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Dissertação apresentada como parte dos requisitos para obtenção do título de Mestre em Ciências Biológicas pela Universidade Federal de Alfenas. Área de concentração: Interação Patógeno-Hospedeiro. Orientador: Prof. Dr. Rômulo Dias Novaes. Coorientador: Prof. Dra. Lívia de Figueiredo Diniz

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A Banca examinadora abaixo-assinada aprova a Dissertação/Tese 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.

Aprovada em: 10 de fevereiro de 2020.

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Documento assinado eletronicamente por **Luiz Felipe Leomil Coelho**, **Professor do Magistério Superior**, em 10/02/2020, às 16:25, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do Decreto nº 8.539, de 8 de outubro de 2015.

Documento assinado eletronicamente por **Rômulo Dias Novaes**, **Presidente**, em 10/02/2020, às 16:27, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do Decreto nº 8.539, de 8 de outubro de 2015.

Documento assinado eletronicamente por **Reggiani Vilela Gonçalves**, **Usuário Externo**, em 10/02/2020, às 16:39, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do Decreto nº 8.539, de 8 de outubro de 2015.

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ABSTRACT

American trypanosomiasis or Chagas disease is caused by the protozoan *Trypanosoma cruzi*. This disease is endemic in Latin America and about 8 million people are infected worldwide. Acute infections are often asymptomatic and deaths are uncommon. Conversely, chronic cardiomyopathy is the most severe clinical manifestation of Chagas disease, which if related to heart failure and death. Currently, the etiological treatment is limited to benznidazole and nifurtmox, which present high toxicity and low cure rates in chronic infections (10-20%). In patients with chronic cardiomyopathy, renin-angiotensin system (RAS) modulating drugs, such as angiotensin-converting enzyme (ACE) inhibitors, are used to improve cardiovascular function. Interestingly, these drugs have also been associated with better parasitological control and attenuation of myocarditis in *T. cruzi*-infected hosts. Although these drugs are immunomodulatory, it is still poorly understood whether antitrypanosomal effects are due to an enhanced immune response or direct antiparasitic effects. Thus, we used a systematic review framework and an *in vitro* and *in vivo* experimental approach to evaluate the impact of RAS modulating drugs on *T. cruzi* infection. According to our systematic review, captopril, losartan and enalapril increase parasite uptake by host cells, upregulate IL-12 and IFN-γ expression and downregulate IL-10 and IL-17 production by leukocytes *in vitro*. In infected animals, the studies reviewed indicated a marked reduction of parasitemia, tissue parasitism, inflammation and mortality. In our experimental models, our *in vitro* findings indicated that ramipril and losartan increased cardiomyocytes infection rate. Surprisingly, the angiotensin-(1-7) antagonist A-779 was able to reduce trypomastigotes viability, cardiomyocytes infection and the endocytic index. The angiotensin's II and 1-7 had no effect on *T. cruzi* infection, while the ECA activator diminazene aceturate (DMZ) had a toxic dose-dependent effect on trypomastigotes. Our *in vivo* findings indicated that ramipril and DMZ did not prevented myocarditis or interfere with parasitemia peak, IgG plasma levels, and animal weight loss. However, both drugs reduced hepatomegaly, cardiomegaly and mortality in infected mice; while ramipril also attenuated skeletal myositis compared to infected untreated animals. Taken together, our findings supports the evidence that RAS modulating drugs, but not angiotensin's II and 1-7, exerts antiparasitic effects *in vitro* potentially mediated by indirect immunomediated processes, as well as by direct toxic effects on infective forms of *T. cruzi*. However, the antiparasitic effect of ramipril and DMZ observed *in vitro* did not manifest in our murine model of *T. cruzi* infection, indicating limited relevance of these drugs to the treatment of experimental Chagas disease.

Key words: Chagas disease. Parasitology. Experimental pathology. Antiparasitic Chemotherapy.

RESUMO

A tripanossomíase americana ou doença de Chagas é causada pelo protozoário *Trypanosoma cruzi*. Esta doença é endêmica na América Latina e cerca de 8 milhões de pessoas estão infectadas em todo o mundo. As infecções agudas são frequentemente assintomáticas e as mortes são incomuns. Por outro lado, a cardiomiopatia crônica é a manifestação clínica mais grave da doença de Chagas, que se relaciona à insuficiência cardíaca e morte. Atualmente, o tratamento etiológico é limitado ao benznidazol e nifurtmox, os quais apresentam alta toxicidade e baixa taxa de cura nas infecções crônicas (10-20%). Em pacientes com cardiomiopatia crônica, drogas moduladoras do sistema renina-angiotensina (SRA), tais como os inibidores da enzima conversora de angiotensina (ECA), são usados para melhorar a função cardiovascular. Curiosamente, esses medicamentos também vem sendo associados a uma melhora do controle parasitológico e atenuação da miocardite em hospedeiros infectados *T. cruzi*. Embora esses fármacos sejam imunomoduladores, ainda não se sabe se os efeitos anti-tripanossômicos se devem a uma resposta imune aprimorada ou a efeitos antiparasitários direto. Assim, utilizamos uma revisão sistemática estruturada e uma abordagem *in vitro* e *in vivo* para avaliar o impacto dos fármacos moduladores do RAS na infecção por *T. cruzi*. De acordo com a nossa revisão, captopril, losartan e enalapril aumentam a captação do parasito pela células hospedeira, regula positivamente a expressão de IL-12 e IFN-γ e regulam negativamente a produção de IL-10 e IL-17 por leucócitos *in vitro*. Nos animais infectados, os estudos revisados indicaram um redução acetuada da parasitemia, parasitismo tecidual, inflamação e mortalidade. Seguindo para nossos modelos experimentais, nossos resultados *in vitro* indicaram que ramipril e losartan aumentaram a taxa de infecção dos cardiomiócitos. Surpreendentemente, o antagonista da angiotensina-(1-7), A-779, foi capaz de reduzir a viabilidade de tripomastigotas, infecção dos cardiomiócitos e o índice endocícito. As angiotensinas II e 1-7 não tiveram efeito na infecção por *T. cruzi*, enquanto o ativador da ECA, aceturato de diminazeno (DMZ), teve um efeito tóxico dose-dependente nos tripomastigotas. Nossos resultados *in vivo* indicaram que o ramipril e o DMZ não preveniu a miocardite não interferiu com o pico de parasitemia, níveis séricos IgG, e perda de peso dos animais. Entretanto, ambos os fármacos reduziram a hepatomegalia, cardiomegalia e mortalidade em camundongos infectados; enquanto ramipril também atenuou a miosite esquelética comparado aos animais infectados e não tratados. Tomados em conjunto, nossos resultados corroboram com as evidências de que os fármacos moduladores do SRA, mas não as angiotensina II e 1-7, exercem enfeitos antiparasitários *in vitro* potencialmente mediados por processos imunomediados indiretos, bem como por efeitos tóxicos diretos nas formas infecciosas de *T. cruzi*. No entanto, o efeito antiparasitário de ramipril e DMZ *in vitro* não se manifestaram em nosso modelo murino de infecção por *T. cruzi*, indicando limitada relevância dessas drogas para o tratamento da doença de Chagas.

Palavras-chave: Doença de Chagas, parasitologia, patologia experimental, quimioterapia antiparasitária.

FIGURES LIST

TABLES LIST

LIST OF ABBREVIATIONS

Ang II	Angiotensin II
Ang-(1-7)	Angiotensin 1-7
ACE	Angiotensin-converting enzyme
Ang I	Angiotensin I
ACE 2	Angiotensin-converting enzyme 2
ACEIn	Angiotensin-converting enzyme inhibitors
AT_1R	Angiotensin II receptor type 1
Bz	Benznidazole
BNP	Brain natriuretic peptide
BSA	Bovine serum album
BUN	Blood urea nitrogen
CCR2	Chemokine receptor 2
CCC	Chronic Chagasic cardiomyopathy
CHO	Chinese hamster ovary cells
CK	Creatine kinase
CK-MB	Creatine kinase isoenzyme MB
CNT	Control
CI	Cardiothoracic Index
ChD	Chagas disease
DMSO	Dimethyl sulfoxide
DMZ	Diminazene aceturate
DC	Dendritic cells
DTH	Delayed-type hypersensitivity
DMEM	Dulbecco's Modified Eagle Medium
ELISA	Enzyme-linked immunosorbent assay
ECG	Electrocardiography
ECHO	Echocardiography
EI	Endocytic Index
FCS	Fetal calf serum
FBS	Fetal Bovine Serum

HClO	Hypochlorous acid
HUVEC	Human primary umbilical vein endothelial cells
H_2O_2	Hidrogen peroxide
IFN-γ	Interferon gamma
IL	Interleukin
IP3	Inosinol triphosphate
Ig	Immunoglobulin
INT	Infected untreated
IDMZ	Infected treated with diminazen aceturate
IRAM	Infected treated with ramipril
LIT	Liver Infusion triptose
LV	Left ventricular
LVDD	Left ventricular end-diastolic diameter
LVSD	Left ventricular systolic dimensions
MCP-1	Monocyte chemoattractant Protein-1
NDMZ	Uninfected treated with diminazen aceturate
NRAM	Uninfected treated with ramipril
NF-κβ	Factor nuclear kappa B
NK	Natural-killer
NO	Nitric oxide
NSVT	Non-sustained ventricular tachycardia
NTD	Neglected tropical disease
ND	Not Detected
NYHA	New York Heart Association
$ONOO^-$	Peroxynitrite
PBS	Phosphate buffered saline
RAM	Ramipril
RPAD	Right pulmonary artery diameter
RAS	Renin-angiotensin system
RBC	Red blood cells
RMD	RAS modulating drugs
RI	Reactivity index
RPAD	Right pulmonary artery diameter

ROS	Reactive oxigen species
RNS	Reactive nitrogen species
SDBP	Systolic and diastolic blood pressure
SAH	Systemic arterial hypertension
TL	T lymphocyte
T. cruzi	*Trypanosoma cruzi*
TNF-α	Tumor necrosis factor-alpha
TCT	Trypomastigote
Vv	Volume density
WBC	White blood cells

SUMMARY

CHAPTER 1

Could angiotensin-modulating drugs be relevant for the treatment of *Trypanosoma cruzi* infection? A systematic review of preclinical and clinical evidence

Manuscript published in *Parasitology* in January 2019. Cite this article: Souza-Silva TG, Diniz LF, Lia Mazzeti A, Mendonça AAS, Gonçalves RV, Novaes RD (2019). Could angiotensin-modulating drugs be relevant for the treatment of *Trypanosoma cruzi*? Asystematic review of preclinical and clinical evidence. *Parasitology* 1-14. doi: 10.1017/S003118201900009X.

ABSTRACT

Although leucocytes are targets of renin-angiotensin system (RAS) effector molecules and RAS-modulating drugs exert immunomodulatory effects, their impact on *Trypanosoma cruzi* infection remains poorly understood. By using the framework of a systematic review, we integrated the preclinical and clinical evidence to investigate the relevance of angiotensin-inhibiting drugs on *T. cruzi* infections. From a comprehensive and structured search in biomedical databases, only original studies were analyzed. In preclinical and clinical studies, captopril, enalapril and losartan were RAS-modulating drugs used. The main *in vitro* findings indicated that these drugs increased parasite uptake per host cells, IL-12 expression by infected dendritic cells and IFN-γ by T lymphocytes, in addition to attenuating IL-10 and IL-17 production by CD8+ T cells. In animal models, reduced parasitemia, tissue parasitism, leucocytes infiltration and mortality were often observed in *T. cruzi*-infected animals receiving RAS-modulating drugs. In patients with Chagas' disease, these drugs exerted a controversial impact on cytokine and hormone levels, and a limited effect on cardiovascular function. Considering a detailed evaluation of reporting and methodological quality, the current preclinical and clinical evidence is at high risk of bias, and we hope that our critical analysis will be useful in mitigating the risk of bias in further studies.

Keywords: angiotensin inhibitors, Chagas disease, Clinical and experimental parasitology, renin-angiotensin system.

INTRODUCTION

Chagas disease is a tropical neglected infection caused by the protozoan parasite *Trypanosoma cruzi* (Lara et al., 2018; Ribeiro et al., 2018). About 6-7 million people are infected by *T. cruzi* worldwide, mainly in Latin American countries, where the disease is a public health problem and at least 25 million people are at risk of infection (WHO, 2018). *T. cruzi* infection is a zoonosis in endemic areas and humans are the main accidental hosts of this parasite (Noireau et al., 2009). Chagas disease exhibits increasing incidence and prevalence in non-endemic areas, especially North America, Europe, Asia and Africa (Jackson et al., 2014; Angheben et al., 2015). In the absence of the insect vector, the spread of Chagas disease in non-endemic countries often occurs due to the migration of infected people from endemic countries, blood transfusion, transplant of infected organs and from infected mother to fetus (vertical transmission) (Rassi Jr et al., 2009).

Chagas disease courses with non-specific clinical manifestations in the acute phase, especially fever, vomit, lethargy and anorexia (Pinto et al., 2008; Malik et al., 2015). About 60-70% of infected people remain asymptomatic during many years after infection (Fresno and Gironès, 2018). However, 30% of infected people may present anatomical and functional cardiac and/or digestive abnormalities while the disease becomes chronic (Rassi and Rezende, 2012). Although the pathogenesis is not completely understood, Chagasic cardiomyopathy is the most frequent and serious manifestation of the symptomatic chronic phase, which is related with high morbidity and mortality rates (Medei et al., 2008; Biolo et al., 2010). The clinical characteristics of chronic Chagas cardiomyopathy (CCC) are associated with four sets of coexisting manifestations: (i) fatigue, dyspnea, venous congestion systemic, anasarca and adynamia; (ii) heart fibrosis, sinus node dysfunction, atrial and ventricular arrhythmias; (iii) microcirculation disorders and angina; and (iv) dilatation of cardiac chambers, vascular aneurysm, thromboembolism and heart failure. Together, these manifestations are dangerous and closely correlated with CCC progression, morbidity and mortality rates in *T. cruzi*-infected patients (Biolo et al., 2010; Simões et al., 2018).

The specific treatment of Chagas disease is currently based on benznidazole and nifurtimox, which are nitroheterocyclic drugs more effective in acute infections, with cure rates ranging from 50-65% (Messenger et al., 2015; Nogueira et al., 2018). However, both drugs induced marked systemic toxicity and low effectivity in chronic infections (cure rates ranging from 0-30%), which are related with the occurrence of side-effects and the high rates of treatment discontinuation, ranging from 14.5-75% (Pérez-Molina et al., 2013; Rassi et al., 2017;

Gulin et al., 2018). Despite these drugs have been produced for 40 years, no additional effective treatments were developed in the last few decades (Rassi et al., 2017; Nogueira et al., 2018). This limitation has been associated with parasite resistance to chemoterapy, which is partially mediated by molecular adaptive mechanisms, which is partially mediated by molecular adaptive mechanisms, such as mutation in the nitroreductase TcNTR gene and upregulation of lipoamide dehydrogenase (Campos et al., 2014; Santos et al., 2016). As the mechanisms associated with host cell parasitism and parasite survival are poorly understood, development of new effective treatments is still an unpromising challenge. In this sense, there is a continuous effort to identify key mechanisms involved in *T. cruzi* infection (Barrias et al., 2013; Romano et al., 2012), which could be potentially relevant to discover new molecular targets useful in the rational design of new effective antitrypanosomal drugs.

There is evidence that molecules of the renin angiotensin system (RAS), especially angiotensins, are involved in the pathogenesis of Chagas disease (Botoni et al., 2007; Teixeira et al., 2011). Considering that molecules such as angiotensin II (Ang II) and angiotensin 1-7 (Ang 1-7) may alter parasitemia and mortality (Leon et al., 2003; de Paula Costa et al., 2010) in *T. cruzi*-infected mice, chemotherapy based on RAS-modulating drugs such as enalapril, captopril and losartan have been suggested as potentially useful in the treatment of Chagas disease (Botoni et al., 2013; Penitente et al., 2015). As the immune response is the main line of defense against *T. cruzi*, the antiparasitic potential of RAS-modulating drugs appears to be dependent on the effect of RAS molecules on innate and acquired immune cells (de Paula Costa et al., 2010; Santos et al., 2010). This aspect becomes even more interesting and complex considering that immune cells are not only targets of RAS molecules, but also are direct effectors of this system (Hoch et al., 2009). In this sense, dendritic and natural-killer (NK) cells, macrophages, neutrophils, eosinophils, mast cells and T lymphocytes ($CD4^+$ and $CD8^+$) express several RAS molecules, such as renin, angiotensinogen, angiotensin converting enzyme (ACE), Ang II and angiotensin receptors (Reilly et al., 1982; Costerousse et al., 1993; Resende and Mill, 2002; Jurewicz et al., 2007). Although the immune response to RAS stimulation is not fully understood, by exerts chemoattractant effects and modulates the survival, production of cytokines and reactive species in leukocytes (Okamura et al., 1999; Hoch et al., 2009; Barroso et al., 2017; Magalhães et al., 2018), angiotensin's activate a set of events whose potential implications on the resistance and/or susceptibility of *T. cruzi*-infected hosts remains overlooked.

Currently, the available evidence on the relation between *T. cruzi* infection and RAS is rather fragmented and a comprehensive analysis on the impact of RAS molecules and their

modulatory drugs on the evolution of *T. cruzi* infection has never been developed. In this context, it remains poorly understood if and to what extent RAS-modulating drugs exert direct trypanocidal effects or if potential biological effects are secondary to modulation of the host immune system. Thus, we used a systematic review framework to integrate the preclinical (*in vitro* and *in vivo*) and clinical evidence to investigate the relevance of angiotensin-modulating drugs in the treatment of *T. cruzi* infections. In addition to evaluating the potential antiparasitic and immunomodulatory effects of RAS-modulating drugs, the methodological quality of the studies reviewed and the risk of bias associated with the current evidence were also critically analyzed.

METHODS

Structured search strategy

The review protocol was based on the PRISMA guideline (Preferred Reporting Items for Systematic Reviews and Meta-analyzes) (Moher et al., 2009). To retrieve all relevant research records, two search strategies were adopted: (i) a primary search in comprehensive electronic databases, and (ii) a secondary search from the reference list of all relevant studies identified in the primary search (Pereira et al., 2017). The databases Pubmed/Medline, Web of Science and Scopus were used in the primary search. The filters developed for the primary search were structured in two levels: (i) Disease model: American trypanosomiasis (Chagas disease) and (ii) Biological target/Pharmacological strategy: RAS-modulating drugs. The search filter for the Pubmed/Medline was based on standardized descriptors obtained from the hierarchical *Thesaurus* MeSH (Medical Subject Headings, www.ncbi.nlm.nih.gov/mesh). In Pubmed/Medline, the commands MeSH and TIAB (title and abstract) were combined for the retrieval of indexed papers and those citations in the indexing process (*epub ahead of print*). The same research descriptors were structured according to the specific search algorithms required in Web of Science (TS=*descriptor*) and Scopus (TITLE-ABS-KEY[*descriptor*]) databases (Pereira et al., 2017).

Specific biological systems (non-human animals *vs.* humans) were intentionally omitted from our search filters to enhance the search sensitivity [designed to find as many relevant papers as possible, often at the cost of much “noise” (much time consumed by screening numerous irrelevant studies)] rather than specificity (designed to find a small set of highly relevant papers, with the risk of omitting numerous relevant papers) (Jenkins, 2004). No chronological or language limits were applied in the primary search. All original full-text

studies published up to December 2018 were included in the systematic review. The search strategy is detailed in the supplementary file (Table S1).

Screening for primary studies

Publication data (journal, volume, number, page and year), title and abstract of all studies identified in the primary and secondary searches were compared and duplicated registers were removed. The title and abstract of all studies were screened and those that were not related to the subject of investigation were excluded. All potentially relevant studies were recovered in full-text and submitted to an eligibility analysis, in which the adequacy to well-defined inclusion and exclusion criteria was analyzed. The inclusion criteria were: Preclinical and clinical original studies evaluating the impact of RAS-modulating drugs on *T. cruzi*-infection. The exclusion criteria were: (i) papers without full-text available, (ii) secondary studies (i.e., literature reviews, comments, letters to the editor, and editorials), (iii) grey literature (studies not peer-reviewed or formally published in indexed journals) and (iv) studies with multiple interventions in which the effect of RAS-modulating drugs cannot be isolated. In the eligibility phase, the researchers independently analyzed all studies and disagreements were solved by consensus. The reference list of all relevant studies identified in the primary search was manually screened considering a potential identification of additional studies (Pereira et al., 2017). The flowchart indicating the process of study selection is presented in Fig. 1.

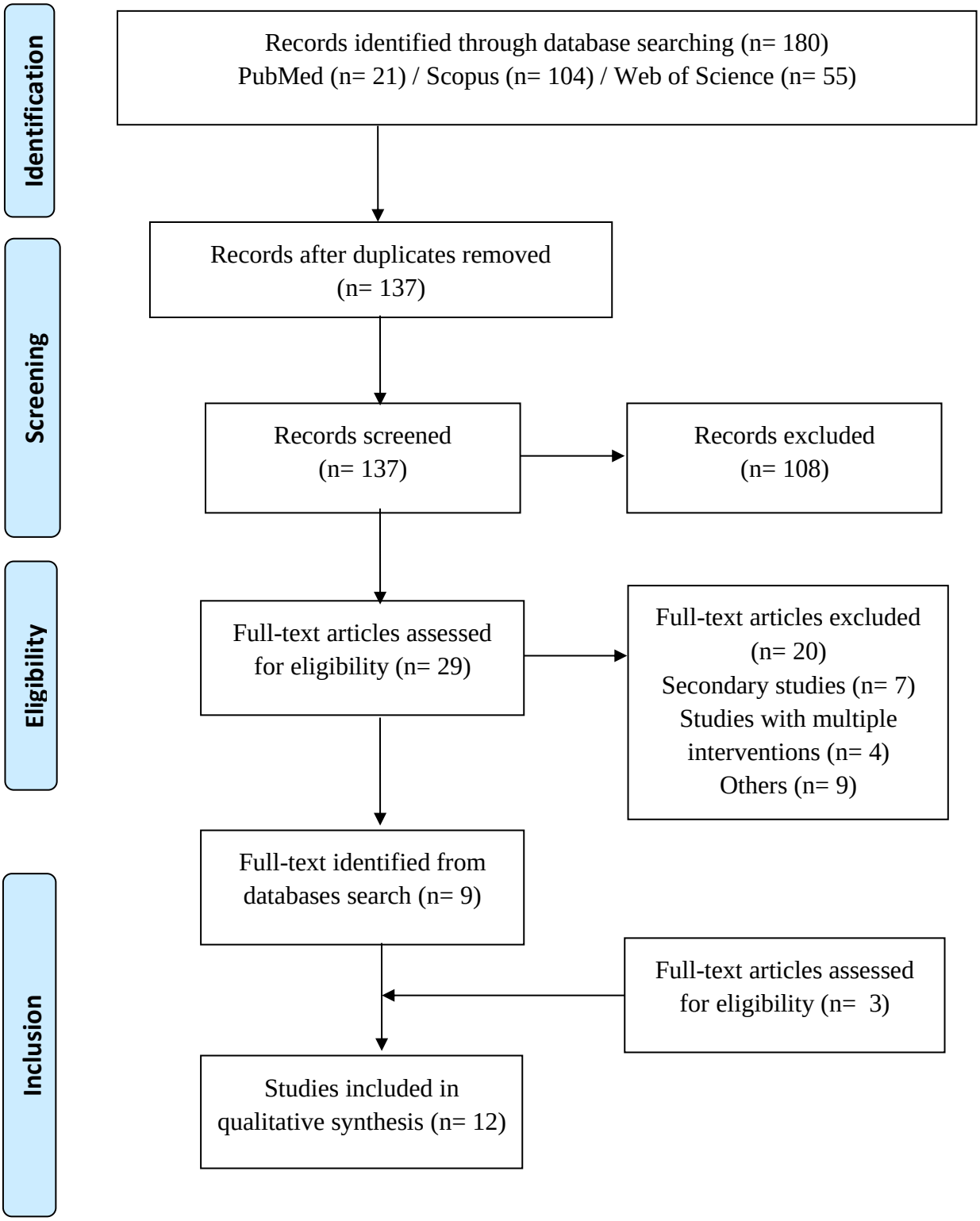

Fig. 1. Flowchart detailing selection of studies included in systematic review. Based on PRISMA statement "Preferred Reporting Items for Systematic Reviews and Meta-Analyses". www.prisma-statement.org

Studies categorization and data extraction

Data extraction was based on basic methodological requirements that should be reported to the proper interpretation of preclinical and clinical evidence. Therefore, the characteristics of all publication such as authors, country in which the study was developed and year of publication were extracted from preclinical and clinical studies. In preclinical studies, additional data were extracted, such as: i) characteristics of the animal model: species, lineage, sex, and age; (ii) disease model: parasitic strain, inoculum (size and route of administration) and duration of infection; (iii) primary outcomes: parasitemia, parasitic load, mortality; and (iv) secondary outcomes: histopathological data, immunological and neuroendocrine markers. In clinical studies, specific data were extracted, such as: i) characteristics of the population: country, age and sex; ii) characteristics of the disease: diagnostic method, time of infection, stage of disease (acute or chronic), form of disease (cardiac, digestive, or undetermined); (iii) primary outcomes: mortality, cardiac function; and (iv) secondary outcomes: neuroendocrine markers.

Analysis of reporting quality and research bias in preclinical and clinical studies

The comprehensiveness of the scientific report in preclinical studies was analyzed by using an analytical instrument described by Pereira et al. (2017). This tool provides a complete screening of all sections of the paper (abstract to acknowledgements and funding) and was developed from basic requirements recommended by the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines (Mcgrath and Lilley, 2015), additionally considering essential elements that should be reported in studies on human trypanosomiasis. The overall mean adherence and the individual quality criteria were expressed as relative and absolute values (Pereira et al., 2017).

The risk of bias in preclinical studies was evaluated by the SYRCLE's risk of bias tool for animal studies (Hooijmans et al., 2014). This instrument is based on the Cochrane Risk of Bias tool and is adjusted for specific aspects of bias playing a relevant impact in animal intervention studies. The SYRCLE's tool is structured in ten topics related to multiple sources of bias such as: (i) selection, (ii) performance, (iii) detection, (iv) attrition, (v) reporting and (vi) additional sources of bias not covered by other domains.

The Downs and Black Measuring Quality was used to evaluate the reporting quality and potential risk of bias in clinical studies based on case-series (Downs and Black, 1998; Nogueira et al., 2018). The scale is based on 27 questions structured in five categories: (i) reporting quality, (ii) external validity, (iii) bias, (iv) confounding and (v) statistical power. This scale

presented high test-retest reliability (r= 0.88) and internal consistency (KR20 formula= 0.89). Due to high ambiguity, statistical power (question 27) was omitted as recommended (Simic et al., 2011).

RESULTS

In vitro models

In *in vitro* assays, heterogeneous cell lineages (i.e., monocytes, dendritic cells (DC), Chinese hamster ovary cells, human primary umbilical vein endothelial cells and T lymphocytes) were used. Although *T. cruzi* Y strain has been applied in most studies, the size of the parasite inoculum and the time of infection was heterogeneous (3 hours to 10 days). Captopril was the drug mainly used, and enalapril was applied in only one study. According to the cell type used, the doses of captopril ranged from 25 to 50 µM, while enalapril was administered between 1 to 3 µM (Table 1).

Table 1. General characteristics of all *in vitro* models of *Trypanosoma cruzi* infection exposed to angiotensin-converting enzyme inhibitors (ACEIn).

Study	Cell lineage	*T. cruzi* strain	Culture medium	Inoculum size	Infection time	ACEIn	Dose
Santos et al. (2010)	Monocyte	Y	RPMI-1640 +5% FCS	5:1	3, 48 or 96 h	Captopril	50 µM
de Paula Costa et al. (2010)	(-)	Y	RPMI-1640	1×10^6	7 days	Enalapril	1-3 $\mu g/mL^{-1}$
Monteiro et al. (2006)	TL and DC	Y	LIT + 10% FCS	5×10^6 for TL, and 5×10^5 for DC	18 h in DC and 10 days in TL	Captopril	$10mg/kg^{-1*}$
Scharfstein et al. (2000)	CHO and HUVEC	Dm28c	DMEM-BSA and M199-BSA	2:1	3 h	Captopril	25 µM

(-): Data not reported or investigated, CHO: Chinese hamster ovary cells, HUVEC: Human primary umbilical vein endothelial cells, TL: T Lymphocytes, FCS: Fetal calf serum, DC: Dendritic cells, BSA: Bovine serum albumin, *dose administered in mice and TL and DC removed later.

In general, the results obtained from *in vitro* systems indicated that captopril increased parasite uptake/parasitic load by host cells (Scharfstein et al., 2000; Santos et al., 2010). Only Santos et al. (2010) investigated ACE expression in host cells, which together with IL-12 and IL-17 was unchanged by captopril in infected monocytes and $CD4^+$ T cells, respectively. Conversely, IL-10 and IL-17 expression in $CD8^+$ T cells was reduced by captopril. Only Monteiro et al. (2006) indicated increased IL-12 expression in infected DC and IFN-γ by T lymphocytes treated with captopril. Cytotoxic activity of enalapril on *T. cruzi* epimastigotes was reported in only one study (Costa et al., 2010). No *in vitro* studies included captopril in direct cytotoxic assays on *T. cruzi* (Table 2).

Table 2. Impact of angiotensin-converting enzyme inhibitors (ACEIn) on *in vitro* models of *Trypanosoma cruzi* infection.

Author	Host Cell	ACEIn Dose	Primary outcomes	Secondary Outcomes
Santos et al. (2010)	Monocytes	Captopril 50 µM	Captopril increased the parasite uptake per host cell, although it did not affect the proportion of infected monocytes.	• Captopril not changed ACE expression in infected monocytes, or IL-12 and IL-17 production by $CD4^+$ T cells. • Captopril reduced IL-10 expression by monocytes and IL-17 expression by $TCD8^+$ cells.
Costa et al. (2010)	-	Enalapril1-3 $\mu g/ml^{-1}$	Enalapril exerted dose-independent cytotoxic on *T. cruzi* epimastigotes	(-)
Monteiro et al. (2006)	TL, DC	Captopril 10 mg/kg^{-1}	(-)	• Captopril increased IL-12 expression by DC and IFN-γ in TL.
Scharfstein et al. (2000)	CHO and HUVEC	25 µM of captopril	Captopril increased the number of intracellular parasite in HUVEC, and in CHO-B_2R^{++}. Captopril had no effect on the invasion of CHO-mock	• Captopril induced intracellular free calcium transients through B_2R in HUVEC infected with *T. cruzi.*

(-): Data not reported or investigated, TL: T Lymphocytes, DC: Dendritic cell, ACEIn: Angiotensin-Converting Enzyme Inhibitors, CHO-B_2R^{++}: Chinese hamster ovary cells with overexpressing B_2 type of bradykinin receptor, HUVEC: Human primary umbilical vein endothelial cells, CHO-mock: Chinese hamster ovary cells without overexpression of receptors.

***In vivo* animal models**

Only five studies evaluating the impact of RAS-modulating drugs on animal models of *T. cruzi* infection were identified in the primary and secondary searches. All studies used isogenic animals (i.e., C57BL/6, BALB/c, and A/J), especially males (n= 4, 80%) ranging from 4 to 10 weeks. The *T. cruzi* strains used to induce the infection were quite heterogeneous and the inoculum size varied from 50 to 10000 parasites per animal, without a direct relation with body mass. The infection time ranged from 25-120 days. Only enalapril, captopril and losartan were used, with doses ranging from 15 to 25 mg kg^{-1} day^{-1} (Table 3).

Table 3. General characteristics of all preclinical animal models of *Trypanosoma cruzi* infection treated with angiotensin-converting enzyme inhibitors (ACEIn).

Author (date)	Animal lineage	Age	Sex	*T. cruzi* strain	*T. cruzi* inoculum [a]	Infection time	ACE inhibitor	Dose/ Frequency
Leite et al. (2017)	C57BL/6	8 w	F	VL-10	5000	120 d	Enalapril	15, 20 and 25 mg kg^{-1} day^{-1}
Penitente et al. (2015)	C57BL/6	10 w	M	VL-10	100	120 d	Enalapril	25 mg $kg^{-1}day^{-1}$
Chumbinho et al. (2012)	BALB/c	8 w	M	Y	1000	30 d	Losartan	200 mg L^{-1}
Costa et al. (2010)	C57BL/6	5-6 w	M	Colombian	50	50 d	Enalapril	25 mg $kg^{-1}day^{-1}$
Leon et al. (2003)	A/J	4-6 w	M	Brazil	10000	25 d	Captopril	5 and 75mg L^{-1}

F: Female, M: Male, d: Days, w: Weeks, *T. cruzi* inoculum: Number of parasites inoculated in each animal.

Most studies indicated that RAS-modulating drugs were effective in reducing parasitemia (Chumbinho et al., 2012; Leite et al., 2017) and mortality (Costa et al., 2010; Chumbinho et al., 2012; Penitente et al., 2015). Conversely, parasitemia was unchanged by enalapril (Penitente et al., 2015), while high doses of captopril (75 mg L^{-1}) increased the animals' mortality with no impact on heart parasitism (Leon et al., 2003). In general, RAS-modulating drugs increased IL-10 plasma levels (Penitente et al., 2015; Leite et al., 2017) and reduced TNF-α, IFN-γ, CCL2 circulating levels and myocarditis severity (Costa et al., 2010; Penitente et al., 2015; Leite et al., 2017). Only Chumbinho et al. (2012) analyzed biochemical markers of organ toxicity (i.e., serum creatinine and urea), indicating that losartan increased kidney injury in *T. cruzi*-infected mice (Table 4).

Table 4. Primary and secondary outcomes in preclinical animal models of *Trypanosoma cruzi* infection treated with angiotensin-converting enzyme inhibitors (ACEIn).

Author (date)	Animal Lineage	ACEIn dose	Primary Outcomes	Secondary Outcomes
Leite et al. (2017)	C57BL/6	Enalapril 15, 20, 25 mg kg^{-1}	Acute effects: only 25 mg kg^{-1} was effective in reducing parasitemia.	• Acute infection: Enalapril at 15 and 25 mg kg^{-1} reduced TNF-α and CCL2, but not CCL5 plasma levels. All doses increased IL-10 production. The treatment was unable to reduce heart inflammation. • Chronic infection: 75-80% of enalapril-treated animals exhibited active colagenogenesis. CCL2 plasma levels were unchanged and CCL5 was reduced at 20 mg kg^{-1}.
Penitente et al. (2015)	C57BL/6	Enalapril 25mg kg^{-1}	Acute effects: enalapril had no impact on the peak of parasitemia, but achieved 60% survival 120 days post-infection.	• Chronic effects (enalapril reduced): heart inflammation, collagen accumulation (fibrosis), and plasma levels of C-reactive protein, CK and CK-MB. CCL2/MCP1 and CCL5/RANTES levels were similar, and IL-10 was increased.
Chumbinho et al. (2012)	BALB/c	Losartan 200 mg L^{-1}	Acute effects: losartan-treated animals presented reduced peak of parasitemia and the mortality rate.	• Acute effects: losartan prolonged the elevated creatinine and urea serum levels, aggravating the acute kidney injury in mice.

ACEIn: angiotensin-converting enzyme inhibitors, CK: Creatine kinase, CK-MB: Creatine kinase isoenzyme MB, DTH: Delayed-type hypersensitivity, BUN: Blood urea nitrogen, AT_1R: Angiotensin II receptor type 1.

Table 4 (*continuation*). Primary and secondary outcomes in preclinical animal models of *Trypanosoma cruzi* infection treated with angiotensin-converting enzyme inhibitors (ACEIn).

Author (date)	Animal lineage	ACEIn dose	Primary outcomes	Secondary outcomes
de Paula Costa et al. (2010)	C57BL/6	Enalapril 25mg kg^{-1}	Acute effects: enalapril reduced the peak of parasitemia, as well as the mean parasitemia 30 days post-inoculation. 100% survival was obtained.	• Acute effects: enalapril reduced: IFN-γ, TNF-α, and CCL5/RANTES serum levels, amastigote nests area, and leucocyte infiltration in the heart. • Acute effects: enalapril increased: circulating neutrophils and monocytes. • Acute effects: the IL-10 circulating levels were unchanged.
Leon et al. (2003)	A/J	Captopril 5, 75mg L^{-1}	Acute effects: captopril at 5mg L^{-1} did not affect parasitemia, heart parasitism or mortality. At 75mg L^{-1}, the treatment had no impact on cardiac parasitism, but increased animals' mortality.	• Acute effects: captopril reduced (75 mg L^{-1}): *T. cruzi* DTH, myosin DTH, heart inflammation, necrosis, fibrosis, heart weight. • Acute effects: captopril reduced (75 mg L^{-1}): increased serum BUN/creatinine ratio. • Acute effects: myocarditis, absolute and relative heart weight, *T. cruzi*-induced T-cell proliferation, and anti-*T. cruzi* IgG circulating levels were unchanged.

ACEIn: angiotensin-converting enzyme inhibitors, CK: Creatine kinase, CK-MB: Creatine kinase isoenzyme MB, DTH: Delayed-type hypersensitivity, BUN: Blood urea nitrogen, AT_1R: Angiotensin II receptor type 1.

Clinical studies

Only four clinical studies were identified in our primary and secondary search. No randomized controlled trials were identified. Clinical studies evaluating the effect of RAS-modulating drugs were exclusively based on case series. In all studies identified, patients between 23 to 64 years old of both sexes were investigated. All studies reported the inclusion of infected patients, but only two studies (Robert et al., 1992; Botoni et al., 2007) reported specific methods for the diagnosis of *T. cruzi* infection (serology and radioimmunoassay). All studies determined the severity of CCC according to the classification established by the New York Heart Association, ranging from I (no symptoms/no limitation) to IV (symptoms at rest/severe limitations). As in studies with the animal model, captopril, enalapril and losartan were exclusively administered in humans. Doses of 5 to 150 mg/day of these drugs were administered in a protocol of treatment ranging from 4 to 120 days (Table 5).

Table 5. General characteristics of all clinical studies with Chagasic patients treated with angiotensin-converting enzyme inhibitors (ACEIn).

Author (date)	Sex / sample	Age (years)	Diagnostic of ChD	Cardiomyopathy severity*	ACE inhibitor	Dose/ frequency	Time of treatment
Botoni et al. (2007)	Male (30) and Female (12)	47.8±10.4	Serology, ECHO and ECG	I, II and III	Enalapril Captopril Losartan	5 to 20 mg/2×day 25 mg day^{-1} 50 mg day^{-1}	120 days
Khoury et al. (1996)	Male (6) and Female (1)	47±10	Cardiothoracic ratio, 2D ECHO, and chest X-rays	III and IV	Enalapril	2.5 mg/2×day	4 days
Szajnbok et al. (1993)	Male (9) and Female (3)	24-64	ECHO	II and III	Enalapril	5-10 mg day^{-1}	60 days
Robert et al. (1992)	Male (9) and Female (6)	23-60	Radioimmunoassay	III and IV	Captopril	12.5 to 50 mg/3×day	49 days

*Classification established according New York Heart Association. ECHO: Echocardiography, ECG: Electrocardiography, ChD: Chagas disease, LV: Left Ventricular, LVDD: LV end-diastolic diameter.

As indicated in Table 6, no parasitological outcome was investigated in all clinical studies identified. However, all studies reported cardiovascular parameters as primary outcomes. Although therapeutic outcomes have been quite heterogeneous, the treatments exerted no or limited impacts on parameters such as heart rate, systolic and diastolic blood pressure, left ventricular shortening and ejection fraction, atrial and ventricular dimensions and the occurrence of non-sustained ventricular tachycardia. Only three studies analyzed plasma markers and only one study reported immunological data. Enalapril and captopril reduced circulating brain natriuretic peptide (BNP) and RANTES circulating levels (Botoni et al., 2007). Captopril also reduced urinary norepinephrine and increased renin plasma levels (Robert et al., 1992).

Table 6. Primary and secondary outcomes in clinical studies with Chagasic patients treated with angiotensin-converting enzyme inhibitors (ACEIn).

Author (date)	Sex / sample	ACEIn / dose	Primary outcomes	Secondary outcomes
Botoni et al. (2007)	M and F / 42	Enalapril /10-40mg, or Captopril 25mg day^{-1}, or Losartan 50 mg day^{-1}	Reduction in SDBP, FS, CI, RPAD, left ventricular end-systolic diameter, and Tei index. Similar heart rate and LVEF.	• Reduction in brain natriuretic peptide (BNP) and RANTES levels. • Similar circulating levels of macrophage inflammatory protein-1α (MIP-1α).
Khoury et al. (1996)	M and F / 6	Enalapril / 2.5 mg/2×day	Similar heart rate and systemic arterial pressure.	• Similar aldosterone, norepinephrine and renin levels
Szajnbok et al. (1993)	M and F / 11	Enalapril / 5-10mg/day	Reduction in the relationship between the amplitude of E/A waves. Similar systolic volume, diastolic and systolic diameter, ejection fraction, LAD, and the incidence of NSVT during the ergometric test.	(-)
Robert et al. (1992)	M and F / 15	Captopril 37.5 mg day^{-1} and increased to 50 mg/3×day	Reduction in heart rate and number of ventricular couplets. Similar SDBP, LVSD, LVDD, LVSD, LVFS, and NSVT episodes.	• Reduction in urinary norepinephrine. • Increase in renin plasma levels. • Similar sodium, magnesium, urea, RBC, WBC and hemoglobin levels.

(-): Data not reported or investigated, M: Male, F: Female, Tei index: Myocardial performance index, LAD: Left atrial diameter, NSVT: Non-sustained ventricular tachycardia, SDBP: Systolic and diastolic blood pressure, FS: Framingham score for heart failure, CI: Cardiothoracic Index LVSD: Left ventricular systolic dimensions, LVDD: Left ventricular end-diastolic dimensions, LVFS: Left ventricular fractional shortening, LVEF: Left ventricular ejection fraction, RPAD: Right pulmonary artery diameter, RBC: Red blood cells, WBC: White blood cells.

Reporting quality and risk of bias in preclinical and clinical studies

From the analysis of reporting quality, no preclinical animal studies met all the quality criteria analyzed, and about 65% of these criteria were completed. The lower adherence to the criteria of reporting quality (60%) was identified for the study developed by Leite et al. (2017) (Fig. 2). The main criteria underreported were related to animals housing and husbandry, strategy of animal allocation in experimental groups, sample size calculation, baseline data, experimental procedures and adverse events, such as indicated in Table S2.

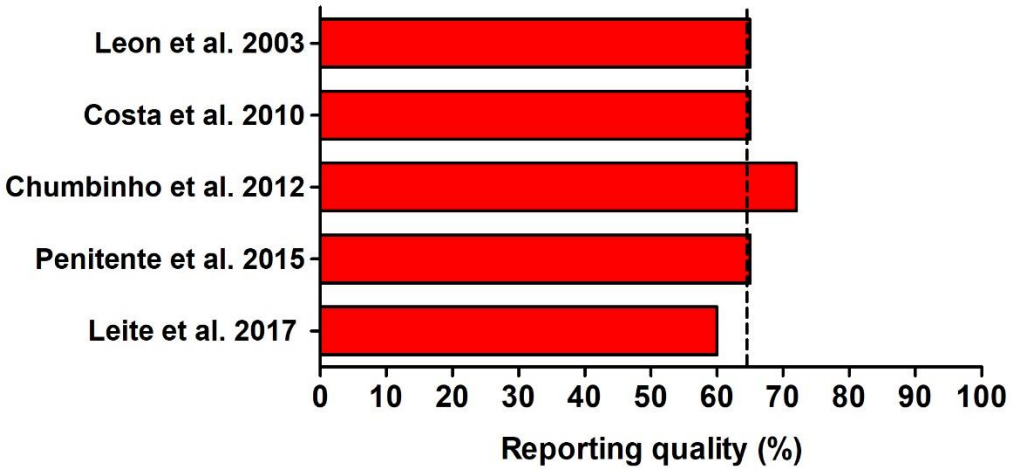

Fig. 2. Reporting quality in preclinical studies investigating the effect of angiotensin-converting enzyme inhibitors on *in vivo* models of *T. cruzi* infection. Based on the reporting quality tool applied to animal models of systemic protozooses (*Parasitology* 144:1275-1287, 2017).

Based on SYRCLE's tool, the risk of bias in preclinical animal studies was variable among clinical studies, with the most frequent bias elements being: (i) Selection bias 1, (ii) Performance bias 2 and (iii) Detection bias 2. In general, the lowest risk of bias was associated with: (i) Reporting bias and (ii) Selection bias 2. Considering the specificities of study design, underreported or incomplete information and the relevance of the information as a criteria of methodological quality; the risk of bias attributed to domains such as attrition bias, detection bias 1, performance bias 1 and selection bias 3, was unclear (Fig. 3).

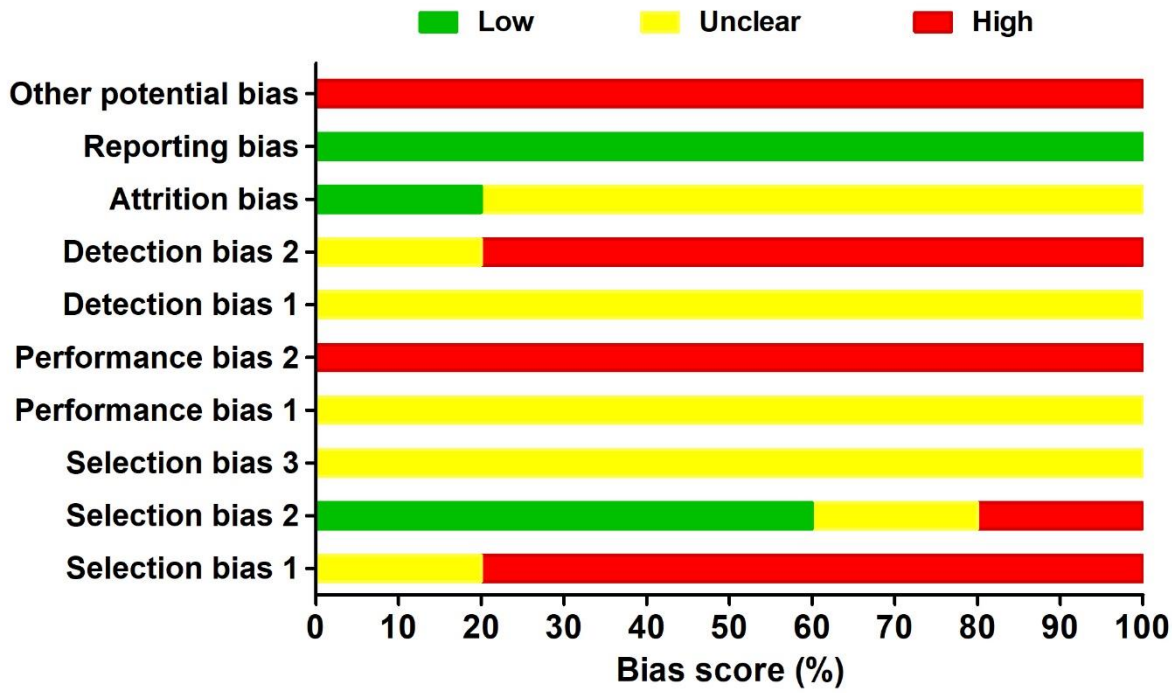

Fig. 3. Risk of bias in preclinical studies investigating the effect of angiotensin-converting enzyme inhibitors on *in vivo* preclinical models of *T. cruzi* infection. Based on SYRCLE's risk of bias tool for animal studies (*BMC Medical Research Methodology* 14:43, 2014).

All clinical studies were also evaluated for the risk of bias. No study met all quality criteria, with an average score of 72%. Two studies (Szajnbok et al., 1993; Khoury et al., 1996) reached a below-average score, which was related to a more limited scientific report (Fig. 4). The main limitations attributed to the potential risk of bias were related with a poor description of clinical outcomes, absence of control groups, adjustment of confounding factors, representativeness of the sample investigated, absence of patients and evaluators blinding and strategies of randomization (Table S4).

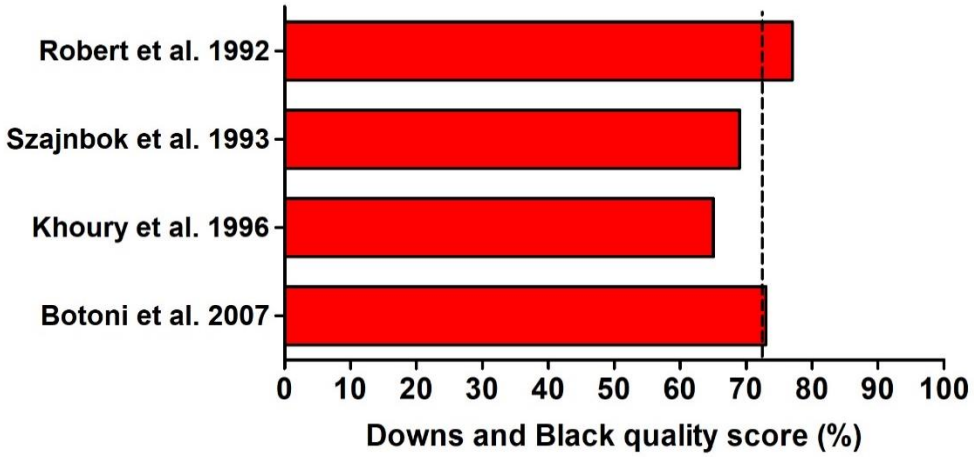

Fig. 4. Risk of bias in clinical studies investigating the effect of angiotensin-converting enzyme inhibitors on Chagasic patients. Based on Downs and Black Measuring Quality in randomized and non-randomized clinical assays (*Journal of Epidemiology and Community Health* 52:377-384, 1998).

DISCUSSION

In vitro models

From a comprehensive search, a limited number of *in vitro* and *in vivo* studies investigating the effect of RAS-modulating drugs on *T. cruzi* infection were identified. Although *T. cruzi* Y strain has been used to induce infection in most *in vitro* studies, the host cells, culture medium, inoculum size, time of infection and drug dose were quite heterogeneous. As these divergences limit the comparison between these studies, a rational and unbiased systematic approach requires that the relationship between experimental models and biological outcomes be individually analyzed.

Even though any nucleated mammal cells can be parasitized by *T. cruzi* (Brener, 1973; Fernandes and Andrews, 2012), the *in vitro* preclinical modeling should ideally consider if the cell lineages used are relevant for the pathophysiology of Chagas' disease. Thus, the application of Chinese hamster ovary cells and human primary umbilical vein endothelial cells impairs the construct validity established by Scharfstein et al. (2000), which presented a greater distance from explanatory models objectively oriented to the understanding of primary targets or effector mechanisms associated with the development of *T. cruzi* infections. However, by using monocytes, DC and T lymphocytes as host cells, Santos et al. (2010) and Monteiro et al. (2006) presented a well-oriented *in vitro* model based on the interaction of *T. cruzi* with effector cells

directly involved in the immunological control of parasite survival and replication (Ferraz et al., 2009; Da Costa et al., 2014; Cardillo et al., 2015). Dendritic cells play a central role as antigen-presenting cells in *T. cruzi* infections, amplifying the antiparasitic immune response from the activation of acquired immune cells, especially lymphocytes (Da Costa et al., 2014; Qu et al., 2014). While the fast response of DC against *T. cruzi* is related to a broad repertoire of membrane receptors and costimulatory molecules (i.e., CD40, CD80, MHC-II, PDL1, CCR5 and CCR7), its innate effectivity is directed by an intense production of immunomodulator molecules, especially TNF-α, IFN-γ, IL-12, IL-22, IL-6, IL-10 and CCL2 (Cunha-Neto and Chevillard, 2014; Cardillo et al., 2015).

Together with DC, classical monocytes ($CD14^+CD16^-$ in humans and $Ly6C^+CD43^-$ in mice) exhibits a marked anti-*T. cruzi* potential, which is induced by a potent Th1 immunological phenotype (Melo and Machado, 2001; Cardillo et al., 2015; Cabral-Piccin et al., 2016). Upon completion of monocytes maturation in activated macrophages, high chemokine receptor 2 (CCR2) expression, marked migratory and pro-inflammatory potential are detected (Pérez-Mazliah et al., 2018). These cells are directly involved in parasitic control, mediating *T. cruzi* death from its phagocytic activity and production of TNF-α, myeloperoxidase, reactive oxygen (ROS: $O_2^{\bullet-}$, $OH^{\bullet-}$, HClO, and H_2O_2,) and nitrogen (RNS: NO, and $ONOO^-$) species, whose production is coupled with intense activation of the endosomal-lysosomal system and oxidative burst (Melo and Machado, 2001; Goes et al., 2016; Paiva et al., 2018). Due to its synergistic roles, DC, monocytes and macrophages exert fundamental relevance against *T. cruzi*, forming an early barrier to infection that orchestrates the assembly and subsequent steps of the adaptive immunity against *T. cruzi* (Cardillo et al., 2015; Da Costa et al., 2014; Poveda et al., 2014). In addition to innate immunity cells, $CD4^+$ and $CD8^+$ T lymphocytes reinforce parasitemia control in acute infections (Ferraz et al., 2009), contributing to the polarization of macrophages from Th1 cytokines (i.e., IFN-γ and TNF-α) (Miranda et al., 2017; Soares et al., 2001). While $CD4^+$ T cells exerts immunomodulatory activities, $CD8^+$ T lymphocytes exhibits direct cytotoxic activity that plays a central role against intracellular *T. cruzi* forms (amastigotes) (Cabral-Piccin et al., 2016). The absence of these cells determines inefficient parasite control, more severe cell parasitism and organ damage, which is often associated with infections with rapid progression and high lethality (Martin and Tarleton, 2004).

Although the effect of RAS-modulating drugs on *T. cruzi* infection remains poorly explored, the identification of metabolic pathways linked to RAS in leukocytes (Fig. 5 and 6) provides an objective rational basis that supports the use of these drugs as immunomodulatory agents in infectious diseases (Costerousse et al., 1993; Jurewicz et al., 2007; Hoch et al., 2009).

In this sense, DC, NK cells, $CD4^+$ T and $CD8^+$ T lymphocytes have high ACE activity and express renin, angiotensinogen AT1 and AT2 angiotensin receptors (Costerousse et al., 1993; Nataraj et al., 1999; Jurewicz et al., 2007). In $CD8^+$ T cells, ACE also participates in MHC-I processing from endoplasmic reticulum, regulating the specificity of the immune response (Shen et al., 2011). Neutrophils and mast cells exhibit an even more complex endogenous RAS, converting Ang I to Ang II by an ACE-independent route, which is mediated by serine protease cathepsin G in neutrophils and chymase in mast cells (Reilly et al., 1982; Resende and Mill, 2002). In addition, the presence of intracellular AT1 receptors indicate an intracrine mechanism of Ang II-induced leucocytes activation (Hoch et al., 2009). As specific responses of RAS activation, Ang II/AT1 stimulation triggers IFN-γ, RANTES and IL-4 secretion by $CD4^+$ T cells (Jurewicz et al., 2007), as well as intense IL-1β, IL-6, NF-κβ, lipoxygenase (Kranzhöfer et al., 1999; Okamura et al., 1999; Silva et al., 2013), and TNF-α and ROS production (Hoch et al., 2009) in macrophages. Furthermore, Ang II/AT1 stimulation also activates IFN-γ production in DC and NK lymphocytes (Jurewicz et al., 2007; Silva et al., 2013).

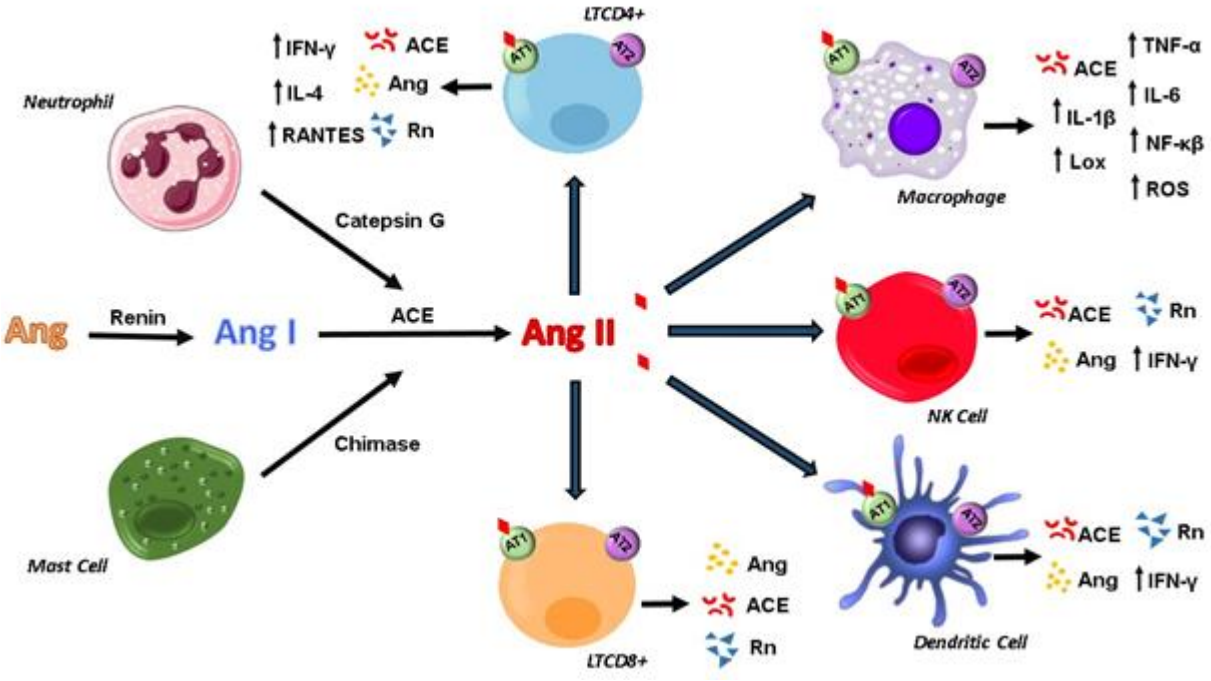

Fig. 5. Synthesis pathways and effects of angiotensin II on immune response cells. Ang: Angitensin, Ang I: Angiotensin I, Ang II: Angiotensin II, ACE: Angiotensin-converting enzyme, TNF-α: Tumor necrosis factor alpha, NF-κβ: Nuclear factor kappa β, Rn: Renin, Lox: Lipoxygenase.

Although the effect of Ang 1-7 on leucocytes is poorly understood, the scarce evidence indicates an opposite effect to Ang II stimulation (Fig. 6). Thus, by activating MAS receptor, Ang 1-7 downregulates NF-κβ-mediated cell signaling in DC, neutrophils and eosinophils (Barroso et al., 2017; Magalhães et al., 2018; Silva et al., 2013), a central pathway involved in the activation of genes encoding pro-inflammatory cytokines (Lawrence, 2009). In addition, Ang 1-7 acts as an anti-inflammatory molecule with potent effect in attenuates TNF-α, IL-6 and MCP-1 expression in macrophages, (Guo et al., 2008; Souza and Costa-Neto, 2012; Thomas et al., 2010), also inducing neutrophils and eosinophils apoptosis (Barroso et al., 2017; Magalhães et al., 2018).

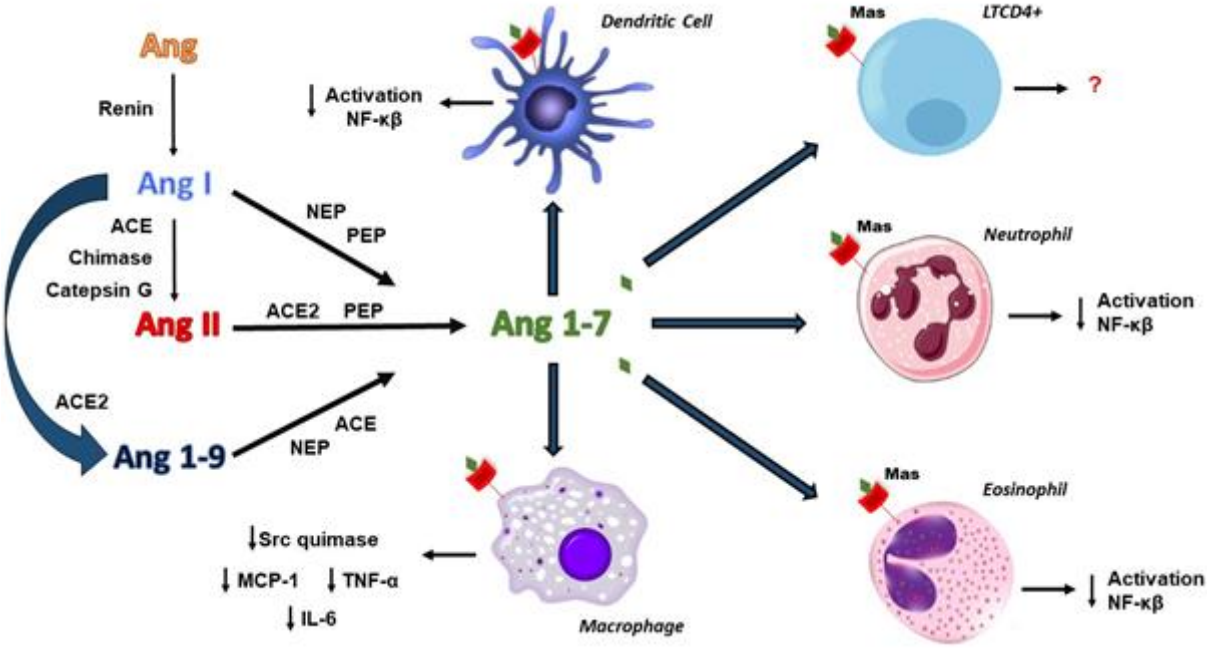

Fig. 6. Synthesis pathways and effects of angiotensin 1-7 on immune response cells. Ang: Angitensin, Ang I: Angiotensin I, Ang II: Angiotensin II, Ang 1-7: Angiotensin 1-7, Ang 1-9: Angiotensin 1-9, ACE: Angiotensin-converting enzyme, ACE2: Angiotensin-converting enzyme type 2, NEP: Neutral Endopeptidase, PEP: Prolylcarboxypeptidase, TNF-α: Tumor necrosis factor alpha, MCP-1: Monocyte chemoattractant protein 1, NF-κβ: Nuclear factor kappa β.

After establishing that the RAS system can modulate important antiparasitic mechanisms in leukocytes, it is important to consider that the alignment between host cell and parasite strain is also relevant in *in vitro* assays of *T. cruzi* infection (Melo and Brener, 1978).

Thus, by using a myotropic strain (Dm28c) to infect host cells with epithelial origin, Scharfstein et al. (2000) dissociates its experimental model from the natural parasite behavior (Contreras et al., 1988). Conversely, Monteiro et al. (2006) and Santos et al. (2010) aligned host cell lineage with the reticulotropic/macrophagotropic characteristic of *T. cruzi* Y strain (Souzaand Alencar, 1984; Santiago et al., 2005), indicating an important element of construct and internal validity of the experimental model used. However, the inoculum size and time of infection were quite heterogeneous in all *in vitro* models, an important factor that makes these models even more divergent, since parasite load and cellular response to infection are profoundly influenced by the inoculum and time of contact between *T. cruzi* and host cells (Vazquez et al., 2015).

Only captopril and enalapril were tested *in vitro* as ACEIn, and only Costa et al. (2010) analyzed direct cytotoxic effect of enalapril on isolated *T. cruzi* epimastigotes. Although enalapril exerted a dose-independent antiparasitic effect, no mechanistic approach was presented in this study. Despite experimental heterogeneity, the main *in vitro* findings indicated that ACE inhibitors might modulate host-pathogen interactions. In this sense, captopril increased parasite uptake per host cells (Scharfstein et al., 2000; Santos et al., 2010), IL-12 expression by infected DC and IFN-γ by T lymphocytes (Monteiro et al., 2006). In addition, IL-10 and IL-17 expression in $CD8^+$ T cells was also reduced by this drug (Santos et al., 2010), a response potentially independent of ACE expression in host cells (Santos et al., 2010). Although no further study on ACEIn and *T. cruzi* is known, the immunomodulatory properties of captopril were indicated from the suppression of TNF-α and IL-1α synthesis in mononuclear cells challenged with LPS (Schindler and Koch, 1995; Peeters et al. 1998). In addition, enalapril and losartan were effective in attenuating the inflammation in mice infected by dengue virus, with reduced IL-1β production by infected peritoneal macrophages (Hernández-Fonseca et al., 2015). By enhance IL-4 and decrease IFN-γ, TNF-α and IL-17 production, losartan was also beneficial in attenuating chronic viral myocarditis, tissue necrosis and mortality in coxsackievirus B3-infected mice, findings attributed to an improved balance between Th1, Th2 and Th17 phenotypes (Zhang et al., 2013).

In vivo animal models

In preclinical animal models, young isogenic male mice were consistently used to induce *T. cruzi* infection. Female mice were used in only one study. Although the current evidence does not indicate a relevant effect of sex hormones on *T. cruzi* infection (Soares et al., 2012, Felizardo et al., 2018), host lineage and age exerts a direct impact on the balance between resistance and susceptibility to *T. cruzi* infections (Pereira et al., 2017; Felizardo et al., 2018).

In this sense, young adult animals are highly susceptible to *T. cruzi* infection than old animals (Felizardo et al., 2018). In addition, the homogenous genetic background of isogenic animals improves the experimental control, providing a lower immunological variability than outbred animals (Trischmann et al., 1978; Vorraro et al., 2014). Furthermore, most studies used *T. cruzi*-resistant C57BL/6 mice (Costa et al., 2010; Penitente et al., 2015; Leite et al., 2017), while only two cases reported susceptible BALB/c (Chumbinho et al., 2012) or A/J (Leon et al., 2003) mice. According to the differential parasitic susceptibility, the choice of these lineages (Silva et al., 2013) should be essentially aligned with three central characteristics of infection, such as evolution time, parasite virulence/pathogenicity (Marinho et al., 1999; Silva et al., 2013) and inoculum size (Trischmann et al., 1978).

An appropriated selection of animal lineage and parasite strain is essential to reproduce the different pathological scenarios typically identified in Chagas disease (Oliveira et al., 2012; Chatelain and Konar, 2015). Thus, longer periods after parasite inoculation are required to induce Chronic infections, in which reduced or absent parasitemia, low-grade inflammation and extensive tissue fibrosis (especially in heart) are pathological outcomes completely divergent to those observed in acute infections (Chatelain and Konar, 2015; Lana, 2017). In general, mice lineage, *T. cruzi* strain and time of infection were aligned in all studies identified in this review. Thus, virulent strains (Y, Brazil and Colombian) (Leon et al., 2003; Costa et al., 2010; Chumbinho et al., 2012) and susceptible mice (BALB/c and A/J) (Leon et al., 2003; Chumbinho et al., 2012) were used in acute models, while a low virulence strain (VL-10) was selected to induce more prolonged infections (Penitente et al., 2015; Leite et al., 2017). As intense parasitemia, tissue parasitism, inflammation and damage are the main desirable characteristics in acute models of *T. cruzi* infection; high inoculum size, highly virulent and pathogenic strains should be a realistic choice for the study of acute outcomes (Lana, 2017; Pereira et al., 2017). However, more resistant animals (Marinho et al., 1999; Silva et al., 2013), lower inoculum and less virulent pathogenic strains should be preferred in chronic models, since assures animals survival for a sufficient period for the installation of chronic manifestations (Trischmann et al., 1978; Pereira et al., 2017).

Unlike *in vitro* assays, enalapril was preferred in animal studies, which also investigated losartan as an ACE receptor inhibitor. In these studies, suitable doses were used, which have been shown to be effective in modulating the production and/or activity of angiotensin and inducing cardioprotection in murine models of endothelial dysfunction and myocardial infarction (Patten et al., 2003; Liu et al., 2006). In addition, both RAS-modulating drugs were effective in reducing parasitemia, tissue parasitism (amastigote nests) and fibrosis, leucocyte

infiltration and mortality in *T. cruzi*-infected animals. In most studies, these effects were partially attributed to the immunomodulatory properties of the drugs tested, which were mainly associated with reduction in TNF-α and IFN-γ levels CCL2/MCP1 and CCL5/RANTES levels (Costa et al., 2010; Leite et al., 2017), as well as the upregulation of IL-10 production (Penitente et al., 2015).

As all studies presented a limited immunological approach, it was difficult to determine to what extent the protective effects reported were a result of the immune adaptations induced by captopril, enalapril and losartan. As there is scant evidence on the effect of these drugs in *T. cruzi*, it is not possible to disregard that the parasitological findings may have been influenced by a direct trypanocidal effect of RAS-modulating drugs, an issue that requires further investigation. However, it is clear that all immunological markers changed by the treatment are directly involved in the pathophysiology of *T. cruzi* infection (Teixeira et al., 2011; Cunha-Neto and Chevillard, 2014; Poveda et al., 2014). Although Th1 molecules are essential in anti-*T. cruzi* immunological responses (Marinho et al., 1999), a Th1 unbalance has been consistently attributed as the central immunopathological mechanism of tissue damage in Chagas disease (Hunter et al., 1997; Poveda et al., 2014). In this sense, downregulation of Th1 effectors (i.e., IFN-γ, TNF-α, CCL2 and CCL5) could be required to reduce the severity of host tissue damage and mortality rates, since high levels of these molecules are associated with intense leukocyte recruitment, heart morphofunctional damage and increased risk of death in chagasic cardiomyopathy (Talvani et al., 2004; YAmauchi et al., 2007; Medeiros et al., 2009). Conversely, IL-10 levels exert a strong protective factor against fatal acute myocarditis, reducing mortality in murine models of *T. cruzi* infection (Reed et al., 1994; Roffê et al., 2012).

Clinical studies

Surprisingly, none randomized-controlled clinical trial was identified. Thus, the available evidence on the effect of RAS-modulating drugs in Chagasic patients is entirely based on case series, indicating that further controlled studies are still needed. Unlike *in vitro* and *in vivo* studies, these drugs were not used with an antiparasitic purpose, but rather to improve the cardiovascular function in patients with CCC. Although all studies have reported including adult Chagasic patients, specific tests (serology and radioimmunoassay) for the diagnosis of *T. cruzi* infection were described in only two papers (Robert et al., 1992; Botoni et al., 2007). However, cardiac function and severity of heart damage were consistently determined in all studies, reinforcing patients' characterization. The underreporting of diagnostic tools represents an important methodological limitation, since different diagnostic strategies are required to

determine the stage of the disease (Balouz et al., 2017; Rodea et al., 2018). Direct parasitological examination (blood smear or microscopic analysis of tissue fragments) is more indicated in detecting acute infections, which can be confirmed with a high sensitivity (around 99%) by serological diagnostic tools, especially indirect hemagglutination, ELISA and indirect immunofluorescence (Andrade et al., 2011; Dias et al., 2016; Nogueira et al., 2018). Since conventional parasitological methods have low sensitivity in cases of sub-patent parasitemia, serological tests are recommended to detect chronic infections (Dias et al., 2016; Balouz et al., 2017).

Similar to animal studies, only captopril, enalapril and losartan were analyzed in clinical investigations. The indication of these drugs was consistent with clinical guidelines for Chagas' heart disease treatment, which recommend administration of ACE inhibitors in all patients with ventricular dysfunction from NYHA I up to IV (Andrade et al., 2011). In general, the doses of each drug tested were coherent with those used in the treatment of systemic arterial hypertension (SAH) in humans (Hodsman et al., 1983; Ikeda et al., 1997; Akat et al., 2010). Dosimetry was also aligned with clinical recommendations, which states that 5-40 mg enalapril (1-2 times/day), 25-150 mg captopril (2-3 times/day) and 50-100 mg losartan (once a day) is suitable to control blood pressure (Mion JR et al., 2004). Although the period of treatment has been quite heterogeneous (4 to 120 days), each study analyzed acute or chronic effects from realistic times of treatment. Interestingly, the study that used a larger number of patients also used a longer time of treatment (Botoni et al., 2007), providing a better methodological control for a longer follow-up.

In general, the treatment of Chagasic patients with captopril, enalapril and losartan exerted limited impact on heart rate, blood pressure, left ventricular shortening and ejection fraction, atrial and ventricular dimensions and frequency of ventricular tachycardia. Surprisingly, immunological markers were investigated in only one study (Botoni et al., 2007), which indicated that RAS-modulating drugs were effective in reducing RANTES levels but without effecting MIP-1α. Conversely, severe cardiovascular dysfunction in Chagasic patients was consistently determined in all studies. Inflammatory infiltrate, progressive fibrosis (Cunha-Neto and Chevillard, 2014) and electromechanical changes (Roman-Campos et al., 2009; Eickhoff et al., 2010) are typical manifestations of CCC, contributing to a 55-65% mortality rate of Chagasic patients (Simões et al., 2018). As heart morphofunctional damage exhibits a progressive behavior, control cardiac overload and complacency is a primary therapeutic goal, representing the most challenge task in the clinical management of patients with CCC (Andrade

et al., 2011; Dias et al., 2016), especially considering that parasitological cure is not a current reality (Biolo et al., 2010; Rassi et al., 2012; Pereira et al., 2017).

Although captopril, enalapril and losartan are effective in regulating blood pressure and cardiovascular remodeling in patients with SAH (Ikeda et al., 1997; Mion et al., 2004; Aakat et al., 2010), the limited effect observed on Chagasic patients remains poorly understood. Although the evidence is still scant, the treatment with RAS-modulating drugs was effective in reduce BNP (Botoni et al., 2007) and norepinephrine levels (Robert et al., 1992) in Chagasic patients. In addition, the treatment had no impact (Khoury et al., 1996) or increased (Robert et al., 1992) renin levels. In addition to exerting an important role regulating cardiovascular function, these molecules are relevant markers of cardiac injury in Chagas disease. Thus, high BNP circulating levels is often detected in patients with severe CCC (Talvani et al., 2004; Garcia-Alvarez et al., 2010). In addition, high norepinephrine levels have been associated with a poor prognosis in Chagasic patients, an aspect potentially related with hemodynamic overload and increased risk of heart failure (Kao et al., 1989; Robert et al., 1992).

Reporting quality and risk of bias

Although our systematic review group and critically analyses the preclinical and clinical evidence on the effect of RAS-modulating in *T. cruzi* infections, the interpretation of the results should consider specific limitations of each study design. Surprisingly, a high proportion of essential criteria reported in animal studies were neglected. There is no doubt that underreported aspects such as animal housing and husbandry, strategy of animal allocation in experimental groups (i.e. randomization), sample size calculation, baseline data and experimental procedures, are serious limitations to the reproducibility and the reliability of preclinical results, indicating a limited internal and external validity of the individual studies. The analysis of methodological bias corroborated the low reporting quality, pointing a high or unknown risk of bias for most preclinical studies in the majority of categories analyzed. In general, the main sources of bias were associated with poor sequence generation (allocation), random outcome assessment, random housing, allocation concealment and experimental blinding. Only selective reporting (reporting bias) did not represented a potential source of bias.

Clinical studies exhibited a better reporting quality than studies with animal models. However, the absence of randomized controlled studies and all methodological limitations observed indicated that the clinical evidence described here should be carefully interpreted. The absence of control groups, a poor description of clinical outcomes, adjustment of confounding factors, small sample size, absence of patients and evaluators blinding and strategies of

randomization were the most important inconsistences identified in clinical studies. Considering that the clinical evidence is based on case series and the heterogeneity in patients' age, cardiac function, drug dose and follow-up period, it is not prudent or advisable to generalize the results to other clinical contexts. However, this review may represent a more comprehensive and up-to-date multilevel analysis on the relationship between RAS-modulating therapy and *T. cruzi* infection.

Although there is a rational basis for the use of RAS-modulating drugs as immunomodulatory agents, the relevance of these drugs as an antiparasitic therapy or as a strategy of cardiovascular support in CCC patients requires more controlled studies with a mechanistic approach. As a simple procedure to improve research quality, essential methodological requirements may be incorporated in preclinical and clinical studies. In this sense, preclinical studies with highest quality can be developed from the use of well-delimited guidelines, including those provided by CAMARADES (Collaborative Approach to Meta-Analysis and Review of Animal Data from Experimental Studies - http://www.camarades.info), and SYRCLE (Systematic Review Centre for Laboratory animal Experimentation - http://www.SYRCLE.nl) initiatives. Similarly, clinical research may benefit from the use of SPIRIT (Standard Protocol Items: Recommendations for Interventional Trials; www.spirit-statement.org) and CONSORT (Consolidated Standards of Reporting Trials; www.consort-statement.org) guidelines, which describes the essential aspects that should be reported when clinical information is publicly disclosed.

ACKNOWLEDGEMENTS

This work was supported by the Brazilian agencies: Fundação do Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG, processes APQ-01895-16 and PPM-00077-18), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, processes 303972/2017-3 and 423594/2018-4), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

CONFLICT OF INTEREST

None to declare.

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Chapter 2

IMPACT OF RENIN-ANGIOTENSIN SYSTEM MODULATING DRUGS ON *Trypanosoma cruzi* INFECTION: *IN VITRO* AND *IN VIVO* STUDIES

ABSTRACT

Renin-angiotensin system (RAS) modulating drugs (RMD) are commonly used to treat heart failure associated with Chronic Chagasic Cardiomyopathy (CCC), the most severe clinical form of Chagas disease. In *Trypanosoma cruzi* infections, these dugs also exhibits anti-inflammatory and antiparasitic properties often attributed to angiotensin II and 1-7, the main RAS effectors. However, it remains poorly understood whether these effects are due to an RMD-boosted immune response or direct antiparasitic effects. Thus, we used *in vitro* and *in vivo* assays to evaluate the impact of RMD on *T. cruzi* infection. Cardiomyocytes, trypomastigote and amastigote *T. cruzi* forms were used to evaluate RMD (angiotensin II and 1-7, ramipril [RAM], losartan and diminazene aceturate [DMZ]) toxicity *in vitro*. The impact of RAM and DMZ were additionally investigated in *T. cruzi*-infected mice. Both angiotensin's exhibited no antiparasitic effects or cardiomyocytes toxicity. Ramipril (9000-500 µM) and losartan (9000-250 µM) reduced trypomastigotes viability, but only low doses of A-779 (2µM) was able in reducing the endocytic index (EI) of parasites and cardiomyocytes infection rate. DMZ induced dose-dependent toxicity against trypomastigotes, reducing cardiomyocytes parasitism. In mice, RAM and DMZ were effective in modulating angiotensin-converting enzyme (ACE) and ACE2 activity and angiotensin II and 1-7 plasma levels. However, did not attenuated myocarditis, parasitemia, and IgG plasma levels. Conversely, both drugs reduced hepatomegaly, cardiomegaly and mortality in infected mice; while RAM also attenuated skeletal myositis compared to infected untreated animals. Our findings supports the evidence that RMD, but not angiotensin's II and 1-7, exerts direct toxic effects on *T. cruzi in vitro*. As the antiparasitic effect of ramipril and DMZ observed *in vitro* did not manifest *in vivo*, these drugs exhibit limited relevance to the treatment of Chagas disease. Considering that ramipril and DMZ were effective in modulating angiotensin II and 1-7 levels, our findings suggest that these molecules are unable in controlling *T. cruzi* infection, which can establish parasitism and damage to the cardiac and skeletal muscles even in the presence of high angiotensin concentrations.

Keywords: Angiotensin-converting enzyme, antiparasitic chemotherapy, Chagas disease, experimental pathology.

INTRODUCTION

American trypanosomiasis is a neglected tropical disease caused by the protozoan *Trypanosoma cruzi*, which is endemic in South and Central America (Lara et al., 2018; Flores-Ferrer et al., 2019; Telleria and Tibayrenc, 2017). About 8 million people are infected worldwide, especially in Latin American countries where oral and vector transmission are the main routs of contamination (Pérez-Molina and Molina, 2018). Due to population migration, vertical transmission, donation of contaminated blood and organs; Chagas disease is on the rise in non-endemic places such as North America, Europe and Asia (Dias et al., 2016).

T. cruzi infection is potentially fatal, and the acute phase is characterized by elevated parasitemia, intense tissue parasitism, systemic inflammation and unspecific symptoms such as fever, myalgia, lymphadenopathy and hepatosplenomegaly (Bilate and Cunha-Neto, 2008; Pérez-Molina and Molina, 2018). About 30% of infected people evolves to a chronic symptomatic form, which courses with gastrointestinal disturbances (mega syndromes) and/or a progressive infectious cardiomyopathy, the most severe and disabling manifestation of Chagas disease (Magalhães et al., 2015; Menezes et al., 2011). Currently, chronic Chagas cardiomyopathy (CCC) is the most common cause of non-ischemic cardiomyopathy and a leading cause of heart transplantation in Latin America (Benatti et al., 2018; Nogueira et al., 2018).

The etiological treatment of Chagas disease is exclusively based on the licensed drugs benznidazole and nifurtmox (Bern et al., 2007; Caldas et al., , 2019). Both have high toxicity and low efficacy in chronic infections, with a cure rate of 10-20% (Bern, 2015; Rassi et al., 2017; Sales Jr et al., 2017). As part of this limitation, about 10,000 people die each year, especially due to CCC (Pérez-Molina and Molina, 2018). As conduction disorders, thromboembolism, angina, as well as chronotropic, inotropic and lusitropic insufficiency are typical manifestations of CCC; Chagasic and non-Chagasic patients with heart insufficiency often receives similar cardiovascular support chemotherapy (Medei et al., 2008; Simões et al., 2018). Thus, beta-blockers, anti-arrhythmic drugs and angiotensin-converting enzyme (ACE) modulators are also beneficial in improving cardiovascular function and survival in patients with CCC (Botoni et al., 2007; Simões et al., 2018).

In addition to its cardiovascular effects, preclinical studies support the evidence that renin-angiotensin system (RAS) modulating drugs (RMD), especially ACE inhibitors (ACEIn), are effective in inducing dose-dependent attenuation of parasitemia, mortality (de Paula Costa et al., 2010; Leite et al., 2017) and systemic inflammatory response (Penitente et al., 2015) in

T. cruzi infected mice. Although not completely understood, these effects has been attributed to ACEIn-induced immunomodulatory effects, especially attenuation of Th1 cytokines biosynthesis and upregulation of mononuclear and polymorphonuclear circulating leukocyte levels (de Paula Costa et al., 2010; Leite et al., 2017). ACE inhibitors also downregulate collagen gene expression by suppressing angiotensin II plasma levels, exerting antifibrotic property (Gallagher et al., 1998), which is potentially relevant in counteracting myocardial fibrosis and heart hypertrophy typically detected in CCC (Biolo et al., 2010; Murphy et al., 2015). This cardioprotective effect has also been attributed to angiotensin 1-7, which is an antifibrotic and vasodilatory heptapeptide produced by angiotensin II converting enzyme (ACEII), an ACE homologous enzyme highly expressed in cardiac tissue and blood vessels (Donoghue et al., 2000; Velkoska et al., 2016).

There is consistent evidence that the immune response is the main line of defense against *T. cruzi*, and that immune cells are direct sources and targets of RAS molecules (Souza-Silva et al., 2019). Thus, the antiparasitic potential of RMD has been systematically associated with an immunomodulatory effect of RAS molecules (de Paula Costa et al., 2010; Santos et al., 2010). However, the impact of RMD on the resistance and/or susceptibility of *T. cruzi*-infected hosts remains overlooked, remaining poorly understood if and to what extent these drugs exert direct trypanocidal effects or if potential antitrypanosomal effects are secondary to RMD-induced immunomediated events. Therefore, we use *in vitro* and *in vivo* models to evaluate the antiparasitic potential of RMD and the impact of ACE activating and inhibiting drugs on the evolution of experimental *T. cruzi* infection.

METHODS

Parasites and drugs

The *Trypanosoma cruzi* Y strain (DTU II), which is partial resistance to the reference drug benznidazole (Dias et al., 2016), was used in all assays *in vitro* and to induce the infection in a murine model of Chagas disease. The stock solutions of angiotensin 1-7 (Ang 1-7), angiotensin II (Ang-II), diminazeno aceturate (DMZ – ACE activator), ramipril (ACE inhibitor), losartan (Ang-II receptor inhibitor), A-779 (Ang 1-7 receptor inhibitor) and benznidazole (Bz – antitrypanosomal reference drug) were purchased from Sigma-Aldrich (St Louis, MO, USA). All drugs were prepared in Mili-Q water or dimethilsulfoxide (DMSO) and stored at -20 ºC. At the time of the use, the reagents were diluted in fresh culture medium and

the maximal final DMSO concentration in the solution equal to or less than 0.06%. Resazurin was diluted in phosphate buffered saline at 1mM (ISO, 2009).

In vitro assays

Cardiomyocytes and parasite cultures

H9c2 cardiomyocytes (American Type Culture Collection, ATCC: CRL 1446) were kept in DMEM culture medium (Gibco, Thermo Fisher Scientific, USA) enriched with 10% fetal bovine serum (FBS), 1% 2mM glutamine (Hepes 2%) and 0.1% penicillin at 200 µg/mL. Culture flasks with 75 cm² containing the cells were kept at 37 ºC and 5% CO_2 and trypsinized when they reached confluence (Tardieux et al., 1992). *T. cruzi* trypomastigotes were obtained from the peripheral blood of previously infected mice. Parasites were propaged in monolayers of H9c2 cells in Dulbecco's modified Eagle medium (DMEM) with 2% FBS, and trypomastigote forms were harvested as previously described (Petersen and Burleigh, 2003; Tardieux et al., 1992). Briefly, H9c2 culture was inoculated with blood tripomastigotes forms. After 3 days of incubation, propaged trypomastigotes were collected from the supernatant, centrifuged at 2500×*g* for 5 min at 4ºC and resuspended in DMEM. Parasites obtained were used in cardiomyocyte parasitism and toxicity assays.

Standard curve of trypomastigotes

Trypanosoma cruzi trypomastigotes were serially plated from 2×10^6 parasites/well and incubated at 37ºC, 5% CO_2 for 24 hours. Resazurina was added at 20µl 1mM to each well and the reduction reaction was read every 2 hours after dye addition until 100% reduction is obtained. Absorbance readings were taken at 570 nm (reduced form detection) and 600nm (oxidized form detection) in a microplate reader (Anthos Zenyth 200, Biochrom, Cambridge, UK) (Ahmed et al., 1994).

Activity of RAS modulators on T. cruzi trypomastigotes

Trypomastigotes were maintained as previously described. To quantify the activity of each drug on the parasite, the previously standardized resazurin-based colorimetric reaction was used. Briefly, trypomastigotes were plated at 1×10^6 in 96-well polystyrene plates. Decreasing drug concentrations in a volume of 100 µL were added to the wells containing the parasites. Seven dilutions of each drug were tested in triplicate, with initial concentration of 90 µM of Ang-II, Ang 1-7, A-779, DMZ, or 9000 µM of losartan and ramipril. The plates were incubated in an oven at 37 ºC and 5% CO_2 for 24 hours. Resazurin (20 µL) was added in each well and

after 7 hours the reaction was read at 570nm and 600nm using a microplate reader (Anthos Zenyth 200, Biochrom, Cambridge, UK).

RAS modulating drugs cytotoxicity on cardiomyocytes

Uninfected H9c2 cardiomyocytes in culture were treated with maximal concentrations of Ang-II, Ang 1-7, A-779, DMZ, Bz, losartan and ramipril. H9c2 were plated in 96-well polystyrene plates at $2x10^3$/mL and incubated at 37 ºC and 5% CO_2 for 24 hours. The culture medium was then removed and replaced with 200 µL of medium containing or not decreasing drug concentrations, with 8 dilutions from 10 µM of Ang-II, Ang 1-7, A-779, DMZ, or 1000 µM Bz, losartan and ramipril. After a 48 hours incubation, 20 µL of 1mM resazurin was added to each well and the plate was read in a microplate reader at 570nm and 600nm as previously described.

RAS modulating drugs cytotoxicity on T. cruzi amastigotes and cardiomyocytes parasitism

H9c2 cardiomyocytes were plated at 1×10^4/mL in 24-well glass-lined plates and incubated at 37 ºC and 5% CO_2 for 24 hours. Then, the culture medium was replaced with 1mL of medium containing or not decreasing drug concentrations, with 4 serial dilutions from 2 µM of Ang 1-7 and A-779, 10 µM of Ang-II, 200 µM of losartan, 20 µM ramipril and 5 µM DMZ and Bz. Host cells were incubated with each drug for 12 hours, followed by trypomastigotes inoculation at 20:1 parasites:H9c2 cells ratio. Drug-containing supernatant and non-internalized parasites were removed after 24 hours of trypomastigotes inoculation, and a fresh culture medium containing the same drug concentrations was added. Cardiomyocytes parasitism was evaluated after 24h and 48h of parasites inoculation by using in triplicates in two independent experiments. Briefly, culture cells were stained with 2% Giemsa solution and cardiomyocytes parasitism was evaluated in bright field microcopy (×400 magnification) by determining the proportion of infected cells in 100 cells counted in each drug concentration. The infection rate was used to calculate the percentage reduction in the number of infected cells compared to infected and untreated cells (Mazzeti et al., 2019). Amastigote load was evaluated from the endocytic index, which was calculated as the percentage of infected cells times the average number of intracellular amastigotes per infected cardiomyocyte (da Silva et al., 2008).

In vivo assays

Animals and infection

Eight-week-old male BALB/c mice weighing 28.44±2.72g were used in the experiment. Mice were randomized in polypropylene boxes kept in animal facility with controlled environment (temperature 22±2 ºC, humidity 60-70%, 12/12 h dark/light cycle). Water and food were provided *ad libitum*. The animals were intraperitoneally infected with 2500 blood trypomastigote forms of *T. cruzi* Y strain. Parasites were collected from the peripheral blood of previously infected mice (Caldas et al., 2008). All experimental procedures involving animal care were approved by Ethics Committee.

Treatment and experimental groups

Mice were randomized into six groups, containing thirteen animals in *T. cruzi*-infected groups and eight animals in uninfected control groups as follows: CNT, control uninfected and untreated; NDMZ, uninfected treated with 1mg/kg diminazene aceturate (Sigma Aldrich, St. Louis, MO, USA); NRAM, uninfected treated with 5 mg/kg ramipril (Biosintetica, Sao Paulo, SP, Brazil); INT, infected untreated; IDMZ, infected treated with 1mg/kg diminazene aceturate (Sigma Aldrich, St. Louis, MO, USA); IRAM, infected treated with 5 mg/kg ramipril. Based on its efficacy in the treatment of *T. brucei* (Pépin and Milord, 1994), the DMZ dose was administered taking into account the best tolerability of the animals and ability to activate ACE2 (Prata et al., 2017). Ramipril dose was established considering efficacy in inhibiting ACE and its typical antihypertensive effect (Crowley et al., 2012; Spindler et al., 2016). Both drugs were diluted in 0.9% NaCl solution and administered orally by gavage at 0.2mL. The treatments were administered daily, starting 4 days before parasite inoculation and for 16 consecutive days post-*T. cruzi* inoculation.

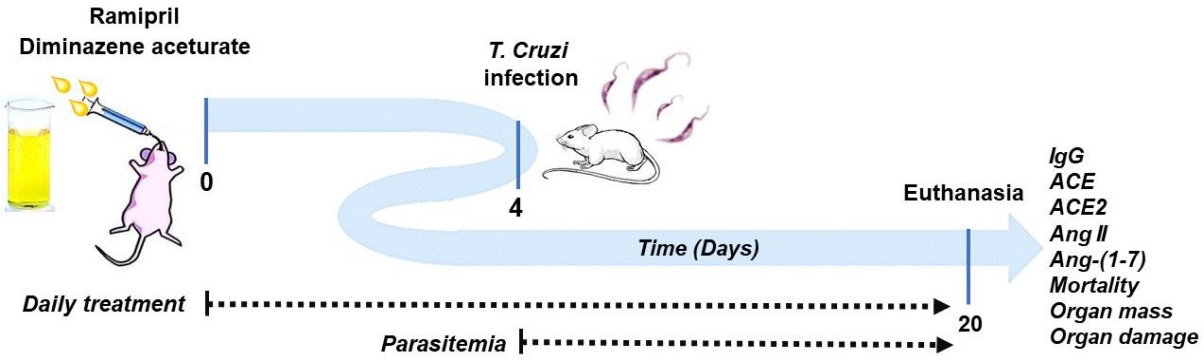

Fig. 7. Experimental design and parameters analyzed to investigate the impact of renin–angiotensin system modulating drugs on *Trypanosoma cruzi* infection in mice. The treatment with ramipril (angiotensin-converting enzyme [ACE] inhibitor) and diminazene aceturate (ACE2 activator) were administered daily for 20 days. The infection progressed for 16 days after four days of starting treatment. IgG, immunoglobulin G; Ang, angiotensin; ACE, angiotensin-converting enzyme; ACE2, angiotensin-converting enzyme2.

Parasitemia, biometry and mortality

After *T. cruzi* inoculation, parasitemia was evaluated daily by microscopic quantification (×400 magnification) of blood trypomastigotes in 5 µl of peripheral blood, collected by venous puncture in the tail of the animals (Brener, 1962). The parasitemia curve of all animals infected was plotted, and initial, peak, mean and final parasitemia were calculated. Treated mice as well as controls, infected or not, were weighed at the beginning of the experiment and every six days after infection until the last day of treatment. After sixteen days of infection, the animals were euthanized by cervical dislocation and the relative organ weight (heart, liver and spleen) was calculated by dividing organ mass by final body mass (Lannes-Vieira et al., 2009). Mortality was recorded throughout the experimental period (Caldas et al., 2008).

Measurement of angiotensin-converting enzymes activity

Blood samples were collected from the orbital venous sinus and centrifuged in the presence of sodium heparin. Plasma was obtained after blood centrifugation at 2500×g for 5 min at 4ºC. Enzymatic activities were measured from fluorometric biochemical kits for ACE (Sigma-Aldrich, St. Luis, MO, USA) and ACE2 (BioVision Inc., Milpitas, CA, USA), according to the procedures provided by the manufacturers. The reactions were read at 320/405 nm excitation/emission for ACE and 320/420 nm excitation/emission for ACE2. Enzyme activity was measured by using a cary eclipse fluorescence spectrophotometer (Santa Clara, CA, USA) and was expressed as relative fluorescence unities.

Measurement of angiotensin levels

Angiotensin II and Angiotensin-(1-7) levels were measured in the same plasma samples used to estimate ACE activity. Angiotensin quantification was based on enzyme-linked immunosorbent assay (ELISA), according to the procedures provided by the manufacturers of Ang II (Enzo Life Sciences Inc., Farmingdale, NY, USA) and Ang-(1-7) (Lifespan Biosciences Inc., Seattle, WA, USA) 96-wells immunoenzymatic kits. Detection range of the assays was 7.8-500 pg/mL for Ang-(1-7) and 3.9-10,000 pg/mL for Ang II.

Heart and skeletal muscle histological processing

Heart and skeletal muscle (gastrocnemius) samples were fixed in 4% buffered paraformaldehyde (0.2M sodium phosphate buffer, pH=7.2) for 48 hours. After dehydration in ethanol, fragments were included in glycol methacrylate histology resin and sectioned in rotatory microtome. Semi-serial sections were obtained at 5µm thickness and 100µm interval to avoid the analysis of the same histological area. Histological sections were stained with hematoxylin and eosin and mounted with coverslips and entellan (Merck Millipore, Kenilworth, NJ, USA). Digital images were obtained using a bright field photomicroscope (Axio Scope A1, Carl Zeiss, Oberkochen, Germany) and a ×40 objective lens (×400 magnification). For each organ and animal, 15 non-coincident histological images were randomly obtained and a total of 6.22×10^5 μm^2 tissue area was analyzed.

Heart and skeletal muscle morphological remodeling

Heart and skeletal muscle inflammation and microstructural remodeling was evaluated according a stereological method previously reported (Novaes et al., 2013, 2017). From digital images, volume density (Vv, %) occupied by cardiac and skeletal myocytes (Vv[my], %), interstitium (Vv [int], %) and interstitial/inflammatory cell nuclei (Vv[nu], %) was estimated by point counting as follows: $Vv= \Sigma P/Pt$; where ΣP represent the number of points of the test system that hits the structure of interest and Pt is the total number of test points. A test system with 100 test points contained in a test area (A_T) of 1.38×10^4 μm^2 was used. The number density of interstitial cells (Q_A, cells/mm^2) was calculated to estimate the intensity of myocarditis (Novaes et al., 2013) and skeletal myositis in *T. cruzi* infected animals (Novaes et al., 2017). The number density was estimated as $Q_A= \Sigma Ic/A_T$; where ΣIc denotes the total number of interstitial cells in the quadratic test area ($A_T = 59.19\times10^2$ μm^2). The Image Pro-Plus 4.5 image

analysis software (Media Cybernetics, Silver Spring, MD, USA) was used to obtain all measurements (Novaes et al., 2011).

Immunoenzymatic assay for anti-T. cruzi antibodies

Blood samples were collected from the orbital venous sinus and centrifuged in the presence of sodium heparin. Plasma was obtained after blood centrifugation at 2500×*g* for 5 min at 4ºC. Plasma samples were used for detection of specific anti-*T. cruzi* antibodies according to the method described by Voller et al. (1976). Briefly, high-affinity immunoassays plates were coated with *T. cruzi* antigens prepared from alkaline extraction of tripomastigote forms exponentially growing in LIT medium. Then, 5 µl plasma samples were added to the wells sensitized for IgG uptake. Peroxidase-conjugated mouse anti-IgG antibodies (Sigma Aldrich, St. Louis MO, USA) were used as detection probes. The cut-off point for discriminating positive and negative results was calculated by the mean absorbance for 10 negative control samples plus two standard deviations (Caldas et al., 2008).

Statistical analysis

The data were presented as mean and standard deviation or median and interquartile interval. The results were compared using One-Way ANOVA followed by the Student-Newman-Keuls *post-hoc* test or Kruskal-Wallis test, according to the Kolmogorov-Smirnov normality test. All the graph were built using the Graph Pad Prism software, version 8.0 (San Diego, CA, USA). Results with P values ≤ 0.05 were considered statistically significant.

RESULTS

***In vitro* results**

Optimization of colorimetric reaction and cytotoxicity in H9c2 cardiomyocytes

The sensitivity of *T. cruzi* to RAS modulating dugs was evaluated after optimization of a resazurin-based colorimetric indicator, which estimates cell proliferation and viability. Thus, the number of parasites to be tested and the optimal incubation time was initially standardized. The correlation between dye reduction and parasites number in all evaluated times is shown in Fig. 8. Resazurin dye reduction was evaluated at 4 different readings until 100% reduction in the wells containing the highest parasite concentration is reached. The minimum limit of detection in the analyzed time range was 0.01×10^6 trypomastigotes/mL (initial concentration). From this method, 1×10^6 parasites/200 µL and the reading time of the 7h after resazurin addition were defined for our assays (Fig. 8A).

The concentration of 2×10³ *H9c2* cells/well and the reading time of the 7h were also used to evaluate cardiomyocytes cytotoxicity. To validate the cytotoxicity assay, cells treated with MiliQ water or toxic DMSO concentrations were evaluated in parallel. The resazurin test was able to identify that the concentration of DMSO and MiliQ-water used to solubilize the drugs was not toxic to the cells, since no significant difference in the percentage of dye reduction by cardiomyocytes was identified (Fig. 8B).

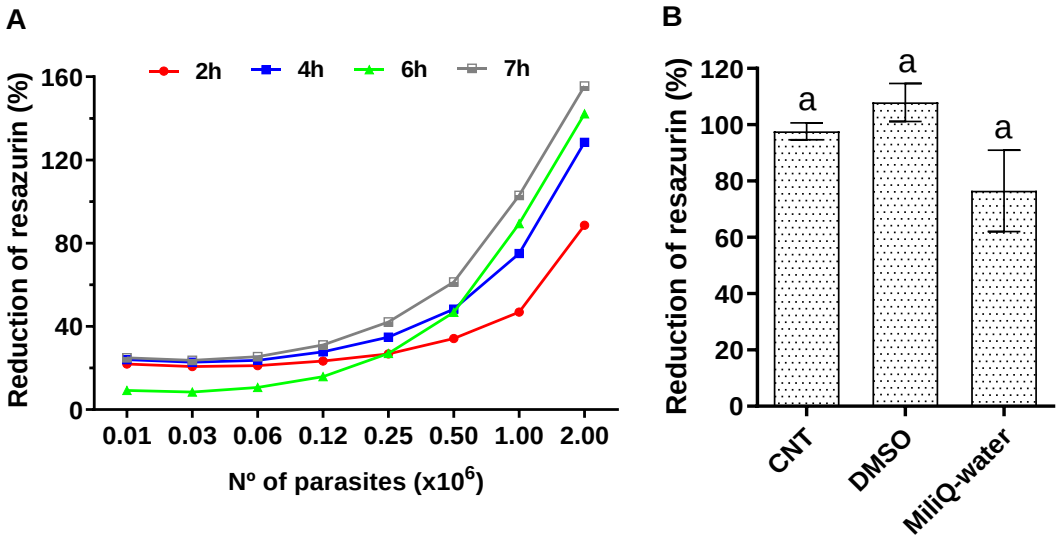

Fig. 8. Relationship between the number of trypomastigotes and reduction of resazurin dye (A) and reduction of the resazurin by H9c2 cardiomyocytes treated with dimethyl sulfoxide (DMSO) or MiliQ water (B). A: *Trypanosoma cruzi* Y strain were serially diluted from 2×10⁶ parasite/well (200µL) and incubated for 24h at 37 ºC. Resazurin reduction was read every 2 hours after dye addition until 100% reduction is obtained. B: H9c2 cells (2×10³) were incubated for 48h with dimethyl sulfoxide (DMSO) and MiliQ water at 1%. In A, data are expressed as mean values. In B, data are expressed as mean and standard deviation, and groups with equal letters are statistically similar (P>0.05).

Cytotoxicity RAS modulating drugs on H9c2 cardiomyocytes

When angiotensins II was administrated at doses between 10 and 0.07 µM no interference in cell viability was detected. Only the highest concentration of A-779 (10µM) reduced cardiomyocytes viability at 12.05%. At 10, 5 and 0.07 µM, DMZ reduced cell viability by 37.4%, 21.36% and 22.45%, respectively (Fig.9).

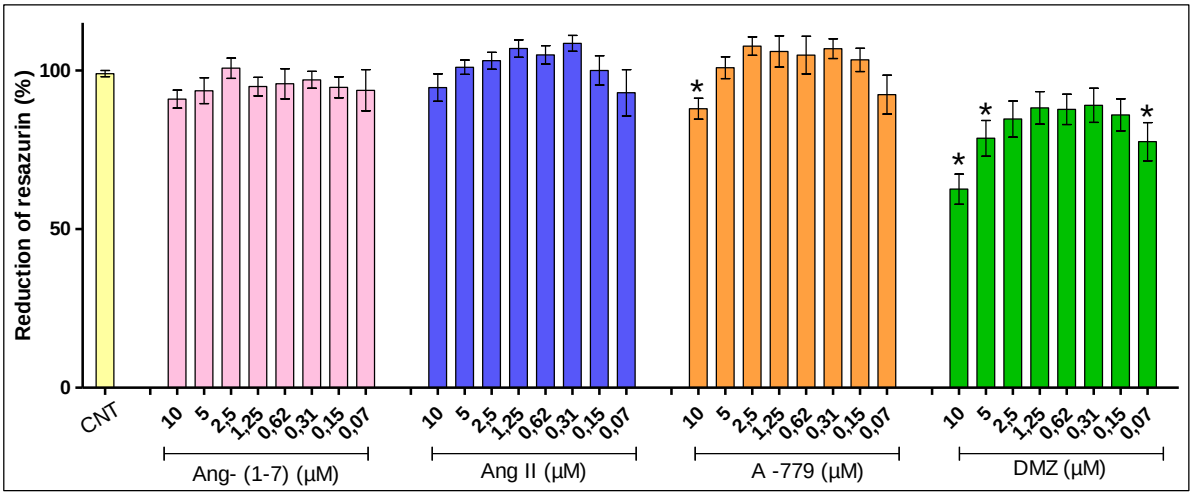

Fig. 9. Effect of angiotensin 1-7 (Ang-(1-7)), angiotensin II (Ang II), A-779 and diminazene aceturate (DMZ) on H9c2 cardiomyocytes viability. Resazurin assay initiated after 48h of cell incubation with each drug in different concentrations. The reaction was read 7h after add resazurin in the culture wells. Data are expressed as mean and standard deviation. *Statistical difference (P≤0.05) compared to the control group (CNT).

At 7.81 to 500 µM, ramipril, losartan and Bz exhibited limited effect on H9c2 cardiomyocytes viability. At 1000 µM losartan reduced cell viability by 31.29%. At 1000 and 7.81 µM Bz, the reference drug for Chagas disease, reduced 17.67% and 8.47% of the cell viability (Fig. 10).

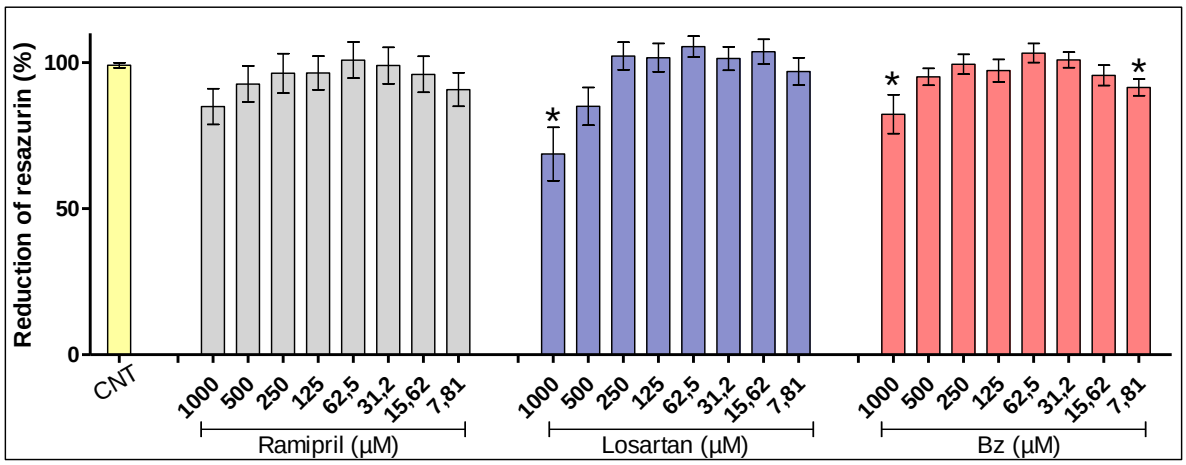

Fig. 10. Effect of ramipril, losartan and benznidazole (Bz) on H9c2 cardiomyocytes viability. Resazurin assay initiated after 48h of cell incubation with each drug in different concentrations. The reaction was read 7h after add resazurin in the culture wells. Data are expressed as mean and standard deviation. *Statistical difference (P≤0.05) compared to the control group (CNT).

Cytotoxicity of RAS modulating drugs on T. cruzi trypomastigotes

When administered at 90 to 1.25µM, only the 45µM concentration of Ang-(1-7) reduced 10.6% *T. cruzi* viability. All doses of Ang-II (90 at 1.25µM) were unable to interfere with trypomastigotes viability. At 90, 45 and 22.5 µM, A-779 reduced 16.35, 10.47 and 11.23% of parasite viability, respectively. All DMZ doses (90 to 1.25µM) significantly reduced *T. cruzi* viability. At 4500 and 9000µM, both losartan and ramipril were effective in reducing trypomastigotes viability by 19.44% and 54.27, respectively. At 1000 and 500 µM, ramipril of increased 18.03 and 14.14% parasite viability, respectively. At 4500 9000, and 2250µM losartan reduced *T. cruzi* viability by 31.32%, 50.66 and 59.28%, respectively. However, at 1000, 500 and 250µM parasite viability was increased to 23.72, 17.75 and 15.12%, respectively (Fig. 11).

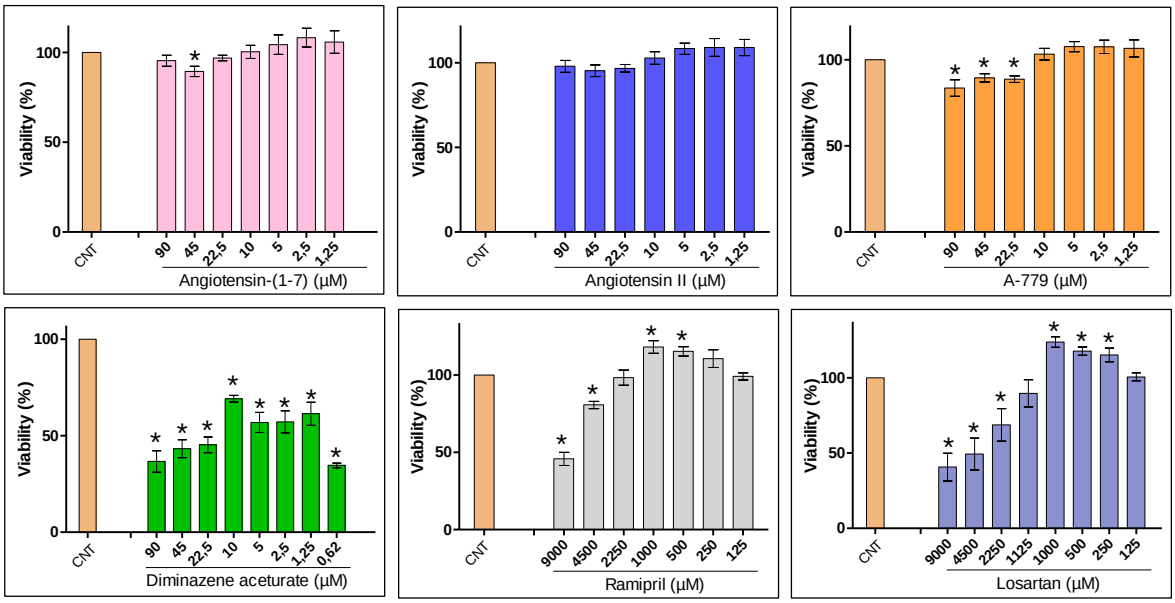

Fig. 11. Effect of RAS modulating drugs on *Trypanosoma cruzi* viability. Trypomastigotes ($1x10^6$) were incubated in the presence or absence of decreasing concentrations of each drug tested for 24 hours. Trypanocidal activity was measured by the resazurin reduction test. The reaction was read 7h after add resazurin in the culture wells. Data are expressed as mean and standard deviation. *Statistical difference ($P \leq 0.05$) compared to the control group (CNT).

Anti-infective activity of RAS modulating drugs on H9c2 cardiomyocytes

The treatment with angiotensin 1-7 and II at 1.25 to 10 µM did not change the infection rate in cardiomyocytes challenge with *T. cruzi* trypomastigotes for 24h. At 1 µM, A-779 reduced the number of infected cells by 16%. Conversely, at 200, 100 and 25 µM; losartan increased infection rate by 23.67, 12.8 and 15.2%, respectively. DMZ was ineffective in modulates cellular infection rate, although a direct toxic effect against trypomastigotes has been detected. At 20, 10, 5 and 2.5µM, ramipril significantly increased the percentage of infection by 26, 22, 28.33 and 20.5%, respectively. As expected for the reference drug, Bz induce a marked reduction of cardiomyocytes parasitism (22.91%) (Fig.12).

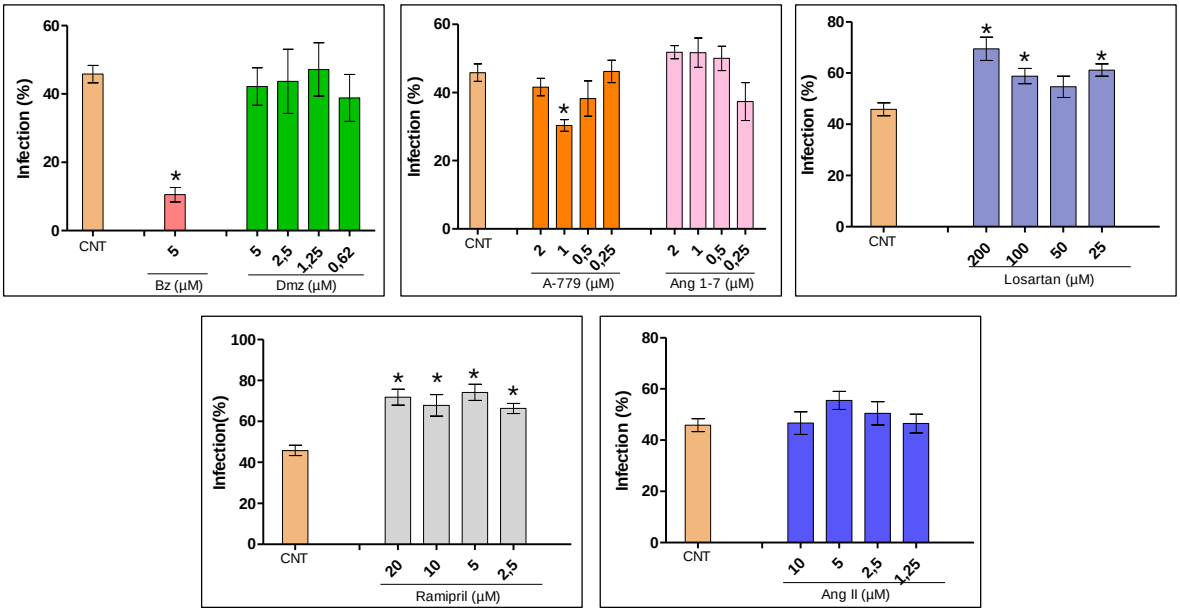

Fig. 12. Effect of RAS modulating drugs and benznidazole on H9c2 cardiomyocytes infection after a 24h-challeng with *Trypanosoma cruzi* trypomastigotes. H9c2 cells ($1x10^4$) were challenge with trypomastigotes ($2×10^5$) after 12 hours of incubation with different concentrations of angiotensin II (Ang II), angiotensin-(1-7) (Ang-(1-7)), losartan, A-779, diminazene aceturate (DMZ), benznidazole (Bz), and ramipril. The time of host-parasite interaction was 24h, and the treatment was maintained in this period. Data are expressed as mean and standard deviation. *Statistical difference ($P \leq 0.05$) compared to the control group (CNT).

When trypomastigotes and H9c2 cells were treated with RAS modulating drugs and co-cultured for 48 hours, reduced cardiomyocytes infection rate was detected. At 1µM, A-779 decreased the number of infection cells by 17.76%. Conversely, 200µM losartan increased 15.5% infection rate. Angiotensin 1-7 and II, DMZ and ramipril had no effect on the number of infected cardiomyocytes (Fig. 13).

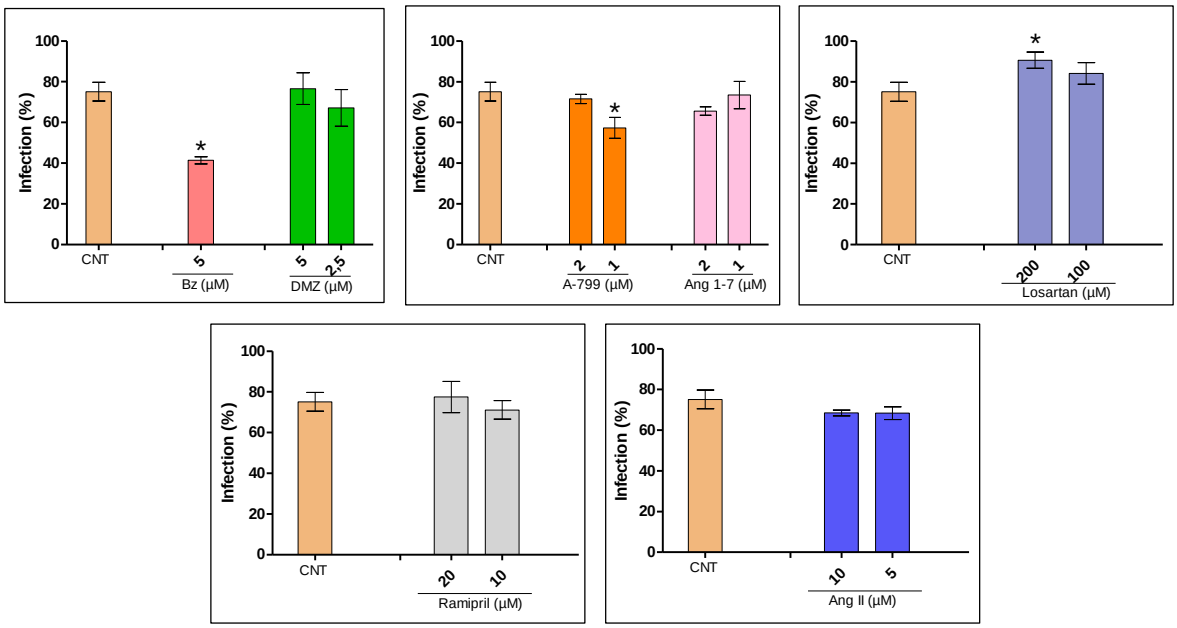

Fig. 13. Effect of RAS modulating drugs and benznidazole on H9c2 cardiomyocytes infection after a 48h-challenge with *Trypanosoma cruzi* trypomastigotes. H9c2 cells ($1x10^4$) were challenge with trypomastigotes (20×10^4) after 12 hours of incubation with different concentrations of angiotensin II (Ang II), angiotensin-(1-7) (Ang-(1-7)), losartan, A-779, diminazene aceturate (DMZ), benznidazole (Bz), and ramipril. The time of host-parasite interaction was 48h, and the treatment was maintained in this period. Data are expressed as mean and standard deviation. *Statistical difference ($P\leq0.05$) compared to the control group (CNT).

The drugs activity was also evaluated by calculating the endocytic index (EI), which takes into account the percentage of infected cells and the amastigote load in each cell. In the 24h-infected assays, control cells (infected and untreated) showed intense parasitism (67.58±2.57 amastigote/cell). At 1µM Ang-(1-7) increased by 10.12% of the EI, and at 0.5 and 0.25 µM decreased this index by 6.55% and 9.5%, respectively. At 1, 0.5 and 0.25 µM, A-779 reduced EI by 9.04, 12.93 and 13.39%, respectively. At 2.5 µM, ramipril increased in 7% the amastigote load in cardiomyocytes. At 10 µM, Ang II reduced EI by 11.22%, whereas 200, 100 and 25 µM

losartan increased Ei by 9.65, 12.3 and 7.78%, respectively. DMZ exerted no impact on amastigote load, while maximal EI inhibition (25.34%) was obtained with Bz (Fig. 14).

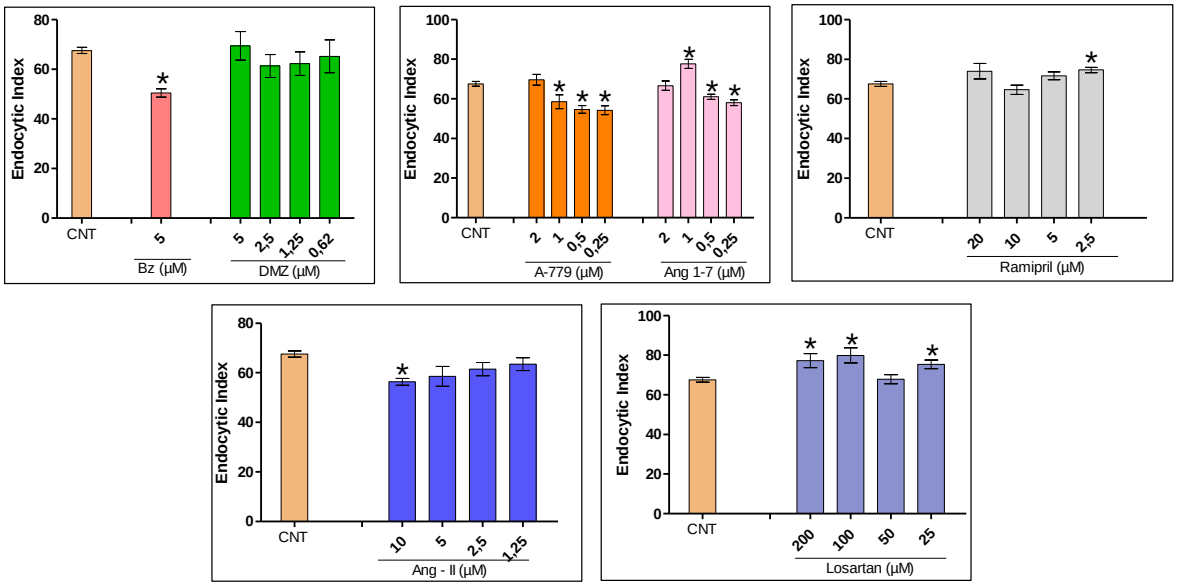

Fig. 14. Effect of RAS modulating drugs and benznidazole on endocitic index in *Trypanosoma cruzi*-infected H9c2 cardiomyocytes after a 24h-challenge with *Trypanosoma cruzi* trypomastigotes. H9c2 cells ($1x10^4$) were challenge with trypomastigotes (20×10^4) after 12 hours of incubation with different concentrations of angiotensin II (Ang II), angiotensin-(1-7) (Ang-(1-7)), losartan, A-779, diminazene aceturate (DMZ), benznidazole (Bz), and ramipril. The time of host-parasite interaction was 24h, and the treatment was maintained in this period. Data are expressed as mean and standard deviation. *Statistical difference ($P\leq0.05$) compared to the control group (CNT).

After 48h of *T. cruzi* infection, untreated control cells presented intense parasitism (406.9±115.73 amastigote/cell). The treatment with Ang II (10 and 5 µM), Ang-(1-7) (2 and 1µM), losartan (200 and 100µM) and ramipril (20 and 10µM) exhibited no impact on amastigote load in cardiomyocytes. However, at 5 and 2.5 µM, DMZ decreased EI by 165 and 171.63%, respectively. Similarly, A-779 at 2 and 1 µM reduced this index by 189.92 and 266.08%, respectively (Fig. 15).

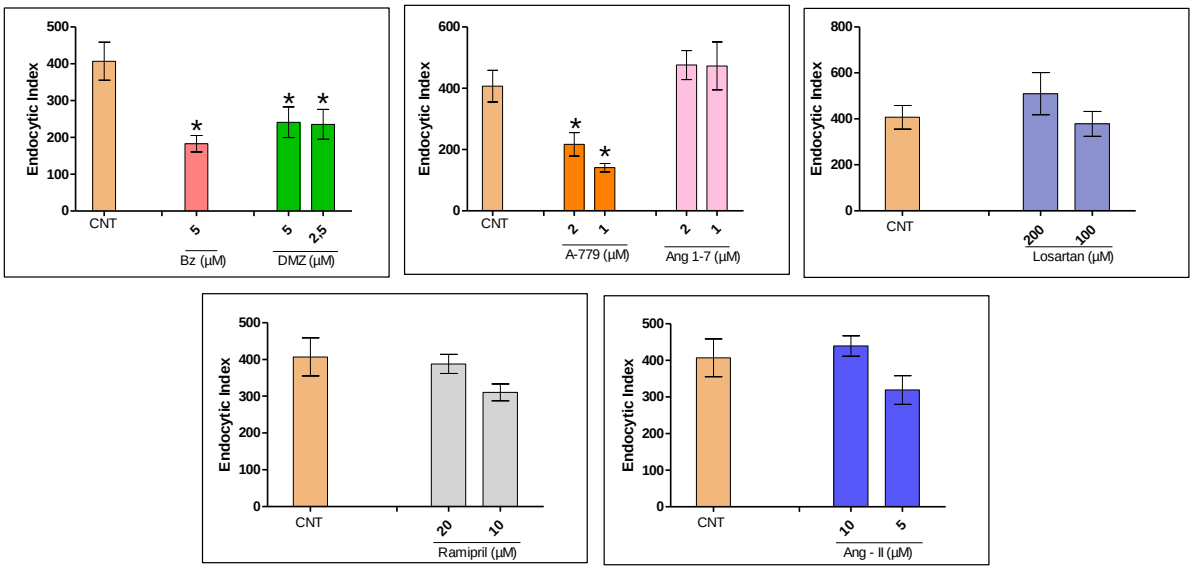

Fig. 15. Effect of RAS modulating drugs and benznidazole on endocitic index in *Trypanosoma cruzi*-infected H9c2 cardiomyocytes after a 48h-challenge with *Trypanosoma cruzi* trypomastigotes. H9c2 cells ($1x10^4$) were challenge with trypomastigotes (22×10^4) after 12 hours of incubation with different concentrations of angiotensin II (Ang II), angiotensin-(1-7) (Ang-(1-7)), losartan, A-779, diminazene aceturate (DMZ), benznidazole (Bz), and ramipril. The time of host-parasite interaction was 24h, and the treatment was maintained in this period. Data are expressed as mean and standard deviation. *Statistical difference ($P\leq0.05$) compared to the control group (CNT).

In vivo results

Parasitemia and mortality

All Infected groups exhibited similar prepatent period (4 days) and synchronized peak or parasitemia. Parasitemia clearance started at day 9, with a progressive parasitemia increase from day 12 (Fig. 16). All infected groups developed similar initial and peak of parasitemia ($P> 0.05$). However, mean parasitemia was higher in animals treated with DMZ compared to infected untreated animals ($P<0.05$). Final parasitemia was similarly higher in both ramipril- and diminazene aceturate-treated mice compared to infected untreated animals ($P<0.05$) (Table 7).

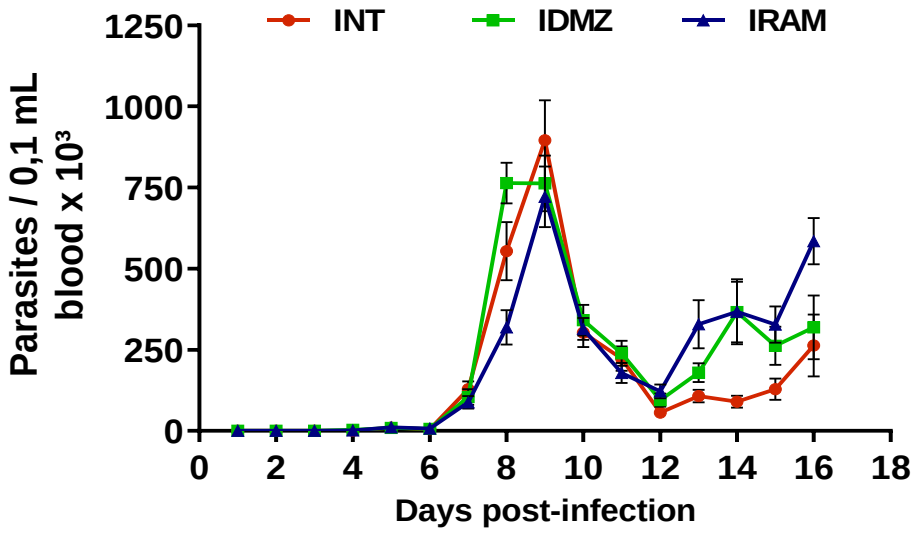

Fig. 16. Curve of the parasitemia in *Trypanosoma cruzi*-BALB/c infected mice untreated and treated with an inhibitor (ramipril - RAM) and activator (diminazene aceturate - DMZ) of angiotensin-converting enzyme. INT, infected untreated (n=13); IDMZ, infected treated with 1mg/kg DMZ (n=13); IRAM, infected treated with 5mg/kg RAM (n=13). Data are expressed as mean and standard deviation.

Table 7. Parasitemia in infected mice with *Trypanosoma cruzi* and treated with RAS modulators.

Groups	Initial Parasitemia (Try/0.1mL blood × 10^3)	Peak parasitemia (Try/0.1mL blood × 10^3)	Mean parasitemia (Try/0.1mL blood × 10^3)	Final parasitemia (Try/0.1mL blood × 10^3)	Mortality (Nº of death animals /total, %)
CNT	ND	ND	ND	ND	0/8 (0%)
NDMZ	ND	ND	ND	ND	0/8 (0%)
NRAM	ND	ND	ND	ND	0/8 (0%)
INT	213.0±309.0	895.0±427.6	213.5±309.00	132.0±118.5	8/13 (61.5%)
IDMZ	263.9±305.5	762.69±298.5	263.9±305.5*	236.7±214.8*	5/13 (38.5%)
IRAM	243.7±272.5	721.53±323.2	243.7±272.5	291.7±241.6*	6/13 (46.2%)

ND: not detected, Try: Trypomastigotes; CNT: uninfected untreated control; NDMZ, uninfected treated with 1mg/kg diminazene aceturate (DMZ); NRAM, uninfected treated with 5 mg/kg ramipril (RAM); INT, infected untreated; IDMZ, infected treated with 1mg/kg DMZ; IRAM, infected treated with 5 mg/kg RAM. Data are expressed as mean and standard deviation. *Statistical difference (P≤0.05) compared to the group INT.

Uninfected untreated animals and those receiving DMZ or ramipril exhibited no mortality (0%). Infected untreated animals exhibited marked mortality (61.5%) compared to the groups treated with ramipril (46.2%) and DMZ (38.5%) (Table 7).

Weight variation and relative organ weight

Uninfected animals treated with ramipril and DMZ presented lower weight gain compared to uninfected untreated mice ($P<0.05$). In infected groups, animals untreated and those treated with ramipril exhibited weight loss compared to the other groups ($P<0.05$). Weight loss was not detected in DMZ-treated animals (Fig. 17).

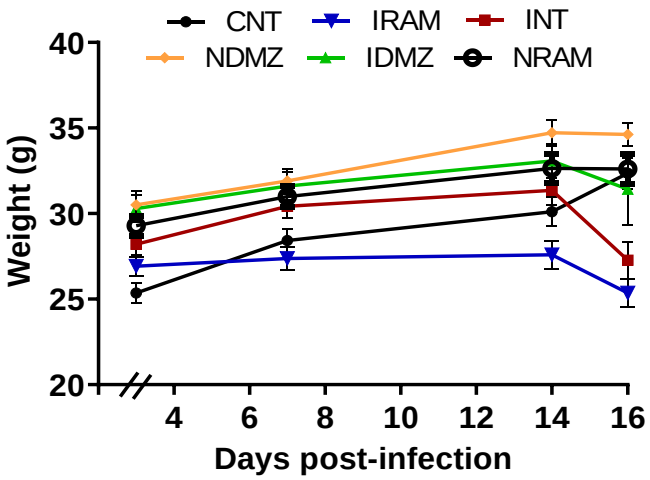

Fig. 17. Body weight curve in *Trypanosoma cruzi*-BALB/c infected mice untreated and treated with an inhibitor (ramipril - RAM) and activator (diminazene aceturate - DMZ) of angiotensin-converting enzyme. CNT, uninfected untreated control (n=8); NDMZ, uninfected treated with 1mg/kg diminazene aceturate (DMZ (n=8)); NRAM, uninfected treated with 5 mg/kg ramipril (RAM (n=8); INT, infected untreated (n=13); IDMZ, infected treated with 1mg/kg DMZ (DMZ (n=13)); IRAM, infected treated with 5 mg/kg RAM (RAM (n=13)).

The organ weight of all animals was collected after the euthanasia. There was no statistical difference in relative weight of liver, spleen and heart between the NDMZ and NRam groups compared to the CNT group. However, the untreated infected animals had a higher relative weight of the liver, spleen and heart compared to the CNT group, indicating that the infection was able of to increase these evaluated parameters ($p <0.05$). Interestingly, the IDMZ

and IRAM animals have a lower relative heart weight compared to INT group (P<0.05), and this effect may be associated with treatments (Fig. 18).

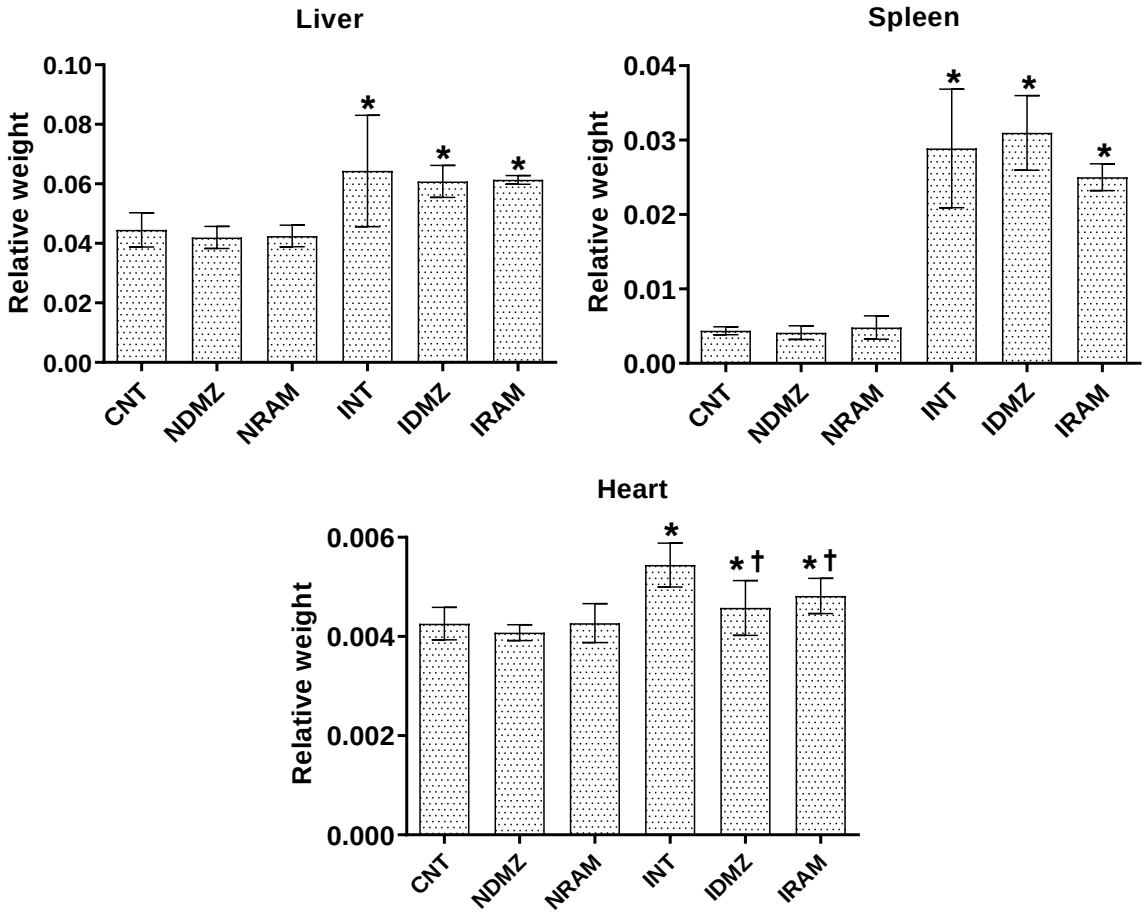

Fig. 18. Relative liver, spleen and heart weight in *Trypanosoma cruzi*-BALB/c infected mice untreated and treated with an inhibitor (ramipril - RAM) and activator (diminazene aceturate - DMZ) of angiotensin-converting enzyme. CNT, uninfected untreated control (n=8); NDMZ, uninfected treated with 1mg/kg diminazene aceturate (DMZ (n=8)); NRAM, uninfected treated with 5 mg/kg ramipril (RAM (n=8)); INT, infected untreated (n=13); IDMZ, infected treated with 1mg/kg DMZ (n=13); IRAM, infected treated with 5 mg/kg RAM (n=13). Data are expressed as mean and standard deviation. * † ‡ Statistical difference (P≤0.05) compared to the groups *CNT, NDMZ and NRAM, †INT.

Enzymatic activity and angiotensin circulating levels

ACE activity and Ang II plasma levels were reduced in both uninfected and infected ramipril-treated animals compared to the other groups (P<0.05). Similarly, DMZ treated animals exhibited higher ACE2 activity and Ang-(1-7) circulating levels compared to the other

groups (P<0.05). Alone, *T. cruzi* infection had no impact on enzymatic activity and angiotensin levels (P>0.05) (Fig. 19).

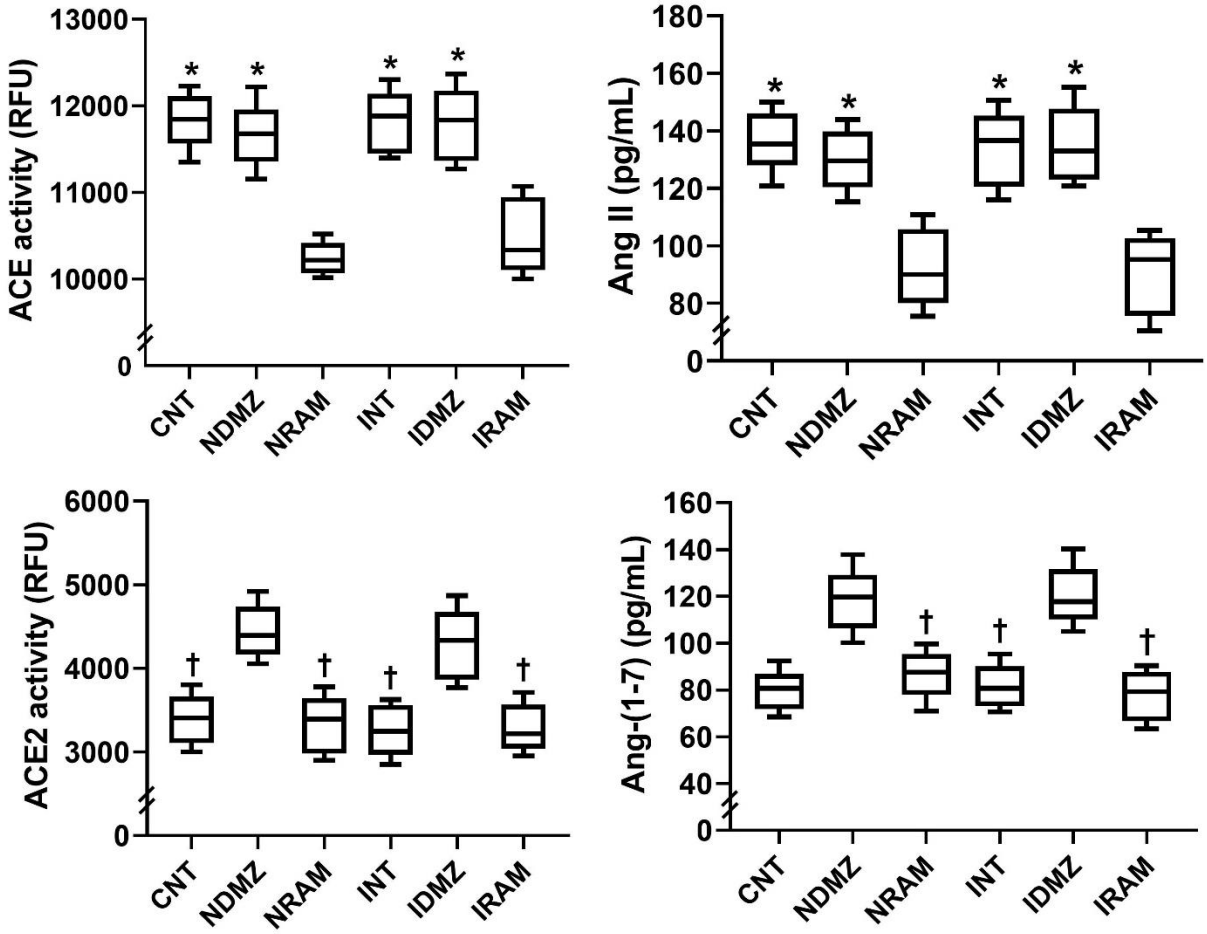

Fig. 19. Plasma levels of renin-angiotensin system components in *Trypanosoma cruzi*-BALB/c infected mice untreated and treated with an inhibitor (ramipril - RAM) and activator (diminazene aceturate - DMZ) of angiotensin-converting enzyme (ACE). ACE2, angiotensin-converting enzyme type 2; Ang II, angiotensin II; Ang-(1-7), angiotensin-(1-7). CNT, uninfected untreated control (n=8); NDMZ, uninfected treated with 1mg/kg diminazene aceturate (DMZ (n=8)); NRAM, uninfected treated with 5 mg/kg ramipril (RAM (n=8)); INT, infected untreated (n=13); IDMZ, infected treated with 1mg/kg DMZ (n=13); IRAM, infected treated with 5 mg/kg RAM (n=13). Data are expressed as mean and standard deviation. * † ‡ § Statistical difference (P≤0.05) compared to the groups *NRAM and IRAM, †NDMZ and IDMZ.

Heart and skeletal muscle microstructural remodeling

All uninfected groups exhibited preserved myocardial microstructure with well-organized and well-defined cardiomyocytes, scarce connective tissue and interstitial cellularity.

Conversely, all infected animals exhibited marked myocardial damage, evidenced diffuse inflammatory infiltrate, stromal expansion, presence of intracellular amastigote nests, and cardiomyocytolysis in necrotic areas (Fig. 20).

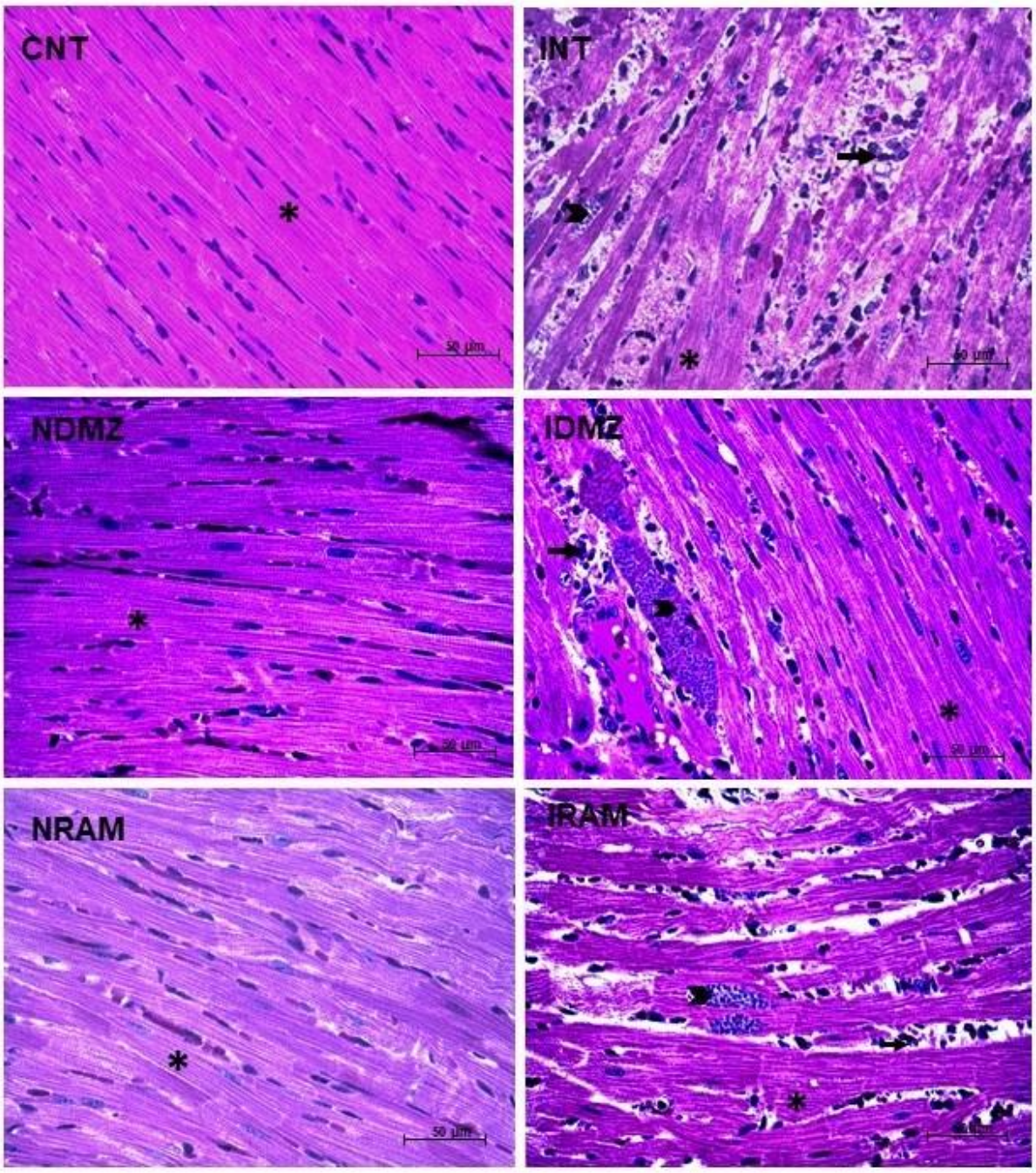

Fig. 20. Representative photomicrography of the cardiac tissue from *Trypanosoma cruzi*-BALB/c infected mice untreated and treated with an inhibitor (ramipril - RAM) and activator (diminazene aceturate - DMZ) of angiotensin-converting enzyme. CNT, uninfected untreated control; NDMZ, uninfected treated with 1mg/kg diminazene aceturate (DMZ); NRAM, uninfected treated with 5 mg/kg ramipril (RAM); INT, infected untreated; IDMZ, infected treated with 1mg/kg DMZ; IRAM, infected treated with 5 mg/kg RAM. Asterisk:

Cardiomyocytes. Arrow: Connective stroma with inflammatory infiltrate. Arrowhead: *T. cruzi* amastigote nest.

Stereological analysis indicated increased stroma distribution, reduced parenchyma proportion and interstitial cellularity in uninfected animals treated with ramipril and DMZ compared to untreated animals (P≤ 0.05). Infected animals presented marked stroma expansion and interstitial cellularity compared to uninfected mice (P≤ 0.05). Among infected animals, connective tissue expansion and compensatory parenchymal loss was similarly identified in mice receiving ramipril and DMZ (Fig. 21).

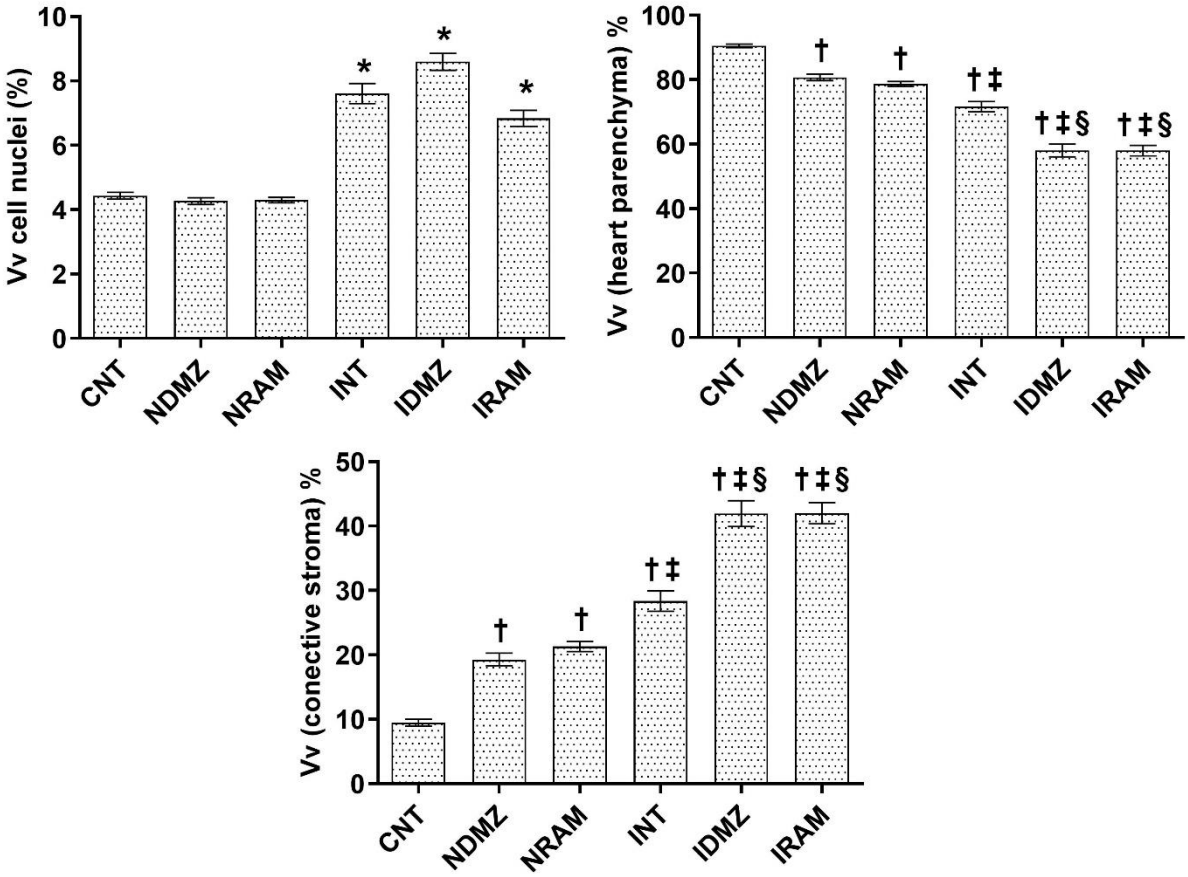

Fig. 21. Heart microstructural remodeling in *Trypanosoma cruzi*-BALB/c infected mice untreated and treated with an inhibitor (ramipril - RAM) and activator (diminazene aceturate - DMZ) of angiotensin converting enzyme. CNT, uninfected untreated control (n=8); NDMZ, uninfected treated with 1mg/kg diminazene aceturate (DMZ (n=8)); NRAM, uninfected treated with 5 mg/kg ramipril (RAM (n=8)); INT, infected untreated (n=13); IDMZ, infected treated with 1mg/kg DMZ (n=13); IRAM, infected treated with 5 mg/kg RAM (n=13). Data are expressed as mean and standard deviation. * † ‡ Statistical difference (P≤0.05) compared to the groups *CNT, NDMZ and NRAM, †CNT, ‡NDMZ and NRAM, and §INT.

The skeletal muscle of all untreated uninfected mice exhibited preserved myocites, scare connective tissue and interstitial celularity. All infected groups presented conective tissue expansion, intense inclammatory infiltrate, intracellular amastigote nests, and focal areas of myonecrosis (Fig. 22).

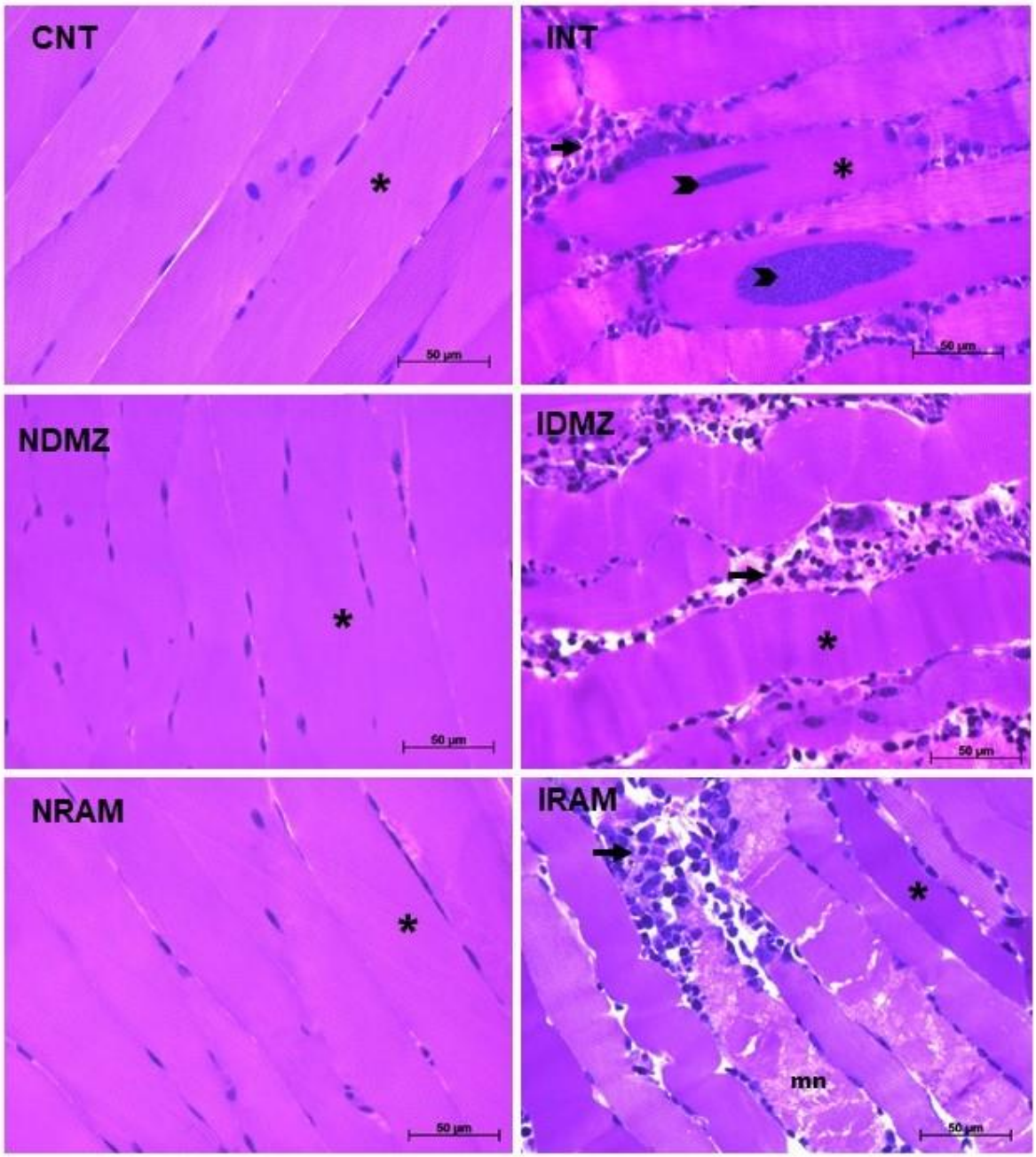

Fig. 22. Representative photomicrography of the skeletal muscle from *Trypanosoma cruzi*-BALB/c infected mice untreated and treated with an inhibitor (ramipril - RAM) and activator (diminazene aceturate - DMZ) of angiotensin-converting enzyme. CNT, uninfected untreated control; NDMZ, uninfected treated with 1mg/kg diminazene aceturate (DMZ); NRAM,

uninfected treated with 5 mg/kg ramipril (RAM); INT, infected untreated; IDMZ, infected treated with 1mg/kg DMZ; IRAM, infected treated with 5 mg/kg RAM. Asterisk: Skeletal myocyte. Arrow: Connective stroma with inflammatory infiltrate. Arrowhead: *T. cruzi* amastigote nest. mn: Myonecrosis**.**

Stereological analysis of uninfected animals indicated incresed cellularity in ramipril-treated animals ($P \leq 0.05$). Interstitial cellylariry and conective tissue expansion was higher ($P \leq 0.05$) and parenchyma distribution was reduced ($P \leq 0.05$) in all infected animals compared to uninfected mice. Among infected animals, both DMZ- and ramipril-treated mice exhibited reduced interstitial cellularity compared do untreated animals ($P \leq 0.05$). Conective tissue expansion was lower in infected mice receiving ramipril compared to infected untreated and DMZ-treated mice ($P \leq 0.05$) (Fig. 23).

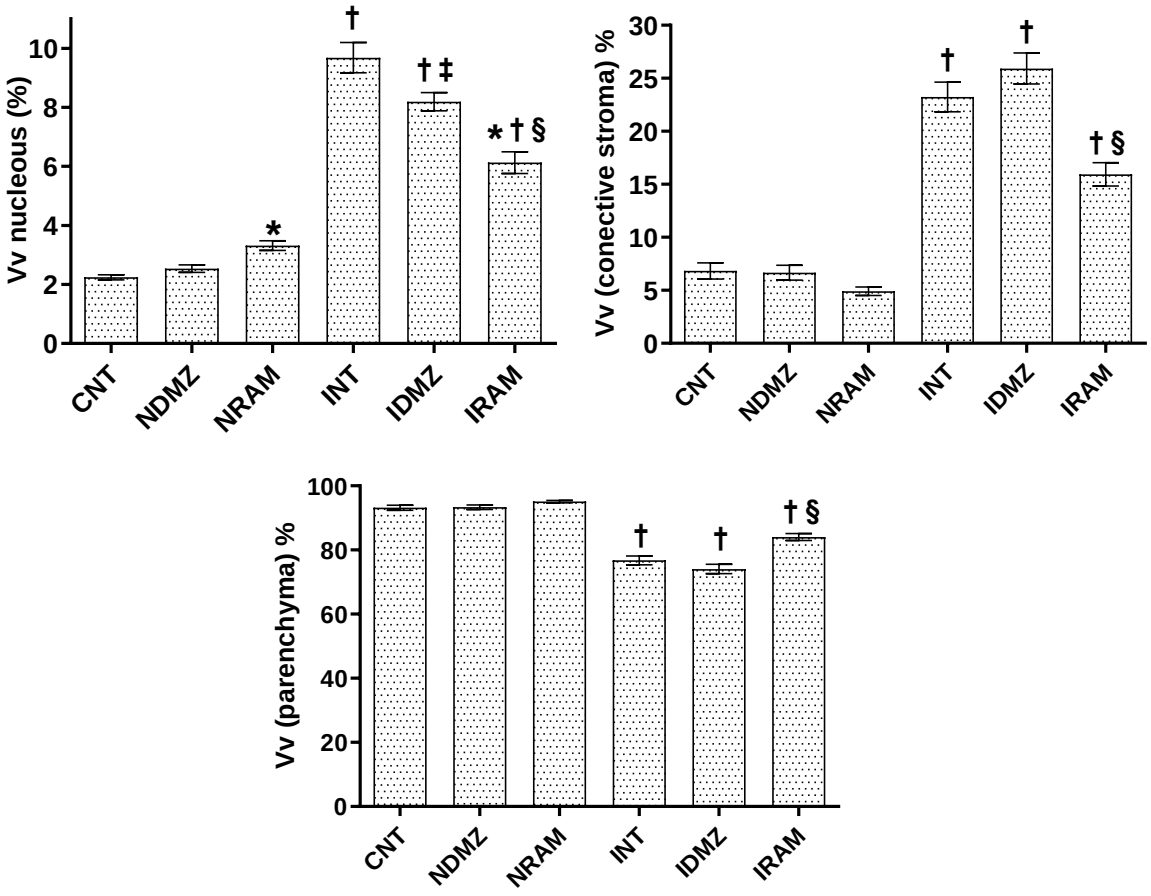

Fig. 23. Skeletal muscle remodeling in *Trypanosoma cruzi*-BALB/c infected mice untreated and treated with an inhibitor (ramipril - RAM) and activator (diminazene aceturate - DMZ) of angiotensin-converting enzyme. CNT, uninfected untreated control (n=8); NDMZ, uninfected treated with 1mg/kg diminazene aceturate (DMZ (n=8)); NRAM, uninfected treated with 5 mg/kg ramipril (RAM (n=8)); INT, infected untreated (n=13); IDMZ, infected treated with 1mg/kg DMZ (n=13); IRAM, infected treated with 5 mg/kg RAM (n=13). Data are expressed as mean and standard deviation. * † ‡ § Statistical difference (P≤0.05) compared to the groups *CNT and NDMZ, †CNT, NDMZ and NRAM, ‡INT, and §INT and IDMZ.

Anti-Trypanosoma cruzi immunoglobulin G plasma levels

In general, all infected groups presented marked anti-*T. cruzi* IgG reactivity compared to uninfected animals (P≤0.05). Among infected animals, animals treated with ramipril developed lower reactivity of IgG total, IgG1 and IgG2b (P≤0.05) compared to infected untreated animals or those treated with DMZ, which exhibited similar results (P>0.05) (Fig. 24).

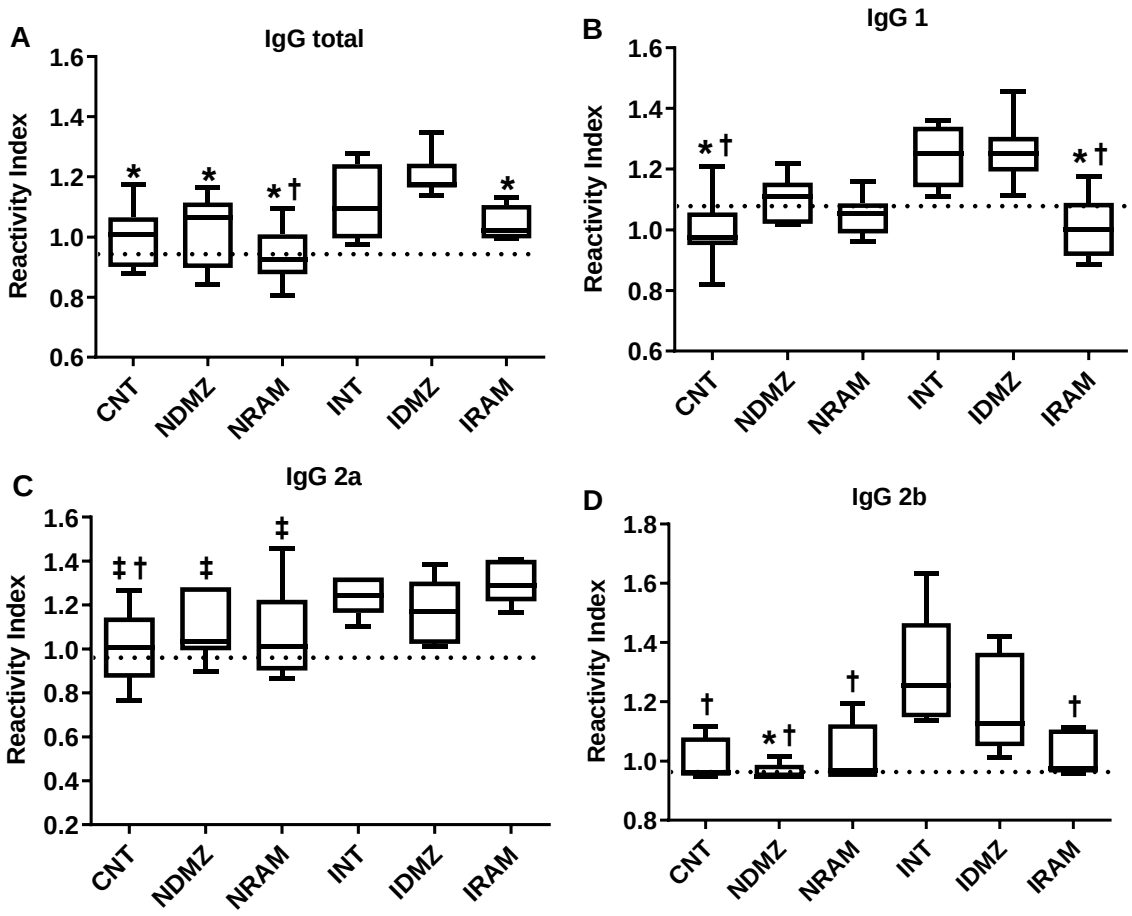

Fig. 24. Anti-*Trypanosoma cruzi* immunoglobulin G plasma levels in *Trypanosoma cruzi*-BALB/c infected mice untreated and treated with an inhibitor (ramipril - RAM) and activator (diminazene aceturate - DMZ) of angiotensin converting enzyme. CNT, uninfected untreated control (n=8); NDMZ, uninfected treated with 1mg/kg diminazene aceturate (DMZ (n=8)); NRAM, uninfected treated with 5 mg/kg ramipril (RAM (n=8)); INT, infected untreated (n=13); IDMZ, infected treated with 1mg/kg DMZ (n=13); IRAM, infected treated with 5 mg/kg RAM (n=13). Data are expressed as median and interquartile interval. * † ‡ Statistical difference (P≤0.05) compared to the groups *IDMZ, †INT and ‡IRAM.

DISCUSSION

In this study, we demonstrated the impact of RMD on the viability of *T. cruzi* infective forms and *T. cruzi* infection *in vitro* and *in vivo*. Our findings indicated a direct parasitic potential for RMD that has been systematically overlooked. Although Ang II and 1-7 showed no antiparasitic effect, drugs such as losartan, ramipril, A-779 and DMZ induced toxicity

against trypomastigotes and amastigotes, modifying the time-dependent infection rates and parasitic load in host cardiomyocytes *in vitro*. When administered *in vivo*, the ACE inhibitor ramipril and activator DMZ were effective in modulating ACE and ACE2 activity, as well as angiotensin II and 1-7 plasma levels. Although these effects were not associated with an improved parasitic and myocarditis control; a limited impact on skeletal muscle inflammation and reduced hepatomegaly, cardiomegaly and mortality was achieved in *T. cruzi*-infected mice receiving these drugs.

From our *in vitro* assays, both angiotensin II and 1-7 had no influence on cardiomyocytes viability. Current evidence is controversial on the effect of RAS effectors on metabolism, proliferation and viability of different cell lineages. By binding AT1 receptors, Ang II was linked to cardiomyocytes death through DNA damage mediated by calcium-dependent endonuclease activation (Cigola et al., 1997; Kajstura et al., 1997). In addition, inhibition of muscle cell proliferation was attributed to Ang II-AT2 receptors interaction (Nakajima et al., 1995; Mogi et al., 2012). In heart disease, Ang II-mediated toxicity is potentially critical, especially considering that high levels of Ang II is closed correlated with heart failure and a worse cardiovascular prognosis (The Consensus Trial Study Group, 1987; Rigatto et al., 2004). Conversely, Ang II is also effective in stimulating proliferation of cardiac fibroblasts and hepatic carcinoma cells (Somanna et al., 2015; Qi et al., 2018). As a Mas receptor agonist, Ang-(1-7) is also a major RAS effector; whose anti-angiogenic, anti-proliferative and cardioprotective effects are reported. Although it have been suggested that these biological properties are linked to Ang-(1-7)-induced immune-inflammatory modulation (Gallagher; Tallant, 2004; Menon et al., 2007; Yang et al., 2018; Chen et al., 2019), the mechanistic basis remains poorly understood and requires further investigation.

Unlike angiotensin, both Mas (A-779) and AT1 (losartan) receptors antagonists reduced H9c2 cardiomyocytes viability at high concentrations. Aligned with these findings, Tambelline et al. (2012) identify that high doses of losartan reduced A7r5 cells proliferation, an effect independent of AT1 receptor since preserved intracellular Ang II was potentially able in activating AT1-induced metabolic pathway (Kumar et al., 2007; Yang et al., 2018). Thus, direct A-779-induced cell toxicity exhibits a marked dose-dependent profile, which needed to be exploited to establish an optimized non-toxic concentration for host cell but with potential toxicity on *T. cruzi*. Although ramipril have been well tolerated by host cells, DMZ reduced cardiomyocytes viability in a dose dependent-way. A similar effect was reported by Tao et al. (2016), which identified higher death rates in DMZ-treated ARPE-19 cells challenge with lipopolysaccharide.

To date, there are no evidence on the expression of angiotensin receptors by different evolutionary forms of *T. cruzi*. Although our findings indicated that angiotensin II and 1-7 did not exhibit toxic effect against host cells or the infective parasite; DMZ, ramipril, A-779 and losartan induced direct dose-dependently toxicity on *T. cruzi* infective forms. As a drug originally used to treat animal (babesiosis) and human (sleeping sickness) trypanosomiasis (Kuriakose and Uzonna, 2014), toxicity against *T. cruzi* was expected. There is evidence that by interacting with adenine-thymine base pairs, DMZ inhibiting protozoan DNA topoisomerase and DNA replication (Riou et al., 1980; Poot et al., 1990). In addition, anti-proliferative effect of enalapril on *T. cruzi* epimastigotes was showed by de Paula Costa et al. (2010). However, the relevance this finding to Chagas disease remains questionable since epimastigotes are non-infective forms to mammalian cells (Romano et al., 2012). Unlike DMZ, the mechanism triggered by RMD to inducing toxic effects on infectious *T. cruzi* forms remains unclear. Thus, identify antiparasitic effects on drugs not intended for this purpose becomes relevant to foster mechanistic studies aimed at identifying new molecules and targets with potential applicability in antitrypanosomal therapies (Sun et al., 2016).

From non-toxic doses for cardiomyocytes, potential anti-infective effects of RMD were investigated *in vitro*. Interestingly, only Ang-(1-7) at lower concentrations was able in reducing parasitic load in host cells (endocytic index) within 24h of treatment. Ang II, once metabolized to Ang-(1-7), has been shown to decrease erythrocyte invasion by *Plasmodium falciparum* (Saraiva et al., 2011; Queiroz et al., 2014). Apparently, decreased parasitism is not mediated by protein G-coupled transmembrane receptors (AT1 and AT2), but by the effect of Ang-(1-7) in inhibiting protein kinase A (PKA) activation (Harrison et al., 2003; Saraiva et al., 2011). This enzyme is also expressed in *T. cruzi* flagella and plasma membrane, sharing with *P. falciparum* a relevant participation in host cell invasion (Harrison et al., 2003; Saraiva et al., 2011).

Unlike its direct toxicity against trypomastigotes, DMZ was surprisingly ineffective in reducing the number of parasitized cardiomyocytes within 24 and 48h of treatment; while ramipril and losartan increased infection rate only within after 24h of treatment. However, DMZ was able in reducing parasitic load in cardiomyocytes after 48h of treatment. The blockage of cellular pathways associated to host cells invasion is proposed to partially explain how RMD interfere in *T. cruzi* infection. In this sense, Scharfstein et al. (2000) showed that the ACE inhibitor captopril potentiates parasitic invasion of non-phagocytic cells. As indicated by the authors, this effect is related with kinin release and free calcium transient upregulation in host cells, a central molecular pathway used by the parasite to establish the infection (Beierwaltes, 2010; Burleigh and Woolsey, 2002). In addition, blockage of bradykinin degradation is also

indicated as a potential mechanism associated with increased parasitism in losartan- and ramipril-treated cells. Therefore, by interacting with bradykinin receptor type 2 in cardiomyocytes, bradykinin stimulates phospholipase C activation and inositol triphosphate biosynthesis, which also trigger an intense intracellular calcium release in host cells (Cahill et al., 1988; Liebmann, 2001).

To investigated the impact of angiotensin modulation on Chagas disease, ramipril and DMZ were administered in mice infected with a *T. cruzi* strain (Y) with marked tropism by cardiac and skeletal muscles (Melo and Brener, 1978; Novaes et al., 2013; 2017) and partially-resistant to the reference drug Bz (Saraiva et al., 2002; Diniz et al., 2018). In this model, ramipril was effective in downregulating ACE activity and Ang II plasma levels; while ACE2 activity and Ang-(1-7) levels were upregulated by DMZ in both uninfected and infected animals. These findings reinforce previous evidence that these drugs are specific in modulating ACE or ACE2 enzymatic activity given distinct substrate-binding pockets, exerting a significant impact on angiotensin biosynthesis (Zang et al., 2005; Velkoska et al., 2011; Chamsi-Pasha et al., 2014; De Maria et al., 2016). Surprisingly, despite angiotensin changes, animals treated with ramipril and DMZ presented similar peak of parasites but higher mean and final parasitemia than untreated mice. Parasitological control by RMD is controversial. In preclinical studies developed by de Paula Costa et al. (2010) and Leite et al. (2017), the treatment with the ACEIn enalapril was effective in attenuating the peak of parasitemia in *T. cruzi*-infected animals. However, these effect was not observed by Leon et al. (2003) and Penitente et al. (2015) when infected mice were treated with captopril and enalapril. As VL-10 strain was used by Penitente et al. (2015) and Leite et al. (2017), and Colombian and Brazil strains and were respectively used by de Paula Costa et al. (2010) and Leon et al. (2003); compare the evidence available is a difficult task. There is consistent evidence showing divergent profiles of pathogenicity and drug resistance (Campos et al., 2014; Sánchez-Guillén et al., 2006) in these strains, which is closed correlated to strain-specific parasitemic responses and pathological outcomes in *T. cruzi* infected mice (Teixeira et al., 2006; Teixeira et al., 2011). In this sense, VL-10 strain is resistant to Bz and typically used to induce chronic infections since determines prolonged parasitemia and exhibits low pathogenicity (Oliveira-Silva et al., 2015). Colombian strain is also resistant to Bz; however, its high virulence and pathogenicity determine intense cell parasitism, myocarditis and mortality in acutely-infected mice (Andrade et al., 1985; Diniz et al., 2018). On the other hand, Brazil strain is associated with intense parasitemia and pathogenicity, but determines marked parasitism of non-muscle organs (Watkins, 1966) in addition to its prominent tropism by skeletal muscles (Watkins, 1966; Cummings and Tarleton et al., 2003).

Therefore, divergent data were not surprising considering variable inoculum size and especially the genetic, phenotypical, biochemical, and immunogenic heterogeneity of *T. cruzi* strains (Manoel-Caetano and Silva, 2007; Chatelain and Konar, 2015; Santi-rocca et al., 2017) used in studies with RMD.

Corroborating previous evidence (Novaes et al., 2013; Erdmann et al., 2016), our findings showed that *T. cruzi* Y strain had marked tropism by heart and skeletal muscle. Organ hypertrophy was also identified in all infected animals, which is coherent with pronounced acute infections (Figueiredo et al., 1985; Lannes-Vieira et al., 2009). Although ramipril and DMZ did not attenuate myocarditis, ramipril reduced skeletal myositis and tissue conjunctivisation in response to parenchyma damage. Parenchyma and stroma pathological remodeling in heart and skeletal muscle are typical manifestations of *T. cruzi* infections (Rossi et al., 2010; Novaes et al., 2013). Although Bz-based etiological treatment are effective in controlling tissue parasitism and organs damage (Toledo et al., 1997; Mendonça et al., 2019), ACEIn such as enalapril, captopril and losartan exerts limited and controversial effects on these parameters (Souza-Silva et al., 2019). While Leite et al. (2017) observed that enalapril was unable in reducing cardiac inflammation; Leon et al. (2003), de Paula Costa et al. (2010) and Penitente et al. (2015) showed that these drugs reduced myocarditis and organ hypertrophy in *T. cruzi* infected mice. In general, these studies were associated with protective RMD-induced immunomodulatory mechanisms. However, preclinical evidence currently available on the antitrypanosomal and anti-inflammatory effects of RMD is potential influenced by the C57BL/6 mice model (de Paula Costa et al., 2010; Penitente et al., 2015; Leite et al., 2017), which is recognizably resistant to *T. cruzi* due to a strong Th1 immunological response (Gazzinelli et al., 1998; Silva et al., 2013). Conversely, the BALB/c mice used in our assays are highly susceptible to this parasite (Pereira et al., 2005), which is a response to a slower and inefficient cellular and humoral immune response against *T. cruzi* (Silva et al., 2013).

Specific immunological reaction was clearly detected in all *T. cruzi* infected mice from anti-*T. cruzi* IgG detection. Although DMZ was not effective in attenuating IgG plasma levels, ramipril-treated mice exhibited attenuated IgG total, IgG1 and IgG2b reactivity; corroborating a potential systemic anti-inflammatory effect reported to ACEIn (de Paula Costa et al., 2010; Penitente et al., 2015; Leite et al., 2017). IgG antibodies are often detected in acute and chronic infections (Bermejo et al., 2010). These immunoglobulins and recognizably involved in protective responses in Chagas disease (Minoprio et al., 1988), especially IgG1 and IgG2 isotypes, which exert potent lytic activity against *T. cruzi* (Stefani et al., 1983; Takehara et al., 1981). Reduced IgG reactivity is a response typically associated with a better parasitological

control and reduced antigenic stimulation achieved from effective antiparasitic drugs (Caldas et al., 2008; Hyland et al., 2007). However, by directly inhibiting leukocytes activation and immunoglobulin biosynthesis, anti-inflammatory molecules can reduce IgG production (Dinarello, 2010), including RMD (ramipril). There is evidence that T and B cell express angiotensin receptors, and that RAS blockade or Ang II deficiency is detrimental to T and B cell activation (Nataraj et al., 1999; Chan et al., 2015; Tawinwung et al., 2018). The potent effect of Ang II on B cells compartment was clearly showed by Chan et al., (2015), which identified a 25% increase in the proportion of activated splenic B cells, 80% increase in plasma cell number, and a 500% increase in IgG circulating levels in mice treated with Ang II (0.7 mg/kg/day per 28 days). Therefore, a potential role of ramipril in inhibiting B-lymphocytes differentiation and function from a reduced angiotensin biosynthesis cannot be ruled out, an intriguing issue that requires further investigation.

Taken together, our findings indicated that angiotensin II and 1,7 are not toxic to H9C2 cardiomyocytes and infective forms of *T. cruzi*. However, RMD such, losartan and especially DMZ and A-779 exhibits some degree of host cells toxicity and direct anti-*T. cruzi* potential *in vitro* that remains poorly explored. However, the antiparasitic effect of ramipril and DMZ observed *in vitro* did not manifest in a murine model of *T. cruzi* infection, indicating limited relevance of these drugs to the treatment of Chagas disease. Considering that ramipril and DMZ were able in modulating ACE and ACE2 enzyme activity and circulating levels of angiotensins II and 1-7, our findings suggest that these molecules do not act as potent modulators of *T. cruzi* infection, which can establish parasitism and damage to the cardiac and skeletal muscles even in the presence of high concentrations of these angiotensins.

ACKNOWLEDGEMENTS

The authors are grateful to the support provided by Fundação do Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG, processes APQ-01895-16, PPM-00687-17 and PPM-00077-18), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, processes 303972/2017-3, 423594/2018-4, 305093/2017-7 and MCTIC 408503/2018-1), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brazil (CAPES, finance code 001).

CONFLICT OF INTEREST

None to declare.

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