UNIVERSIDADE FEDERAL DE ALFENAS

DIEGO FERNANDES VILAS BOAS

AVALIAÇÃO DO EFEITO DO COMPOSTO 4-NITROBENZOILCUMARÍNICO EM CAMUNDONGOS EXPERIMENTALMENTE INFECTADOS POR *Trypanosoma cruzi*

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Orientador: Prof. Dr. Ivo Santana Caldas
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A Banca Examinadora, abaixo assinada, aprova a Dissertação apresentada como parte dos requisitos para a obtenção do título de Mestre em Ciências Biológicas pela Universidade Federal de Alfenas.
Área de concentração: Interação Patógeno-Hospedeiro

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RESUMO

Estudos prévios mostram que compostos cumarínicos apresentam atividades anticancerígenas e antiparasitárias e recentemente foi demonstrada importante atividade *in vitro* do composto 4-nitrobenzoilcumarínico (4NBC) especificamente contra o *Trypanosoma cruzi*. Este trabalho descreve o efeito do tratamento com 4NBC em monoterapia e combinado com benznidazol em camundongos experimentalmente infectados pela cepa Y do *T.cruzi*. Foram utilizados 56 camundongos Swiss, divididos em 8 grupos. O índice de cura foi verificado pela reativação natural da parasitemia, hemocultura e qPCR. Para verificação do possível efeito tóxico do 4NBC, os camundongos foram pesados semanalmente e foi realizada a dosagem de AST e ALT como marcadores de toxicidade hepática e análise do tecido hepático. Para avaliar a resposta imune humoral, foram quantificados anticorpos da classe IgG e os isotipos IgG1, IgG2a e IgG2b. Ao final do experimento os animais foram eutanasiados e o tecido cardíaco foi utilizado para análise da miocardite. Todos os esquemas terapêuticos foram eficazes em prevenir a morte de 100% dos animais. De forma geral, o tratamento foi bem tolerado pelos animais, evidenciado pelo acompanhamento do peso, pela dosagem das enzimas AST e ALT e pela análise do tecido hepático. O benefício da utilização da cumarina em monoterapia ou em combinação foi evidenciado pela redução da área sob a curva de parasitemia em relação aos animais infectados e não tratados. Só foi observada cura parasitológica entre os animais tratados com Bz em monoterapia e na combinação de 100mg/kg de 4NBC com 50mg/kg de Bz. Todas as combinações de Bz com 4NBC se mostraram mais eficazes na prevenção da miocardite e redução da produção de IgG e IgG2a, se comparados com o grupo tratado com 50mg/kg de Bz. Os resultados obtidos mostram que o composto 4NBC apresenta atividade tripanocida, que é bem tolerado pelo hospedeiro e que este pode interferir positivamente na prevenção da miocardite típica da doença de Chagas.

Palavras-chave: Tripanossomíase americana. Compostos cumarínicos. Benznidazol.

ABSTRACT

Previous studies have shown that coumarin compounds exhibit anticancer and antiparasitic activities and have recently demonstrated important *in vitro* activity of the 4-nitrobenzoylcoumarin compound (4NBC) specifically against *Trypanosoma cruzi*. Here, we evaluated the trypanocidal effect of 4NBC alone and in combination with benznidazole (Bz) using fifty-five Swiss mice experimentally infected with *T. cruzi* Y strain, divided into 8 groups. Cure rates were checked by microscopic examination of fresh blood, as well as blood culture and real time PCR. Cardiac inflammation of treated animals was checked after euthanasia compared to uninfected group and infected and untreated mice in tissue sections stained with hematoxylin & eosin. Treatment toxicity assessments were performed considering (i) weekly animal weights, (ii) dosages of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) liver injury markers, and (iii) histopathological analyzes of liver tissues. The involvement of humoral immune response in experimental therapy was investigated by assessing the levels of G immunoglobulins class (IgG) and their IgG1, IgG2a and IgG2b isotypes. All therapeutic regimens were effective in preventing the death of 100% of the animals. Overall, the treatments were well tolerated, as evidenced by monitoring of animal weights, AST and ALT enzymes measurements and analysis of the hepatic tissue. The benefit of using coumarin alone or in combination was evidenced by the reduction of the area under the parasitaemia curve in relation to infected and untreated animals. Parasitological cure was observed only between the animals treated with Bz in monotherapy and in the combination of 100mg/kg of 4NBC with 50mg/kg of Bz. All combinations of Bz with 4NBC showed to be more effective in preventing myocarditis and reducing the production of imunoglobulins compared to the group treated with 50mg/kg of Bz. The results show that compound 4NBC has trypanocidal activity, which is well tolerated by the host and that it can positively interfere in the prevention of typical myocarditis of Chagas disease.

Keywords: American trypanosomiasis. Coumarin compounds. Benznidazole.

SUMÁRIO

INTRODUÇÃO GERAL

A doença de Chagas, também conhecida por tripanossomíase americana, é uma enfermidade infecciosa, considerada negligenciada pela Organização Mundial da Saúde (WORLD HEALTH ORGANIZATION, 2012). Foi descoberta e caracterizada em 1909 por Carlos Justiniano Ribeiro das Chagas (CHAGAS, 1909) e tem como agente etiológico o parasito *Trypanosoma cruzi*. No mundo existe por volta de 6-7 milhões de pessoas infectadas pelo *T. cruzi*, a maioria na América Latina, onde 21 países, incluindo o Brasil, são considerados endêmicos para a doença de Chagas. Porém a enfermidade tem se espalhado para outros continentes (WORLD HEALTH ORGANIZATION, 2019). Atividade migratória desordenada, instabilidade socioeconômica, urbanização e desequilíbrio ambiental estão entre os principais fatores relacionados à transmissão do parasito ao homem (COURA, 2015).

A doença possui duas fases, aguda e crônica. A fase aguda, inicial, tem a duração de 4-8 semanas e apresenta elevada parasitemia, ou seja, alto número de parasitos circulantes no sangue. Os sintomas, geralmente, são brandos e inespecíficos. Quando sintomática, o enfermo, pode apresentar, entre muitas manifestações, febre, hepatoesplenomegalia, edema subcutâneo, mal estar. A pessoa infectada, através da transmissão vetorial, pode apresentar chagoma de inoculação e/ou sinal de Romaña que são os indícios da entrada do parasito pela pele e pelas mucosas oculares, respectivamente (BERN, 2015; RASSI; RASSI; MARIN-NETO, 2010). Ocasionalmente pode ocorrer miocardite grave, e a insuficiência cardíaca é responsável por grande parte das mortes durante a fase aguda da doença em crianças menores de dois anos. A meningoencefalite pode ser também a responsável pela morte durante esta fase inicial da infecção (RASSI; RASSI; MARCONDES DE REZENDE, 2012).

O início da fase crônica se dá 2-3 meses após a infecção, onde cerca de 60% a 70% das pessoas infectadas irão apresentar a forma indeterminada, sem apresentar sintomas, da doença. E cerca de 30% a 40% irão desenvolver a forma determinada, sintomática, da enfermidade (RASSI; RASSI; MARCONDES DE REZENDE, 2012). As formas sintomáticas são caracterizadas em três: cardíaca, digestiva ou cardiodigestiva. A forma cardíaca é a condição clínica mais importante da enfermidade, em consequência da sua frequência e gravidade. Entres os sintomas da cardiomiopatia chagásica estão a insuficiência cardíaca, angina, arritmias e tromboembolismo. Comumente, a insuficiência biventricular é a primeira manifestação clínica. É notável a ocorrência de morte súbita nessa fase da doença (BENZIGER; DO CARMO; RIBEIRO, 2017).

Outra possibilidade na doença de Chagas, crônica e sintomática, é o desenvolvimento de formas digestivas, que afetam predominantemente o esôfago, cólon ou ambos. As manifestações esofágicas variam de distúrbios de motilidade ao megaesôfago grave, com sintomas como perda de peso, refluxo esofágico e tosse. O megacólon se caracteriza por constipação prolongada, podendo causar isquemia intestinal. A doença de Chagas gastrointestinal é menos frequente do que a cardíaca (BERN, 2015; PINAZO, 2010). A combinação das manifestações clínicas digestivas (megaesôfago e megacólon) com as cardíacas origina a forma cardiodigestiva (RASSI; RASSI; MARCONDES DE REZENDE, 2012).

Quimioterápicos para o tratamento da doença de Chagas vêm sendo pesquisados desde a descoberta da enfermidade em 1909. Na década de 1970 dois compostos foram lançados no mercado para o tratamento da doença, o nifurtimox (Nfx) (Lampit®, Bayer) e o benznidazol (Bz) (LAFEPE), os quais são utilizados atualmente como quimioterápicos de referência (DIAS *et al.*, 2009; SALES JUNIOR *et al.*, 2017; URBINA; DOCAMPO, 2003). Em pacientes com doença de Chagas na fase aguda e aqueles com infecção congênita precoce, tanto o Bz quanto o Nfx são capazes de diminuir a gravidade dos sintomas, encurtar o curso clínico da enfermidade, diminuir o número de parasitos no sangue e até mesmo curar os indivíduos na maioria dos casos. A taxa de cura na fase aguda é estimada de 80 a 90% (BERN, 2015), entretanto, quando o tratamento ocorre durante a fase crônica a taxa de cura é menor. É consenso que com a realidade dos fármacos hoje disponíveis quanto mais cedo é realizado o diagnóstico e o tratamento é iniciado, maiores são as chances de sucesso terapêutico. Contudo outros fatores interferem na eficiência dos fármacos, como a existência de grande variedade de cepas do parasito que apresentam diferentes graus de resistência aos medicamentos (URBINA; DOCAMPO, 2003). Os dois fármacos apresentam efeitos colaterais, sendo os do Bz mais brandos quando comparados aos do Nfx (BERN, 2015). Para pacientes com danos cardíacos, durante a fase crônica, o tratamento com Nfx pode ser mais perigoso do que o tratamento com o Bz, pois o enfermo corre maior risco de apresentar insuficiência cardíaca, por esse motivo o Nfx não é escolhido para o tratamento da doença, no Brasil e em grande parte dos países onde a doença é endêmica (BERMUDEZ *et al.*, 2016).

Portanto a descoberta e desenvolvimento de novos fármacos são necessários, e as buscas por novos compostos para o tratamento da doença de Chagas têm crescido nas últimas décadas, principalmente pelo sequenciamento do genoma de *T. cruzi*, que possibilitou a identificação de genes presentes no parasito e ausentes no homem. A identificação de diversos alvos biológicos promissores, sendo a maioria deles enzimas, permite uma busca racional de

novas estratégias terapêuticas (DIAS *et al.*, 2009). A procura por inibidores das enzimas da via glicolítica é importante para o desenvolvimento de fármacos para a doença de Chagas, pois o *T. cruzi* é dependente da via glicolítica para a produção de adenosina trifosfato (ATP). Três enzimas tem se destacado como possíveis alvos biológicos, a gliceraldeído-3-fosfato-desidrogenase (GAPDH), a hexoquinase e a fosfofrutoquinase. A GAPDH, que possui grande relevância nos estudos em química medicinal, é uma enzima tetramérica que catalisa a conversão do substrato gliceraldeído-3-fostato em 1,3-bisfosfoglicerato, na presença do cofator NAD^+ e fosfato inorgânico e exerce a função de controlar o fluxo glicolítico, uma vez que as formas amastigotas possivelmente dependem da glicólise para a produção de ATP, a sua inibição pode causar problema na multiplicação do parasito e previne potencialmente o *T. cruzi* de ser infeccioso (ALVIM JR. *et al.*, 2005; DIAS *et al.*, 2009). Muitos inibidores, da GAPDH, de origem natural e sintética, com boa diversidade química, têm sido descobertos. Entre eles destacam-se as cumarinas, flavonoides, os reativos intermediários de nitrogênio (óxido nítrico, nitroxil e peroxinitrito) e mais recentemente alguns complexos doadores de óxido nítrico (DIAS *et al.*, 2009).

As cumarinas são metabólitos secundários presentes em várias espécies de plantas e óleos essenciais, e podem ser isoladas naturalmente ou produzidas sinteticamente. Devido à variedade de atividades biológicas que esses compostos exibem, os estudos, desde o início, focaram na possibilidade de sua atividade terapêutica e têm mostrado que compostos cumarínicos possuem ações anticarcinogênicas, antivirais, antifúngicas, antibacterianas, antioxidantes, inibidoras de enzimas, anti-inflamatórias e antiparasitárias (BORGES *et al.*, 2005; FIGUEROA-GUIÑEZ *et al.*, 2015). A cumarina do tipo mammea A/BB, extraída das folhas da planta *Calophyllum brasiliense*, apresentou potente atividade contra as formas promastigotas e amastigotas de *Leishmania (L.) amazonensis*, assim como, maior atividade contra as formas epimastigotas do que as tripomastigotas, de *T. cruzi*, mas em geral é considerada nociva para ambas (BRENZAN *et al.*, 2007; REYES-CHILPA *et al.*, 2008) Também é conhecida por sua ação moluscicida contra *Biomphalaria glabrata* (GASPAROTTO JR. *et al.*, 2005). Cumarina extraída de *Kielmeyera albopunctata*, espécie vegetal do cerrado brasileiro, apresentou uma taxa de mortalidade de 80% para tripomastigotas sanguíneos no período de 24 horas (SCIO *et al.*, 2003). Estudos vêm mostrando que os compostos cumarinícos são capazes de inibir a enzima GAPDH, importante na via glicolítica dos tripanossomatídeos, como mostrado por Freitas e colaboradores (2009) e no crescimento de *Leishmania amazonensis*, causando mudanças importantes na estrutura do parasito (BRENZAN *et al.*, 2007). Portanto o estudo dos compostos cumarínicos como

possíveis agentes para a quimioterapia da doença de Chagas é relevante, visto que a enfermidade é um grande problema de saúde pública e apresenta várias falhas terapêuticas.

A atual quimioterapia específica para a doença de Chagas, os fármacos Bz e Nfx, apresentam limitações devido aos efeitos adversos, tempo de tratamento e variabilidade de sensibilidade de algumas populações de *T. cruzi*. Várias estratégias são utilizadas a fim de diminuir essas limitações, entre elas encontra-se a terapia combinada, que vem sendo cada vez mais sustentada como forma de melhorar a eficácia e a tolerância ao tratamento, além de diminuir a toxicidade e resistência ao mesmo (ASSÍRIA *et al.*, 2015; VIVAS *et al.*, 2008).

Logo, através da informação exposta, torna-se clara a importância de pesquisar o uso das cumarinas como possível estratégia terapêutica para a doença de Chagas, devido a sua atividade antiparasitária além de suas atividades anti-inflamatórias e antioxidantes. Neste trabalho também foi utilizada a estratégia de combinação de fármacos, considerando que a combinação pode melhorar a eficácia do tratamento, diminuir a toxicidade, evitar aumento de resistência do parasito, além de poder atuar em diferentes vias de sinalização do *T.cruzi*.

COUMARIN 8-METHOXY-3-(4-NITROBENZOYL)-6-PROPYL-*2H*-CHROMEN-2-ONE REDUCES PARASITISM, PREVENTS MYOCARDITIS AND POTENTIATES THE TRYPANOCIDAL ACTIVITY OF BENZNIDAZOLE

ABSTRACT

Previous studies have shown that coumarin compounds exhibit anticancer and antiparasitic activities and have recently demonstrated important *in vitro* activity of the 4-nitrobenzoylcoumarin compound (4NBC) specifically against *Trypanosoma cruzi*. Here, we evaluated the trypanocidal effect of 4NBC alone and in combination with benznidazole (Bz) using fifty-five Swiss mice experimentally infected with *T. cruzi* Y strain, divided into 8 groups. Cure rates were checked by microscopic examination of fresh blood, as well as blood culture and real time PCR. Cardiac inflammation of treated animals was checked after euthanasia compared to uninfected group and infected and untreated mice in tissue sections stained with hematoxylin & eosin. Treatment toxicity assessments were performed considering (i) weekly animal weights, (ii) dosages of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) liver injury markers, and (iii) histopathological analyzes of liver tissues. The involvement of humoral immune response in experimental therapy was investigated by assessing the levels of G immunoglobulins class (IgG) and their IgG1, IgG2a and IgG2b isotypes. All therapeutic regimens were effective in preventing the death of 100% of the animals. Overall, the treatments were well tolerated, as evidenced by monitoring of animal weights, AST and ALT enzymes measurements and analysis of the hepatic tissue. The benefit of using coumarin alone or in combination was evidenced by the reduction of the area under the parasitaemia curve in relation to infected and untreated animals. Parasitological cure was observed only between the animals treated with Bz in monotherapy and in the combination of 100mg/kg of 4NBC with 50mg/kg of Bz. All combinations of Bz with 4NBC showed to be more effective in preventing myocarditis and reducing the production of imunoglobulins compared to the group treated with 50mg/kg of Bz. The results show that compound 4NBC has trypanocidal activity, which is well tolerated by the host and that it can positively interfere in the prevention of typical myocarditis of Chagas disease.

Keywords: American trypanosomiasis. Coumarin compounds. Benznidazole.

1 INTRODUCTION

Chagas disease, also known as American trypanosomiasis, is a parasitic disease caused by the protozoan *Trypanosoma cruzi*, discovered in 1909 by Carlos Justiniano Chagas, in the northern region of Minas Gerais State, Brazil (CHAGAS, 1909). Worldwide, there are around 7 million people infected with the parasite, most of them in Latin America, where the protozoan *T. cruzi* is endemic in 21 countries, including Brazil. The drugs used for the treatment of Chagas disease are the nitro heterocyclic compounds, nifurtimox (Lampit®, Bayer), a nitrofuran, and benznidazole (Bz) (LAFEPE), a nitroimidazole derivative (URBINA; DOCAMPO, 2003). The drug of choice for the treatment of Chagas disease in Brazil is Bz (CANÇADO, 1999), which has been very effective during the acute phase of infection, but has shown low cure rates when administered in the chronic phase. In addition,

other limitations of the available chemotherapy have been related to adverse effects, treatment time, and variable sensitivity of some *T. cruzi* populations (SALES JUNIOR *et al*., 2017). Several strategies have been evaluated to reduce these limitations, including drug combination, which has been increasingly sustained as a way to improve treatment efficacy and tolerance by decreasing parasite resistance and host toxicity (ASSÍRIA *et al.*, 2015; VIVAS *et al.*, 2008). Recent studies have pointed to the benefits of combining drugs, often as complementary results of different mechanisms of action (SUN; SANDERSON; ZHENG, 2017).

Coumarins derived from phenolic acids, which have a benzene ring combined with oxygen heterocycle, are found in several plant species and essential oils. Many studies using coumarins have shown anticarcinogenic (CAROCHO; FERREIRA, 2013), antiviral (HUPFELD; EFFERTH, 2009) and antioxidants activities, in the latter case, reducing or eliminating reactive oxygen species (ROS) and protecting tissues from possible damage (FIGUEROA-GUIÑEZ *et al*., 2015). Other studies have investigated the antiparasitic action of coumarins and have suggested that some coumarins are able to inhibit GAPDH, an important protein of the glycolytic pathway of trypanosomatids (FREITAS *et al*., 2009). Moreover, in experiments against *Leishmania major*, morphological variations and reduced motility and parasite size have also been observed (MANDLIK *et al*., 2016).

In previous studies in our laboratory, we found that the IC_{50} of 4-nitrobenzoylcoumarin (4NBC) (IC_{50} 28±3) is similar to that observed with Bz (IC_{50} 25±10) against epimastigote forms and that the parasitaemia of mice infected with the *T. cruzi* Y strain is reduced with administration of this coumarin (BRANCAGLION *et al*., 2018). Considering these antecedents, we propose to verify the antiparasitic potential of 4NBC monotherapy in mice experimentally infected with *T. cruzi* and to evaluate the influence of combined treatment with Bz on cure rates, toxicity and reduction of heart damage typically observed in the experimental Chagas disease.

2 MATERIAL AND METHODS

2.1 PARASITE, ANIMALS AND INFECTION

Trypanosoma cruzi Y strain (DTU II), which is characterized as partially resistant to experimental chemotherapy with benznidazole (FILARDI; BRENER, 1987) was used here for mouse infections. Fifty-six Swiss mice, 4-5 weeks old, weighing around 30 g, were kept in an

environment with temperature control (21 +/- 2ºC), photoperiod of 12h / 12h, and food and water *ad libitum.* Mice were infected intraperitoneally with 5,000 blood trypomastigotes obtained from previously infected mice. Parasitaemia was performed by microscopic fresh blood examination, using 5 µL of blood collected from the mouse's tail and the number of parasites was estimated according to the technique described by Brener (1962). This study was approved by the Animal Research Ethics Committee at Federal University of Alfenas (Protocol number 59/2017).

2.2 EXPERIMENTAL TREATMENTS

The benznidazole (Bz) produced by the Pharmaceutical Laboratory of Pernambuco State (LAFEPE-Brazil) and the 8-methoxy-3-(4-nitrobenzoyl)-6-propyl-*2H*-chromen-2-one compound (4NBC) obtained by the Pharmaceutical Chemistry Research Laboratory of the Federal University of Alfenas (BRANCAGLION *et al*., 2018) were used for the treatments. Animal infections were confirmed by parasitaemia within 5 days after inoculation. The treatments were performed by gavage once a day, for 20 consecutive days, with Bz and 4NBC, alone and in combination, using concentrations of 50 mg/kg and 100 mg/kg body weight for both drugs. The 20-day therapeutic regimen was based on the protocol described for Bz by Filardi and Brener (1987).

The fifty-five Swiss mice were divided into the following experimental groups: infected and untreated control, uninfected control, and infected groups undergoing monotherapy with 100 mg/kg Bz, 50 mg/kg Bz, 100 mg/kg 4NBC and 50 mg/kg 4NBC, as well as combinations of 100 mg/kg 4NBC + 50 mg/kg Bz and 50 mg/kg 4NBC + 50 mg/kg Bz. Aliquots of Bz (10 mg/mL) were previously prepared from macerated Bz tablets resuspended in water with carboxymethylcellulose 0,4% m/v. Aliquots of 4NBC were prepared with 5% Cremophor in water. Both drugs were stored at -20 ° C until required for use.

2.3 EVALUATION OF TREATMENT BENEFITS

2.3.1 Toxicity analyses

The average weight gain of the animals during the experiment was used as one of the parameters in the toxicity analysis. From the beginning of the treatments, the animals were

weighed weekly and the average weight changes in each experimental group were calculated by the difference of the weekly weights by the initial weights (in the first day of treatment). In addition, histopathological analyzes of liver tissue (described below) and the Aspartate Amino Transferase (AST) and Alanine Amino Transferase (ALT) tests (Bioclin Quibasa, Belo Horizonte, MG, Brazil), were also used as complementary toxicity parameters.

2.3.2 Cure definition

The cure control of the treated animals was determined based on three tests: fresh blood test, blood culture, and real-time PCR (RT-PCR). Animals that showed negative results in all tests were considered cured. The fresh blood test was performed for another 20 days after the end of treatments so that the natural reactivation of *T. cruzi* was observed in the treated and uncured animals. For the blood culture test, 200 μL of blood was collected from the retro-ocular plexus with the aid of Pasteur pipette and added in conical base tubes containing 2 mL of LIT medium. The tubes were maintained in BOD at 28°C and homogenized twice weekly. After 30, 60 and 90 days of blood culture, 5 μL of this medium were collected in order to detect the parasites using an optical microscope. RT-PCR was performed in blood samples of all experimental groups. Wizard genomic DNA purification kit (Promega Corp., Madison, WI) was used to extract the genomic DNA from the blood samples following the manufacturer's instructions.

The RT-PCR was performed, using the multiplex TaqMan system targeting the *T. cruzi* satellite DNA and murine TNF-alpha DNA. Reactions were carried out with 50 ng of genomic DNA, 10 nM of primer oligonucleotides specific for *T. cruzi* satellite DNA, (CzFw 5 "-CCACCATTCATAATTGGAAACAAA -3" and CzRv 5 "-CTCGGCTGATCGTTTTCGA-3" which amplifies a 76 bp product) and murine TNF-alpha (TNF-F 5 "-GCCCAGACCCTCACACTCA-3" and TNF-R 5 "AACTGCCCTTCCTCCATCTTAAA-3", which amplifies a 69 bp product), 6 nM of *T. cruzi* oligonucleotide probe (5 'FAM-ACCACAACGTGTGATGC-3'MGB -NFQ) and TNF-alpha oligonucleotide probe (5 "VIC-TAAGTGTTCCCACACCTC-3'MGB-NFQ), 5 μL of 2x TaqMan® Universal Master Mix II (Thermo Fisher Scientific, Runcorn, UK) and 1.3 μL of 0.25 mg/mL BSA, totalizing a volume of 10 μL/reaction. PCR cycling conditions were: 95° C for 10 min, followed by 40 cycles at 95° C for 15 s and 60° C for 1 min. The samples were tested in duplicate. Each plate contained a negative and a positive control.

2.3.3 Immunological analyses

Prior to euthanasia, 200 µL of blood were collected from the retro-ocular plexus of the animals to determine the levels of total immunoglobulin G (IgG) and IgG1, IgG2a and IgG2b subclasses by enzyme-linked immunosorbent assay (ELISA) according to Voller et al., 1976 (VOLLER; BIDWELL; BARTLETT, 1976). Briefly: ELISA plates were coated with *T. cruzi* antigen prepared from alkaline extraction of the Y strain epimastigotes at exponential growth in the LIT medium. Anti-mouse IgG, IgG1, IgG2a and IgG2b peroxidase conjugated antibodies (Sigma Chemical Co.) were used. The mean absorbance for ten negative control samples plus two standard deviations were used as cutoff to discriminate positive and negative results. The reactivity index was calculated from dividing each sample by the cutoff value.

2.3.4 Histopathological analysis

For the histopathological evaluation, 20 days after the end of treatments, the animals were weighed and then euthanized with isoflurane. Heart and liver were collected and dehydrated at increasing concentrations of ethanol (70^{o} GL to absolute), followed by xylol diaphanization and paraffin inclusion. Blocks were cut into 5 µm sections and stained by Hematoxylin and Eosin (H&E) for inflammation assessment. Images were visualized at 40x magnification (Axioscope A1, Carl Zeiss, Oberkochen, Germany) and scanned by the Axion Vision LE software (Carl Zeiss, Oberkochen, Germany).

For each animal 10 and 15 cardiac and liver tissue images were obtained respectively. The average density of cardiac cell nuclei was determined over an area of $74x10^{3}$ μm^{2} using Axiovision 4 software (Carl Zeiss do Brasil Ltda). Hepatic tissue sections were used for stereological analysis. Volumetric density of sinusoidal capillaries and numerical density (hepatocytes and interstitial/inflammatory cells) were estimated according to the stereological concepts presented by Novaes *et al*. (2013). The volume density (Vv,%) was estimated by the counting points according to the formula $Vv = \Sigma P/Pt$; in which ΣP represents the number of test points that reach the structure of interest and Pt is the total number of points in the test system. A quadratic test system containing 100 points included in a test area of $53.5x10^{3}\mu m^{2}$ (At) at the tissue level was used. The numerical density of hepatocytes and interstitial cells per unit area (QAG, n/mm^{2}) was evaluated using the stereological formula $QAG = \Sigma G/At$, where ΣG is the sum of the number of hepatocytes/interstitial cells and At is the size of the

test area ($34x10^3$ μm^2). The mean nuclear diameter of the hepatocytes was obtained through the formula Dm = dma + dme/2, where dma represents the largest nuclear diameter and dme represents the smaller nuclear diameter, which were obtained using Image-Pro plus 4.5 (Media Cybernetics Inc., Silver Spring, Md., USA). The mean nuclear volume of the hepatocytes was estimated by the formula $Vm = \pi * (dm/2)^2$, where dm represents the mean nuclear diameter.

2.4 STATISTICAL ANALYSES

Parasitological, serological and histopathological data were analysed using the nonparametric version of Tukey's multiple-comparison test. Differences were considered significant if the *P* value was less than or equal to 0.05. All tests were performed using the GraphPad Prism version 7.00 statistical software (GraphPad Software Inc., La Jolla, CA, USA).

3 RESULTS

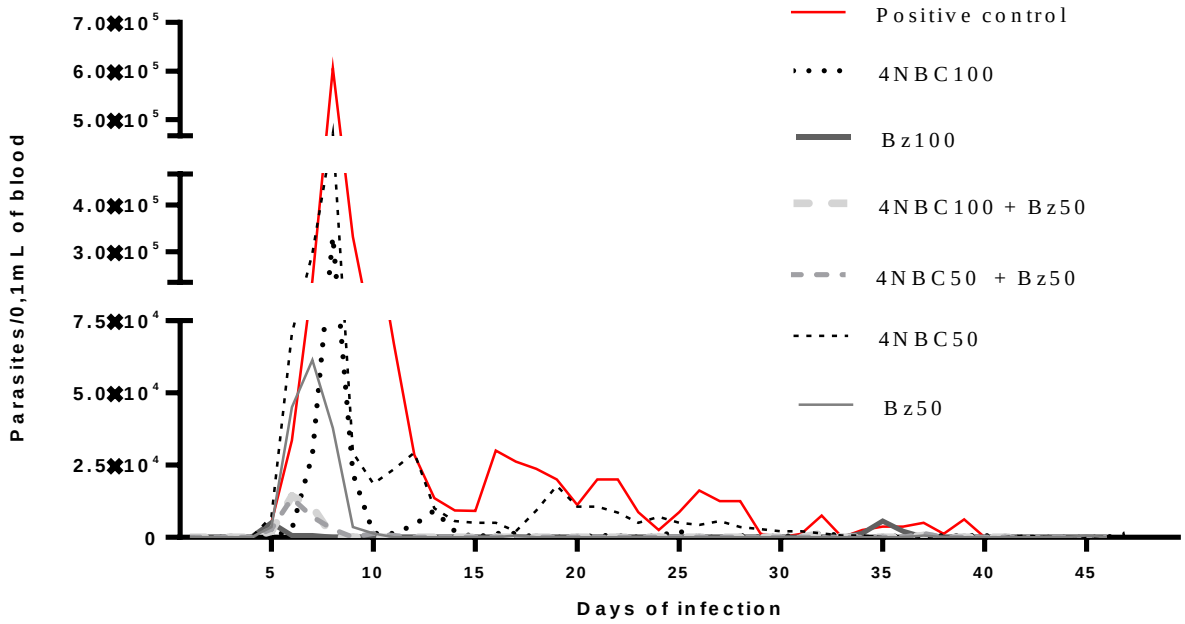
3.1 4-NITROBENZOYLCOUMARIN COMPOUND REDUCES PARASITAEMIA AND ENHANCES TRYPANOCIDAL ACTIVITY OF BENZNIDAZOLE HALF-DOSE

All treatments showed some level of protection when compared to infected and untreated animals (positive control), which showed 43% mortality rate with peak of parasitaemia and area under the curve (AUC) equal to 605,714 parasites/0.1 mL of blood and $16,9x10^5$ parasites/0.1 mL of blood respectively (Figure 1 and Table 1). The group treated with 4NBC 50 mg/kg showed no significant reduction in the peak of parasitaemia and AUC compared to the positive control, but was able to prevent the death of all infected animals. At 100 mg/kg, 4NBC showed reduction of parasitaemia peak to 332,443 parasites/0.1 mL of blood and AUC to $4.1x10^5$ parasites/0.1 mL of blood inducing parasitaemia suppression at levels not detectable by fresh blood test after 12 days of treatment.

More sharp reductions in blood parasitism were observed with the combined therapies. The association of 4NBC 50 mg/kg with Bz 50 mg/kg half-dose showed a more significant reduction of parasitaemia (peak = 13,571 parasites/0.1 mL of blood and AUC = $0,30x10^5$ parasites/0.1 mL of blood) when compared to 4NBC or Bz monotherapies at the same

concentrations. Although the number of doses for blood parasitism suppression was slightly higher (7 doses) for the combination of 4NBC 50 mg/kg with Bz 50 mg/kg compared to Bz 50 mg/kg alone (6 doses), a more significant reduction in blood parasite levels (peak = 13,571 parasites/0.1 mL of blood and AUC = $0{,}30x10^5$ parasites/0.1 mL of blood) was observed when compared with 4NBC or Bz monotherapies at the same concentrations. On the other hand, the combination of 4NBC 100 mg/kg with Bz 50 mg/kg showed similar levels of parasitaemia peak and AUC than those presented for the experimental reference treatment (Bz at 100 mg/kg), as well as previous association, standing out by suppressing the parasitaemia with only three doses, similar to that observed for Bz monotherapy in its experimental reference dose.

Figure 1 - Parasitaemia evaluation of mice treated with benznidazole (Bz) and 4-nitrobenzoylcoumarin compound (4NBC) alone and in combination.



The lines represent the means of parasitaemia values obtained in peripheral blood samples from mice infected with 5,000 trypomastigotes of the *Trypanosoma cruzi* Y strain. Positive control - group of animals infected and untreated. 4NBC100 - animals treated with 4NBC 100mg/kg, Bz100 - animals treated with benznidazole 100mg/kg, 4NBC100 + Bz50 - animals treated with the combination 4NBC 50mg/kg + Bz 50mg/kg, 4NBC50 + Bz50 - animals treated with the combination 4NBC 50 mg/kg + Bz 50mg/kg, 4NBC50 - animals treated with 4NBC 50mg/kg, Bz50 - animals treated with benznidazole 50mg/kg.

Table 1 - Mortality rate, doses required for parasitaemia suppression, parasitaemia peak and area under the curve (AUC), obtained from mice experimentally infected with *Trypanosoma cruzi* Y strain and treated with 4-nitrobenzoylcoumarin (4NBC) and benznidazole (Bz) alone or in combination during the acute phase of the infection.

Experimental groups	Mortality rate	Doses required for parasitaemia suppression	Parasitaemia Peak*	AUC*
Positive control	43%	NA	605,714 [a]	$16{,}9x10^{5}$ [a]
4NBC 50mg/kg	0%	-	471,429 [a]	$10{,}7x10^{5}$ [a]
4NBC 100mg/kg	0%	12	332,443 [b]	$4{,}1x10^{5}$ [b]
Bz 50mg/kg	0%	6	61,429 [d]	$1{,}55x10^{5}$ [d]
Bz 100mg/kg	0%	3	5,000 [c]	$0{,}15x10^{5}$ [c]
4NBC 50mg/kg + Bz 50mg/kg	0%	7	13,571 [c]	$0{,}30x10^{5}$ [c]
4NBC 100mg/kg + Bz 50mg/kg	0%	3	14,286 [c]	$0{,}29x10^{5}$ [c]

* Parasites/0.1 mL of blood
NA - Not applicable.
AUC - area under the curve. Different letters represent statistical difference ($p < 0.05$)

Cure control of mice after the different treatments was accessed by the following three tests: fresh blood test, blood culture and RT-PCR. Blood culture was used as a complementary test applied only to animals with negative parasitological results on fresh blood examination, and the molecular RT-PCR test was performed on all animals regardless of previous results. As can be seen in Table 2, the positive control group showed consistently positive results in both parasitological and molecular tests. Similar parasitological and molecular data were observed for 4NBC monotherapy, differing only in relation to the natural reactivation of the parasitaemia, where 14.3% (1/7) of the animals treated with 4NBC at 100 mg/kg required blood culture for parasite detection. Bz monotherapy at 50 mg/kg showed 71.4% (5/7) of the animals with positive parasitological tests (two positive results by fresh blood test and three by blood culture). In addition, RT-PCR showed that this half-dose Bz was not able to induce parasitological cure in any of the experimental animals. On the other hand, treatment with Bz at the reference dose induced cure in 57.14% (4/7) of the mice, with natural reactivation of the parasitaemia in 42.8% (3/7) of them, and absence of positive complementary results by blood culture or RT-PCR.

When analyzing the results from combination of 4NBC 50 mg/kg with Bz 50 mg/kg, no additional gain of trypanocidal activity was observed and even higher blood culture positivity was detected compared to monotherapy with Bz at 50 mg/kg. However, although the combination of Bz 50 mg/kg with 4NBC 100 mg/kg showed 71.4% (5/7) of positive parasitological results, similar to Bz monotherapy at 50 mg/kg, here RT-PCR confirmed the cure of 14.3% (1/7) of experimental mice.

Table 2 - Cure control: assessing the number of cured animals using fresh blood test, blood culture and real-time PCR (RT-PCR). For each test, data represent the number of animals positive in relation to the total number of mice in the experimental group.

Experimental groups	Fresh blood test[1]	Positive blood culture[2]				Positive RT-PCR	Total cured[3]
		30	60	90	Total		
Positive control	4/4 (100%)	-	-	-	-	4/4 (100%)	0/4 (0%)
4NBC 50mg/kg	7/7 (100%)	-	-	-	-	7/7 (100%)	0/7 (0%)
4NBC 100mg/kg	6/7 (85,7%)	1/1	1/1	1/1	1/1 (100%)	7/7 (100%)	0/7 (0%)
Bz 50mg/kg	2/7 (28,6%)	2/5	3/5	3/5	3/5 (60%)	7/7 (100%)	0/7 (0%)
Bz 100mg/kg	3/7 (42,8%)	0/4	0/4	0/4	0/4 (0%)	3/7 (42,8%)	4/7 (57,14%)
4NBC 50mg/kg + Bz 50mg/kg	2/7 (28,6%)	3/5	4/5	4/5	4/5 (80%)	7/7 (100%)	0/7 (0%)
4NBC 100mg/kg + Bz 50mg/kg	3/7 (42,8%)	2/4	2/4	2/4	2/4 (50%)	6/7 (85,7%)	1/7 (14,3%)

[1]Fresh blood test was used to check the natural reactivation of the parasitaemia.
[2]Cumulative number of positive results at 30, 60 and 90 days after blood collection.
[3]Animals with negative results in all tests were considered parasitologically cured.

3.2 4-NITROBENZOYLCOUMARIN COMPOUND WAS WELL TOLERATED BY ANIMALS

Animal weight gain, plasma levels of liver enzymes AST and ALT, and stereological analyzes of liver tissues were the parameters used to infer treatment toxicity. Figure 2 shows the average weight gain of the animals in relation to the initial average weight in each experimental group. Uninfected group showed greater weight gain in the first month of the experiment compared to infected mice, regardless of treatment, which showed delayed weight gain during the treatment period, clearly perceived in the fourth week of infection. However, after the fourth week (period corresponding to the end of treatment) all groups showed ascending average weight gains.

Figure 2 - Mean weight gain of the mice infected with *Trypanosoma cruzi* Y strain and treated with 4-nitrobenzoylcoumarin (4NBC) and benznidazole (Bz), alone and in combination. The arrows indicate the beginning and end of the treatments.

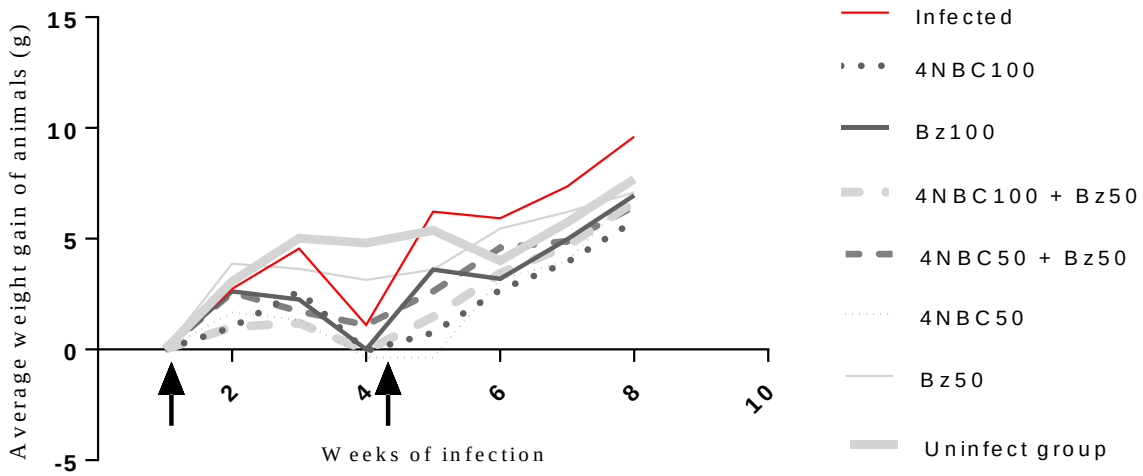
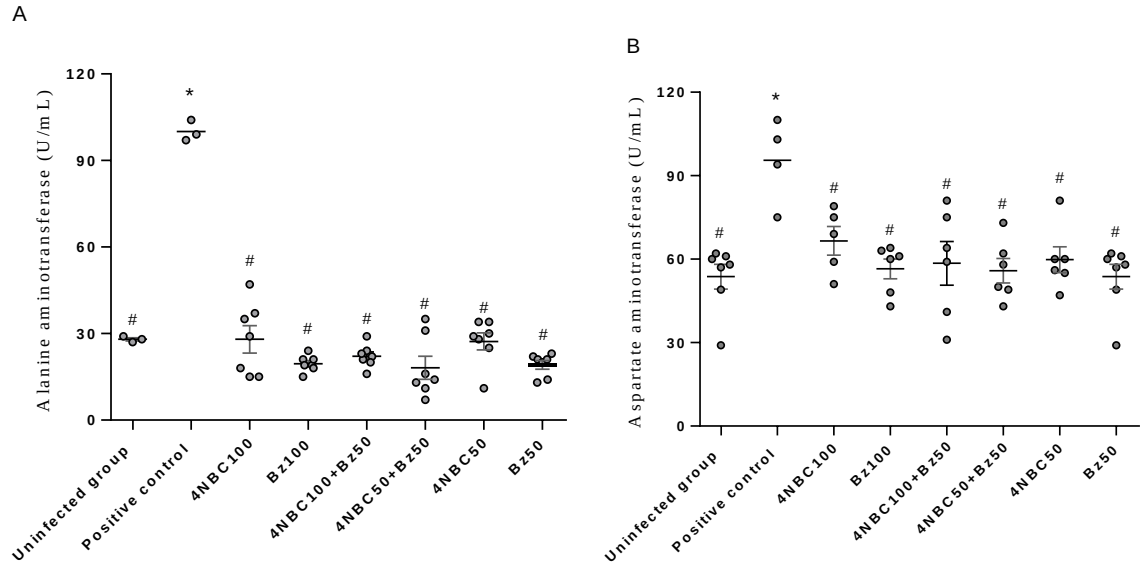


Figure 3 shows that the infected and untreated animals (positive control) presented an increase of both enzymes, being the only one to have difference in relation to uninfected group. All groups submitted to treatment showed a low amount of AST/ALT enzymes, resembling uninfected animals (Figure 3A and 3B).

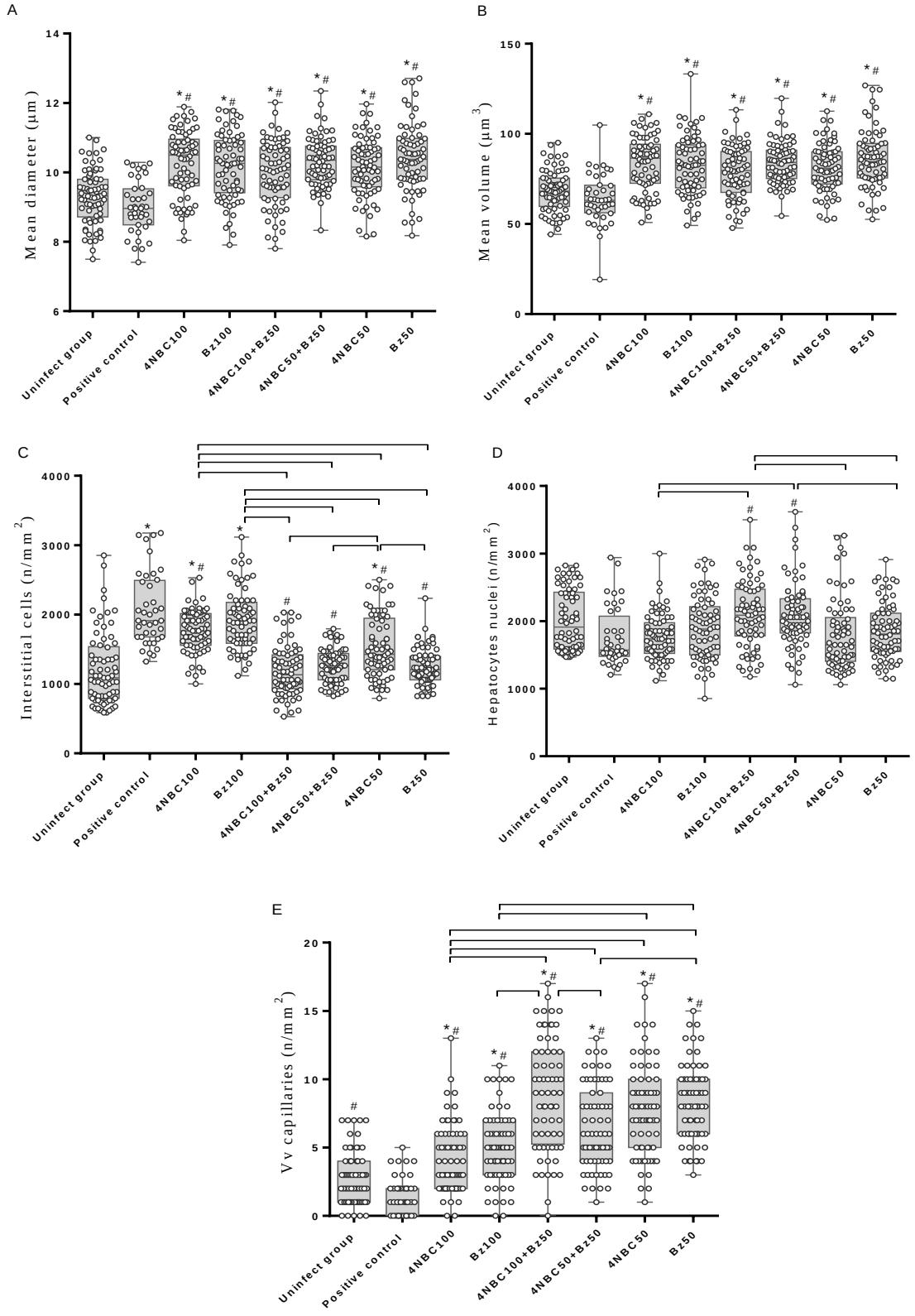
Figure 3 - Quantification of the enzymes Aspartate Amino Transferase and Alanine Amino Transferase in the serum of the mice experimentally infected with *Trypanosoma cruzi* Y strain treated with 4-nitrobenzoylcoumarin (4NBC) and benznidazole (Bz) alone and in combination.



*Represents statistical difference in relation to uninfected animals.

The results obtained from the stereological analysis of liver tissue showed that all therapeutic regimens presented hepatocytes with mean nuclear diameter (Figure 4A) and mean nuclear volume (Figure 4B), significantly higher than the positive control as well as the uninfected group. The amount of interstitial cells in an area of $34x10^3$ mm^2 (Figure 4C) was higher in the hepatic tissue of infected and untreated animals, while those treated with both combinations and Bz 50mg/kg had lower numbers of cells per mm^2, with difference in comparison to the other treatments. In relation to the number of hepatocyte nuclei found in the same area (Figure 4D), the groups treated with the combinations were the only ones presenting a statistical difference in relation to the other groups, which had similar results. The volumetric density of sinusoidal capillaries in an area of $53.5x10^3$ μm^2 (Figure 4E) was lower in the positive control, showing a difference with the other groups. Among all groups, the highest amount of capillaries found was in the combination of 4NBC 100mg/kg with Bz 50mg/kg.

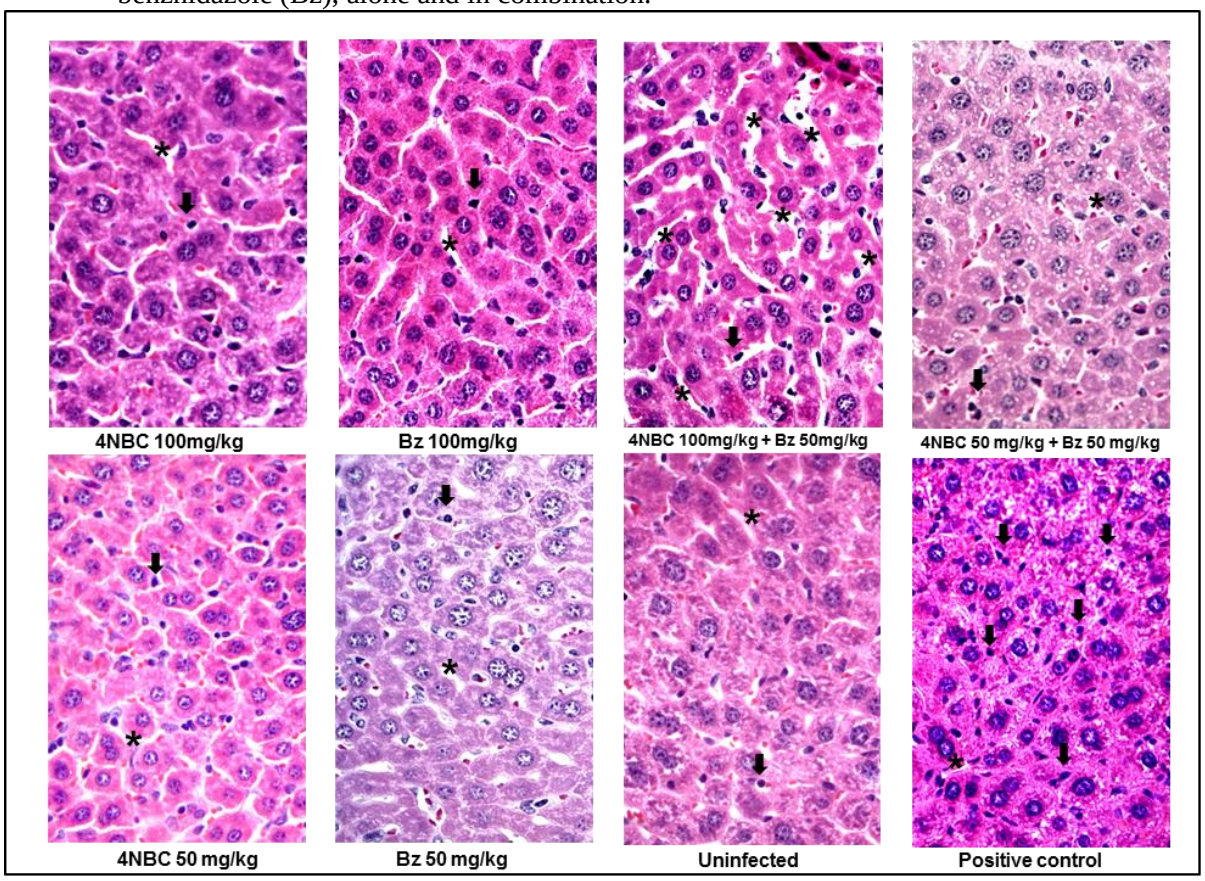
Figure 4 – Stereological analysis of hepatic tissue. Diameter (A) and nuclear volume (B) of hepatocytes; quantity of interstitial cells (C) and nuclei of hepatocytes (D) and volume density of sinusoidal capillaries (E) of mice experimentally infected with *Trypanosoma cruzi* Y strain treated with 4-nitrobenzoylcoumarin (4NBC) and benznidazole (Bz) alone and in combination.



* Represents statistical difference in relation to uninfected animals. # Represents statistical difference in relation to infected and untreated animals. The bars connecting the columns represent statistical difference between the other groups.

Figure 5 shows representative images from histological sections of hepatic tissue from mice submitted to the different therapeutic schemes, according to the data shown in Figure 4.

Figure 5 - Images obtained from histological sections of liver tissue from mice experimentally infected with *Trypanosoma cruzi* Y strain and treated with 4-nitrobenzoylcoumarin (4NBC) and benznidazole (Bz), alone and in combination.



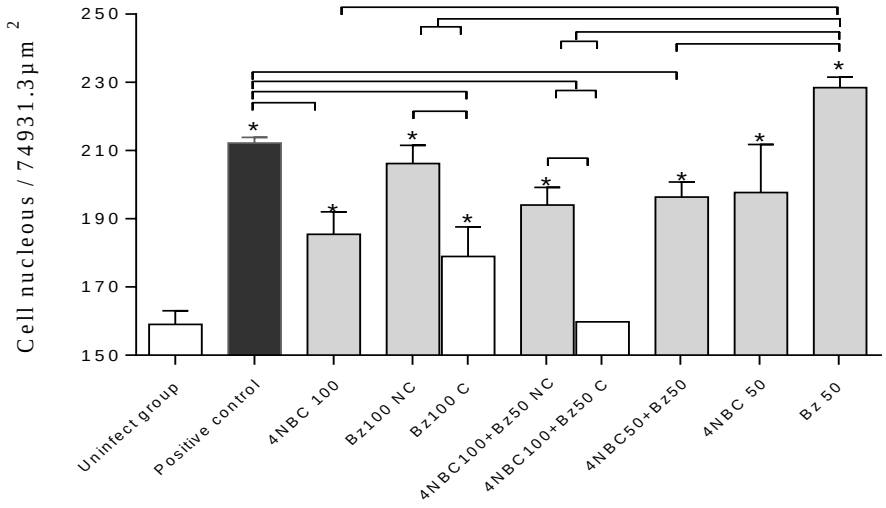
*Sinusoid capillaries. Arrows indicate interstitial cells.

3.3 4-NITROBENZOYLCOUMARIN COMPOUND IS ABLE TO REDUCE MYOCARDITIS

The experimental animals had myocarditis estimated by quantification of cell nuclei from cardiac tissues. All infected groups presented statistical difference in relation to the uninfected animals, showing a greater number of cellular nuclei (Figure 6). The highest number of cardiac cell nuclei was observed in the animals treated with Bz 50mg/kg, presenting the mean of 228 cell nuclei/74931.3 μm^2, followed by infected and untreated mice,

which showed the mean of 212 nuclei/74931.3 μm^2. The lowest number of nuclei was observed in the uninfected group, which presented the mean of 159 cell nuclei/74931.3 μm^2. Four animals from the group treated with Bz 100mg/kg were considered cured and showed difference in relation to the uncured animals of the same group. The same could be observed in the group treated with the combination 4NBC 100mg/kg + Bz 50mg/kg, where one animal was considered cured.

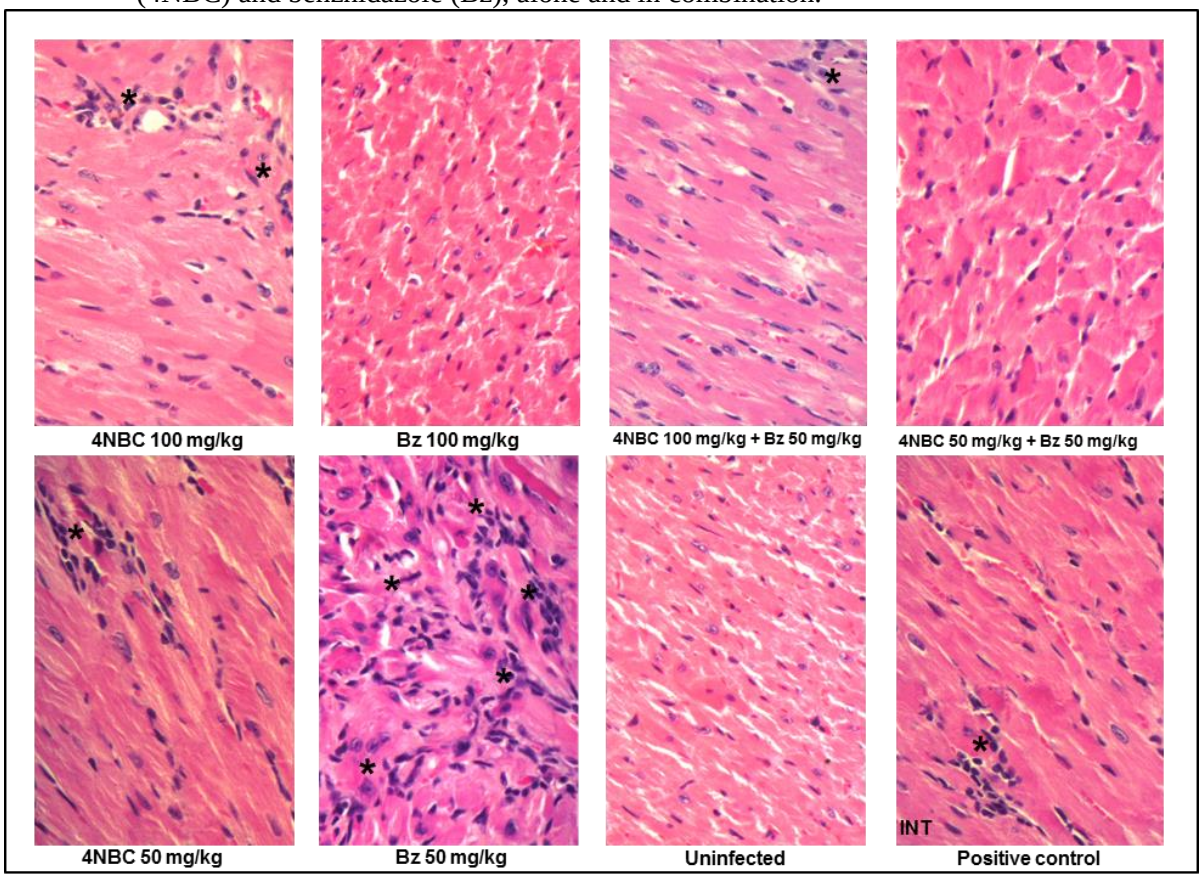
Figure 6 - Quantitative myocardial evaluation of mice experimentally infected with *Trypanosoma cruzi* Y strain and treated with 4-nitrobenzoylcoumarin compound (4NBC) and benznidazole (Bz), isolated and in association.



* Represents statistical difference in relation to uninfected animals. The bars connecting the columns represent statistical difference between the other groups.

Figure 7 shows representative images of cardiac histological sections from mice submitted to the different therapeutic schemes. In general, no large amount of inflammatory infiltrates was observed in the cardiac tissue of the animals, including those infected and untreated. The highest number of inflammatory nuclei was observed in the heart of the animals treated with Bz 50 mg/kg, whereas in the group treated with the combination 4NBC 50 mg/kg + Bz 50 mg/kg, four of seven mice had no accumulation of inflammatory cells in their cardiac tissues. The combination 4NBC 100 mg/kg + Bz 50 mg/kg showed quantification of inflammatory nuclei similar to that observed in the previous combination. Four animals from the Bz treated group (100 mg/kg) were considered cured and did not present inflammatory foci in their tissues.

Figure 7 - Images obtained from histological sections of heart tissue from mice experimentally infected with *Trypanosoma cruzi* Y strain and treated with 4-nitrobenzoylcoumarin (4NBC) and benznidazole (Bz), alone and in combination.

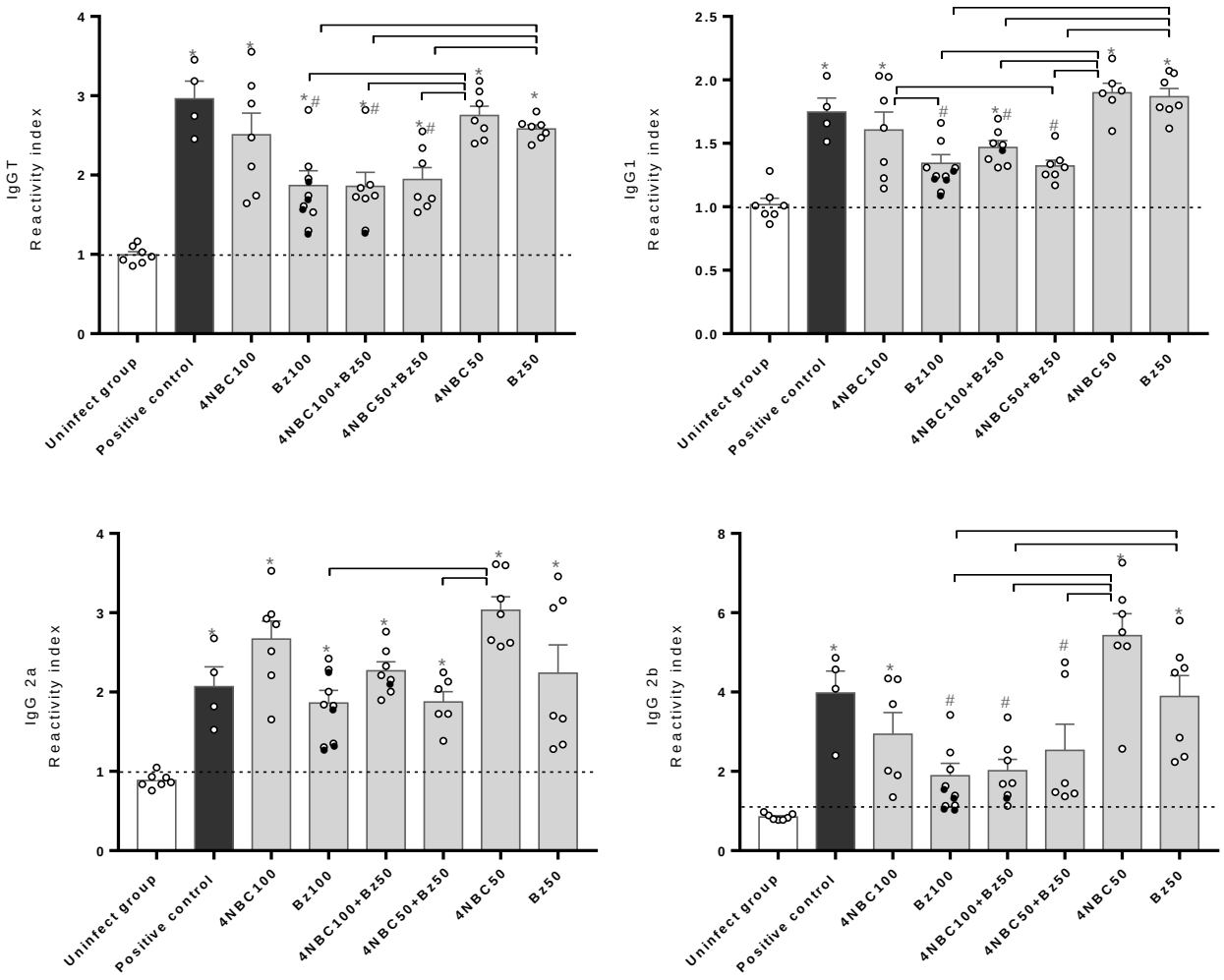


*Areas with inflammatory infiltrates representing acute myocarditis.

3.4 TREATMENT WITH 4NBC IS ABLE TO INFLUENCE THE HUMORAL IMMUNE RESPONSE

Total immunoglobulin G (IgG) and IgG1, IgG2a and IgG2b subclass levels were determined by ELISA from the plasma of experimental animals 20 days after the end of treatments (Figure 8). It was observed that the total IgG and all subclasses showed higher reactivity index in all infected groups when compared to the uninfected control. Regarding IgG and IgG2a, the two strategies of drug combinations were able to reduce the production of these immunoglobulins, considering that they presented lower levels than those presented by the group treated with Bz at 50mg/kg alone. Similar profile was observed in the reactivity index of the IgG1 subclass. A different pattern was observed for IgG2a, which the animals treated with 4NBC alone had the highest reactivity indexes.

Figure 8 - Reactivity index of total immunoglobulins of class G (IgG) and IgG1, IgG2a and IgG2b subclasses, obtained by ELISA, from plasma of mice infected with *Trypanosoma cruzi* Y strain, treated with 4-nitrobenzoylcoumarin (4NBC) and benznidazole (Bz), alone and in combination.



* Represents statistical difference in relation to uninfected animals. # Represents statistical difference in relation to infected and untreated animals. The bars connecting the columns represent statistical difference between the other groups. The dark points represent animals considered cured.

4. DISCUSSION

In previous work our group has identified anti-*Trypanosoma cruzi* activity of 8-methoxy-3-(4-nitrobenzoyl)-6-propyl-*2H*-chromen-2-one compound (4NBC), a new synthetic coumarin, using in vitro assays against amastigotes as well as in vivo assays against blood trypomastigotes in experimentally infected mice (BRANCAGLION *et al*., 2018). Here, this work aims to expand previous studies by evaluating the trypanocidal activity of 4NBC in association with Bz, compared to the isolated treatments, using mice experimentally infected with *T. cruzi* Y strain.

Through the daily evaluation of parasitaemia, it was possible to verify that the treatment with 4NBC, in monotherapy, was not highly effective against the blood trypomastigote forms, since in the lower concentration, 50 mg/kg, there was no suppression of the parasitaemia and the number of parasites at the peak day was the highest among the treated groups ($4.7x10^5$ parasites/0.1 mL of blood). With the dose of 100 mg/kg the parasitaemia suppression occurred on the 13th day of treatment, and the parasite peak (8th day of infection) showed a significant reduction ($3.3x10^5$ parasites/0.1 mL of blood) in relation to the previous dose and to uninfected control animals. The benefit of 4NBC treatment was assumed considering that it was able to reduce the number of circulating parasites in relation to infected and untreated mice and prevented death, since the untreated group had a mortality rate of 42.8% (3/7). In the groups treated with Bz 100 mg/kg, which is the reference drug for Chagas disease (URBINA; DOCAMPO, 2003), only a few doses were required for the suppression of parasitaemia. When using Bz at half the previous concentration, the number of doses required to suppress blood parasitism increased from three to six, with the parasite peak increasing by about 10-fold from $0.05x10^5$ to $0.61x10^5$ parasites/0.1 mL of blood. Regarding drug combination, animals treated with the combination of 4NBC with Bz 50 mg/kg also required few administrations to suppress parasitaemia (seven doses for the combination with 4NBC 50mg/kg and three for 4NBC100mg/kg). In both associations, 4NBC enhanced Bz trypanocidal activity, reducing parasitaemia intensity by more than 30-fold compared to Bz monotherapy at 50 mg/kg. In addition, the combination of Bz 50 mg/kg with 4NBC 100 mg/kg induced a marked reduction in the amount of doses required for parasitaemia suppression (from 6-12 doses to only 3 doses) when compared to both compounds alone at the same concentrations.

Accordingly, previous study using curcumin demonstrated limited antiparasitic effect of curcumin monotherapy, but when combined with Bz, a similar potentiation of its

therapeutic effect was observed, with reduced parasitism and increased survival of experimental mice (Novaes et al., 2016). Another study associating Bz with itraconazole showed that the elimination of the parasite was more efficient in the combination than with each drug alone (ASSÍRIA FONTES MARTINS *et al*., 2015). However, Santos et al (2015) when evaluating the association of Bz with suramin, an antiparasitic drug, observed an increase in parasitaemia peak in mice treated with the drug combination compared to the infected and untreated group as well as the group treated with Bz 100 mg/kg alone

The RT-PCR performed on blood samples from all infected animals, treated or not, showed ~43% (3/7) of positive results for those treated with 100 mg/kg Bz, whereas the combination Bz 50mg/kg + 4NBC 100mg/kg showed positivity in 86% (n = 6) of the mice. This result was interesting, since no cure was observed among the animals that received 50 mg/kg of Bz in monotherapy, evidencing the contribution of 4NBC in the potentiation of Bz anti-*T.cruzi* activity. In the other groups, 100% positivity was observed among treated animals. Parasitological cure, often not achieved in Chagas disease, could be attributed to the occurrence of parasite populations naturally resistant to chemotherapy or even due to the possibility of parasites remaining quiescent in different tissues and organs. This possibilities may explain at least partially why even if treatment is quite effective during the acute phase of the disease, it can often fail to eliminate all the parasites from the vertebrate host (CALDAS *et al.*, 2008; NAGAJYOTHI *et al.*, 2013; TEIXEIRA *et al.*, 2011).

Considering that drug toxicity is a concern in the development of new therapeutic alternatives, in this work, during the treatment and post-treatment period the animals were weighed weekly to verify possible toxic effects caused by the different treatments. The uninfected group was the only one gaining weight progressively, up to the fifth week of infection. The other groups, which were infected, presented lower weight gain during the initial phase of the infection regaining weight as they progressed to the chronic phase. Supposedly the reduced gain of weight is related to reduced food intake and high energy expenditure typical of systemic infections (NOVAES *et al.*, 2015). Another parameter used to verify toxicity due to the treatments was the dosage of liver enzymes ALT and AST. The enzyme ALT is a biomarker, highly specific and sensitive in animals, whose increased levels result in hepatocyte necrosis, and its therefore the most widely used indicator to detect hepatotoxicity (OZER *et al*., 2008; SABAINI PAVAN *et al*., 2018). ALT levels increased only in the infected and untreated animals, while in the treated groups the values presented were similar to the uninfected mice, suggesting that there was no hepatic damage. On the other hand, the levels of AST in the group treated with 4NBC 100mg/kg, alone or in

combination, showed no difference with the infected and untreated animals, which had an increase in the enzyme, whereas the other groups presented lower levels, similar to the healthy animals. The levels of AST were higher than that of ALT in all groups, presumably because the enzyme was found in several tissues such as heart, skeletal muscle, kidneys, lungs, pancreas and brain, thus decreasing their specificity for liver damage (AL-BUSAFI; HILZENRAT, 2013).

In mammals the liver is the main organ responsible for detoxifying the organism (DAVIES *et al*., 2014), so stereological analyzes of the hepatic tissue were also performed to verify the occurrence of liver damage. The mean nuclear volume and diameter of the hepatocytes were higher in the treated groups. The presence of defense cells (Kupffer cells and inflammatory cells) can be considered as mediators of hepatic toxicity after administration of drugs and chemical compounds (BISSELL; GREGORY J. GORESLASKIN; HOOFNAGLE, 2011). In our study, the mice treated with 4NBC in the two concentrations, in monotherapy, also those treated in combination with 50 mg/kg of Bz had significantly smaller amounts of interstitial cells when compared to the infected and untreated control that obtained the largest amount among all the groups, mostly by inflammatory cells. Through the inflammatory mediators macrophages exert a protective and pathological function in situations of hepatic toxicity (BISSELL; GREGORY J. GORESLASKIN; HOOFNAGLE, 2011). In general, the nuclear density of hepatocytes was similar in almost all groups, except for animals treated with both combinations, which were higher than infected and untreated animals and monotherapies with 4NBC at both doses of 100mg/kg and 50mg/kg, as well as Bz 50mg/kg. The increase in nuclear density of hepatocytes corresponds to mechanisms of reaction to tissue damage in the hepatic parenchyma, in which polyploidy and nuclear division events can be representative of the compensation of metabolic losses in macromolecular synthesis, as well as replacing the functions of dead cells (IKEDA *et al*., 2012). The infected control presented the lowest volume of sinusoidal capillaries, showing difference in relation to all other groups. In contrast, the mice treated with Bz 50mg/kg + 4NBC 100mg/kg had the highest volume. The deposition of extracellular matrix elements (glycoproteins, elastic fibers and collagens, proteoglycans), may be responsible for the volumetric decrease of the sinusoidal capillaries (GRESSNER, 1994). Damage to the cells constituting the bile duct as well as fibrosis of the bile duct may be correlated with the reduction of sinusoidal capillary volume (CUPERTINO *et al*., 2013). Interestingly, the uninfected group also presented a reduction in the volume of sinusoidal capillaries. In light of

these observations, we have found that treatment with 4NBC alone or in combination is well tolerated by mice.

It is agreed that the evolution of Chagas disease is influenced by both parasite and host, and that immune response is decisive for the control or escape of the parasite as well as the development of typical lesions of parasite infection. Along with the cellular immune response, humoral immunity is also very important for the control of parasitic infection (MALAGA-MACHACA *et al*., 2017). Different immunoglobulin isotypes (IgM, IgG1, IgG3, IgG2a and IgG2b) are found in the anti-*T. cruzi* response during the acute phase of infection (BERMEJO *et al*., 2010; SPINELLA; LIEGEARD; HONTEBEYRIE-JOSKOWICZ, 1992). Thus, the influence of 4NBC in monotherapy and associated in the production of total immunoglobulin G and its subclasses IgG1, IgG2a and IgG2b was evaluated by ELISA in plasma samples of the mice collected 20 days after the end of treatment. It was observed that the groups treated with Bz 100mg/kg and with the two combinations showed the lowest antibody reactivity indices presenting a difference with infected and untreated animals, which presented high reactivity when compared to the uninfected control, and mainly with the treatment in monotherapy with 50mg/kg Bz, evidencing again the influence of the combination with 4NBC. A possibility for this result would be that treatment with Bz administered in the first week of infection is able to reduce the intensity of humoral immunity (HYLAND *et al*., 2007). Another possibility for the reduction of the level of IgG in the mice would be related to the decrease of the circulating parasites in the blood, caused by the treatment, since the immunoglobulins G respond the presence of trypomastigote forms, and in these groups the natural reactivation occurred in a few mice and the number of parasites on the peak day was low. The profile of animals treated with 4NBC alone was not different from those infected and untreated, showing that coumarin alone was not able to reduce the amount of IgG. The IgG1 and IgG2b isotypes presented similar results to that obtained by IgG, high reactivity in 4NBC treatments alone, presenting difference with animals treated with Bz 100mg/kg and the combinations, as well as with the uninfected group. Novaes *et al* (2016), observed that mice treated with curcumin combined with Bz showed negative reactivity for IgG1 and IgG2a antibodies resembling the uninfected control, whereas curcumin administered alone showed similar results to that of infected and untreated group.

Caldas *et al*. (2017), demonstrated that the high production of IgG, and especially IgG1 subtype, are related to a higher occurrence of myocarditis in dogs and mice infected with different strains of the parasite. Here, we observed that the group treated with Bz 50 mg/kg presented the greatest amount of inflammatory infiltrate in the cardiac tissue, also

showed high reactivity of all IgGs evaluated. 57% (4/7) of the animals treated with Bz 100mg/kg and 14.3% (1/7) of those treated with the combination Bz 50mg/kg + 4NBC 100mg/kg were considered cured, showing no inflammatory nuclei in their tissues, as also observed in uninfected group. Mice treated with 4NBC alone had high reactivity for total IgG and subclasses, as well as few inflammatory foci in their tissues, suggesting that 4NBC treatment may positively interfere with the prevention of myocarditis typical of Chagas disease.

Finally, the global analysis of our data suggests that although 4NBC was not effective in eliminating the host parasite when used alone, it was able to reduce the parasitism of the animals during the period evaluated and still had the ability to potentiate the antiparasitic effect of Bz when in combination. This is particularly interesting considering that the side effects of Bz are responsible for the discontinuation of treatment by many individuals. Therapeutic strategies using lower doses of Bz may contribute to better acceptance and treatment success. In cases of treatment failure, the initial control of parasitism levels would not be sufficient to prevent the persistence of the parasite during the course of the infection and neither heart injury. It has also been found that 4NBC compound is well tolerated by the host and that it can influence the humoral immune response and interfere positively with the prevention of myocarditis.

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6 COMPETING INTERESTS

None.

7 ETHICAL APPROVAL

This study was approved by the Ethics Committee in Animal Research at UNIFAL-MG [number 59/2017].

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