados com 20 mg/kg/dia de nanocápsulas de benznidazol via oral por 8 dias consecutivos, em intervalos de 24h;

Grupo 8: Animais infectados com a cepa Y e tratados com 20 mg/kg/dia nanocapsulas de benznidazol via oral por 8 dias consecutivos, em intervalos de 24h.

Os tratamentos iniciaram após a confirmação da infecção, através de análise de parasitemia, o que se dá, aproximadamente, entre o 3º e 5º dia após a infecção (experiência do grupo de pesquisa). A dose do benznidazol prevista nesse estudo é amplamente usada em estudo com camundongos, isto é, a dose de 100 mg/kg/dia (FILARDI, BRENER, 1987, FRACASSO *et al*, 2021). Como a nanotecnologia costuma potencializa o efeito de drogas, foram realizados experimentos prévios, para o ajuste das doses, verificação do potencial e citotoxicidade das formulações em nanoestruturas.

Figura 5 - Desenho experimenta III - os procedimentos experimentais iniciam-se com a reativação da cepa em animais, via intraperiotoneal. Após a reativação o sangue, com formas tripomastigotas, será inoculado via intraperitoneal nos grupos experimentais para realizar a infecção. O acompanhamento da parasitemia é realizado ao longo de todo o período experimental. Os tratamentos foram administrados via gavagem. Ao final do tratamento os animais foram submetidos a eutanásia para a coleta de amostras para a realização de análises.



Fonte: Autora.

4. MATERIAIS, MÉTODOS E RESULTADOS

A metodologia e os resultados referentes a esta tese estão apresentados sob a forma de um artigo e dois manuscritos científicos. Os itens Materiais e Métodos, Resultados e discussão, e conclusão encontram-se descritos nos próprios manuscritos.

O artigo apresentado nesta tese está baseado na versão publicada na revista "*Molecular and Celullar Biochemistry"*, sob o título "*Chagas disease: modulation of the inflammatory response by acetylcholinesterase in hematological cells and brain tissue*".

A apresentação do primeiro e segundo manuscrito é referente às versões a serem submetidas a revistas ainda não definidas.

4.1 Artigo I

Título: “Chagas disease: modulation of the inflammatory response by acetylcholinesterase in hematological cells and brain tissue”

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Chagas disease: Modulation of the inflammatory response by acetylcholinesterase in hematological cells and brain tissue

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Abstract

Chagas disease is an acute or chronic illness that causes severe inflammatory response, and consequently, it may activate the inflammatory cholinergic pathway, which is regulated by cholinesterase, including the acetylcholinesterase. This enzyme is responsible for the regulation of acetylcholine levels, an anti-inflammatory molecule linked to the inflammatory response during parasitic diseases. Thus, the aim of this study was to investigate whether *Trypanosoma cruzi* infection can alter the activity of acetylcholinesterase and acetylcholine levels in mice, and whether these alterations are linked to the inflammatory cholinergic signaling pathway. Twenty-four mice were divided into two groups: uninfected (control group, n=12) and infected by *Trypanosoma cruzi,* Y strain (n=12). The animals developed acute disease with a peak of parasitemia day 7 post-infection (PI). Blood, lymphocytes and brain were analyzed on days 6 and 12 post infection. In the brain, acetylcholine and nitric oxide levels, myeloperoxidase activity and histopathology were analyzed. In total blood and brain, acetylcholinesterase activity decreased at both times. On the other hand, acetylcholinesterase activity in lymphocytes increased on day 6 PI compared to the control group. Infection by *T. cruzi* increased acetylcholine and nitric oxide levels and histopathological damage in the brain of mice associated to increased myeloperoxidase activity. Therefore, an intense inflammatory response in mice with acute Chagas disease in the central nervous system caused an anti-inflammatory response by the activation of the cholinergic inflammatory pathway.

Keywords: Chagas disease; acetylcholine, acetylcholinesterase, inflammatory process.

INTRODUCTION

Chagas disease (CD) is an endemic illness in the Americas caused by the protozoan *Trypanosoma cruzi* [1], and it is related to rural areas, poverty, immigration, and poor work conditions [2]. In Brazil, endemic regions include areas from the South, Southeast, Northeast and Midwest of the country mainly by chronic disease, which evolves to clinical disease with cardiac failure, meningitis and encephalitis [3].

CD causes an exacerbated inflammatory process in the brain, leading to several microvasculopathy due to increased leukocytes adhesion in the brain and arteriolar endothelial

dysfunction in the central nervous system (CNS) [4]. These pathological changes may affect different mechanisms, such as the cholinergic system [5], that is involved in many physio pathological events, such as those described by Wolkmer *et al.* [6] during the inflammatory response caused by *Trypanosoma evansi*.

The cholinergic system is notably involved in anti-inflammatory reactions [7], and the cholinesterases are enzymes present in cholinergic and non-cholinergic tissues. The acetylcholinesterase (AChE) is a specific cholinesterase enzyme involved in the immune response by modulating acetylcholine (ACh) levels, a molecule with anti-inflammatory properties [8, 9]. The relation between AChE activity and ACh concentration can characterize a pro or anti-inflammatory response, i.e. when AChE activity increases, there is a reduction in ACh levels in the brain extracellular space [9, 10], reflecting a pro-inflammatory profile, which contributes to disease pathophysiology.

The ACh is the main neurotransmitter, but also an anti-inflammatory molecule that controls the release of pro-inflammatory cytokines [10]. These cytokines (IFN-γ (interferon-gamma) and TNF (tumor necrosis factor)) triggered by *T. cruzi* acute infection also stimulate infected macrophages to produce large amounts of nitric oxide (•NO) via the enzymatic activity of inducible nitric oxide synthase (iNOS) [11]. Therefore, considering the importance of cholinergic system in the immune response, this study aimed to investigate whether *T. cruzi* infection can alter the AChE activity and ACh levels in mice, and if these alterations are linked to the inflammatory cholinergic signaling.

MATERIALS AND METHODS

Strain Y

Trypomastigotes of *T. cruzi* (strain Y) [12] cryopreserved in liquid nitrogen was used in the experimental model. Initially, a mice (M1) was infected by *T. cruzi* in order to reactivate the strain, and to obtain enough parasites for the infection.

Animal model and experimental design

Twenty-four Swiss mice (45 days of age, 20 ± 3 g of body weight) from the Central Animal House of the Universidade Federal de Santa Maria (UFSM) were used in this experiment. They were maintained in a room with constant temperature (23 ± 1 ºC, relative humidity 70 %)

on a 12 h light/dark cycle with access to feed and water *ad libitum*. They were divided into two groups with 12 animals each: uninfected group (the control) and infected group. Infection was through the intraperitoneal route using 0.2 mL of blood containing 1.05 x 10^4 trypomastigotes of *T. cruzi* (strain Y) from a mice previously infected (M1).

Blood parasitemia evaluation

The infection was monitored by counting the number of motile parasites in 5 μL of fresh blood sample drawn from the lateral tail vein, as recommended by a standard protocol [13]. The number of trypomastigotes was recorded in a two-day interval from 2 to 12 days post-infection (PI), and expressed as parasites/mL of blood.

Sample collection

The animals were anesthetized with isoflurane and euthanized by decapitation on days 6 (n=6 mice per group) and 12 (n=6 mice per group) PI. Blood samples were collected in tubes containing EDTA as anticoagulant for lymphocytes isolation, or hemolyzed with phosphate buffer (pH 7.4) containing Triton X-100, followed by storage at − 30 °C for 1 week. The brain structure was homogenized in a glass potter in 10 mM of Tris–HCl buffer (1:10 w/v), centrifuged at 7500 *x g* during 10 min, and the supernatants were stored at −80 °C until utilization.

Lymphocyte isolation

Lymphocytes-rich mononuclear cells were isolated from peripheral blood collected with 129 mM of sodium citrate as anticoagulant, and separated in Ficoll-Histopaque density gradient, as described by Böyum [14]. Protein in lymphocytes was measured by the Comassie Blue method according to Bradford [15], using serum albumin as standard.

AChE activities

Whole blood AChE activity was determined by the method of Ellman *et al.* [16] modified by Worek *et al.* [17]. The specific activity of whole blood AChE was calculated from the quotient between the AChE activity, and hemoglobin content. The results were expressed as mU/μmol Hb. Already, the AChE activity in lymphocytes was determined according to the method described by Ellman *et al.* [16] modified by Fitzgerald and Costa [18]. Briefly, proteins of all

samples were adjusted to 0.1–0.2 mg/mL. Volume 0.2 mL of intact cells were added to a solution containing 1.0 mM of acetylthiocholine (ATC), 0.1 mM of 5.5´–dithio bis (2-nitrobenzoic acid) (DTNB), and 0.1 mM of phosphate buffer (pH 8.0). Immediately before and after incubation for 30 min at 27 º C the absorbance was read on a spectrophotometer at 412 nm. AChE was calculated from the quotient between lymphocytic AChE activity and protein content, and the results were expressed as µmol/AcSCh/mg of protein.

The cerebral AChE enzymatic assay was determined by Ellmann *et al.* [16] as previously described by Rocha *et al.* [19]. The reaction mixture (final volume of 2 mL) contained 100 mM of phosphate buffer (pH 7.5) and 1 mM of DTNB. The method is based on the formation of the yellow anion, 5,5′-dithio-bis-acid-nitrobenzoic, measured by absorbance at 412 nm during 2 min incubation at 25 °C. The enzyme (40–50 µg of protein) was pre-incubated for 2 min. The reaction was initiated by adding 0.8 mM of acetylthiocholine iodide (AcSCh). All samples were run in triplicate and the enzyme activity was expressed in µmol AcSCh/h/mg of protein.

Acetylcholine levels in the brain

Acetylcholine levels were assayed using the EnzychromTM Acetylcholine (ACh) assay kit. In this assay, acetylcholine is hydrolyzed by acetylcholinesterase to choline, which is then oxidized by choline oxidase to betaine and hydrogen peroxide. The resulting hydrogen peroxide reacts with a specific dye to form a pink-colored product. The color intensity at the 570 nm spectrum wavelength is directly proportional to the acetylcholine concentration in the sample [20]. The absorbance of each sample was compared to a standardized curve and the concentration was calculated in micromolars (µM).

Myeloperoxidase assay

Tissue myeloperoxidase (MPO) assay was assessed according to the techniques described by Suzuki *et al.* [21]. Brain samples were homogenized with a motor-driven homogenizer in Tris-HCl (50mM, Ph 7.5, 1% Triton-X 100) buffer. Brain homogenate was centrifuged at 10.000 *x g* at 4 °C for 15 min, and the supernatant was stored at 4 °C until utilization. For evaluation of MPO activity, 30 µL of the supernatant was incubated with 390 µL of aminoperimidina (25 mM) and 450 µL of H_2O_2 (1.7 mM) at 37 °C for 30 min. The reaction was stopped in a cold bath, and colorimetrically read at 492 nm on a microplate reader. The results were expressed as optical

density (OD)/mg of protein.

Nitric oxide (nitrite/nitrate - NOx) levels

Cerebral NO levels were measured using an indirect method by evaluation of nitrite/nitrate (NOx) levels. For NOx determination, an aliquot (200 µL) was homogenized in 200 mM of Zn_2SO_4 and acetonitrile (96%, HPLC grade). After, the brain homogenate was centrifuged (Eppendorf centrifuge 5415 R, Germany) at 15,000 *x g* for 30 min at 4 °C, the supernatant was separated for analysis of the NO content as previously described [22]. NO content in brain tissue was estimated in a medium containing 70 µL of 2% vanadium chloride (VCl_3) in 5% HCl, 70 µL of 0.1% N-(l-naphthyl) ethylenediamine dihydrochloride and 2% sulphanilamide (in 5% HCl) in 1:1 ratio. After incubating at 37 °C for 60 min, nitrite level, which corresponds to an estimative level of NO, was determined spectrophotometrically at 570 nm, based on the reduction of nitrate to nitrite by VCl_3. The nitrite and nitrate levels were expressed as nanomole of NO/mg of protein.

Histopathology

Heart and brain samples were collected, fixed in 10% buffered formalin, embedded in paraffin wax and routinely processed for hematoxylin and eosin (HE) staining followed by histopathological examinations. Heart and brain sections were examined under an optical microscope in a blind way, and lesions were scored as mild, moderate, or severe.

Statistical analysis

First, the data were submitted to the normality test, which proved to be normal. Then, the data obtained were analyzed statistically by the Student's t test for independent samples, and considered significant when probability (P) was <0.05. Variables were expressed as mean ± standard deviation.

RESULTS

Course of infection: parasitemia

Trypomastigotes in *T. cruzi* infected mice were observed four days PI, and the peak of parasitemia occurred on day 7 PI (Figure 1). During the experimental period, infected animals showed no apparent clinical signs.

AChE activities

The results of AChE activities in total blood, lymphocytes and brain tissue were showed in Figure 2. AChE activity decreased in total blood and brain tissue (Figures 2A and 2C) in animals infected by *T. cruzi* on days 6 and 12 PI compared to the uninfected control group. The AChE activity in lymphocytes increased compared to the control group on day 6 PI (Figure 2B).

ACh levels

Results of cerebral ACh levels can be seen in Figure 3. ACh levels increased in brain of animals infected by *T. cruzi* on days 6 and 12 PI compared to the control group.

Myeloperoxidase

Results regarding cerebral MPO activity were shown in Figure 4A. The cerebral MPO activity increased in animals infected by *T. cruzi* on day 12 PI compared to the control group.

NOx levels

The results of NOx levels in brain were shown in Figure 4B. NOx levels increased in the brain of animals infected by *T. cruzi* on days 6 and 12 PI compared to the control group.

Histopathology

Uninfected animals did not show macroscopical or microscopical alterations in heart and brain tissues (data no shown). Macroscopically, infected animals showed no cardiac or brain alterations. However, the heart of mice experimentally infected by *T. cruzi* showed several pseudocysts containing amastigotes within cardiomyocytes, and necrosis associated with diffuse moderate to severe inflammatory infiltrate of lymphocytes (Figure 5A). In the meninges, light focal lymphoplasmacytic infiltrate was observed, as well as mild to moderate multifocal gliosis in the cerebral cortex (Figure 5B).

DISCUSSION

Cholinesterases in the CNS have been extensively studied not only because they are involved in the cholinergic neurotransmission [8, 23], but also due to their role on immune response during parasitic infections [5]. In this sense, several studies have reported that

cholinergic system are important in the modulation of the inflammatory and immune responses [24]. In this study, alterations in the enzymatic activity involved in the regulation of ACh levels were found in animals infected by *T. cruzi*, similarly as observed during *T. evansi* infection [6, 25].

A significant decrease in AChE activity in the brain was observed in animals infected by *T. cruzi*, in agreement with Da Silva *et al.* [5] while studying rats infected by *T. cruzi*. Decreased AChE activity in the brain consequently increased ACh levels in the synaptic cleft, which may lead to increased anti-inflammatory responses. An anti-inflammatory response may have occurred through a cholinergic pathway in mice infected by *T. cruzi*, using nicotinic acetylcholine receptors in macrophages [7, 26]. These receptors modulate interactions between the nervous system and the immune system, and these modulations causes an anti-inflammatory response [9].

The ACh is an important neurotransmitter for learning and memory activities [27], and when in high levels in the brain of chagasic mice, it contributed to reduce the inflammatory damage, since this molecule is a cholinergic neurotransmitter with anti-inflammatory profile. It is important to emphasize that ACh is capable to inhibit cytokine proliferation, NO and lysosomal enzymes, which are mediators of inflammatory processes [9, 28, 29].

In summary, decreased cerebral AChE activity exerts an anti-inflammatory profile since increased ACh levels is an attempt to reduce cerebral inflammatory damage. In addition, an increase in NOx levels on day 6 PI in brain of infected mice corroborate to the pathological changes founded in brain. The NO is produced during macrophage activation in order to destroy the parasite, exerting an important role in the host defense through the anti-inflammatory system when released at low concentrations for a brief period of time, but high concentrations of NO cause damage to adjacent cells and tissues [30], contributing to the appearance of neurological clinical signs observed in cases of trypanosomosis by *T. evansi* and *T. brucei* [31, 32]. Furthermore, the cytotoxicity of NO may be due to its ability to generate peroxynitrite, initiating a variety of oxidative reactions, including modifications of nucleic acids, lipids and proteins, leading to brain injury [31].

Nevertheless, the Y strain of *T. cruzi* investigated in this study also affected the cholinergic signaling in non-neural cells. The AChE activity was increased in lymphocytes, and decreased in whole blood, demonstrating that in the acute phase (day 6 PI), when the mice showed a high number of circulating parasites, an increase was observed in the AChE activity in

lymphocytes compared to uninfected animals. These results suggest an inflammatory response against *T. cruzy* and are in accordance with Da Silva *et al.* [33] that also showed an increase in AChE activity in lymphocytes attached to an increased number of circulating lymphocytes in *T. evansi* infected rats. Several researches with patients infected by *T. cruzi*, showed the increase in cytokines like IL-6, IL-10 and TNF- α in human as an inflammatory response to *T. cruzi* infection [34, 35, 36]. The cytokines shown an increase in patients with infection by *T. cruzi* when compared to healthy patients. Furthermore, some cytokines demonstrated higher levels in asymptomatic individuals with Chagas disease, when compared to patients with cardiomyopathy by CD [34].

During CD, myeloperoxidase is released during the degranulation of neutrophils and monocytes [37], and this process occurs to recruit these cells to defend the host against *T. cruzi* invasion that may cause injury in cells and tissues [37-40]. The results show an increase in myeloperoxidase activity in the brain of mice infected by *T. cruzi*, that might be related to the inflammatory process caused by *T. cruzi* as demonstrated by histopathology, i.e. infiltration of granular cells in the CNS. The infected mice showed multiple pseudocysts containing amastigotes within cardiomyocytes, extensive focal necrosis associated with diffuse moderate to severe inflammatory infiltration of lymphocytes, and light focal lymphoplasmocytic infiltrate in the meninges.

Based on these results, enzymatic activities can act modulating the anti-inflammatory response in the brain of mice infected by *T. cruzi*, through higher ACh levels. While a pro-inflammatory response in lymphocytes is responsible for the recruitment of granular cells and increase NOx levels against amastigotes in cases of CD. These findings reinforce the hypothesis that cholinesterase's may have pro- and anti-inflammatory characteristics during *T. cruzi* infection. Thus, AChE and MPO activities, as well as the NOx levels may be used as inflammatory markers due to a significant increase of them during the acute phase of CD, in addition to the fact that increased ACh levels showed a negative feedback against the inflammatory process. The literature shows the inflammatory process in human related to cytokines levels alterations during Chagas disease, but not related to cholinergic system alteration, this can be a new possible focus to continue researches.

Ethical Committee

The present study was approved by the Ethics Committee for Use of Animals (CEUA) of the Universidade Federal de Santa Maria (UFSM) under protocol number 3960110915.

Conflict of interest

The authors have declared no conflict of interest.

Acknowledgments

Professor Mario Steindel from the Protozoology Laboratory of Universidade Federal de Santa Catarina (UFSC) for providing the *T. cruzi* strain used in this study. The funding agency CNPq for the research productivity grants and financial resource.

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Figures and Legends

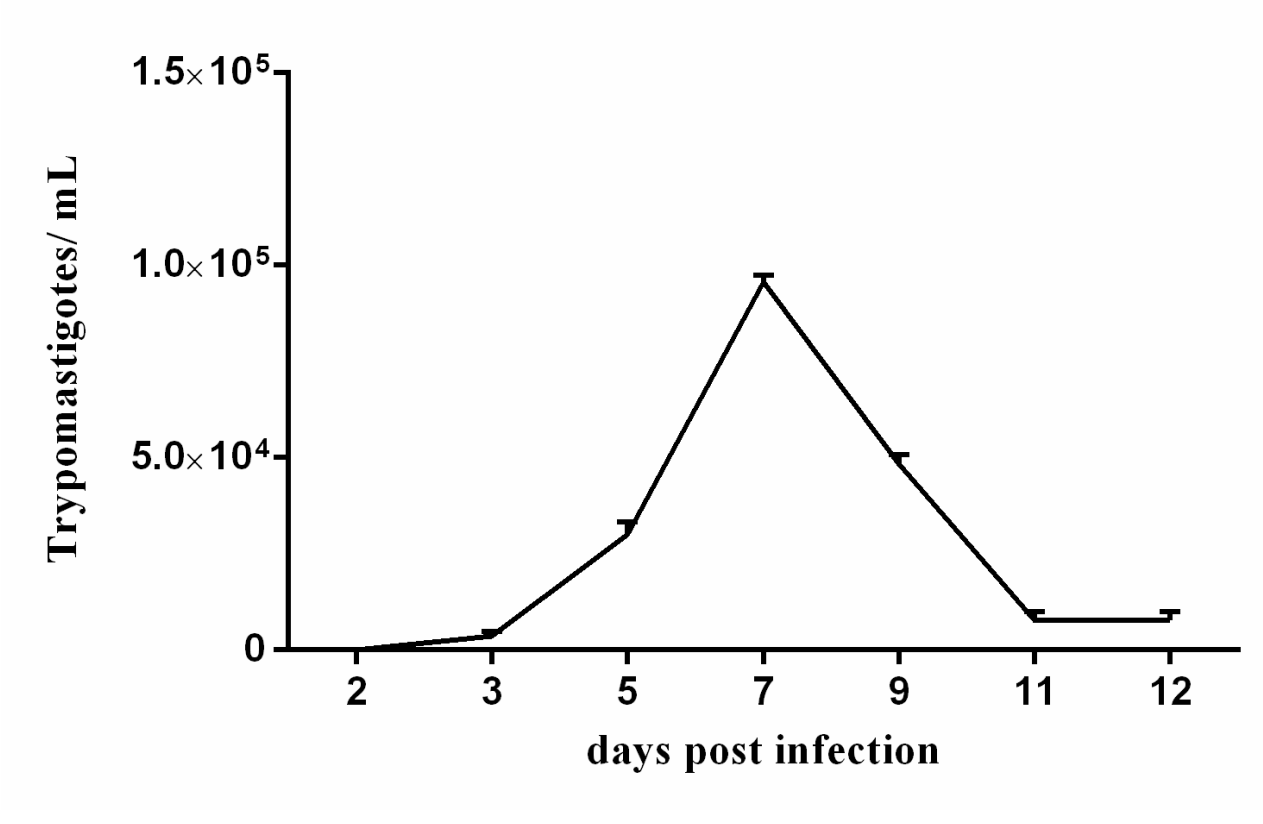


Figure 1. Parasitemia of mice experimentally infected by *Trypanosoma cruzi* (Y strain) during 12 days of the experiment.

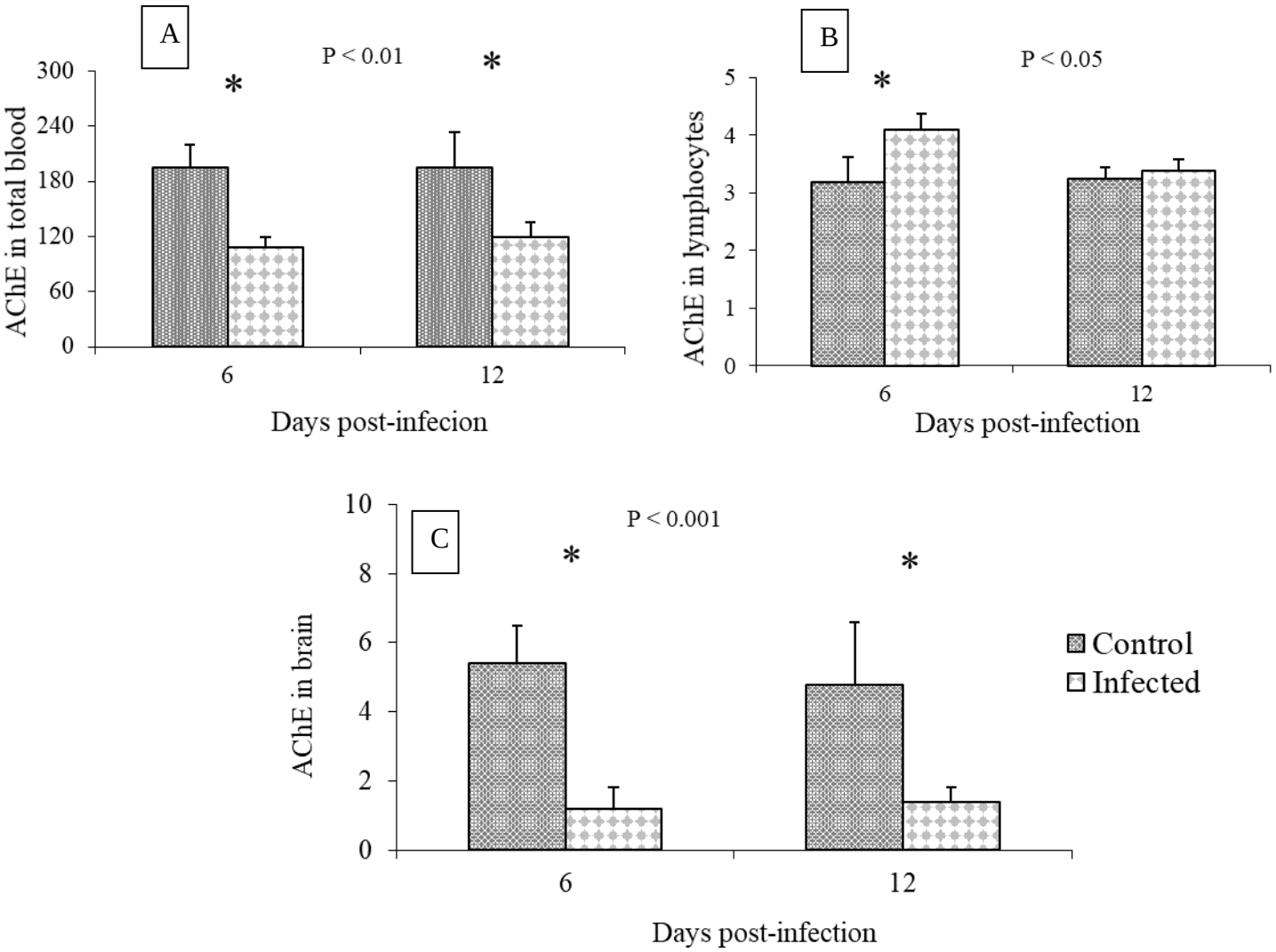


Figure 2. Mean and standard deviation of acetylcholinesterase (AChE) activity in total blood (mU/µmol of wole blood), lymphocytes (µmol/AcSCh/mg of protein) and brain (µmol AcSCh/h/mg of protein) of mice experimentally infected by *Trypanosoma cruzi* compared to uninfected control group on days 6 and 12 post-infection (PI). Groups with * are statistically different using the Student's *t* test for independent samples.

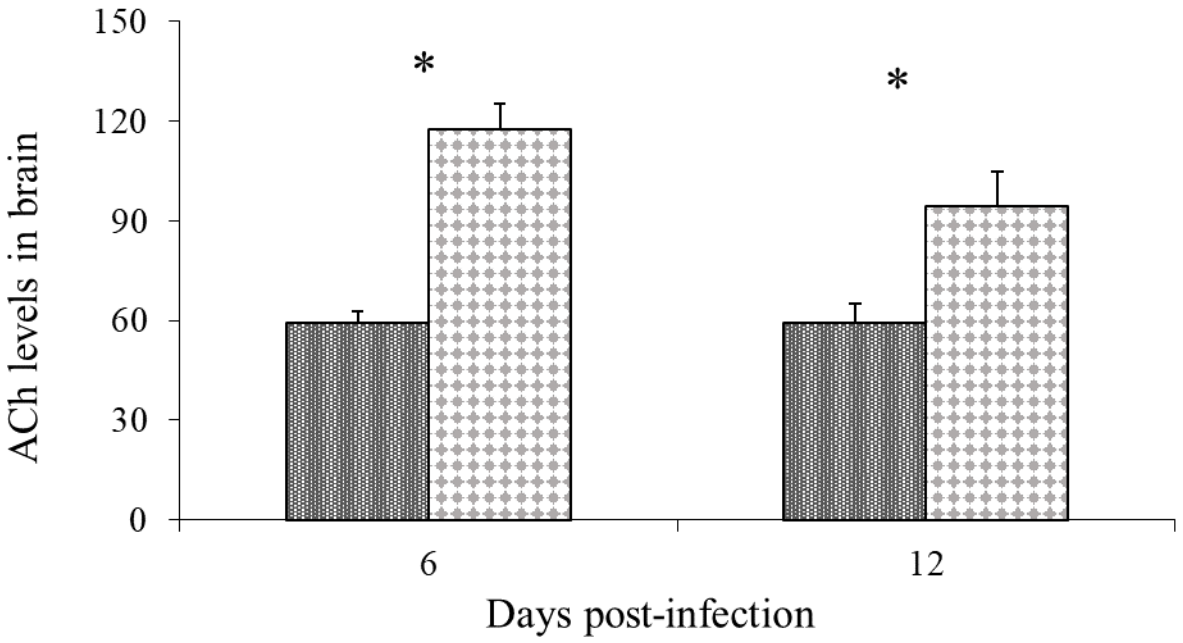
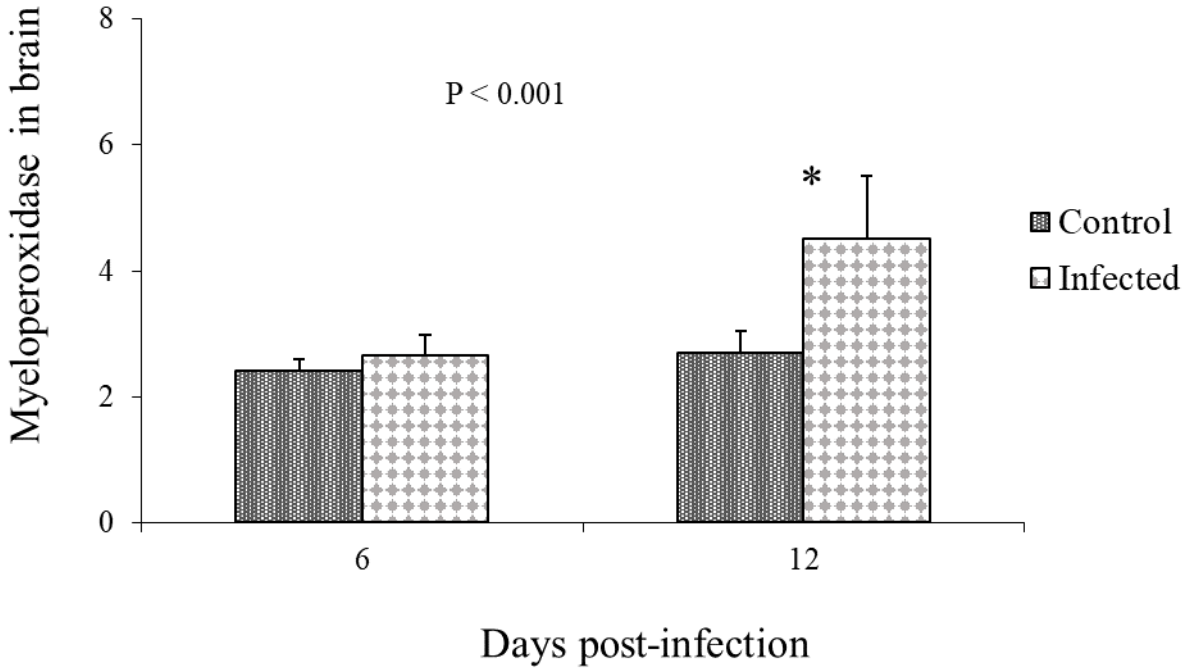


Figure 3. Acetylcholine (ACh) levels in brain of mice experimentally infected by *Trypanosoma cruzi* (white column) compared to uninfected (control - black column). The results were expressed as mean and standard deviation. Student's t test for independent samples was used for all the analyses (*P<0.001).



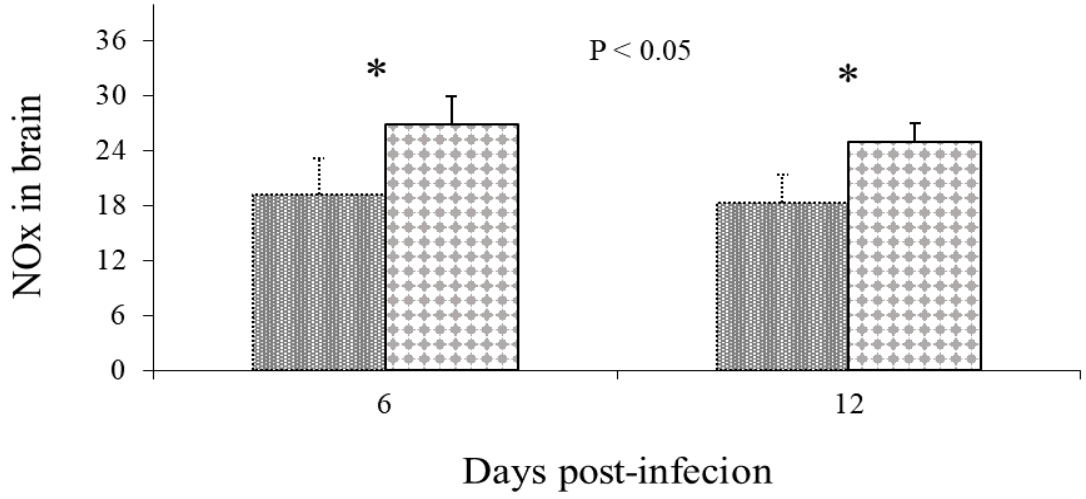


Figure 4. Myeloperoxidase activity and nitrite/nitrate (NOx) levels in the brain of mice experimentally infected by *Trypanosoma cruzi* compared to uninfected. The results were expressed as mean and standard deviation. Student's t test for independent samples was used for all the analyses.

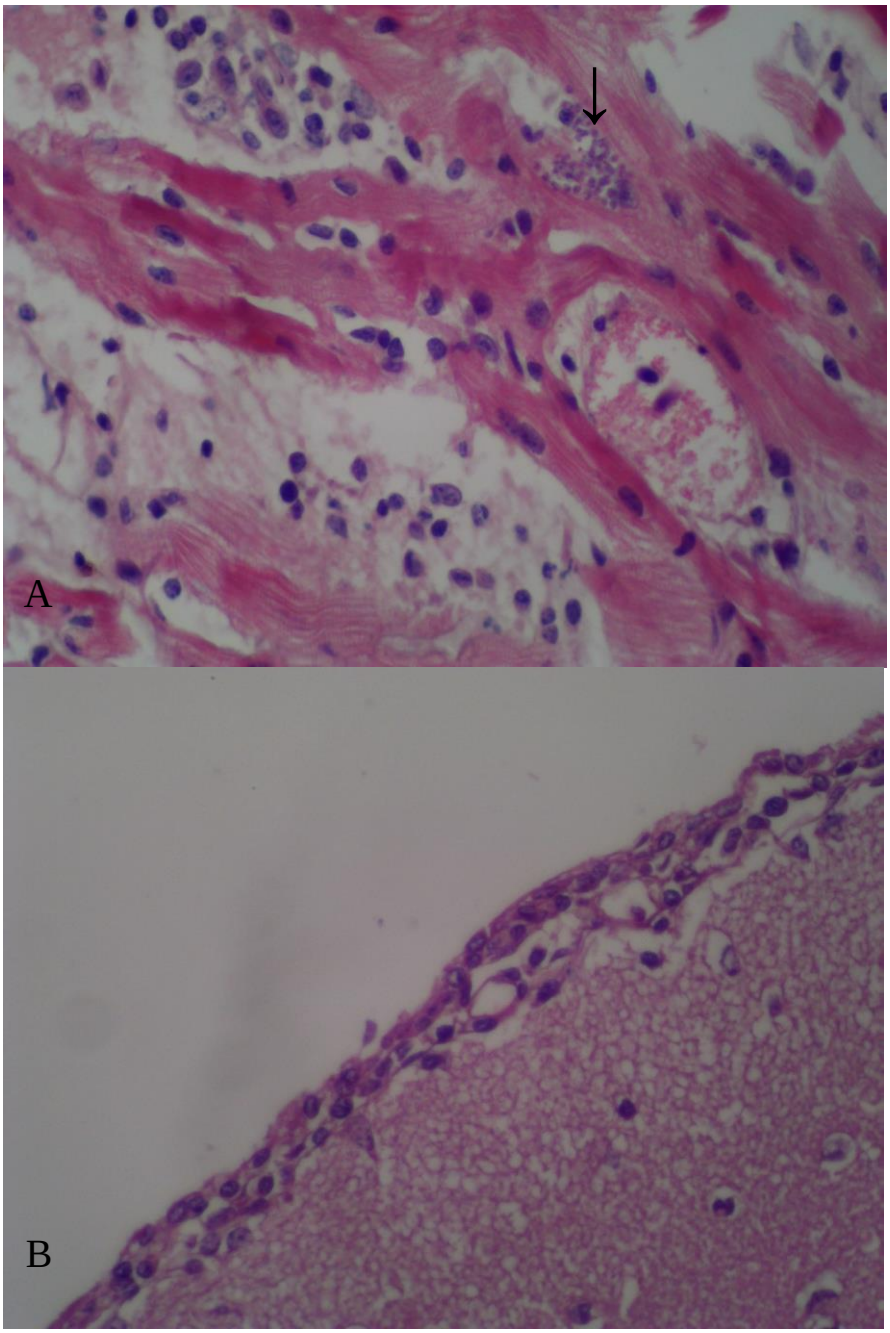


Figure 5. Mice experimentally infected by *Trypanosoma cruzi*. (A) Multiple pseudocysts containing amastigotes inside cardiomyocytes, focally extensive necrosis associated with diffuse and moderate to severe inflammatory infiltrate of lymphocytes. (B) Light focal lymphoplasmocytic infiltrate in the meninges.

4.2 Manuscrito I

Does the intake of nanoencapsulated benznidazole control acute *Trypanosoma cruzi* infections?

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Abstract

The Chagas Disease (CD) affects around eight million people worldwide. It is considered a neglected disease that presents few treatment options with efficacy only in the acute phase. Nanoparticles have many positive qualities for treating parasite infections and may be effectively and widely employed in clinical medicine. This research aimed to evaluate the nanoencapsulated benznidazole treatment in animals experimentally infected with *Trypanosoma cruzi*. To analyze the treatment efficacy, we evaluated survival during thirty days, parasitemia, genotoxicity, and heart and liver histopathology. Thirty-five female Swiss mice were organized into seven groups characterizing a dose curve: A – Negative control (uninfected animals), B – Positive control (infected animals), C – Benznidazole (BNZ) 100 mg/kg (infected animals), D – 5 mg/kg Benznidazole nanocapsules (NBNZ) (infected animals), E – 10 mg/kg Benznidazole nanocapsules (infected animals), F – 15 mg/kg Benznidazole nanocapsules (infected animals), G – 20 mg/kg Benznidazole nanocapsules (infected animals). The animals were infected with the Y strain of *T. cruzi* intraperitoneally. The treatment was administered for eight days by oral gavage. It was possible to observe that the treatment with the highest NBNZ dose presented efficacy similar to the standard benznidazole drug. The 20 mg/kg NBNZ dose was able to reduce parasitemia, increase survival, and drastically reduce heart and liver tissue damage compared to the 100 mg/kg BNZ dose. Moreover, it showed a lower DNA damage index than the BNZ treatment. In conclusion, the nanoencapsulation of BNZ promotes an improvement in parasite proliferation control with a five times smaller dose relative to the standard dose of free BNZ, thus demonstrating to be a potential innovative therapy for CD.

Keywords: acute phase, nanocapsules, Chagas Disease, treatment, pathogenesis.

1. Introduction

Trypanosoma cruzi is a hemoflagellate protozoan that causes Chagas Disease (CD), a neglected disease first described in 1909 [1]. The World Health Organization estimated that eight million people are infected worldwide, leading to 12,500 deaths per year, mainly in Latin America [2]. The route of infection is via insect vectors from the Triatominae subfamily (Order: Hemiptera, Family: Reduviidae), blood transfusions, accidents, oral infections, and congenital transmission [3-5].

CD is often asymptomatic. However, specific symptoms such as myalgias and fever may occur in the acute phase of the disease. Following the acute phase, CD progresses to the chronic phase and may remain asymptomatic for years or be characterized by cardiac, digestive, and/or neurological pathologies that may lead to systemic and pulmonary embolisms and, in the most severe cases, sudden death [6-7].

There are currently two drugs to control CD: Benznidazole (BNZ), a first-choice drug for treating the disease, and Nifurtimox, the second-line option to benznidazole. However, the Brazilian Health Regulatory Agency (ANVISA) only clears benznidazole for drug treatment in Brazil. According to the BNZ package insert, numerous side effects of the drug have been reported, such as skin reactions, nausea, tingling or nerve inflammation symptoms, headaches, vertigo, fatigue, and changes in blood, besides relative time treatments [2, 8-9].

In addition to the aforementioned side effects, another point that hinders the treatment is the dosage of the drug. Currently, it is marketed by the Pharmaceutical Laboratory of the State of Pernambuco (LAFEPE) in two 100 mg or 12.5 mg formulations, making treatment difficult in children, for example, for whom the dose is 5 mg/kg of body weight to 10 mg/kg for sixty consecutive days according to the manufacturer's recommendations. Thus, the efficacy of the drug therapy declines with the duration of the infection and is still a matter of debate in the late chronic phase of CD [7, 10-11].

Nanoparticulate drug delivery systems have emerged as a promising area of research in the therapy and prevention of neglected tropical diseases such as CD. These delivery systems provide novel mechanisms for targeted drug delivery within the host, maximizing therapeutic effects while minimizing systemic side effects [12]. However, as the study of nanocapsules is increasing, we find several types of nanocapsules.

Several studies have characterized encapsulated formulations of natural compounds or drugs. [13] showed that the use of Eudragit® nanocapsules potentiates the compound actions at doses ten times smaller than the usual dose. Vinuesa *et al.* examined the efficacy of a novel BZN-carrier nanostructure in fibroblasts *in vitro* and *in vivo* murine models, finding reduced mammalian cell cytotoxicity, decreased parasite viability, and increased infected mice survival rates [14]. Similarly, lychnopholide encapsulated in polymeric nanocapsules has been shown to be effective in *T. cruzi* infections and just as effective as the standard BZN treatment during the acute phase *via* intravenous administration in murine models [15]. This study aimed to

characterize BNZ Eudragit nanocapsules and evaluate their efficacy in an acute infection by the Y strain of *Trypanosoma cruzi*.

2. Material and Methods

2.1 Benznidazole nanocapsule development and characterization

Benznidazole (N-Benzyl-2-nitro-1H-imidazole-1-acetamide) (97%) was acquired from Sigma-Aldrich (São Paulo, Brazil), and the Eudragit L100 polymer was kindly donated by the Evonik company (São Paulo, Brazil).

2.1.1 Development of the nanocapsules

Nanocapsules containing benznidazole (1 mg/mL) were developed using the preformed polymer interfacial deposition method [16], with modifications. The formulation consisted of an organic phase containing the Eudragit L100 polymer (0.25 g), benznidazole (0.025 g), sorbitan monooleate (0.19 g), medium-chain triglycerides (413 μL), ethanol (67 mL), and an aqueous phase containing polysorbate 80 (0.19 g) and ultrapure water (134 ml). The organic phase was homogenized for 60 min at 40 °C, and the aqueous phase was homogenized for 15 min without heating, both separately, with the aid of a magnetic stirrer. Subsequently, the organic phase was poured into the aqueous phase and kept under stirring for another 15 min, and then the formulation was taken to the rotaevaporator to evaporate the organic solvent under reduced pressure to the final volume (25 mL), with temperature control at 40 °C.

2.1.2 Characterization of the nanocapsules

The mean size and polydispersion index of the nanocapsules were determined by the dynamic light scattering technique (Zetasizer®, nano-ZS model ZEN 3600, Malvern) after diluting the sample 500 times in ultrapure water. The zeta potential was determined using the electrophoretic mobility technique (Zetasizer®, nano-ZS model ZEN 3600, Malvern) after 500 times dilution of the sample in an aqueous sodium chloride solution (10 mM). The pH of the nanocapsules was determined using a potentiometer (DM-22, Digimed®) previously calibrated with a standard solution, and readings were taken directly on the formulations. All readings were taken in triplicate, and the results are expressed as mean ± standard deviation [17].

2.1.3 Determination of benznidazole content and encapsulation efficiency

The benznidazole content and encapsulation efficiency were determined by High-Performance Liquid Chromatography (HPLC) (LC-20A, Prominence, Shimadzu, Japan). The analyses were performed using a LiChroCART® Purospher® STAR RP-18 endcapped column (5 µm – 250 mm × 4.6 mm) (Merck KGaA, Germany) with pre-column, by isocratic elution, with the mobile phase composed of acetonitrile:water (50:50 v/v), a flow rate of 0.8 mL/min, oven at 30 °C, detection at 322 nm, and injection volume of 20 µL. The method presented linearity from 10 µg/mL to 50 µg/mL ($y = 48740x + 70742$, $r = 0.9996$) [18].

The benznidazole content was determined after dissolving an aliquot of the nanocapsules in 10 mL of acetonitrile. Samples were sonicated for 30 min, and then an aliquot of the supernatant was diluted into a mobile phase. Samples were filtered (0.45 µm) and injected into the chromatographic system. The encapsulation efficiency was determined by the ultrafiltration-centrifugation technique with ultrafiltration devices (Microcon 10 kDa, Millipore). The ultrafiltrate was obtained by centrifuging a 400 µL aliquot of the nanocapsules for 10 min at 5000 rpm (2548 ×g). The encapsulation efficiency was calculated from the difference between the total amount of benznidazole in the nanocapsule (content) and the amount of free benznidazole (ultrafiltered). The amount of free benznidazole present in the ultrafiltrate was determined by HPLC under the same chromatographic conditions described above.

2.1.4 Stability Assessment

For the preliminary evaluation of the physicochemical stability, nanocapsules containing benznidazole ($n = 3$) were produced and stored at room temperature (25 °C ± 2 °C) and characterized according to the mean particle size, polydispersity index, zeta potential, pH, benznidazole content, and encapsulation efficiency shortly after preparation and 7, 15, and 30 days after preparation.

2.2 Experimental design

2.2.1 Animal infection

For this study, female Swiss mice were infected with the Y strain of *T. cruzi* according to the guidelines established by the Brazilian National Council on Animal Experimentation Control (CONCEA). The Y strain reactivation procedure was through intraperitoneal (i.p.) route infection

with 1×10^4 trypomastigotes from the Y strain. The parasitemia was evaluated two days post-infection to confirm the success of the procedure. All animal procedures were approved by the Ethics Committee on Animal Experimentation of the Federal University of Santa Maria, Brazil (approval number 2842070618).

2.2.2 Experimental groups

The animals were submitted to a week of acclimatization before the infection and treatment. During the experiment period, the animals were kept in 12 h light/dark cycles with controlled temperature and humidity (25 °C and 70%, respectively) and water and food *ad libitum*.

Thirty-five female Swiss mice were distributed into seven groups as follows: NC – Negative control (uninfected animals), PC – Positive control (infected animals), BNZ – 100 mg/kg Benznidazole (BNZ) (infected animals), NBNZ 5mg/kg – 5 mg/kg Benznidazole nanocapsules (infected animals), NBNZ 10mg/kg – 10 mg/kg Benznidazole nanocapsules (infected animals), NBNZ 15mg/kg – 15 mg/kg Benznidazole nanocapsules (infected animals), NBNZ 20mg/kg – 20 mg/kg Benznidazole nanocapsules (infected animals).

The treatment protocols were performed using benznidazole (Sigma Aldrich) for NBNZ administration and BNZ ($C_{12}H_{12}N_4O_3$ - LAFEPE) at 100 mg/kg for free administration. The treatment was initiated on the first day of confirmed parasitemia and was given for eight consecutive days.

2.3 Survival and parasitemia control

The infections were confirmed by quantifying trypomastigotes in whole blood from the caudal vein of the mice every two days [19]. All animals were evaluated daily to identify the number of animals that had died.

2.4 Sample collection and preparation

On day 30 post-infection (PI), the mice that had survived at the end of the experiment were anesthetized using isoflurane in a controlled inhalation box and euthanized by cardiac punction. The hearts, brains, kidneys, and livers were removed and stored at -80 ºC for genetic analysis and in 10% formol for histological analyses until use.

2.5 Alkaline Comet Assay

The samples were collected by puncturing the tail vein before the start of treatment and after the eighth day of treatment for comparison. The samples were resuspended in agarose (0.7%), placed on slides pre-coated with agarose (1%), dipped in a lysis solution, and stored at 4 °C in the dark. After the incubation period, the slides were submitted to the electrophoresis step in a horizontal vat using a specific buffer (1 mM EDTA and 300 mM NaOH, pH = 13) for 20 min. Later, the slides were neutralized, fixed, and stained with a silver nitrate solution [20].

Finally, the analysis was performed using a score defined for each damage, ranging from the least damage (0) to the maximum damage (4). All results will be presented as damage indices relative to the control.

2.6 Histopathology assay

Heart and liver fragments were collected, fixed in a 10% formalin buffer, embedded in paraffin. Then, microsope slides of 6μm thick sections were made, stained with hematoxylin and eosin (H&E) and analyzed under an optical microscope (Zeiss Axioscope A1). The slides were scored for inflammation, defined by the degree of infiltration. The injuries were classified as mild, moderate, or severe.

2.7 Statistical analysis

First, the data were subjected to normality testing (Shapiro-Wilk). All variables had a normal distribution, and parametric testing was performed. All statistical analyses were assessed using a two-way analysis of variance (ANOVA) followed by the Tukey test as a post-test using the Graph Pad Prism (Version 6.0) software. The results were expressed as mean ± standard error of the mean (SEM). The results were considered statistically significant when $p < 0.05$.

3. Results

3.1 Benznidazole nanocapsule characterization

The suspension of the benznidazole nanocapsules (NBNZ) had a final concentration of 1 mg/mL. A physicochemical analysis of the parameters size, polydispersity index, zeta potential, and pH was performed and is presented in Table 1.

Through the analysis, a size of 146 mm ± 0.62 nm was obtained on NBNZ synthesis, in addition to a polydispersity index of 0.085 ± 0.002. The zeta potential evaluated was negative, -12.8 ± 0.87 mV, and the pH of the NBNZ was 5.00 ± 0.01, characterizing an acid pH of the nanocapsules. After obtaining and characterizing the suspension of nanocapsules, the protocol for evaluating the appropriate dose for the treatment was initiated.

After preparation, the nanocapsules were maintained at room temperature for thirty days, and the stability parameters were evaluated after seven, fifteen, and thirty days. In Table 1, one may observe that the average size presents a slight increase over time, remaining around 163 nm. The drug loading showed the opposite standard, presenting a decrease in benznidazole content during this time. On the other hand, the encapsulation efficiency values remained above 90%. The polydisperse index, zeta potential, and pH of the NBNZ did not present significant changes over time.

3.2 Parasitemia and survival evaluation

The parasitemia was evaluated after the infection by the *T. cruzi* Y strain and monitored up to twenty days post-infection (PI) through blood obtained from the tail vein (Fig. 1A). The treatments were well tolerated during eight days of treatment.

The parasitemia peak occurred on the fifth day PI, after this, a reduction in parasitemia was observed (Fig 1A). One may observe a significant reduction in parasitemia in the animals infected and treated with BNZ (100 mg/kg) and 20 mg/kg NBNZ compared to the positive group. In addition, the parasitemia reduction standard was similar in these groups.

Relative to the survival percentage (Fig. 1B), it was possible to observe that the treatments with 100 mg/kg BNZ (100%) showed survival indices similar to those of the uninfected group (100%). In additon 20 mg/kg NBNZ (50%) showed the major survivel percentage of NBNZ treatments. Furthermore, the survival was directly related to the NBNZ doses: 5 mg/kg (0%), 10 mg/kg (20%) and 15 mg/kg (33.33%), i.e., the lowest dose had the lowest survival compared to the uninfected group.

3.3 Alkaline Comet assay

Treatment cytotoxicity was evaluated through a comet assay, in which it was possible to rank the DNA damage generating a damage index. This parameter was evaluated in two

experimental moments, before and after the treatment protocol (Table 2). No significant differences were observed before the treatment protocol among all groups compared to the uninfected group ($p > 0.05$).

On the other hand, the 100 mg/kg BNZ group showed an increase in the damage (983%) index after eight days of treatment compared to the index before the treatment. In addition, the 100 mg/kg BNZ group had a significantly higher damage index after the treatment than the uninfected (1473%) group, and the infected (421%) groups treated with NBNZ.

3.4 Histopathological evaluation

The histopathology of the cardiac and hepatic tissues was evaluated through microscopy using H&E stains. The cardiac samples showed amastigote forms of the parasite in Fig. 2B (infected and untreated) and Fig. 2C (infected and treated with 5 mg/kg of NBNZ). No parasite forms were observed in the infected animals treated with BNZ at 100 mg/kg or infected and exposed to different higher doses of NBNZ (Fig. 2 D, E, F).

In the infected and untreated group, sinusoidal congestion and dilation may be observed in the hepatic tissue, along with swelling of nuclei hepatocytes, cytoplasmic vacuolization, and many inflammatory cells (Fig. 3B). It was possible to observe the amastigote form of the parasite in the 5 mg/kg NBNZ group (Fig. 3C), and this group also showed degenerate hepatocytes, inflammatory cells, and activated macrophages.

Leukocyte infiltrations may be observed in the groups treated with 10 mg/kg, 15 mg/kg, and 20 mg/kg of NBNZ (Fig. 3D, E, F), which decrease as the treatment doses increase. However, no parasite forms in the hepatic samples were observed in the infected groups treated with NBNZ at 10 mg/kg, 15 mg/kg, and 20 mg/kg (Fig. 3A, D, E, F, G).

4. Discussion

The treatment of CD is influenced by a series of prominent factors such as clinical evolution, the natural resistance of *T. cruzi* strains, and drug toxicity. Despite the high efficacy in acute infections, the treatment has a high rate of adverse effects. Hence, the use of nanocapsules as alterative therapy would improve the biodistribution and efficacy of drugs such as BNZ. Here, we evaluated the oral efficacy of different dosages of Eudragit nanocapsules of benznidazole against experimental acute *T. cruzi* infections using the Y strain.

Currently, benznidazole is a drug administered orally, available in 100 mg tablets for treating adults and 12.4 mg tablets for treating children [10-11]. The oral administration of medications is widely used since it facilitates patient adherence to treatments. However, these tablets are of fast release, leading to the need for multiple administrations [21]. Davanço *et al.* [22] demonstrated the importance of using slow-release formulations to treat Chagas disease, but twice the usual dose was employed for this evaluation. In addition, the authors demonstrated that the pediatric formulation of the dose had low drug absorption, which may compromise the effectiveness of the treatment [22].

The use of a suspension of Eudragit L-100 nanocapsules with benznidazole in the treatment may improve the drug release process, which may increase its bioavailability, in addition to enabling a reduction in the dosage used throughout the treatment. Eudragit L-100 may improve the absorption and action potential of compounds used in nanocapsules produced with this polymer [13].

The stability evaluation of NBNZ demonstrated through the PDI values, which were stable during de time analyzed, that the formulation has a good homogeneity. This parameter may be related to the uniformity of particle sizes [23]. In addition, the zeta potential remained around -15 mV, this parameter may be related to a significant system stability when its values are different from zero [24]. Furthermore, the encapsulation efficiency remained over 90%, even with the drug loading decreasing during the days.

The Eudragit L-100 nanocapsules demonstrated few variations during the evaluation period but remained in proper condition for administration. The pH data demonstrated the acid characteristic of the NBNZ suspension. This polymer was used in nanocapsules produced and presents promising characteristics similar to NBNZ [13]. The formulation of BNZ nanocapsules showed good indices for *in vivo* administration and a final suspension concentration of 1 mg/mL. This formulation made it possible to obtain results similar to the standard treatment using a drug nanoencapsulation system with a dose five times smaller than usual. In addition, the administration as a nanocapsule suspension allows a liquid formulation for oral administration. The possibility of oral administration and dose adjustment with greater ease represents an important tool, especially for treating children affected by the disease, maintaining the effectiveness of the treatment.

Furthermore, the parasitemia evaluation was used as an important tool to monitor the course of the *T. cruzi* infection. The Y strain demonstrated the course infection, with the peak parasitemia occurring on the fifth day PI followed by a continuous reduction in the number of circulating parasites, which signals a referral to the chronic phase of the disease in the absence of treatment (Fig. 1A). Animals treated with NBNZ at 10 mg/kg, 15 m/kg, and 20 mg/kg were able to reduce parasitemia compared to the positive control (Fig. 1A). In addition, the high dose of NBNZ reset the parasitemia, as did the 100 mg/kg dose of BNZ. Treatment with benznidazole is widely used and promotes a reduction in parasitemia and cure in the acute phase of the disease, as demonstrated by several studies [5, 25-26].

Here, we also analyzed the survival curve to monitor the death frequency on several treatment protocols for acute *T. cruzi* infections. The infected and untreated animals showed a significant decrease in the survival percentage. On the other hand, the NBNZ treatment with the highest dose (20 mg/kg) extended the survival rate in a manner similar to the 100 mg/kg dose of BNZ (Fig. 1B). The effects observed here showed that NBNZ may be a very promising treatment option during the acute phase of Chagas disease, and can present better results if was administered during a major period, since that a low concentration of NBNZ for a short time significantly improved the animal survival rates. Moreover, no cytotoxic effects were observed for the assessed doses, showing that NBNZ is a lower toxic administration system during the acute phase of CD. The BNZ showed a hight toxicity, because one of its action mechanisms is based in ROS and NOX species generation. These molecules promotes several cell damages [27].

Several adverse effects related to BNZ have been described in the literature and reported by patients that may lead to treatment dropout [22]. The nanoencapsulation system is an efficient delivery approach to administer better and limit the toxic side effects of drugs. In general, nanoparticulate systems may have multiple synergistic functions while maintaining high specificity and selectivity [12]. Our results revealed that oral treatment with 100 mg/kg of BNZ increases DNA damage in infected mice after eight days. On the other hand, all NBNZ doses decreased the DNA damage in the infected animals (Table 2) compared to the beginning of treatment. In addition, the results showed a reduction in the inflammatory process caused by the *T. cruzi* infection.

The parasitological cure was reinforced by the histological analysis of the presence or absence of tissue parasitism, inflammation, and macrophage activation. In the histological

evaluation of the cardiac tissue, the infected animals presented amastigotes nests and inflammatory infiltrates. In the groups treated with the highest NBNZ dose or BNZ, no parasitic forms were observed (Fig. 2), probably achieved because the parasitemia was suppressed in all treated animals. Furthermore, Rial *et al.* [28] demonstrated a similar reduction in the inflammatory process and heart damage during the infection with lower doses of nanoencapsulated BNZ.

Abriata *et al*. [29] carried out an *in vivo* study in mice infected with the Y strain, which is partially resistant to treatment, and treated with ursolic acid loaded into nanoparticles by the oral route. They found that the treatment had efficacy similar to that of BNZ in controlling parasitemia and that the ursolic acid loaded into nanoparticles produced a better reduction in parasitemia than free ursolic acid. They attributed this effect to an improvement in the bioavailability of the encapsulated drug. The nanoencapsulation process contributes to considerable improvements to the absorption, distribution, metabolism, and excretion properties of molecules [13, 28, 30]. Our study showed that no amastigote forms were observed in the liver section when the animals were treated with NBNZ (Fig. 3). A surprising finding was that, after oral administration, the nanocapsules seem to be able to pass through gastrointestinal barriers while transiting through the enterocytes by surface adsorption, enhancing the permeability. Moreover, the NBNZ proved to reduce leukocyte infiltration, potentializing the anti-inflammatory effects of BNZ. Also, this approach takes advantage of the endothelial leakage of inflamed tissues, which results in nanoparticle perfusion to the extravascular region [30]. Long-circulating NBNZ may exhibit this property because the Eudragit polymer increases its ability to circulate longer and extravasate to inflamed tissue, where the parasites are located after they are released from host cells. In addition, these nanocapsules have a small hydrodynamic diameter (approximately 163 nm), which could facilitate their passage through the fenestrate spaces of the endothelium of leaky vessels. Therefore, our experimental design showed that oral Eudragit NBNZ administration is a safe and efficient treatment during the acute phase of CD. The data showed an absence of *T. cruzi* amastigote nests inside the heart and hepatic tissues mediated by controlled leucocyte immune response.

5. Conclusion

This study reported a secure oral nanocapsule dose of BNZ against infections caused by *T. cruzi* during the acute phase of the disease. These results show the enormous potential of BNZ nanoencapsulation. It represents a potential innovative therapy for CD and offers a great prospective treatment to be explored by public or private pharmaceutical health care using lower doses of BNZ, avoiding toxicity, and improving biodistribution in target organs. During treatment period the NBNZ was not efficient as BNZ treatment in total control of *T. cruzi* infection. However, this treatment reduces de tissue damage in infection.

Ethics Committee

The animal experiments were approved by the Ethics Committee on Animal Experimentation of the Federal University of Santa Maria (UFSM) under protocol number 2842070618.

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Conflict of interest

The authors declared no conflict of interest.

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Highlights

Benznidazole is a cytotoxic compound to cell DNA.

Nanoencapsulated benznidazole improve treatment with lower dose and reduced citotoxycity.

Chagas disease promotes tissue damage in several organs.

Benznidazole may increase the survival in *T. cruzi* infection

Nanoencapsulated benznidazole protect cardiac tissue invasion in *T. cruzi* infection.

Figures and Tables

Table 1 – Physicochemical stability of nanocapsules containing benznidazole.

Time (days)	Size (nm)	Polydispersity index	Zeta potential (mV)	pH	EE (%)	Drug loading (mg/mL)
0	149.73 ± 1.40	0.15 ± 0.01	-14.07 ± 0.74	3.50 ± 0.04	96.33 ± 0.58	1.02 ± 0.01
7	166.57 ± 1.53	0.18 ± 0.01	-16.03 ± 0.76	3.71 ± 0.04	93.00 ± 1.00	0.83 ± 0.01
15	171.90 ± 0.40	0.18 ± 0.01	-17.40 ± 0.62	3.62 ± 0.05	92.33 ± 1.15	0.79 ± 0.01
30	163.67 ± 1.65	0.19 ± 0.01	-15.73 ± 0.96	3.51 ± 0.06	91.33 ± 1.53	0.63 ± 0.01

The data are shown as mean ± SD.

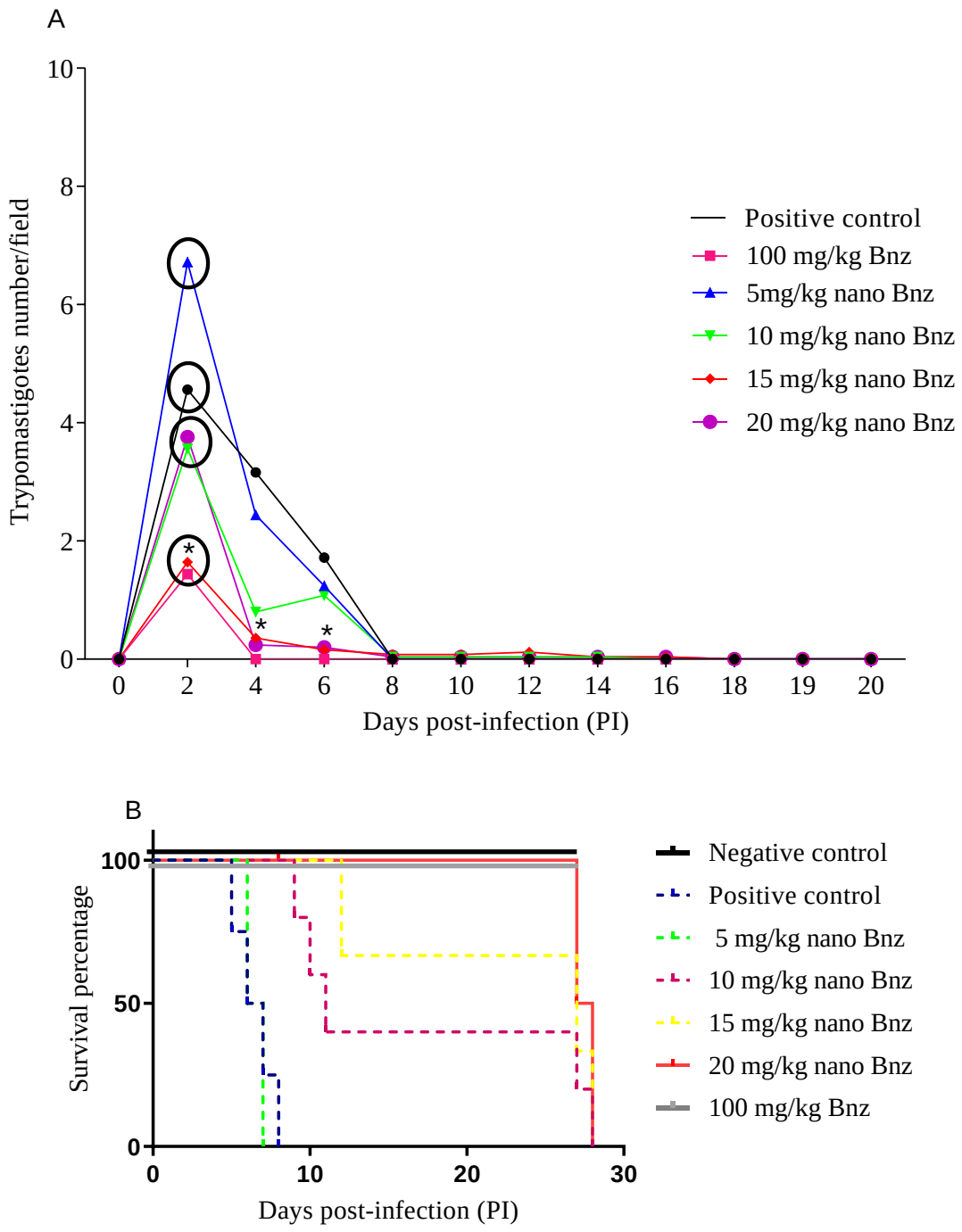

Figure 1. Parasitemia and survival curve. (A) Parasitemia assessment. (B) Survival curve of animals infected by *T. cruzi* (Y strain) exposed to BNZ and/or NBNZ treatments. Differences were considered statistically significant when *$p < 0.05$ when comparing the treated groups with the positive control or negative control groups.

Table 2. DNA damage index during acute *T. cruzi* infections

	Uninfected	Infected	Infected 100 mg/kg BNZ	Infected 5 mg/kg NBNZ	Infected 10 mg/kg NBNZ	Infected 15 mg/kg NBNZ	Infected 20 mg/kg NBNZ
Before	0.074 ±0.033	0.123 ±0.038	0.077 ±0.021****	0.124 ±0.014	0.120 ±0.033	0.083 ±0.042	0.084 ±0.019
After	0.053 ±0.026	0.160 ±0.032	0.834 ±0.070 ####	0.082 ±0.022 [a]	0.069 ±0.008 [a]	0.130 ±0.021 [a]	0.094 ±0.024 [a]

The data are shown as mean ± SD. * Means significant differences when comparing one group at different times, # Means significant differences between all groups and the uninfected group at a given time, [a] Means significant differences between the 100 mg/kg BNZ group and the NBNZ doses groups at a given time.

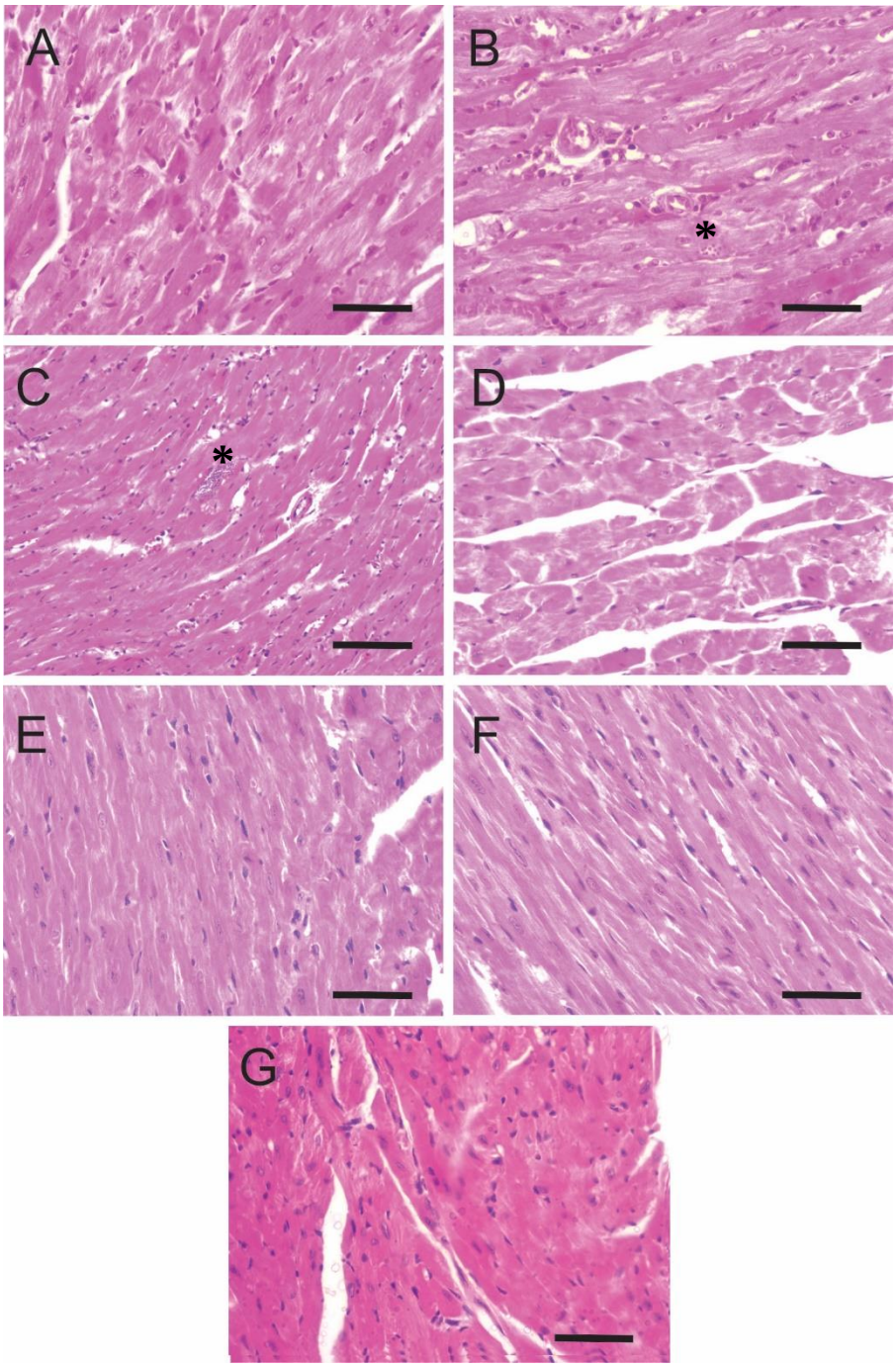

Figure 2 – Photomicrography of the cardiac tissue of mice experimentally infected by *T. cruzi*. A: uninfected and untreated), B: infected and untreated, C: infected and treated with 5 mg/kg of NBNZ, D: infected and treated with 10 mg/kg of NBNZ, E: infected and treated with 15 mg/kg of NBNZ, F: infected and treated with 20 mg/kg of NBNZ, G: infected and treated with 100 mg/kg of BNZ. * Amastigote nest. Stained by H&E. Scale bar 50mm.

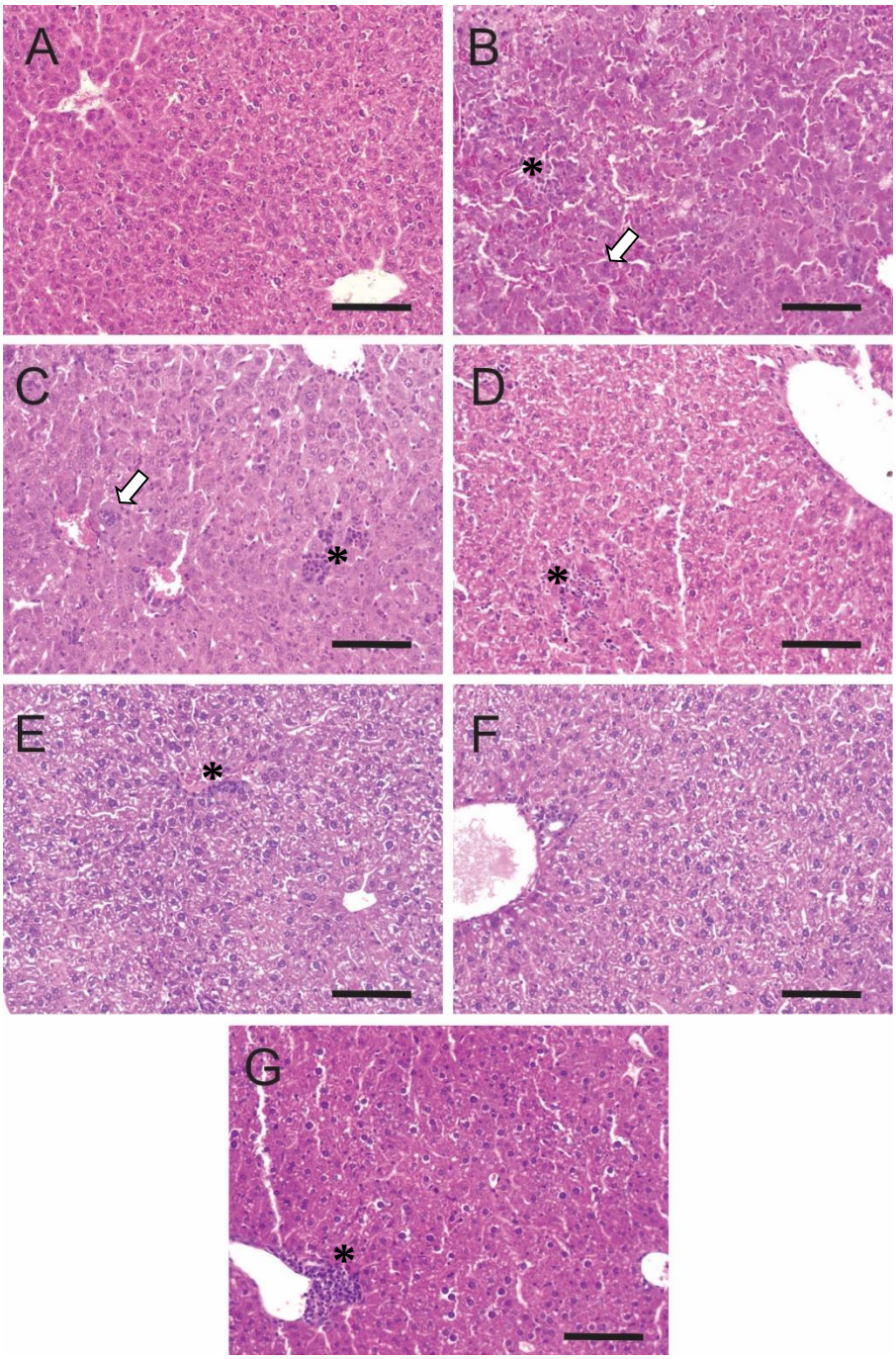

Figure 3 – Photomicrography of the hepatic tissue of mice experimentally infected by *T. cruzi*. A: uninfected and untreated, B: infected and untreated, C: infected and treated with 5 mg/kg of NBNZ, D: infected and treated with 10 mg/kg of NBNZ, E: infected and treated with 15 mg/kg of NBNZ, F: infected and treated with 20 mg/kg of NBNZ, G: infected and treated with 100 mg/kg of BNZ. * Inflammatory infiltrate; arrows showed activated macrophages. Stained by H&E. Scale bar 100 mm.

4.3 Manuscrito II

Effects of free benznidazole and nanoencapsuled in acute *Trypanosoma cruzi*-infection: role of cholinergic pathway and redox status

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Abstract

The *T. cruzi* infection promotes an intense inflammatory process in affect tissue, and the cholinergic system may exert a regulatory process of immune response and control the inflammatory process. The aim of this study was evaluated whether the protocol with nanoencapsulated benznidazole enhances curative efficacy in acute *T. cruzi* infection compared to free benznidazole, as well as the effects of therapies on the cholinergic system response, hematological, biochemical parameters and oxidative status. For this, fifty female Swiss mice were distributed in eight groups, uninfected and infected animals under four treatment protocols: untreated (CT), vehicle treatment (EL-100), benznidazole treatment (BNZ) and nanoencapsulated benznidazole treatment (NBNZ). After eight treatment days, the animals were submitted to euthanasia, for sample collect. It was possible observe the peak of parasitemia at day 7 p.i., the BNZ and NBNZ treatment was able to control and reduced parasitemia, but was not efficacy to total elimination of parasite, as the genomic DNA of parasite was detected by RT-PCR in both infected groups. The infection promotes a significative anemia, leukopenia and thrombocytopenia, that was prevents by BNZ treatment. There was an increase in AChE activity during infection leading to a pro-inflammatory response, the same occurs in a expression of AChE, M1 and M2 mAChR in BNZ group, showing that the treatment interacts with this regulation via, assisting to maintain the pro-inflammatory. In addition, a pro-oxidative response was characterized in the infection and mainly in the infected BNZ and NBNZ groups. and the histopathology analyze showed a significative splenomegaly, a tissue inflammatory infiltrate in heart, liver and spleen. In conclusion, both the infection and treatments, promotes changes in the cholinergic signalization, altering the immunomodulation. The infection is characterized by anemia, thrombocytopenia, granulocytopenia and lymphocytosis, as well as hepatic and renal alterations, that can be controlled by BNZ treatment. The oxidative/antioxidant status is feature by pro-oxidative status. In addition, BNZ and NBNZ was able to prevents the tissue damage during acute chagas disease. In summary the BNZ treatment demonstrate an interaction in immunoregulation via cholinergic anti-inflammatory pathway, stimulating the cholinergic components to a pro-inflammatory response during acute *T. cruzi infection*.

Keywords: nanocapsule; AChE; oxidant; benznidazole; Chagas disease.

1. Introduction

Trypanosoma cruzi is the etiological agent of Chagas disease (CD), transmitted in a vector way in America, it also has forms of infection by maternal-fetal transmission, orally, accidentally and blood transfusion. Naturally, the protozoan has tropism for organs such as the heart, brain, intestine and esophagus and affects many people worldwide (WHO, 2020). In Brazil, the highest mortality of people with CD is concentrated mainly in the northern and northeastern states (Martins-Melo *et al.* 2021). Nifurtimox and benznidazole (BNZ) are option of treatment to acute and chronic stage of disease. CD has many obstacles to its treatment, such as the dose used, the scarce study of new drugs and especially the adverse effects caused by BNZ, such as hepatotoxicity, skin reactions, changes in hematological parameters among others. Therefore, it is necessary new therapeutical approaches that regard to effectiveness against *T. cruzi*. In this sense, nanotechnology becomes an important tool for reducing the dose and a possibility to improve the solubility, absorption and bioavailability of the drugs.

During intracellular infection by the parasite, an innate immune response is triggered, with the release of pro-inflammatory cytokines, which may induce enteric, cardiac and neuronal damages (CHUENKOVA, PERRIN, 2011). Once into intracellular space *T. cruzi* leads to an increase in the formation of reactive oxygen species (ROS) by host cells, due to the stimulus of inflammatory mediators, such as cytokines and chemokines, which results in oxidative stress (GUPTA *et al.*, 2009). Oxidative stress is considered a host defense mechanism against the parasite in the acute phase of infection, besides contributes to development of tissue damage (WEN *et al.*, 2004; ZACKS *et al.*, 2005). In contrast to *T. cruzi* presence, persistence and inflammatory processes, anti-inflammatory responses limit excessive inflammation, cellular damage and apoptosis (Borges *et al.*, 2013).

The anti-inflammatory cholinergic pathway is a regulatory system involved into immune responses in CD, reported in previous study group our (Silva *et al.*, 2017). Immune cells have a complete cholinergic system, composed of acetylcholine (ACh), choline acetyltransferase (ChAT), nicotinic and muscarinic receptors (nAChR and mAChR) and acetylcholinesterase (AChE) enzyme (Kawashima and Fujii, 2003; Tayebati *et al.*, 2002). ACh acts as a neurotransmitter in both the central and peripheral nervous systems (Tucek, 1988). In addition, this molecule is involved in the regulation of immune functions (Kawashima and Fujii, 2003), showing anti-inflammatory properties (Soreq and Seidman, 2001; Das, 2007). On the other hand,

the enzyme butyryl cholinesterase (BChE: EC 3.1.1.8) is present in the intestine, liver, kidneys, heart, lung and serum (Ecobicon and Corneau, 1973). Despite hydrolyzing esters such as butyrylcholine, BChE can act on the degradation of ACh when there is a situation of inhibition or absence of AChE (Taylor and Brown, 1999; Li *et al.*, 2000). Studies in the literature have already demonstrated the occurrence of changes in the activity of acetyltransferase and AChE enzymes related to trypanosomiasis (Zanella *et al.* 2014; Silva *et al.* 2017).

Therefore, the aim of this study was evaluated whether the protocol with nanoencapsulated benznidazole enhances curative efficacy in acute *T. cruzi* infection compared to free benznidazole, as well as the effects of therapies on the cholinergic system response, hematological, biochemical parameters and oxidative status.

2. Material e Métodos

2.1 Benznidazole nanocapsule development

Benznidazole (N-Benzyl-2-nitro-1H-imidazole-1-acetamide) (97%) was acquired from Sigma-Aldrich (São Paulo, Brazil), and the Eudragit L100 polymer was kindly donated by the Evonik company (São Paulo, Brazil).

To development of nanocapsules containing benznidazole (1 mg/mL) were used the preformed polymer interfacial deposition method (Ribeiro *et al.* 2016), with modifications. The organic phase containing the Eudragit L100 polymer (0.25 g), benznidazole (0.025 g), sorbitan monooleate (0.19 g), medium-chain triglycerides (413 µL), ethanol (67 mL), and the aqueous phase containing polysorbate 80 (0.19 g) and ultrapure water (134 ml). Then the formulation was submitted to evaporate the organic solvent under reduced pressure to the final volume (25 mL), with temperature control at 40 °C. The characterization and evaluation of this nanocaspsule is presented in manuscript II.

Benznidazole (N-Benzyl-2-nitro-1H-imidazole-1-acetamide) (97%) was acquired from Sigma-Aldrich (São Paulo, Brazil), and the Eudragit L100 polymer was kindly donated by the Evonik company (São Paulo, Brazil).

2.1.1 Development of the nanocapsules

Nanocapsules containing benznidazole (1 mg/mL) were developed using the preformed polymer interfacial deposition method [16], with modifications. The formulation consisted of an

organic phase containing the Eudragit L100 polymer (0.25 g), benznidazole (0.025 g), sorbitan monooleate (0.19 g), medium-chain triglycerides (413 μL), ethanol (67 mL), and an aqueous phase containing polysorbate 80 (0.19 g) and ultrapure water (134 ml). The organic phase was homogenized for 60 min at 40 °C, and the aqueous phase was homogenized for 15 min without heating, both separately, with the aid of a magnetic stirrer. Subsequently, the organic phase was poured into the aqueous phase and kept under stirring for another 15 min, and then the formulation was taken to the rotaevaporator to evaporate the organic solvent under reduced pressure to the final volume (25 mL), with temperature control at 40 °C.

2.2 Experimental design I

Fifty female *Swiss* mice were distributed in eight groups: CT – Negative control (uninfected animals, n=6); EL-100 - EL-100 nanocapsule (uninfected animals, n=6); BNZ – free benznidazole 100mg/kg (uninfected animals, n=6); NBNZ – benznidazole nanocapsule 20mg/kg (uninfected animals, n=6); infected CT – Positive control (infected animals, n=8); infected EL-100 - EL-100 nanocapsule (infected animals, n=6); infected BNZ – free benznidazole 100 mg/kg (infected animals, n=6); infected NBNZH – benznidazole nanocapsule 20mg/kg (infected animals, n=6). The groups E to H were infected with *T. cruzi* (Y strain), with $1x10^4$ trypomastigote forms, by intraperitoneal injection.

Treatments

Protocols treatment were made using benznidazole ($C_{12}H_{12}N_4O_3$, Sigma Aldrich) nanoencapsualted (20mg/kg) (dose defined in pilot study) and benznidazole pills of 100 mg (LAFEPE). After confirming of infection, the groups received treatment by orally (gavage) over 8 days, and the parasitemia was analyzed each two days.

Animals and infection

The animals were submitted to a week of acclimatation before the infection and treatment. During the experiment period animals were kept in light/dark cycles (12-h) with controlled temperature and humidity (25 °C and 70% respectively), and water and food *ad libtum*. At day 11 post-infection, the mice were submitted to euthanasia. Animals were anesthetized using isoflurane chamber and were euthanized by cardiac punction according to Ethics Committee Protocols. All animals' manipulation were approved by animal welfare ethics committee from

Federal University of Santa Maria (approval number 2842070618). Blood was collected on EDTA tubes, samples were processed immediately after collected. Cortex, heart, liver, spleen and kidney samples were collected and stored at -80°C until analysis. A fragment of tissues was kept in 10% formalin solution to histological analysis.

2.3 Monitoring of parasitemia evolution

Parasitemia was proceeded by quantification of trypomastigotes in total blood, according to methodology of Brener (1962). Blood sample was collected of the caudal vein of infected mice each two days.

The confirmation of infection, Real-time Polymerase Chain Reaction (qPCR) was performed in cardiac and cortex samples from three mice of each group at day 12 p. i. DNA was extracted from the samples using the method described by Lachaud *et al.* (2001). The detection of *T. cruzi* was performed by the amplification of a 188 bp nuclear fragment of the parasite DNA using two specific primers: TCZ-1 (5'- CGA GCT CTT GCC CAC ACG GGT GCT -3') and TCZ-2 (5'- CCT CCA AGC AGC GGA TAG TTC AGG -3') (Virreira *et al.*, 2003). The RT-PCR was realized using KAPA SYBR Fast qPCR Master Mix (2X) (Kapa Biosystems) with 10 ng of genomic DNA (gDNA). The samples were amplified with a Rotor-Gene Q (Quiagen) thermal cycler.

2.4 Hematological parameters evaluation

Hematological analysis was performed in an electronic counter (BC-2800 Vet – Auto Hematology Analyzer, Mindray®) from samples packed in tubes containing EDTA, and the following parameters were determined: total erythrocyte concentration (/µL), hemoglobin concentration (g/dL), platelet concentration (/µL) and total leukocyte concentration (/µL). The leukocyte differential was performed under immersion microscopy, at 1.000x magnification, from blood smears made on a glass slide and stained with rapid Panoptic (Diff-Quick®). Total plasma protein (PPT) was verified by refractometry, using the plasma portion of the microhematocrit capillary.

2.5 Biochemical parameters evaluation

Biochemical analyzes (AST, ALT, creatinine and urea) were performed in serum using a commercial kit (Bioclin®) from the animals and according to the manufacturer's instructions. The samples were processed in an automatic biochemical analyzer (Mindray BS-120®).

2.6 Mononuclear-rich lymphocytes isolation from spleen

Isolation of mononuclear-rich lymphocytes from spleen was performed follow Doleski *et al* (2014). The organs were kindly homogenized in cold PBS-EDTA, then were centrifuged at 1500 rpm for 10 minutes. One wash was performed suspending the pellet in PBS-EDTA. To form the lymphocytes-rich mononuclear band, Ficoll-Hypaque was used, after centrifugation de band was collected. This isolate was submitted to washing process and the resultant pellet was washed and kept in ice.

2.7 Protein determination

Protein content was determined using the Coomassie blue method according to (Bradford, 1976) with bovine sera albumin as standard. The protein supernatant of tissue were maintained at 0.6–0.8 mg/mL.

2.8 Cholinergic system evaluation

2.8.1 AChE assay in lymphocytes

After the isolation of the lymphocytes, the AChE activity was determined according to the method described by Ellman *et al.* (1961) modified by Fitzgerald and Costa (1993). Briefly, proteins of all samples were adjusted to 0.1–0.2 mg/ml. The amount of 0.2 ml of intact cells was added to a solution containing 1.0 mM acetylthiocholine, 0.1 mM of DTNB, and 0.1 M phosphate buffer (pH 8.0). Immediately before and after incubation for 30 min at 27 °C, the absorbance was read on a spectrophotometer at 412 nm. The results are expressed as μmol ACh/h/mg of protein.

2.8.2 AChE assay in whole blood

Whole blood AChE activity was determined by the method modified by Worek *et al.* (1999). Samples were hemolyzed with phosphate buffer, pH 7.4 containing Triton X-100 and stored at −30 °C for 1 week. The specific activity of whole blood AChE was calculated from the

quotient between the AChE activity and hemoglobin content and the results are expressed as mU ACh/μmol Hb.

2.8.3 Cerebral AChE enzymatic assay

The AChE enzymatic assay was determined by a modification of the spectrophotometric method of Rocha *et al.* (1993). The reaction mixture (300 µl final volume) contained 100 mM K+-phosphate buffer, pH 7.5 and 1 mM 5,5′-dithiobisnitrobenzoic acid (DTNB). The method is based on the formation of the yellow anion, 5,5′-dithio-bis-acid-nitrobenzoic, measured by absorbance at 412 nm during 2-min incubation at 25°C. The enzyme (40–50 µg of protein) was pre-incubated for 2 min. The reaction was initiated by adding 0.8 mM acetylthiocholine iodide (AcSCh). All samples were run in duplicate or triplicate and the enzyme activity was expressed in µmol AcSCh/h/mg of protein.

2.8.4 **BuChE**

Plasmatic, liver and cortex tissue BuChE (EC 3.1.1.8; BUChE) activity was determined as described by Ellman et al. (1961) The BUChE activity was assayed in a medium containing sodium phosphate buffer 0.1 mM, pH 7.4, DTNB 0.30 mM and 15 µL of serum. After 3 min of pre-incubation at 37 °C, the reaction was started with 1 mM of butyrylthiocoline (BuSCh), and the reading was performed for 2 min at intervals of 20 s in a spectrophotometer at 412 nm. The specific activity was expressed in µmol BuSCh/h/mg protein

2.9 Lymphocytes cytometry

The lymphocytes were fixed for 20 min in ice-cold 1% PFA in PBS, washed with PBS supplemented with 2% FBS, and incubated for 30 min with primary antibodies against AChE (1:200 Santa Cruz), AchM1 (1:200, Santa cruz), AchM2 (1:2000, Santa Cruz) receptors. The cells were incubated with Alexa Fluor 488- or 647-conjugated secondary antibodies (1:500) (Life Technologies, Carlsbad, CA) and analyzed by flow cytometry (BD FACS Calibur; BD Biosciences, La Jolla, CA). Thirty thousand events were acquired per sample. Data were analyzed by using the FlowJo V10 software (FlowJo, BD Biosciences) and plotted in histogram format.

2.10 Oxidative parameters

Carbonylation of serum proteins was determined using a modified method (Levine *et al.*, 1990). Carbonyl content was calculated using 22×10^3 mmol/cm as the molar extinction coefficient, and the results were expressed as ηmoles of carbonyl groups per milligram protein.

Lipid peroxidation was measured as TBARS and expressed in terms of malondialdehyde (MDA) content. MDA, an end-product of fatty acid peroxidation, reacts with TBA to form a colored complex. The TBARS was analyzed in serum according (Jentzsch *et al.*, 1996). The results were expressed as ηmoles of malondialdehyde/mg of protein.

Nitric oxide levels were measured using an indirect method by evaluation of nitrite/nitrate (NOx) content (Miranda and Espey, 2001). For NOx determination, an aliquot (200 µL) was homogenized in 200 mM of Zn_2SO_4 and acetonitrile (96%, HPLC grade). The results were expressed as levels of NO/mg protein.

Reactive species (RS) were measured using 2´-7´-dichlorofluorescein (DCFH) fluorescence levels as an index of peroxide production by cellular components according to (Halliwell and Gutteridge, 2007). Liver protein (0.8 µg) was added to a medium containing Tris–HCl buffer (10 mM; pH 7.4) and DCFH-DA (1 mM). The mixture medium was incubated in the dark for 1 h until the start of fluorescence measurement procedure (excitation at 488 nm and emission at 525 nm, and both slit widths were 1.5 nm). The results were expressed as U DCF/mg protein.

2.11 Antioxidant determination

CAT activity in tissue liver was determined by the decomposition of H_2O_2 at 240 ηm, according to the method described by Nelson and (Kiesow, 1972) and modified by (Aebi, 1984). The results were expressed as ηmol CAT/mg protein. SOD activity was measured according to the method of (Misra and Fridovich, 1972). The results are expressed as U SOD/mg protein. GST activity was assayed spectrophotometrically at 340 ηm using the method of (Habig *et al.*, 1974). The activity was expressed as ηmol/h/mg of protein.

Thiols was measured spectrophotometrically using method (Ellman, 1959). An aliquot of 100 µL for liver in a final volume of 900 µL of solution was used for the reaction. The reaction product was measured at 412 nm after the addition of 10 mM 5-5-dithio-bis (2-nitrobenzoic acid)

(DTNB) (0.05 mL). A standard curve using cysteine was added to calculate the content of thiol groups in samples,and was expressed as μmol TSH/mg protein.

For NPSH determination subsequent to precipitation of protein, the resulting solution was centrifuged at 10.000 × *g* for 5 min at 4 °C and the free SH groups were determined in the supernatants. The reaction mixture consisting 50 μL of sample, 450 μL phosphate buffer and 1.5 mL of 0.1 mM of 5′,5′-dithiobis 2-nitro benzoic acid was incubated for 10 min at 37 °C. The absorbance was measured at 412 nm using a SpectraMax plate reader (Molecular Devices, CA, USA). NPSH levels were expressed as μmol NPSH/mL.

2.12 Histopathology assay

Heart and liver fragments were collected, fixed in a 10% formalin buffer, embedded in paraffin. Then, microscope slides of 6μm thick sections were made, stained with hematoxylin and eosin (H&E) and analyzed under an optical microscope (Zeiss Axioscope A1). The slides were scored for inflammation, defined by the degree of infiltration. The injuries were classified as mild, moderate, or severe.

2.13 Statistical analysis

First, the data were subjected to normality testing (Shapiro-Wilk). All variables had a normal distribution, and parametric testing was performed. All statistical analyses were assessed using a two-way analysis of variance (ANOVA) followed by the Tukey's test as post-test using the Graph Pad Prism (Version 6.0) software. The results were expressed as mean ± standard error of the mean (SEM). The results were considered statistically significant when $p < 0.05$.

3. Results

3.1 *T. cruzi* confirm infection

The parasitemia evolution (Fig. 1A) was monitored through microscope parasitic count in blood sample, collected by a punction of tail vein. The measurement was performed every day until confirm the infection, after this it was execute each two days. The first positive parasitemia analyze was obtained on day 3 p.i. and the peak of parasitemia occur between the days 5 and 7 p.i., after this period was possible observed a decrease of parasitemia. The BNZ and NBNZ groups present a similar standard whit low parasitemia arrived to zero on days 5 and 11 p.i.

respectively. The BNZ group presented diminished parasitic load than the NBNZ group. There was a significant decrease of parasitemia on BNZ and NBNZ groups when compared to CT group on days 5, 7 and 11 p.i., the same was observed in comparison to E L-100 group at this time points. Besides, no differences were observed between CT and E L-100 groups ($p>0.05$).

In addition, the RT-PCR was assessed to confirm parasite infection was evaluated and genomic DNA were quantified in cardiac (Fig. 1B) and cortex (Fig. 1C) tissue. The quantification parasitic DNA copies were plotted in a standard curve based on Ct values in relation the log of DNA copy in the sample. The coefficient of determination (R^2) was 0.9919. It was possible observe significative DNA parasite copies lower in EL-100, BNZ and NBNZ when compared to infected CT group in both, heart and cortex tissues. In cardiac tissue all treatments present significative difference between them, but the lower quantities of parasites occurred in BNZ treatment followed by NBNZ treatment. In cortex sample the lowest parasite DNA copies was present in BNZ and NBNZ groups, that present no difference when compared between them.

3.2 *T. cruzi* infection promotes anemia, thrombocytopenia and granulocytopenia

The erythrogram parameters are showed in Table 1. There was a significant difference between uninfected and infected groups. The total erythrocytes, hemoglobin and hematocrit were lower at infected animals, when compared to uninfected animal. Besides, the BNZ (100 mg/kg) infected group had a high in these parameters compared to other infected treatment groups, but there was no difference between uninfected and infected BNZ 100 mg/kg groups. Furthermore, the platelets number was lower on positive control (infected group) in comparison to uninfected group. The same can be observed in number of platelets NBNZ 20 mg/kg.

The leukocytes number and its differential count (Table 1) were evaluated in animals uninfected and infected exposed to BNZ treatments. The CT group demonstrate a tendency of lower in white cell number on infected animals when compared to uninfected animals in all treatments. Total leucocytes demonstrate no differences in CT, EL-100 and BNZ treatments in uninfected groups when compared to infected groups. The total leukocytes number was superior of NBNZ group on infected group in comparison with uninfected NBNZ group and infected CT group.

The granulocytes number was lower in infected CT, EL-100, groups compared to their uninfected groups, this parameter also demonstrated major in infected NBNZ when compared to

uninfected NBNZ and infected CT group. The lymphocytes number didn't have significative differences between uninfected and infected animals of CT, EL-100 and BNZ treatments groups. On the other hand, the NBNZ treatment showed major on lymphocytes number in infected group when compared to its uninfected group and infected CT group. There were no differences in monocytes number of CT, EL-100, BNZ and NBNZ infected groups regarding to their uninfected groups.

3.3 Hepatic and renal alterations occur even with treatment

The biochemical parameters related to hepatic and renal function were analyzed in serum sample (Table 2). It was possible to note that the AST and ALT activities of infected groups was superior when compared to uninfected groups of each treatment protocol. The creatinine plasmatic level was major in infected CT and BNZ group regarding to their uninfected groups.

The infected CT group showed major of urea serum levels compared to uninfected group, the same condition was observed in comparison between uninfected and infected E-L100 groups. However, the NBNZ treatment promotes lower urea levels in infected group when compared its uninfected group. At the same time, all treatments protocol showed lower of urea levels in infected groups in relation infected CT group.

3.4 Benznidazole improve the cholinergic signalization

To evaluate the interaction of cholinergic activity in *T. cruzi* infection, the acetylcholinesterase and butyrilcholinesterase activities was assessed in lymphocytes, total blood and cortex. The lymphocytes AChE enzyme activity was superior in infected CT group when compared to uninfected CT group, the same situation was observed in the comparison between infected EL-100 with uninfected EL-100 and infected BNZ with uninfected BNZ group (Fig 2A). However, the NBNZ treatment promotes a lower the AChE activity in infected group in relation to uninfected group and to infected CT group.

On the other hand, the AChE blood activity (Fig. 2B) demonstrates no alterations between infected and uninfected groups of CT, EL-100 and BNZ treatments. Although, the infected NBNZ group showed major of AChE enzyme activity when compared to uninfected NBNZ group. It was no observed differences between treatments of infected groups. The cortex enzyme

activity (Fig. 2C) showed an elevated only between infected and uninfected groups of CT treatment.

Moreover, the flow cytometry was used to analyze the AChE expression on lymphocytes and the muscarinic receptors (M1 and M2) involved in immune response. The AChE expression (Fig. 3A and 4) showed lower in infected CT group in comparison to uninfected CT group, as can be observed on representative histograms. However, there was a major of AChE expression in infected BNZ group when compared to infected CT group.

The muscarinic receptor M1 and M2 (Fig. 3B, C and Fig. 4) showed major expression of infected CT and EL-100 groups, in relation to their uninfected groups. The infected BNZ group, showed major receptors expression compared to infected CT group. Besides, the NBNZ treatment the M1 and M2 expression was superior when compared to its uninfected group and infected CT group.

The plasmatic BuChE activity (Fig. 5A) was lower on CT and EL-100 infected when compared to CT and EL-100 uninfected. The infected BNZ and NBNZ groups demonstrate major of enzyme activity in comparison with infected CT group, keeping to basal levels.

The liver enzyme activity (Fig. 5B) demonstrates lower in the infected CT, EL-100 and BNZ in comparison to their uninfected groups. Moreover, the infected NBNZ there was no difference in relation to uninfected NBNZ, but showed major of enzyme activity when compared to infected CT group, as occur with infected BNZ in relation to infected CT. In cortex (Fig. 5C) was observed lower of BuChE activity in infected CT and NBNZ group in comparison to their uninfected groups. In addition, the infected + NBNZ showed lower compared to infected CT group.

3.5 Oxidative status on acute *T. cruzi* infection

The oxidative stress was evaluated on liver, kidney and cortex tissue, through several oxidative parameters. Protein carbonylation, in the liver (Fig. 6A) was major in the infected CT group when compared to the uninfected CT group. However, the infected EL-100 and BNZ were lower in comparison to their uninfected groups and to infected CT groups. The infected NBNZ had no difference compared to its uninfected group, but was lower compared to the infected CT group.

The renal carbonylation protein (Fig. 6B) was lower in infected groups, when compared to uninfected groups. This lower was significant mainly in infected CT and BNZ comparing to their uninfected groups. The NBNZ treatments groups had no difference on the comparison between uninfected and infected groups. In cortex (Figure 6C) the protein carbonylation concentration only was superior in infected CT group compared to uninfected CT group, the others comparison had no significant differences.

Malondialdehyde levels in liver tissue (Fig. 7A) was superior in all infected groups when compared to uninfected groups. The infected CT, EL-100, BNZ and NBNZ showed a major of MDA levels when compared to uninfected CT, EL-100, BNZ and NBNZ, respectively. In addition, infected EL-100 and NBNZ demonstrate major TBARS levels in relation to infected CT group.

In kidney (Fig. 7B) analyzes it was possible observed a similar standard to liver, with a major of MDA levels in infected CT, EL-100 and BNZ groups when compared to uninfected groups. However, the infected NBNZ groups had no significant difference in relation to its uninfected groups, but showed lower of MDA levels in comparison to infected CT group. The cortex MDA levels (figure 7C) evaluation showed no significant differences between all groups.

Nitrite levels in liver sample (Fig. 8A) was superior in infected CT, BNZ and NBZ when compared to their uninfected groups. In addition, The BNZ and NBNZ promotes major of NOx levels in infected groups compared to infected CT group. There were no differences between treatments within uninfected groups and within infected groups. A similar result to liver was observed for NOx kidney (Fig. 8B). The NOx levels in infected (CT, EL-100, BNZ and NBNZ) was superior comparing to respective uninfected groups. Moreover, the infected BNZ group showed major NOx levels related to other infected groups. The cortex NOx levels (Fig. 8C) were major in infected BNZ and NBNZ in relation to infected CT and EL-100 groups. The same can be observed on the comparison between uninfected groups.

The liver ROS levels (Fig. 9A) were superior in infected EL-100, BNZ and NBNZ when compared to their uninfected groups. Besides, the infected EL-100, BNZ and NBNZ demonstrate more ROS levels compared to infected CT group. In kidney evaluation (Fig. 9B) was possible to observe major ROS levels in all infected groups in comparison to its respective uninfected groups. The cortex ROS levels (Fig.9C) were major in infected groups, that was significant between infected and uninfected groups of CT and BNZ treatments.

3.6 Evaluation of the antioxidant capacity of free and nanocapsuled benznidazole during *T. cruzi* infection

The antioxidant enzymes catalase and superoxide dismutase were evaluated. The liver CAT activity (Fig. 10A) was major of infected groups when compared to their uninfected groups of treatments. In kidney (Fig. 10B) was observed superior of CAT enzyme activity in infected (CT and EL-100) in comparison to uninfected groups, respectively. The mice of BNZ and NBNZ treatment showed lower CAT activity in infected groups, with similar levels to uninfected groups.

The SOD activity there was no significant difference between groups on liver tissue (Fig. 11A). However, in kidney tissue (Fig 11B) was observed major SOD activity in infected CT and EL-100 compared to their uninfected groups. On the other hand, the animals of BNZ and NBNZ treatment have lower SOD activity in infected groups in relation to infected CT and EL-100 groups. The cortex SOD activity (Fig. 11C) was major in infected (CT, BNZ and NBNZ groups) when compared to respective uninfected groups.

The glutatione-S transferase activity, TSH and NPSH were evaluated in liver, kidney and cortex sample (supplementary material – Fig S1). The GTS activity in liver (Suppl. 1A) was superior only in infected BNZ compared to uninfected BNZ, other groups present no differences. The kidney (Suppl.1B) and cortex (Suppl. 1C) enzyme activity had no differences in groups comparison. The liver THS levels (Suppl. 2A) were major in infected (CT, EL-100 and NBNZ) in comparison to their uninfected groups. The kidney (Suppl. 2B) of THS levels were superior in infected BNZ comparing to its uninfected group. The cortex (Suppl.2C) presents no differences between groups. The NPSH levels was evaluated in liver, kidney, cortex and there were no differences between groups (Suppl. 3A, B, C).

3.6 Histopathological analysis

The analyze of splenomegaly (Fig. 13) was assessed through a relation between spleen weight and body weight percentage. It was possible to evidence that there was a major spleen weight in all infected groups in relation to uninfected groups. A lower percentage of body weight in infected BNZ when compared to infected CT group. The infected NBNZ showed a major spleen weight compared with infected CT group.

The histopathology of spleen tissue (Fig. 12) was evaluated through microscopy using H&E stain. No parasitic forms were observed on the microscopic slides analyzed. Tissue samples of CT group demonstrated preservation of tissue architecture, without microscopic changes. The

EL-100, BNZ and NBNZ groups showed marked lymphocyte clonal expansion and presence of activated macrophages scattered throughout the organ. Emphasizing that in the BNZ and NBNZ groups these cells were formed in a more abundant quantity.

The cardiac histopathology (Fig. 13) showed the presence of parasites was observed in the CT, EL-100 and NBNZ groups. In the biopsies of the CT and EL-100 groups, extensive areas of inflammatory infiltrate, amastigote nests and diffuse necrosis of cardiac tissue were observed. The samples from the other groups showed no alterations.

In photomicrography of liver tissue (Fig. 14), it was possible observe in the CT group, loss of integrity of hepatocyte cords, leukocyte clusters, presence of activated macrophages and areas of necrosis was observed. In the samples of the EL-100 group, a vast leukocyte infiltration with the presence of activated macrophages, hepatic cords with structural loss and vacuolated hepatocytes close to the centrilobular vein can be observed.

In the liver of animals from the BNZ group, leukocyte infiltration was observed around the vessels and maintenance of structural integrity. In samples from the NBNZ group, abundant inflammatory infiltrate and the presence of activated macrophages are observed.

4. Discussion

The chagas disease shown treatment efficacy only in acute phase. Benznidazole is the first-choice drug for the treatment protocol in Brazil. However, this treatment promotes many adverse effects, that can lead to treatment interruption (Pinazo *et al*, 2010; Sales Junior *et al*, 2017; Pavan *et al*, 2018). In this context the present study investigates the influence of conventional treatment and nanotechnology in order to suggest an aprimorate and efficiently therapeutically drug in the CD. For this, hematological, biochemistry parameters, oxidant/antioxidant status, histology analysis, and cholinergic molecular pathway were evaluated.

T. cruzi is an obligate intracellular parasite, metacyclic trypomastigotes and bloodstream trypomastigotes are the infective form (Barrias *et al.* 2013). In addition, the bloodstream trypomastigote can be identified to confirm the infection and accompanist parasitemia host. In curse of parasitemia, the peak of infection occurred at 7 p.i. Our results were also observed in others study (Moreira *et al.* 2014; Silva *et al.* 2017; Fracasso *et al.* 2019).

To diminished side effects, nanotechnology has been used as tool. Here, nanostructure containing BNZ were produced in stable nanocapsules of Eudragit. In addition, drug nanocarriers

against *T. cruzi* are being used in sense of decrease the dose, and increase the effects against parasite as reported by Sousa and collegues (2021). Which showed that nanoformulations demonstrate low cytotoxicity when compared with conventional treatment. These findings are consistent with our study and data.

It is knowledge that the toxicity of BNZ is derived by formation of nitro radical anion and reactive products generation when interacting with molecular DNA (Caldas, Santos and Novaes 2019; Castro, DeMecca and Bartel 2006). In this sense, we investigate whether NBNZ, could also diminished parasitemia trough qPCR technique. It was notorious the presence of genomic DNA of *T. cruzi* in cardiac and nervous tissues, of animals treated with BNZ free reflating low efficiency of treatment *in situ*. This data is in accordance with previously study, that describe BNZ efficiency was about 76% in acute phase (Cançado, 1999; Cançado 2002). The NBNZ treatment showed lower efficacy than BNZ in heart, and similar efficacy to BNZ treatment in brain, but in both tissue of NBNZ treated infected animals have a genomic DNA of *T. cruzi*.

The hematological parameters analyze have a substantial role to evaluated and to accompany the CD and treatment evolution. This analyze demonstrated an anemia state with reduction in erythrocytes, hemoglobin and hematocrit in current research (Chagas, 1909; Roberts, 2016; Villalba-Alemán, 2018). This condition was observed in our study in infected groups. However, the BNZ treatment was able to prevents this hematological alteration, but the NBNZ treatment doesn't present this action. Furthermore, the leukocytes seem present a reduction during acute *T. cruzi* infection accompanied by granulocytopenia. However, the treatment with NBNZ was able to promotes a leukocytosis with a granulocytosis and lymphocytosis. Besides, it was observed a thrombocytopenia in *T. cruzi* infection, that was reversed by BNZ and NBNZ treatment.

The anemia, leukopenia and thrombocytopenia physiopathology can be related to complex mechanisms, involving the increase of cell destruction or a decrease in cell production. The infection may be associated with a reduction number of cell precursors in the bone marrow, mainly erythroblast and megakaryoblast. The decrease in the numbers of erythrocytes, platelets, and leukocytes may result of a suppression in cell production in bone marrow, that can be ralted to a side effect of BNZ. On the other hand, the anemia can be related to interaction with parasite molecules in blood, as well as to an autoimmunity action of organism, that can occur during the infection. (Marcondes, 2000; Ferraz 2014, Villalba-Alemán, 2018).

Function parameters as AST and ALT are considered hepatic damage marker, but it is to related to cardiac tissue damage, when altered. These parameters were evaluated to verify hepatotoxicity in CD and the effects of treatments. There was a pronounced augment in both markers in all experimental groups infected. Besides, BNZ is a choice drug for acute CD treatment, this drug is metabolized in the liver and may promotes hepatic toxicity, which can be related to increase of AST and ALT activities at the initial weeks of treatment (Pavan *et al.* 2018; Dos Santos *et al.* 2020) such as observed here. The other treatment groups can reflects, with these alterations, cardiac and hepatic damage promotes by parasite infection in these tissues.

In addition, creatinine and urea are used as a renal injury marker. These parameters showed an increase in infected groups. However, the treatments were able to control these alterations. The BNZ infected animal presents an increase of creatinine levels, but there were no alterations of tissue in the histopathological analyzes, that can be related with the BNZ metabolism that is partially renal. It has been related the renal injury in acute CD, but the intensity of inflammatory process is may be linked with the parasites load of infection (Lemos *et al.* 2013). Some research related that the renal alterations and compromising are not related to the parasite presence, but with the infiltrate inflammatory present as a complication of disease. The cardiovascular disfunction is one of mainly complications in acute CD, and can lead to a decrease renal blood flow, that promotes several alterations in tissue conditions (Lemos *et al.* 2013; Silva Junior *et al.* 2017; Oliveira e Brito, 2019).

The cholinergic system it has been related to immune response in parasite infections (da Silva *et al.* 2011; Silva *et al.* 2017). This system plays an important role in inflammatory process as well as in immune response modulation (Kawashima & Fujii, 2004; Fujii *et al.* 2017; Fujji *et al.* 2017a; Silva *et al.* 2017). Here, we evaluated some components of cholinergic system in lymphocytes isolated of spleen. This organ is an essential target involved in immune responses. Furthermore, AChE enzyme was evaluated, in total blood and cortex. BuChE activity were analyzed in plasm, liver and cortex.Our data showed an increase in AChE activity in lymphocytes, blood and cortex in the acute *T. cruzi* infection. The NBNZ treatment promotes a reduction of AChE in uninfected lymphocytes, but in blood this treatment promotes an increase of enzymatic activity. AChE is responsible to hydrolyze acetylcholine, that has inflammatory response. Once the AChE is increase it’s possible suggest that ACh levels can be reduced, by an accelerate hydrolysis. The Ach is a neurotransmitter, but is too produced by mononuclear

immune cells, and may acts by autocrine and paracrine form to immune regulation, interacting with AChR muscarinic and nicotinics (Kawashima *et al.* 2012; Fujii *et al.* 2017a, Fujii *et al.* 2017 b).

The expression of M1 and M2 mAChR was evaluated and demonstrate a decrease during acute *T. cruzi* infection. However, the infected animals and treated with BNZ present an increase expression. The AChE expression demonstrates the same pattern of mAChR. The reduced may be linked to a control mechanism of inflammatory process, since the reduction of AChE levels can attenuate the inflammation promotes by immune cells. This reduction is associated with AChE binding to M1 and M2 receptors. The M1 mAChR suppression may be associated a reduction of TNF-α, INF-ɤ and IL-6, that is, these receptor types are involved in regulating pro-inflammatory response and modulating antibody class transition from IgM to IgG, for example (Fujji *et al.* 2017a; Fujii *et al.* 2017b).

The M1, is a muscarinic receptor coupled to Gq/11, when stimulated they mediate the phospholipase C activation that result in the increase of Ca^{2+}intracellular. The M2 mAChR subtypes are coupled to Gi/o, under stimulation they promote the inhibition of adenylyl cyclase, resulting in a cAMP synthesis reduction (Kawashima & Fujii, 2004). However, the M2 are little explored in anti-inflammatory cholinergic pathway studies, not having a clearly described role, but in this research, it showed an expression pattern very similar to M1 mAChR. Some researchers showed that M1/M5 KO mice demonstrate a suppression in AChE gene expression, suggesting that M1 and/or M5 mAChR may modulates AChE transcription, and it occur in an independent form of α7 nAChRs. Thereby suggest that up-regulates of immunological stimulation occurs both by the synthesis of ACh and by its degradation (Fujii *et al.* 2007a; Fujii *et al.* 2007b).

The AChE activity in blood and brain was previously evaluated and showed a reduction in enzyme activity, but this was related to a reduction of parasitemia in infected and untreated animals (Silva *et al.* 2017). The cholinergic modulation showed a different pattern in blood and brain, that can be related to a second increase of parasitemia, that occurs after day 9 p.i. This increase of parasitemia may be promotes a major inflammatory response in these cells. Since the process and presentation of antigen is a factor that may stimulates the pro-inflammatory modulation in these cells (Fujii *et al.* 2017a; Fujii *et al.* 2017b). In addition, the BuChE activity showed a reduced activity in infected groups. This enzyme also hydrolyzes the ACh, but the

AChE had higher catalytic efficiency than BuChE in substrate hydrolyze (Reale *et al.* 2018). This reduced activity may be a compensatory mechanism to regulates the inflammatory response in acute infection, once ACh perform an anti-inflammatory response.

Redox status was measured in present study. Carbonyl levels are associated with protein oxidation (Dalle Donne and col. 2003), and was elevated this study. Fracasso *et al.* (2021) also showed decreased carbonyl levels in liver in acute phase of CD, corroborant with our data to infected and treated groups. Besides the infected and untreated group evidence the opposite pattern. In addition, TBARS levels indicate the lipid peroxidation related to membrane integrity and loss of membrane protein function (Droge 2002). Is notary the major TBARS in liver and kidney in our findings and also described by Barbosa *et al.* (2016) and Fracasso *et al.* (2021).

NOx and ROS are molecules direct relationship with inactivation of trypomastigotes, mediated by BNZ molecule. The BNZ exerts its trypanocidal effects after enzymatic activation by trypanosomal type I nitroreductases (NTRs) according Petterson and Wylle (2014). Furthmore, *T. cruzi*-derived inflammation, recruits immune cell and produces ROS/RNS (Martins and col. 1998; Youne´s-Chennoufi *et al.* 1998). Findings reported by Fracasso *et al.* (2021) founded similar results to those shown here. Therefore, trypomastigotes forms are likely the targets of NOx, resulting in killing of the parasite (Vespa, Cunha and Silva, 1994). In addition to the ROS/RNS levels being turned on increases interact with production of other inflammatory cytokines that contribute to exacerbation of inflammatory responses mediated by *T. cruzi* responses, as demonstrated in other studies (Silva *et al.* 2017).

Redox status also is balanced by antioxidant enzymes. Catalase according Anschau (2011) and Baldissera *et al.* (2014) is an enzyme that acts decompounding hydrogen peroxide. While superoxide dismutase enzyme acts dismuting the superoxide radical into hydrogen peroxide (Halliwell 1987; Halliwell 1990). Here the data shown elevated CAT activity in infected animals, corroborating previous results (Anschau, 2011; Baldissera *et al* 2014; Fracasso *et al.* 2021). The increase in SOD activity in *T. cruzi*-infected animals may depend on the type of tissue involved with activation of other O^{-2} dismutation pathways and evertheless, SOD activity could not reverse ROS levels (Floreano *et al.*, 2003; Colato *et al.* 2018). Levels of GST, TSH and NPSH did not statistical significance, but may be associated one compensatory mechanism, were the host uses of other ways of control the parasitemia levels and acts to reduce the pro-inflammatory process by *T. cruzi*-induced.

The *T. cruzi* infected the host thought mucosa invasion, the trypomastigote form of parasite invade mainly macrophages, but they can infect several cells types in tissues, like heart, smooth muscle in digestive tract, esophagus, lungs, kidney, spleen, liver, brain, placenta, bone marrow and other (Pereira *et al.* 2019). In this experiment protocol the brain, heart, liver, kidney and spleen were histopathological evaluated.

The parasite amastigote forms were found only in cardiac tissue. However, this tissue, liver and spleen showed a significant inflammatory infiltrate and tissue damage. No significative alterations were observed in brain, heart, liver, kidney and spleen. The treatment with BNZ prevents the cardiac tissue invasion by parasite. The treatment with NBNZ prevents a tissue damage, but was not efficient in prevents parasite invasion in heart. The lesions extension and parasite ability to invade tissues is not involved only with genotype of parasite, but is too related with host genetic characteristics and immune response (de Oliveira *et al.* 2016).

5. Conclusion

In summary, we study the cholinergic signaling pathways in immunomodulation and redox status during acute chagas disease. *T. cruzi* infection and benznidazole treatment improve the ACh hydrolyze, leading to a pro-inflammatory response. corroborating with this, the lymphocytes increase a cholinergic components expression seems keeping the pro-inflammatory profile of response, evidence the interaction of drug with cholinergic pathway. The redox status had a very organ specific response pattern, in a general observation, there was a pro-oxidant response of organism in the infection and treatments, that is related to exacerbate inflammatory process, to control the parasite proliferation, induced by immune response and drug action. Nevertheless, even with highest dose, free BNZ was not 100% efficacy in this treatment period, but continuous been a better option to treatment, and can be associated with antioxidant components in future research, to protect the host organism. In addition, the nanotechnology needs be continuous tested front this disease, because represents an opportunity to improve the treatment and the live quality of infected people.

Declaration of competing interest

The authors declare no competing or financial interests.

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Figures and Tables

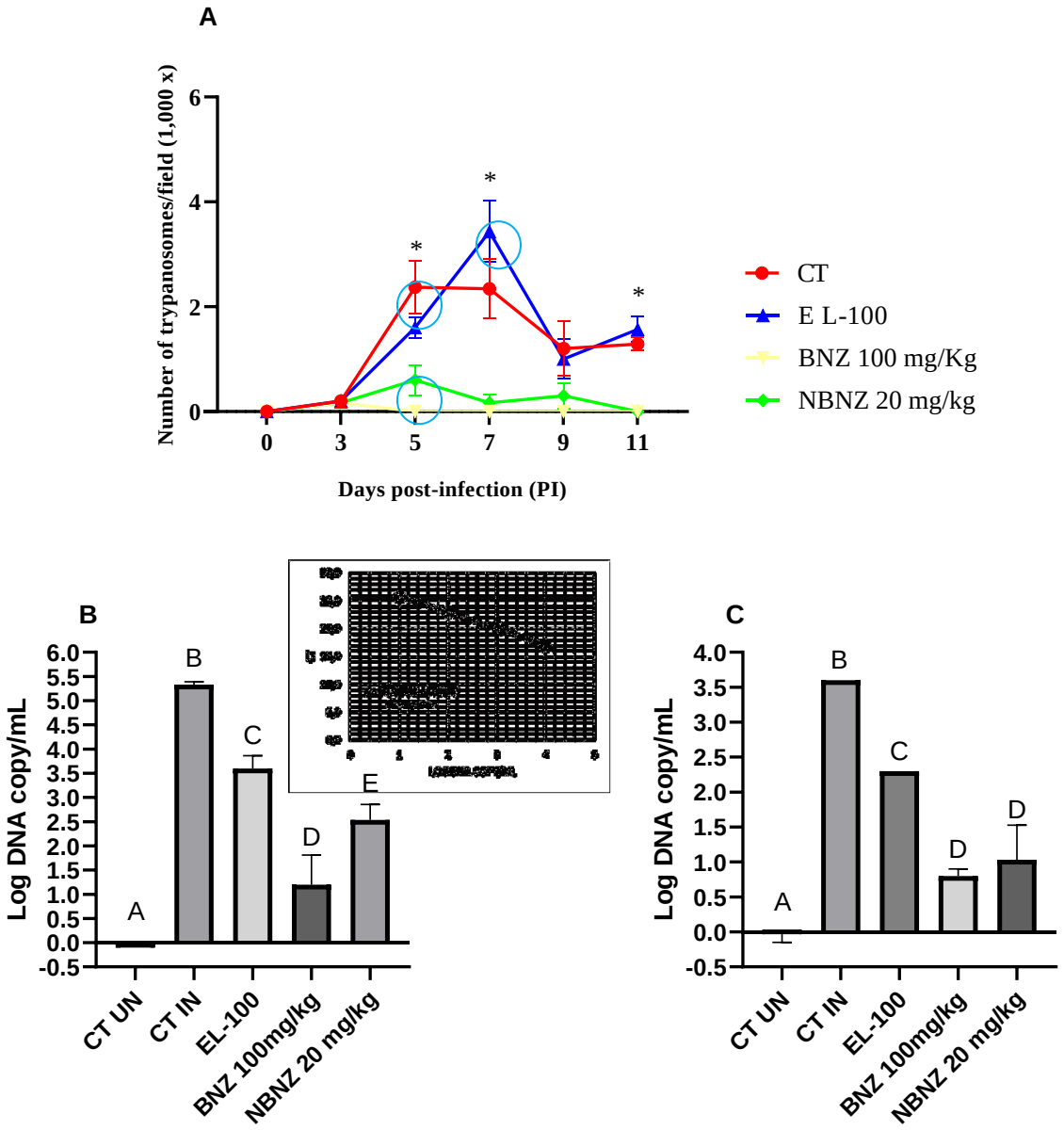

Figure 1. ***Trypanosoma cruzi*** **confirm infection. A.** Parasitemia assessment of animals infected by *T. cruzi* (Y strain) exposed to BNZ treatments protocol. **B.** qPCR of cardiac tissue; **C.** qPCR of cortex tissue. Differences were considered statistically significant when *$p < 0.05$. Data are presented as mean± S.EM.

Table 1. Mean and SD of hematological parameters in *Trypanosoma cruzi* infection

Treatments		Erythrocytes (x 10^6 /µL)	Hemoglobin (g/dL)	Hematocrit (%)	Platelets (/µL)	Total leukocytes (/µL)	Granulocytes (/µL)	Lymphocytes (/µL)	Monocytes (/µL)
CTL	**UN**	8.17^{A} ± 0.26	13.22^{A} ± 0.44	39.40^{A} ± 1.41	798666.67^{A} ± 207258.93	1833.33^{AB} ± 1427.81	426.33^{AB} ± 239.53	1351.67^{AB} ± 1090.71	55.33^{A} ± 104.50
	IN	5.17^{B} ± 0.17	8.16^{B} ± 0.26	24.40^{B} ± 0.69	392200.00^{B} ± 144771.19	1240.00^{AB} ± 536.65	163.20^{B} ± 81.15	1026.00^{AB} ± 494.17	50.80^{A} ± 43.47
E L-100	**UN**	8.00^{A} ± 0.54	12.95^{A} ± 0.76	38.48^{A} ± 2.48	791000.00^{A} ± 229249.50	2900.00^{AB} ± 1739.73	1017.50^{AC} ± 708.64	1706.00^{AB} ± 1056.41	176.50^{A} ± 161.68
	IN	5.38^{B} ± 0.74	8.375^{B} ± 1.34	25.02^{B} ± 3.26	425500.00^{B} ± 176722.94	3575.00^{B} ± 464.57	425.25^{B} ± 518.04	2882.50^{B} ± 666.54	267.25^{A} ± 214.43
BNZ 100 mg/Kg	**UN**	7.77^{A} ± 0.48	12.40^{A} ± 0.91	37.04^{A} ± 2.55	748000.00^{AB} ± 68025.73	2220.00^{AB} ± 1465.26	491.60^{AB} ± 201.37	1678.20^{AB} ± 1236.35	50.20^{A} ± 50.88
	IN	7.72^{A} ± 0.58	12.68^{A} ± 0.97	37.57^{A} ± 2.76	835833.33^{A} ± 116214.31	3116.67^{AB} ± 941.09	424.67^{B} ± 130.03	2639.17^{AB} ± 929.64	52.83^{A} ± 31.56
NBNZ 20 mg/Kg	**UN**	7.76^{A} ± 0.43	12.80^{A} ± 1.09	37.62^{A} ± 2.89	913500.00^{A} ± 141778.34	1216.67^{A} ± 552.87	306.67^{B} ± 112.91	859.00^{A} ± 413.83	51.00^{A} ± 37.79
	IN	6.01^{B} ± 0.60	9.45^{B} ± 1.01	28.92^{B} ± 2.80	780666.67^{A} ± 101653.66	5916.67^{C} ± 994.81	832.50^{C} ± 457.93	4902.50^{C} ± 957.21	181.67^{A} ± 112.77

The data are shown as mean ±SD. Statistical differences are considered when $p<0,05$, and represents by different letters.

Table 2. Mean and SD liver and kidney function marker in serum

Treatments		AST	ALT	Creatinine	Urea
CTL	UN	126.50^{A} ± 9.81	31.00^{A} ± 1.15	0.43^{A} ± 0.049	52.00^{A} ± 3.46
	IN	448.25^{B} ± 5.5	174.5^{C} ± 5	0.625^{BC} ± 0.05	123.5^{C} ± 3.31
E L-100	UN	147.75^{A} ± 1.5	32.25^{A} ± 2.5	0.43^{A} ± 0.049	43.75^{B} ± 1.5
	IN	2764.25^{C} ± 10.5	241^{D} ± 4.76	0.53^{AB} ± 0.05	87.25^{D} ± 1.5
BNZ 100 mg/Kg	UN	132.33^{A} ± 15.37	24.25^{B} ± 3.40	0.56^{A} ± 0.12	43.75^{B} ± 2.5
	IN	144.5^{A} ± 3	37.75^{E} ± 1.5	0.68^{C} ± 0.05	45.25^{B} ± 2.87
NBNZ 20 mg/Kg	UN	99.00^{A} ± 6.92	23.50^{B} ± 5.19	0.45^{A} ± 0.05	36.50^{B} ± 0.57
	IN	807^{D} ± 34.43	61^{F} ± 1.63	0.53^{AB} ± 0.05	27.00^{E} ± 0.81

The data are shown as mean ± SD. The significative differences between groups as demonstrate through different letters. The differences are considered statistically significant when $p < 0.05$.

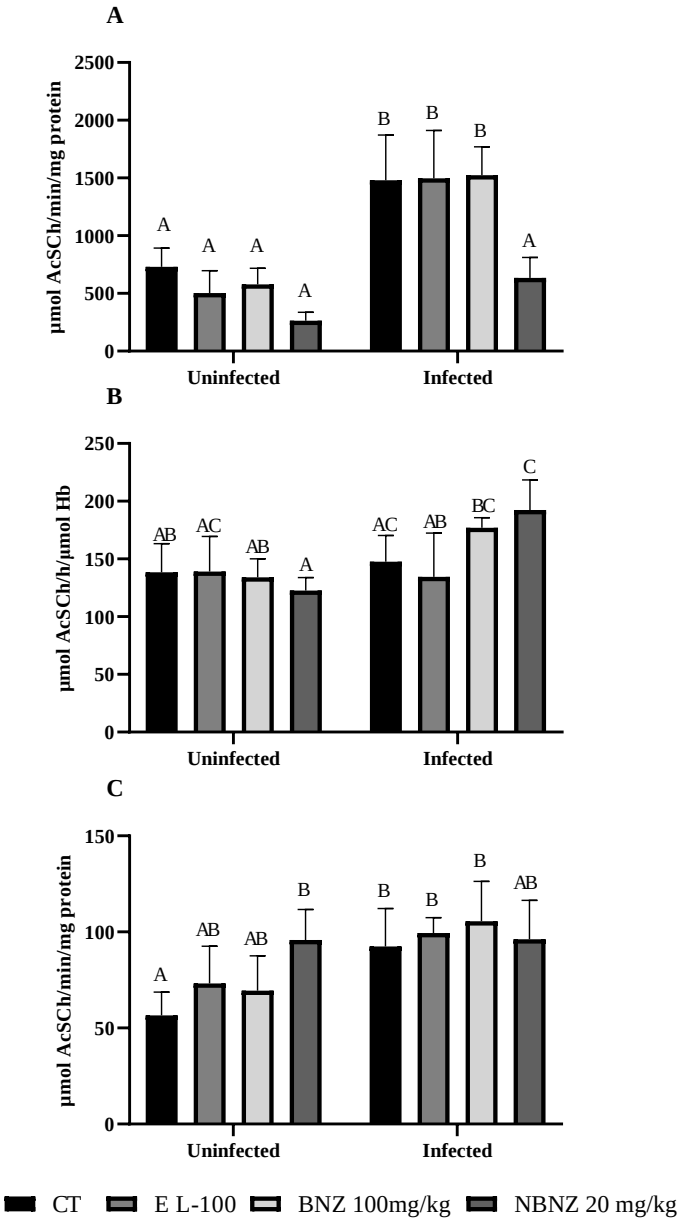

Figure 2. Acetylcholinesterase activity during experimental *T. cruzi* infection. Swiss mice lymphocytes (A), erythrocytes (B) and cortex (c) enzyme activity during Y strain infection. Bars indicates mean ± SD. The differences are considered statistically significant when $p<0.05$ and these are demonstrated by different letters, same letters represent no difference between groups.

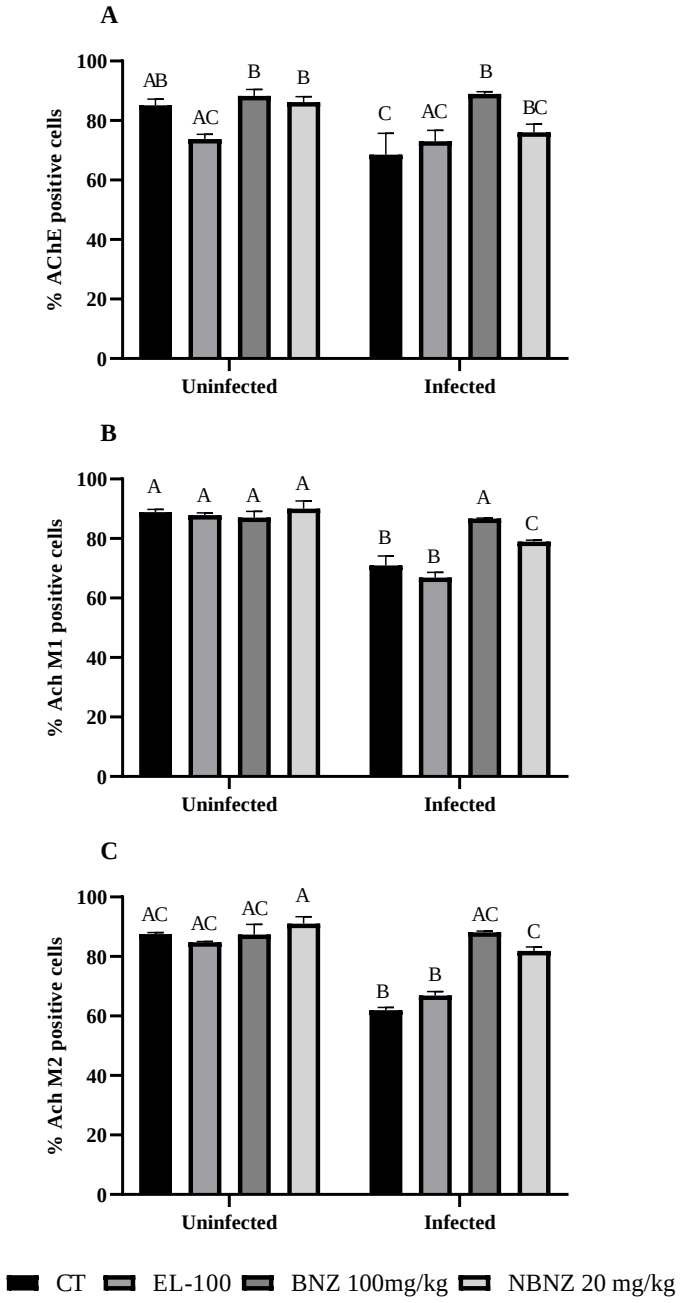

Figure 3. Cytometer lymphocytes cholinergic enzyme and muscarinic receptors expression during experimental *Trypanosoma cruzi* infection. Swiss mice lymphocytes acetylcholinesterase (A), M1 receptor (B) and M2 receptor (C) expression during Y strain infection. Bars indicates mean ± SD. The differences are consider statistically significant when $p<0.05$ and these are demonstrate by different letters, same letters represents no difference between groups.

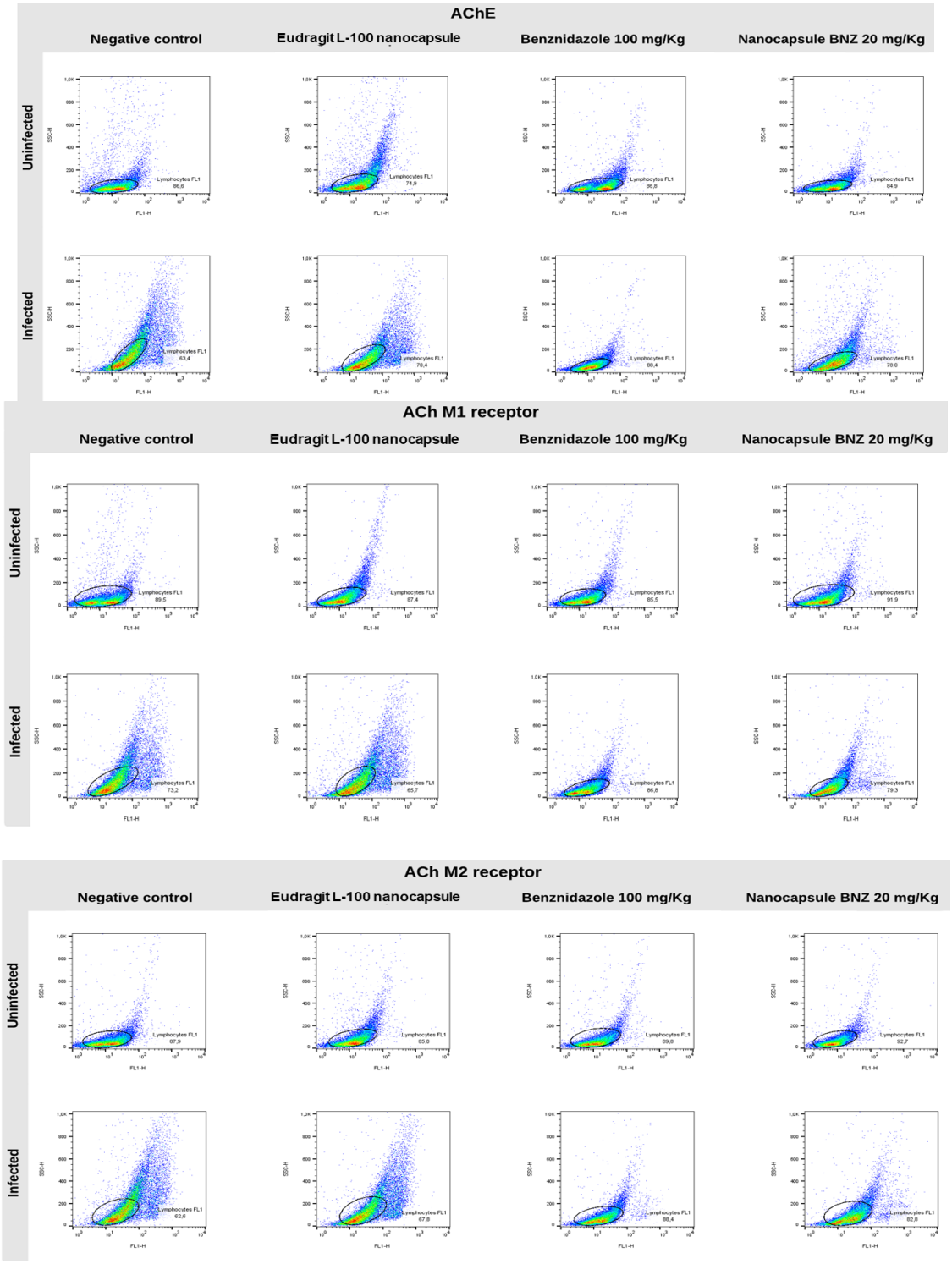

Figure 4. Representative lymphocytes cytometry marked with cholinergic enzyme and muscarinic receptors during experimental *Trypanosoma cruzi* infection. Ploted gates representation of AChE, AchM1 and AchM2 markers events. Bars indicates mean ± SD. The differences are consider statistically significant when $p<0.05$ and these are demonstrate by different letters, same letters represents no difference between groups.

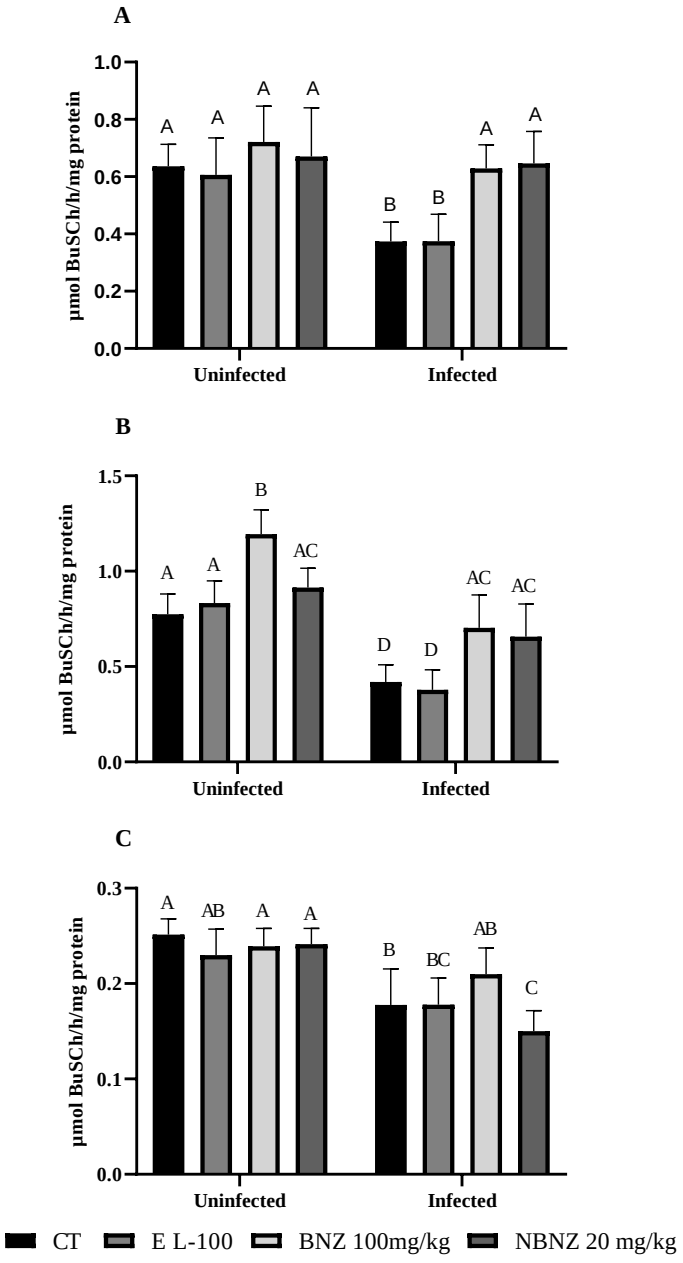

Figure 5. Butirylcholinesterase activity during experimental acute *T. cruzi* infection. Swiss mice plasmatic (A), liver (B) and cortex (C) enzyme activity during Y strain infection. Bars indicates mean ± SD. The differences are considered statistically significant when $p<0.05$ and these are demonstrated by different letters, same letters represent no difference between groups.

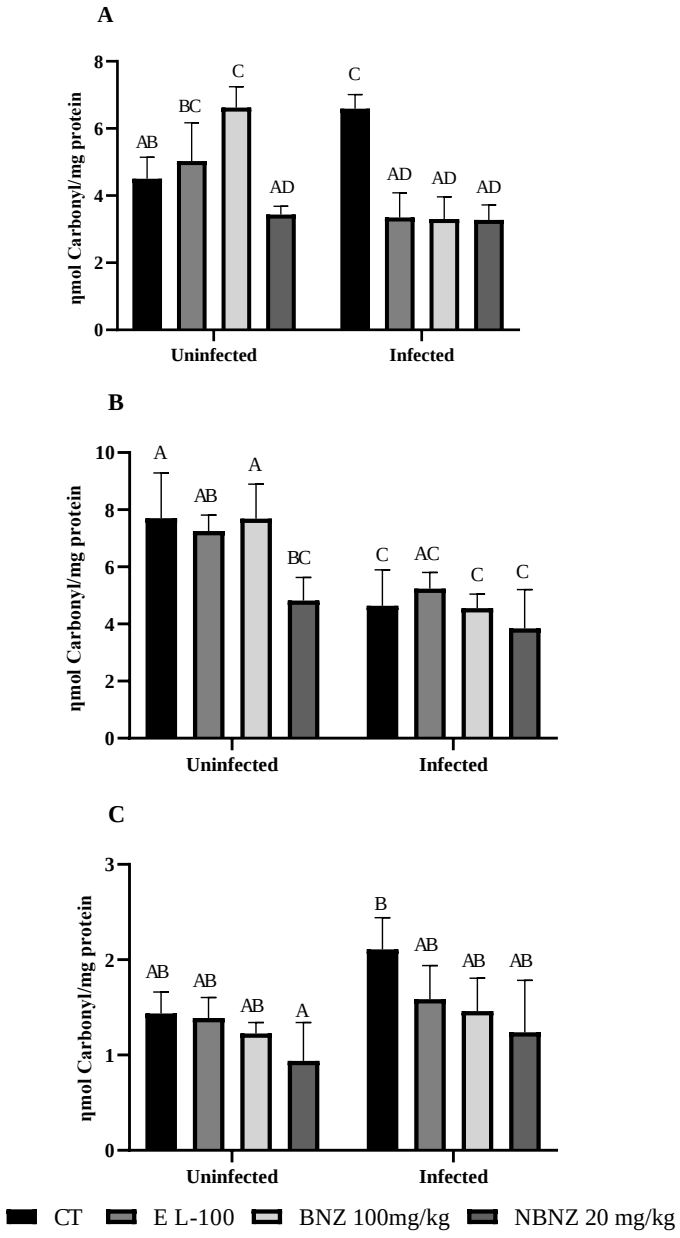

Figure 6. Evaluation of protein carbonylation during experimental acute *T. cruzi* infection. Swiss mice liver (A), kidney (B) and cortex (C) parameter during Y strain infection. Bars indicates mean ± SD. The differences are considered statistically significant when $p<0.05$ and these are demonstrated by different letters, same letters represent no difference between groups.

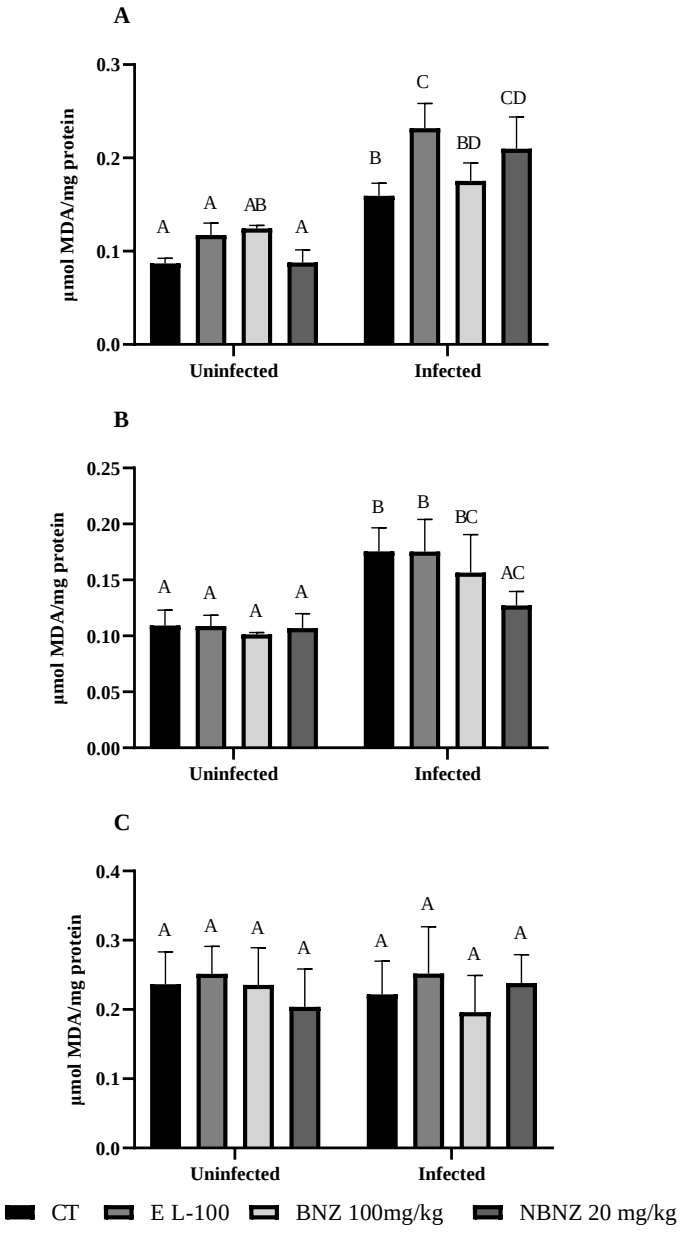

Figure 7. Evaluation of MDA levels during experimental acute *T. cruzi* infection. Swiss mice liver (A), kidney (B) and cortex (C) parameter during Y strain infection. Bars indicates mean ± SD. The differences are considered statistically significant when $p<0.05$ and these are demonstrated by different letters, same letters represent no difference between groups.

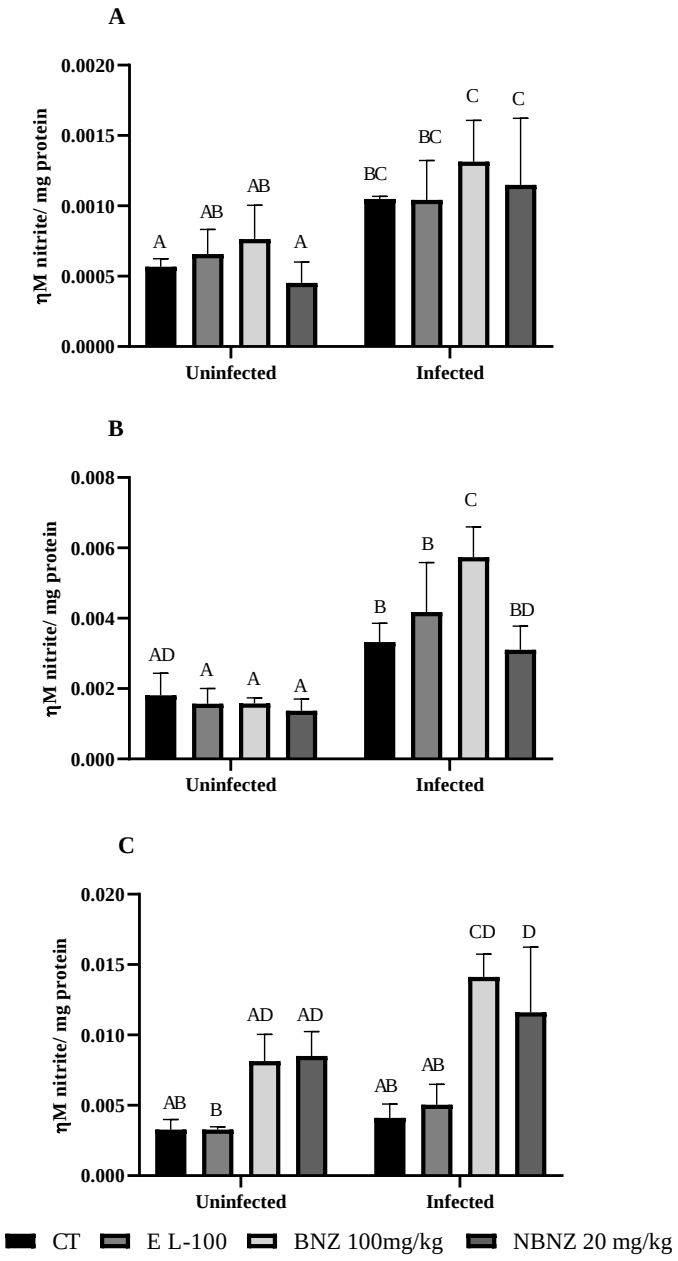

Figure 8. Evaluation of nitrate levels during experimental acute *T. cruzi* infection. Swiss mice liver (A), kidney (B) and cortex (C) parameter during Y strain infection. Bars indicates mean ± SD. The differences are considered statistically significant when $p<0.05$ and these are demonstrated by different letters, same letters represent no difference between groups.

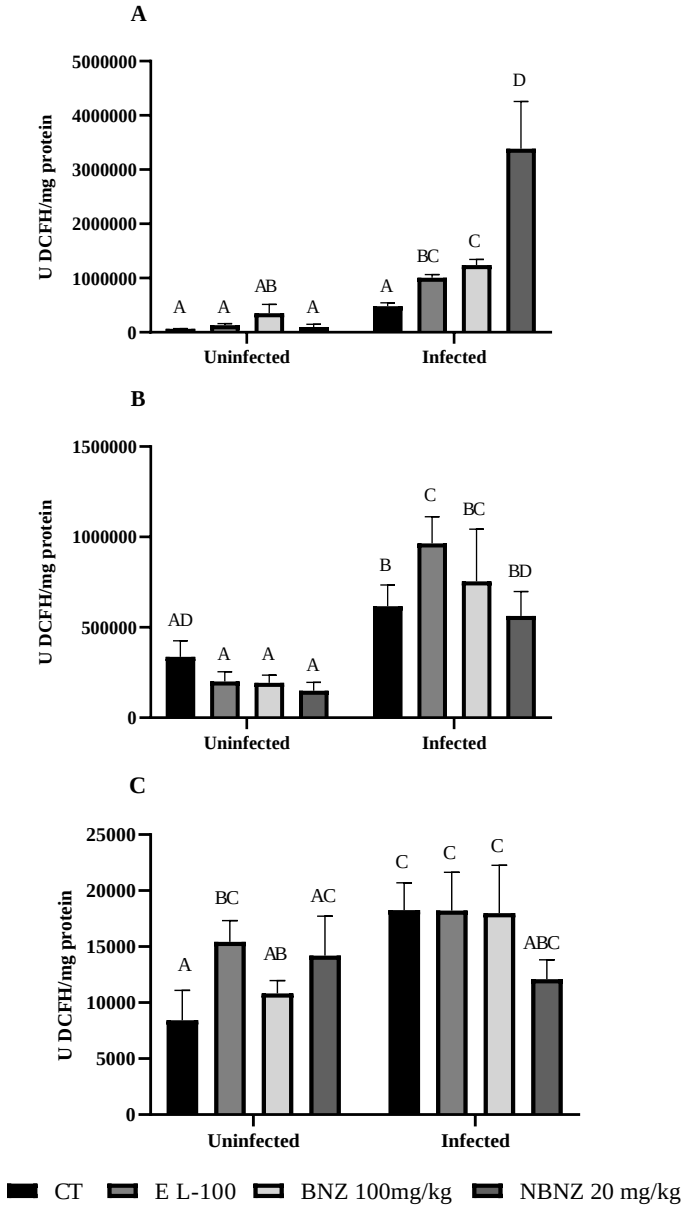

Figure 9. Evaluation of reactive oxygen species levels during experimental acute *T. cruzi* infection. Swiss mice liver (A), kidney (B) and cortex (C) parameter during Y strain infection. Bars indicates mean ± SD. The differences are considered statistically significant when $p<0.05$ and these are demonstrated by different letters, same letters represent no difference between groups.

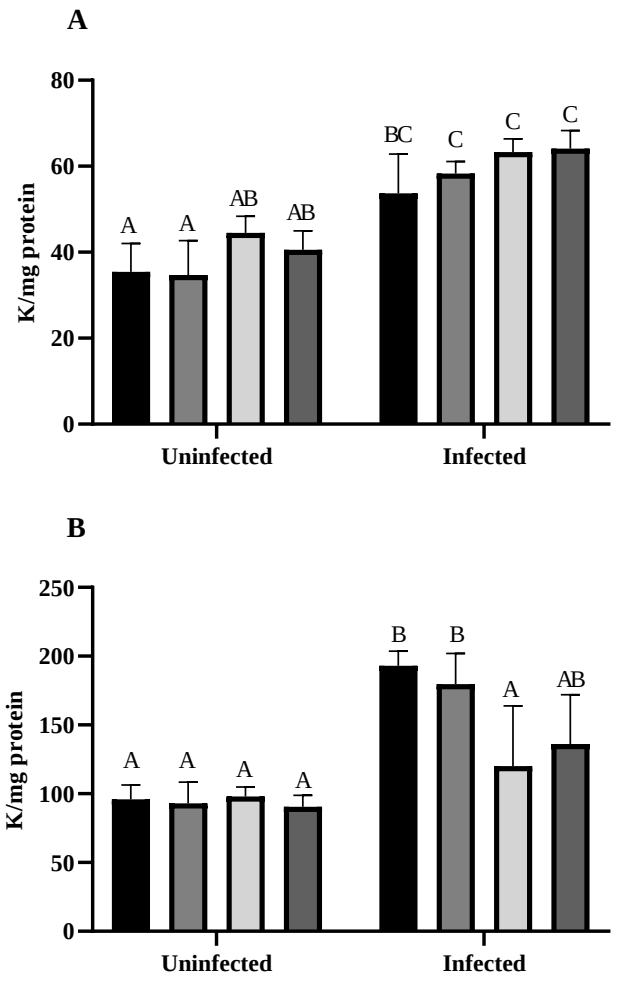

Figure 10. Catalase activity assay in experimental acute *T. cruzi* infection. Swiss mice liver (A), kidney (B) enzyme activity during Y strain infection. Bars indicates mean ± SD. The differences are considered statistically significant when $p<0.05$ and these are demonstrated by different letters, same letters represent no difference between groups.

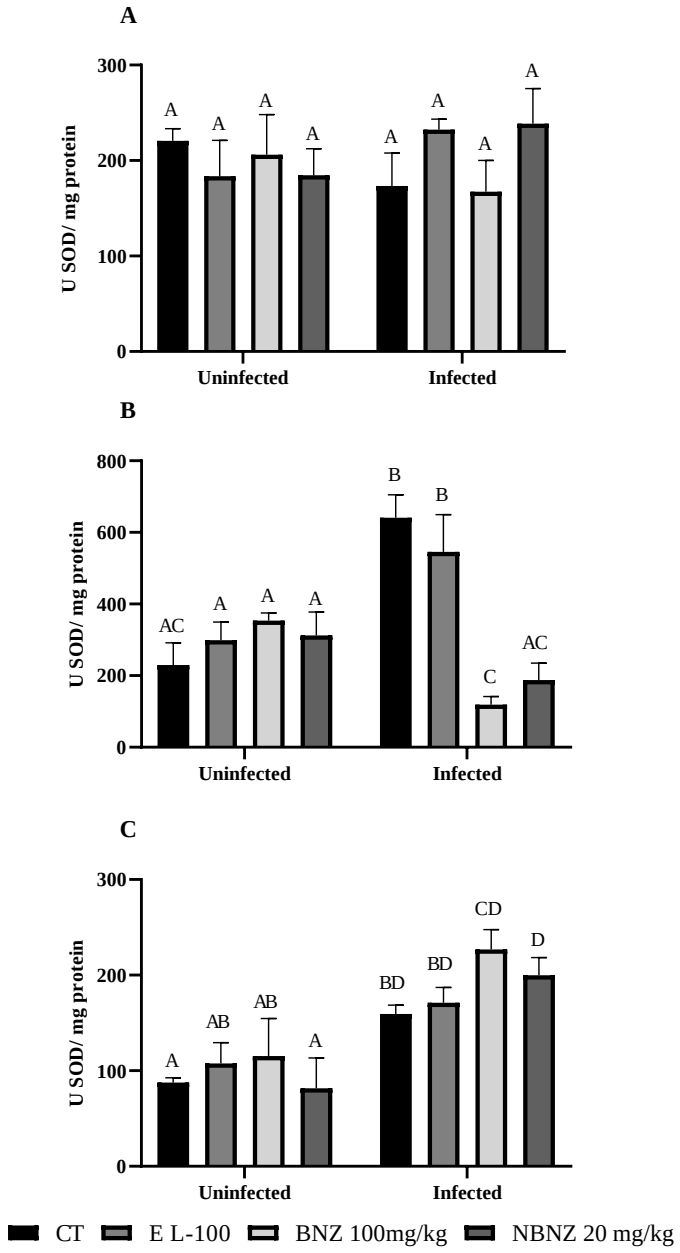

Figure 11. Superoxide dismutase activity assay in experimental acute *T. cruzi* infection. Swiss mice liver (A), kidney (B) and cortex (C) enzyme activity during Y strain infection. Bars indicates mean ± SD. The differences are considered statistically significant when $p<0.05$ and these are demonstrated by different letters, same letters represent no difference between groups.

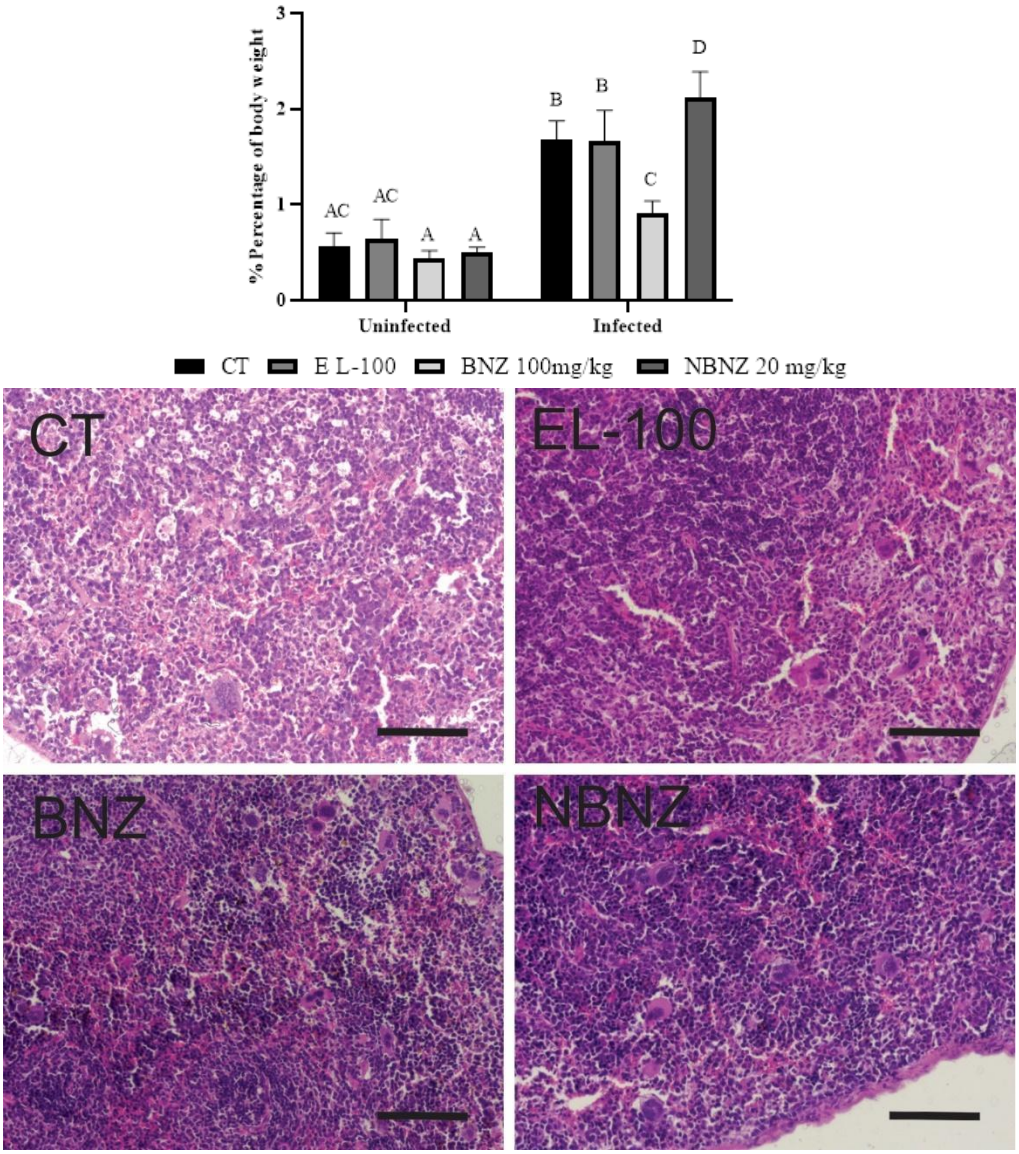

Figure 12. Spleen evaluation of mice experimentally infected by *Trypanosoma cruzi*. Graphic demonstration the spleen percentage of body weight. Photomicrography of the spleen tissue of mice experimentally infected by *T. cruzi*. CT: infected and untreated, EL-100: infected and treated with vehicle, BNZ: infected and treated with 100 mg/kg of BNZ, D: infected and treated with 20 mg/kg of NBNZ. Stained by H&E. Scale bar 100mm.

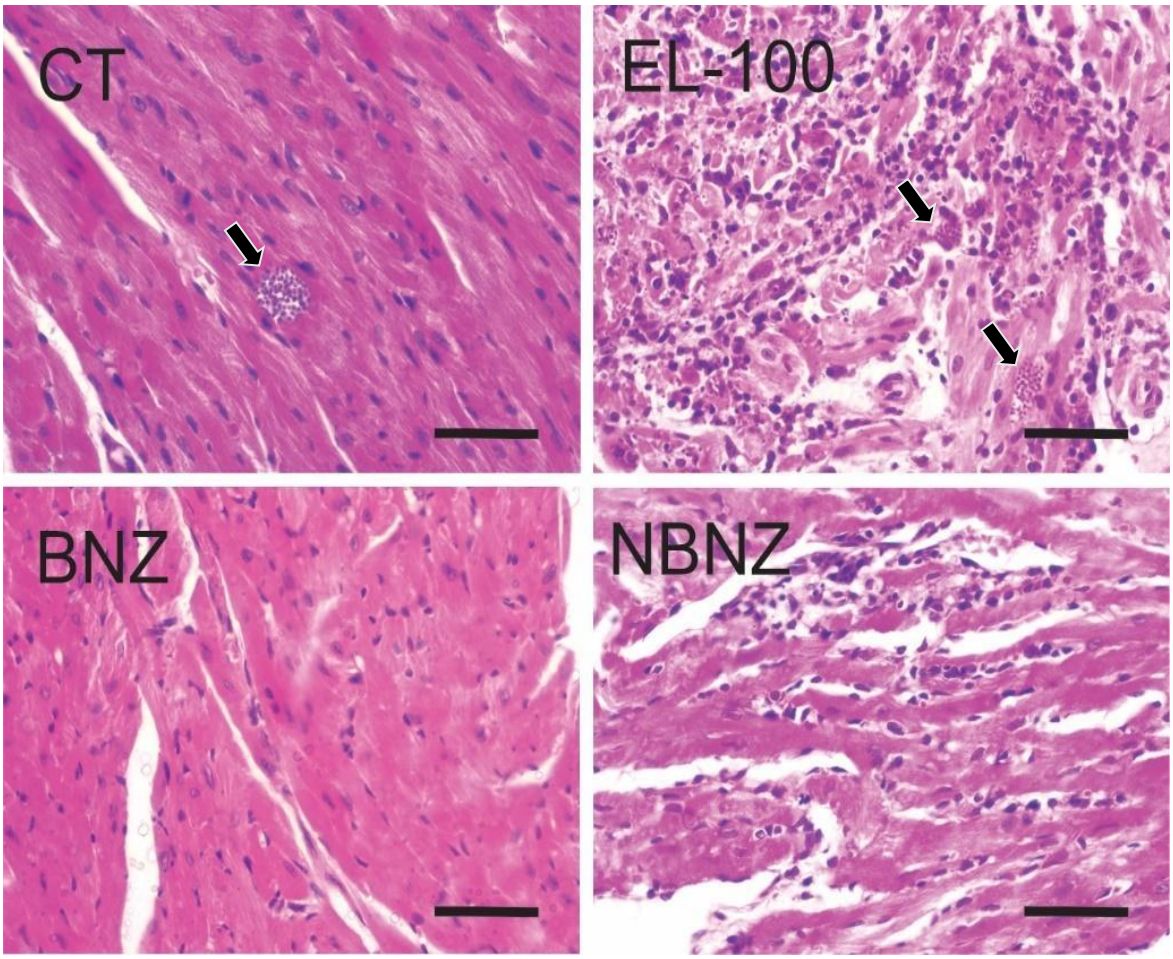

Figure 13. Histopathological images of the cardiac tissue of mice experimentally infected by *T. cruzi*. CT: infected and untreated, EL-100: infected and treated with vehicle, BNZ: infected and treated with 100 mg/kg of BNZ, D: infected and treated with 20 mg/kg of NBNZ. Arrows showed amastigote nests. Stained by H&E. Scale bar 50mm.

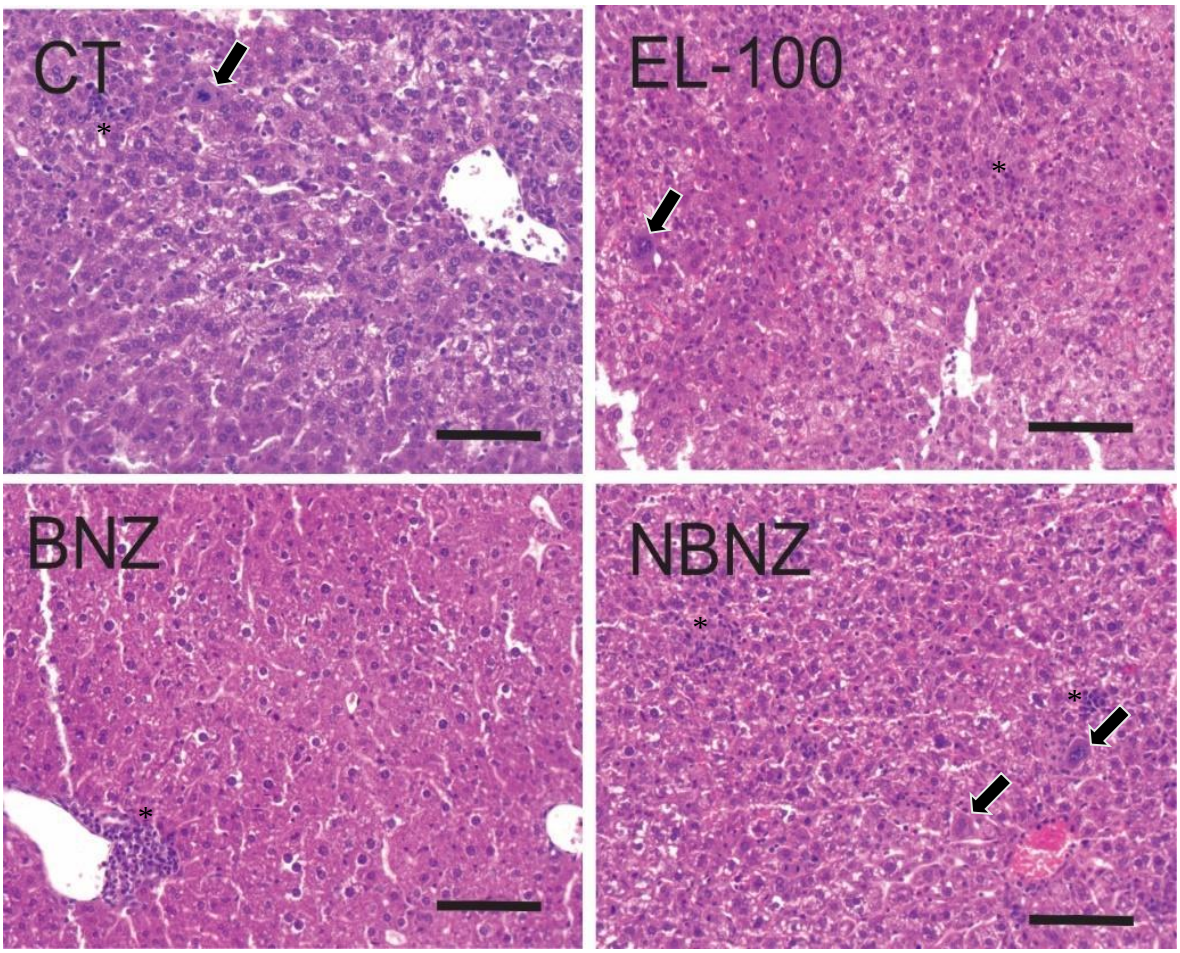

Figure 14. Histopathological images of the liver tissue of mice experimentally infected by *T. cruzi*. CT: infected and untreated, EL-100: infected and treated with vehicle, BNZ: infected and treated with 100 mg/kg of BNZ, D: infected and treated with 20 mg/kg of NBNZ. * Inflammatory infiltrate; arrows showed activated macrophages. Stained by H&E. Scale bar 100mm.

Suplementar Material

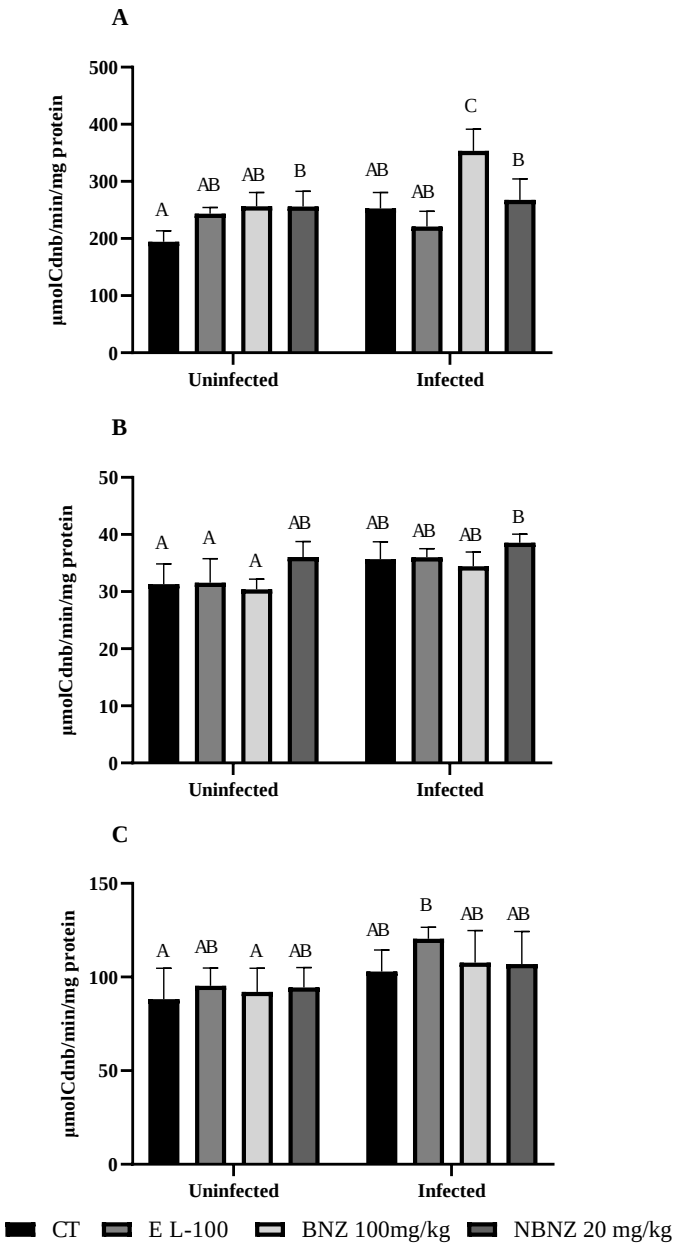

Figure S1. Glutathione s transferase activity assay in experimental acute *Trypanosoma cruzi* infection. Swiss mice liver (A), kidney (B) and cortex (C) enzyme activity during Y strain infection. Bars indicates mean ± SD. The differences are considered statistically significant when $p<0.05$ and these are demonstrated by different letters, same letters represent no difference between groups.

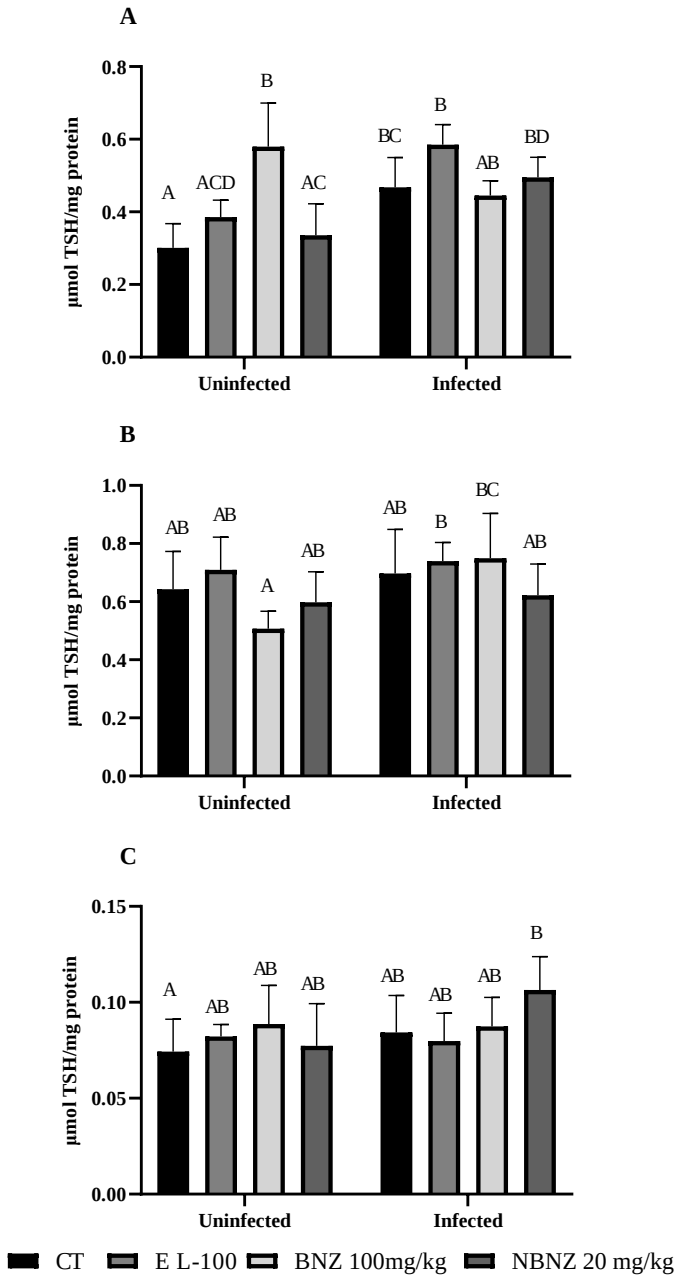

Figure S2. TSH leves in experimental acute *T. cruzi* infection. Swiss mice liver (A), kidney (B) and cortex (C) parameter during Y strain infection. Bars indicates mean ± SD. The differences are considered statistically significant when $p<0.05$ and these are demonstrated by different letters, same letters represent no difference between groups.

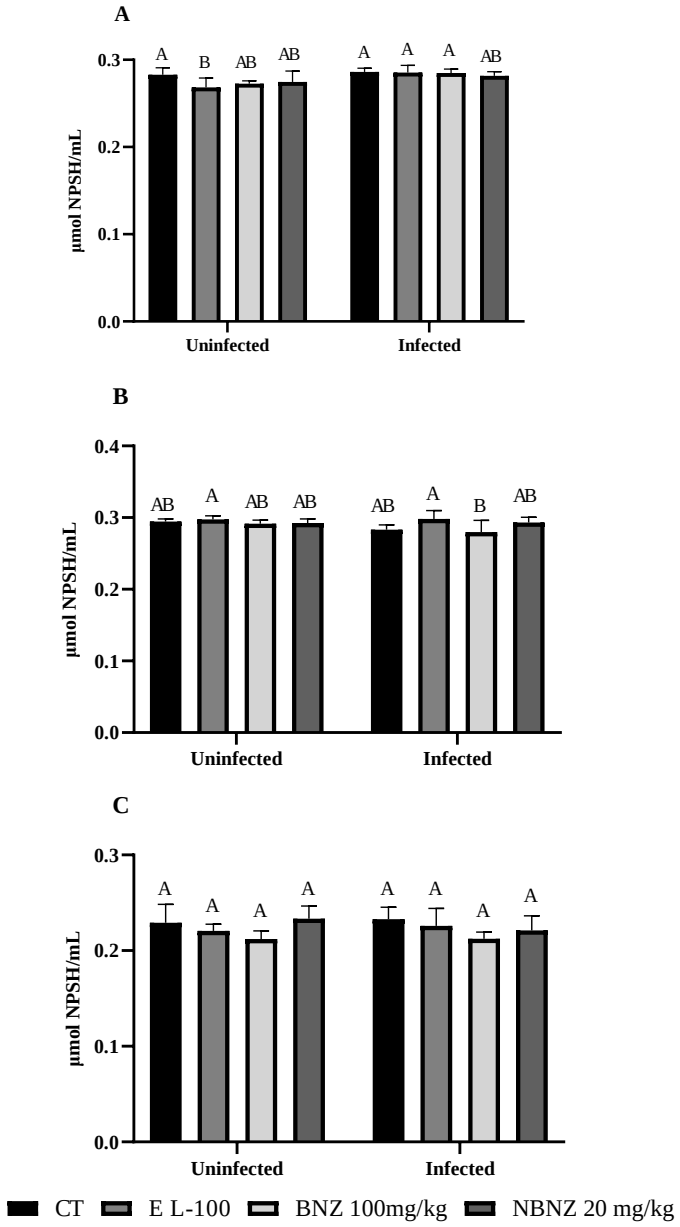

Figure S3. NPSH leves in experimental acute *T. cruzi* infection. Swiss mice liver (A), kidney (B) and cortex (C) parameter during Y strain infection. Bars indicates mean ± SD. The differences are considered statistically significant when $p<0.05$ and these are demonstrated by different letters, same letters represent no difference between groups.

5. DISCUSSÃO

A DC apresenta no Brasil como fármaco de escolha o benznidazol, até o momento é o fármaco com maior eficácia no tratamento, porém sua ação ocorre frente aos parasitos circulantes, fazendo com que o mesmo tenha sucesso na eliminação do parasito somente na fase aguda. O efeito tripanocida do BNZ está relacionado a uma indução do aumento da produção de espécies reativas de oxigênio e nitrogênio, sejam provenientes do metabolismo do parasito ou os gerados pela resposta imune do hospedeiro (BATISTA *et al.*, 2020; GOMEZ, 2017; SALES JUNIOR *et al.*, 2017). A terapia farmacológica tem como objetivo reduzir a parasitemia, impedir a reativação e promover uma melhora dos sintomas relacionado da doença, porém o uso do BNZ está associado a uma alta frequência de efeitos adversos (PÉREZ-MOLINA & MOLINA, 2018; PAVAN *et al.*, 2018; SALES JUNIOR *et al.*, 2017).

Considerando fatores como a baixa solubilidade do benznidazol, que fazem com que a dose necessária para o tratamento seja mais alta. O uso de nanotecnologia e formação de partículas menores com o fármaco, representam uma boa estratégia para que se permaneça com um fármaco eficaz, porém reduzindo a dose administrada (MAXIMIANO *et al.*, 2011; FERRAZ *et al.*, 2018; QUIJIA QUEZADA *et al.*, 2019). No presente estudo aplicou-se o processo de nanoencapsulação do benznidazol, utilizando o polímero Eudragit L-100, o mesmo foi administrado no tratamento experimental na fase aguda da infecção. Através do qual foi possível reduzir da parasitemia e do processo inflamatório nos tecidos. Porém o tratamento não apresentou 100% de eficácia no período adotado. Considerando utilização de uma baixa dosagem e que a administração do tratamento foi por pouco tempo, não pode se descartar totalmente o uso do benznidazol nanoencapsulado, visto que adaptações experimentais podem ser realizadas, visando verificar o desempenho da formulação em um período mais longo. Rial *et al.* (2017) realizaram um estudo de eficácia de micropartículas de BNZ administradas na forma de spray. A partir desse estudo demonstraram a eliminação do parasito, bem como a redução do processo inflamatório após trinta dias de tratamento.

A DC é caracterizada por uma grande geração de EROs e ERN, durante a fase aguda. A resposta inicial do organismo para controlar o parasito é a geração dessas espécies reativas que podem interromper a proliferação do mesmo. O tratamento com BNZ e NBNZ promoveu um aumento significativo nos níveis de espécies reativas de nitrogênio e oxigênio, e de MDA,

evidenciando um status pró-oxidante. As EROs podem oxidar proteínas, lipídios e DNA do parasito, matando o mesmo. Podendo ser geradas a partir de danos mitocondriais, destruição tecidual pelo parasito e reações citotóxicas imunomediadas. Já o NO pode afetar a sobrevivência do parasito ainda nos macrófagos infectados, inibindo a atividade de enzimas do parasito, modificando proteínas que contenham cisteína e impedindo a ligação a metaloproteínas. Essas ERs podem, ainda, reagir gerando moléculas oxidantes potentes e citotóxicas, as quais apresentam grande efetividade contra o *T. cruzi*, como por exemplo o oxido nítrico pode reagir com o ânion superóxido, formando peroxinitrito (CARDOSO *et al.*, 2016).

Na infecção aguda por *T. cruzi* há intensa inflamação, muito tecidos são afetados, podendo levar a degeneração de conexões do sistema nervoso autônomo. Esses danos estão relacionados as principais complicações presentes na evolução cardíaca ou gastrointestinal da doença. (TEIXEIRA *et al.*, 2011; MACHADO *et al.*, 2012). A resposta imunológica e inflamatória pode sofrer regulação via componentes do sistema colinérgico. Atividade e expressão de componentes do sistema colinérgico em células imunes, desempenha um importante papel na regulação da resposta imune, seja ela inata ou adaptativa (MACHADO *et al.*, 2012; FUJII *et al.*, 2017a; FUJII, 2017b). Através de suas mudanças com o hospedeiro, o parasito, pode retardar a resposta imune facilitando a invasão de diferentes tecidos (TRUYENS & CARLIE, 2017; BORGES *et al.*, 2013; FUJII *et al.*, 2017a). O que foi observado através da imunossupressão ocasionada pela infecção, esse processo também ocorreu na via de sinalização colinérgica, pois a expressão da AChE e dos receptores muscarínicos M1 e M2 estavam reduzidas no quadro da infecção aguda, essa redução culmina para uma modulação de resposta anti-inflamatório, o que beneficia o parasito. A inibição de receptores muscarínicos está atrelada a uma redução na produção de citocinas pro-inflamatórias e de imunoglobulinas (FUJII *et al.* 2017a). Por outro lado, o tratamento com benznidazol livre mostrou atuar na via colinérgica, levando a um aumento na expressão desses componentes, e prevenindo a ocorrência da leucopenia, modulando uma resposta pró-inflamatória, e controlando parcialmente os danos teciduais relacionados a esse processo.

Dessa forma foi possível demonstrar que o sistema colinérgico desempenha importante papel na modulação da resposta imune e inflamatória na fase aguda da DC e que o tratamento com benznidazol promove importante regulação dessa resposta bem como da modulação da via

colinérgica, que estimula a produção de citocinas pró-inflamatórias levando a um aumento na geração de espécies reativas. Além disso, foi possível demonstrar um potencial forma de administração do fármaco benznidazol, com apresentação em forma líquida, que ainda deve ser avaliada em diferentes protocolos de tratamento, pois permite ajustes de dosagens e adaptações na administração do fármaco, principalmente considerando o tratamento em crianças.

6. CONCLUSÃO

- A infecção aguda por *T. cruzi* interferiu na modulação da sinalização colinérgica. No SNC essa modulação apresentou uma resposta anti-inflamatória, porém em linfócitos evidenciou-se uma modulação pró-inflamatória; demonstrando que a regulação da via colinérgica se dá principalmente pela ação desse sistema em células imunes.

- O status oxidativo/antioxidante está em desequilíbrio durante a DC em fase aguda, sendo observado danos oxidativos no córtex, tecidos hepático e renal; além disso, o tratamento com beznidazol induziu a um aumento na resposta pró-oxidante em animais infectados com *T. cruzi.*

- A produção de nanocápsulas de BNZ, utilizando um polímero de alta afinidade intestinal, apresentou boa eficiência de encapsulação, relativamente estável, sem sofrer muitas alterações ao longo do tempo avaliado. Caracterizando-se como uma formulação adequada para administração oral, através de ajustes de doses e períodos de tratamento.

- O tratamento feito com NBNZ não foi eficaz para a eliminação do parasito durante o período avaliado, porém promoveu uma redução da parasitemia; além disso, ressalta-se que o tratamento com BNZ também não apresentou total eficácia no mesmo período.

- O BNZ foi citotóxico para as células, mostrando importante danos às células através do ensaio cometa, porém o processo de nanoencapsulação demonstrou reduzir essa citotoxicidade.

- O tratamento com BNZ exerceu estimulação na via colinérgica, promovendo um aumento na expressão da AChE, M1 e M2 mAChR, acompanhado de aumento da atividade da AChE; o que caracteriza uma resposta pró-inflamatória; onde a atividade linfocitária da AChE pode ser uma ferramenta como marcador do processo inflamatória na DC aguda.

- A utilização da terapia com BNZ e NBNZ levou a uma redução dos danos teciduais característicos da DC avaliados em tecidos cardíaco, hepático, renal, cerebral e esplênico; além disso na infecção aguda os danos cardíacos e hepáticos foram acentuados.

- Através do presente trabalho foi possível demonstrar o envolvimento da via colinérgica com o processo inflamatório presente na DC, via esta que se mostrou como um potencial alvo farmacológico para regulação dos danos teciduais ocasionados pelo parasito, tanto na fase aguda,

quanto na fase crônica da doença, representando uma melhora na qualidade de vida de pacientes infectados.

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8. ANEXOS

8.1 Carta de aprovação do CEUA:

Comissão de Ética no Uso de Animais
da
Universidade Federal de Santa Maria

CERTIFICADO

Certificamos que a proposta intitulada "INFECÇÃO EXPERIMENTAL COM Trypanosoma cruzi EM CAMUNDONGOS: PAPEL DO SISTEMA COLINÉRGICO E MECANISMOS BIOQUÍMICOS ENVOLVIDOS NA PATOGENIA DA DOENÇA DE CHAGAS, ASSIM COMO USO DA NANOTECNOLOGIA PARA POTENCIALIZAR O TRATAMENTO COM BENZONIDAZOL ", protocolada sob o CEUA nº 2842070618, sob a responsabilidade de **Aleksandro Schafer da Silva** *e equipe; Aniélen Dutra da Silva* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Universidade Federal de Santa Maria (CEUA/UFSM) na reunião de 19/07/2018.

We certify that the proposal " EXPERIMENTAL INFECTION WITH Trypanosoma cruzi IN MICE: ROLE OF THE CHOLINERGIC SYSTEM AND BIOCHEMICAL MECHANISMS INVOLVED IN CHAGAS DISEASE PATOGENIA, AND THE USE OF NANOTECHNOLOGY TO POTENTIATE BENZONIDAZOLE TREATMENT", utilizing 262 Heterogenics mice (262 females), protocol number CEUA 2842070618, under the responsibility of **Aleksandro Schafer da Silva** *and team; Aniélen Dutra da Silva* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the Federal University of Santa Maria (CEUA/UFSM) in the meeting of 07/19/2018.

Finalidade da Proposta: Pesquisa (Acadêmica)

Vigência da Proposta: de 06/2018 a 07/2019 Área: Bioquimica Toxicológica

Origem:	Biotério Central UFSM						
Espécie:	Camundongos heterogênicos	sexo:	Fêmeas	idade:	50 a 60 dias	N:	262
Linhagem:	Swiss			Peso:	30 a 45 g		

Resumo: A doença de chagas é um antropozoonose com ampla distribuição na América Latina. Diversas lesões tardias ocasionadas pela infecção do parasito estão relacionadas ao desenvolvimento da doença durante fase aguda. O fármaco benzonidazol é utilizado para o tratamento da doença, porém apresenta diversos efeitos colaterais e alta toxicidade. Portanto, torna-se de extrema importância a busca por amenizar tantos esses efeitos quanto a toxicidade do fármaco, visando melhora na qualidade de vida dos indivíduos que necessitam fazer uso do mesmo. A utilização da biotecnologia tem sido grande aliada na busca por estratégias que permitam desenvolver formas de administração de medicamentos com maior eficácia e menos prejuízos para o organismo, sendo as nanocápsulas uma dessas alternativas. O sistema colinérgico apresenta diversas atividades no sistema nervoso central, porém a nível sérico pode atuar modulando respostas inflamatórias frente a diversos parasitos. Sendo assim, objetiva-se analisar o funcionamento sérico do sistema colinérgico durante a infecção por Trypanosoma cruzi em modelos experimentais de camundongos, além de analisar a eficácia da utilização de nanoencapsulados de benzonidazol na infecção por T. cruzi para fins de potencializar o tratamento. Os experimentos objetivam conhecer a participação do sistema colinérgico na patogenia da doença, assim como trazer novas possibilidades no tratamento da Doença de Chagas, buscando uma melhora na qualidade de vida de pacientes acometidos por essa doença, que é considerada negligenciada, apesar de ocasionar um número significativo de mortes em âmbito mundial.

Local do experimento: Os animais serão mantidos no Laboratório de Parasitologia Veterinária (LAPAVET), prédio 20, em um ambiente com temperatura controlada (23°C ± 1) e com um ciclo de 12 horas claro/escuro, o ambiente possui 2 exaustores de ar para uma completa renovação do ar interno do biotério. A ração que os animais recebem tem balanceamento de nutrientes essenciais, proteínas, carboidratos e lipídeos, componentes básicos e iguais aos utilizados no biotério central, sendo a água e a ração sólida fornecidas ad libitum. Antes do início do experimento, os animais passarão por um período de adaptação de 7 dias e o fundo das caixas receberão maravalha, que após o uso, será descartada como contaminante. Além disso, os animais serão distribuídos em 6 a 10 camundongos por caixa (protocolo I) e 10 camundongos por caixa (protocolo II), durante todo o período dos tratamentos, e todas as caixas receberão objetos (rolos de papel e/ou PVC) para enriquecimento ambiental. BIOSSEGURANÇA: O Trypanosoma cruzi é um microorganismo listado na classe de risco 2, definida pelas seguintes características: é capaz de causar doenças em seres humanos ou animais de laboratório sem apresentar risco grave aos trabalhadores, à comunidade ou ao ambiente; não é transmissível pelo ar; há tratamento e medidas preventivas disponíveis. O Nível de Biossegurança 2 (NB2) precisa ser implementado para o trabalho com T. cruzi e as medidas obrigatórias a serem adotadas são as seguintes: I. Quanto à área física 1. Será realizada a identificação do NB e do microorganismo nas portas de todas as salas e áreas de trabalho com T. cruzi; 2.

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CEUA N 2842070618

O laboratório é separado de passagens públicas; 3. O laboratório funcionará em sala própria, sem superposição com outras atividades; 4. O laboratório tem acesso restrito às pessoas autorizadas que manipularão o parasita ou os animais infectados. O acesso de crianças será estritamente proibido; 5. O laboratório é separado por antecâmara, com portas trancáveis interdependentes; 6. As janelas são vedadas, inquebráveis, e com telas; as portas devem ter sistema de fechamento automático; 7. As paredes, teto e chão são lisos, íntegros, de fácil limpeza, sem juntas e resistentes a desinfetantes. Os ralos são vedados; 8. Existem barreiras seguras para evitar a fuga de animais ou insetos infectados; 9. O laboratório possui um mínimo de móveis e equipamentos, todos de fácil limpeza; II. Quanto às instalações 1. Os dutos de fiação elétrica são acessíveis para manutenção; 2. O laboratório possui iluminação de emergência; 3. Possui pia no laboratório ou perto da saída; 4. O laboratório possui lava-olhos, que são verificados diariamente, pois é comum o entupimento por ferrugem ou desuso. III. Quanto à manipulação 1. É indispensável treinamento adequado antes do início do trabalho. Apenas pessoal treinado devera trabalhar com T. cruzi, seja in vivo ou in vitro, ou seja, pessoal bem treinado em procedimentos gerais de laboratório, que conheça tanto as técnicas especiais necessárias para o trabalho com o parasito como a sua biologia. 2. É obrigatório manter no laboratório cópia de procedimentos de trabalho no laboratório, bem como de procedimentos para emergência; 3. É essencial não trabalhar sozinho; o trabalho com material patogênico deve ser feito sempre em equipe, para evitar que, na eventualidade de um acidente, o indivíduo se veja sozinho e não possa tomar as providências necessárias à situação; 4. É obrigatório usar equipamento individual de proteção; este equipamento deve estar acessível a quem for trabalhar com animais ou com os parasitas: avental longo e de mangas compridas (deve estar disponível na antecâmara); sapatos fechados (nunca sandálias ou sapatos abertos); luvas (se possível, luva dupla) em todos os procedimentos que envolverem contato direto da pele com culturas, sangue, extratos ou animais infectados. Anéis ou outros adereços de mão que interferem com o uso da luva devem ser retirados. As luvas devem ser removidas com cuidado, para evitar a formação de

Santa Maria, 29 de outubro de 2018

Prof. Dr. Denis Broock Rosemberg
Coordenador da Comissão de Ética no Uso de Animais
Universidade Federal de Santa Maria

Prof. Dr. Saulo Tadeu Lemos Pinto Filho
Vice-Coordenador da Comissão de Ética no Uso de Animais
Universidade Federal de Santa Maria