UNIVERSIDADE ESTADUAL DE MONTES CLAROS

Patrícia Natalicia Mendes de Almeida

Alterações clínicas e cardíacas em cães sintomáticos com leishmaniose visceral na cidade de Montes Claros, Minas Gerais, Brasil.

Montes Claros – Minas Gerais

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Co-orientadora: Profª. Drª. Thallyta Maria Vieira

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Prof.ª Dr.ª Thallyta Maria Vieira COORIENTADOR (participação à distância por videoconferência)

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“Além do amor e da simpatia, os animais exibem
outras qualidades ligadas aos instintos sociais que,
em nós, seriam chamados de morais.”
(Charles Darwin)

RESUMO

Infecções causadas por *Leishmania* spp. representam uma das maiores causas de problemas de saúde pública no mundo relacionadas a infecções por protozoários. Os cães e os humanos são os indivíduos mais frequentemente infectados pelos agentes da leishmaniose visceral, que corresponde à forma mais grave da doença. Entretanto, pouco é conhecido sobre as alterações cardíacas associadas aos quadros dessa protozoonose. Neste estudo, o objetivo foi identificar alterações cardíacas nos cães sintomáticos com leishmaniose visceral naturalmente infectados. Foram selecionados 20 cães machos, adultos, sintomáticos e positivos para leishmaniose visceral, diagnosticados através do exame clínico e no teste de ELISA. Dentre os sinais clínicos, houve maior ocorrência de lesões cutâneas, esplenomegalia e linfadenopatia periférica. Apos sedação e anestesia, avaliou-se a eletrofisiologia cardíaca, seguida de mensuração da pressão arterial. Coletou-se sangue em frasco com EDTA para dosagem da fração MB da creatina fosfoquinase (CK-MB). Posteriormente foi promovida eutanásia dos animais. Coletou-se, então, um segmento da musculatura cardíaca do ventrículo direito e a extirpação completa da válvula mitral, para avaliação histopatológica. Lâminas, em duplicata, foram preparadas, uma silanizada corada por picrosirius, para análise da presença de fibras colágenas, e outra em lâmina convencional, coradas com HE para estudo de alterações celulares, proliferativas e pesquisa de amastigotas de *Leishmania infantum.* Ao avaliar os eletrocardiogramas, o segmento QT apresentou duração média acima do limite máximo fisiológico e a onda R apresentou amplitude superior aos índices de normalidade para todos os animais avaliados. Onze cães (55%) apresentaram pressão arterial sistêmica acima da média esperada. A dosagem de CK-MB estava elevada em todos. Oitenta por cento (16/20) dos cães apresentaram alterações cardíacas ao exame histopatológico. Nos cortes corados por HE, infiltrado lipídico isolado ou associado a infiltrados inflamatórios e necrose da banda de contração foram as lesões mais frequentemente detectadas (80%), seguida pela necrose da banda de contração, única ou associada a outras lesões em 66,66% dos animais. Amastigotas foram identificadas nos tecidos cardíacos de dois animais. Sob a coloração picrosirius verificou-se aumento de fibra colágena em 95% dos cães. Nove dos animais apresentaram nódulos na válvula mitral e destes, em oito confirmou-se valvulopatia à histolopatologia, sendo fibrose valvar e metaplasia adiposa as mais frequentes. Portanto, neste estudo a leishmaniose visceral canina provavelmente desencadeou alterações cardíacas.

Palavras-chave: Leishmaniose visceral canina; Cardiopatia crônica; Eletrocardiograma.

ABSTRACT

Infections caused by *Leishmania* spp. represent one of the major causes of public health problems in the world related to the protozoan community. Dogs and humans are the individuals most frequently infected by visceral leishmaniasis agents, which correspond to the most severe form of the disease. However, little is known about the cardiac alterations associated with this protozoonosis. In this study, the objective was to identify cardiac alterations in symptomatic dogs with naturally infected visceral leishmaniasis. Twenty adult male dogs, symptomatic and positive for visceral leishmaniasis, were selected through clinical examination and ELISA test. Among the clinical signs, there was a higher occurrence of skin lesions, splenomegaly and peripheral lymphadenopathy. After sedation and anesthesia, evaluating cardiac electrophysiology, followed by measurement of blood pressure. Blood was collected in a vial with EDTA for measurement of the MB collection of creatine phosphokinase (CK-MB). the animals were subsequently euthanized. Then, a segment of the heart muscle of the right ventricle and the complete removal of the mitral valve were collected for histopathological evaluation. Slides, in duplicate, were prepared, one silanized stained by picrosirius, for analysis of the presence of collagen fibers, and another in a conventional slide, stained with HE for the study of cellular and proliferative alterations and research of amastigotes of *Leishmania infantum*. When evaluating the electrocardiograms, the QT segment had an average duration above the maximum physiological limit and the R wave had an amplitude greater than the normality indices for all evaluated animals. Eleven dogs (55%) had systemic blood pressure above the expected mean. The CK-MB dosage was high in all of them. Eighty percent (16/20) of the dogs showed cardiac alterations on histopathological examination. In stained with HE, lipid infiltrate or isolated associated with inflammatory infiltrates and necrosis of the contraction band were the most frequently detected lesions (80%), followed by necrosis of the contraction band, alone or associated with other lesions in 66.66% of the sections of the animals. Amastigotes were identified in the cardiac tissues of two animals. Under the colors picrosirius tolerated increased collagen fiber in 95% of dogs. Nine of the animals had nodules on the mitral valve and of these, in eight, valvulopathy was confirmed by histolopathology, with valve fibrosis and adipose metaplasia being the most frequent. Therefore, in this study, canine visceral leishmaniasis probably triggered cardiac alterations.

Keywords: Canine visceral leishmaniasis; Chronic heart disease; Electrocardiogram.

LISTA DE ILUSTRAÇÕES

metaplasia (thick arrows) under picrosirius staining ....

LISTA DE ABREVIATURAS E LISTA DE SIGLAS

CCZ – MOC	Centro de Controle de Zoonoses do município de Montes Claros, MG, Brasil
CK-MB	Creatino fosfoquinase Fração MB
ECG	Eletrocardiograma
ELISA	Ensaio Imunoenzimático
Fiocruz	Fundação Osvaldo Cruz
HE	Hematoxilina e Eosina
ICB-UFMG	Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais
LV	Leishmaniose visceral
LVC	Leishmaniose visceral canina
LVH	Leishmaniose Visceral Humana
MS	Ministério da Saúde
OMS	Organização Mundial de Saúde
OPAS	Organização Pan-Americana da Saúde
PCR	Reação em cadeia da polimerase
RIFI	Reação de Imunofluorescência Indireta
WHO	World Health Organization

SUMÁRIO

1. INTRODUÇÃO

1.1. A leishmaniose

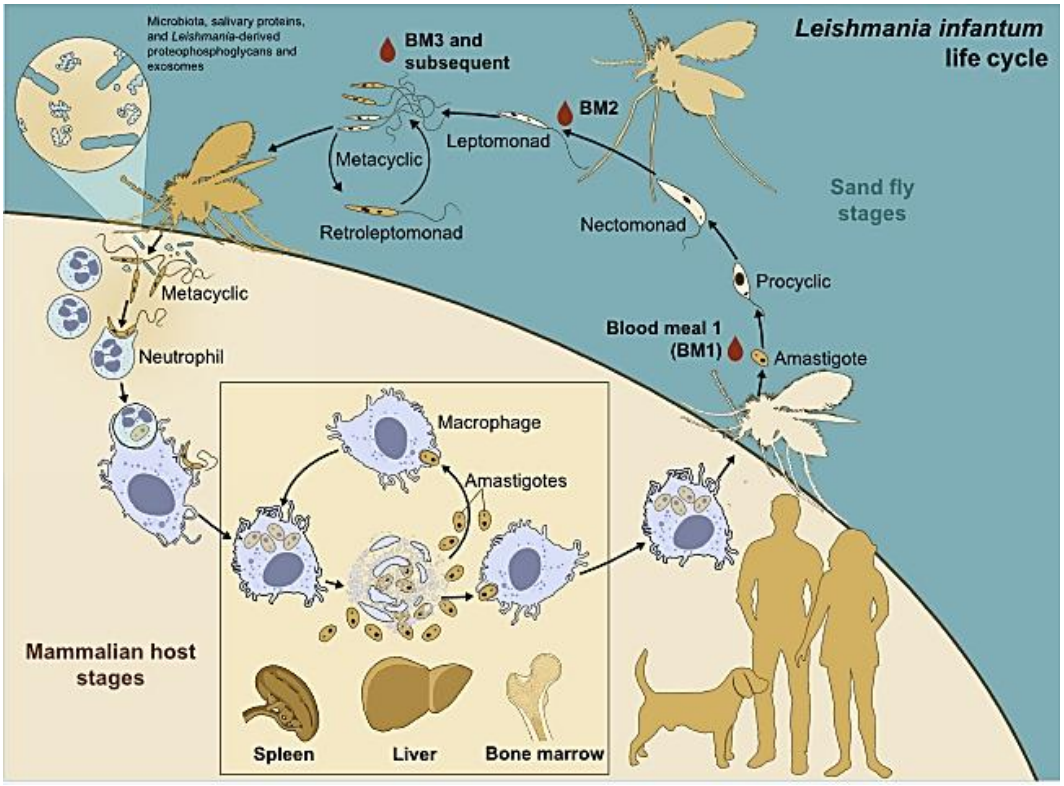
As leishmanioses são um grupo de doenças causadas por protozoários de mais de 20 espécies de *Leishmania*. Esses parasitos são transmitidos aos seres humanos pelas picadas da fêmea infectada de um flebotomíneo. Estima-se que 50.000 a 90.000 novos casos de leishmaniose visceral (LV) ocorram anualmente em todo o mundo, entretanto apenas 25 a 45% são relatados à Organização Mundial da Saúde (OMS). Em 2020, mais de 90% dos novos casos notificados ocorreram em 10 países: Brasil, China, Etiópia, Eritreia, Índia, Quênia, Somália, Sudão do Sul, Sudão e Iêmen segundo a *World Health Organization* (WHO, 2022).

A doença ocorre principalmente em pessoas socialmente menos favorecidas na África, Ásia e América Latina, e está associada ao sistema imunológico debilitado, desencadeado por desnutrição ou outros fatores, como deslocamento da população para áreas próximas às matas, moradia precária com acúmulo de matéria orgânica (locais de proliferação do agente transmissor), e falta de recursos. Dos 200 países e territórios que se reportam à Organização Mundial da Saúde, 98 eram endêmicos para leishmaniose em 2018 segundo a Organização Pan-Americana de Saúde (OPAS, 2019). Na América Latina, dos casos de leishmaniose visceral humana (LVH) apontados em 2021, 93,5% ocorrem no Brasil (OPAS, 2022). Deve-se ter atenção para um diagnóstico correto e precoce, fatores fundamentais para a sobrevida do paciente a uma patologia na qual o número de óbitos é elevado.

O cão doméstico é o principal hospedeiro definitivo do parasito, e para adquirir a leishmaniose visceral canina (LVC) é necessário que a fêmea do vetor *Lutzomia longipalpis* sugue o sangue de um hospedeiro infectado e que nesse sangue tenha a presença de macrófagos infectados com amastigotas de *Leishmania spp.* (BHATIA; GOLI, 2017). Essas células serão rompidas no trato digestivo anterior da fêmea. Com a lise dessas células, as amastigotas se reproduzem por divisão binária e mudam de fase, sendo a primeira delas a promastigota, que é flagelada. Com o passar dos dias, se tornam promastigotas metacíclicas, forma infectante do parasito, que migram para as probóscides. Essa fêmea infectada pica um novo hospedeiro vertebrado, inoculando no momento do repasto sanguíneo, as promastigotas metacíclicas junto com a saliva. No vertebrado, a forma metacíclica será engolfada pelas células do sistema fagocítico mononuclear. Aqui a promastigota irá se diferenciar em amastigota e multiplicar-se no vacúolo parasitóforo de macrófagos até o seu rompimento. Posteriormente, outros

macrófagos irão fagocitar esses parasitos, ocorrendo a disseminação hematogênica para todo o organismo (Figura 1) (BANETH; SOLANO-GALLEGO, 2015).

Figura 1 - Ciclo de vida da *Leishmania infantum*, causando a leishmaniose visceral em vertebrados. Fonte: adaptado de Serafim et al. (2020).



1.2. Hospedeiros definitivos da *Leishmania infantum*

Os hospedeiros mais comuns da LV são os cães e os humanos (JAMBULINGAM et al., 2017). Dentre os animais silvestres são também hospedeiros os canídeos *Cerdocyon thous* (raposa cinzenta), *Pseudalopex* **(***Lycalopex***)** *vetulus* (raposa do pé seco) (COSTA; COURTENAY, 2003), *Spheotos venaticos* (cachorro-do-mato), *Chrysocyon brachyurus* (lobo-guará) (LUPPI et al., 2008), e os *Didelphis albiventris* (marsupiais) (JORGE et al., 2010).

No cão, o período de incubação pode variar de 2 meses até 6 anos, porém os primeiros anticorpos podem ser encontrados 45 dias após a infecção. No flebótomo, o período entre a contaminação e a eliminação da forma infectante é de 4 a 21 dias (RIBEIRO, 2007).

1.3. Vias de transmissão da leishmaniose visceral canina

O principal agente transmissor da *Leishmania infantum* nas Américas é o *Lutzomia longipalpis*. Popularmente conhecido como mosquito palha, tatuquiras ou birigui, apresenta

hábitos crepusculares e pós-crepusculares. Seus *habitats* são lugares úmidos, sombrios e bem protegidos dos ventos. São encontrados na natureza em tocas de animais, buracos de pau e ocos de bambu. Possui vasta distribuição nos climas quentes e temperados e as fêmeas são hematófagas. Todavia, em cães outras rotas são conhecidas, como a transmissão vertical e a participação de outros ectoparasitas (MARQUES, 2008).

1.4. Sinais clínicos da leishmaniose visceral canina

Por infectarem vários órgãos, as manifestações clínicas são diversas. Os sinais clínicos da LV em cães são linfadenomegalia, hepatoesplenomegalia, emagrecimento, prostração, febre, palidez, hemorragias (epistaxe), poliartrites, lesões oculares (blefaroconjuntivites/ ceratoconjuntivite), reação local associado à picada do vetor, dermatite seborreica, alopecia periorbital, hiperqueratoses, nódulos subcutâneos, onicogrifose, erosões e úlceras (pontas de orelha/focinho) não-pruriginosas. Porém, em 60% dos casos, os cães com a doença podem ser assintomáticos. A diversidade, assim como a ausência de sintomas são fatores que torna dificultado o diagnóstico clínico (BRAZ et al., 2015; SILVA; WINCK, 2018; SOLANO-GALLEGO et al., 2009).

1.5. Creatino fosfoquinase, fração MB em cães com LVC

Dentre os exames auxiliares, aumentos nos níveis séricos da fração MB da creatino fosfoquinase (CK-MB) indicaram lesões em cardiomiócitos, além de alterações eletrocardiográficas (MENDES et al., 2014; GODOY et al., 2016; ALVES et al., 2010).

1.6. Diagnóstico da leishmaniose visceral canina

O exame parasitológico é o método com maior especificidade para diagnóstico da leishmaniose em caninos domésticos. Nele observa-se a presença de formas amastigotas do parasito no material coletado. Esse material pode ser obtido através de punções hepática, de linfonodos, esplênica, de medula óssea e biópsia ou escarificação de pele. As provas de sorologia comumente utilizadas são: reação de imunofluorescência indireta (RIFI); ensaio imunoenzimático (ELISA); fixação do complemento, aglutinação direta, testes rápidos para calazar rK39 (GUSMÃO et al., 2009) e mais recentemente os testes moleculares, como a reação em cadeia da polimerase - PCR (AKHOUNDI et al., 2017).

No Brasil, o Ministério da Saúde em conjunto com o Conselho Federal de Medicina Veterinária (CFMV) recomenda, para animais, o teste rápido imunocromatográfico em plataforma de duplo percurso (TR-DPP) na triagem e o Ensaio Imunoenzimático (Elisa) como confirmatório, ambos produzidos pelo laboratório público Bio-Manguinhos/Fiocruz (BRASIL, 2020). Caso o médico veterinário ou o tutor do animal queiram, existe a possibilidade de realizar outros testes, como a Imunofluorescência Indireta (IFI); parasitológico direto de lesões de pele, punção de linfonodo ou de aspirado de medula óssea; reação de cadeia de polimerase (PCR); entre outros (AKHOUNDI et al., 2017).

Embora a literatura científica considere o diagnóstico parasitológico o método de certeza, esses processos tendem a ser mais invasivos, enquanto os sorológicos são realizados com soro sanguíneo coletado em veias periféricas, preferencialmente na jugular (BRASIL, 2020).

Apesar dos achados em seu relato, Mendes et al. (2014) afirmaram não ser possível inferir categoricamente o mecanismo envolvido no comprometimento cardíaco do cão por eles estudados, devido a outras condições sob as quais o animal se encontrava, sugerindo a necessidade de maior volume e aprofundamento em estudos dessa ordem.

2. OBJETIVOS

2.1.Objetivo geral

Identificar alterações clínicas e cardíacas em cães sintomáticos com leishmaniose visceral naturalmente infectados.

2.2.Objetivos específicos

- Identificar alterações clínicas em cães naturalmente infectados por *Leishmania infantum*.
- Avaliar a eletrofisiologia cardíaca de cães naturalmente infectados por *Leishmania infantum* através de eletrocardiograma.
- Identificar a presença e os tipos de alterações celulares e proliferativas em musculatura cardíaca e válvula mitral de cães sintomáticos naturalmente infectados por *Leishmania infantum*.
- Identificar coinfecções por hemoparasitos e avaliar os sinais clínicos e hematológicos encontrados em cães naturalmente infectados no município de Montes Claros, MG, Brasil.

3. METODOLOGIA

Essa dissertação foi composta por dois produtos, sendo que cada um empregou um percurso metodológico diferente para atingir o objetivo de identificar alterações clínicas e cardíacas em cães sintomáticos com leishmaniose visceral naturalmente infectados

O primeiro estudo, "Sinais clínicos e hematológicos em cães naturalmente coinfectados com leishmaniose visceral e hemoparasitoses– comunicação breve", foi conduzido no município de Montes Claros, situado na Região Norte de Minas Gerais, no ano de 2020. Participaram do estudo 20 cães com diagnóstico positivo para leishmaniose visceral pelo teste ELISA. Foram realizadas análises de sangue para verificação da concentração de enzimas marcadoras de lesão cardíaca (CK-MB) e também realizado diagnóstico diferencial para *Babesia* spp. e *Erlichia* spp., ambos através de PCR convencional. De acordo com as patologias concomitantes identificadas, obteve-se quatro grupos de animais, sendo eles animais com *L. infantum*, animais com *L. infantum*. e *Babesia* spp., animais com *L. infantum*. e *Erlichia* spp. e animais com *L. infantum*., *Babesia* spp. e *Erlichia* spp. Foi realizado teste ANOVA com os quatro grupos de animais; também o Teste não-paramétrico de Kruskal-Wallis com comparações em pares (Dwass-Steel-Chritchlow-Fligner) dos grupos de animais, sendo confrontadas todas as variáveis; e por fim, calculou-se o coeficiente de correlação dos postos de Spearman (ρ de Spearman). O artigo foi formatado e submetido de acordo com as normas do periódico Veterinary Microbiology (ISSN: 0378-1135, classificação Qualis A1 na área Interdisciplinar no quadriênio 2017-2020) (Link das normas: https://www.elsevier.com/journals/veterinary-microbiology/0378-1135/guide-for-authors).

O segundo estudo, "Alterações cardíacas em cães com leishmaniose visceral na cidade de Montes Claros, Minas Gerais, Brasil", também foi conduzido no município de Montes Claros no ano de 2020. Foram selecionados 20 cães machos, adultos, positivos no exame ELISA para leishmaniose visceral, nos quais foram avaliados os sinais clínicos e alterações cardíacas, tanto eletrocardiográficas quanto histopatológicas. Os sinais clínicos (exceto pressão arterial), caracterização do segmento ST, eixo elétrico cardíaco, ritmo cardíaco e os cortes histológicos foram analisados através de estatística descritiva. Já os parâmetros mensuráveis numericamente (pressão arterial, ondas eletrocardiográficas e parâmetros hematológicos) foram quantificados e analisados de acordo com os níveis fisiológicos recomendados pela literatura científica para cada um deles. A seguir, calculou-se o coeficiente de correlação dos postos de Spearman para

todos os parâmetros, comparados de dois em dois. O artigo foi formatado conforme normas do periódico Veterinary Parasitology (ISSN: 0304-4017, classificação Qualis A1 na área Interdisciplinar no quadriênio 2017-2020) (Link das normas: https://www.elsevier.com/journals/veterinary-parasitology/0304-4017/guide-for-authors).

Detalhes dos métodos e análises conduzidas estão descritos na metodologia de cada um dos produtos apresentados a seguir.

4. PRODUTOS TÉCNICO-CIENTÍFICOS GERADOS

5.1 Produto 1:

Clinical and hematological signs in dogs naturally co-infected with visceral leishmaniasis and hemoparasites – brief communication

Patrícia Natalicia Mendes de Almeida[1, 2]
Chrystian Iezid Maia e Almeida Feres[3]
Rebeca Mendes Rocha[1]
Agostinho Gonçalves Viana[4]
Guilherme Rafael Gomide Pinheiro[4]
Ricardo Toshio Fujiwara[4]
Jamille Fernandes Lula [1]
Leandro de Freitas Teles [1]
Thallyta Maria Vieira[1]
Sílvio Fernando Guimarães de Carvalho[1]

[1]Postgraduate Program in Health Sciences at the State University of Montes Claros (PPGCS-UNIMONTES) - Montes Claros-Minas Gerais, Brazil.
[2] Pitágoras Integrated Colleges University Center (UNIFIPMOC) - Montes Claros-Minas Gerais, Brazil.
[3] Graduate Program in Horticulture at the São Paulo State University "Júlio de Mesquita Filho" (UNESP) – São Paulo – São Paulo, Brazil.
[4] Institute of Biological Sciences of the Federal University of Minas Gerais (ICB-UFMG) – Belo Horizonte – Minas Gerais, Brazil.

Highlights

• *Babesia* spp. and *Erlichia* spp. can co-infect dogs with leishmaniasis;

• Dogs that are co-infected or do not show the same clinical and hematological signs.

• Dogs from endemic areas should be tested for various hemoparasites.

Summary

Canine visceral leishmaniasis, as well as hemoparasitoses, have significant mortality rates in dogs that are not properly or not treated at all. The objective was to analyze the clinical and hematological signs in dogs naturally co-infected with visceral leishmaniasis and hemoparasitosis, in the municipality of Montes Claros, MG, Brazil. Twenty dogs with CVL

were evaluated, and blood samples were collected with and without EDTA; clinical signs and systolic blood pressure were evaluated. After conventional PCR to verify co-infections, the animals were grouped into four groups according to the infecting hemoparasites: 1: *L. infantum*; 2: *L. infantum* + *Babesia* spp.; 3: *L. infantum* + *Erlichia* spp.; 4: *L. infantum* + *Babesia* spp. + *Erlichia* spp. An ANOVA test was performed with the four groups seeking interference from different parasites on the evaluated parameters. There was coinfection with *Leishmania* spp. with *Erlichia* spp. or *Babesia* spp. or both in 70% (14/20) of the dogs, the most common being the association of the three microorganisms studied. These concomitants did not interfere with the presentation of clinical signs and/or hematological parameters. Studies with a greater number of observations of animals over time (longitudinal studies) are recommended to categorically clarify such interactions.

Keywords: Canine visceral leishmaniasis. Hematological changes. *Babesia* spp.. *Erlichia* spp..

Introduction

Among the hemoparasites of dogs mentioned in the literature, *Erlichia* spp., *Babesia* spp. and *Leishmania* spp. are considered pathogens with a worldwide distribution, especially in regions with a tropical climate (Buddhachat et al., 2020; Brasil, 2022), such as the north of the state of Minas Gerais, Brazil (Alvares et al., 2013). Co-infections of dogs by these agents are possible thanks to the overlapping areas of activity of their vectors and the large population of both vectors and vertebrate hosts (Mekuzas et al., 2009; Valente, 2014).

Leishmaniasis is a group of diseases caused by protozoa of more than 20 species of *Leishmania* (OPAS, 2023). The domestic dog is the main definitive host of canine visceral leishmaniasis (CVL).

Microorganisms of the genus Ehrlichia are transmitted to dogs by the infected tick Rhipicephalus sanguineus, being obligate intracellular blood cells, although they have a predilection for the mononuclear phagocytic system (Valente, 2014).

Canine babesiosis is caused by protozoa of the genus *Babesia*, with the species B. canis and B. gibsoni identified as infecting dogs (Valente, 2014). The geographic distribution of the pathology coincides with endemic regions for the tick R. sanguineus, its main transmitting agent (Solano-Gallego et al, 2009).

Both canine VL and hemoparasitoses have high morbidity and mortality rates if not properly treated (Sykes, 2014).

Thus, the present study aimed to analyze the clinical and hematological signs in dogs naturally co-infected with visceral leishmaniasis and hemoparasitosis, in the municipality of Montes Claros, MG, Brazil.

Material and methods

This experiment was carried out in the municipality of Montes Claros, which is located in the north of the state of Minas Gerais, and is recognized as endemic for visceral leishmaniasis (Brasil, 2022).

Twenty adult male dogs positive for visceral leishmaniasis in the ELISA test were evaluated. The project for this research was approved by the Ethics and Animal Welfare Commission of UNIMONTES / CEEBEA-UNIMONTES, having been approved in accordance with opinion number 192, of July 12, 2019, and changes in the project were also approved in accordance with opinion certificate number 234, of October 24, 2021.

The animals were sedated with 2ml of 1% acepromazine intramuscularly and then 1g sodium thiopental diluted in sterile saline solution (final concentration: 2.5%) at a dose of

1ml/kgBW intravenously (Viana, 2014) . The animals were clinically evaluated according to Feitosa (2014).

Blood was collected by venipuncture, in three 5ml bottles, one with EDTA and the other two without additives, which were identified and packed in an isothermal box with recyclable ice, at a temperature of around 22°C.

In blood with EDTA, a complete blood count was performed using a Bio 2900 Vet® device (Alere, São Paulo, Brazil). Cardiac muscle injury enzyme (CK-MB) was also analyzed in a BS 120® model equipment (Shenzhen Mindray Bio-Medical Electronics Co. Ltd., China).

The blood without reagent was centrifuged and the serum was sent to the laboratory of the Institute of Biological Sciences of the Federal University of Minas Gerais – ICB-UFMG for identification of *Babesia* spp. and *Erlichia* spp. through conventional PCR adopting the following methodology.

PCR for Babesia sp.

PIRO-A (AATACCCAATCCTGACACAGGG) and PIRO-B antisense (TTAAATACGAATGCCCCCAAC) primers were used, which amplified approximately 410 bp of the ssu-rDNA portion of *Babesia* spp. (Carret et al., 1999; Olmeda et al., 1997).

PCR used Supermix (Invitrogen, Brazil), 4 μL of DNA (approximately 200 ng) and 1 μL of each primer (10 pmol), in a total volume of 25 μL. PCR conditions consisted of an initial denaturation at 94°C for 4 minutes, 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 60 seconds, followed by a final extension at 72°C for 7 minutes.

The amplified products were subjected to electrophoresis on a 2% agarose gel (Invitrogen, Brazil) in Tris-Borate EDTA running buffer (TBE - 50 mM Tris, 50 mM boric acid, 2.5 mM EDTA, pH 8.0), stained with SYBR safe DNA gel strain (Invitrogen, Brazil) and

examined on an ultraviolet light transilluminator. The bands were compared with the molecular size plus DNA leadder standard (Invitrogen, Brazil) of 100 bp.

For RFLP analysis of Piro-A / B-PIRO amplification products, 1/5 of each amplification product was digested for 3 hours with Hinfl restriction enzyme (10 U) or TaqI restriction enzyme (10 U) in its appropriate buffer (Termo Scientific). Digestion products were visualized on a 2% gel system (Invitrogen, Brazil).

PCR for Ehrlichia sp.

The primers EHCA sense (CAATTATTTATAGCCTCTGGCTATAGC) and EHCA antisense (TATAGGTACCGTCATTATCTTCCCTAT) were used, which amplified approximately 389bp of the portion of the 16S rRNA from Ehrlichia canis (Wen et al., 1997).

PCR used supermix (Invitrogen, Brazil), 4 μL of DNA (approximately 200 ng) and 1 μL of each primer (10 pmol), in a total volume of 25 μL. PCR conditions consisted of an initial denaturation at 94°C for 10 minutes, 40 cycles of denaturation at 94°C for 60 seconds, annealing at 60°C for 60 seconds, and extension at 72°C for 60 seconds, followed by a final extension at 72°C for 4 minutes.

The amplified products were subjected to gel electrophoresis under the same conditions described in the previous technique (PCR for *Babesia* spp.).

PCR for Leishmania infantum

The Leish F kDNA primers (CGTGGGGGAGGGGCGTTCT) and the Leish R kDNA primer (CCGAAGCAGCCGCCCCTATT) were used, which amplify approximately 135pb of the *Leishmania* ssp. kDNA portion (CARDOSO et al., 2019)

PCR used supermix (Invitrogen, Brazil), 2 μL of DNA (approximately 100 ng) and 1 μL of each primer (2 pmol), in a total volume of 25 μL. PCR conditions consisted of an initial denaturation at 94°C for 5 minutes, 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds, followed by a final extension at 72 °C for 7 minutes.

The amplified products were subjected to electrophoresis on a 1.5% agarose gel (Invitrogen, Brazil) in Tris-Borate EDTA running buffer (TBE - 50 mM Tris, 50 mM boric acid, 2.5 mM EDTA , pH 8.0), stained with SYBR safe DNA gel strain (Invitrogen, Brazil) and examined under an ultraviolet light transilluminator. The bands were compared with the molecular size plus DNA leadder standard (Invitrogen, Brazil) of 100 bp.

For all PCR analyses, positive and negative controls were used as an internal reaction control.

Blood pressure measurement was obtained with the animals in a deep anesthetic plane throughout the evaluation. The measurement was performed in five repetitions in each animal, using a DeltaLife veterinary sphygmomanometer and Portable Vascular Doppler dv 610V. The technique adopted was presented by Feitosa (2014).

2.1 Statistical analyzes

By grouping the animals according to the identified pathologies, four groups of animals were obtained: 1: *L. infantum*.; 2: *L. infantum*. and *Babesia* spp.; 3: *L. infantum*. and *Erlichia* spp.; 4: *L. infantum*, *Babesia* spp. and *Erlichia* spp.

An ANOVA test was performed with the four groups, and the parameters were analyzed using the statistical program Jamovi® version 2.3.17 for Windows, with 95% significance ($p<0.05\%$).

Table 1

Variables analyzed in dogs naturally infected with *Leishmania infantum* in the municipality of Montes Claros, Minas Gerais, Brazil. (n=20).

Parameter	Variable
Clinical signs	Systolic blood pressure Skin lesions Weight loss and loss of muscle mass Splenomegaly Hepatomelagia Peripheral lymphadenopathy Onychogryphosis (big nails) Uveitis
Hematological parameters	Hemoglobin in percentage Platelets per cubic millimeter Total leukocytes per cubic millimeter Total protein in grams per deciliter Albumin in grams per decilitre Globulin in grams per decilitre Albumin / globulin ratio Creatine in milligrams per deciliter Creatine kinase MB isoenzyme (CK-MB)

With the aid of the same statistical program, the Kruskal-Wallis non-parametric test was adopted with pairwise comparisons (Dwass-Steel-Chritchlow-Fligner) of groups of animals, with all variables being compared (Table 1). Finally, the Spearman rank (Spearman's ρ) correlation coefficient was calculated, using the transformed variable value [$x'= (\sqrt{(x + 0.5)}$].

Results

It was verified, associated with *L. infantum*, the presence of *Erlichia* spp. or *Babesia* spp. or both in 70% (14/20) of the dogs in this study (Table 2).

Table 2

Proportion of co-infection with hemoparasites in naturally infected dogs with visceral leishmaniasis in the municipality of Montes Claros, MG (n=20).		
Pathogenic agentes	%	n
L. infantum. + *Babesia* sp + *Erlichia* sp.	45%	9
L. infantum	30%	6
L. infantum. + *Babesia* sp.	15%	3
L. infantum. + *Erlichia* sp.	10%	2

Of the evaluated animals, 100% were symptomatic, 5% (1/20) oligosymptomatic and 95% (19/20) polysymptomatic. Among the identified clinical signs, there was a higher prevalence of skin lesions and splenomegaly, each of which was observed in 70% (14/20) of the dogs. Another highly prevalent clinical sign was peripheral lymphadenopathy, present in 65% (13/20) of the animals.

The means of the hematological parameters and the presence/absence of each of the groups of animals in this study, classified according to the species of parasites found, are shown in Table 3. The statistical analysis of multiple comparisons showed that there was no significant difference in the presentations either of clinical signs and hematological parameters between groups ($p \leq 5\%$).

Table 3

Means, within groups, of hematological parameters and clinical signs verified in dogs naturally infected with visceral leishmaniasis and other hemoparasites in the municipality of Montes Claros, MG. The reference value for clinical signs is represented by the number zero, since they should be absent (n=20).

Parameters	**Infective microorganisms ***				**Reference values**[**]
	L	**L + B**	**L + E**	**L+B+E**	
Hemoglobin (%)	11,67	12,60	10,9	8,79	8,5 - 13
Hematocrit (%)	32,03	35,03	31,3	24,71	26 - 40
Platelets (/mm3)	271.833	277.666	196.500	254.333	175.000 a 500.000
Total leukocytes/mm3	8.016	7.933	6.650	10.244	8.500 a 17.300

Total protein (g/dl)	9,27	9,03	9,05	8,37	5,30 a 7,80
Albumin (g/dl)	3,74	3,13	3,05	3,49	2,30 a 3,80
Globulin (g/dl)	5,53	5,90	6	4,88	2,30 a 5,20
Albumin/globulin ratio	0,68	0,54	0,515	0,74	-
Creatinine (mg/dl)	0,98	1,00	0,95	1,24	0,5 a 1,5
Ck-mb (i.u./l)	289,67	339,00	263	313,33	84,11 a 97,39
Dermatitis	0,17	0,33	0,00	0,78	0
Slimming	0,00	0,33	0,00	0,33	0
Splenomegaly	0,67	0,33	1,00	0,78	0
Hepatomegaly	0,17	0,33	0,50	0,22	0
Peripheral lymphadenopathy	0,83	0,00	1,00	0,67	0
Onychogryphosis	0,50	0,00	0,00	0,67	0
Ulcerations in Cushions	0,17	0,00	0,00	0,11	0
Ulcerations on tips of ears	0,33	0,33	0,00	0,44	0
Ulcerations at mucocutaneous junctions (lips and muzzle)	0,17	0,00	0,50	0,44	0
Ulcerations on extremities and areas of bony protuberances	0,33	0,00	0,00	0,22	0
Uveitis	0,17	0,00	0,00	0,33	0
Systolic blood pressure	138,00	109,70	120,00	136,40	120,00

***** L: *Leishmania infantum*; B: *Babesia* spp.; E: *Erlichia* spp. ** Kaneko, Harvey e Bruss (2008); Sousa (2012); Carvalho (2015); Gama-Melo et al. (2019); Eregowda et al., (2020).

It is observed that the average total protein of the four evaluated groups was above the normality standards for the canine species (Gama-Melo et al., 2019), as well as the activity of the CK-MB enzyme (Pino et al., 2008).

Discussion

The lack of significance in the comparison tests of means between the groups of animals according to the concomitant agents shows that multiple infestation with *L. infantum*, *Babesia* sp. and *Erlichia sp.* does not interfere with the presentation of clinical and/or hematological

signs. This shows the need for multiple tests in animals from endemic regions for more than one hemoparasite, due to the possibility of co-infection.

Although there are publications reporting co-infections with both *Babesia* spp. as with *Erlichia* spp., no reports of interference in clinical or laboratory signs were found, since previous research only sought to identify the parasites (Lopes et al., 2005; Medeiros et al, 2008).

Valente (2014) identified 63.5% (33/93) of monoinfected dogs, 18% of them with *Leishmania chagasi* (6/33), while in this present study, this agent was identified, alone, in 30% (6/20) of the dogs. Co-infection with *Leishmania* and *Erlichia* canis was similar in both studies, having been verified in 10% (2/20) of the animals in the latter and in 10.5% (2/19) in the former.

In another study, *Leishmania* spp. was identified, at the ELISA test, in 0.7% of the analyzed dogs (Santos et al, 2009). Of these, one animal was monoinfected, another co-infected with *Babesia canis*, and the third with *Anaplasma phagocytophilum* and *Erlichia canis*.

In the only longitudinal study found, Mekuzas et al. (2009) found that *E. canis* infection preceded *L. infantum* in naturally infected animals. They claim that clinical signs are more evident in dogs with dual infections, proving a synergistic pathological effect between pathogens. This condition was not verified in this present study.

Researchers claim that *Erlichia sp.* is a contributing factor to the infection and establishment of *Leishmania* sp. in dogs (Mekuzas et al., 2009). Therefore, treatment for canine ehrlichiosis should be started as soon as possible after confirming the diagnosis in the animal, which would reduce the possibilities of co-infection.

The CK-MB enzyme activity of the dogs in this research showed, in the four analyzed groups, high values in relation to the reference ones (Pino et al., 2008). There is agreement with the fact that CK-MB is a reliable biomarker of cardiac muscle injuries in dogs (Gama-Melo et

al., 2019; Pino et al., 2008; Eregowda et al., 2020), being directly related to this factor in this research.

The hyperproteinemia observed in the animals in this study is compatible with results published in the scientific literature evaluating dogs with CVL (Lopes et al., 2005; Medeiros et al, 2008), although correlations with coinfections are not reported.

To the best of our knowledge, this is the first study to evaluate hemoparasite co-infections in dogs positive for *Leishmania* spp. and their intercurrences on hematological and clinical parameters. Therefore, the findings described here may be relevant for future studies and decision-making.

Conclusion

Coinfections in dogs positive for *L. infantum* occurred in most of the evaluated animals, the most common being the association of *L. infantum*, *Erlichia* spp. and *Babesia* spp. The presence of more than one parasite did not interfere with the clinical signs or the hematological parameters of the animals in this research. Studies with a greater number of observations of animals over time (longitudinal studies) are recommended to categorically clarify such interactions.

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5.2 Produto 2

Clinical and cardiac alterations in symptomatic dogs with visceral leishmaniasis in the city of Montes Claros, Minas Gerais, Brazil

Patrícia Natalicia Mendes de Almeida[a *], Jamille Fernandes Lula [b], Leandro de Freitas Teles [b], Chrystian Iezid Maia e Almeida Feres[c], Roberto Rodrigues Ferreira[d], Marcelo Meuser-Batista[d], Thallyta Maria Vieira [e], Silvio Fernando Guimaraes de Carvalho [e]

[a] *Doctoral student - Postgraduate Program in Health Sciences at the State University of Montes Claros (PPGCS-UNIMONTES), Av. Cula Mangabeira, 562 - Santo Expedito - Montes Claros-Minas Gerais, Brazil. CEP 39401-002.*
[b] *Research - Postgraduate Program in Health Sciences at the State University of Montes Claros (PPGCS-UNIMONTES), Av. Cula Mangabeira, 562 - Santo Expedito - Montes Claros-Minas Gerais, Brazil. CEP 39401-002.*
[c] *Research - Universidade Estadual Paulista "Júlio de Mesquita Filho" (UNESP) – São Paulo – São Paulo, Brasil.*
[d] *Research - Fundação Oswaldo Cruz (Fiocruz), Av. Brasil, 4365 - Manguinhos, Rio de Janeiro, RJ, Brasil*
[e] *Research and Adjunt teacher - Postgraduate Program in Health Sciences at the State University of Montes Claros (PPGCS-UNIMONTES), Av. Cula Mangabeira, 562 - Santo Expedito - Montes Claros-Minas Gerais, Brazil. CEP 39401-002*

* Corresponding author at: Postgraduate Program in Health Sciences at the State University of Montes Claros (PPGCS-UNIMONTES), Av. Prof. Cula Mangabeira, 562 - Santo Expedito - Montes Claros-Minas Gerais, Brazil. CEP 39401-002.
e-mail adress: pnobre01@gmail.com (P. Almeida)

Highlights

• Symptomatic dogs with visceral leishmaniasis may have myocarditis and cardiomyocyte degenerations, signaled by increases in CK-MB.

• Electrocardiographic changes that predict the severity of cardiac injury can be observed in animals with CVL.

• Nodular, inflammatory, and degenerative lesions have been observed in the mitral valve of dogs with CVL.

Summary

Infections caused by agents of the genus *Leishmania* are one of the major causes of public health problems in the world. The most common hosts for visceral leishmaniasis are dogs and humans. In this research, the objective was to identify alterations in the heart of symptomatic

dogs with naturally infected VL. Twenty adult male dogs, positive in the ELISA test for visceral leishmaniasis, were selected. Among the clinical signs, there was a higher prevalence of skin lesions, splenomegaly and peripheral lymphadenopathy. On the electrocardiogram, the QT segment had an average duration above the maximum recommended limit. The R wave presented an amplitude greater than the normality indices in 100% of the evaluated animals. Eleven dogs had systemic blood pressure above the recommended mean. CK-MB levels were elevated in all animals, while 80% (16/20) of them showed cardiac alterations at histopathological examination. In this exam, fibrosis alone or associated with lipid infiltration was more prevalent in the myocardium, followed by necrosis of the contraction band. Other changes identified were intramuscular (30%) and perivascular (10%) infiltrates. Under picrosirius staining, there was an increase in collagen fiber in the myocardium of 95% of the dogs. In the mitral valve, valve fibrosis and (a) adipose metaplasia were the most prevalent (95% of the animals). It is concluded, then, that *Leishmania infantum*. triggers alterations in the peripheral blood, in the cardiac musculature and in the mitral valve of symptomatic dogs with VL

Keywords: Canine visceral leishmaniasis. Clinical changes. Chronic heart disease.

1. Introduction

Leishmaniases are a group of diseases caused by protozoa from 21 species of *Leishmania* (Akhoundi et al., 2017). These parasites are transmitted to humans through the bite of an infected female of the Lutzomia longipalpis vector (OPAS, 2019), with the domestic dog being the main definitive host (Baneth; Solano-Gallego, 2015).

Canine VL presents various clinical signs (Ribeiro, 2007; Baneth; Solano-Gallego, 2015, Leishvet, 2018). However, in 60% of cases, dogs with the disease may be asymptomatic. The diversity, as well as the absence of symptoms, are factors that make the clinical diagnosis difficult (Ribeiro, 2007).

Among the organs reported with alterations caused by *Leishmania* spp., the heart has been rarely reported (Mendes et al., 2014), both in the macroscopic and histopathological aspects and, even less, in terms of electrocardiographic parameters. Studies in dogs with VL have registered cases of lymphoplasmocytic myocarditis, myonecrosis, increased interstitial collagen, Virchowian-type granulomatous myocarditis, fibrinoid vascular alteration and vasculitis, in addition to the presence of the parasite in the amastigote form. Among the auxiliary tests, electrocardiographic alterations such as reduction in P wave amplitude (Santos et al., 2015) and low voltage QRS complex are possible to be observed, in addition to second-degree atrioventricular block and electrical alternation (Godoy et al., 2016).

Recurrent elevations in the serum levels of the MB fraction of the enzyme creatine phosphokinase (CK-MB), indicating lesions in cardiomyocytes, were also observed (Mendes et al., 2014; Godoy et al., 2016; Alves et al., 2010).

Despite the findings in their report, Mendes et al. (2014) state that it is not possible to categorically infer the mechanism involved in the cardiac impairment of the dog they studied, due to other conditions under which the animal was found, suggesting the need for greater volume and depth in studies of this order. This suggestion reinforces how little is known about cardiac alterations associated with this protozoonosis.

In this study, the objective was to identify alterations in the heart of symptomatic dogs naturally infected with VL.

2. Material and methods

This experiment was carried out in the municipality of Montes Claros, which is located in the north of the state of Minas Gerais, within the region known as "Drought Polygon". The geographical coordinates of the municipal seat correspond to 16º50'52" south latitude, 43º55'29" west longitude and it is located at an altitude of 781 m. The climate, according to the Koppen-Geiger classification, is considered tropical subhumid dry, with a well-defined precipitation regime, with a rainy period in the summer and a long dry period for the rest of the year (Alvares et al., 2013). This region is recognized as endemic for visceral leishmaniasis (Brasil, 2022).

Twenty symptomatic dogs with *L. infantum*, male, adult, positive for visceral leishmaniasis were evaluated. All evaluated animals were received at the Montes Claros Zoonoses Control Center (CCZ-MOC) to be euthanized, since they were positive in the rapid test of the Dual Path Platform chromatographic immunoassay type (TR DPP®, Bio-Manguinhos, Rio de Janeiro, Brazil) and the ELISA test for the presence of anti-*Leishmania* antibodies.

It was decided, in this research, to sedate and anesthetize the animals so that they would all be under the same conditions when being analyzed, removing the individual behavioral responses that the environment could trigger in each of them.

Initially, the animals were sedated with 2 mL of 1% acepromazine intramuscularly and, when showing little reactive behavior, they received, intravenously, sodium thiopental 1g

diluted in sterile saline solution (final concentration: 2.5%) at the dose of 1mL/kgPV (Viana, 2014). The team of this research started the procedures with the animal as soon as the deep anesthetic plane was verified, clinically evaluating them according to Feitosa (2014), noting the clinical findings in spreadsheets suitable for this purpose.

2.1. Systolic blood pressure

The measurement of the animals' blood pressure was obtained immediately after the electrocardiogram was performed, following the methodology presented by Feitosa (2014).

The measurement was performed on the left anterior limb, using a palm-type aneroid sphygmomanometer with a set of five cuff sizes: 1, 2, 3, 4 and 5 reusable one-way (DeltaLife, São Paulo, Brazil), and Portable Vascular Doppler dv 610V (Medmega, São Paulo, Brazil).

Five sequential measurements were taken, and the values were recorded in specific spreadsheets for this purpose and, later, the average of the measurements of each animal was analyzed.

2.2. Electrocardiogram

The electrocardiogram showed electrical activity through seven leads using a veterinary electrocardiograph model DL650® (DeltaLife, São Paulo, Brazil). The information was obtained through data collection electrodes connected to a notebook with the ECGDelta 1.0® software (DeltaLife, São Paulo, Brazil), for about three uninterrupted minutes.

2.3. Tissue collection

2.3.1. Blood

A collection of 5ml of blood was performed by venipuncture, in the jugular vein or in the cephalic vein, in a vial with EDTA for vacuum blood collection. After collection, the vials were identified and placed in an isothermal box with recyclable ice, at a temperature of around 22°C.

Subsequently, the animals were euthanized in accordance with the guidelines of Resolution No. 1000 of 05/11/2012 (CFMV, 2012) and Normative Resolution Nº 37, of February 15, 2018, of the National Council for the Control of Animal Experimentation - CONCEA (Brazil, 2018).

Then followed with the next collections.

2.3.2 Mitral valve and cardiac muscle tissue

Immediately after euthanasia, the sternum was removed and the heart was exposed, which was evaluated after incising the pericardial sac and cutting the great vessels. Then, a cubic-shaped segment was removed, with an edge of approximately 2 cm on the right ventricular wall, formed by the epicardium, myocardium and endocardium. The complete mitral valve was also collected, connected to the margin of cardiac muscle tissue (Fenoglio et al., 1972).

All fragments were placed individually in identified vials containing 10% buffered formalin in sufficient quantity to completely cover the fragment.

2.4. Laboratory analysis

2.4.1 Blood with EDTA

The EDTA blood tubes were sent to an analysis laboratory within a maximum period of six hours after collection. CK-MB dosage was performed to assess cardiac muscle damage in a BS 120® model equipment (Shenzhen Mindray Bio-Medical Electronics Co. Ltd., China).

2.4.2. Heart and mitral valve fragments

The samples obtained were fixed in 10% neutral buffered formalin for 24 hours and then dehydrated, cleared, embedded in paraffin and microtomized according to the methodology of Caputo, Gitirana and Manso (2009).

Slides were stained with hematoxylin and eosin (HE) to verify cellular and proliferative alterations (ALVES et al., 2010). Observations were performed using the Olympus FSX100® inverted microscope and its own software FSX-BSW V02.01® (Olympus Latin America, Inc., Miami, U.S.A.).

Silanized slides also prepared for staining by picrosirius, for analysis and evaluation of the presence of collagen fibers in the structures (JUNQUEIRA et al., 1979). The slides were scanned and digitized using the Motic VM 3.0 – Motic Digital Slide Assistant program (MoticEurope S.L.U., Barcelona, Spain).

2.5. Statistical analyzes

The variables presented in Table 1 were analyzed using descriptive statistics. Clinical signs (except blood pressure), characterization of the ST segment, cardiac electrical axis, cardiac rhythm and histological sections were analyzed according to the presence or absence of alterations. The numerically measurable parameters (blood pressure, electrocardiographic waves and CK-MB) were quantified and analyzed according to the physiological levels recommended by the scientific literature for each one of them.

The silanized slides were stained with the picrosirius technique, scanned using the ImageJ software (National Institutes of Health, Maryland, USA) and statistically analyzed using the GraphPad Prism® software (GraphPad Software, California, USA), adopting the test Mann Whitney ($p \leq 0.0001$).

Next, the Spearman rank correlation coefficient (Spearman's ρ) was calculated using the transformed value of all variables [$x' = (\sqrt{(x + 0.5)}$].The parameters were analyzed using the statistical program Jamovi® version 2.3.17 for Windows, with 95% significance ($p<0.05\%$).

Table 1
Variables analyzed in symptomatic dogs naturally infected by *L. infantum* in the municipality of Montes Claros, Minas Gerais, Brazil. (n=20).

Sample / analysis	**Variable**	
Clinical signs	Systolic blood pressure	
	Skin lesions	
	Weight loss and loss of muscle mass	
	Splenomegaly	
	Hepatomelagia	
	Peripheral lymphadenopathy	
	Onychogryphosis (big nails)	
	Uveitis	
Electrocardiogram	P-wave duration in milliseconds	
	P wave amplitude in millivolts	
	Pr segment duration in milliseconds	
	Qt segment duration in milliseconds	
	Qrs segment duration in milliseconds	
	Qrs segment amplitude in millivolts	
	R wave amplitude in millivolts	
	S-wave amplitude in millivolts	
	St segment characterization	Sublevel
		Isoelectric
		Overpass
	Cardiac electrical axis	Normal
		Left detour
		Right turn
	Heart rate	Sinus arrhythmia
		Sinus arrest
		Sinus
		Atrial arrhythmia
		Ectopic activity
		Atrial flutter
		St coving; sinus arrhythmia
		St coving; sinus arrest
Hematological parameters	Creatine kinase MB isoenzyme (CK-MB)	
Histological section of cardiac muscle stained with hematoxylin and eosin, observed under an optical microscope	Perivascular inflammatory infiltrate	
	Diffuse intramuscular inflammatory infiltrate	
	Lipid infiltrate similar to adipose metaplasia	
	Amastigotes forms	
	Necrosis in cardiomyocyte contraction bands	
Histological section of cardiac muscle stained with picrosirius, observed under an optical microscope	Deposition of collagen fibers in the myocardium	
Histological section of the mitral valve stained with hematoxylin and eosin, observed under an optical microscope	Adipose metaplasia (similar to lipid degeneration)	
	Neovascularization in the valve material	
Histological section of the mitral valve stained with picrosirius, observed under an optical microscope	Collagen fiber deposition between valve leaflets	

3. Results

3.1 Clinical evaluation of animals

All animals in this present study were symptomatic, with 95% (19/20) being polysymptomatic and 5% (1/20) being oligosymptomatic, showing peripheral lymphadenopathy. The verified clinical signs are shown in Figure 1.

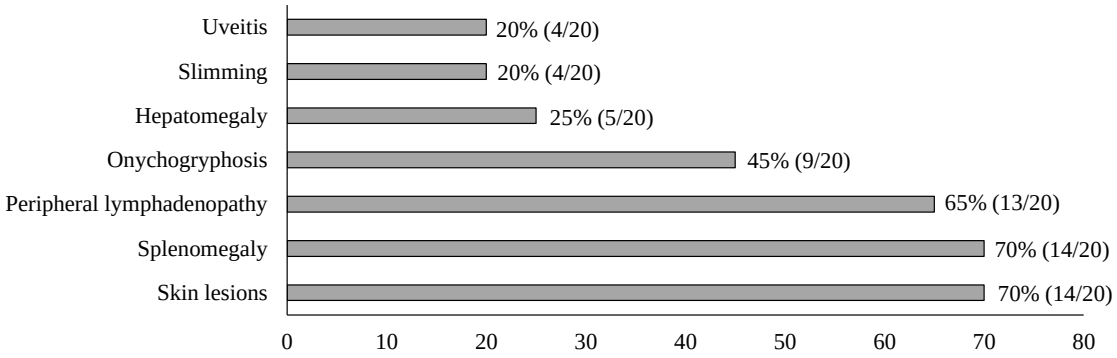

Fig. 1. Clinical signs observed in symptomatic dogs with naturally infected visceral leishmaniasis in the municipality of Montes Claros, MG (n=20). More than one signal was observed in 19 animals.

3.2 Systolic Blood Pressure

The means of the five individual systolic blood pressure measurements of each animal are shown in Table 2, as well as the mean pressures of all animals and the standard deviation of these measurements.

Table 2
Systolic blood pressure in symptomatic dogs naturally infected with *L. infantum* (n=20). Average of five measurements in each animal.

Parameter	Mean (n=20)	Standard deviation	Reference value *
Systolic blood pressure (mmHg)	131,25	35,04	120

*****Reece et al. (2017)

3.3 Electrocardiogram

The electrocardiographic data of three animals were invalidated for technical reasons, therefore, data were obtained from 17 dogs. Table 3 presents the averages obtained from the measurements of the data of the animals in this study.

The electrocardiographic changes observed in the animals are shown in Figure 2.

Table 3
Electrocardiographic parameters of symptomatic dogs naturally infected with *L. infantum* (n=17) in the municipality of Montes Claros, MG, Brazil.

Parameter	Mean	Standard deviation	Reference value *
P-wave duration (milliseconds)	49,00	7,68	30 - 50*****
P-wave amplitude (millivolts)	0,16	0,05	≤ 0,4*****
Duration of the pr complex (milliseconds)	123,29	19	60 - 140*****
Qt complex duration (milliseconds)	230,12	26,6	150 - 230*****
QRS complex duration (milliseconds)	67,06	9,46	30 - 70*****
R-wave amplitude (millivolts)	1,24	0,7	≤ 0,3*****
Heart rate (beats per minute)	97,76	44,5	70 - 120******

***** Richig, Sleeper (2019). ****** Reece et al. (2017).

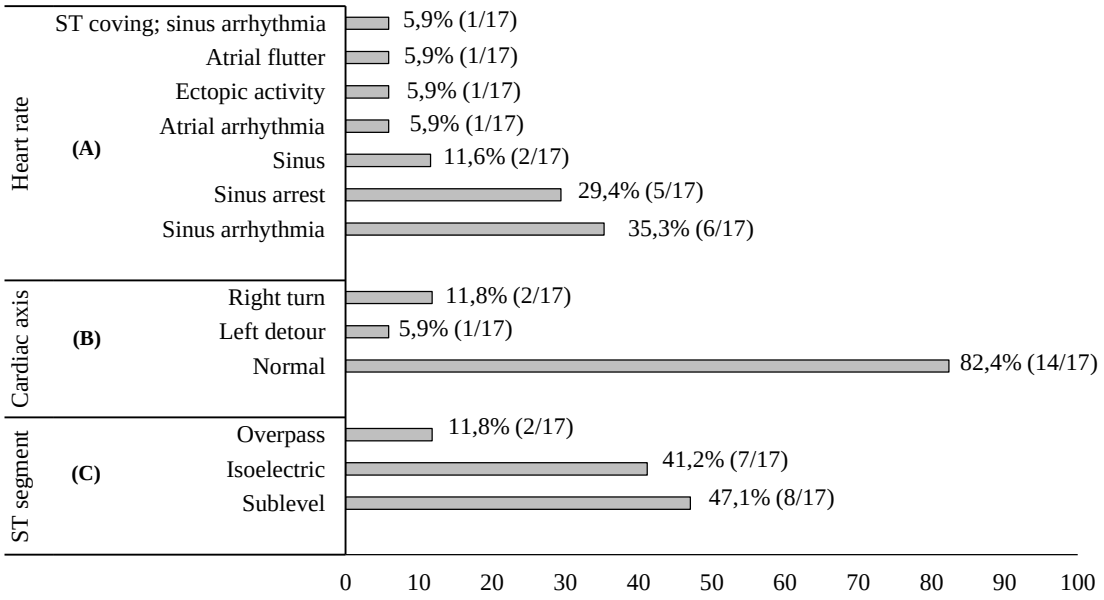

Fig. 2. Electrocardiographic changes observed in symptomatic dogs naturally infected with *L. infantum* (n=20) in the municipality of Montes Claros, MG. (A) Characteristic of heart rhythm; (B) characteristic of the cardiac electrical axis; (C) characteristic of the lower wave of the ST segment. (n=17).

3.4 CK-MB enzyme activity

The values measured showed that all animals (n=20) had CK-MB enzyme levels above the reference values presented in the scientific literature, which range from 84.11U.I./L to 97.39U.I. /L (Eregowda et al., 2020). The mean measurement of the animals in this study was 305.05I.U./L, with a standard deviation of 134.15I.U./L.

3.5 Histopathology of cardiac musculature

Under picrosirius staining, only one animal (5%) did not show collagen fiber deposition, while other changes could be seen under HE staining (Figure 3).

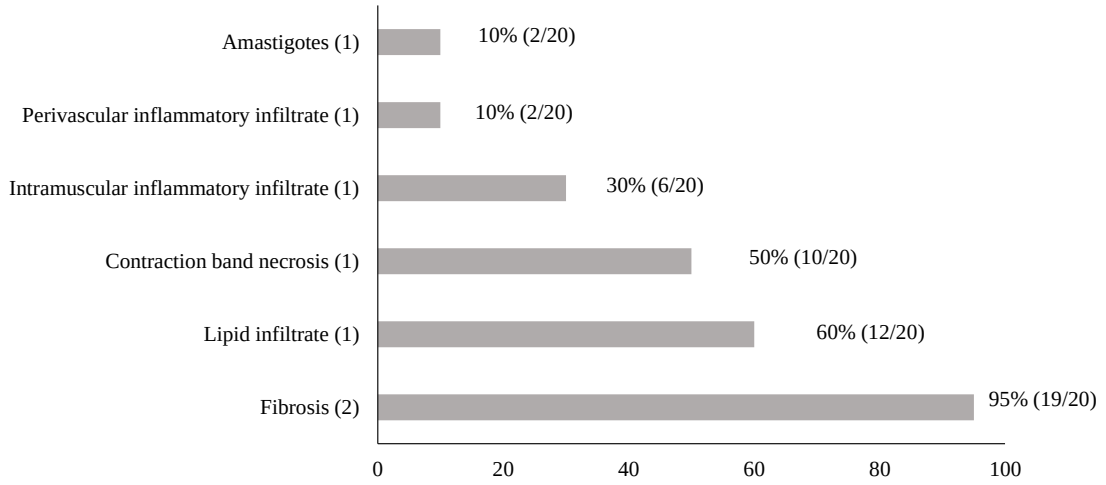

Fig. 3. Alterations observed in histological sections of cardiac muscle (right myocardium) of symptomatic dogs with naturally infected visceral leishmaniasis in the municipality of Montes Claros, MG (n=20). More than one alteration was observed in some animals. (1) staining with hematoxylin and eosin; (2) staining with picrosirius.

Figure 4 proves the existence of a statistically significant difference in the quantification of collagen fiber deposits in the myocardium of the dogs in this study when compared to the heart of a healthy dog ($p \leq 0.0001$), demonstrating that dogs with CVL had a higher rate of deposits of collagen fibers.

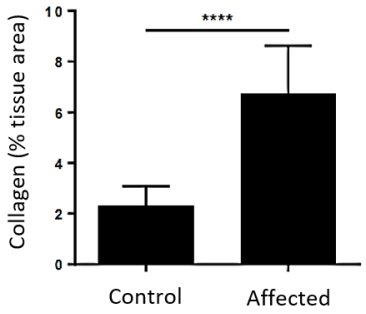

Fig. 4. Statistical analysis of the quantification of tissue area occupied by collagen in histological sections of cardiac muscle (right myocardium) of symptomatic dogs with naturally infected visceral leishmaniasis in the city of Montes Claros, MG (n=20), using ImageJ software and statistical analysis performed in the Graph Prism software. *$p \leq 0.0001$ by the Mann Whitney test. (n=20).

It was verified that there was concomitant histopathological alterations in 60% (12/20) of the analyzed animals, while 20% (4/20) presented only one alteration and another 20% (4/20) did not present any type of alteration to the staining. HE (Figure 5).

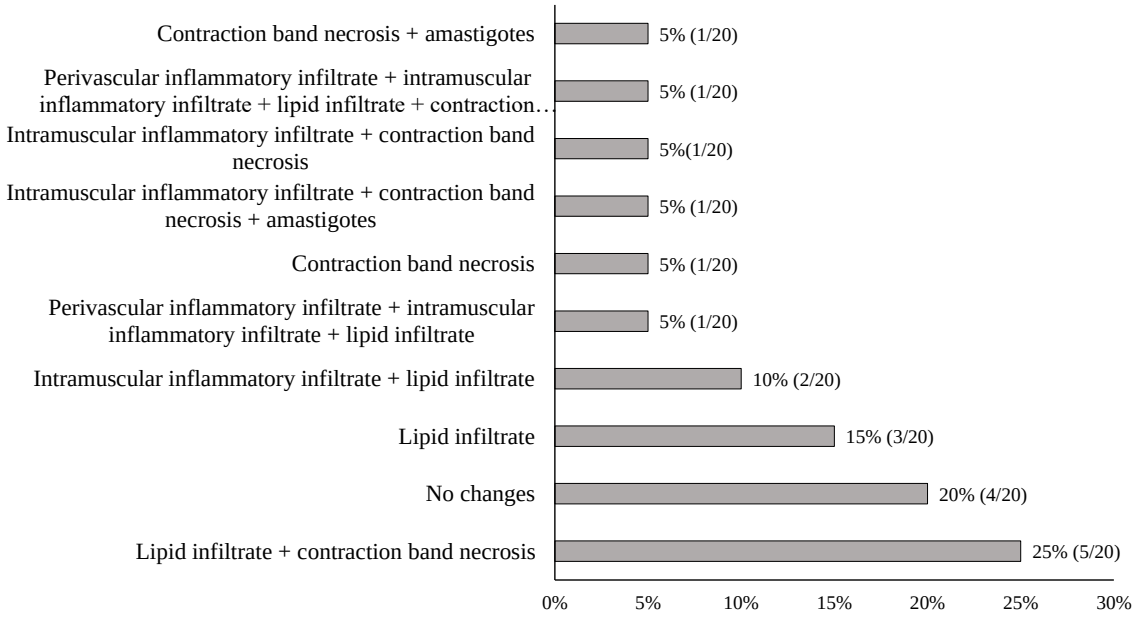

Fig. 5. Associations of alterations observed in histological sections of cardiac muscle (right myocardium) stained with HE from symptomatic dogs with naturally infected visceral leishmaniasis in the municipality of Montes Claros, MG (n=20).

3.6 Mitral valve histopathology

Macroscopic analysis showed that 45% (9/20) of the animals had nodules on the mitral valve (Fig. 6), compatible with endocardiosis. These nodules had a smooth and shiny aspect, firm but not rigid, adhered to the valve leaflet.

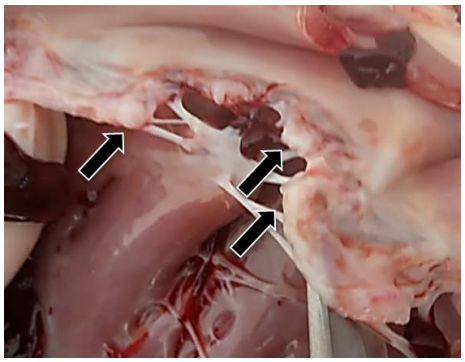

Fig. 6. Mitral valve in the heart of a symptomatic dog with CVL, showing nodules (arrows) compatible with endocardiosis.

Histopathological analysis of the mitral valve blades, stained with HE and picrosirius, identified valvulopathy in 95% (19/20) of the dogs, the most frequent being valvular fibrosis and adipose metaplasia (Fig. 7).

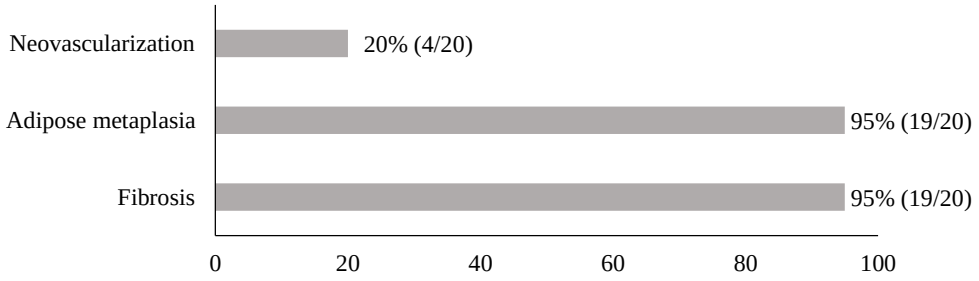

Fig. 7. Alterations observed in histological sections of the mitral valve of symptomatic dogs with naturally infected visceral leishmaniasis in the municipality of Montes Claros, MG (n=20). More than one alteration was observed in some animals.

It was found that 20% of the animals (4/20) had concomitant valvular fibrosis, adipose metaplasia and neovascularization (Figures 8 and 12). However, the highest proportion of associations was between adipose metaplasia and fibrosis, which was observed in 75% (15/20) of the animals (Figure 8).

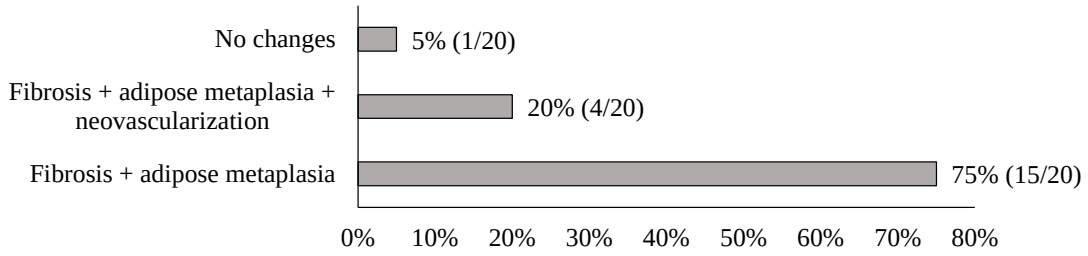

Fig. 8. Associations of alterations observed in histological sections of the mitral valve of symptomatic dogs with naturally infected visceral leishmaniasis in the municipality of Montes Claros, MG (n=20).

4. Discussion

4.1. Clinical evaluation of animals

Macroscopic cutaneous lesions of CVL can be diverse, even in the same dog. Granulomatous or pyogranulomatous inflammatory processes associated or not with immunocomplex deposition are the main causes of cutaneous symptoms. Thus, among the lesions, there are histological differences, different parasite densities and immunophenotyping of inflammatory cells and skin residents (Saridomichelakis; Koutinas, 2014).

Among the clinical signs identified in the animals in this research (Fig. 1), it was possible to verify a higher prevalence of skin lesions and splenomegaly, each of which was observed in 70% (14/20) of the dogs. Another highly prevalent clinical sign was peripheral lymphadenopathy, present in 65% (13/20) of them. Similar results were found by Pacheco (2016) in the municipality of Araçatuba, São Paulo, Brazil. This author found a higher incidence of skin lesions and lymphadenopathy in animals with CVL. Animals evaluated in Campo Grande, Mato Grosso do Sul, Brazil, showed a predominance of dermatopathies, lymphadenopathy and cachexia (Godoy et al., 2016).

In the aforementioned studies, as well as in this research, there is a higher frequency of skin lesions. This has been the most common finding on physical examination and may be the only clinical manifestation of dogs with CVL, in addition to the fact that it seems to be the most common reason that leads tutors to seek veterinary medical help for their animals, even if they do not yet know of infection by *Leishmania* spp. (Saridomichelakis; Koutinas, 2014).

Exfoliative dermatitis, ulcerative dermatitis, nodular dermatitis, sterile pustular dermatitis, paw hyperkeratosis, nasal hyperkeratosis and onychogryphosis were the skin lesions most cited by Saridomichelakis and Koutinas (2014) in their literature review. The presence of *Leishmania* amastigotes on the skin is an important factor, since it is from the ingestion of peripheral blood that contamination of sandflies occurs, allowing the dissemination of the parasite (CRMV-PR, 2015; Brasil, 2019).

Baneth and Solano-Gallego (2015) cite both the clinical signs found in this present investigation and others in dogs with the pathology. However, many infected dogs do not manifest the disease due to their efficient immune response. This variation can be seen in the categorization of animals into four classes, according to the symptoms presented, both clinical and laboratory, ranging from the absence of clinical signs to lesions at an advanced and irreversible stage, with an unfavorable prognosis (Solano-Gallego et al., 2009; Leishvet 2018).

While in this study, 100% (20/20) of the evaluated animals were symptomatic, the results observed by Santos et al (2015) in Uruguaiana, RS, Brazil, were different, with 8.3%

(n=3) of the positive dogs for CVL classified as asymptomatic, while 66.6% (n=24) and 25% (n=9) were classified as polysymptomatic and oligosymptomatic, respectively. The most commonly observed symptom in that study was generalized lymphadenopathy, followed by dry seborrhea and ulcers and skin crusts.

Differently from this study, face, paws and abdomen edema were recorded by López-Peña et al. (2009), in addition to non-ulcerated skin nodules, generalized mild lymph node enlargement of superficial lymph nodes and rigid gait. Also, Sebastián-Marcos et al. (2019) verified, in a dog with CVL, severe abdominal distension with palpable fluid wave, as well as jugular distention, muffled heart sounds on auscultation and palpable paradoxical pulse, having been diagnosed pericardial effusion with consequent chronic cardiac tamponade.

Osteoarticular symptomatology has been reported in the scientific literature (Silva et al., 2021; Wallborn et al., 2016) with cases diagnosed both clinically and by radiography and tomography. However, no case was diagnosed in the animals in this study, since the evaluation was restricted to the clinical symptoms of the animals under anesthesia, making it impossible to analyze ambulation.

4.2. Systolic Blood Pressure

In this research, 55% (11/20) of the dogs had systolic blood pressure above the average parameter recommended by the academic literature, which is 120 mmHg (Reece et al., 2017) (Table 2).

The hypertensive condition presented by most of them was compatible with the information presented by Fantoni et al. (2017), who reported that normovolemic dogs, after administering a standard anesthetic dose of thiopental, showed an increase in mean arterial pressure and cardiac output, although they did not report on the association between this barbiturate and acepromazine, which were used in this study. .

Despite the use of the anesthetic, dogs with CVL are prone to developing hypertensive conditions due to cardiac alterations caused by the protozoan. This is due to the fact that, indirectly, cardiac lesions that cause a reduction in cardiac output, such as fibrosis, myocarditis and valvular lesions, conditions observed in the animals in this study, trigger triggering of the renin-angiotensin-aldosterone system (RAAS) in the kidneys. This mechanism results in systemic vasoconstriction both of central origin by activation of the sympathetic portion of the

nervous system, as well as by the release of vasopressin and by greater sodium retention by the kidneys, increasing fluid retention (Reece et al., 2017). In addition to this route, hypertension can be triggered directly by renal lesions caused by the protozoan (Leishvet, 2018; Solano-Gallego et al., 2009), which trigger the RAAS and also impair the elimination of excreta, such as urea.

Mendes et al., (2014), in a case report, associated the condition of hypertension in a dog with CVL with initial compensatory cardiac mechanisms such as the release of catecholamines, resulting from the possible contractile impairment of the myocardium due to the inflammatory and infectious condition. These authors concluded that cardiac lesions caused by *Leishmania* spp. may be related to the direct or "reactive" action of the tissue to the aggression caused by the parasite. However, Rosa et al., (2014) found no significant correlation between positivity for CVL and hypertension among the 30 dogs studied, as well as this present study.

4.3. Electrocardiogram

The mean heart rates (Table 3) of the animals in this research were within the normal range for the canine species (Richig, Sleeper, 2019; Reece et al., 2017). However, 29.4% (5/17) of the evaluated animals had a heart rate below the minimum recommended by the scientific literature. This condition may have been caused by the use of sodium thiopental as anesthetic medication, which leads to bradycardia when applied slowly (Fantoni et al., 2017).

Table 3 presents the characteristics of the electrocardiographic waves of the animals in this research. The amplitude of the P wave in milliVolts (mV) and the duration of the PR segment in milliseconds (msec) are within the physiological parameters recommended by the scientific literature (Richig, Sleeper, 2019).

The duration of the P wave (msec) and the duration of the QRS segment (msec), although they had their averages within the normal range, there were seven (41%) and six (35%) animals, respectively, that presented data above the maximum parameter of normality, which meant that the standard deviation of these parameters was above that recommended by the literature. The widening of both waves indicated dilatation of cardiac chambers. Increased P wave width is an indicator of left atrial dilation, also called P mitrale, while increased QRS segment width is a result of left ventricular dilation (Tilley et al., 2008).

The QT segment (msec) had an average duration above the maximum limit recommended by science, indicating an enlargement at this point on the electrocardiogram in most of the evaluated animals. This interval tends to increase with bradycardia and decrease with tachycardia (Tilley et al., 2008). These events may occur due to interventricular conduction disturbances that are associated with prolongation of QRS complexes, ethylene glycol toxicity, strenuous activity, or CNS disorders (Fox et al., 1999). In the case of the animals in this research, the alterations presented in the integrity of the cardiac musculature may have caused interference in electrical conduction, which may have corroborated this result.

The R wave amplitude was greater than the normal indices in 100% (17/17) of the animals evaluated in this study. It then presented a marked alteration, as well as the entire QT segment. As the R wave is part of the QT complex, it suffers interference from the same factors as the latter (Tilley et al., 2008). According to Willis et al. (2018), left ventricular hypertrophy in dogs is one of the factors that causes this response to the ECG, in addition to pericardial effusion. Both alterations were described in animals with CVL, but emphasizing that their correlation with the pathology is not definitive (Shrivastava et al., 2007), with the need for further scientific investigations to clarify these findings. The animals studied in this research were not evaluated for these parameters, although they presented valvular alterations, which may incur in the secondary development of hypertrophy (Santos, Alessi, 2016).

Since the QRS segment is the moment of depolarization of the interventricular septum and the walls of the right and left ventricles (Tilley et al., 2008), changes in electrical conduction in any of these segments lead to changes in the characterization of these waves on the electrocardiogram, as demonstrated by López -Peña et al. (2009) in their case report.

Fibrosis, myocarditis and degeneration of cardiomyocytes due to several factors (microinfarcts, heart infections, etc.) are conditions that interfere with the conduction of electrical stimuli both in the myocardium and in the mitral valve (Tilley et al., 2008). Both alterations were detected in the animals of this investigation (Fig. 5), and may have been the cause of the marked alteration in the R wave and in the entire QT segment.

Honse (2014), in an electrocardiographic and histopathological evaluation of the heart of 41 animals with CVL, identified similarities with this study in the measures of P wave duration and of the PR and QRS segments, as well as in the amplitudes of the P and R waves. differences in the duration of the QT interval and in the heart rate, both variables being smaller in their study than in this present research. Histopathological findings were also similar,

identifying chronic myocarditis, which suggested the possibility that myocarditis was related to electrocardiographic changes. However, it is recommended that the claim that *L. infantum*being the definitive causal factor of myocarditis must be confirmed after identifying this agent in the heart muscle (Rosa et al., 2014; Santos et al., 2015).

Cardiac injury could be developed by deposition of antibodies against *Leishmania* in the myocardium, inducing an inflammatory process (Lakhdhir et al., 2020). This mechanism is recognized and well described as causing chronic myocarditis triggered by an autoimmune alteration by the formation of anti-*Trypanosoma cruzi* antibodies in humans, which attack cardiomyocytes (De Albba-Alvarado et al., 2023).

The cardiac axis of the evaluated animals showed the characteristics shown in Figure 2. It is observed that, of the 17 animals analyzed, three showed deviations, two animals (11.8%) with deviation to the right and one (5.9%) to the right. left.

Left and right electrical deviations in dogs have different causes. Left shifts are characteristic of left anterior fascicular block, and may be caused by diseases associated with left ventricular hypertrophy, hyperkalemia, ischemia, and postoperative cardiac surgery (Tilley et al., 2008). On the other hand, deviations to the right are one of the consequences of structural heart disease (valvular alterations and persistence of fetal shunts, for example), Chagas disease, heartworm disease, acute pulmonary thromboembolism and hypokalemia (Fox et al., 1999). It is noteworthy that the presence of blockages does not directly impair cardiac performance, but it is a significant marker of heart disease, and attention should be paid to the causes of these blocks (Tilley et al., 2008). In this present study, the animal with the deviation to the left presented, at cardiac histopathology, necrosis of the cardiomyocyte contraction band and diffuse mixed inflammatory infiltrate. He also presented fibrosis and adipose metaplasia in the mitral valve. The aforementioned injuries could trigger left ventricular concentric hypertrophy.

Among the animals that showed deviation to the right, one had necrosis of the contraction band and areas of fibrosis of the cardiomyocytes, in addition to lesions in the mitral valve (fibrosis, adipose metaplasia and neovascularization). The second animal had fibrosis in areas of the myocardium, and in the mitral valve fibrosis and adipose metaplasia were identified. Both groups of changes can trigger loss of contractile strength and compensatory hypertrophy of the ventricles (Reece et al, 2017).

The characteristics of the ST segment were analyzed, and it was verified that 58.82% of the animals presented alterations and, of these, 47.06% showed depression on the

electrocardiogram. These alterations are identified, in studies with human patients, as predictors of myocardial infarction with an important risk of death (Namana et al., 2018; Zeijlon et al., 2022). Studies of this order in animals indicate similar results, but evaluating other parameters (Macêdo et al., 2019; Almeida et al., 2006). Therefore, it can be inferred that, among the animals in this study, at least 47.1% (8/17) are likely to have heart disease.

When evaluating the cardiac rhythm, sinus alterations were observed in most of the animals studied, especially sinus arrhythmia and sinus arrest (Figure 2). Sinus arrhythmia is classified as a physiological alteration, being observed, especially, due to the change in pressure in the thoracic cavity triggered by respiratory movements (Richig, Sleeper, 2019; Willis et al., 2018). Sinus arrest is one of the changes described in Sinoatrial Node Syndrome, which is related to weight loss, ataxia and syncope in affected animals, which can lead to death (Willis et al., 2018).

In this study, deposits of fibrous and lipid tissue in the cardiac musculature were observed in 100% and 60% of the dogs, respectively, leading to difficulty or interruption of the transmission of sinus stimuli between the SA node and the underlying cardiac musculature. Similar results were observed by Nakao et al. (2012), although their studies were carried out in the right atrium of animals without CVL, while this study analyzed the right ventricular myocardium. Despite this difference, the findings of this study suggest that electrocardiographic alterations in dogs with visceral leishmaniasis may be caused by ultrastructural lesions of the cardiac muscles, which points to the need for specific studies in the region of the sinoatrial node and other cardiac regions of animals in this condition. Also, a described cause of sinus arrest is the increase of these deposits, causing difficulty or interruption of the transmission of sinus stimuli between the sinoatrial node and the underlying heart muscle (Nakao et al., 2012).

Ectopic activity, observed in animals in this research (Fig. 2), both atrial and ventricular, refers to disturbances in the generation and conduction of electrical discharges in the heart. In the right atrium, the most common form of presentation is the premature atrial complex, which is often associated with structural atrial abnormalities (atrial dilatation, for example) or other cardiac diseases, such as cardiomyopathies, metabolic disease (neoplasia, for example), diseases systemic or inflammatory diseases (sepsis) (Miller et al., 1999; Tilley et al., 2008). Although no studies were found relating ectopic activity to myocardial degeneration (both fibrous and fatty), this possibility should be considered, since these alterations compromise the

architecture of the cardiac muscle and may interfere with the mechanisms of generation and transport of electrical stimuli.

Premature ventricular complexes, another form of presentation of cardiac ectopic activity, may be associated with multiple factors, both cardiac and extracardiac, as described in Tilley et al., (2008). Possible causes include structural heart disease, inherited cardiovascular disease, gastric dilatation-volvulus, neoplasia, splenic torsion, pancreatitis, and drug induction. Systemic changes such as hypokalemia, hypoxemia, anemia or excessive concentrations of circulating catecholamines (triggered by fear, pain, anxiety and anger) can also increase the propensity to form such complexes (Richig, Sleeper, 2019).

In this research, animals with CVL show clinical signs of important systemic alterations (Fig. 1). Thus, it is possible to infer that these animals are part of the group prone to ectopic activity, confirming the findings of this study.

4.4. CK-MB enzyme activity

The CK-MB enzyme activity of the dogs that took part in this research (Table 4) showed, in 100% of the animals, higher values in relation to the reference values presented in the scientific literature, which is 90.75 ± 6.64 IU/ mL (Eregowda et al., 2020). It should be noted that a reference value for this enzyme is still difficult to find, with considerable divergence among researchers about exact maximum and minimum levels. However, there is agreement with the fact that CK-MB is a reliable biomarker of cardiac muscle injuries in dogs, being directly related to this factor (Kaneko et al., 2008; Pino et al., 2008; Lopes et al., 2005; Eregowda et al., 2020). In this research, we opted for the most recent reference among those found.

In an analysis of dogs with CVL, Godoy et al. (2016) and Silva et al. (2016) found differences between CK-MB measurements in dogs according to the stage of presentation of clinical signs, with the increase in enzymatic activity being directly proportional to the amount of symptoms presented. A positive and significant correlation was also observed between the serum activity of the CK-MB enzyme and histological lesions in cardiac muscle in the studies by Santos et al. (2015) and Mendes et al. (2014).

The electrocardiographic alteration was not verified in all the animals in this investigation, despite the increase in CK-MB levels. Furthermore, there was an indirect and

significant correlation between the dimension of the QT segment on the electrocardiogram and the levels of CK-MB in the blood. However, in 5% (1/20) of the evaluated dogs, histopathological changes in the heart were not observed, but even in this animal, the serum level of the enzyme was found to be increased. These situations point to the need for studies with other cardiac variables to clarify the causes of these results. Further studies are suggested, associating CK-MB activity with confirmation of the presence of *Leishmania* spp in tissues through immunohistochemistry.

4.5. Histopathology of cardiac musculature

The analysis of histological sections stained with hematoxylin and eosin (HE) showed a higher prevalence of lipid infiltrates (60%, being 12/20) and necrosis of the contraction band (50%, being 10/20), and under picrosirius staining they were significant deposits of collagen fibers were observed (95%, being 19/20), indicating chronic inflammatory processes. Mixed diffuse inflammatory infiltrate was seen in 30% (6/20) (Figures 3 and 10) of the dogs, while perivascular infiltrate (Figure 3) and the presence of amastigotes (Figures 3 and 11) were identified in 10% (2/20) of the cases each.

It appears that cardiac fibrosis was the most prevalent type of alteration among the evaluated animals, being present in 95% (19/20) of them (Figure 9). By the Mann Whitney test, a statistically significant difference was verified when comparing the levels of collagen fibers between the histological sections (under picrosirius staining) of the animals in this study and that of a healthy animal (Figure 4).

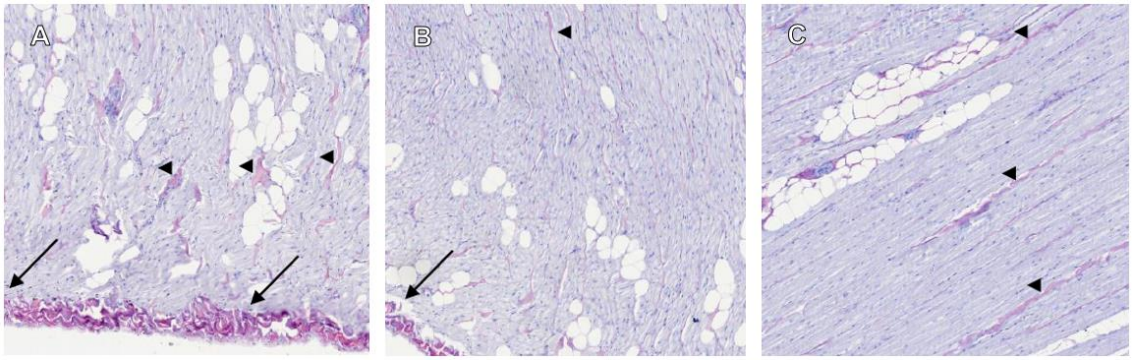

Fig. 9. Collagen deposition in the cardiac tissue of dogs naturally infected with *Leishmania*. The organs were collected, submitted to histological processing in paraffin and stained using the Picrosirius method. (A, B and C) Representation of areas with deposition of collagen fibers both along the pericardium (arrows) and in regions of the myocardium (arrowheads).

The term fibrosis is used to denote the excessive deposition of collagen and other components of the interstitial matrix in a tissue, which may be responsible for severe functional impairment and even organ failure. Fibrotic disorders include several chronic and debilitating diseases (Kumar et al., 2021), such as CVL.

The scientific literature presents cardiac alterations that can be triggered by areas of myocardial fibrosing, such as sinus arrest (electrocardiographic signal presented by 29.4% of the animals in this experiment), first and/or third degree ventricular blocks, left bundle branch block and and finally, complete heart block (Tilley et al., 2008). Despite that, this study did not find any significant correlation between cardiac muscle fibrosing and electrocardiographic changes.

Inflammatory infiltrates in the myocardium have been recurrently identified in dogs with CVL, although predominantly mononuclear cells are observed (Rosa, 2012; López-Peña et al, 2009; Godoy et al., 2016; Santos et al., 2015; Pacheco, 2016; Soares et al., 2015; Alves et al., 2010, Mendes et al., 2014), although mixed infiltrates were identified in this study (Figure 10-B and C).

The influx of inflammatory cells into the myocardium may be related to tissue reaction mechanisms to the presence of *L. infantum* (Godoy et al., 2016), although there is no consensus on this type of response (Rosa, 2012; Mendes et al., 2014) .

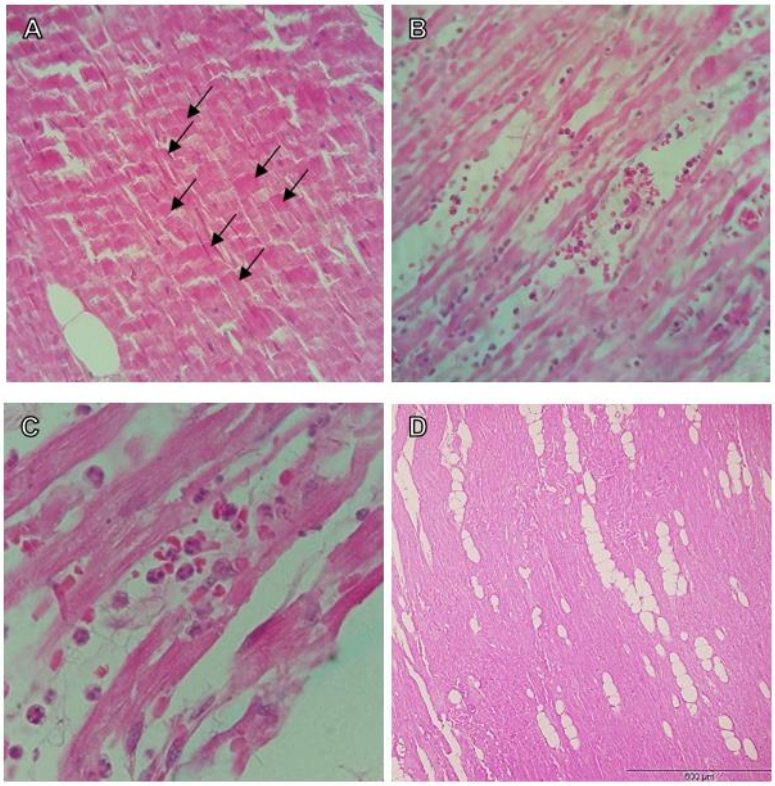

Fig. 10. Histological sections of heart muscle (right myocardium wall) from dogs naturally infected with *L. infantum* in the municipality of Montes Claros, MG, Brazil, stained with HE and visualized under an optical microscope. (A) Necrosis of the contraction bands (arrows) at 400x magnification. (B) Mixed diffuse inflammatory process at 400x magnification. (C) Mixed diffuse inflammatory process at 1,000x magnification. (D) Lipid infiltrate at 100x magnification.

In their literature review and study on myocarditis, Molesan et al (2019) reported multiple factors related to the inflammatory process, both isolated and together. These authors reported as causes of myocarditis in dogs viral, bacterial, protozoal, fungal, algae and even agents with secondary inflammatory effects on the cardiac muscles. Granulomatous and lymphoplasmacytic myocarditis with loss of cardiomyocytes, associated with vasculitis, fibrosis and amastigotes within myocardial macrophages have also been reported in dogs with *Leishmania* spp. (Torrent et al., 2005).

Lipid infiltrate was verified in 16.7% of the animals analyzed in Araçatuba, São Paulo (Rosa, 2012), while 60% (12/20) of the dogs showed the same type of alteration in this present study (Figure 10-D). However, in the study by Rosa (2012), this infiltrate was only visualized in the atria, both right and left, and in this research it was verified in the right ventricle, since fragments from other cardiac chambers were not collected. In other studies on the histopathological characterization of the heart of dogs positive for CVL, lipid alterations in the cardiac musculature were not verified (Pacheco, 2016; Santos et al., 2015; Oliveira, 2009;

Soares et al, 2015). Almeida (2019), in a study of human cases of VL, also did not identify this type of alteration.

According to Kumar et al. (2021), lipid degeneration, also called steatosis, is the result of excessive entry of free triglycerides into cells and is related to intoxication, protein malnutrition, diabetes mellitus, obesity and tissue anoxia. However, these same authors state that this is a reversible alteration. Therefore, the presence of this lesion in the cardiac muscles in the animals in this study may be an indicator that the causal factor was still present.

Knowing that the S wave and the ST segment are related to the depolarization of the ventricles, electrocardiographic changes in these regions are identified when there is myocardial hypoxia, hyper or hypokalemia, myocardial infarction or digoxin intoxication (Tilley et al., 2008). Having already proven the association between ST-segment depression on electrocardiogram and myocardial infarction pressing for death (Namana et al., 2018; Zeijlon et al., 2022), it is inferred that the presence of lipid infiltrates may predispose patients to severe risk of dying. Thus, 47.1% of the dogs in this study could be classified in this group, since they presented that electrocardiographic alteration.

Rosa (2012), when analyzing 55 cardiac fragments from 30 dogs, identified necrosis of the contraction band in 29.1% (16/55) of them, as opposed to the 50% (10/20) observed in this research. In a study with induction of cardiac hypoxia by clamping the ascending aorta for 120 minutes, necrosis of the contraction band was observed in 100% (5/5) of the evaluated dogs and all of them died. In these animals, anatomopathological analyzes revealed intense alterations compatible with myocardial necrosis (Dias et al., 2002).

In experimental studies with animals in which catecholamines (epinephrine, norepinephrine, salbutamol, terbutaline and ephedrine) were administered, multifocal areas of myocardial necrosis were observed, predominantly in the left ventricle (Greaves, 2012). This group of amines increases both the influx of Ca++ ions and the sensitivity of sarcomeres of cardiac muscle fibers to this ion, which is responsible for triggering sarcomere contraction (Reece et al., 2017). Microscopically, this damage is visualized as muscle fiber necrosis associated with contraction band necrosis and inflammatory infiltrate, the last stage being tissue fibrosis (Greaves, 2012).

Contraction band necrosis was also observed in the heart after acute myocardial infarction, in areas of necrosis. Microscopically, irreversible damage to myocytes with development of necrosis has been described, even after myocardial reperfusion. In this

pathological process, there is an influx of calcium through the plasma membrane reaching the myofibrils. In the absence of ATP resulting from tissue hypoxia, sarcomeres cannot relax, entering a state of tetany, which is visualized in the form of eosinophilic bands due to hypercontraction of sarcomeres (Kumar et al., 2021).

In this study, the necrosis of the contraction bands presented a positive and significant correlation with the amount of serum globulins, but the correlation was negative with the dimension of the PR segment of the electrocardiogram.

It is noteworthy that the animals in this study showed concomitant histopathological changes, with the association between lipid infiltrate and necrosis of the contraction band being the most frequent. These changes are compatible with areas of acute myocardial infarction (Greaves, 2012).

In this study, of the 16 animals that presented histopathological alterations, only one presented, in isolation, necrosis of the contraction band, while nine had this necrosis associated with other alterations and another six animals did not present necrosis, but inflammatory infiltrates. Possibly, these last animals developed myocardial alterations more recently when compared to the others in the group, which would explain the absence of contraction band necrosis.

It can be inferred, then, that tissue hypoxia promotes important tissue changes in the cardiac muscles, starting with lipid and/or inflammatory infiltrate, progressing to necrosis of the contraction band and ending with myocardial fibrosis. In this study, this hypoxia could have been caused by alterations in the mitral valve, which was verified in 95% (19/20) of them (Figures 7 and 8). This type of alteration normally triggers left congestive heart failure, which culminates in respiratory failure, the main consequence of which is generalized tissue hypoxia. This hypoxia could trigger injury to cardiomyocytes, which would worsen the condition, thus initiating a positive feedback cycle, which could cause cardiac decompensation and consequent death.

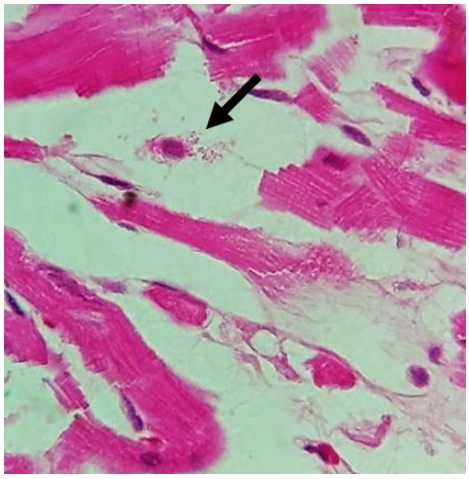

Fig. 11. Macrophage infected with amastigotes of *L. infantum* (arrow) in a histological section of the heart muscle (right myocardium wall) of a dog naturally infected with *L. infantum* in the municipality of Montes Claros, MG, Brazil, stained with HE and viewed under an optical microscope at 1,000x magnification.

The presence of amastigotes of *L. infantum* (Figure 11) were verified in the cardiac musculature of 10% (2/20) of the dogs in this investigation. Despite this, few studies in dogs describe similar findings (Alves et al., 2010; Mendes et al., 2014; López-Peña et al, 2009). However, these authors used the immunohistochemical technique to verify amastigotes in the myocardium, while this team adopted the HE and picrosirius stains, making it impossible to perform the analysis with specific stains. Thus, the type of staining used was not favorable to the identification of the parasites, which reinforces the need for further investigations of this type not only in the heart, but also in other organs and structures to elucidate the pathogenesis of *Leishmania* in mammals.

4.6. Mitral valve histopathology

Although studies about the mitral valve in dogs with VL have not been found in the scientific literature, reports of changes such as those found in this research, even triggered by other causal factors, allow for a brief discussion.

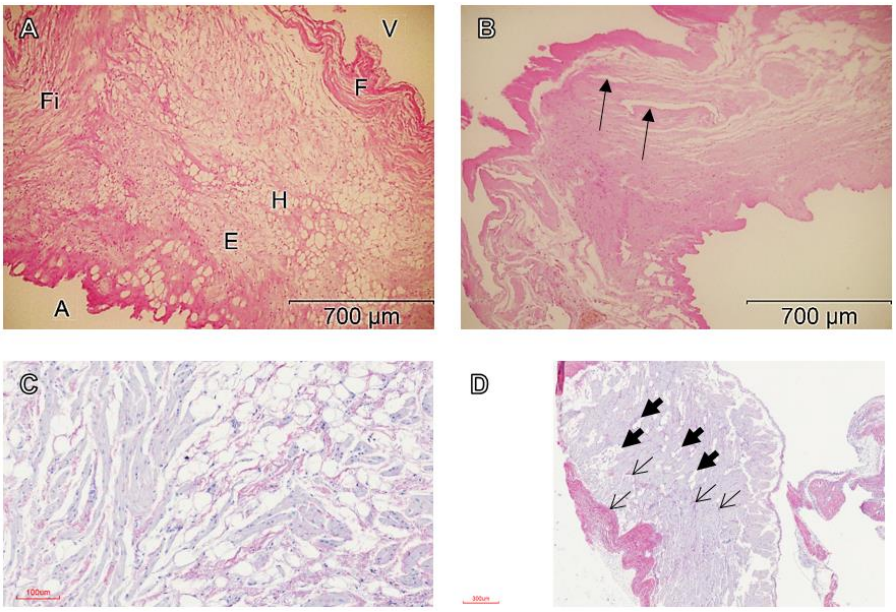

Fig. 12. Histological sections of the mitral valve of dogs naturally infected with *L. infantum* in the municipality of Montes Claros, MG, Brazil. (A) Atrial (A), spongy (E), fibrous (F) and ventricular (V) regions, and distribution of lesions as fibrous regions (Fi) and hydropic degeneration (H) under HE staining. (B) neovascularization (arrows) under HE staining. (C) Adipose metaplasia under picrosirius staining. (D) Areas of fibrosis (thin arrows) and adipose metaplasia (thick arrows) under picrosirius staining.

In a review of the literature on the histopathological characteristics of the mitral valve with myxomatous degeneration in dogs, expansion of the extracellular matrix with glycosaminoglycans and proteoglycans, changes in valve interstitial cells, and attenuation or loss of the collagen-laden fibrous layer were reported (Fox, 2012). Unlike these findings, this present study identified an increase in the deposition of collagen fibers, in addition to adipose metaplasia and areas of neovascularization (Figure 12), factors related to various chronic injuries and reactional responses of the organism, in an attempt to recover, as reported by Kumar et al. (2021). This type of reaction has already been reported in dogs with CVL, although the valve apparatus was evaluated, but the cardiac musculature (Mendes et al., 2014).

Cardiac murmur resulting from reflux in the mitral valve of dogs with VL was verified in 5.56% (2/36) of the animals in the studies by Santos et al. (2015). However, these authors, unlike this present study, did not find valve alterations in the animals, suggesting that the reduction in blood viscosity due to anemia would be the causal factor of valve reflux, which would generate the sound.

5. Conclusion

On the electrocardiogram, the R wave was increased in all animals analyzed, as well as the mean systolic blood pressure of the animals. The presence of fibrous degeneration, lipid infiltrate and contraction band necrosis in the right myocardium was also verified. Fibrosis and adipose metaplasia were also observed in the animals' mitral valve, characterizing myxomatous degeneration.

Ethical Care

The project for this research was submitted to the Ethics and Animal Welfare Commission of UNIMONTES / CEEBEA-UNIMONTES, having been approved in accordance with opinion number 192, of July 12, 2019, and changes to the project were also approved. according to opinion certificate number 234, of October 24, 2021.

Authorship Contribution Statement CRediT

Patrícia Almeida: conceptualization, methodology, formal analysis, investigation, data curation, writing - original draft, visualization, project administration; **Thallyta Vieira:** conceptualization, resources; writing - proofreading and editing, supervising, acquiring funding; **Jamille Lula:** writing - proofreading and editing; supervision, resources; **Leandro Teles:** writing - proofreading and editing, supervision, resources; **Chrystian Iezid Feres:** writing – review, methodology, complete statistical analysis; **Roberto Ferreira:** partial methodology; **Marcelo Meuser-Batista:** partial methodology; **Sílvio Carvalho:** conceptualization, resources, writing - proofreading and editing, supervision, project administration, funding acquisition

Declaration of Conflict of Interests

The authors declare that they have no conflict of interest.

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5. CONSIDERAÇÕES FINAIS

A infecção por *Leishmania infantum* causando leishmaniose visceral canina podem desencadeia alterações cardíacas importantes nos cães, mesmo que esses animais estejam assintomáticos. Manifestações eletrocardiográficas, como aumento da onda R, podem ser adotados como preditores dessa patologia.

Manifestações de insuficiência cardíaca congestiva esquerda devem ser consideradas como fator preditor para suspeita de LVC, tendo em vista a grande cadeia de alterações cardíacas observadas nesta pesquisa. Recomenda-se, portanto, atenção a sinais clínicos, laboratoriais e de demais exames auxiliares que apontem para lesões cardíacas.

Em contrapartida, postula-se atenção ao sistema cardiovascular em animais diagnosticados com LVC, objetivando-se reduzir ou evitar a evolução da sintomatologia cardíaca e melhorando a qualidade e o prognóstico de vida desse paciente.

Como diversas variáveis foram fortemente correlacionadas com a presença da *Leishmania infantum*, analisá-las pode ser útil na prática clínica para auxiliar no diagnóstico da LVC e no acompanhamento da progressão da doença. Uma limitação deste estudo é a falta de informações sobre coinfecções por *Babesia* spp. e *Ehrlichia canis*. Portanto, estudos futuros poderiam avaliar a influência de tais coinfecções nas associações estudadas por métodos multivariados com amostras maiores.

Recomenda-se, por fim, estudos onde haja maior esquadrinhamento das alterações cardíacas tanto em animais quanto em pessoas, objetivando-se identificar vias mais responsivas, eficazes e de efeito mais duradouro para seus tratamentos.

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APÊNDICE

APÊNDICE A – Certificado de aprovação do projeto pelo CEEBEA / UNIMONTES

Universidade Estadual de Montes Claros
Comissão de Ética em Experimentação e Bem-Estar Animal da Unimontes
CEEBEA

Unimontes

CERTIFICADO

Certificamos que o protocolo nº **234,** relativo ao projeto intitulado "**Desenvolvimento de lesões cardíacas, pulmonares e renais em cães com Leishmaniose visceral na cidade de Montes Claros, MG"** sob coordenação do Coordenador Prof. Dr. Sílvio Fernando Guimarães Carvalho está de acordo com os princípios éticos na experimentação animal, adotados pela Comissão de Ética em Experimentação e Bem-Estar Animal da Unimontes, e encontra-se **APROVADO** referente a reunião de 01/10/2021.

A quantidade total de animais pelo CEEBEA para este projeto foi de 50 animais.

Este certificado é válido por cinco anos após sua aprovação.

Montes Claros, 28 de Outubro de 2021.

Profª Drª Antonia de Maria Filha Ribeiro
Coordenadora do CEEBEA/UNIMONTES

Profª Antonia de Maria F. Ribeiro
Coordenadora da CEEBEA da Unimontes