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"JÚLIO DE MESQUITA FILHO"
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Vitaminas A e D, e Zinco regulam a atividade leishmanicida em leucócitos esplênicos caninos

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Vitaminas A e D, e Zinco regulam a atividade leishmanicida em leucócitos esplênicos caninos

Tese apresentada à Faculdade de Medicina Veterinária de Araçatuba da Universidade Estadual Paulista "Júlio de Mesquita Filho" – UNESP, como parte dos requisitos para a obtenção do título de Doutor em Ciência Animal (Área de Medicina Veterinária Preventiva e Produção Animal).

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Araçatuba, 14 de fevereiro de 2020.

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Araçatuba, 14 de fevereiro de 2020.

*Aos meus pais **Vanda** e **Geraldo***

*Ao meu marido **Miguel***

*Á minha filha **Luísa***

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Thomas Wolfe

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RESUMO

A leishmaniose visceral é uma doença parasitária endêmica no Brasil, que representa 90% dos casos humanos nas Américas, com aumento da letalidade expressivo a cada ano. A supressão da imunidade celular, com predomínio da imunidade humoral ineficaz no combate ao parasito representa a maioria dos casos de leishmaniose em cães. As limitações quanto ao tratamento convencional, muitas vezes ineficaz no combate ao parasita, instiga a busca por novas alternativas, como o uso coadjuvante de nutrientes imunomoduladores, tais como vitamina A e D, e zinco. O objetivo desse estudo foi avaliar os níveis séricos de vitamina A (retinol), vitamina D (25-hidroxi vitamina D3 ou 25(OH)VD_3) e zinco (Zn) em cães com leishmaniose (CanL), e o efeito da suplementação *in vitro* nas suas formas ativas *all trans* ácido retinoico (ATRA), 1,25-dihidroxi vitamina D3 (1,25$(OH)_2VD_3$) e sulfato de zinco hepahidratado (SZn) em leucócitos esplênicos de CanL na resposta imune a doença. Baixo nível sérico de retinol e Zn, e alto de 25(OH)VD_3 foi observado na CanL. A suplementação *in vitro* com ATRA, 1,25$(OH)_2VD_3$ e SZn em leucócitos esplênicos de CanL na presença de antígeno solúvel de *Leishmania* spp. (SLA) aumentou óxido nítrico e espécie reativa de oxigênio. Observamos que o interferon-gama diminuiu no sobrenadante de cultura celular após a estimulação dos leucócitos esplênicos com 1,25$(OH)_2VD_3$ e SZn. Por outro lado, o fator de necrose tumoral-alfa aumentou no sobrenadante de cultura celular após a estimulação dos leucócitos esplênicos com ATRA e SZn, e a interleucina-10 diminui no sobrenadante de cultura celular na estimulação dos leucócitos esplênicos com ATRA, 1,25$(OH)_2VD_3$ e SZn, e carga parasitária diminuiu na cultura celular na estimulação dos leucócitos esplênicos com SZn. Concluímos que a leishmaniose em cães está associada a deficiência de retinol e Zn, e sugerimos que a ATRA, 1,25$(OH)_2VD_3$ e SZn estão envolvidos com a regulação imunológica associada a resposta efetora leishmanicida, com um grande potencial de investigação em modelos *in vivo,* especialmente quanto ao Zn.

Palavras-chave: Retinol*. All trans* ácido retinóico. Vitamina D3. Zinco. Leishmaniose.

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ABSTRACT

Visceral leishmaniasis is a parasitic disease endemic in Brazil, which represents 90% of cases in the Americas, with an expressive increase in lethality each year. Suppression of cellular immunity, with a predominance of humoral immunity without combating the parasite represents the majority of cases of leishmaniasis in dogs such as vitamin A and D, and zinc. The objective of the present study was to determine the serum levels of vitamin A (retinol), the levels of vitamin D (25-hydroxy vitamin D3 ($25(OH)VD_3$)), and zinc (Zn) levels in dogs with leishmaniasis (CanL), as well as the effect of *in vitro* supplementation with the active forms of all trans retinoic acid (ATRA), 1,25-dihydroxy vitamin D3 ($1,25(OH)_2VD_3$) and zinc sulfate heptahydrate (SZn) on spleen leukocytes of CanL dogs regarding the immune response to the disease. We detected low serum levels of retinol and Zn, and high levels of $25(OH)VD_3$ in CanL dogs. *In vitro* supplementation with all-trans retinoic acid (ATRA), $1,25(OH)_2VD_3$ and SZn of CanL spleen leukocytes dogs in the presence of the soluble antigen of *Leishmania* spp. (SLA) increased the levels of nitric oxide and reactive oxygen species. We also observed that interferon-gamma was reduced in the supernatant of spleen leukocytes stimulated in vitro with $1,25(OH)_2VD_3$ and SZn, while tumor necrosis factor-alpha was increased after stimulation with ATRA and SZn. Interleukin-10 was reduced after stimulation with ATRA, $1,25(OH)_2VD_3$ and SZn, and parasite load was reduced after stimulation with SZn. We conclude that canine leishmaniasis is associated with retinol and Zn deficiency and we suggest that ATRA, $1,25(OH)_2VD_3$ and SZn are involved in the immunological regulation of the leishmanicidal effector response, providing a high potential for investigation in *in vivo* models, especially regarding Zn.

Keywords: Retinol. *All trans* retinoic acid. Vitamin D3. Zinc. Leishmaniosis.

LISTA DE FIGURAS

Figure 1 – Serum retinol, 25(OH)VD_3 and Zn levels of CanL animals and healthy dogs 31

Figure 2 – NO production *in vitro* after supplementation with ATRA, 1,25$(OH)_2VD_3$ and SZn of spleen leukocytes of CanL animals and healthy dogs 33

Figure 3 – ROS production *in vitro* after supplementation with ATRA, 1,25$(OH)_2VD_3$ and SZn of spleen leukocytes of CanL animals and healthy dogs 34

Figure 4 – IFN-γ, TNF-α e IL-10 production *in vitro* after supplementation with ATRA, 1,25$(OH)_2VD_3$ and SZn of spleen leukoytes of CanL animals and healthy dogs 35

Figure 5 – Parasite load *in vitro* after supplementation with ATRA, 1,25 $(OH)_2VD_3$ and SZn of spleen leukoytes of CanL animals 37

Figure 6 – Graphical Abstract 42

LISTA DE ABREVIATURAS

$1,25(OH)_2VD_3$	1,25-dihydroxyvitamin D3
$25(OH)VD_3$	25-hydroxyvitamin D3
ATRA	*All trans* retinoic acid
CanL	Canine leishmaniosis
IFN-γ	Interferon gamma
IL-10	Interleukin 10
IL-12	Interleukin 12
iNOS	Induced nitric oxide synthase
NO	Nitric oxide
PBMC	Peripheral blood mononuclear cells
ROS	Reactive oxygen specie
SLA	Soluble leishmania antigen
SZn	Zinc sulfate heptahydrate
TNF-α	Turmor necrosis factor
VDR	Vitamind D receptor
Zn	Zinc

SUMÁRIO

1 INTRODUÇÃO GERAL 16

1.1 Imunopatogênese 17

1.2 Vitamina A 19

1.3 Vitamina D 20

1.4 Zinco 21

2 CAPÍTULO 1 - VITAMINS A AND D, AND ZINC REGULATE THE LESHMANICIDAL ACTIVITY OF CANINE SPLEEN LEUKOCYTES 23

2.1 Resumo 24

2.2 Abstract 25

2.3 Introduction 26

2.4 Materials and Method 27

2.4.1 Animals and sample collection 27

2.4.2 Serum micronutrient analysis 28

2.4.3 Isolation and culture of spleen cells 28

2.4.4 Flow cytometry 29

2.4.5 ELISA 29

2.4.6 Parasite load 30

2.4.7 Reagents and antibodies 30

2.4.8 Statistical analysis 30

2.5 Results 31

2.5.1 Reduction of retinol and Zn and increase of $25(OH)VD_3$ in CanL serum 31

2.5.2 *In vitro* supplementation of spleen leukocytes with ATRA, $1,25(OH)_2VD_3$ and SZn increased production of NO an ROS in the CanL group 32

2.5.3 *In vitro* supplementation of CanL spleen leukocytes stimulated with 1,25$(OH)_2VD_3$ and SZn reduced IFN-γ; ATRA and SZn increased TNF-α. ATRA, 1,25$(OH)_2VD_3$ and SZn reduced IL-10 34
2.5.4 *In vitro* supplementation of spleen leukocytes of CanL animals stimulated with ATRA, 1,25$(OH)_2VD_3$ and SZn reduced parasite load, although only SZn continued to have this effect after the addition of SLA 37
2.6 Discussion 37
2.7 Conclusion 41
2.8 Acknowledgments 41
2.9 References 43
APÊNDICE – Referências da Introdução Geral 51
ANEXOS 56

1. INTRODUÇÃO GERAL

A leishmaniose é considerada uma doença infecto parasitária característica de países subdesenvolvidos e em desenvolvimento, incluindo o Brasil que representa 90% dos casos da América Latina [1]. Apesar de importantes avanços na ciência, ela ainda permanece endêmica no país e representa grave problema de saúde pública. Além disso, a letalidade da doença vem aumentando gradativamente nos últimos anos e atinge cada vez mais áreas urbanas de médio e grande porte [2].

A leishmaniose visceral, considerada a forma mais grave e letal da doença, é causada pela espécie *Leishmania infantum* (sinônimo de *L. chagasi).* Em cães, a doença é mais prevalente comparada aos seres humanos, e normalmente a infecção no homem é precedida de casos caninos, que apresentam maior quantidade de parasitas na pele, favorecendo a infecção dos vetores [2]

No ciclo biológico, durante o repasto sanguíneo, os insetos fêmeos de flebotomíneos ingerem os parasitas sob a forma amastigota, sem flagelo externo e presentes no interior das células do sistema fagocítico mononuclear do hospedeiro vertebrado. No vetor (fêmeas de flebotomíneos), o parasito se transforma na forma promastigota (com flagelo externo), se multiplica e adere ao tubo digestivo do inseto. Após um novo repasto sanguíneo, a forma promastigota (forma infectante) é inoculada na pele do hospedeiro vertebrado, onde são englobados por células fagocitárias, como os macrófagos, e se transformam novamente na forma amastigota. Em seguida, a intensa proliferação das amastigotas no interior do macrófago provoca o rompimento da membrana celular e liberação dos parasitos, tornando o hospedeiro como reservatório [3].

Do ponto de vista epidemiológico, cães infectados podem permanecer sem sinais clínicos por um longo período de tempo, mas ainda assim, continuam sendo reservatórios do parasita, especialmente em áreas urbanas [2]. O tratamento medicamentoso, muitas vezes com limitações e ineficácia na morte do parasita, também implica na permanência do cão como reservatório [4,5], e por isso a eutanásia é preconizada em muitos casos.

Dados de 2018 mostram que as regiões Norte e Nordeste do Brasil permanecem liderando o coeficiente de incidência por 100.000 mil habitantes da leishmaniose visceral (4,05 e 3,06, respectivamente), seguida das regiões Centro-Oeste (0,76), Sudeste (0,49) e Sul (0,04) [6]. No estado de São Paulo, foram notificados

68 casos confirmados em 2019 (7 deles no município de Araçatuba) com 9 óbitos (3 também em Araçatuba) [7].

Os cães são considerados os mais suscetíveis à infecção pelo parasito Leishmania, sendo a doença de evolução lenta e início insidioso, com manifestações tardias graves que podem evoluir ao óbito. As manifestações clínicas variam de acordo com a resposta imunológica do hospedeiro, e nos estágios avançados, é comum observar esplenomegalia, linfadenopatia, alopecia, dermatites, diarreia e hemorragia intestinal. Na fase final, observa-se caquexia, inanição e óbito [2].

1.1 Imunopatogênese

Já é bem estabelecido que a supressão da imunidade celular, com predomínio da imunidade humoral ineficaz na eliminação do parasito, é um dos aspectos mais importantes na imunopatogênese e progressão doença [8]

A interleucina 12 (IL-12) produzida por células dendríticas (CD) é determinante para o desenvolvimento de células T CD4+ e interferon gama (IFN-γ), levando à um padrão de resistência à doença [9]. A IL-12 também pode agir sobre células *natural killer* (NK) para produzirem IFN-γ, induzindo a respostas dos linfócitos TCD4+ Th1 e citotóxicos CD8+ [10]. Já a ausência de IL-12 permite uma expansão progressiva de células Th2, regulação positiva da arginase nos macrófagos e, portanto, determina um padrão de suscetibilidade e proliferação dos parasito [9].

Em cães naturalmente infectados por *L. infantum*, injeção intradérmica de antígenos de *Leishmania* spp. induziu maturação de CDs com efeito adicional numa maior expressão de moléculas de superfície do complexo de histocompatibilidade de classe II (MHCII) [11].

Quanto aos macrófagos, estudos em cães e em outros modelos experimentais destacam o seu papel como principal atividade microbicida do parasita da leishmania. Uma vez ativados pelas citocinas IFN-γ e fator de necrose tumoral alfa (TNF-α), os macrófagos irão provocar a morte das amastigotas intracelulares através da produção de óxido nítrico (NO) pela enzima óxido nítrico sintase induzível (iNOS) [12,13].

Destaca-se também o estresse oxidativo com produção de superóxidos observado em cães no estágio moderado à grave da doença, e a viabilidade diminuída de neutrófilos nos estágios finais [14].

Assim como na resposta imune inata, na resposta adaptativa a secreção de citocinas também é determinante para o tipo de resposta. Em cães resistentes com leishmaniose foram descritas citocinas como a interleucina 2 (IL-2), TNF-α e INF-γ [15], características do padrão Th1 efetivo para o combate do parasita e que leva a cura da doença. Em cães sintomáticos e suscetíveis à doença, é observada uma resposta imune do tipo Th2, com alta produção de anticorpos e permissiva a sobrevivência e disseminação das amastigotas no interior dos macrófagos e vísceras, especialmente o baço e fígado [16].

Citocinas associadas à resposta do tipo Th2 incluem as interleucinas 4 (IL-4), 10 (IL-10) e 13 (IL-13), além do fator de crescimento transformador (TGF-β) [17]. Em cães, as citocinas Th1 e Th2 são comumente observados nos tecidos de cães sintomáticos com leishmaniose [18] e as respostas são variáveis nos diferentes órgãos acometidos. Por fim, a suscetibilidade canina também foi atribuída a taxas aceleradas de apoptose das células T, desorganização da polpa branca do baço e níveis reduzidos de células T no sangue periférico de cães [8].

Apesar do avanço dos estudos que tratam da resposta imune, pouco ainda se sabe sobre os determinantes dessa resposta durante a infecção [19], sendo os nutrientes possíveis gatilhos de causa e/ou consequência e com grande potencial de investigação.

A relação entre doença, nutrição e imunidade, ainda que não elucidada completamente, já é reconhecida há centenas de anos e hoje extensivamente investigada sob diferentes aspectos. Como consequencia de uma má nutrição, destacam-se algumas alterações imunológicas tais como prejuízo na estrutura e função do timo, redução da função das células T, redução nos componentes do sistema do complemento, prejuízo da fagocitose, da resposta de citocinas, da produção e afinidade de anticorpos, entre outros [20].

Micronutrientes (vitaminas e minerais), embora necessários em quantidades traços no organismo, desempenham papéis essenciais em inúmeros processos bioquímicos, fisiológicos e imunológicos. Como por exemplos, diversos componentes do sistema antioxidante são micronutrientes, como as vitaminas E e C, ou são dependentes deles, como o mineral selênio na enzima glutationa peroxidase e os minerais zinco (Zn), cobre e manganês na enzima superóxido dismutase. São também componentes estruturais e/ou funcionais de uma série de outras metaloenzimas e

metaloproteínas, e assim participam do metabolismo celular, da homeostase e de diversos eventos imunológicos [21,22].

1.2 Vitamina A

A vitamina A é um termo genérico utilizado para designar uma série de compostos retinóides com atividade biológica de *all-trans* retinol. É considerada um micronutriente essencial numa série de funções fisiológicas, dentre elas a visão (sua deficiência causa xeroftalmia), reprodução, hematopoese, integridade epitelial e imunidade [23].

Nos tecidos, *all trans* retinol e β-caroteno são oxidados à *all trans* retinal pela enzima álcool desidrogenase, que por sua vez é oxidado à *all trans* ácido retinóico pela enzima retinal desidrogenase (RALDH) através de uma reação irreversível e estritamente controlada. Em mamíferos adultos, foi encontrada RALDH nos enterócitos, células dendríticas (CD) da placa de Peyer e de linfonodos mesentéricos. O organismo é capaz de armazenar essa vitamina no fígado, principalmente nas células estreladas sob a forma de ésteres de retinil [24].

O mecanismo de ação da vitamina A se dá através da sua ligação com a família de receptores nucleares de ácido retinóico (RAR) em suas isoformas principais α, β e γ. O RAR forma héterodímeros com o receptor retinóico X (RXR), os quais interagem com os elementos de resposta do ácido retinóico localizados na região promotora de genes alvos [24].

A literatura mostra que sua deficiência (hipovitaminose A) resulta em aumento de risco de morbimortalidade por sarampo, infecções diarreicas, cegueira e anemia em crianças, e em gestantes há risco aumentado de mortalidade. Tais riscos podem estar associados à sua função imunológica [23].

Diversos estudos vêm demonstrando o papel imunomodulador da vitamina A, como por exemplo, estímulo à fagocitose por células fagocitárias, ativação da citoxicidade mediada por células e aumento na resposta de timócitos a mitógenos específicos, sendo esse aumento associado à expressão de receptores para IL-2 em suas células precursoras [24,25].

Em crianças com e sem leishmaniose visceral de área endêmica do nordeste brasileiro, células Treg e monócitos foram suplementadas com *all trans* ácido retinóico. Os resultados mostraram aumento de IL-10 e TGF-β após estímulo ao antígeno no

grupo sem LV, evidenciando um perfil de resposta regulatória. Já no grupo com LV, *all trans* ácido retinóico preveniu aumento de IL-10 nas células Treg e monócitos [19]

1.3 Vitamina D

A vitamina D é considerada um hormônio esteroide cuja principal função envolve o metabolismo ósseo (regulação da homeostase do cálcio e formação/ reabsorção óssea) através da interação com as paratireoides, rins e intestino [26]. Pode-se apresentar sob diversas formas, com por exemplos, calcifediol ou calcidiol ou 25-hidroxivitamina D3 [25(OH)D_3] (principal forma circulante pois possui meia-vida de 2 a 3 semanas, e por isso indicada para exames laboratoriais) e calcitriol ou 1,25-diidroxivitamina D3 [1,25$(OH)_2D_3$] (principal forma ativa no organismo com meia-vida curta de 4 horas, e por isso não indicada para exames laboratoriais, até porque na sua deficiência o organismo lança mão de mecanismos para manutenção de níveis normais). A 1,25$(OH)_2D_3$ é sintetizada na pele a partir do 7-dehidrocolesterol, processo dependente da exposição solar (radiação ultravioleta B 270-300nm) e é considerada a principal fonte de vitamina D no organismo [24,27].

Já é bem estabelecido na literatura que a deficiência crônica da vitamina D pode levar ao raquitismo, osteomalácia e osteoporose, além de risco aumentado de fraturas em adultos e idosos [26]. Entretanto, achados recentes vêm demonstrando que seu papel vai muito além do metabolismo ósseo e que há uma forte relação com a imunidade, especialmente em doenças autoimunes como diabetes melito tipo 1, esclerose múltipla, doenças inflamatórias intestinais, lúpus eritematoso sistêmico e artrite reumatoide [26] , devido a sua ação antinflamatória e supressora de IFN-γ [28].

No organismo, a vitamina D é hidroxilada no fígado à 25(OH)D_3 (forma circulante) através da enzima CYP27A1, e em seguida nos rins à 1,25$(OH)_2VD_3$ (forma ativa) através da enzima CYP27B1. Ambas as enzimas supracitadas pertencem ao grupo do citocromo P450 e também podem ser sintetizadas por células dendríticas (CD), macrófagos, além de linfócitos T e B (esses apenas CYP27B1), o que denota a capacidade do sistema imune em metabolizar a vitamina D [24].

O mecanismo de ação da 1,25$(OH)_2VD_3$ se dá através da sua ligação com receptores nucleares de vitamina D (VDR), semelhantes aos receptores para esteróides, hormônios tireoidianos e retinóides. O VDR é capaz de interagir com a família do RXR (isoformas α, β e γ) formando héterodímeros, os quais interagem com

os elementos de resposta da VD3. O VDR é expresso em diversos tipos celulares como epitélio do intestino delgado, células ósseas e pancreáticas, além das células do sistema imune [24,26]. Por isso, atualmente, o papel da vitamina D vem sendo extensivamente estudado em diversas condições clínicas não calcêmicas.

Quanto ao sistema imune, o VDR é expresso em macrófagos, CD, NK, linfócitos T e B, e em maior concentração nas células imaturas do timo e nos linfócitos CD8 maduros. Os principais achados sobre os efeitos imunomoduladores da vitamina D incluem efeitos anti-inflamatórios como diminuição na produção de IL-2, INF-γ e TNF-α, inibição da expressão de IL-6 e da secreção e produção de anticorpos pelos linfócitos B [26] e aumento dos níveis de IL-10 [29].

Estudos sobre ações não calcêmicas da vitamina D na leishmaniose são escassos e com resultados contraditórios. Suplementação *in vitro* ($1,25(OH)_2VD_3$ à 40nM) em cultura de macrófagos de camundongos infectados com *Leishmania major*, mostrou redução da produção de NO e sobrevivência intracelular do parasita no grupo selvagem em comparação ao grupo *knockout* para VDR (VDR-KO) [28]. Apesar da maior resistência à infecção em animais VDR-KO [30], mais estudos são necessários para extrapolar os resultados em linhagens selvagens [29].

Camundongos infectados com *Leishmania mexicana* e tratados com $1,25(OH)_2VD_3$ obtiveram resultados positivos em análises histológicas de lesões causadas pela doença, tais como limitações de áreas das lesões, arquitetura celular e tecidos preservados, no entanto, sem efeito quanto a sobrevivência do parasita [29].

1.4 Zinco

O Zinco (Zn) é um mineral que teve sua essencialidade definida no início do século XX, e dentre suas inúmeras funções biológicas, destacam-se ação catalítica (mais de 100 enzimas são dependentes de Zn como catalisador, dentre elas álcool desidrogenase, fosfatase alcalina e RNA polimerase), ações estruturais e reguladoras, como antioxidante [25,31].

A literatura destaca o Zn como essencial ao sistema imunológico, afetando vários aspectos desde a barreira da pele até a regulação gênica em linfócitos. É crucial para o desenvolvimento e função das células que medeiam a imunidade inata e adquirida e seus efeitos estão associados à papéis miríades de funções celulares básicas, tais como a replicação de DNA, transcrição de RNA, divisão e ativação celular [32].

Embora o papel do zinco no sistema imunológico esteja bem documentado, na leishmaniose os estudos são escassos. Estudo recente avaliou suplementação oral de Zn (2,2 mg/ peso/ dia durante 12 meses) associado à terapêutica (antimoniato de megluemina associado ou não com alopurinol) em cães infectados com *L. infantum* de uma área endêmica no sul da Itália. Os autores observaram uma resposta mais rápida à terapêutica e prolongamento na recidiva da doença nos grupos que receberam a suplementação com Zinco [33].

Por fim, mais estudos são necessários para elucidar o papel dos micronutrientes na leishmaniose, visto que eles possuem papel chave no sistema imunológico.

O objetivo desse estudo foi avaliar os efeitos da suplementação *in vitro* de micronutrientes imunomoduladores, como vitamina A, vitamina D e zinco na resposta imunológica de leucócitos esplênicos de cães com leishmaniose.

2. CAPÍTULO 1 - VITAMINS A AND D, AND ZINC REGULATE THE LESHMANICIDAL ACTIVITY OF CANINE SPLEEN LEUKOCYTES

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2.1 Resumo

A leishmaniose visceral é uma doença infecciosa crônica causada pelo parasita do gênero *Leishmania* spp., endêmica em muitos países subdesenvolvidos e em desenvolvimento, representando grave problema de saúde pública. As limitações quanto ao tratamento convencional, muitas vezes ineficaz no combate ao parasita, instiga a busca por novas alternativas, como o uso coadjuvante de nutrientes imunomoduladores. Nosso objetivo foi avaliar os níveis séricos de vitamina A (retinol), vitamina D (25-hidroxi vitamina D3 ou 25(OH)VD_3)) e zinco (Zn) em cães com leishmaniose (CanL), e o efeito da suplementação *in vitro* nas suas formas ativas *all trans* ácido retinoico (ATRA), 1,25-dihidroxi vitamina D3 (1,25$(OH)_2VD_3$) e sulfato de zinco hepahidratado (SZn) em leucócitos esplênicos de CanL na resposta imune a doença. Observamos baixo nível sérico de retinol e Zn, e alto de 25(OH)VD_3 em CanL. A suplementação *in vitro* com ATRA, 1,25$(OH)_2VD_3$ e SZn em leucócitos esplênicos de CanL na presença de antígeno solúvel de *Leishmania* spp. (SLA) aumentou óxido nítrico e espécie reativa de oxigênio. Observamos também que o interferon-gama diminuiu no sobrenadante de cultura celular na estimulação dos leucócitos esplênicos com 1,25$(OH)_2VD_3$ e SZn, o fator de necrose tumoral-alfa aumentou no sobrenadante de cultura celular na estimulação dos leucócitos esplênicos com ATRA e SZn, a interleucina-10 diminui no sobrenadante de cultura celular na estimulação dos leucócitos esplênicos com ATRA, 1,25$(OH)_2VD_3$ e SZn, e a carga parasitária diminuiu na cultura celular na estimulação dos leucócitos esplênicos com SZn. A leishmaniose em cães está associada a deficiência de AR e Zn, e sugerimos que a ATRA, 1,25$(OH)_2VD_3$ e SZn estão envolvidos com a regulação imunológica associada a resposta efetora leishmanicida, com um grande potencial de investigação em modelos *in vivo,* especialmente quanto ao Zn.

Palavras-chave: Retinol. Todo o ácido trans retinóico. Vitamina D3. Zinco. Leishmaniose.

2.2 Abstract

Visceral leishmaniasis is a chronic infectious disease caused by a parasite of the genus *Leishmania* spp., which is endemic in many underdeveloped and developing countries, representing a serious public health problem. The limitations of conventional treatment, often ineffective in the fight against the parasite, encourages the search for new alternatives such as the coadjutant use of immunomodulatory nutrients. The objective of the present study was to determine the serum levels of vitamin A (retinol), vitamin D (25-hydroxy vitamin D3 or ($25(OH)VD_3$)) and zinc (Zn) in dogs with leishmaniasis (CanL), as well as the effect of *in vitro* supplementation with the active forms of all trans retinoic acid (ATRA), 1,25-dihydroxy vitamin D3 ($1,25(OH)_2VD_3$) and zinc sulfate heptahydrate (SZn) on spleen leukocytes of CanL dogs regarding the immune response to the disease. We detected low serum levels of retinol and Zn, and high levels of $25(OH)VD_3$ in CanL dogs. *In vitro* supplementation with all-trans retinoic acid (ATRA), $1,25(OH)_2VD_3$ and SZn of CanL spleen leukocytes dogs in the presence of the soluble antigen of *Leishmania* spp. (SLA) increased the levels of nitric oxide and of reactive oxygen species. We also observed that interferon-gamma was reduced in the supernatant of a spleen leukocyte stimulated with $1,25(OH)_2VD_3$ and SZn, while tumor necrosis factor-alpha was increased after stimulation with ATRA and SZn. Interleukin-10 was reduced after stimulation with ATRA, $1,25(OH)_2VD_3$ and SZn, and parasite load was reduced after stimulation with SZn. We conclude that canine leishmaniasis is associated with retinol and Zn deficiency and we suggest that ATRA, $1,25(OH)_2VD_3$ and SZn are involved in the immunological regulation of the leishmanicidal effector response, providing a high potential for investigation in *in vivo* models, especially regarding Zn.

Keywords: Retinol. *All trans* retinoic acid. Vitamin D3. Zinc. Leishmaniosis.

2.3 Introduction

Visceral leishmaniasis is a chronic infectious disease characteristically occurring in underdeveloped and developing countries, caused by a parasite of the genus *Leishmania* ssp., with a broad spectrum of clinical and immunopathological manifestations [1]. Dogs are considered to be the main reservoirs of the parasite in urban centers and, despite the efforts in the fight against the disease, many endemic sites of canine leishmaniasis (CanL) persist, preceding human infection and representing a serious public health problem [2].

It has been well established that one of the most important aspects of the pathogenesis and progression of CanL is the suppression of Th1 cell immunity, with a predominance of Th2 cell immunity which is ineffective in the elimination of the parasite [3]. The absence of a T cell response to *Leishmania* ssp. antigens has been observed in infected dogs based on negative skin tests. On the other hand, a Th1 cell effector response in the fight against the parasite is detected in resistant animals [3,4].

The Th1 cell effector response is characterized by the action of TCD4+ lymphocytes which secrete Th1 type cytokines such as interleukin 12 (IL-12), interferon gamma (IFN-γ) and tumor necrosis factor (TNF-α). In contrast, humoral immunity is characterized by the secretion of type Th2 cytokines such as interleukin 10 (IL-10) and associated with parasite survival and disease persistence [5,6].

The therapeutic arsenal for the treatment of leishmaniasis is limited by toxicity, a high cost and inefficacy in some cases [7,8]. Treatment failure implies the permanence of dogs as reservoirs of the parasite, with further aggravation of the public health problem, indicating that new therapies should be evaluated in order to increase treatment efficacy.

The relationship between the disease, nutrition and immunity, although not yet fully elucidated, has been recognized for many years and is currently being investigated under different clinical conditions experimental models [9]. Nutrients are necessary for immune system function [10], they may act synergistically with drug treatment [11] and can help prevent a series of infectious contagious diseases [12].

The importance of nutrients for the regulation of immunity has been little studied in visceral leishmaniasis. Zinc (Zn) is an essential mineral for innate and adaptive immune response [13].

In dogs, Zn supplementation in combination with standard therapy has elicited a more rapid therapeutic response and the delay in relapse [11], whereas vitamin D deficiency was associated with disease progression[14]. In patients with visceral leishmaniasis, serum vitamin A has been found to be reduced and its *in vitro* supplementation has promoted a reduction of IL-10 in Treg cells and monocytes [15]. In mice infected with *L. donovani*, vitamin A and D supplementation improved immunity and reduced parasite load in the spleen [16].

Despite the evidence of the role of nutrients in the immune system and in leishmaniasis, few studies have been conducted on dogs. Thus, the objective of the present study was to determine the serum levels of vitamin A (retinol), vitamin D (25-hydroxy vitamin D3 or 25(OH)VD_3)) and Zn in dogs with leishmaniasis, and to assess *in vitro* the ability of these nutrients to regulate the production of nitric oxide (NO), reactive oxygen species (ROS) and cytokines that regulate adaptive response (IFN-γ, TNF-α and IL-10), as well as the parasite load in CanL.

2.4 Materials and Method

2.4.1 Animals and sample collection

The study was approved by the Ethics Committee for the Use of Animals of Sao Paulo State University (Unesp), School of Veterinary Medicine, Araçatuba (Protocol N° 00165-2017).

The group of dogs with leishmaniasis (CanL) consisted of 15 adult animals (8 males and 7 females) seropositive for the *L. infantum* antigen as determined by indirect ELISA [17], with the diagnosis confirmed by the detection of *Leishmania* ssp. DNA by real time PCR (qPCR) [18]. Animals were obtained from the Center of Zoonosis Control, aged 2 to 5 years old and varied breed and weight, with disease staging ranging from discrete (15%) to moderate (80%) and severe (5%) according to the parameters proposed by Solano and Galego [8]. They were symptomatic and exhibited at least 3 of the following clinical signs: onychogryphosis, cachexia, alopecia, skin and periocular lesions, and lympho-hepatosplenomegaly.

The control group (CG) consisted of 5 healthy dogs (2 males and 3 females) residing in an endemic area and negative for *Leishmania* spp. as determined by indirect

ELISA and qPCR, and with blood count and biochemical parameters within normal range for the species.

Blood samples were collected by puncture of the jugular vein and placed in dry tubes, with serum being obtained immediately for later determination of vitamins A and D, and Zn levels.

Spleen tissue samples were obtained from the CanL group after euthanasia performed according to state legislation with an intravenous injection of a barbituric anesthetic (thiopental, Cristália Itapira, SP), followed by a 19.1% solution of potassium chloride. A spleen fragment was obtained from CG animals by surgical excision according to the protocol described by Lima et al. [3].

2.4.2 Serum micronutrient analysis

Serum retinol was determined based on its circulating form (retinoic acid, RA) by HPLC using a 4.6 x 25 cm C-18 column (Shimpack CLC-ODS), a 4 mm x 1 cm precolumn and a flow of 2.0 mL/min[19]. Serum ($25(OH)VD_3$) was determined by competitive capture ELISA using the commercial Dog 25-hydroxy vitamin D3 kit (BiorByt, Cambridge, UK) according to manufacturer instructions. Serum Zn was determined by inductively coupled plasma mass spectrometry (ICP-MS) [20].

2.4.3 Isolation and culture of spleen cells

Whole spleen cells were obtained from a 3 cm^3 macerated fragment and added to 10 mL RPMI-1640 medium supplemented with fetal bovine serum inactivated to 10% (Gibco, Waltham, MA, USA), 0.03% L-glutamine (Sigma, St. Louis, MO, USA), 100 IU/mL penicillin (Sigma), and 100 mg/mL streptomycin (Sigma). After removal of cell debris through a BD Falcon Cell filter (San Diego, CA, USA), cell suspension was processed with 5 mL erythrocyte lysis buffer containing 7.46 g/L ammonium chloride (NH_4ClO_3) at 4°C for 10 min, centrifuged at 2000 rpm for 5 min and washed three times with phosphate buffered saline, pH 7.2.

Spleen leukocyte cultures, 2.5 x 10^6 cells in 400 μL per well, were incubated in an oven at 37°C with 5% CO_2 for 20 h for the detection of reactive species and for 72 h for the detection of cytokines and of parasite load.

The following treatment conditions were used: basal medium, addition of 10 µg/ml soluble leishmania antigen (SLA) [15], addition of the active form of vitamin A (0.5 nM all-trans retinoic acid or ATRA), addition of the active form of vitamin D, 4 nM 1,25-dihydroxy vitamin D3 ($1,25(OH)_2VD_3$), addition of 0.05 nM zinc sulfate heptahydrate (SZn), and addition of 10 µg/ml phytohemagglutinin [15].

Concentrations of ATRA, $1,25(OH)_2VD_3$ and SZn used in the canine spleen leukocyte cultures were based on literature [15,21,22] and previously obtained in a pilot study in which percent cell death by apoptosis was titrated by flow cytometry using the Guava Nexin kit (Millipore®) according to manufacturer instructions. In the cytometry analysis we considered the percentage of cells in late apoptosis that were positive for PE- conjugated annexin and 7AA-D, respectively collected from the FL2 and FL3 channels of the flow cytometer. We chose the concentration that promote lower cell death by apoptosis.

2.4.4 Flow cytometry

NO and ROS levels in spleen leukocytes were determined after 20h of cell culture. For ROS determination, cell suspension was treated and stained with 10 µM H2DCFDA (Invitrogen-Leiden Molecular Probes, The Netherlands), and then incubated for 30 minutes at 37ºC in the presence of 5% CO_2. For NO determination, the same cell suspension was treated with 2 µM DAF-2DA for 30 minutes at 37°C in the presence of 5% CO_2. Unlabeled samples were used as a negative control in order to define the negative populations in the samples analyzed. FITC fluorescence (FL1) was measured in a log scale and was expressed as mean fluorescence considering 10,000 closed events. Data were analyzed using the BD Accuri C6 software, version 1.0.264 (BD Biosciences, CA).

2.4.5 ELISA

The cytokines IFN-γ, TNF-α and IL-10 were quantitated in the supernatant of dog leukocytes after 72 h of culture using commercial ELISA capture Duo Set® Canine kits (R&D System, Minneapolis, MN, USA) according to manufacturer instructions. Plate readings were obtained with a Spectra Count spectrophotometer (Packard Bio Science Company, USA) with a 450 nm filter.

2.4.6 Parasite load

Parasite load was determined in canine spleen leukocytes after 72h of incubation by cytocentrifugation at 1000 rmp for 5 minutes at room temperature. Slides were stained with a commercial hematological dye (Instant-Prov, Newprov, Pinhais, PR, Brazil) for parasite count inside the macrophages under a light microscope (Eclipse E800, Nikon, Tokyo, Japan), with differentiation of infected and noninfected cells. We counted 50 infected macrophages (when the infection rate was low we considered the maximum number of infected macrophages) and the quantity of amastigotes inside the cells. Parasite load was determined by dividing the number of amastigotes by the number of infected macrophages counted.

2.4.7 Reagents and antibodies

In vitro cultures were supplemented with vitamin A (all-trans retinoic acid, Biomedicals MP, Canada), vitamin D (1α,25-dihydroxyvitamin D3, Sigma) and Zn (zinc sulfate heptahydrate, Sigma). Serum vitamin A was determined using synthetic retinol, dichloromethane, acetonitrile, methanol, ethanol and hexane (Sigma) and serum vitamin D was determined using the commercial kit Dog 25-hydroxy vitamin D3 (BiorByt, Cambridge, UK). DNA was extracted using the commercial kit DNAeasy® (Quiagen, Valencia, CA, USA). NO and ROS were quantitated using the DAF-2DA probe (2 µM) and the H2DCFDA probe (10 µM) (Invitrogen, Leiden, The Netherlands), respectively. IFN-γ, TNF-α and IL-10 were quantitated with commercial Duo Set® Canine kits (R&D System).

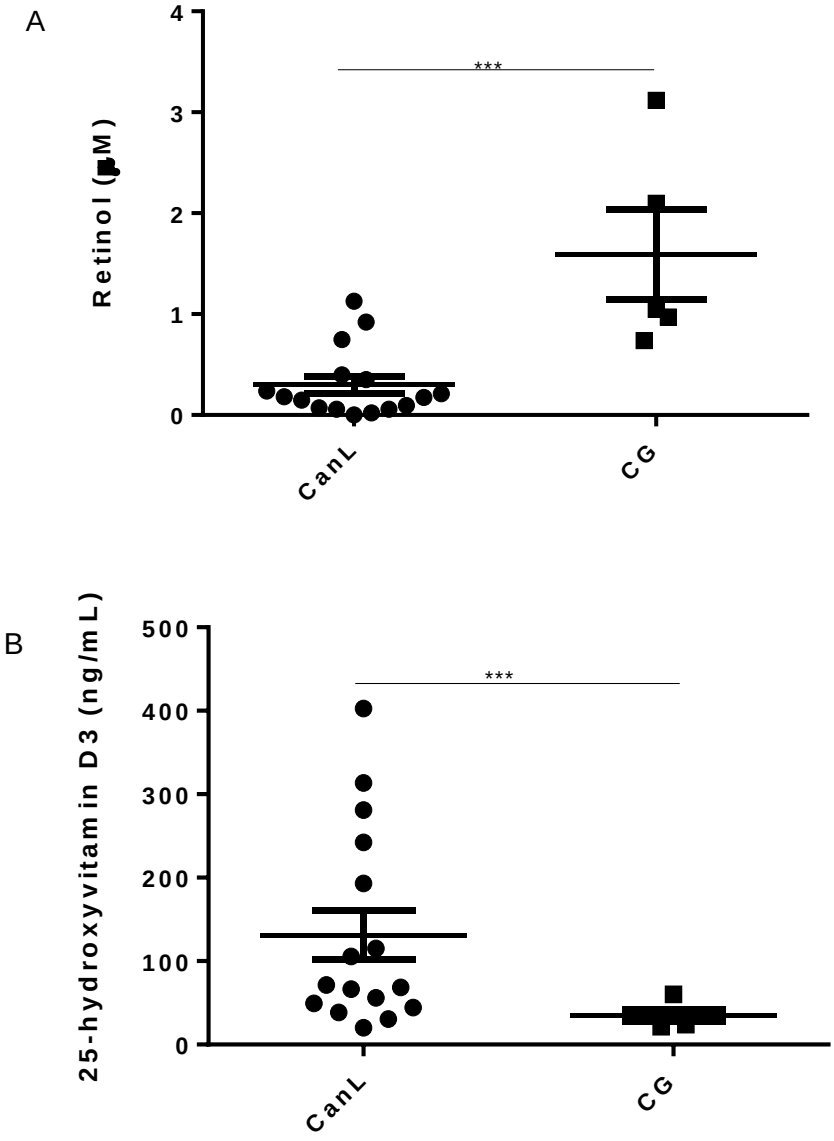
2.4.8 Statistical analysis

Data were tested for normality and the Mann-Whitney test was used to compare serum retinol, 25(OH)VD_3 and Zn between the CanL group and CG. Wilcoxon test was used to compare the production of NO and ROS, the cytokines IFN-γ, TNF-α and IL-10 and parasite load between treatments. All analyses were carried out and graphs constructed using the GraphPad Prisma 6 software (La Jolla, CA, USA), with the level of significance set at $p < 0.05$.

2.5 Results

2.5.1 Reduction of retinol and Zn and increase of 25(OH)VD_3 in CanL serum

Vitamin D deficiency has been associated with the progression of CanL [14] and low serum levels of vitamin A [15] and Zn [23] have been detected in patients with leishmaniasis [15]. On this basis, in the present study we quantitated the serum levels of these nutrients in the dogs under study and observed low levels of retinol (Fig.1a) and Zn (Fig.1c) in the CanL group compared to CG and, conversely, higher levels of 25(OH)VD_3 in the CanL group (Fig.1b).



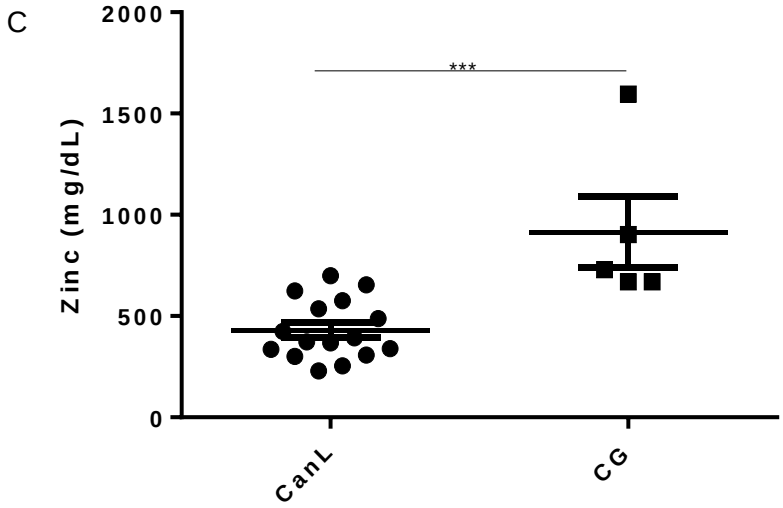


Figure 1. Serum retinol (A), 25(OH)VD_3 (B) and Zn (C) levels of the CanL group and CG. Data are reported as mean ± SEM (n=15 and 5 for CanL and CG, respectively). ***p<0.001, Mann-Whitney test.

2.5.2 *In vitro* supplementation of spleen leukocytes with ATRA, 1,25(OH)$_2$VD$_3$ and SZn increased production of NO and ROS in the CanL group

The reactive species NO and ROS are important for leishmanicidal activity [24], NO having been previously demonstrated in CanL [25]. On this basis, we determined the production of NO and ROS in spleen leukocytes of CanL after *in vitro* supplementation with the above nutrients and observed an increased production of NO in the spleen leukocytes of CanL with ATRA, 1,25(OH)$_2$VD$_3$ and SZn (Fig.2a), but no change in ROS production (Fig. 3a). Addition of SLA, ATRA, 1,25(OH)$_2$VD$_3$ and SZn increased NO (Fig.2a) and ROS (Fig.3a) production in cells. NO production was increased in CG spleen leukocytes only after the addition of ATRA (Fig.2a), whereas no effect was observed for the remaining treatment conditions or for ROS production (Fig.3a).

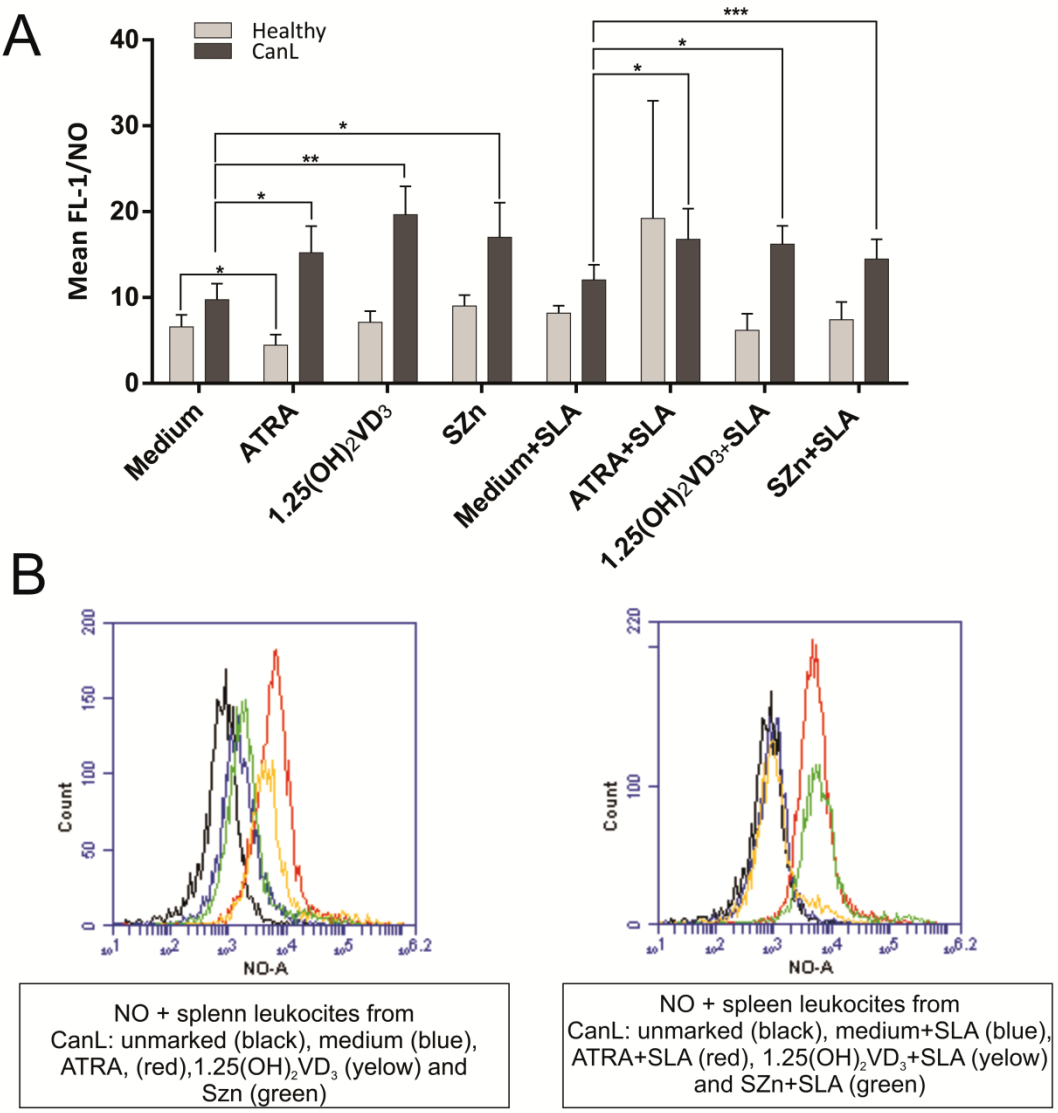


Figure 2. NO (A) production increased with *in vitro* supplementation with ATRA, 1,25(OH)$_2$VD$_3$ and SZn of spleen leukocytes of CanL animals, and NO production increased with *in vitro* supplementation with ATRA of spleen leukocytes of healthy dogs. Flow cytometry histogram showing the production of NO (B) in spleen leukocytes of CanL. Data are reported as mean ± SEM (n=15).*p<0.05, **p<0.01, ***p<0.001, Wilcoxon test.

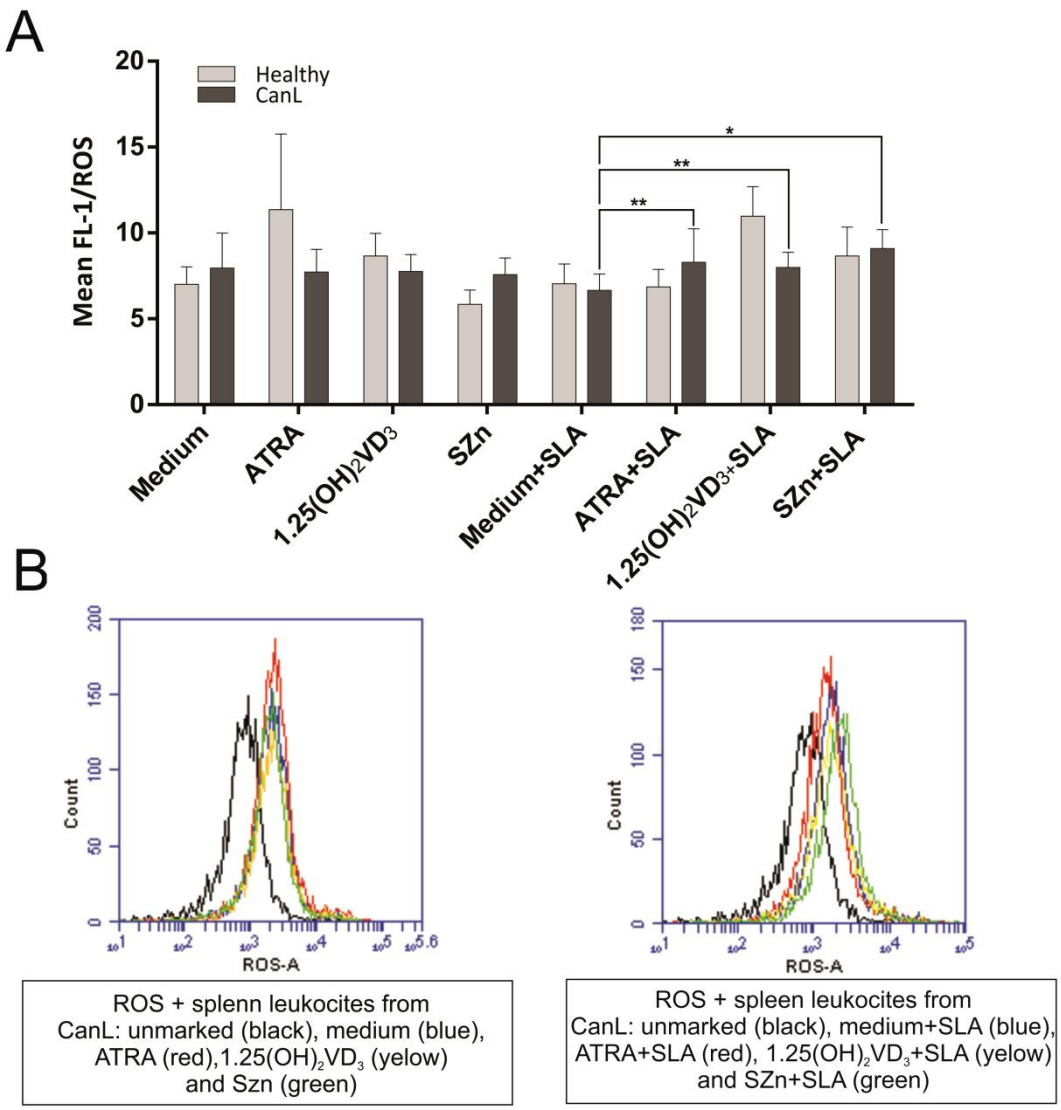


Figure 3. ROS (A) production increased with *in vitro* supplementation with ATRA, 1,25$(OH)_2VD_3$ and SZn of spleen leukocytes of CanL animals. Flow cytometry histogram showing the production of ROS (B) in spleen leukocytes of CanL. Data are reported as mean ± SEM (n=15).*p<0.05, **p<0.01, ***p<0.001, Wilcoxon test.

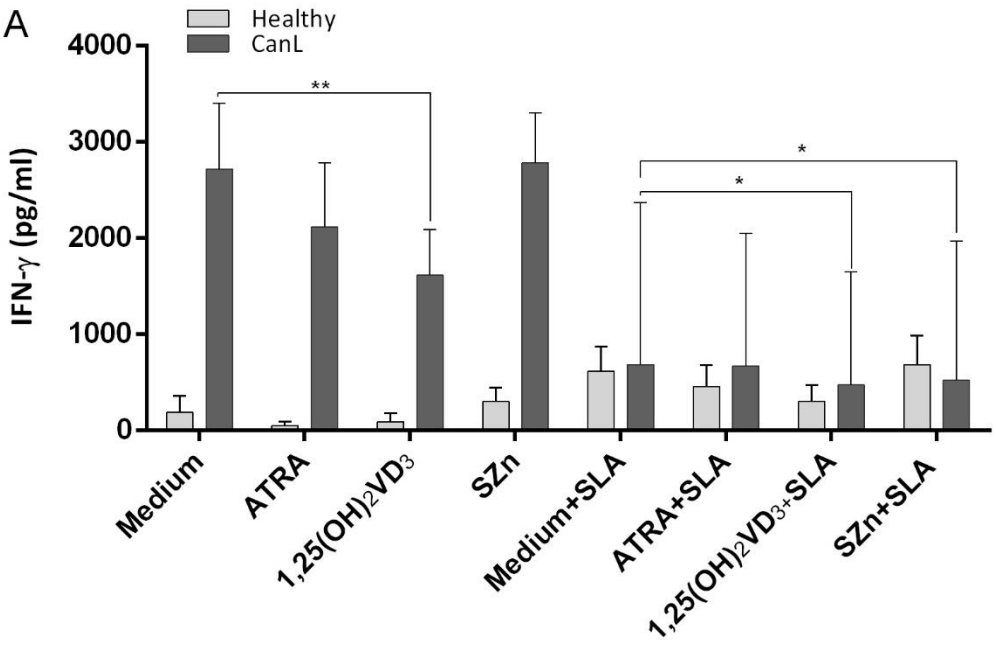
2.5.3 *In vitro* supplementation of CanL spleen leukocytes stimulated with 1,25$(OH)_2VD_3$ and SZn reduced IFN-γ; ATRA and SZn and increased TNF-α. ATRA, 1,25$(OH)_2VD_3$ and SZn reduced IL-10

We also determined whether *in vitro* supplementation with the above nutrients would interfere with production of immunity regulating cytokines such as IFN-γ, TNF-α and IL-10 in CanL animals. We observed that IFN-γ was reduced in the cell

culture supernatant of the CanL group following treatment with 1,25$(OH)_2VD_3$, but not after treatment with ATRA or SZn (Fig. 4a). IFN-γ was also reduced in the cell culture supernatant of the CanL group after spleen leukocyte stimulation with SLA, 1,25$(OH)_2VD_3$ and SZn (Fig. 4a). Again, no effect observed for ATRA. In CG, supplementation of spleen leukocytes with ATRA, 1,25$(OH)_2VD_3$ and SZn did not interfere with the production of IFN-γ under any treatment conditions studied.

TNF-α was increased in the cell culture supernatant of spleen leukocytes of both CanL and CG with stimulation with ATRA and SZn, whereas it was increased only in the CanL group after stimulation with SLA (Fig. 4b). No effect was observed in CG after stimulation with 1,25$(OH)_2VD_3$.

No change in IL-10 production was observed in the supernatant of the spleen leukocyte culture of CanL animals after stimulation with ATRA, 1,25$(OH)_2VD_3$ and SZn, whereas IL-10 production was reduced after stimulation with SLA plus the addition of ATRA, 1,25$(OH)_2VD_3$ and SZn (Fig. 4c). In CG, IL-10 was increased in the supernatant of the spleen leukocyte cell culture supplemented with 1,25$(OH)_2VD_3$, while no other effect was observed with the remaining treatment conditions (Fig. 4c).



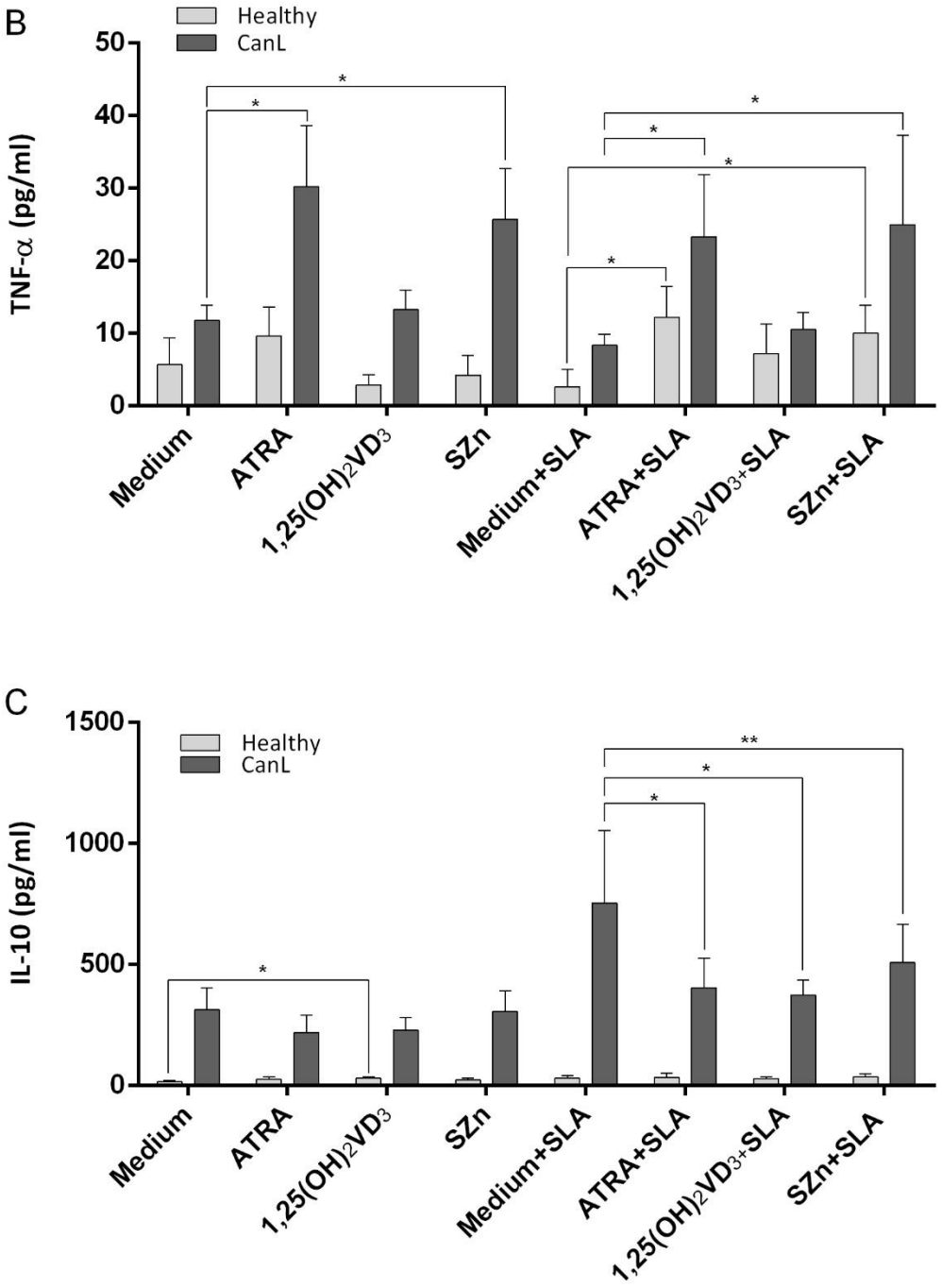


Figure 4. (A) IFN-γ production was reduced by *in vitro* supplementation with 1,25(OH)$_2$VD$_3$ and SZn stimulated with SLA in spleen leukocytes of CanL dogs; (B) TNF-α production was increased by *in vitro* supplementation with ATRA and SZn with and without SLA stimulation in spleen leukocytes of CanL dogs, and was increased only with SLA stimulation in healthy dogs; (C) IL-10 production was reduced by *in vitro* supplementation with ATRA, 1,25(OH)$_2$VD$_3$ and SZn stimulated with SLA in spleen leukocytes of CanL animals and was increased by 1,25(OH)$_2$VD$_3$ in healthy dogs . Data are reported as mean ± SEM (n=10, 7 and 15 for IFN-γ, TNF-α and IL-10, respectively). *p<0.05, **p<0.01, Wilcoxon test.

2.5.4 *In vitro* supplementation of spleen leukocytes of CanL animals stimulated with ATRA, 1,25(OH)$_2$VD$_3$ and SZn reduced parasite load, although only SZn continued to have this effect after the addition of SLA

Intracellular Zn can influence phagocytosis and microbicidal activity [26]. On this basis, we determined whether supplementation of a spleen leukocyte culture of the CanL group with ATRA, 1,25(OH)$_2$VD$_3$ and SZn would affect parasite load. Parasite load was reduced by supplementation with ATRA, 1,25(OH)$_2$VD$_3$ and SZn (Fig. 5); however, in the presence of stimulation with SLA, only the treatment with SZn continued to have this reducing effect on parasite load (Fig. 5).

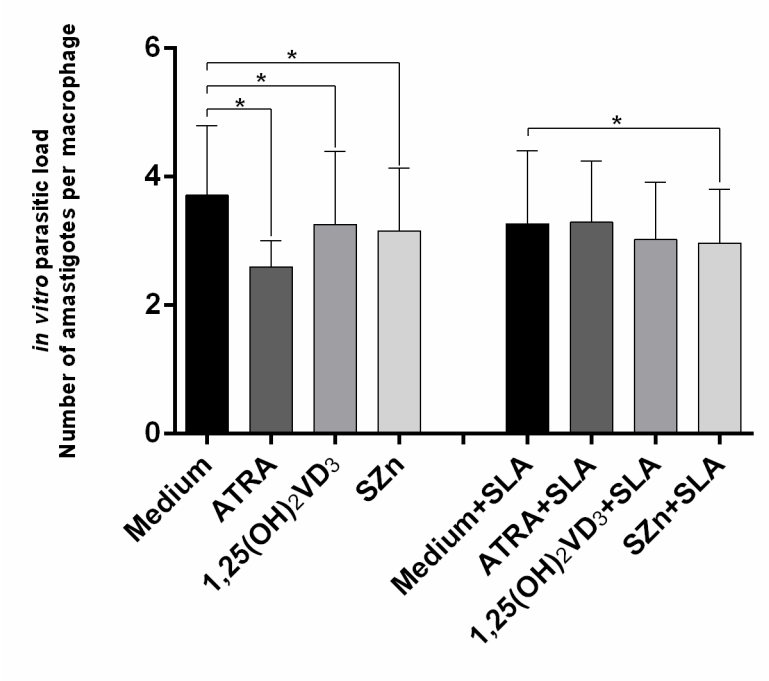


Figure 5. Parasite load was reduced by *in vitro* supplementation of spleen leukocytes of CanL animals with ATRA, 1,25(OH)$_2$VD$_3$ and SZn. Parasite load was quantitated by counting the amastigotes present inside the macrophages on slides obtained by cytocentrifugation. Data are reported as mean ± SEM (n=15). *p<0.05, **p<0.01, Wilcoxon test.

2.6 Discussion

In the present study we observed low retinol and Zn levels and increased 25(OH)VD$_3$ levels in the serum of dogs with leishmaniasis (CanL group) compared to uninfected dogs. *In vitro* supplementation of CanL spleen leukocytes with ATRA, 1,25(OH)$_2$VD$_3$ and SZn and treatment with an antigen caused an increase in NO and

ROS levels. Supplementation with 1,25$(OH)_2VD_3$ and SZn and treatment with an antigen reduced IFN-γ in the culture supernatant, while the addition of ATRA and SZn increased TNF-α levels and only SZn reduced the parasite load (ENCLOSURE 1). These findings suggest an important role of these immunomodulatory nutrients in CanL, with positive effects that favor an effector Th1 response (Fig. 6).

Low serum level of retinol observed in dogs with leishmaniasis confirmed previous results regarding this nutrient in the disease, such as a low serum vitamin A level in children with visceral leishmaniasis in an endemic area of Brazil [15]. Vitamin A deficiency affects the immunity of mice, reduces Th1 memory cells [27], and provokes an abnormal expansion of myeloid cells [28], and in symptomatic dogs the histopathological changes associated with the increased myeloid cell levels in the spleen [29] may be related to the low serum level of retinol observed here in the CanL group.

Low serum Zn levels observed in dogs with leishmaniasis confirmed previous findings reported for dogs [30–32] and for human with chronic visceral leishmaniasis living in endemic areas of India [33] and Blangadesh [34]. Zn deficiency shows effects on immunity such as reduction of peritoneal macrophages in mice and a lower microbicidal activity of these cells, while Zn addition restores the ability of macrophages to capture and kill *Trypanosoma cruzi* parasites [35]. In addition, Zn is essential for thymus function, with atrophy of the organ occurring under Zn deficiency, as well as lymphopenia and depression of both immune and adaptive response [13,36]. Adaptive response is suppressed in dogs with leishmaniasis [3] and part of this suppression may be related to Zn deficiency.

In addition, Zn status influences vitamin A status because this mineral is involved in several aspects such vitamin A absorption, transport and utilization[37]. Also, vitamin A [38] and Zn [13] are antioxidants and their use in the fight against the oxidative stress characteristic of leishmaniasis [39] may be at the root of the observed deficiency in this disease.

Since vitamin A and Zn deficiency has been extensively reported in leishmaniasis, we emphasize the need for supplementation with these nutrients as a preventive strategy or as a coadjutant treatment in view of their importance for the immune system.

On the other hand, in the present study we did not observe 25$(OH)VD_3$ deficiency, but rather a high serum level in the CanL group compared to uninfected

dogs, in contrast to a study in an endemic area of Spain in which dogs with visceral leishmaniasis had low serum vitamin D levels [14]. This difference may be explained by the high incidence of solar radiation in Brazil compared to Spain, since most vitamin D is produced on the skin by exposure to UVB radiation [40]. In addition, our control group consisted of dogs belonging to private owners who usually reside inside their homes protected from the sun, possibly justifying the low serum 25(OH)VD_3 levels of this group.

Supplementation with all three nutrients tested in the present study, in the presence or absence of SLA, increased NO levels in the spleen leukocytes of the CanL group, although these nutrients only induced an increase in ROS when the cells were stimulated with SLA. NO and ROS have a microbicidal activity in canine leishmaniasis [24,41], suggesting that these micronutrients may potentiate the leishmanicidal effect of the Th1 response.

In vitro increase of NO in the spleen leukocytes of CanL animals supplemented with ATRA may have been due to activation of the promoter region of the induced nitric oxide synthase (iNOS) gene, as previously demonstrated *in vitro* in humans [42] and *in vivo* in mice [43]. In agreement with our results, the addition of 1,25$(OH)_2VD_3$ also increased NO production in murine macrophages activated by IFN-γ and infected with *L. major* [21]. Future studies are needed to elucidate the mechanism by which ATRA, 1,25$(OH)_2VD_3$ and Zn increase NO levels in dogs with leishmaniasis.

We also investigated cytokine levels of spleen leukocytes from CanL animals after supplementation with ATRA, 1,25$(OH)_2VD_3$ and SZn and the presence or absence of SLA. *In vitro* supplementation with 1,25$(OH)_2VD_3$ reduced IFN-γ levels in the cell culture supernatant, an effect that was not observed with ATRA or SZn. In the presence of SLA, 1,25$(OH)_2VD_3$ and SZn also reduced IFN-γ levels in the supernatant ot the leukocyte culture, whereas ATRA had no effect. High IFN-γ levels have been observed in canine leishmaniasis, with high spleen parasitism and worsening of the disease [44–46], and the reduction of IFN-γ observed in the present study may have been favorable to the regulation of effector immunity, since we also detected a low parasite load in the presence of 1,25$(OH)_2VD_3$ and SZn.

Reduction of IFN-γ in the supernatant of spleen leukocytes observed in the CanL group after supplementation with 1,25$(OH)_2VD_3$ confirmed the anti-inflammatory role of vitamin D, also observed in experimental leishmaniasis [47,48] and in other infectious-parasitic [49,50] and autoimmune diseases [51].

On the other hand, ATRA and SZn increased TNF-α levels in the cell culture supernatant with or without the use of SLA, and 1,25$(OH)_2VD_3$ had no effect. TNF-α is associated with the effector Th1 response for the fight against the disease[52], and its increase after supplementation with SZn agreed with data reported in an *in vitro* study using peripheral blood mononuclear cells (PBMC) from healthy humans [53]. Regarding the effect of ATRA on the increased levels of TNF-α, we suggest a positive effect on the Th1 response of dogs with leishmaniasis, in contrast to the results obtained *in vitro* in mouse PBMC, in which supplementation with ATRA reduced the TNF-α mRNA, a difference that may be related to the different host and experimental designs used in the studies. The modulation of TNF-α production by ATRA and SZn suggests that these nutrients may regulate the adaptive response of the Th1 cell profile.

Production of IL-10 was reduced by supplementation with all three nutrients tested in the present study. In agreement with our results, *in vitro* supplementation with ATRA of PMBC (T-reg and monocytes) from human infected with *L. infantum* reduced expression of IL-10 [15], indicating a possible regulatory role of vitamin A in the production of IL-10 in visceral leishmaniasis. The IL-10 cytokine in the spleen seems to be an indicator of susceptibility[54] and its reduction observed in the CanL group after supplementation with ATRA, 1,25$(OH)_2VD_3$ and SZn may favor the regulation of immunity.

Finally, supplementation with all three nutrients tested in this study reduced parasite load, although only SZn continued to have this effect when the leukocytes were stimulated with SLA. *In vitro* addition of SLA reproduces the condition of the dogs in the endemic area, since the animals are in constant contact with antigens of *Leishmania* sp., and the fact that only SZn reduced the parasite load in this condition suggests that this mineral has a more potent leishmanicidal effect with different pathways of immunological activation compared to ATRA and 1,25$(OH)_2VD_3$.

In agreement with these results, mice infected with *L. donovani* and receiving a diet enriched with ATRA and 1,25$(OH)_2VD_3$ also showed a reduced spleen parasite load [16]. In addition, Zn supplementation has proved to be beneficial in various infectious diseases including leprosy, tuberculosis and others [55]. Therapeutic response of dogs with leishmaniasis treated with standard therapy in combination with Zn was more rapid, with a longer interval before relapse [11], supporting the important role of this mineral in the disease. These findings suggest that Zn may be directly linked

to the leishmanicidal activity of macrophages, although further studies are needed to elucidate the mechanisms involved.

2.7 Conclusion

In summary, we showed that canine leishmaniasis is related with vitamin A and Zn deficiency, and we suggest that ATRA, $1,25(OH)_2VD_3$ and SZn are involved in the immunological reaction associated with the leishmanicidal effector response, with great potential for investigation *in vivo* models, especially regarding Zn.

2.8 Acknowledgments

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Enclosure - Graphical Abstract

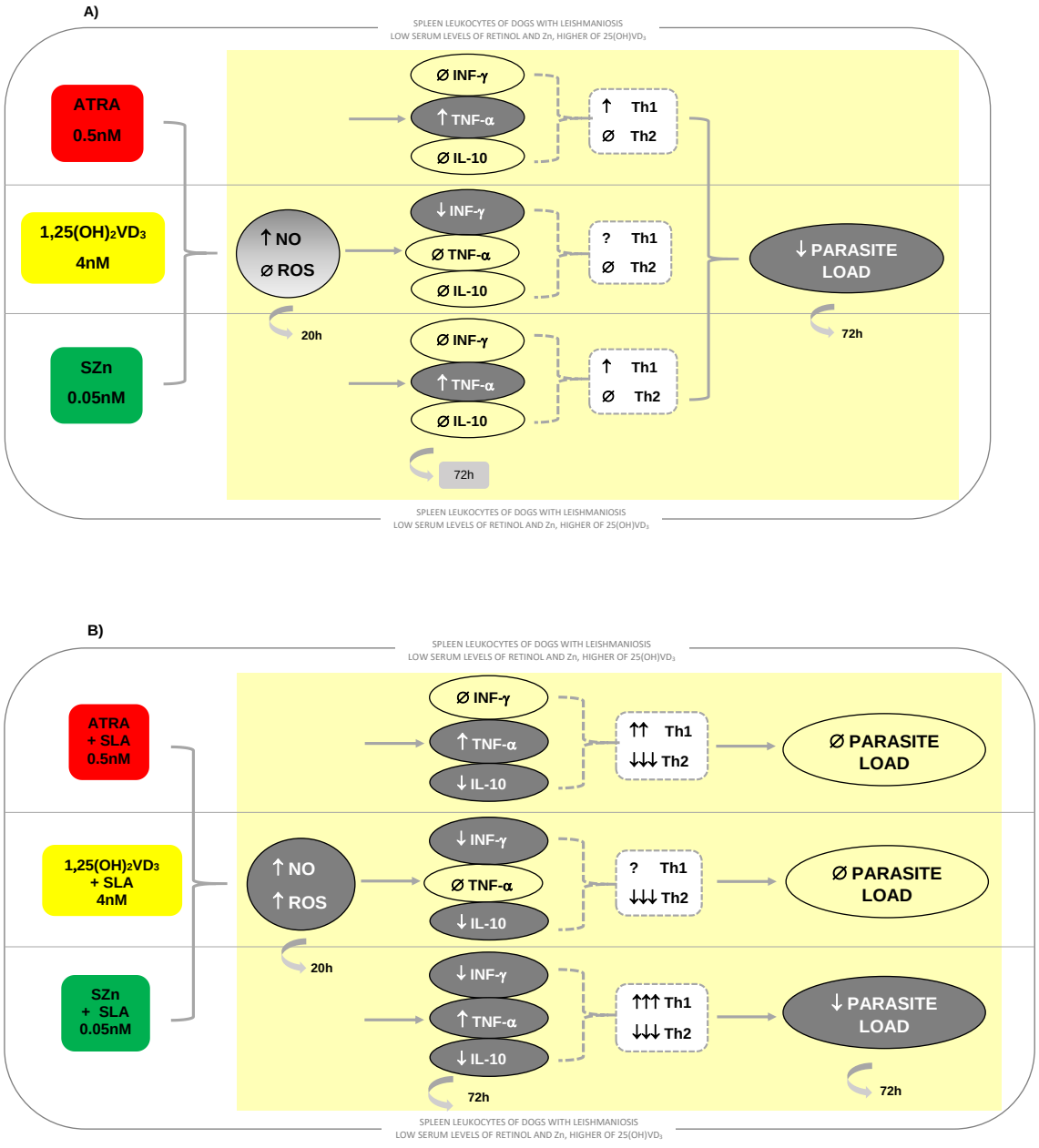

Figure 6. (A) *In vitro* supplementation of spleen leukocytes of CanL stimulated with ATRA, 1,25(OH)$_2$VD$_3$ and SZn promoted an increase of NO and reduction of parasite load; 1,25(OH)$_2$VD$_3$ reduced IFN-γ; ATRA and SZn increased TNF-α; with no effect on IL-10. **(B)** *In vitro* supplementation of spleen leukocytes of CanL stimulated with ATRA, 1,25(OH)$_2$VD$_3$ and SZn in the presence of SLA promoted an increase in NO, ROS and IL-10; 1,25(OH)$_2$VD$_3$ and SZn reduced IFN-γ; ATRA and SZn increased TNF-α; SZn reduced parasite load.

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ANEXO A – Protocolo de aprovação pelo Comitê de Ética no Uso de Animais

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CEUA - Comissão de Ética no Uso de Animais
CEUA - Ethics Committee on the Use of Animals

CERTIFICADO

Certificamos que o Projeto de Pesquisa intitulado **"Papel dos micronutrientes na resposta imunológica da leishmaniose visceral canina"**, Processo FOA nº 00165-2017, sob responsabilidade de Valéria Marçal Félix de Lima apresenta um protocolo experimental de acordo com os Princípios Éticos da Experimentação Animal e sua execução foi aprovada pela CEUA em 22 de Fevereiro de 2017.

VALIDADE DESTE CERTIFICADO: 01 de Março de 2021.
DATA DA SUBMISSÃO DO RELATÓRIO FINAL: até 01 de Abril de 2021.

CERTIFICATE

We certify that the study entitled **"The role of micronutrients in the immune response of canine visceral leishmaniasis"**, Protocol FOA nº 00165-2017, under the supervision of Valéria Marçal Félix de Lima presents an experimental protocol in accordance with the Ethical Principles of Animal Experimentation and its implementation was approved by CEUA on February 22, 2017.

VALIDITY OF THIS CERTIFICATE: March 01, 2021.
DATE OF SUBMISSION OF THE FINAL REPORT: April 01, 2021.

Prof. Ass. Dr. Leonardo Perez Faverani
Coordenador da CEUA
CEUA Coordinator

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ANEXO B – Guide for Auhors

FREE RADICAL BIOLOGY AND MEDICINE

Aims and scope

*Free Radical Biology and Medicin*e is the premier forum for publishing groundbreaking research in the redox biology of both health and disease. We focus on signal transduction and redox signaling; oxidative stress; reductive stress; redox stress; nitrosative stress; aging and age-related diseases; metabolic regulation and metabolic diseases; mitochondrial function and signaling; homeostatic mechanisms and adaptive responses; redox chemistry and mechanisms; materials & nanomaterials; non-thermal plasmas; microorganisms, fungi, plants, insects, animals, and humans; and antioxidant enzymes, pathways, and networks. We welcome both full-length and short Research Communications, Hypothesis Papers, Reviews, Mini Reviews, Graphical Reviews, and Critical Methods Papers. *Free Radical Biology and Medicine* also commissions themed Special Issues aimed at highlighting recent advances in both basic and clinical fields, with a particular focus on mechanisms underlying altered metabolism and redox signaling.

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Introduction

Free Radical Biology and Medicine is the premier forum for publishing groundbreaking research in the redox biology of both health and disease. We focus on signal transduction and redox signaling; oxidative stress; reductive stress; redox stress; nitrosative stress; aging and age-related diseases; metabolic regulation and metabolic diseases; mitochondrial function and signaling; homeostatic mechanisms and adaptive responses; redox chemistry and mechanisms; materials & nanomaterials; non-thermal plasmas; microorganisms, fungi, plants, insects, animals, and humans; and antioxidant enzymes, pathways, and networks.

We welcome both full-length and short Research Communications, Hypothesis Papers, Reviews, Mini Reviews, Graphical Reviews, and Critical Methods Papers.

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