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PROGRAMA DE PÓS GRADUAÇÃO EM BIOCIÊNCIA ANIMAL

AVALIAÇÃO DOS ACHADOS CLÍNICOS, HEMATOLÓGICOS E BIOQUÍMICO SÉRICOS EM CÃES NATURALMENTE INFECTADOS POR *Leishmania infantum* SUBMETIDOS A TRATAMENTO EXPERIMENTAL

VÍCTOR JESÚS HUARINGA PAYANO

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Dissertação apresentada ao Programa de Biociência Animal da Universidade Federal Rural de Pernambuco, como pré-requisito parcial para obtenção do grau de Mestre em Biociência Animal.

Orientador: Prof. Dr. Leucio Câmara Alves

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Aprovada em __ de _____ de 2018

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“Kanmi rurakuna huk punchaw atipaq, allin runakunam kanku. Kantaqmi wakin, watantin atipaq; alli-allinmi kanku. Kantaqmá pikuna achka watantin atipaq, llumpay allinmi kanku. Ichaqa kankutaqmi, pikuna tukuy kawsakuynimpi atipaq; paykunam chay mana qipanchanakuna"

“Hay hombres que luchan un día y son buenos. Hay otros que luchan un año y son mejores. Hay quienes luchan muchos años, y son muy buenos. Pero hay los que luchan toda la vida, esos son los imprescindibles.”

“Há homens que lutam um dia e são bons, há outros que lutam um ano e são melhores, há os que lutam muitos anos e são muito bons. Mas há os que lutam toda a vida e estes são imprescindíveis.”

Bertolt Brecht.

RESUMO

A Leishmaniose visceral canina (LVC) é uma antropozonoose causada pelo parasito de tipo intracelular *Leishmania infantum.* Os cães podem ser classificados em assintomáticos ou sintomáticos, sendo os sinais dependentes da resposta imune de cada animal. O tratamento continua sendo um desafio, assim como a interpretação dos achados hematológicos e da bioquímicos sérica, pela falta de uniformização dos resultados. O objetivo deste trabalho foi a avaliar achados clínicos, hematológicos e da bioquímicos sérica de cães naturalmente infectados com *Leishmania infantum*, submetidos a tratamento experimental. Foram utilizados 14 cães com diagnóstico parasitológico positivo para *Leishmania spp.* Estes foram separados em dois grupos de tratamento, um com alopurinol associado a domperidona (DOA) (n=7) e o outro grupo alopurinol associado a miltefosina (MIA) (n=7). Os animais foram monitorados a cada 30 dias até o dia 90 pós tratamento, e realizou-se exame físico, clínico, pesquisa parasitológica de formas amastigotas de *Leishmania* sp. de aspirado de medula óssea, linfonodo e citologia esfoliativa de pele, além do hemograma e bioquímica sérica: ureia, creatinina, ALT, AST, proteínas totais, globulina e albumina. Todos os animais a partir do dia 60 apresentaram melhora clínica e negativaram na pesquisa parasitológica. Os dois grupos de tratamento no início do estudo revelaram trombocitopenia seguida por hiperproteinemia plasmática e anemia, os resultados da bioquímica sérica revelaram hiperglobulimenia, hipoalbuminemia, AST elevado e azotemía. Após 90 dias de tratamento as alterações hematológicas e bioquímicas diminuíram, mas no grupo DOA ainda apresentou anemia (14,29%), trombocitopenia (28,57%), hiperproteinemia (71,43%) e leucocitose (42,86%) no hemograma, e azotemía (14,29%), hipoalbuminemia (71,43%) e hiperglobulinemia (71,43%) na bioquímica sérica. No grupo MIA apresentou linfocitose (28,57%), eosinofilia (14,29%), trombocitopenia (42,86%) e hiperproteinemia plasmática (71,43%) no hemograma, e azotemia (42,86%), hiperglobulinemia (100%) e hipoalbuminemia (85,71%) na bioquímica sérica. Conclui-se que, o tratamento de alopurinol associado a domperidona é o melhor protocolo favorecendo a remissão dos achados clínicos e laboratoriais.

Palavras-chave: Leishmaniose Visceral Canina, Tratamento, Hematologia, Bioquímica sérica, Clínica médica

ABSTRACT

Canine Visceral Leishmaniose (CVL) is an anthropozoonosis caused by a intracelular parasite *Leishmania infantum.* The canines can be classified by asymptomatic or symptomatic, the clinical signs depend on the immune response of each animal. The treatment is still a challenge, also the interpretation of hematologic and serum biochemical findings, because the results has not a consensus. The aim of this study was evaluate the clinical, haematological and serum biochemical findings of canines naturally infected with *Leishmania infantum*, submitted to experimental treatment. Were used 14 canines with positive parasitological diagnostic to *Leishmania spp.* them were separated in two treatment groups, one allopurinol associate with domperidone (DOA) (n=7) and other allopurinol associated with mitelfosine (MIA) (n=7). The animals were monitored every 30 days until 90 days of treatment, were made physical and clinical exam, parasitological analysis to find amastigote forms of *Leishmania sp.* on bone marrow, lymph node and skin cytology, also were made an complete blood count (CBC) and serum biochemistry (urea, creatinine, ALT, AST, total proteins, globulin and albumin). Al the animals showed clinical improve and were negative to parasitological exam since day 60. Both treatment groups revealed thrombocytopenia, plasmatic hyperproteinemia and anaemia on CBC, serum biochemistry revealed hyperglobulinemia, hipoalbuminemia, azotaemia and increased AST. After 90 days of treatment CBC and biochemistry alterations decreased, DOA treatment still has Anaemia (14,29%), thrombocytopenia (28,57%), hyperproteinemia (71,43%) and leucocytosis (42,86%) at CBC, and e azotaemia (14,29%), hipoalbuminemia (71,43%) and hyperglobulinemia (71,43%), at serum biochemistry. MIA group still present lymphocytosis (28,57%), eosinophilia (14,29%), thrombocytopenia (42,86%) e plasmatic hyperproteinemia (71,43%)at CBC, and azotaemia (42,86%), hyperglobulinemia (100%) and hipoalbuminemia (85,71%) at serum biochemistry. Was concluded that allopurinol associated with domperidone treatment is the best protocol because it decrease clinical signs and laboratorial findings.

Key words: Canine Visceral Leishmaniose, Treatment, Haematology, Serum biochemistry, Medical clinic.

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1 INTRODUÇÃO

A Leishmaniose Visceral (LV) é uma antropozoonose de distribuição mundial que acomete os animais silvestres e domésticos e o homem em áreas rurais e urbanas (SASANI *et al.*, 2016), sendo transmitido aos hospedeiros susceptíveis mamíferos por insetos dípteros da família Psychodidade (BRITO, FLÁVIO GONÇALVES *et al.*, 2016) do gênero Phlebotomus no velho mundo, e Lutzomyia no novo mundo (GHARBI *et al.*, 2015; QUINNELL; COURTENAY, 2009).

No Brasil, o agente responsável pela LV é o protozoário da espécie *Leishmania infantum*, cuja transmissão é realizada pelo *Lutzomyia longipalpis* (LAINSON; RANGEL, 2005; NOLI; AUXILIA, 2005) durante o repasto sanguíneo, tendo o cão como principal reservatório em áreas urbanas (FRAGA, DEBORAH B M *et al.*, 2012; MOREIRA *et al.*, 2016).

A LV nos cães é uma doença complexa, sistêmica, crônica e até mesmo fatal, caracterizada por alterações clínicas muito variáveis, envolvendo quase todos os órgãos, em consequência da multiplicidade de mecanismos patogênicos do protozoário, da diversidade de respostas imunológicas desenvolvidas nos hospedeiros e do longo período de incubação, que pode variar de alguns meses até vários anos (BANETH *et al.*, 2008).

Considerada como doença imunomediada, a progressão da infecção nos cães apresenta acentuada resposta humoral com títulos de imunoglobulinas e uma depressão da resposta imunológica celular levando o aparecimento de animais assintomáticos ou sintomáticos (BANETH *et al.*, 2008; CARDOSO *et al.*, 2007; MAIA; CAMPINO, 2008).

Sendo assim os cães infetados podem apresentar uma variedade de sinais clínicos inespecíficos, devido às interações complexas do parasito com o sistema imune do hospedeiro (DE ALMEIDA LEAL *et al.*, 2014; NICOLATO *et al.*, 2013; PAPADOGIANNAKIS; KOUTINAS, 2015). No exame clínico dos cães com LV pode-se observar lesões de pele, linfadenomegalia, hepato-esplegnomegalia, perda de peso, intolerância ao exercício, letargia, poliúria, polidipsia, lesões oculares, epistaxe, onicogrifose, debilidade, vômito e diarreia (SOLANO-GALLEGO, LAIA *et al.*, 2017; SYKES, 2013).

Os achados laboratoriais mais comuns nestes aninais são hiperproteinemia com hipoalbuminemia e hiperglobulinemia, azotemia, elevação das enzimas hepáticas, anemia, leucocitose ou leucopenia, e trombocitopenia, (BRASILICA *et al.*, 2015; TORRECILHA *et al.*, 2016; ULCHAR *et al.*, 2015).

O tratamento contra LV nos cães tem sido realizado na Europa, seguindo distintos protocolos (NOLI; SARIDOMICHELAKIS, 2014; REGUERA *et al.*, 2016). No Brasil o tratamento de LVC não era uma medida recomendada, sendo indicado a eutanásia dos cães soro positivos para *L. infantum* (MINISTÉRIO DA SAÚDE, 2014).

A partir de 2016, o Ministério de Agricultura, Pecuária e Abastecimento autorizou o uso de mitelfosina, agente antiprotozoario, para o tratamento da LV em cães no Brasil (MAPA, 2016), regulamentando assim o tratamento para a espécie canina. Apesar disto atualmente o protocolo mais recomendado para tratamento nestes animais é a combinação da mitelfosina e alopurinol.

Por mais que tenha sido realizado um protocolo de tratamento específico para um canino com infecção natural por *L. infantum poucos* são os dados existentes na literatura sobre a avaliação dos achados clínicos e patológicos durante e após o tratamento. Sendo assim o objetivo deste trabalho foi avaliar a os achados clínico-patológicos e citológicos, em cães naturalmente infectados com *L. infantum submetidos* a diferentes protocolos de tratamento experimental.

2 REVISÃO DE LITERATURA

2.1 Leishmaniose Visceral

A LV é uma enfermidade de distribuição mundial, endêmica na Europa, Ásia, África e Américas, (BRITO, FLÁVIO GONÇALVES *et al.*, 2016; GREENE, 2012; OMS, 2017). No Brasil, a doença estava distribuída nas regiões Norte, Centro-oeste, Sudeste, e Nordeste, sendo esta última região com a maior quantidade de casos reportados anualmente (DANTAS-TORRES; BRANDÃO-FILHO, 2006; DO NASCIMENTO RAMOS, 2012; MINISTÉRIO DA SAÚDE, 2014). Em 2009 casos humanos autóctones na cidade de São Borja (RS) foram registrados (PACHECO *et al.*, 2013), com presença do principal *vetor Lutzomyia longipalpis* neste estado (SOUZA; DOS SANTOS; FILHO, 2009), passando assim a enfermidade a ser endêmica em todas as regiões brasileiras.

2.2 Agente etiológico

O agente etiológico da Leishmaniose é um protozoário bifásico do gênero *Leishmania*, da classe Kinetoplastida e família Trypanosomatidae, o qual divide-se nos subgêneros Vianna e Leishmania (GREENE, 2012; OMS, 2017; SYKES, 2013).

Considerada uma zoonose crônica, a LV é causada por protozoários intracelulares do gênero Leishmania, pertencentes ao complexo *Leishmania* (*Leishmania*) *donovani* (LAINSON; SHAW, 1987) encontrada principalmente na Índia, Paquistão e Bangladesh e *L. infantum* encontrada no Brasil e alguns países do Mediterrâneo África Oriental, norte da Ásia e da China (DANTAS-TORRES; BRANDÃO-FILHO, 2006), acometendo o homem e diferentes espécies de mamíferos silvestres (ORYAN; AKBARI, 2016; ROQUE; JANSEN, 2014), e cuja transmissão ocorre através da picada de insetos vetores (QUINNELL; COURTENAY, 2009) da família Psychodidae, (MARTINS; WILLIAMS; LIMA FALCAO, 1978; TAFURI *et al.*, 2004).

No Brasil, o agente responsável pela LV é o protozoário da espécie *Leishmania infantum*, cuja transmissão é realizada pelo *Lutzomyia longipalpis* (LAINSON; RANGEL, 2005; NOLI; AUXILIA, 2005) durante o repasto sanguíneo, tendo o cão como principal reservatório em áreas urbanas (FRAGA, DEBORAH B M *et al.*, 2012; MOREIRA *et al.*, 2016).

2.3 Ciclo biológico

A biologia parasitária ocorre com participação de dois hospedeiros, o flebotomíneo, que apresenta a forma promastigota no seu intestino, e um hospedeiro definitivo mamífero, que possui a forma ovoide ou redondeada, não móvel, denominada de amastigota (GREENE, 2012; KASPER *et al.*, 2015; SASANI *et al.*, 2016; TREVISAN *et al*, 2015).

As formas promastigotas são inoculadas no organismo vertebrado mediante a picada da fêmea do flebotomíneo durante o repasto sanguíneo. Dentro do organismo vertebrado as promastigotas são fagocitadas pelos macrófagos, onde o parasito se transforma na forma amastigota, a qual resiste ao fagolisossoma e replica-se por divisão binária rompendo os macrófagos e infectando outros macrófagos, os quais disseminaram a infecção por via sanguínea e linfática (GREENE, 2012; KUMAR, 2013; SYKES, 2013).

Quando os insetos vetores se alimentam do sangue do hospedeiro infectados contendo as formas amastigotas, ingerem os macrófagos, parasitados as quais irão se transformar na forma promastigota, completando assim, o ciclo biológico deste protozoário (GREENE, 2012; KUMAR, 2013; SYKES, 2013).

2.4 Hospedeiros susceptíveis e reservatório

Dentro dos hospedeiros vertebrados estão os animais silvestres, domésticos e o homem (KASPER *et al.*, 2015; MINISTÉRIO DA SAÚDE, 2014; REGUERA *et al.*, 2016). No Brasil o cão tem sido considerado o principal reservatório para a leishmaniose visceral nas áreas urbanas e secundariamente o gato (DANTAS-TORRES; BRANDÃO-FILHO, 2006; GREENE, 2012; REGUERA *et al.*, 2016).

2.5 Leishmaniose Visceral Canina (LVC)

É uma síndrome imunomediada, gerada pela interação do parasito com o sistema imune do cão (BANETH *et al.*, 2008; PEREIRA JUNIOR, 2014; SILVA, FRANCINE MARIA DE FRANÇA, 2014). Esta associação é um fator determinante na LVC, uma vez que, a resposta imune do cão no momento da

infecção é crucial na eliminação ou persistência do parasito hospedeiro canino (GREENE, 2012; NASCIMENTO, 2015; PEREIRA JUNIOR, 2014), com apresentação clínica ou não, sendo em ambos os casos considerados reservatórios (DANTAS-TORRES *et al.*, 2012; GREENE, 2012; SOLANO-GALLEGO, L. *et al.*, 2009)

2.6 Sinais clínicos

Como a LVC é considerada uma doença imunomediada a progressão dos sinais clínicos ocorre em um intervalo de meses a vários anos (CIARAMELLA P., 2003; FERRER, 1999). Neste sentido, os cães com LV apresentam uma doença multissistêmica e pódem apresentar quadros clínicos variados desde aparente estado sadio a um severo estágio final (FERRER, 1999).

Entre os sinais clínicos, mais frequentes destacam-se as oftalmopatias, dermatopatias, onicogrifose, hepatoesplenomegalia e linfoadenopatia (GÓMEZ-OCHOA, P. *et al.*, 2009; LAURENTI *et al.*, 2013; PALTRINIERI *et al.*, 2010), além de perda de peso, vômito, diarreia, poliúria, polidipsia, epistaxe, melena (GHARBI *et al.*, 2015; GREENE, 2012; NELSON; COUTO, 2014; SYKES, 2013).

Embora não estejam necessariamente presentes em todos os animais (REIS, ALEXANDRE B. *et al.*, 2006), alguns achados clínicos laboratoriais podem ser encontrados como a anemia, trombocitopenia, e alterações nas funções renal e hepática (TORRECILHA *et al.*, 2016).

As dermatopatias são as manifestações clínicas mais frequentes na LVC (ORDEIX *et al.*, 2005; SOLANO-GALLEGO, L. *et al.*, 2004), sendo a dermatite descamativa a alteração mais comum, reportada entre 56 a 91% dos casos (PAPADOGIANNAKIS *et al.*, 2005), além de alopecia não pruriginosa, ulceras indolores geralmente localizadas na cabeça ou membros, dermatite pustular estéril, dermatite nodular mucocutanea e onicogrifose (ORDEIX *et al.*, 2005), podendo aparecer na ausência de outros sinais clínicos em 56% ou 90% dos cães (GREENE, 2012; NASCIMENTO, 2015; PAPADOGIANNAKIS; KOUTINAS, 2015).

A hiperqueratose pode surgir na cabeça, focinho e patas, além de um crescimento desordenado das unhas (onicogrifose) associado a paroníquia (GHARBI *et al.*, 2015; NELSON; COUTO, 2014; SYKES, 2013).

Outro sinal clínico frequente é a linfadenomegalia (GHARBI *et al.*, 2015; GREENE, 2012), os linfonodos afetados podem estar aumentados de dois até seis vezes o tamanho normal, podendo ser o único sinal clínico (GHARBI *et al.*, 2015; GREENE, 2012; NELSON; COUTO, 2014).

Os sinais oculares são comuns em 16% a 80,5% dos cães com LVC (GREENE, 2012), os quais são: conjuntivite difusa ou nodular, blefarite nodular ou ulcerativa, esclerite difusa ou nodular, ceratoconjuntivite, glaucoma, panoftalmia e uveite anterior ou posterior a qual pode ser granulomatosa ou difusa (BRITO, F L C *et al.*, 2006; GHARBI *et al.*, 2015; GREENE, 2012; NELSON; COUTO, 2014).

A insuficiência renal pode estar presente em cães sem os sinais clássicos sistêmicos de LVC. A presença persistente de proteína na urina pode levar ao aparecimento de sinais clínicos compatíveis com síndrome nefrótica, tais como hipoalbuminemia, ascite, edema periférico e hipercolesterolemia (KOUTINAS; KOUTINAS, 2014; PIERANTOZZI *et al.*, 2013).

Surgimento dos sinais clínicos estão associados à densidade de parasitas na pele, baço, linfonodos e medula óssea (TORRECILHA *et al.*, 2016), podendo contribuir nas alterações dos achados hematológicos e bioquímicos (MANNA *et al.*, 2009; TORRECILHA *et al.*, 2016).

2.7 Diagnóstico

Muitas vezes, o diagnóstico da LVC é complexo, devido à variedade de sinais clínicos inespecíficos (GHARBI *et al.*, 2015; SYKES, 2013; WILLARD; TVEDTEN, 2011). O diagnóstico da LVC tem sido realizado para a confirmação da doença em animais que apresentem sinais clínicos compatíveis com a enfermidade, ou para investigar a presença da infecção em cães clinicamente saudáveis provenientes de regiões endêmicas (MIRÓ *et al.*, 2008).

Acredita-se que 40% a 60% dos animais infectados são assintomáticos (MELO LIMA, 2013), motivo pelo qual o diagnóstico por métodos laboratoriais é de suma importância (ABRANTES *et al.*, 2016).

O diagnóstico laboratorial é realizado através de técnicas parasitológicas, sorológicas e moleculares (BEST *et al.*, 2014; RODRÍGUEZ-CORTÉS *et al.*, 2010; SOLANO-GALLEGO, LAIA *et al.*, 2017).

2.7.1 Técnicas parasitológicas

Dentre as formas de diagnóstico da doença, o teste “padrão ouro” é a detecção do parasito através de punção aspirativa da medula óssea, linfonodos, baço, fígado, além da citologia esfoliativa da pele íntegra e/ou lesionada (DE MELLO *et al.*, 2016; MOMO *et al.*, 2014; SILVA, KATHLENN LIEZBETH OLIVEIRA *et al.*, 2014). Estes baseiam-se na pesquisa de formas amastigotas da *L infantum* dentro dos macrófagos ou livres no esfregaço do aspirado de medula óssea, linfonodo, baço, fígado e na citologia esfoliativa da pele (CIAN; FREEMAN, 2016; COWELL; VALENCIANO, 2014; GHARBI *et al.*, 2015; GREENE, 2012).

Esses exames apresentam alta especificidade demostrando-se seguros quanto à positividade dos casos, porém, possuem baixa sensibilidade (BEVILACQUA; ALVES, 2004).

Para a detecção do parasito, outros testes como o histopatológico e a imunohistoquímica podem ser utilizados (ABRANTES *et al.*, 2016) os quais permitem a avaliação da carga parasitária e o tipo de resposta inflamatória frente à infecção (MAXIE, 2015; NASCIMENTO, 2015; SILVA, FRANCINE MARIA DE FRANÇA, 2014; ZACHARY; MCGAVIN, 2016).

2.7.2 Técnicas sorológicas

Os métodos sorológicos utilizados para o diagnóstico de LVC detectam presença de IgG anti *L infantum* (GREENE, 2012; NASCIMENTO, 2015; SILVA, FRANCINE MARIA DE FRANÇA, 2014; SYKES, 2013).

O título de anticorpos vai depender do tipo de resposta imune do cão infectado, sendo que animais sintomáticos apresentam níveis elevados de imunoglobulinas IgG e uma baixa ou nula resposta celular (SOLANO-GALLEGO, LAIA *et al.*, 2016; TORRECILHA *et al.*, 2016).

Entre os métodos sorológicos destaca-se a Reação de Imunofluorescência Indireta (RIFI), Ensaio de Imunoadsorção Enzimática

(ELISA), Fixação do Complemento (FC), Teste de Aglutinação Direta (TAD) (BRASIL, 2006) e Imunoeletroforese (MARCONDES *et al.*, 2000) e o ensaio imunocromatográfico denominado de “Dual-Path Platform” (DPP), o qual se mostrou eficaz e com alta especificidade para diagnóstico da LVC em cães sintomáticos (GRIMALDI *et al.*, 2012).

2.7.2.2a Reação de Imunofluorescência Indireta (RIFI)

A RIFI mostrou sensibilidade e especificidade inferior a 68 e74% respectivamente , produzindo uma grande quantidade de falsos positivos e falsos negativos (ABRANTES *et al.*, 2016; FIGUEIREDO, F. B. *et al.*, 2010; FIGUEIREDO, FABIANO B *et al.*, 2010).

2.7.2.2b Ensaio de Imunoabsorção Enzimática (ELISA)

O ELISA permite a avaliação de um grande número de amostras, apresentando alta sensibilidade, variando de 71% a 100%, e especificidade de 85% a 100% (MARCONDES *et al.*, 2013) Mesmo que o ELISA seja considerado um método de alta sensibilidade, não é útil para o monitoramento da enfermidade (MANNA *et al.*, 2015). Embora, seja atualmente é a prova confirmatória de LVC (MINISTÉRIO DA SAÚDE DO BRASIL, 2011).

2.7.2.2c Teste rápido: Dual Path Platform (DPP® Bio Manguinhos)

É o método de triagem recomendado pelo Ministério de Saúde por sua simplicidade, rapidez, sensibilidade (93 a 100%) e especificidade (99 a 100%) (ALVAR *et al.*, 2004; MINISTÉRIO DA SAÚDE DO BRASIL, 2011). O teste baseia-se na detecção da proteína recombinante quimérica rk28, o qual da os altos valores de especificidade e sensibilidade, além de permitir a obtenção de resultados em 15 minutos, utilizando pequenas quantidades de amostra (FRAGA, DEBORAH BITTENCOURT MOTHÉ *et al.*, 2016).

2.7.3 Técnicas moleculares

Os métodos moleculares para detecção de DNA de *L infantum* no paciente canino tem um melhor desempenho comparadas com as provas diretas, imunohistoquímica e sorologia (SANTOS *et al.*, 2014). A Reação da Cadeia em Polimerase (PCR) é útil para detecção da enfermidade em cães assintomáticos e soronegativos (ASCHAR *et al.*, 2016; COURA-VITAL *et al.*, 2014).

A sensibilidade da técnica depende do tipo de amostra, sendo o material obtido da medula óssea, linfonodos e biopsia de pele os mais indicados e sensíveis (CORPAS-LÓPEZ *et al.*, 2016; NASCIMENTO, 2015; NOLI; SARIDOMICHELAKIS, 2014). Além destes, é possível usar sangue periférico (ASCHAR *et al.*, 2016; CORPAS-LÓPEZ *et al.*, 2016; LOMBARDO *et al.*, 2012). A quantificação do DNA do cinetoplasto dos parasitos mostrou utilidade no monitoramento da enfermidade (ROURA *et al.*, 2013).

A PCR em tempo real permite um diagnóstico quantitativo sendo possível a avaliação do tratamento usado, pela mensuração da carga parasitária (MANNA *et al.*, 2009; TORRECILHA *et al.*, 2016), tendo maior vantagem em especial quando se usa alvos multicópia, como DNA de kinetoplasto (REIS, LEVI EDUARDO SOARES *et al.*, 2013).

2.7.4 Exames complementares

Outros testes laboratoriais podem ter valor no diagnóstico da LVC, como o hemograma, bioquímica e mielograma (PALTRINIERI *et al.*, 2010, 2016; RODRÍGUEZ-CORTÉS *et al.*, 2010; TLAMCANI, 2016; VÍCTOR ACERO *et al.*, 2015).

O achado hematimétrico mais comuns na LVC são: anemia, presente em 57 a 94,2% dos casos (LATIMER; DUNCAN, 2011; STACY; HARVEY, 2017), leucocitose ou leucopenia e trombocitopenia (NICOLATO *et al.*, 2013; PALTRINIERI *et al.*, 2016; SONODA *et al.*, 2013).

Em se tratando da bioquímica sérica, a hiperproteinemia com hiperglobulinemia com as elevação de proteínas de fase aguda e hipoalbuminemia (GREENE, 2012; NELSON; COUTO, 2014; SYKES, 2013), além da elevações nas enzimas hepáticas (DE FREITAS *et al.*, 2016; DE PÁDUA COSTA *et al.*, 2015; MELO *et al.*, 2009).

No mielograma tem sido observado displasia eritroide, com maturação anormal e morfologia anormal de eritrócitos, núcleos fragmentados, células multinucleadas, asincronia nuclear, citoplasmática, mitose anormal (FOGLIA MANZILLO *et al.*, 2006), além de eritrofagocitose elevada (FOGLIA MANZILLO *et al.*, 2006; MOMO *et al.*, 2014).

2.8 Tratamento

O tratamento da LVC é uma questão complexa. Os tratamentos disponíveis podem atingir apenas a cura clínica, reduzindo os sinais clínicos, títulos de anticorpos e a transmissão para os vetores em função da diminuição do parasitismo cutâneo (PINHÃO, 2009). Na maioria dos animais tratados ocorre a cura clínica mas não a parasitológica (PEREIRA JUNIOR, 2014; REGUERA *et al.*, 2016).

2.8.1 Antimonial pentavalente

Antimoniato de meglumina e Estibogluconato de sódio são antimoniais pentavalentes utilizados na medicina veterinária (PLUMB, 2011; REGUERA *et al.*, 2016). Ambos fármacos precisam ser metabolizados pelo parasito para serem ativos (REGUERA *et al.*, 2016), uma vez ativos, inibem os processos metabólicos essenciais do parasitos, como síntese de ATP e GTP, possibilitando a redução da carga parasitaria nas primeiras quatro semanas de tratamento (MANNA *et al.*, 2009; TRAVI, 2014).

A dose é 75-100 mg/kg/dia por 30 dias, devendo ser administrado por via intravenosa, subcutânea ou intramuscular (NOLI; SARIDOMICHELAKIS, 2014). visando obter concentrações plasmáticas adequadas nos órgãos alvo (REGUERA *et al.*, 2016). Não se recomenda o uso como monoterapia, porque pode não eliminar completamente o parasito e apresentar reincidência, em especial se o tratamento é curto (CROFT, SIMON L.; SUNDAR; FAIRLAMB, 2006; REGUERA *et al.*, 2016). Nos locais onde os antimoniais pentavalentes são injetados o paciente pode apresentar complicações como dor intensa, flebotoxicidade, tromboflebites, fibroses muscular e abcessos além de distúrbios hemáticos, nefrotoxicidade e hiperproteinemia esporádica (REGUERA *et al.*, 2016).

2.8.2 Alopurinol

É um análogo de purina, hipoxantina, usado como inibidor da xantina oxidase para reduzir a concentração de urato no soro sanguíneo (PAPICH, 2015; PLUMB, 2011; REGUERA *et al.*, 2016). *Leishmania* sp. metaboliza o alopurinol e o converte em inosina uma forma inativa o qual é incorporado no seu RNA (PLUMB, 2011; REGUERA *et al.*, 2016), inibindo a hipoxantina-guanina fosforibosiltransferase (HGPRT) assim interrompendo a sínteses de RNA e em

consequência a síntese de proteínas do parasito (PAPICH, 2015; REGUERA *et al.*, 2016; YASUR-LANDAU *et al.*, 2016).

A dose é de 15-30 mg/kg/dia divido em duas ou três doses diárias (NOLI; AUXILIA, 2005). Bons resultados foram obtidos em tratamentos de longo prazo entre quatro a dez semanas.

É administrado associado a outros fármacos (MATTIN *et al.*, 2014), como antimoniais pentavalentes, meglumine antimoniato ou estibogluconato de sódio (MANNA *et al.*, 2015; NOLI; SARIDOMICHELAKIS, 2014; PAPICH, 2015; TORRES *et al.*, 2011; YASUR-LANDAU *et al.*, 2016). A dose recomendada é 20-40 mg/Kg/dia dividida em duas doses associadas com meglumine antimoniato numa dose de 100mg/Kg/dia via intramuscular de três a quatro semanas (REGUERA *et al.*, 2016), e seguido da administração de alopurinol por pelo menos seis meses até um ano (SOLANO-GALLEGO, L. *et al.*, 2009).

2.8.3 Domperidona

É um benzimidazol antiemético usado em pacientes humanos, além de ser um galactogogo, promove a produção de prolactina, como antagonista do receptor D2, desde a hipófise que melhora a resposta inata Th1 e posterior liberação de IFN γ, IL 2, IL 12 e TNF α (REGUERA *et al.*, 2016).

A dose é 0.5 mg/Kg/dia por quatro semanas como profilaxia contra leishmaniose, e pode ser usado em fases iniciais da enfermidade (REGUERA *et al.*, 2016). A remissão total da enfermidade não é atingida (GÓMEZ-OCHOA, PABLO *et al.*, 2012), mas segundo um ensaio clínico a medicação por 30 dias a cada quatro meses diminui o risco de desenvolver a enfermidade (SABATÉ *et al.*, 2014)

2.8.4 Sulfato de aminosidina

É um antibiótico amino glucósido e mostrou efetiva atividade associado ao mitelfosine (CROFT, S. L.; OLLIARO, 2011). Este fármaco inibe a síntese de proteínas ribosomais do parasito (REGUERA *et al.*, 2016). A dose recomendada é de 15-20 mg/Kg/dia por três semanas, após este período o tratamento deve ser interrompido para evitar toxicidade (ATHANASIOU *et al.*, 2013).

2.8.5 Anfotericina B

É um fungicida, que se une ao ergosterol na membrana celular do fungo alterando a membrana, promovendo desequilíbrio osmótico e morte celular, além da atividade contra protozoários (PAPICH, 2015; PLUMB, 2011), apresentando atividade leishmanicida, formando poros aquosos na membrana do parasito (REGUERA *et al.*, 2016).

2.8.6 Miltefosina

O princípio ativo é a hexadecilfosfocolina (REGUERA *et al.*, 2016), sendo este fármaco liberado no Brasil no outubro do 2016 para tratamento de LVC segundo a Nota Técnica Nº 11/2016/CPV/DFIP/SDA/GM/MAPA, devido a não utilização em tratamento humano.

O mecanismo de ação não está esclarecido totalmente, mas estudos in vitro mostram que altera o metabolismo de ácidos graxos e esterol, ativa o mecanismo similar a apoptoses da célula no cinetoplasto e disfunção da mitocôndria (REGUERA *et al.*, 2016). Além disto promove a resposta tipo Th1, elevando os níveis de IFN γ assim estimulando a produção de NO e radicais reativos de oxigênio dentro dos vacúolos dos macrófagos, eliminando os parasitos (MANNA *et al.*, 2009; REGUERA *et al.*, 2016).

Tem a vantagem de ser o único princípio ativo que pode ser administrado via oral (REGUERA *et al.*, 2016). Pode ser usado na dose de 2 mg/kg/dia por 30 dias (NOLI; SARIDOMICHELAKIS, 2014) permitindo eliminar o parasito na maioria de órgãos infectados mas não elimina a totalidade destes na medula óssea (MATEO *et al.*, 2009) e linfonodos (MANNA *et al.*, 2009), por tanto é recomendável associar a medicação com outros fármacos anti leishmania sinérgicos (REGUERA *et al.*, 2016).

O Alopurinol também pode ser usado associado a miltefosina (MANNA *et al.*, 2015; TORRES *et al.*, 2011). Num ensaio clínico o uso da dose de 10mg/Kg/dia de alopurinol associado a 2 mg/kg/dia de miltelfosina por 30 dias, mostrou efetividade similar a associação alopurinol antimonial penta valente (MANNA *et al.*, 2008). Reincidências depois do tratamento com mitelfosina/alopurinol foram maiores em comparação com as reincidências do tratamento com meglumine antimoniato/alopurinol (MANNA *et al.*, 2015).

2.8.7 Outros fármacos

Entre outros produtos utilizados a pentamidina, um antifúngico usado para tratar LVC (SOEIRO *et al.*, 2013), inibe a replicação e transcrição do DNA do parasito (CALONGE *et al.*, 1996) e não apresentou efeitos adversos em cães tratados (NOLI; AUXILIA, 2005)

Marbofloxacina é uma fluoroquinolona de segunda geração, tem atividade bactericida (REGUERA *et al.*, 2016), melhorando a resposta do sistema imune favorecendo a produção de NO, lL 6 e TNF α (VOULDOUKIS *et al.*, 2006). A dose de 2 mg/Kg/dia por 28 dias produz remissão dos sinais clínicos, mas em 50% dos casos tem reincidência (ROUGIER *et al.*, 2012). Num ensaio de um ano em cães mostrou remissão mas estes se mantem positivos ao PCR de linfonodo (ROUGIER *et al.*, 2012).

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Objetivos

Geral

Avaliar os achados clínicos, hematológicos e da bioquímica sérica de cães naturalmente infectados com *Leishmania infantum,* submetidos a tratamento experimental.

Específicos

Avaliar o perfil hematológico de cães naturalmente infectados com *Leishmania infantum,* durante o tratamento experimental.

Avaliar o perfil da bioquímica sérica de cães naturalmente infectados com *Leishmania infantum*, durante o tratamento experimental.

CAPÍTULO I

CLINICAL AND HAEMATOLOGICAL FOLLOW-UP IN DOGS NATURALLY INFECTED WITH *Leishmania infantum* AND TREATED WITH MILTEFOSINE AND DOMPERIDONE, AND ALLOPURINOL AND DOMPERIDONE

CLINICAL AND HAEMATOLOGICAL FOLLOW-UP IN DOGS NATURALLY INFECTED WITH *Leishmania infantum* AND TREATED WITH MILTEFOSINE AND DOMPERIDONE, AND ALLOPURINOL AND DOMPERIDONE

Abstract

This study was made to assess the haematological findings in dogs submitted a two different experimental treatments: Allopurinol (10 mg/kg/per day/ sine die, PO) associated with Domperidona (1 mg/kg/per day/30 day, PO) (DOA), and Allopurinol (10 mg/kg/per day/ sine die, PO) associated with Miltefosine (2 mg/Kg/per day/28 days, PO) (MIA). For this were recruited 14 dogs naturally infected with *Leishmania sp.,* all the dogs were positive to detection of amastigotes forms at the bone marrow, lymph node or skin ulcer smears samples. Seven dogs were submitted to DOA treatment and other seven were submitted to MIA treatment. In all the dogs were made a clinical and physical exam, a parasitological exam, and a Complete Blood Count (CBC) before the treatment and 30, 60 and 90 days after the beginning of the treatment. The most common haematological alterations at beginning of the DOA treatment were anaemia (28.57%), lymphocytosis (14.29%) and eosinophilia (28.57%), thrombocytopenia (85.71%) and hyperproteinemia (85.71%). The most common alterations in MIA treatment were anaemia (42.86%), neutropenia (14.29%) or neutrophilia (14.29%), lymphopenia (14.29%), eosinophilia (14.29%), thrombocytopenia (71.43%) and hyperproteinemia (100%). At 90 days of treatment DOA group still showed anaemia (14.29%), lymphocytosis (42.86%), eosinophilia (42.86%) and thrombocytopenia (28.57%) and hyperproteinemia (71.43%). MIA group had not anaemic dogs, but still present lymphocytosis (28.57%), eosinophilia (14.29%), thrombocytopenia (42.86%) and hyperproteinemia (71.43%). Both treatments have an action against CVL, but domperidona associated to allopurinol showed a better effect decreasing clinical signs and improve the normalization of CBC alterations in dogs with CVL.

Keywords: Canine Visceral Leishmaniasis, Experimental treatment, Haematology parameters, Protozoa, Canine medicine

Introduction

Visceral leishmaniasis (VL), also known as kala-azar (VL) is an important vector-borne disease caused by different species of genus *Leishmania,* which occurs in 98 countries in tropical and subtropical areas, especially in India, Bangladesh, Sudan, Ethiopia and Brazil (WHO, 2010; CANTOS-BARREDA et al., 2017; ZULFIQAR; SHELPER; AVERY, 2017).

In Brazil *Leishmania infantum* is the etiological agent of the disease that affects both humans and dogs in endemic areas, where this animal has been considered as reservoir in urban areas (COIRO *et al.*, 2017; MILLER *et al.*, 2013).

The infected dogs developed a persistent infection, and different clinical forms which ranges from symptomatic or *asymptomatic* (GHARBI *et al.*, 2015; GREENE, 2012; SYKES, 2013) can be observed *depending on the* host immune response and the parasite load (TORRECILHA *et al.*, 2016).

The *diagnosis* of Canine Visceral *Leishmaniasis (CVL)* is difficult because the clinical signs are variable and nonspecific (GHARBI *et al.*, 2015; MINISTÉRIO DA SAÚDE, 2014; SOLANO-GALLEGO *et al.*, 2017). The parasitological, diagnosis are based on detection of amastigotes forms in ~~of~~ bone marrow, lymph node and skin ulcer smears. Due to the sensitivity of this test, the presence of antibodies is routinely used as a marker of infection.

In addition to the serologic tests, PCR-based assays have also been used for detecting *L. infantum* DNA in diagnostic samples (GHARBI *et al.*, 2015; QUARESMA *et al.*, 2009).

The treatment of CVL still a challenge in veterinary medicine and miltefosine has just become available in Brazil for the treatment of CVL, and clinical relapses have also been reported when this drug is used alone (MATEO *et al.*, 2009; REGUERA *et al.*, 2016). On the other hand the efficacy of this molecule improves when given in association with allopurinol and domperidona (MANNA *et al.*, 2015; NOLI; SARIDOMICHELAKIS, 2014; REGUERA *et al.*, 2016).

The evaluation of the complete blood count parameters are still not clearly understood during the treatment of dogs with natural infection of *L infantum*. The purpose of this study was to evaluate changes in CBC parameters in dogs

naturally infected with visceral leishmaniasis and treated with miltefosine and domperidona.

Materials and methods

Ethical Aspects

This study was approved under number H12, Licence 137m data 05/12/2016 by The Research Ethics Committee of UFRPE.

Animals

Were recruited fourteen domiciled dogs, from one year to seven years old and different breeds. All the dogs were positive to detection of amastigotes forms of *L. infantum* at least in one of the bone marrow, lymph node or skin ulcer smears samples.

In addition, Clinical evaluation was performed in all dogs and blood were collected and tested for *Ehrlichia canis*, *Anaplasma platys, Dirofilaria immitis* and *Babesia canis* and gastro intestinal parasites by FLOTAC technique before the beginning of the treatment to exclude positive animals.

Treatment

The study was performed on two groups of animals naturally infected admitted to the Small animal Hospital of the Universidade Federal Rural de Pernambuco (UFRPE), with the clinical signs of *CVL.*

All animals were randomly allocated to these groups of the treatment: Group 1 (G1) compound of seven dogs treated with domperidona (1 mg/kg/per day/30 day, PO) associated to allopurinol (10 mg/kg/12 hours/ sine die, PO), (DOA). Group 2 (G2) compound of the same number of dogs, treated with miltefosine (2 mg/Kg/per day/28 days, PO) associated to allopurinol (10 mg/kg/12 hours/ sine die, PO) (MIA).

Follow-up study

Animals in both groups were monitored for clinical haematological changes at day 0, and 30, 60 and 90 post treatments.

Clinical evaluation

In every monitoring all the animals were evaluated for the presence of systemic, cutaneous and ocular signs suggestive of CVL and were annotated in an individual medical history.

Collection of samples and blood analysis

A sample of peripheral blood was drawn from each animal through puncture of the cephalic vein and placed in labelled tubes with EDTA, to make a quantification of erythrocytes and leucocytes using an automatic cell counter (Labtest SDH-3 vet). Differential leucocyte counts were performed in *Wright-Giemsa*-stained blood smears by shilling method in optic microscopy. The calculation of hematimetric indices was carried out according to WEISS; WARDROP, 2011.

Data analysis

A descriptive analysis was made for all the variables, clinical signs, parasitological analysis and CBC. Statistical analyses were performed with the aid of R project for statistical computing (R CORE TEAM, 2017). ANOVA followed by Tukey's Test were used to compare quantitative variables, CBC parameters, of both groups at the same time. The Pearson's correlation test was used to evaluate if the quantitative variables of this study are correlated.

A Principal Component Analysis (PCA), was made to make a distinction between the similarity or difference between the treatments, DOA and MIA, and health status evaluation day.

The histograms from quantitative variables were processed with the statistical software GraphPad Prism 6® (2012).

To compare qualitative variables, clinical among groups at the same time was used Kruskal Wallis Tests. Spearman's correlation test were calculated to evaluate correlation between variables from both groups.

In all the analysis were considered p-value ≤ 0.05.

Results.

Efficacy assessment

The most common clinical signs in DOA treatment group (alopecia, descale, ulcerative dermatitis, nodular dermatitis and onychogryphosis, lymphadenopathy, lethargy and ocular signs) and in MIA treatment group (alopecia, descale, pyodermatitis, paronychia, lymphadenopathy, lethargy and ocular signs) decrease in more than 50% in 60 days after the beginning of the treatment. A positive correlation was observed between alopecia and descale

(r=0.51) and lethargy and lymphadenopathy (r=0.55). The most persistent signs where onychogryphosis and ocular disease in G1 and pyodermatitis in G2

No statistical differences was found between DOA and MIA treatment action, but both treatments showed a statistical difference at the interaction with the day of dog health status evaluation.

Also all animals from G1 and G2 group were negative to parasitological evaluations in bone marrow, lymph node and skins 60 days post-treatment, and the analysis showed no significant difference between both groups.

On day 0 before the treatment, 28.57% of the animals (2/7 dogs) in DOA treatment showed anaemia, 14.29% of animals (1/7 dogs) with microcytic hypochromic anaemia and 14.29% of animals (1/7 dogs) with microcytic normochromic anaemia and reticulocytoses. At day 90 post-treatment only 14.29% of animals (1/7 dogs) showed anaemia microcytic normochromic and absolute reticulocytoses.

On MIA group on day 0, 42.86% of the animals (3/7 dogs), showed anaemia, 14.29% of animals (1/7 dog) showed microcytic hypochromic anaemia with absolute reticulocytoses and 28.57% of the animals (2/7 dogs) showed normocytic hypochromic anaemia, only one dog showed absolute reticulocytoses. At the day 90% no one has anaemia.

The relation between variables erythrocyte and packed cell volume (r=0.74), showed a strong positive correlation. A moderate positive correlation (values between 0.5 to 0.7) between platelets and erythrocytes, platelets with haemoglobin, and haemoglobin with packed cell volume. And moderate negative correlation between erythrocyte and reticulocytes (r=0.55). Also it was observed a strong positive correlation (r=0.89) between CBC anaemia and hypochromia.

On the other hand, DOA group presented 14.29% of animals (1/7 dogs) with lymphocytosis and 28.57% of animals (2/7 dogs) with eosinophilia at day 0 of treatment, and at the end of the study 42.86% of animals (3/7 dogs) presented lymphocytosis and 42.86% of animals (3/7 dogs) present eosinophilia.

MIA group 14.29% of animals (1/7 dogs) present segmented neutrophil elevated, 14.29% of (1/7 dogs) animals with segmented neutrophil decreased, 14.29% of animals (1/7 dogs) with lymphopenia and 14.29% of animals (1/7 dogs) with eosinophilia at beginning of the treatment. Ninety days post-treatment

28.57% of animals (2/7 dogs) presented lymphocytosis and 14.29% of animals (1/7 dogs) present eosinophilia.

Hypersegmented neutrophils were elevated at day 0 in both groups, 85.71% of animals (6/7 dogs) in DOA and MIA group, at day 90 of treatment this alteration was still elevated 71,43% of animals (5/7 dogs) in DOA group and 100% of animals (7/7 dogs) in MIA group.

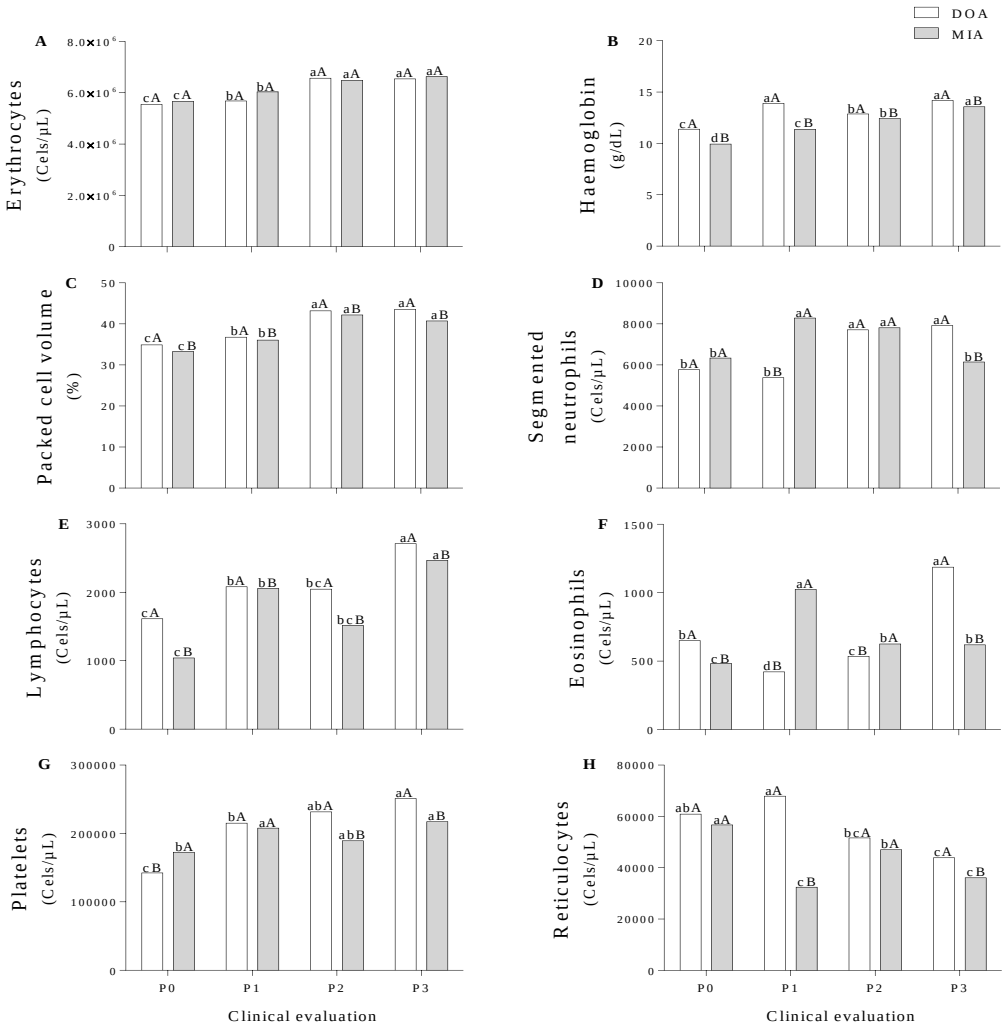

Figure 2 .- Effect of two experimental treatments domperidone+allopurinol (DOA) and miltefosine+ allopurinol (MIA) under different periods of dog health status evaluation day P0 (day 0), P1 (Day 30), P2 (day 60) and P3 (day 90) evaluating erythrocytes (A), haemoglobin (B), packed cell volume (C), segmented neutrophils (D), lymphocytes (E), eosinophils (F), platelets (G) and reticulocytes (H). Different letters, lower case letter for periods and capital letter for medicament, its show significative differences (P≤0,05) according to Tukey's Test.

On DOA treatment 85.71% (6/7 dogs) present thrombocytopenia before the treatment, after 90 days 28.57% (2/7 dogs) show thrombocytopenia. On MIA

treatment 71.43% (5/7 dogs) show thrombocytopenia at the beginning of the treatment and 42.86% (3/7 dogs) show thrombocytopenia at day 90 of treatment. Platelets and packed cell volume show a moderate positive correlation (r=0.54).

At the beginning of the treatment plasmatic hyper proteinemia was showed in DOA treatment group 85.71% of animals (6/7 dogs) and MIA treatment group 100% of animals (7/7 dogs). At the day 90 of treatment both treatment groups showed 71.43% of animals (5/7 dogs) with plasmatic hyper proteinemia.

Effect of both treatments MIA and DOA has a statistical difference . Also the CBC variables show statistical difference between them at different health status evaluations days (Day 0, 30, 60 and 90) (Figure 1A-H).

Multivariate analysis

The Principal component Analysis (PCA) (figure2) of CBC show that 80,21% of the variance in this study can be explained by the parameters of CBC. In general the first dimension group together in function to dog health status evaluation day (day 0, 30, 60 and 90), while the second dimension distinguish in function of Treatment DOA and MIA.

The Interaction of DOA treatment at day 90 was in the first dimension by the variables erythrocyte, packed cell volume, platelets, lymphocyte and haemoglobin, these variables contribute strongly with a variance showing an elevation on the production of blood cells and improve the response of white cells and a negative correlation with reticulocytes, confirming the adequate values of blood cells.

In contrast to DOA treatment, MIA not show the same comportment, because the variables during the health status evaluation day had a lower relation with the same variables. The interaction of MIA treatment at day 90 is similar to DOA treatment at day 60.

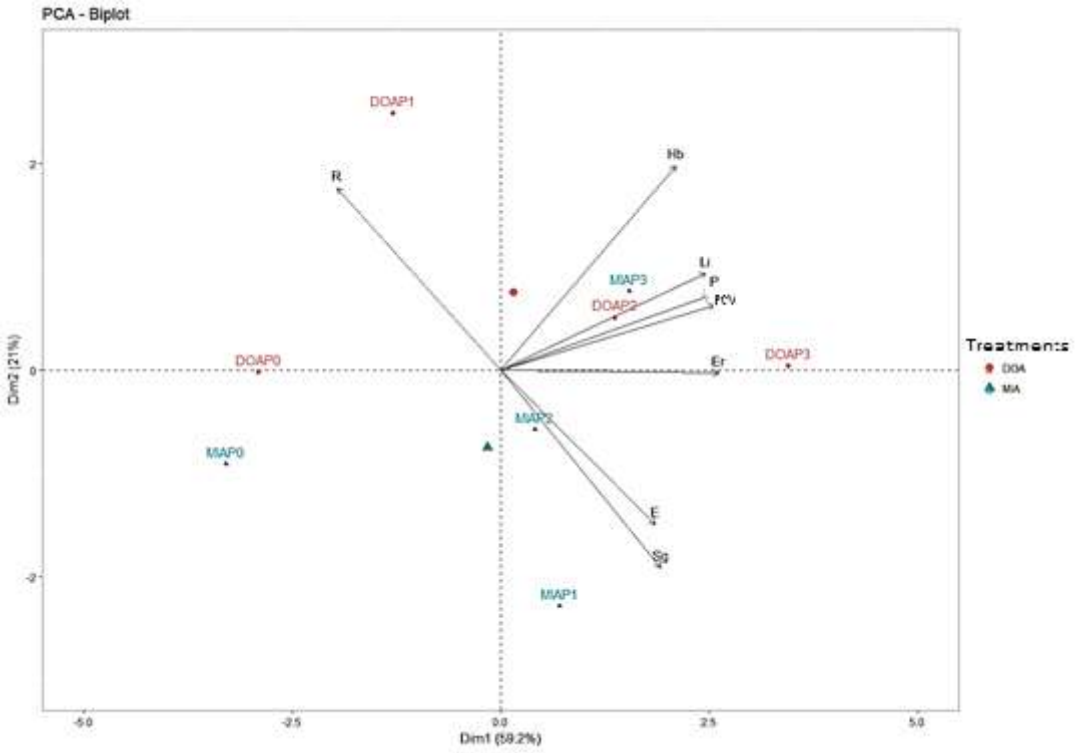

Figure 3.- Principal components analysis (PCA) of dog health status evaluation day (P0: day 0; P1: day 30; P2: day 60; P3: day 90) and CBC parameters (R: reticulocytes, Hb: haemoglobin; Li: lymphocytes; P: platelets; PCV: packed cell volume; Er: erythrocyte; E: eosinophil; Sg: Segmented neutrophils.) under two experimental treatments domperidone+allopurinol (DOA) and miltefosine+ allopurinol (MIA).

Discussion

The frequency and spectrum of clinical signs found in this study is related with previous studies in dogs with visceral leishmaniasis (FREITAS *et al.*, 2012; NOLI; SARIDOMICHELAKIS, 2014; PALTRINIERI *et al.*, 2016). The major clinical signs observed in all the dogs before the treatment were alopecia, descale, ulcerative dermatitis, onychogryphosis, lymphadenopathy, lethargy and ocular secretion. These dermatologic aspects are the most frequent clinical changes of CVL, and it reflects the host-parasite relationship (SARIDOMICHELAKIS; KOUTINAS, 2014). The ophtalmopaties in this study could be related to immune complex associated to Leishmania infection, as was reported previously (ABBAS; LICHTMAN; PILLAI, 2017; NASCIMENTO, 2015).

This study show a clinical improve in dogs 60 days post-treatment of both groups and no relapses were seen until the end of treatment. This results are in agreement with previous reports with mitelfosine, allopurinol and domperidone

improve the health status of the dogs with CVL (GÓMEZ-OCHOA *et al.*, 2009; MANNA *et al.*, 2015; MIRÓ *et al.*, 2009; PASSOS *et al.*, 2014; TORRES *et al.*, 2011).

In addition at day 60 all the dogs from MIA and DOA treatment were negative to detection of amastigotes forms in bone marrow, lymph node and skin ulcer smears. Similar result was reported by MATEO et al., 2009.

Although dogs with clinical cure can be a potential reservoirs of parasite (FRANCINO *et al.*, 2006; MANNA *et al.*, 2015; MATEO *et al.*, 2009). Because of this, parasitological techniques does not seem to be a secure technique to monitor treatment efficacy.

Erythrocytes values obtained in this study as the type of anaemia have been described in CVL (MANNA *et al.*, 2015; SASANI *et al.*, 2016). Anaemia, particularly no regenerative, normocytic or normochromic in different stages of infection is a major haematological disorder associated to CVL (GHARBI *et al.*, 2015; MANNA *et al.*, 2015; MOREIRA *et al.*, 2016). These values could be originated by a bone marrow hypoplasia, that made it non regenerative and it depends of the stage of the disease (PALTRINIERI *et al.*, 2016; TRÓPIA DE ABREU *et al.*, 2011).

DOA and MIA treatment improve the production of erythrocytes, but in DOA treatment on dog remain with anaemia. It can be explained by the chronicity of the dog (erythrocytes: 2.8 $x10^6$ cels/µL) at the beginning of the treatment.

Additionally in this study reticulocytes values of anaemic dogs at the beginning of the treatment .were elevated in both groups, showing a response in bone marrow to a erythropenia (LATIMER; DUNCAN, 2011; WEISS; WARDROP, 2011). Meanwhile the erythrocyte values were increasing, the reticulocytes values decreasing at day 90 of treatment on both treatment groups, only the same dog in DOA treatment group that still present anaemia, still present reticulocytoses.

Hypersegmented neutrophils are related to delayed emigration of neutrophils from the vasculature into tissues (normally related to increase in endogenous or exogenous administrated corticosteroids), also to chronic inflammation, and hyperadrenocorticism (HARVEY, 2012; WEISS; WARDROP, 2011). All the dogs in both groups show hypersegmented neutrophils at the

beginning of the treatment and at day 30, 60 and 90 pos-treatment. In this study can be related at the beginning by the chronic inflammation caused by CVL, and in the following evaluations can be related to increased in endogenous corticosteroids.

The thrombocytopenia was the most common haematological finding in DOA and MIA treatments groups during every dog health status day. Secondary immune-mediated *thrombocytopenia* in dogs naturally infected by *L. infantum* has been described (FOGLIA MANZILLO, VALENTINA *et al.*, 2013; PALTRINIERI *et al.*, 2016). Also thrombocytopathy may result to vasculitis, changes in thrombocytopoiesis, increase in platelet destruction and megakaryocytic hipoplasia (HOSEIN *et al.*, 2015; NOLI; SARIDOMICHELAKIS, 2014; TURINELLI *et al.*, 2015).

DOA and MIA treatment increased the production of platelets in this study. In both groups still remain some dogs with thrombocytopenia at day 90 post-treatment, that can be originated by the damage of LV in the bone marrow (FOGLIA MANZILLO, V *et al.*, 2006; MOMO *et al.*, 2014). New studies must be done in order to evaluate the platelets patterns for a better evaluation of the efficacy of the treatment.

DOA and MIA treatment group presented animals with lymphocytosis at day 90, in this case lymphocytosis can be related with a chronic inflammation and trypanosomal infections (HARVEY, 2012; LATIMER; DUNCAN, 2011; WEISS; WARDROP, 2011) but it could be necessary to make a subset analysis to differentiate if it is a humoral or a cellular response. Also MIA treatment group presented one animal with lymphopenia that can be caused by sequestration of lymphocytes in organs in infected by LV, the release of endogenous glucocorticoids in response to LVC, (HARVEY, 2012; NICOLATO *et al.*, 2013; WEISS; WARDROP, 2011).

MIA treatment group presented one dog with neutrophilia. this finding can be explained by the systemic inflammation (PALTRINIERI *et al.*, 2016; TORRECILHA *et al.*, 2016). And 1 dog present neutropenia, that can be induced by the recruitment of neutrophils to inflammation site or decrease of release from bone marrow (HARVEY, 2012; WEISS, 2008). Dogs with eosinophilia are present

in both groups, this was related as a occasionally in CVL by other studies (IKEDA-GARCIA *et al.*, 2008; PALTRINIERI *et al.*, 2016).

Both treatments treatment groups, DOA and MIA, showed a plasmatic hyper proteinemia during all the study. Plasmatic hyper proteinemia is related to increased globulin synthesis and dehydration (HARVEY, 2012; THRALL *et al.*, 2012). The hyper proteinemia in this study could be related not only to dehydration, but also by the globulins increased by the gammaglobulin production in consequence for the VL.

During the 90 days of experimental treatment, dogs from DOA treatment showed progress in clinical status and haematological results. Similar results, with similar protocols, were obtained by previous studies (CAVALIERO *et al.*, 1999; GÓMEZ-OCHOA *et al.*, 2009; NASCIMENTO, 2015; PEREIRA JUNIOR, 2014). Dogs from MIA treatment also showed progress in clinical status and haematological status as were reported previously (MANNA *et al.*, 2009, 2015; MIRÓ *et al.*, 2009).

Conclusion

In conclusion, after 90 days follow up of dogs submitted to DOA and MIA DOA treatments, the data of this study suggest that DOA treatment has a better efficacy to improve clinical status and haematological parameters of dogs with CVL.

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CAPITULO II

CLINICAL AND BIOCHEMICAL CHANGES IN DOGS NATURALLY INFECTED WITH *Leishmania infantum* SUBMITTED TO EXPERIMENTAL TREATMENT

CLINICAL AND BIOCHEMICAL CHANGES IN DOGS NATURALLY INFECTED WITH *Leishmania infantum* SUBMITTED TO EXPERIMENTAL TREATMENT

Abstract

Serum biochemistry had a limited application in the diagnosis of Canine Visceral Leishmaniasis (CVL), but it can be a tool to the monitoring the health status of dogs and to evaluate the recovery of dogs under treatment. The aim of this study was to evaluate the clinical and biochemical changes in dogs naturally infected with *leishmania infantum* submitted to an experimental treatment. Fourteen dogs were recruited with clinical signs of CVL, and were separated in two groups one group (n=7) were submitted to a treatment with Allopurinol (10 mg/kg/per day/ sine die, PO) associated with Domperidona (1 mg/kg/per day/30 day, PO)/(DOA). A second group were submitted to a treatment with Allopurinol (10 mg/kg/per day/ sine die, PO) associated with Miltefosine (2 mg/Kg/per day/28 days, PO)/(MIA). A clinical, parasitological and serum biochemistry follow up was performed at the beginning of the treatment and after 30, 60 and 90 days after treatment. Improvement in clinical signs was achieved after 60 days of treatment in both groups of treatment, also all the dogs were negative to parasitological exam after 60 days of treatment in both groups. At serum biochemistry analysis the noteworthy alterations were in DOA group: azotaemia (14.29%), elevated AST (28,57%), hyperglobulinemia (85,71%) and hipoalbuminemia (57,14%) , after 90 days present azotaemia (14.29%), hyperglobulinemia (71,43%), and hipoalbuminemia (71.43%). MIA group showed azotemia (42,86%), elevated AST (14,29%), hyperglobulinemia (100%) and hipoalbuminemia (85.71%), after 90 days present azotaemia (42.86%), elevated AST (71,43%), hyperglobulinemia (100%) and hipoalbuminemia (85.71%). With the results obtained and analysed data the treatment domperidona associated with allopurinol show a better efficacy in normalize the serum biochemistry alterations of the dogs with CVL.

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Keywords: Canine Visceral Leishmaniasis, Serum Biochemistry, Experimental treatment.

Introduction

In urban areas of Brazil the Canine Visceral Leishmaniasis (CVL) has been associated with human disease (DANTAS-TORRES, 2007), infected dogs may present a range of clinical signs, from apparently healthy to symptomatic diseased depending the immune responses (RODRÍGUEZ-CORTÉS *et al.*, 2016; TIZARD, 2013).

The symptomatic form has a wide spectrum of clinical signs as fever, lymphadenopathy, weight loss, anaemia, anorexia, hyperglobulinemia (SOLANO-GALLEGO, L. *et al.*, 2009). And affects different organs like spleen, liver, bone marrow, lymph nodes (RODRÍGUEZ-CORTÉS *et al.*, 2016).

The *diagnosis* of *CVL can be performed by* parasitological methods, based on detection of amastigotes forms in bone marrow, ~~or~~ lymph node and skin ulcer smears, serological tests, and PCR-based assays for detecting *L. infantum* (GHARBI *et al.*, 2015; QUARESMA *et al.*, 2009).

Miltefosine has just become available in Brazil for the treatment of CVL (MINISTÉRIO DE AGRICULTURA PECUÁRIA E ABASTECIMENTO, 2016). However the efficacy of this molecule improves when given in association with allopurinol (MANNA *et al.*, 2015; NOLI; SARIDOMICHELAKIS, 2014; REGUERA *et al.*, 2016).

The serum biochemical profile, particularly the renal and liver function n *L. infantum*–infected dogs are not useful for disease diagnosis (DE PÁDUA COSTA *et al.*, 2015), but it can be very important biomarkers for evaluating the treatment and animal status (DE PÁDUA COSTA *et al.*, 2015; PALTRINIERI *et al.*, 2016).

Increase of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity, creatinine, blood urea nitrogen (BUN) and total serum protein are the most common changes in serum biochemical profile in dogs with CVL (NOLI; SARIDOMICHELAKIS, 2014; PEREIRA JUNIOR, 2014).

Although the CVL was described for *more than 100 years*, the biochemical profile in treated dogs still not clearly understood. The purpose of this study was to evaluate changes in the biochemical profile in dogs naturally infected with visceral leishmaniasis and treated with miltefosine and domperidona.

Materials and methods

Ethical Aspects

The Research Ethics Committee of UFRPE, under number H12, Licence 137, data: 05/12/2016 approved the study.

Animals

Were recruited fourteen domiciled dogs, from one year to seven years old and different breeds. All the dogs were positive to detection of amastigotes forms of *L. infantum* at least in one of the bone marrow, lymph node or skin ulcer smears.

In addition, Clinical evaluation was performed in all dogs and blood were collected and tested for *Ehrlichia canis*, *Anaplasma platys, Dirofilaria immitis* and *Babesia canis* and gastro intestinal parasites by FLOTAC technique before the beginning of the treatment to exclude positive animals.

Treatment

The study was performed on two groups of animals naturally infected admitted to the Small animal Hospital of the Universidade Federal Rural de Pernambuco (UFRPE), with the clinical signs of *CVL.*

All animals were randomly allocated to these groups of the treatment : Group 1 (G1) compound of seven dogs treated with domperidona (1 mg/kg/per day/30 day, PO) associated to allopurinol (10 mg/kg/12 hours/ sine die, PO),(DOA). Group 2 (G2) compound of the same number of dogs treated with miltefosine (2 mg/Kg/per day/28 days, PO) associated to allopurinol (10 mg/kg/12 hours/ sine die, PO),(MIA).

Follow-up study

Animals in both groups were monitored for clinical and serum biochemical profile changes at day 0, and 30, 60 and 90 post treatments.

Clinical evaluation

In every monitoring all the animals were evaluated for the presence of systemic, cutaneous and ocular signs suggestive of CVL and were annotated in an individual medical history.

Collection of samples and serum analysis

A sample of peripheral blood was drawn from each animal through puncture of the cephalic vein and placed in labelled sterile tubes, after the serum

were separated and the ALT, AST, creatinine, BUN, total serum protein, albumin and globulin assays were performed in an automated analyser TP-ANALYZER BASIC.

Data analysis

A descriptive analysis was made for all the variables. Statistical analyses were performed with the aid of R project for statistical computing (R CORE TEAM, 2017). ANOVA followed by Tukey's Test were used to compare quantitative variables, serum biochemistry variables, of both groups at the same time. The Pearson's correlation test was used to evaluate if the quantitative variables of this study are correlated.

A Principal Component Analysis (PCA), was made to make a distinction between the similarity or difference between the treatments, DOA and MIA, and health status evaluation day.

The histograms of quantitative variables were processed with the statistical software GraphPad Prism 6® (2012).

To compare qualitative variables, clinical signs and parasitological exam, among groups at the same time was used Kruskal Wallis Tests. Spearman's correlation test were calculated to evaluate correlation between variables from both groups.

In all the analysis were considered p-value ≤ 0.05.

Results

Evaluation of the efficacy of the treatment

During the clinical follow up was observed a reduction on more than 50% of clinical signs in DOA treatment group (alopecia, descale, ulcerative dermatitis, nodular dermatitis and onychogryphosis, lymphadenopathy, lethargy and ocular signs) and MIA treatment group (alopecia, descale, pyodermatitis, paronychia, lymphadenopathy, lethargy and ocular signs) at day 60 of treatment. Lethargy has a positive correlation with lymphadenopathy (r=0.55), also alopecia has a positive correlation with descale (r=0.51). The most persistent signs where onychogryphosis and ocular disease in G1 and pyodermatitis in G2.

DOA and MIA treatment has not statistic difference in the efficacy related to clinical signs, but evaluating the interaction of treatments with the dog health status evaluation day, present a statistical difference.

Parasitological evaluations not showed significant difference between both treatment groups. All the animals were positive at the beginning of the treatment, but after 60 days of treatment both groups, MIA and DOA treatment, were negative to parasitological evaluations in bone marrow, lymph node and skin.

Elevated urea and BUN values were observed, at the beginning of the treatment, 14.29% of animals (1/7 dogs) on DOA treatment group and 42.86% of animals (3/7 dogs) at MIA treatment group, and at day 90 of treatment both groups maintain the same quantity of dogs with urea and BUN elevated values. Decreased urea and BUN values were observed in 14.29% of animals (1/7 dogs) at the beginning of the treatment on MIA treatment group, at day 90 of treatment no one was found.

On MIA treatment group at the beginning of the treatment 14.29% of animals (1/7 dogs) showed creatinine value decreased and another 28.57% of animals (2/7 dogs) showed elevated creatinine values. At day 90 of treatment no one dog showed abnormal values.

DOA treatment showed 14.29% of animals (1/7 of dogs) with azotaemia at the beginning of the treatment and 14.29% of animals (1/7 of dogs) at the day 90 of treatment. MIA treatment group had 42,86% of animals (3/7 of dogs) with azotaemia at the beginning of the treatment and 42.86%of animals (3/7 dogs) of animals dogs with azotaemia at the day 90 of the treatment. BUN/creatinine ratio were higher on 42.86% (3/7dogs) of animals at day 0 of MIA treatment and 71.43% of animals (5/7 of dogs) at the day 90 of MIA treatment.

DOA and MIA treatments showed 14.29% of animals (1/7 dogs) at the beginning of treatment with elevated ALT value respectively and at day 90 all the dogs has not abnormal values. In addition both treatments show elevated values of AST at the beginning of the treatment, 28.57% animals (2/7 dogs) in DOA treatment and 71.43% animals (5/7 dogs) in MIA treatment, and at day 90 all the dogs has not abnormal values. No one dog has ALT and AST values elevated at the same time.

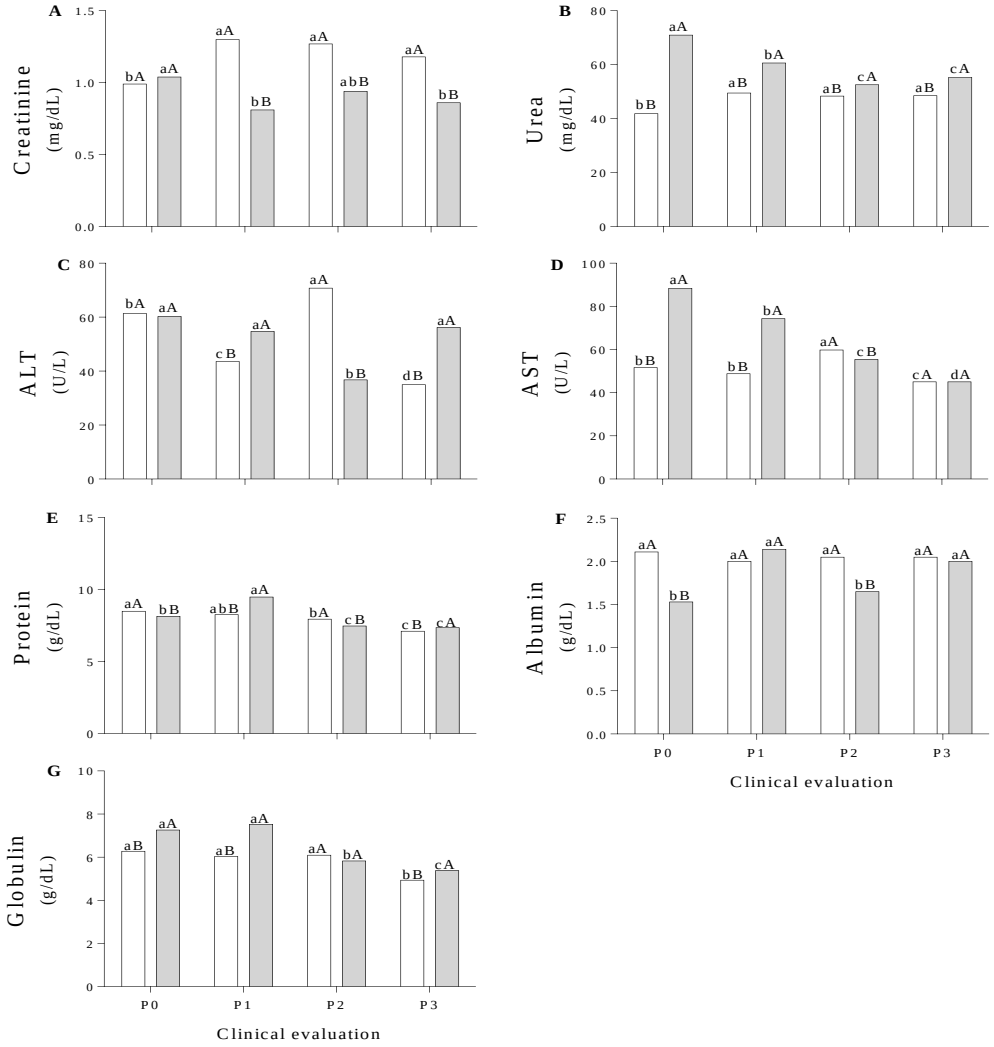

Figure 1 Effect of two experimental treatments domperidone+allopurinol (DOA) and miltefosine+ allopurinol (MIA) under different periods of dog health status evaluation P0 (day 0), P1 (Day 30), P2 (day 60) and P3 (day 90) evaluating creatinine(A), Urea (B), ALT (C), AST (D), Protein (E), Albumin(F) and globulin (G). Different letters, lower case letter for periods and capital letter for medicament, its show significative differences (P≤0,05) according to Tukey's Test

In the DOA treatment group, at the beginning of the treatment 85.71% of animals (6/7 dogs) showed total serum protein and globulin values elevated and 57.14% of animals (4/7 dogs) showed low values of albumin. After 90 days of treatment 57.14% of animals (4/7 dogs) showed total serum protein, 71.43% of animals (5/7 dogs) showed elevated globulin values and 71.43% of animals (5/7 dogs) showed low values of albumin. The Albumin/Globulin (A/G)

ratio at the beginning were low in 85.71% of animals (6/7 dogs) and were elevated in 71.43% of animals (5/7 dogs) after 90 days of treatment.

In MIA treatment group at the beginning of the treatment 100% of animals (7/7 dogs) showed total serum protein and globulin values elevated and 85.71% of animals (6/7 dogs) showed low values of albumin. After 90 days of treatment 42.86% of animals (3/7 dogs) showed total serum protein, 100% of animals (7/7 dogs) showed elevated globulin values and 85.71% of animals (6/7 dogs) showed low values of albumin. The A/G ratio at the beginning and after 90 days of treatment were low in all the dogs.

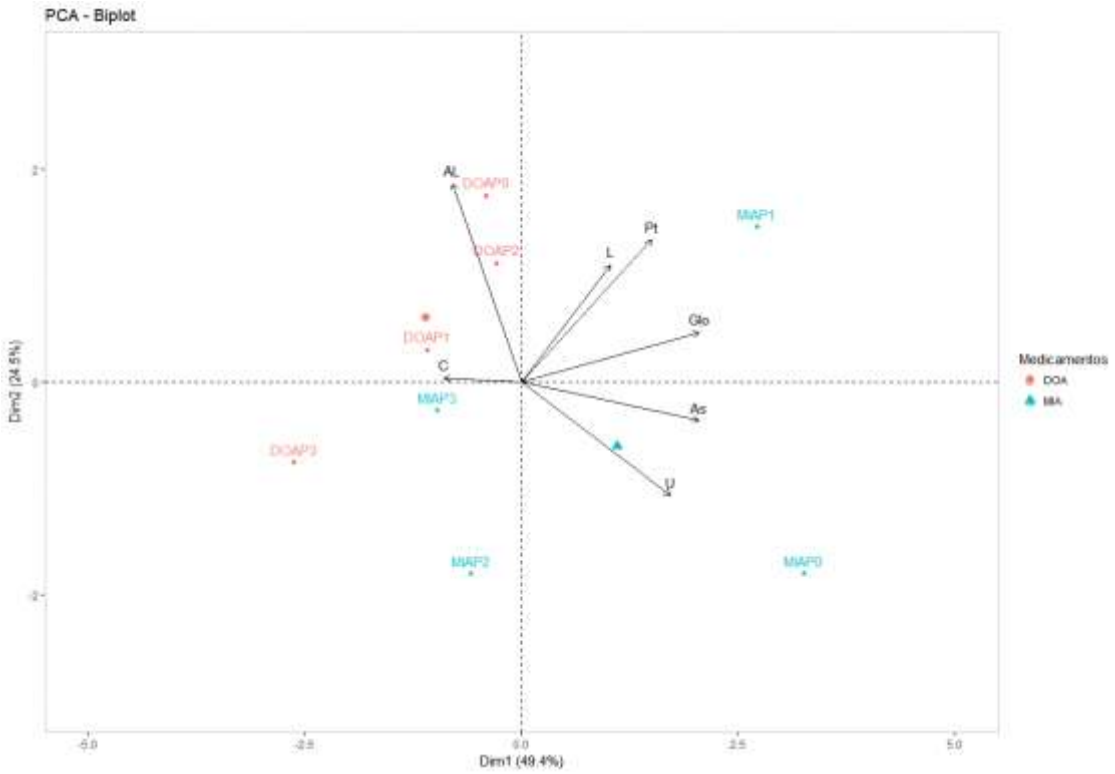

Figure 2 Principal components analysis (PCA) of dog health status evaluation period (P0: day 0; P1: day 30; P2: day 60; P3: day 90) and biochemical parameters (c: creatinine, Al: albumin; L: ALT, Pt: protein; Glo: globulin; As: AST; U: urea) under two experimental treatments domperidone+allopurinol (DOA) and miltefosine+ allopurinol (MIA).

A strong positive correlation between protein and globulin (r=0.78), also AST and globulins(r=0.83) was observed.

Biochemical analysis at the different periods of dog health status evaluation and different experimental treatments DOA and MIA show statistical significant differences (Figure 1A-G).

Multivariate Analysis

The Principal component Analysis (PCA) (Figure 2) show that 73,87% of the variance in this study can be explained by the parameters of explained by the biochemical parameters. In general the first dimension group together in function of Treatment DOA and MIA, while the second dimension distinguish in function of dog health status evaluation day (P1, P2 and P3, P4). The interaction DOA treatment at day 90 is in the first dimension by the variables urea, AST, protein and globulin, these variables contribute strongly with a variance showing a decrease in the value of this analytes, confirming the return to a good health status of the dogs under this treatment.

The interaction of MIA treatment at day 0 and day 30 were influenced by the variables urea, AST, protein and globulin, they has a positive correlation with them, but in the interactions at day 60 and 90 has a low influence by the aforementioned variables, this meaning that in clinical health status evaluation at day 60 and 90 the values of this variables decrease barely in contrast to DOA treatment.

Discussion

The higher values of urea and BUN found in dogs during the treatment could be explained because allopurinol has the side effect to elevate urea values (PAGANA; PAGANA, 2006). Low values of urea that also found and can be by a hepatic damage (LATIMER; DUNCAN, 2011; THRALL *et al.*, 2012).

On the other hand, high creatinine values were found in two dogs. The possible causes of these values are dehydration, renal failure and kidney inflammation (LATIMER; DUNCAN, 2011; THRALL *et al.*, 2012).

The low value of creatinine in one dog can be related to caquexia (low protein intake), liver damage and muscle loss (LATIMER; DUNCAN, 2011; THRALL *et al.*, 2012).

BUN/creatinine ratio help to classify the type of azotaemia (LATIMER; DUNCAN, 2011; THRALL *et al.*, 2012), Azotaemia is a common alteration in dogs with CVL, that indicates a high levels of nitrogen containing compound in the blood, it is related to dysfunction in blood filtering by the kidneys (PALTRINIERI *et al.*, 2010, 2016). According to BUN/creatinine ratio in DOA treatment group a

dog at the beginning of the treatment and one dog at day 90 of treatment were classified as a pre renal azotaemia. In MIA treatment group one dog showed renal azotaemia and the other four showed pre renal azotaemia, and at the day 90 of treatment three animals were classified as a renal azotaemia.

The hepatic enzymes ALT was not found elevated in dogs of this study as other reports (PENNISI, 2015; VÍCTOR ACERO *et al.*, 2015), but AST was elevated and both groups. These two analytes are related to liver damage and also muscle damage (LATIMER; DUNCAN, 2011; NELSON; COUTO, 2014). This could be related to a liver damage or a muscle damage (ETTINGER; FELDMAN; CÔTÉ, 2016; THRALL *et al.*, 2012). The value of these analytes decreased 90 post-treatment. Similar results were reported in other studies (MANNA *et al.*, 2015; PASSOS *et al.*, 2014), is necessary a complementary exams, as a ultrasonography, biopsy and other liver biomarkers in serum biochemistry, to confirm the improvement of liver (ETTINGER; FELDMAN; CÔTÉ, 2016; GÓMEZ-OCHOA, P. *et al.*, 2009).

Proteins and globulins were elevated during all the dogs health status evaluation periods. Hyperproteinemia are related to dehydration and hyper globulinemia can be caused by the strong humoral response after the beginning of the treatment and the chronic inflammation of the organism (FREITAS, JOSÉ CLÁUDIO CARNEIRO DE *et al.*, 2012; LATIMER; DUNCAN, 2011).

Hyperglobulinemia and hypoalbuminemia are reported as a common clinical sign in CVL, it is related with the elevated production of immunoglobulin and inflammatory proteins (DE PÁDUA COSTA *et al.*, 2015; NELSON; COUTO, 2014; ULCHAR *et al.*, 2015). The following analysis after the beginning the treatment were still with hyperglobulinemia. It could be explained by the immunoglobulins decreased approximately after 90 days of treatment (ABBAS; LICHTMAN; PILLAI, 2017; TIZARD, 2013).

Albumin showed lower values after the beginning of the treatment, in concordance with other reports (FREITAS, JOSÉ CLÁUDIO CARNEIRO DE *et al.*, 2012; NELSON; COUTO, 2014). During the treatments the albumin values showed a soft increased Hipoalbuminemia is related to liver damage of the parasite, anorexia, proteinuria by renal disease (DE PÁDUA COSTA *et al.*, 2015; LATIMER; DUNCAN, 2011; SILVA, LUCELIA C. *et al.*, 2013; THRALL *et al.*,

2012). The increase in blood albumin at 90 days post-treatment in all the dogs could be possible by the increase in the function of the liver and the increase in aliment ingestion on all the dogs of this study.

The A/G ratio is elevated in most of the dogs of this study because albumin values are low and globulin values are elevated. Is it necessary a serum proteinogram to quantify the protein fractions in both treatment groups.

Conclusion

After 90 days of DOA and MIA treatments, DOA treatment has a better efficacy because during the follow up decrease the values of AST, protein and globulin and improve the health status of the dog.

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