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Dogs as a model for chemotherapy of Chagas disease and Leishmaniasis

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Abstract: ***Background:*** Dogs are natural reservoir of Chagas disease (CD) and Leishmaniasis and have been used for studies of these infections as they develop different clinical forms of these diseases similar to humans.

Objective: This revision describes publications in dog model relative to CD and Leishmaniasis chemotherapy.

Methods: The search of articles was based in PubMed, Scopus and MESH using the keywords: dog, *Trypanosoma cruzi*, treatment (*T. cruzi* chemotherapy analysis) in addition to dog, *Leishmania chagasi*, *Leishmania infantum*, canine visceral leishmaniasis, treatment (*Leishmania* chemotherapy evaluation).

Results: Benznidazole and nifurtimox were used as reference in the treatment of CD and associated with other compounds. Eleven out of the fifteen studies have authors from the same team, using similar protocols and post-treatment evaluations, which assured more reproducibility and credibility. Twenty Leishmaniasis studies, especially in visceral leishmaniasis, presenting at least one parasitological analysis tested in distinct monochemotherapy and polychemotherapy approaches were accessed. Data demonstrated that polychemotherapy was more effective in improving the clinical signs and parasitism control.

Conclusion*:* The benefits of treatment in terms of reducing or eliminating lesions and/or cardiac dysfunctions were demonstrated at acute and/or chronic phases relative to parasite load and/or the *T. cruzi* strain resistance to treatment. BZ presented better therapeutic results than the two EBI compounds evaluated. Although treatment of the canine visceral leishmaniasis was not able to induce complete parasite clearance, it can improve clinical recovery. Thus, the dog is a good model for CD and Leishmaniasis studies of chemotherapy and may be indicated for pre-clinical trials of new treatments.

Keywords: Chagas disease, visceral leishmaniasis, cutaneous leishmaniasis, canine visceral leishmaniasis, chemotherapy, dog model

1. INTRODUCTION

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1.1 Dogs as an experimental model for Chagas disease

Before discussing dogs as an experimental model for Chagas disease chemotherapy, it is important to remember that dogs have been used as experimental models for Chagas disease studies since 1909. Chagas (1909) [1] used them for the first experimental infection studies at Fundação Oswaldo Cruz (FIOCRUZ) in Rio de Janeiro, after the discovery and description of *Trypanosoma (Schyzotrypanum) cruzi* as the etiological agent of the disease, which subsequently received his name.

The most important advantages of dogs when compared to other animal models is the advanced knowledge and similarity of the canine cardiac morphology and physiology of the cardiac conduction system with that of human beings [2]. For this reason, this animal model is ideal for studies of electrocardiographic and echocardiographic changes, an important consideration when the cardiac clinical form of Chagas disease in the chronic phase is evaluated and compared with results observed in humans. Another advantage is the relatively long life span of this animal (11 to 18 years) or still more in experimental conditions, which naturally allows the disease to evolve to its later clinical forms. Moreover, dogs are easy to breed and handle because they are generally docile. Together, these aspects are fundamental for evaluating the impact of etiological treatment on the histopathogical and clinical evolution of the disease in this animal species. Consequently, dogs have been used as an experimental model for CD studies because they are easily infected by *T. cruzi* of distinct origin with different inoculum sources and routes of inoculation, mainly when young [3-5], and reproduce the acute phase of the infection with the same clinical features and electrocardiographic changes observed in humans [4, 6].

As in humans, dogs survive the acute phase and evolve toward the chronic phase. In general, the indeterminate clinical form of chronic CD is easily reproduced in this species [4]. However, the typical cardiac clinical form of the disease in humans may also be reproduced in this animal model, being *T. cruzi* strain dependent [7], which explains the unpredictability and late evolution of this clinical form in dogs, as reported [4]. Interestingly, the histopathological and physiological changes typical of the disease are very similar to those in humans in both phases of the infection, including those regarding electrocardiographic changes [8, 9]. Important cardiac lesions, such as diffuse fibrotic chronic chagasic cardiopathy, have been reproduced in dogs [10]. Several publications have reported denervation, an important consequence of CD related to cardiac and hollow viscera dysfunctions (megaesophagus and megacolon) [11-13]. Evidence of host immune participation in CD pathology has also been demonstrated in mongrel and beagle dogs [14, 15] with IgM and IgG profiles similar to those observed in humans [16].

Together, these findings indicate the dog as the best model for studying Chagas disease, according to the requisites established by the World Health Organization [17]. The only exception is the inconsistent reproduction in dogs of the digestive clinical form of the disease present in humans (mainly megaesophagus and megacolon), which has been demonstrated in mice and other animal models. Therefore, we propose the dog as a good model for chemotherapy as a way of treating Chagas disease because it is phylogenetically closer to human, easy to handle and maintain, as compared to primates, which also reproduce different aspects and clinical forms of the disease [7]), but are difficult and dangerous, to handle. Moreover, dogs are a natural reservoir of *T. cruzi*.

1.1.1 Chemotherapy studies in dog

The search for published studies used MeSH terms according to Medline criteria. The criteria for inclusion in chemotherapy against *T. cruzi* used the terms: dog, *Trypanosoma cruzi*, treatment. Treatments using substances such as immunomodulatory drugs, which could interfere with disease pathology and not against *T. cruzi* infection, were not considered.

A similar approach was used to select studies in dogs against *Leishmania* infection using the MeSH terms: dog, *Leishmania chagasi*, *Leishmania infantum*, canine visceral leishmaniasis, treatment. Studies that included treatment using antigen compounds and those without parasitism quantification were excluded.

1.1.2 Cure control

The cure criteria adopted in most of the studies were: (i) The most conservative criterion: Simultaneous negativation of all parasitological tests (FBE, HC, blood PCR, and/or qPCR) in the blood or heart tissue, conventional serology (ELISA), and non-conventional serology CML (Complement-Mediated Lysis Mediated [18], which was employed only in publication [19], and (ii) Classic cure criteria (Second Brazilian Consensus on Chagas Disease, 2016) [20]: Simultaneous negativation of parasitological tests and ELISA, (iii) Parasitological cure: Negativation of parasitological tests (FBE, HC, PCR, and/or qPCR), and (iv) Negativity of qPCR in the blood and/or heart tissue).

As in humans [21], the natural order of negativation of the examinations used in monitoring therapeutic efficacy was: FBE, HC, blood PCR, qPCR, non-conventional serology, CML, and serological test (ELISA).

1.1.3 Use of benznidazole, nifurtimox and in association with other compounds

Details of each study are shown in Table **1**.

1.1.3.1 Comments on each study

The first investigations into the experimental treatment of Chagas disease were published in 1972) [22] and 1975 [23] using Nifurtimox (NF) and Nitroimidazolacetamide compounds. In a later study, [4] six dogs were treated with the association of NF+Dexamethazone (corticoid) and nine with NF. As a control, the same number of infected, untreated dogs and ten healthy dogs were evaluated in parallel. Although the description of the results were global, without distinction between the treatment

used in each group of infected dogs, the parasitemia in the treated animals was greatly reduced and tissue parasitism showed characteristics of the indeterminate chronic clinical form of the human disease. Combined treatment led to the complete elimination of inflammation and electrocardiographic changes. Dogs treated with only Dexamethazone showed increased inflammation and ECG changes, whereas NF led to a progressive decline in lesions and a decrease in ECG changes (Table **1**).

Table 1. Therapeutic efficacy, clinical, histopathological and immunological features in dogs infected with *Trypanosoma cruzi* and etiologically treated

Reference/ Author(s)	Chemotherapy scheme/ Follow-up months/ *T. cruzi* strain/ Inoculum/ Phase of Infection	Percentage of Cure		Clinical features Cardiomegaly, ECG, Echocardiography		Heart histopathology (% of change/Absence)	
		Classic Criterion/ Alternative (FBE, HC, PCR, qPCR, and ELISA/LMC^{o} negative)	Parasitological Cure: FBE, HC, PCR, and/qPCR negative and positive serology	ECG	Echocardiography	Inflammation	Fibrosis
[4] Andrade and Andrade, 1980	NF (60 mg/Kg/24 h for 5 or 10 days) and Dexamethasone (1 mg/Kg/24 h for 5 or 10 days)/ 12SF (4.0 x 10^5) Colombian (6.0 x10^5) Acute and Chronic (IND)	Strong reduction of mortality		Reduction and/or absence	–	Reduction	Reduction
[23] Andrade *et al*.; 1980	NF (60 mg/Kg/24 h for 5 or 10 days) and Bethametasone (1 mg/Kg/24 h for 5 or 10 days) 12SF (4.0 x 10^5)^{AP} (4.0 x 10^5)^{CP} Acute and Chronic	Reduction of mortality and evolution for chronic phase observed in some animals		Reduction and/or absence	–	Increase (NF) Almost abolished (Drug association)	Reduction^{CP}
[24] Guedes *et al*.; 2002	BZ (7 mg/Kg/12 h for 45 days) 6/Acute Phase: 24/Chronic Phase: Y, Be-78, Colombian (2.0 x 10^3)	Acute Phase: Global (68.75%) Y: 50% Be-78: 87.5% Colombian: 0%	Chronic Phase: Global (37.5%)	–	–	–	–
[26] Santos *et al*.; 2012	BZ (7 mg/Kg/12h for 60 days) Be-78 (4.0 x 10^3) Acute		Negative blood/heart PCR: 1 month: 80% blood/60% heart 6 months: 40% blood/20% heart 12 months: 0% blood/0% heart	Cardiomegaly similar in T and NT dogs	Decline of systolic function: (LVEF, LVFS), Cardiopathy without cardiomegaly	Heart/PBMC expression: Treated and Note Treated: Reduction of IL-10 correlated with cardiac alterations Increase of TNF-α correlated with cardiac alterations	

[28] Caldas *et al*.; 2013	BZ (7 mg/Kg/12 h for 60 days) Be-78, AAS, VL10 (2.0×10^3) Acute		HC and blood PCR: Be-78 (100% negative) AAS (0% negative) VL10 (0% negative)	Be-78: Reducion AAS: Reduction VL10 Progressive ECG changes (75%)	_	Be-78: Fibrosis reduction, but no scar area AAS: Reduction of fibrosis and scar area VL10: Fibrosis and scar area increased progressively	
[30] Daliry *et al*.; 2014	BZ (7 mg/Kg/12 h for 60 days) Y, AAS, VL10 (4.0×10^3)	_	_	Y: 0 AAS: 20 VL10: 100	IgG antibodies/Autoantibodies: Anti-β1-AR AAb: Similar profiles and levels for all strains throughout the infection.Y (89%), AAS (75%), VL-10 (100%) Anti-M_2-CR AAb: Distinct profiles and levels for each strain throughout the infection Anti-M_2-CR AAb:VL-10 (100%) > AAS (78%) > Y (78%)		
[27] Santos *et al*.; 2016	BZ (7 mg/Kg/12 h for 60 days) Be-78 (4.0×10^3) Chronic		Negative HC and blood PCR: (82%) Negative blood PCR/qPCR: (60%/20%) Negative blood/heart qPCR: (40%/20%)	_	Increase in E/E' Diastolic dysfunction: Early improvement, but not at 12^{th} month	Increased cardiac damage following treatment (12^{th} month) revealed by inflammation and fibrosis levels, which were similar to NT control	
[29] Caldas *et al*.; 2019	BZ 7 (2 doses) Be-78, AAS, VL10 (2.0×10^3)	Be-78 (100%) AAS (0%) VL10 (0%)	qPCR Negative: Be-78: 100% Cured AAS: 0% NC VL10: 0% NC	_	_	Be-78 (Absence) AAS ($Reduction^{AP}$) VL10 (Presence)	Be-78 (Absence) AAS ($Reduction^{AP}$) VL10: Increased $presence^{CP}$
[31] Carvalho *et al*.; 2019	BZ (7 mg/Kg/12h for 60 days) VL10 (2.0×10^3) Chronic			_	Systolic dysfunction: 28% of dogs (17 – 67%) Significant Reduction (LVEF, LVFS)	_	_
[33] Cunha *et al*.; 2019[a]	BZ (2 doses/7 mg/Kg/12h for 60 days) ITZ (6 mg/Kg/12h for 60 days) BZ+ITZ (equal) VL10 (2.0×10^3) Acute	BZ (0%) ITZ (0%) BZ+ITZ (20%)	BZ (0%) ITZ (20%) BZ+ITZ (40%)	BZ: 40% of mortality ITZ: 20% of mortality BZ+ITZ: 0% of mortality		BZ+ITZ: Reduction > BZ and ITZ	BZ+ITZ: Reduction: 67% > ITZ: 60% > BZ 0%
[36] Madigan *et al*.; 2019	ITZ (10 mg/Kg/24 h for 12 months) and Amiodarone hydrochloride (7.5	_	PCR in blood: 98.91%	General clinical signs: 98% T x 0% NT		_	

	mg/Kg/24 h for 12 months) Natural Infection Chronic			Treated: 18% of mortality NT: 50% of mortality		
[37] Guedes *et al*.; 2004	Albaconazole (1.5 mg/Kg/12 h for 60, 90, or 150 days) BZ (5 mg/Kg/12 h for 60, 90, or 150 days) / 1, 6 Y, Be-78 (2.0×10^3) Acute	Albaconazole: No cure, but suppression of the parasitemia in the AP in dogs infected with both strains in all regimes of treatment	FBE, HC and blood PCR Negative: Y 25% (ttm for 60 days), Y 100% (ttm for 90 days) Be-78: 0% (in all regimes) BZ: 100% (Y, Be-78)	Albaconazole: 0% of mortality Minimal toxicity BZ: 0% of mortality weight loss (signal of toxicity)	–	
[39] Diniz *et al*.; 2010	Ravuconazole (6 mg/Kg/12 h for 90 days) BZ (7 mg/Kg/12 h for 90 days) Y, Be-78 (2.0×10^3) Acute	Ravuconazole: Y, Be-78: 0% of cure 0% of Mortality: BZ: Be-78: 100% of cure 0% of Mortality	Negative HC and PCR: Ravuconazole: Y (40%), Be-78 (20%) BZ : Y, Be-78 (100%):	Cytokine mRNA expression: Ravuconazole and BZ: INF-ɣ: Y reduction; Be-78, increase; BZ: Lower levels IL-10: Y reduction, Be-78: Increase, BZ: Lower levels IgG1 showed similar profiles in animals infected with Y strain T, NT and healthy, indicating that IgG1 titers might not to be a general marker of cure. Total IgG is correlated to IgG2 and not to IgG1	Ravuconazole and BZ: Y: 20% of Reduction Be-78: (No reduction) BZ: (> Reduction)	Ravuconazole and BZ: Y: Significant Reduction Be-78: No reduction BZ: > Reduction
[40] Zao *et al*.; 2019	ITZ (10 mg/Kg/24 h for 12 months) and Amiodarone (7.5 mg/Kg/24 h for 12 months) / Unknown Follow-up Natural Infection Inoculum unknown Chronic	–	qPCR-kDNA: better for post-treatment evaluation qPCR-nDNA confirmation of cure	Mortality and toxicicity not determined	–	–

NF: Nifurtimox, BZ: benznidazole, ITZ: Itraconazole, AP: Acute Phase, CP: Chronic Phase, IND: Indeterminate clinical form of Chagas disease, NT: Not treated, Alternative criteria: Kretlli & Brener (1982); FBE: Fresh Blood Exammination, HC: Hemoculture, PCR: Polymerase Chain Reaction, qPCR: Quantitative PCR or real time PCR, ELISA: Enzime-linked-Immunosorbent-Assay, CML: Complement Mediated Lysis, ECG: Electrocardiogram, LVEF: left ventricle ejection fraction, LVFS: left ventricle fractional shortening, RA: Right Atria, LV: Left Ventricle, INF-ɣ: Interferon-ɣ, IL-10: Interleukine 10, Anti-β1-AR AAB: Anti-adrernergic receptor autoantibody, Anti-M_2-CR AAb: Anti-muscarinic receptor autoantibody, TNF-α: Tumor Necrosis Factor, T: Treated, NT: Not treated.
BZ or NF were used as reference drug in the studies in two doses of 7 mg/kg/day (Brener, 1962) or 60 mg/kg/day, respectively.

Study [24] used a combination of NF (2-methyl-4-[5'nitrofurfurylideneamino]-tetrahydro-4H-1,4-thiazine-1,1-dioxine) and bethametasone to verify the results of an antinflammatory and imunossupressor in myocarditis and electrocardiographic changes in dogs with the indeterminate clinical form of the disease infected with 12SF, São Felipe, Bahia, and Colombian *T. cruzi* strains. Histopathological and electrocardiographic changes were evaluated. The authors confirmed that the association of a nitrofuranic drug with a corticoid in acute infection led to parasite destruction and inhibited the inflammatory response with a consequent reduction in mortality and electrocardiographic changes (Table **1**).

Over the following two decades, there were no publications concerning Chagas disease chemotherapy in the dog model. Then, 22 years later the study [19] reported on the treatment of dogs at acute and chronic phases of the infection with Colombian (TcI), Y and Be-78 (TcII), prototypes of resistant, partially susceptible, and sensitive *T. cruzi* strains to treatment with BZ and NF, respectively [25]. FBE, HC, blood PCR, ELISA, and CML were evaluated post-treatment. The cure rates for each *T. cruzi* strain considering the classic and parasitological cure criteria, are presented in Table **1**. Interestingly, the cure rate and behavior of all tests used in the post-treatment evaluation were similar to those in human studies. The cure rates were also similar to those

observed in infected mice and treated under similar conditions, although better results were obtained with the Y strain. Thus, the authors proposed that the dog could be a relevant model for chemotherapy studies of Chagas disease.

With the aim of investigating the efficacy of the etiological treatment for preventing disease progression and cardiac lesions, an aspect still very controversial in human Chagas disease, study[26] used the experimental dog model treated with BZ in the chronic phase. The animals were evaluated by echodopplercardiogram, cytokines level (TNF-α, IL-10) in PBMC in supernatant by ELISA, PCR in blood eluate and in the heart tissue (Table **1**). The authors concluded that the temporary suppression of parasitemia by BZ administered in the early chronic infection was effective at reducing the systolic cardiac function, but not for preventing cardiomyopathy.

The same animals infected with Be-78 strain [26] were studied regarding diastolic dysfunction and cardiac damage by Santos *et al.* (2016) [27] and evaluated by parasitological (HC, blood PCR), histopathological (cardiac inflammation and fibrosis), and echocardiographic exams. The findings strongly suggested that the temporary reduction in the parasite load induced by BZ treatment was not able to prevent myocardial lesions and diastolic dysfunction 12 months after treatment.

Study [28] evaluated the electrocardiographic changes in 35 young mongrel dogs, four months old, experimentally infected with the susceptible Be-78 and resistant (AAS and VL10) strains to BZ and NF treatments in mice. Treatment efficacy was evaluated by HC and blood PCR one month and six months after treatment. An electrocardiogram (ECG) was recorded prior to infection, four months and nine months post-treatment. Histopathological analysis and fibrosis quantification were performed at the end of the experiments nine months after treatment (Table **1**). The findings demonstrated that an effective treatment at the acute phase of infection led to a significant reduction in the intensity and severity of cardiac disease even if the parasites were not completely eliminated. This study advanced in previous observations of the study [19] regarding the use of the dog model to explore the benefits of etiological treatment in the clinical evolution of Chagas disease and demonstrated that the effect of treatment on the clinical evolution is *T. cruzi* strain dependent.

To understand the role of host parasitemia in etiological treatment efficacy, pathogenicity, and disease progression, study [29] evaluated the same mongrel dogs that had been previously studied in [28]. Parasitemia was previously evaluated by FBE, HC, and blood PCR. After necropsy, heart tissues were evaluated by qPCR [35] and histopathology (parasitism, inflammation, and fibrosis). All of these results are shown in Table **1**. No correlation between parasitic load and cardiac fibrosis was observed between the acute and chronic phases of infection. The findings suggest that parasite burden is a limited predictor for disease progression in dogs after treatment and show that BZ, although it did not induce parasitological cure, led to total prevention of fibrosis in the early stages of infection and the complete prevention of cardiac damage when parasites are eliminated at the onset of infection.

Study [30] investigated the appearance and progress of anti-β1-AR and anti-M2-CR autoantibodies in sera from 60 young mongrel dogs infected with the VL10, AAS, and Y *T. cruzi* strains and then submitted to BZ treatment. The analysis was performed 30, 90, and 270 days post-infection by ELISA test using synthetic peptides comprising the extracellular loops of the β1 and M2 cardiac receptors. Dogs infected with the VL10 strain showed a high level of anti-M2-CR AAb throughout the entire infection. These AAbs were present in only 44% of the animals and at different levels in the dogs infected with each strain (Table **1**). Both AAbs appeared early in dogs infected for all strains and a strain-specific modulation of anti-M2-AAb titers was verified. These results strengthen the hypothesis that the AAbs are produced in response to the animals'parasitemia rather than as a consequence of heart damage, being able to cross-react with host antigens causing autoimmunity. This study further showed that the modulation of AAb levels by BZ treatment is *T. cruzi* strain dependent and associated with ECG abnormality modulation.

As the literature regarding echocardiography in dogs with chagasic cardiopathy is scarce, study[31] established some parameters in the evaluation of young mongrel dogs infected with 2.0×10^3 blood trypomastigotes of VL10 strain, which is cardiotropic and pathogenic for dogs [28]. Using an ejection fraction (EF) cut-off value of 40%, established for dilated chagasic cardiomyopathy (DCM) in dogs, 28% of the infected animals were affected by the chronic infection with *T. cruzi* (Table **1**). This percentage is similar to that observed in human chronic infections [32]. The main conclusion of this study is that the chronic dog model with *T. cruzi* infection mimics human chronic chagasic cardiopathy (CCC). Thus, it is mandatory to include echocardiography parameters in the experimental design of preclinical studies in the dog model to account for the variable effect of chagasic chronic infection on systolic function.

Following the recommendations of specialists in using chemotherapy for the treatment of Chagas disease, various drug combinations, including BZ or NF in association with BZ+ITZ, were evaluated in the dog model [33]. ITZ was included because, like other azolic compounds, it inhibits cytochrome P450-dependent lenosterol C14 demethylase, thus reducing ergosterol synthesis. Moreover, ITZ had been used for treating patients in the indeterminate chronic form of Chagas disease and CCC, leading to parasitological negativation in a sizable number of patients, although the serology remained positive. For this reason, ITZ is not recommended as first-line therapy for Chagas disease [34]. However, in study [33], three groups of five young mongrel dogs of both genders were infected with 2.0×10^3 blood trypomasitgotes/kg of the VL10 *T. cruzi* strain, treated at the acute phase with BZ, Itraconazole (ITZ), and BZ+ITZ, and then evaluated in parallel with five infected, untreated dogs (INT). The post-treatment evaluations were carried out over a period of 1, 6, 12, and 18 months. As the VL10 strain is resistant to treatment, B+ITZ was not effective in inducing a sustained parasitological cure in all dogs. However, this association had several positive effects on infection evolution during the follow-up, such as reduction of the parasitemia, inflammation, fibrosis, and mortality. Details of all methods used in post-treatment evaluations in dogs, including step-by-step illustrations, were published in [35] for the same team.

Study [36] evaluated the ITZ+Amiodarone hydrochloride association for treatment of 105 dogs from Texas that had been naturally infected with *T. cruzi* over a period of 12 months, in tandem with a control group comprising 16 healthy, untreated dogs. Adverse effects were discreet and reversible and the results also suggested efficacy of this trypanocidal drug combination for the treatment of dogs.

1.1.3.2 Use of ergostherol biosynthesis inhibitors (EBI) as treatment

Taking into account that several ergostherol biosynthesis inhibitor (EBI) compounds used in mycoses treatment are potentially active in *T. cruzi*, two of them were evaluated in the dog model as chemotherapeutic agents because this parasite is also dependent on endogenous ergostherol biosynthesis. The first EBI studied was triazole derivative albaconazole [37], which has a remarkably long half-life in dogs as it does in humans. Albaconazole suppressed parasitemia (FBE) and prevented mortality when administered in a daily oral dose of 1.5 mg/kg/day in all treatment regimes. Other parasitological (HC and PCR) and serological (ELISA) tests were carried out at 1 to 6 months after treatment. The difference in therapeutic efficacy between albaconazole and BZ suggests that these compounds have different action mechanisms. It was demonstrated the potential anti-*T. cruzi* activity of albaconazole when used for 60 to 150 days with minimal toxicity under study conditions. Moreover, its favorable pharmacokinetics (half-life of 51 h and large volume of distribution) in dogs [38] may explain the results obtained.

Study [39] studied the effects of Ravuconazole in mongrel dogs infected with Y and Be-78 *T. cruzi* strains during the acute phase. Parasitological evaluations included FBE (during the acute phase) and HC, blood PCR, and ELISA and were carried out four and six months after treatment. Total IgG, IgG1, and IgG2 were evaluated before and in parallel to parasitological tests (Table **1**). After necropsy, the profile of heart cytokine expression by semiquantitative reverse transcription-PCR (RT-PCR), cardiac inflammation, and fibrosis were evaluated. A reduction in heart lesions was associated with low IFN-mRNA and high IL-10 mRNA levels. High IFN- mRNA and low IL-10 mRNA levels were detected in the heart tissue of the infected and nontreated control animals. A similar correlation was observed in animals infected with the EBI-resistant Be-78 strain. The results showed that the efficacy of RAV treatment in preventing cardiac lesions is related to early modifications in the humoral and cellular immune responses. Taken together, these findings support a link between the effectiveness of the specific treatment in preventing cardiac chronic lesions and the quality of the immune response. It is possible that a fine balance of pro- and anti-inflammatory cytokines could be the key in controlling morbidity following treatment. The final evaluations demonstrated that Ravuconazole was not curative, probably due to its unfavorable pharmacokinetics when compared with that in humans (8.8 h - lower half-life vs. 4 to 8 days).

Study [40] investigated the use of qPCR as a diagnostic tool and assessment of treatment efficacy. PCR (rtPCR) with two targets, kDNA [41] and nuclear satellite DNA (nDNA) [42], were used to detect *T. cruzi* in whole blood of 131 naturally-infected and untreated dogs from Texas with natural *T. cruzi* infection conditions. The results were similar and the target kDNA was slightly more sensitive for the diagnosis and less specific than nDNA target. Both tests were then employed in 137 itraconazole+amiodarone-treated dogs and the results compared with the ELISA test. It was concluded that the kDNA-based qualitative rtPCR may be used for parasitemia screening of infected and treated dogs, which is typically used in post-treatment evaluations, while nDNA-based qualitative rtPCR may be used for confirmation of the previous results (Table **1**).

1.1.3.3 Nanocarrier improvement of BZ activity in dogs

It is well known that successful treatment is related to the controlled and sustained release of the drug in order to achieve ideal plasmatic concentration, better volume of distribution, and mean residence time (MRT), which prevents toxic effects with simultaneous improvement in therapeutic efficacy. BZ, which is the most widely available drug for treating human Chagas disease in Brazil, presents very low solubility, [43] making its bioavailability more difficult. Some studies have focused on interpolyelectrolyte complexes (IPECs), which have the ability to achieve more sustained drug release, such as BZ, with improved drug delivery [44, 45].

Study [46] published the first pharmacokinetics study in the dog model. The authors evaluated IPECs composed of polymetacrylates (EE-EL-BZ) and polysaccharides (Ch-AA-BZ) of both origins (Abarax, Argentine vs Radanil, Roche®) in six young healthy dogs (4 males and 2 females) treated with one oral dose of 100 mg/kg/day or with multiple doses twice/day of 100 mg/kg/day, washout of 15 days. The HPLC-UV method [47] was validated for BZ quantification in plasma (collected 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 12, 19, 36, and 48 h post-administration). The Tmax values for both IPECs were higher ($p<0.05$) and the areas under the curve were 25% greater than for the BZ Roche ($p<0.01$). The authors concluded that these formulations, using a capsule as a carrier, prolonged drug release in dogs and improved BZ performance *in vivo* (bioavailability and Tmax) when compared to the reference BZ, and that these carrier systems offer new possibilities for oral doses of BZ in the treatment of Chagas disease.

1.1.4 Discussion

Several factors make it difficult to compare experimental results in animal models. Some of them are related to standardization of animal conditions (lineage or breed, sex, age) and comparison between the results from males and females, age, weight, origin, and microbiologic status. Other important factors are the experimental conditions, such as *T. cruzi* sample/strain, parasite genetics, parasite origin (geographic, host species), number and infective forms of the inoculum, inoculation route, dose, treatment administration route, treatment scheme, methods used for post-treatment evaluations (interval between the evaluations, duration of the follow-up), number of animals in the experimental groups and control groups, cure criteria, and statistical analysis methods. These details may explain, in part, the differences in the results between animal experimentations (especially when different

animal species are used) and clinical trials involving new candidates for Chagas disease treatment. Furthermore, the guidelines for animal experimentation have, over time, included an array of regulations and guidelines governing the handling of animals and the conditions under which they are kept so as to avoid stress, suffering, and mortality [48], following the 3Rs Principles (Replacement, Reduction, and Refinement). The lack of standardization and accurate description of materials and methods, experimental design, and failure to report the results has serious scientific, ethical, and economic implications for the scientific community and public opinion. Following these rules helps to avoid unnecessary repetitions of experiments and simplify comparisons among different studies. In this context, the ARRIVE (Animals in Research: Reporting In Vivo Experiments) guidelines were published [49] to improve the quality of animal experimentation and its validation to promote translational research for human benefit, which presents a formidable challenge [50].

There is a paucity of studies on chemotherapy for the treatment of Chagas disease in dogs. This review included 15 publications in experimental treatment, eleven of them from the same institution/team (UFOP) and four from other institutions. Six publications evaluated drug associations (two with NF, two with BZ and two with ITZ), two studied EBI compounds and one study is referent to BZ pharmacokinetics. This situation may lead to some bias, but the scarcity of publications is likely due to the high cost of dog maintenance, difficulty in handling, and the space needed for kennels when compared to rodents such as mice [7]. The majority of the studies making up this review were carried out by the same research team (UFOP). This situation naturally minimized the variations in both animal and experimental conditions. Additionally, the great majority of *T. cruzi* strains used are already susceptible to the customary drugs, BZ and NF, previously known in the standard conditions in murine model [25], according to a pioneering study of Chagas disease chemotherapy [51]. Interestingly, the profile of drug resistance or susceptibility of the same *T. cruzi* strain to BZ or other compounds, including NF, is very similar in both dogs and mice with few exceptions. This may be due to small differences in the methods used in post-treatment evaluations and the relativity between the times of follow-up and the life span of the animal species used. In other words, animals with a longer life span need to be assessed over a longer period of time in order to detect cure, regardless of the cure criterion adopted. Clinical cure was not effectively demonstrated in dogs due the absence of a strong and safe biomarker, as occurs in treated humans. However, electrocardiography and echodopplercardiography were used in post-treatment evaluations in some studies. Some clinical improvement was observed, but there is insufficient data to demonstrate clinical cure.

It is important to highlight the studies in which treatment with BZ had a positive effect on disease evolution when histopathological lesions and cardiac functions were evaluated in treated dogs [26, 28, 29, 31, 33], in which improvement or regression of inflammatory and fibrotic lesions, electrocardiographic (various) and echocardiographic (LVFE, LVFS, and systolic and diastolic dysfunctions) changes were recorded. These studies also showed correlations between a reduction in the parasite load and histopathological lesions and clinical changes as revealed by ECG and echodopplercardiography. These findings suggest that the same benefits may be observed in humans in function of the type of the *T. cruzi* strain used, host immune response, and clinical parameters evaluated. The absence of a positive effect of the treatment on the clinical evolution was observed in some studies conducted in animals infected with a *T. cruzi* strain resistant to drugs, such as VL10 [28, 29], and also more pathogenic. The lack of success of the BENEFIT project [52] in demonstrating improvement in the clinical aspects of the disease could be explained by the fact that the patients involved had already presented miocardiopathy. Experimental studies in animal models, including dogs [6, 10, 28, 29, 33], have clearly shown that only highly pathogenic *T. cruzi* strains more resistant to treatment in animal experimentation caused the severe cardiac clinical form of the disease. The discrepancies in the experimental results with the studies in humans may also be related to the time of post-treatment observation, which needs to be directly proportional to the life span of the host [7, 21] and the unfamiliarity of the *T. cruzi* strain involved. It would, therefore, expected that a longer period of evaluation would be necessary in humans. These studies expanded and reinforced the value of the dog as an experimental model for the study of Chagas disease [17] since the results are similar to data obtained in human patients [52- 54].

The six studies in dogs that used drug associations (NF+bethametazone [4], NF+bethametazone [24], BZ+ITZ [33], administered at the acute or chronic phase [4, 24, 35]), ITZ+Amiodharone [36, 40]) exhibited some benefits for dogs that had been experimentally [4, 24, 33] or naturally infected [36, 40]). These benefits were recorded as reduction of inflammation and fibrosis, reduction of clinical signs and cardiac alterations observed in ECG and/or Echocardiogram and decrease in mortality rate.

Two studies evaluated EBI compounds. One of them studied Albaconazole (UR-9825), with worse results of parasitological cure than those of BZ and without demonstrating cure by the classic cure criteria [20], which includes negative serology. The second azolic derivative studied was Ravuconazole [39], but the results were also worse than those of BZ because it failed to demonstrate cure in the animals. Unfavorable pharmacokinetics, mainly related to short half-life, explain the failure of this compound in the dog model, which were different from the results reported in humans. However, the E1224 compound, a prodrug of Ravuconazole, is being investigated in the clinical essay BENDITA [55] in patients from Bolivia, in association with BZ and employing different therapeutic regimes.

The use of Beagle dogs as an experimental model led to expectations of more homogenous results, especially when evaluating immunological parameters that need more refinement. However, the use of this breed was not advantageous. Beagles are very expensive and difficult to be maintained in experimental conditions due their low resistance to common infections. When study [15] evaluated the production of IL-10, INF-ɣ and TNF-α in Beagles infected and treated with BZ during the acute phase, the results revealed that these dogs are good models for studying the immunopathogenic mechanism of Chagas disease since they reproduced cardiac lesions and clinical signs similar to those in humans. Data further suggested that the development of the

chronic cardiac form of the disease was linked to a strong Th1 response during the acute phase, while the development of the indeterminate form was the result of an early blend of Th1 and Th2 responses after infection.

Another aspect to be considered is the pharmacokinetics (PK) of the drug or new compounds for Chagas disease treatment, including the dog model. The PK parameters of each compound are different in each host species (animals or human). This fact is responsible for failures in clinical trials of new candidates for Chagas disease treatment that presented excellent results in pre-clinical studies, primarily in mice. PK parameters, such as volume of distribution and half-life of the drug, are particularly important to be considered. In this context, nanotechnology offers a promising opportunity to improve the pharmacokinetics of a compound in function of the physicochemical nature of its molecule, which needs to be appropriated to a particular nanocarrier [56], as well as to the host species to be treated. The correct use of nanotechnology might prevent the precocious elimination of substances or compounds that could be used in Chagas disease treatment. Numerous studies in the murine model have demonstrated advances in the therapeutic efficacy of several new compounds that could be potentially useful in the treatment of *T. cruzi* infections or Chagas disease [57].

1.2 Dog as a model for chemotherapy in the treatment of leishmaniasis

1.2.1 General aspects of leishmaniasis and dogs as a model for experimental chemotherapy

Leishmaniasis is a neglected disease complex subgrouped in tegumentary and visceral clinical forms and presenting distinct *Leishmania* species in human infections [58]. Visceral leishmaniasis (VL), caused by *Leishmania donovani* and *Leishmania infantum* (syn. *L. chagasi*), is the more severe form and potentially fatal in untreated human [59]. The dog is considered an important domestic reservoir of *L. infantum* in transmitting the parasite to humans [59, 60]. The VL caused by *L. infantum* represents an important public health problem and is considered a disease that employs a number of complex and challenging controls [59, 60]. For this reason, the main challenge in effective VL control is dependent on new vaccines and treatments for human and canine disease [61, 62]. The human VL treatment is based on pentavalent antimonials (sodium stibogluconate or meglumine antimoniate), considered an important therapeutic option in many countries since the 1920s, with increased resistance being reported in human treatment since the 1980s [63]. The option of treating human relapses in the 2000s was polyene antibiotic amphotericin B deoxycholate in spite of adverse side effects such as nephrotoxicity [63, 64]. The reduction in toxicity, along with increased efficacy, was obtained with a liposomal amphotericin B formulation that has been safe, albeit expensive, to use for VL treatment [65]. The rationale for developing new drugs for leishmaniasis treatment needs to consider: (i) safety, (ii) practical administration (preferably oral), (iii) short term treatment, and (iv) high efficacy.

In this scenario, the analysis of innovative treatment approaches applied to human leishmaniasis needs to consider an appropriate experimental model for preclinical trials [66]. In this context, the dog model presents a metabolism, drug kinetics, and responses similar to those in humans [66]. In fact, the dog's genetic characteristics are considered very similar to humans [67, 68], supporting the use of this experimental model in trial vaccines and chemotherapies before proceeding with traditional clinical trials. Although the canine experimental model presents additional advantages when compared to rodents (particularly mice and hamsters for leishmaniasis studies), the requirement of specialized human resources and appropriate facilities is restrictive to their widespread application.

Additional interest in *Leishmania infantum*-infected dogs is due to the fact that canine visceral leishmaniasis (CVL) is a health problem for veterinary medicine [61], resulting in extensive efficacy studies against VL in dogs (Tables **2** and **3**). CVL is characterized by numerous clinicopathological presentations, including abnormalities in biochemical and hematological parameters [69-71], in addition to histopathological changes in liver, spleen, lymph nodes, bone marrow, and skin [61, 62, 69, 71-74]. Moreover, CVL natural infection has demonstrated that the clinical symptoms are directly associated with parasite load in the target organs [61, 62, 73]. Notably, VL presents important clinicopathological manifestations that are similar to those in human disease [75]. These findings further support dogs being used as a suitable experimental model in leishmaniasis studies [61, 62].

1.3 Chemotherapy treatment of canine visceral leishmaniasis

The studies on chemotherapy efficacy against *L. infantum* infection in dogs have been described as a common pattern regarding the promotion of parasite clearance in the entire organism. From an epidemiological point of the view, parasite clearance is required to block VL transmission. However, since the dog can also be used as an experimental model to evaluate drug efficacy, the search for parasite clearance represents another target to be evaluated in new chemotherapeutic agents. Indeed, complete parasite clearance is a challenge that has not been obtained in studies of therapeutic schemes [61, 76, 77]. However, the reduction in parasite load has been considered important to reducing the risk of parasite transmission in therapeutic evaluations against CVL (Table **2** and **3**). For this reason, the major studies on CVL chemotherapy included in Tables **3** and **4** were selected taking in account an evaluation of parasitism as a minimal requirement for standardizing the quality of the studies.

In this sense, the first line of defense used in CVL treatment in Europe is based on antimonial pentavalent (meglumine antimoniate or sodium stibogluconate) chemotherapy, which is intravenously administered once or twice a day for 21 days (Table **2**). The antimonial pentavalent used in monochemotherapy has demonstrated limited clinical recovery and parasitism control [78-81]. In contrast, polychemotherapy (Table **3**) using antimonial pentavalent and allopurinol or antimonial pentavalent and spiramycin plus metronidazole induced better parasite control and reduction in clinical relapses [82, 83]. Notably, liposomal

meglumine antimoniate formulation and/or allopurinol combined chemotherapy (Table **3**) performed well in inducing early clinical recovery, decreased IgG levels, and a marked reduction in parasite load [84, 85]. Widely used in Brazil, pentavalent antimonial is the first drug of choice in the chemotherapeutic treatment of humans and, therefore, is prohibited from being used to treat CVL.

Table 2. Preclinical trial for analysis of monochemotherapy agents against infection of *Leishmania infantum* in dogs.

Reference	Chemotherapy scheme/number of dogs	Criterion of dog inclusion in the study	Type of infection	Immunological biomarkers	Treatment efficacy (clinical improvement/reduction in parasite load)
[80] Ikeda-Garcia *et al*.; 2007	Meglumine antimoniate (75 mg/kg/every 12h, subcutaneously for 21 days) /n=7	Positivity in *Leishmania*-specific IgG, amastigotes detection in bone marrow and lymph node	Naturally infected dogs	--	Clinical recovery 60 days after the treatment in all symptomatic dogs (5/5) Clinical relapses: 150 days after the treatment (2/5) Parasitism observed 180 days after the treatment in 4/7 dogs in lymph node, bone marrow, spleen, or liver (smears or parasite culture)
[88] Andrade *et al*.; 2011	G1: Miltefosine (100 mg⁄dog/every 24h, orally for 28 days) /n=5 G2: Miltefosine (200 mg⁄dog/every 24h, orally for 28 days) /n=5 G3: Miltefosine (100 mg⁄dog/every 24h, orally for 45 days) /n=5	Positivity in *Leishmania*-specific IgG, PCR, and amastigotes (smears) detection in bone marrow	Naturally infected dogs	Cytokines production after *L. infantum* stimulation on peripheral blood mononuclear cells at the end of follow up in all of the groups: decreased IFN-γ levels, IL-4 remained in low levels while IL-10 increased	Clinical recovery after 24 months of follow-up: complete clinical recovery in 7/14 treated dogs, 5/14 were polysymptomatic, 2/14 died Parasitism in bone marrow by PCR: 100% positivity, all of the dogs presented increased parasite load 6 months after treatment; Positivity in 13/14 spleen samples by qPCR
[86] Woerly *et al*.; 2009	Miltefosine (2 mg/kg/ every 24h, orally for 28 days) / n=96	Presence of CVL clinical signs, positivity in *Leishmania*-specific IgG or bone marrow smear or bone marrow PCR; negative for ehrlichiosis	Naturally infected dogs	--	Reduction of clinical score: 61.2±44.9% Parasitism in bone marrow smears: 17/33 initially positive became negative
[87] Dos Santos Nogueira *et al*.; 2019	Miltefosine (2 mg/kg/every 24h, orally for 28 days) /n=35	Presence of CVL clinical signs and infection status proven by serological, parasitological, and/or molecular diagnosis	Naturally infected dogs	--	Clinical recovery 12 weeks after starting the treatment: reduction from average 16.29 (before treatment) to 5.17 (after treatment) Parasitism in skin by PCR 12 weeks after starting the treatment: 98.7% of reduction in parasite load; Xenodiagnosis: 18/35 dogs were infective before treatment and 9/35 after 12 weeks after starting treatment
[93] Sabaté *et al*.; 2014	G1: Domperidone (0.5 mg/kg/every 24h, orally for 30 days, every 4 months) /n=44 G2: untreated dogs/n=46	Negative serology to *L. infantum*; without use of synthetic pyrethroids, permethrin or	Non-infected dogs to check if the preventive domperidone treatment was	IgG positivity: G1 (5/44), GII (22/46)	CVL clinical signs (lymphadenomegaly, exfoliative dermatitis, and weight loss): G1 (5/44), GII (22/46)

		deltamethrin. Dogs were analyzed in a field open-label, controlled, randomized clinical trial in a region with 20% of CVL prevalence	able to induce reduction in the disease		Parasitism in lymph nodes and/or bone marrow smears: G1 (5/44), G2 (22/46) Prevention failure (indicative of active infection and incipient disease progression): G1 (5/44), G2 (22/46)
[94] Vexenat *et al*.; 1998	G1: Aminosidine (20 mg/kg/every 24h, intramuscular, for 15 days) /n=3 G2: Aminosidine (80 mg/kg/every 24h, intramuscular, for 20 days) /n=6 G3: Aminosidine (40 mg/kg/ every 24h, intramuscular, for 30 days) /n=12	*L. infantum* infection was confirmed in the skin and/or bone marrow smears	Naturally infected dogs	--	Died within one month of treatment: G1 (0/3), G2 (3/6), G3 (2/12) Clinical relapsed and died: G1 (3/3), G2 (2/6), G3 (7/12) Cured: G1 (0/3), G2 (1/6), G3 (3/12)
[96] Athanasiou *et al*.; 2013	Aminosidine (15 mg/kg/every 24 h, subcutaneously for 21 days) /n=12	Positivity in *Leishmania*-specific IgG and parasitological analysis (PCR and microscopy in lymph node and bone marrow)	Naturally infected dogs	IgG levels: reduction in all dogs 3 months after treatment	Lymphadenomegaly and total clinical scores were significantly lower 3 months after treatment Reduction in parasitism 3 months after treatment: 3/12 (lymph node and bone marrow smears), 7/12 (negative in lymph node PCR), 4/12 (negative in lymph node PCR)
[95] Passos *et al*.; 2014	G1: Aminosidine (10 mg/kg/every 12h, subcutaneously, for four weeks) /n=12 G2: Sodium stibogluconate (150mg/kg/ every 24h, intravenously for 4 weeks) / n=indeterminate	Positivity in *Leishmania*-specific IgG or parasite isolation in dogs; presence of at least one CVL clinical, the absence of azotemia, proteinuria, ehrlichiosis and dirofilariosis	Naturally infected dogs	Reduction in IgG levels: 30 and 60 days after treatment in G1 and G2	Clinical recovery: G1 (11/12) and G2 (all dogs) Parasitism in lymph nodes by culture isolation: G1 (100%), G2 (100%)
[91] Pineda *et al*.; 2017	Marbofloxacin (2 mg/Kg/every 24h, orally for 28 days) /n=28 .	At least one clinical CVL sign, evidence of chronic kidney disease, negative for coinfections (*Ehrlichia canis*, *Dirofilaria immitis*, *Anaplasma phagocytophilum*, *Anaplasma platys* and *Borrelia burgdorferi*), presence of *Leishmania*-specific IgG and PCR positivity in lymph nodes	Naturally infected dogs	--	Clinical recovery: 18/28 Reduction in parasite load in lymph node: 18/25
[97] Rhalem *et al*.; 1999	G1: Dimethasulfonate pentamidine (4 mg/kg, intramuscularly with 2 courses of treatment at 3-week intervals: each course consisted of 8 injections at 3-day intervals) /experimentally infected /n=5 G2: Control – untreated experimentally infected /n=5	Positivity in *Leishmania*-specific IgG and parasitological analysis (spleen smears). G1, G2 and G3 were polysymptomatic (emaciation, thinness, onychogryphosis, and splenomegaly)	Naturally infected dogs and *L. infantum* experimentally infected (10^5 amastigotes/kg body weight by	G1 and G3: reduction in IgG levels until 6 months after treatment; G2: high IgG levels throughout 6 months; G4: low IgG levels	Clinical recovery: 6 months after treatment all infected dogs presented as asymptomatic Parasitism negative 6 months after treatment in G1 (2/2) in spleen smears and culture

	G3: Treated as G1, naturally infected /n=3 G4: Control – untreated asymptomatic / experimentally infected /n=2 G5: Control – untreated noninfected /n=2		intravenous route)	throughout 6 months G1, G3, and G4: increased lymphoproliferation response using *Leishmania* antigens 6 months after treatment	
[98] Marques *et al.;* 2008	Liposomal trifluralin (10 mg/Kg/every 24h, intravenously for 10 days) /n=5	Clinically healthy (physical examinations, routine haematological tests, and the absence of detectable levels of *Leishmania*-specific antibodies)	Experimental *L. infantum* infection: 10^6 amastigotes/kg with treatment started 6 months later – all dogs were positive in *Leishmania*-specific IgG and positive in bone marrow and culture for *L. infantum*	Reduction in IgG levels: 3 months after treatment (2/4)	Clinical recovery 1 month after treatment (2/4) Parasitism at 3 months after treatment: high parasite load in 1/4

Sb: Sodium stibogluconate

Table 3. Preclinical trial for analysis of polychemotherapy agents against infection of Leishmania infantum in dogs.

Reference	Chemotherapy scheme/number of dogs	Criterion of dog inclusion in the study	Type of infection	Immunological biomarkers	Treatment efficacy (clinical improvement/reduction in parasite load)
[81] Pennisi *et al.*; 2008	G1: Meglumine antimoniate (55-100 mg/kg/every 12 h, subcutaneously for 60 days), and Allopurinol (10 mg/kg/every 12h, orally for 90 days) /n=6 G2: Meglumine antimoniate (55-100 mg/kg/every 12h, subcutaneously for 30 days), and Allopurinol (10 mg/kg/every 12h, orally for 90 days) /n=9 G3: Meglumine antimoniate (55-100 mg/kg/every 12h, subcutaneously for 60 days), and Spiramycin (150,000 UI/kg), plus Metronidazole (25 mg/kg/every 12h, orally for 90 days) /n=8 G4: Meglumine antimoniate (55-100 mg/kg/every 12h, subcutaneously for 30 days), and Spiramycin (150,000 UI/kg), plus Metronidazole (25 mg/kg/every 12h, orally for 90 days) /n=6	Presence of CVL clinical signs, positivity in *Leishmania*-specific IgG, PCR (lymph node/bone marrow and blood) and amastigotes detection in lymph node, bone marrow, or skin smears	Naturally infected dogs	Reduction in IgG levels: in all dogs from 3 to 11 dilutions	Clinical relapses 9 months after treatment: G1 (2/6), G2 (5/9), G3 (2/8)
[82]	G1: Meglumine antimoniate (50 mg/kg/ every 12h up to clinico-pathological recovery, subcutaneously)/n=6	Positivity for amastigotes detection in lymph node smears;	Naturally infected dogs	--	Clinical recovery: G1: 5/6 at 1 month after starting treatment, G2: 6/6 at 1-3

Paradies *et al*.; 2012	G2: Meglumine antimoniate (50 mg/kg/ every 12h for 8 weeks or up to clinic-pathological recovery) and/or followed by Allopurinol (15 mg/kg/every 12h, orally – administered for 6 months after Meglumine antimoniate discontinuing) / n=6 G3: Allopurinol (15 mg/kg/every 12h, orally for 12 months /n=6 G4: Meglumine antimoniate (37.5 mg/kg/ every 6h up to clinico-pathological recovery, subcutaneously for 21 days) / n=6	negative for ehrlichiosis and renal damage			months after starting treatment, G3: 2/6 at 2 months after starting treatment, G4: 6/6 at 1.5 months after starting treatment Clinical relapses one year after treatment: G1 (3/6), G2 (0/6), G3 (4/6); G4 (6/6) Parasitism in lymph node smears: G1 (3/6), G2 (0/6), G3 (5/6), G4 (6/6)
[83] da Silva *et al*.; 2012	G1: Liposomal meglumine antimoniate formulation (6.5 mg Sb/kg/dose, intravenously 6 doses at 4-day intervals) and Allopurinol (20 mg/kg/ every 24h, orally for 140 days starting from the first dose of liposomal formulation) /n=8 G2: Liposomal meglumine antimoniate formulation (6.5 mg Sb/kg/dose, intravenously 6 doses at 4-day intervals) / n=8 G3: Allopurinol (20 mg/kg/every 24h, orally for 140 days) and 6 doses of saline given at the same time intervals as liposomal formulation of G1 group /n=8 G4: Empty liposomes (given at the same time intervals as liposomal formulation of G1 group) and Allopurinol (20 mg/kg/ every 24h, orally for 140 days) /n=8 G5: Empty liposomes (given at the same time intervals as liposomal formulation of G1 group) G6: Saline (6 doses of saline given at the same time intervals as liposomal formulation of G1 group /n=12	Presence of CVL clinical signs, positivity in *Leishmania*-specific IgG, PCR in bone marrow	Naturally infected dogs	Reduction in IgG levels 60 days after the end of treatment compared to time before treatment: G1 (20.3-fold reduction); G2 (1.9-fold reduction); G3 (1.3-fold reduction); G4 (2.5-fold reduction); G5 (1.4-fold reduction); G6 (2.5-fold reduction);	Clinical recovery 60 days after the end of treatment: lower clinical scores in G1, G2, G3, and G4 compared to G5 and G6 Reduction of parasitism (parasite load) in skin, bone marrow, and spleen by PCR showed G1 as the most effective treatment; parasite load by PCR in liver demonstrated negative results in G1 and G2; frequency of *L. infantum*-infected sand flies by Xenodiagnosis: G1 (0), G2 (1.9), G3 (0), G4 (2.5), G5 (30.0), G6 (16.4)
[84] Dos Santos *et al*.; 2020	G1: Mixture of conventional and PEGylated liposomal meglumine antimoniate formulations (6.5 mg Sb/kg/dose, intravenously at 4-day intervals, in 2 cycles of 6 doses with an interval of 40 days between both cycles) and Allopurinol (30 mg/kg/every 12h, orally for 130 days starting 30 days before the first dose of liposomal formulation) / n=9 G2: Conventional liposomal meglumine antimoniate formulation (6.5 mg Sb/kg/dose, intravenously at 4-day intervals, in 2 cycles of 6 doses with an interval of 40 days between both cycles) and Allopurinol (30 mg/kg/every 12h, orally for 130 days starting 30 days before the first dose of liposomal formulation) / n=9 G3: Allopurinol (30 mg/kg/every 12h, orally for 130 days) / n=8 G4: Dogs without treatment /n=11	Positivity in *Leishmania*-specific IgG, PCR in bone marrow	Naturally infected dogs	Reduction in IgG levels: G1 and G2 displayed lower IgG levels compared to G3	Clinical recovery after 4 months of treatment: higher proportion of dogs with a low clinical score in G1 compared with G2 Parasitism: reduction in parasite load in liver, spleen, and bone marrow after 4 months of treatment (G1 and G2 - compared to time before treatment); G3 presented high parasite load in comparison to G1 and G2; Skin: reduction in parasitism in G1 compared to G4

[90] Miró *et al*.; 2009	G1: Miltefosine (2 mg⁄kg/every 24h, orally for 28 days) and Allopurinol (10 mg⁄kg/ every 12h, orally for 7 months) / n=37 G2: Meglumine antimoniate (50 mg⁄kg/ every 12h, subcutaneously for 28 days) and Allopurinol (same dose as G1) for 7 months /n=36	Presence of CVL clinical signs; positivity in *Leishmania*-specific IgG; PCR positivity for *L. infantum*; absence of azotemia, proteinuria, negative for specific ehrlichiosis serology	Naturally infected dogs	--	Clinical recovery after 7 months – reduction of clinical score: G1 (89.9%) and G2 (84.1%) Parasitism demonstrated reduction in parasite load greater than 80% after 7 months: G1 (88%) and G2 (86%)
[89] Manna *et al*.; 2015	G1: Meglumine antimoniate (100 mg⁄kg/ every 12h, subcutaneously for 30 days) and Allopurinol (10 mg⁄kg/every 12h, orally for 30 days) /n=9 G2: Miltefosine (2 mg⁄kg/every 24h, orally for 30 days) and Allopurinol (10 mg⁄kg/ every 12h, orally for 30 days)/ n=9 After 30 days G1 and G2 treatment, Allopurinol was continued for 72 months	Presence of CVL clinical signs; positivity in *Leishmania*-specific IgG; PCR positivity for *L. infantum*; absence of renal failure and ehrlichiosis	Naturally infected dogs	Reduction in score of IgG levels: after 72 months (G1: 0.3, G2: 0.8) in comparison to time before treatment (G1: 2.8, G2: 3.3)	Clinical recovery – improvement of clinical score after 72 months (G1:0, G2: 0.4) in comparison to time before treatment (G1: 6.2, G2: 6.0) Reduction in lymph node parasite load by PCR: after 72 months (G1:16, G2: 98) when compared with time before treatment (G1: 4952, G2: 5222)
[79] Pennisi *et al*.; 2005	G1: Metronidazole (25 mg/kg/every 24h) and Spiramycin (150,000 iu/kg/every 24h, orally)/(n=13) G2: Meglumine antimonate (55 to 100 mg/kg/every 12h, subcutaneously) and Allopurinol (20 mg/kg/every 12h, orally)/ n=14 G1 and G2 received 90-day course of treatment antimonial therapy	Negative serology to *Ehrlichia canis*; no concurrent tickborne disease, normal levels for creatinine; Positivity in *Leishmania*-specific IgG; PCR positivity for *L. infantum* in bone marrow and/or lymph node and/or blood; presence of CVL clinical signs	Naturally infected dogs	--	Clinical recovery: at day 90: G1 (10/12), G2 (8/10); at day 210: G1 (9/9), G2 (8/8) PCR Positive: at day 90: G1 (9/12); G2 (8/10); at day 210: G1 (9/9); G2 (6/8)
[78] Bianciardi *et al*.; 2004	G1: Enrofloxacin (20 mg/kg/every 24h, orally for 30 days) /n=12 G2: Enrofloxacin (20 mg/kg/every 24h, orally for 30 days) plus metronidazole (10 mg/kg/ every 24 h, orally for 30 days) / n=12 G3: Meglumine antimoniate (50 mg/kg/ every 12h, subcutaneously for 30 days) / n=12	Negative serology to *Ehrlichia canis*, Positivity in *Leishmania*-specific IgG; positivity (microscopy and/or culture) for *L. infantum* in bone marrow and/or lymph node aspirate; presence of one or more CVL symptoms	Naturally infected dogs	Reduction in IgG levels: G1 (7/12), G2 (5/12); G3 (6/12);	Clinical relapses: 60–90 days after treatment: G1 (5/12) and G2 (6/12), 120 days after treatment: G3 (2/12) Clinical recovery: skin lesions healed: G1 (3/6), G2 (9/11), G3 (10/12); normalization of lymph node size: G1 (7/12), G2 (8/15), G3 (6/8) Parasitism reduction in all groups (G1, G2, G3)

Sb: Sodium stibogluconate

Miltefosine was previously described as an anti-tumor drug and has been presented as an alternative form of chemotherapy with the advantage of being administered orally [85, 86]. CVL miltefosine chemotherapy has been used in oral route, 2 mg/kg/day, for 28 days (Table **2**) and is capable of inducing clinical recovery and parasitism reduction [87, 88]. However, Andrade *et al.* [88] demonstrated a limited effect of monotherapy using miltefosine in CVL treatment with all treated dogs showing parasitism in the bone marrow by PCR (Table **2**). Moreover, combined chemotherapy using miltefosine and allopurinol induced an additional and prominent effect in clinical recovery [80, 90] and a reduction in IgG levels [89] and parasite load [89, 90]. Interestingly, combined chemotherapy using miltefosine and allopurinol (Table **3**) was able to induce similar levels of clinical recovery and reduction in the parasite load when compared to the association with meglumine antimoniate and allopurinol [80, 90, 91]. Moreover, marbofloxacin, another orally administered chemotherapy (Table **2**), has demonstrated important results in CVL clinical recovery and decreased parasite loads [92].

Another treatment option against CVL is domperidone, a benzimidazole compound presenting a selective dopamine D2 receptor antagonist with gastrokinetic and antiemetic activity [93]. It increases prolactin serum levels and triggers a pro-inflammatory type 1 immune response with the production of IFN-γ, IL-12, and TNF-α. Since CVL resistance is related to asymptomatic disease and low parasite burden associated with triggering a type 1 immune response [61, 62], domperidone treatment could be useful for inducing a pro-inflammatory immune profile. In healthy dogs maintained in a *L. infantum* endemic area, domperidone was also able to reduce infection in treated animals [94] and contribute to minimizing partial clinical relapses in CVL [95] (Table **2**). The treatment of *Leishmania braziliensis*-naturally infected dogs was analyzed using furazolidone at 35 mg/kg, two oral doses daily for 21 consecutive days, interspersed with domperidone (1 mg/kg, two oral doses daily for 10 days), resulting in clinical cure of skin lesions without the presence of parasites [96].

Aminosidine, an aminocyclitol aminoglycoside antibiotic, has also been used in CVL treatment (Table **2**). Although clinical recovery and the impairment of IgG levels have been reported after aminosidine treatment, the persistence of parasitism is a frequent finding [78, 97]. Similar results have been described for dimethasulfonate pentamidine CVL [98]. Furthermore, liposomal trifluralin formulation was described as having limited ability to control the parasite burden [99]. Similarly, an important clinical relapse was described in enrofloxacin, with or without being associated with metronidazole [79]; in addition, a high positivity rate in PCR was reported in metronidazole and spiramycin CVL treatment [80] (Table **3**).

Successful variable treatment among the distinct chemotherapies described in this section could be partially explained by drug clearance. Remarkably, after the administration of N-methylglucamine antimoniate, more than 80% of the antimony was excreted in the urine in the first nine hours [100, 101]. Similarly, metronidazole excretion in dogs (4.5h) is about twice as fast as it is in humans (8.7h) [102]. Moreover, aminosidine administration in *L. infantum*-infected dogs showed that the pharmacokinetics was not affected during treatment [103], indicating that any limitation on efficacy is probably due to low parasite response to the drug. Since chemotherapy in CVL is not enough to induce parasite clearance, new approaches need to be associated with drug treatment in order to impede *L. infantum* transmission. In fact, the association of chemotherapy with immunotherapy, including *Leishmania* antigens, could result in better performance in parasite load reduction [62]. Moreover, the association of sand fly antigens in immunotherapy has been considered as a major strategy in blocking CVL transmission since the treatment in dogs has not been successful in preventing it [62].

Table 4. Preclinical trial for analysis of polychemotherapy agents against infection of *Leishmania infantum* in dogs.

Reference		Chemotherapy scheme/number of dogs	Criterion of dog inclusion in the study	Type of infection	Immunological biomarkers	Treatment efficacy (clinical improvement/reduction in parasite load)
[81] Pennisi *et al.*; 2008		G1: Meglumine antimoniate (55-100 mg/kg/every 12 h, subcutaneously for 60 days), and Allopurinol	Presence of CVL clinical signs, positivity in *Leishmania*-specific IgG, PCR (lymph	Naturally infected dogs	Reduction in IgG levels: in all dogs from 3 to 11 dilutions	Clinical relapses 9 months after the treatment: G1 (2/6), G2 (5/9), G3 (2/8)

		(10 mg/kg/every 12h, orally for 90 days) /n=6 G2: Meglumine antimoniate (55-100 mg/kg/every 12h, subcutaneously for 30 days), and Allopurinol (10 mg/kg/every 12h, orally for 90 days) /n=9 G3: Meglumine antimoniate (55-100 mg/kg/every 12h, subcutaneously for 60 days), and Spiramycin (150.000 UI/kg), plus Metronidazole (25 mg/kg/every 12h, orally for 90 days) /n=8 G4: Meglumine antimoniate (55-100 mg/kg/every 12h, subcutaneously for 30 days), and Spiramycin (150.000 UI/kg), plus Metronidazole (25 mg/kg/every 12h, orally for 90 days) /n=6	node/bone marrow and blood) and amastigotes detection in lymph node, bone marrow or skin smears			
[82] Paradies *et al.*; 2012		G1: Meglumine antimoniate (50 mg/kg/ every 12h up to clinico-pathological recovery, subcutaneously)/n=6 G2: Meglumine antimoniate (50 mg/kg/ every 12h for 8 weeks or up to clinic-pathological recovery) and/or followed by Allopurinol (15 mg/kg/every 12h, orally – administered for 6 months after Meglumine antimoniate discontinuing) / n=6 G3: Allopurinol (15 mg/kg/every 12h, orally for 12 months /n=6 G4: Meglumine antimoniate (37.5 mg/kg/ every 6h up to clinico-	Positivity for amastigotes detection in lymph node smears; negative for ehrlichiosis and renal damage	Naturally infected dogs	--	<u>Clinical recovery:</u> G1: 5/6 at 1 month after starting treatment, G2: 6/6 at 1-3 months after starting treatment, G3: 2/6 at 2 months after starting treatment, G4: 6/6 at 1.5 months after starting treatment <u>Clinical relapses</u> one year after the treatment: G1 (3/6), G2 (0/6), G3 (4/6); G4 (6/6) <u>Parasitism</u> in lymph node smears: G1 (3/6), G2 (0/6), G3 (5/6), G4 (6/6)

		pathological recovery, subcutaneously for 21 days) / n=6				
[83] da Silva *et al*.; 2012		G1: Liposomal meglumine antimoniate formulation (6.5 mg Sb/kg/dose, intravenously 6 doses at 4-day intervals) and Allopurinol (20 mg/kg/ every 24h, orally for 140 days starting from the first dose of liposomal formulation) /n=8 G2: Liposomal meglumine antimoniate formulation (6.5 mg Sb/kg/dose, intravenously 6 doses at 4-day intervals) / n=8 G3: Allopurinol (20 mg/kg/every 24h, orally for 140 days) and 6 doses of saline given at the same time intervals as liposomal formulation of G1 group /n=8 G4: Empty liposomes (given at the same time intervals as liposomal formulation of G1 group) and Allopurinol (20 mg/kg/ every 24h, orally for 140 days) /n=8 G5: Empty liposomes (given at the same time intervals as liposomal formulation of G1 group) G6: Saline (6 doses of saline given at the same time intervals as liposomal formulation of G1 group /n=12	Presence of CVL clinical signs, positivity in *Leishmania*-specific IgG, PCR in bone marrow	Naturally infected dogs	Reduction in IgG levels 60 days after the end of treatment compared to time before treatment: G1 (20.3-fold reduction); G2 (1.9-fold reduction); G3 (1.3-fold reduction); G4 (2.5-fold reduction); G5 (1.4-fold reduction); G6 (2.5-fold reduction);	Clinical recovery 60 days after the end of treatment: lower clinical scores in G1, G2, G3, and G4 compared to G5 and G6 Reduction of parasitism (parasite load) in skin, bone marrow, and spleen by PCR showed G1 as the most effective treatment; parasite load by PCR in liver demonstrated negative results in G1 and G2; frequency of *L. infantum*-infected sand flies by Xenodiagnosis: G1 (0), G2 (1.9), G3 (0), G4 (2.5), G5 (30.0), G6 (16.4)
[84]		G1: Mixture of conventional and PEGylated liposomal meglumine antimoniate	Positivity in *Leishmania*-specific IgG, PCR in bone marrow	Naturally infected dogs	Reduction in IgG levels: G1 and G2 displayed lower	Clinical recovery after 4 months of treatment: higher proportion of dogs

Dos Santos *et al*.; 2020		formulations (6.5 mg Sb/kg/dose, intravenously at 4-day intervals, in 2 cycles of 6 doses with an interval of 40 days between both cycles) and Allopurinol (30 mg/kg/every 12h, orally for 130 days starting 30 days before the first dose of liposomal formulation) / n=9 G2: Conventional liposomal meglumine antimoniate formulation (6.5 mg Sb/kg/dose, intravenously at 4-day intervals, in 2 cycles of 6 doses with an interval of 40 days between both cycles) and Allopurinol (30 mg/kg/every 12h, orally for 130 days starting 30 days before the first dose of liposomal formulation) / n=9 G3: Allopurinol (30 mg/kg/every 12h, orally for 130 days) / n=8 G4: Dogs without treatment /n=11			IgG levels compared to G3	with a low clinical score in G1 compared with G2 Parasitism: reduction in parasite load in liver, spleen and bone marrow after 4 months of treatment (G1 and G2 - compared to time before treatment); G3 presented high parasite load in comparison to G1 and G2; Skin: reduction in parasitism in G1 compared to G4
[90] Miró *et al*.; 2009		G1: Miltefosine (2 mg⁄ kg/every 24h, orally for 28 days) and Allopurinol (10 mg⁄kg/ every 12h, orally for 7 months) / n=37 G2: Meglumine antimoniate (50 mg⁄ kg/ every 12h, subcutaneously for 28 days) and Allopurinol (same dose as G1) for 7 months /n=36	Presence of CVL clinical signs; positivity in *Leishmania*-specific IgG; PCR positivity for *L. infantum*; absence of azotemia, proteinuria, negative for specific ehrlichiosis serology	Naturally infected dogs	--	Clinical recovery after 7 months – reduction of clinical score: G1 (89.9%) and G2 (84.1%) Parasitism demonstrated as reduction of the parasite load greater than 80% after 7 months: G1 (88%) and G2 (86%)

[89] Manna *et al*.; 2015		G1: Meglumine antimoniate (100 mg⁄kg/ every 12h, subcutaneously for 30 days) and Allopurinol (10 mg⁄kg/every 12h, orally for 30 days) /n=9 G2: Miltefosine (2 mg⁄kg/every 24h, orally for 30 days) and Allopurinol (10 mg⁄kg/ every 12h, orally for 30 days)/ n=9 After 30 days G1 and G2 treatment, Allopurinol was continued for 72 months	Presence of CVL clinical signs; positivity in *Leishmania*-specific IgG; PCR positivity for *L. infantum*; absence of renal failure and ehrlichiosis	Naturally infected dogs	Reduction in score of IgG levels: after 72 months (G1: 0.3, G2: 0.8) in comparison to time before treatment (G1: 2.8, G2: 3.3)	Clinical recovery – improvement of clinical score after 72 months (G1:0, G2: 0.4) in comparison to time before treatment (G1: 6.2, G2: 6.0) Reduction in lymph node parasite load by PCR: after 72 months (G1:16, G2: 98) in comparison to time before treatment (G1: 4952, G2: 5222)
[79] Pennisi *et al*.; 2005		G1: Metronidazole (25 mg/kg/every 24h) and Spiramycin (150,000 iu/kg/every 24h, orally)/(n=13) G2: Meglumine antimonate (55 to 100 mg/kg/every 12h, subcutaneously) and Allopurinol (20 mg/kg/every 12h, orally)/ n=14 G1 and G2 received 90-day course of treatment antimonial therapy	Negative serology to *Ehrlichia canis*; no concurrent tickborne disease, normal levels for creatinine; Positivity in *Leishmania*-specific IgG; PCR positivity for *L. infantum* in bone marrow and/or lymph node and/or blood; presence of CVL clinical signs	Naturally infected dogs	--	Clinical recovery: at day 90: G1 (10/12), G2 (8/10); at day 210: G1 (9/9), G2 (8/8) PCR Positive: at day 90: G1 (9/12); G2 (8/10); at day 210: G1 (9/9); G2 (6/8)
[78] Bianciardi *et al*.; 2004		G1: Enrofloxacin (20 mg/kg/every 24h, orally for 30 days) /n=12 G2: Enrofloxacin (20 mg/kg/every 24h, orally for 30 days) plus metronidazole (10 mg/kg/ every 24 h, orally for 30 days) / n=12 G3: Meglumine antimoniate (50 mg/kg/ every 12h, subcutaneously for 30 days) / n=12	Negative serology to *Ehrlichia canis*, Positivity in *Leishmania*-specific IgG; positivity (microscopy and/or culture) for *L. infantum* in bone marrow and/or lymph node aspirate; presence of one or more CVL symptoms	Naturally infected dogs	Reduction in IgG levels: G1 (7/12), G2 (5/12); G3 (6/12);	Clinical relapses: 60–90 days after treatment: G1 (5/12) and G2 (6/12), 120 days after treatment: G3 (2/12) Clinical recovery: skin lesions healed: G1 (3/6), G2 (9/11), G3 (10/12); normalization of lymph node size: G1 (7/12), G2 (8/15), G3 (6/8) Parasitism reduction in all analyzed groups (G1, G2, G3)

Sb: Sodium stibogluconate

CONCLUSION

Studies using the dog model for chemotherapy of Chagas disease are still rare. The therapeutic efficacy or parasitological cure with BZ in dogs was similar to that observed in the murine model for most *T. cruzi* strains using the same post-treatment evaluations. The parasitological and serological tests showed similar results to the ones reported in human. Benefits of the etiological treatment on disease evolution were demonstrated at acute and chronic phases with BZ, drug associations, and ravuconazole. BZ in dogs presented better therapeutic results than the two EBIs studied. The cytokine profile was compatible with the parasitological, histopathological, clinical, and therapeutic results, suggesting immune participation in infection modulation. Dogs are considered an appropriate experimental model because they are evolutionarily closer to humans, presents similar cardiac physiology and disease evolution, and allow follow-up over a longer period of time, making it possible to reproduce the later clinical changes of Chagas disease. Furthermore, they are docile and easy for management under experimental conditions. Globally, the findings demonstrated to be the dog an excellent model for studies involving the use of chemotherapy for Chagas disease and recommend its use for preclinical studies of new drug candidates. With regard to Leishmaniasis, the biggest challenge to developing new effective chemotherapeutical approaches against *Leishmania* infection using the dog as experimental model or as a patient in a veterinary clinic concerns the ability of this animal species to maintain effective levels of the drug. Dogs present physiological characteristics in their metabolism that directly affect drug excretion, which likely contributes to limiting parasite clearance, resulting in frequent clinical relapses regardless of treatment. Taking into account the high excretion rate observed in dogs using different chemotherapeutic agents, the development of strategies aimed at retaining the minimal drug concentration becomes critical to be overcome. In this sense, drug-vectoring systems that target drug delivery, as observed in liposomal formulations, could provide a suitable approach that might lead to important advances in CVL treatment. Lastly, the inclusion of sand fly antigens in the CVL treatment protocols could provide a relevant strategy for blocking *L. infantum* transmission without the need for public health interference.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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