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Expression of TLR2, FOXP3, and COX2 in the synovial membrane of dogs with canine leishmaniasis-induced arthritis[1]

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ABSTRACT.- Souza-Filho F.C., Martins C.S., Ferreira T.C., Carvalho-Sombra T.C.F., Lopes-Neto B.E., Ferreira T.M.V., Girão V.C.C. & Nunes-Pinheiro D.C.S. 2024. **Expression of TLR2, FOXP3, and COX2 in the synovial membrane of dogs with canine leishmaniasis-induced arthritis**. *Pesquisa Veterinária Brasileira 44:e07412, 2024*. Programa de Pós-Graduação em Ciências Veterinárias, Faculdade de Veterinária, Universidade Estadual do Ceará, Av. Dr. Silas Munguba 1700, Fortaleza, CE 60714-903, Brazil. E-mail: tiago.cunha@uece.br

Canine leishmaniasis (CanL) is a multifaceted disease triggered by the protozoan *Leishmania infantum*, characterized by diverse clinical presentations, including osteoarticular complications. Immune-mediated joint diseases invariably initiate at the synovial membrane, implicating its pivotal role in arthritis pathogenesis. This study aimed to investigate the influence of natural *L. infantum* infection on synovial fluid characteristics and the expression of immune markers, including TLR-2, FOXP3, and COX-2, in the synovial membrane. Twenty naturally infected dogs (NID) with *L. infantum* were sourced from the Zoonosis Surveillance Unit (ZSU). Clinical-orthopedic assessments were conducted, encompassing lameness, joint edema, crepitus, patellar luxation, and the drawer test. Synovial fluid (SF) parameters, including volume, appearance, viscosity, total nucleated cell count (TNC), neutrophil count, and total protein (TP) content, were determined. After anesthesia and euthanasia, synovial membrane specimens were obtained. SF protein concentrations categorized dogs into three groups: GI (2 to 2.5g/dL), GII (2.5 to 6.0g/dL), and GIII (>6g/dL). Inflammatory infiltrates and synovial membrane changes were assessed, and immunohistochemistry evaluated TLR-2, FOXP3, and COX-2 marker expressions. Clinical evaluations revealed various osteoarticular abnormalities in NID dogs, including lameness (55%), joint edema (25%), crepitus (30%), patellar luxation (20%), and positive drawer test (25%). *Post mortem* examinations revealed bilateral subchondral bone, meniscus, and trochlea erosion in 30% of cases. Amastigotes of *L. infantum* were identified extracellularly and within macrophages (60%). An inflammatory infiltrate was predominant in 70% of dogs, with varying intensity among the groups. Mononuclear cells, chiefly macrophages and lymphocytes, and neutrophils comprised the infiltrate. TLR-2 and COX-2 expression levels were elevated in GIII compared to GII and GI. Conversely, FOXP3 showed moderate expression in GI and minimal expression in GII and GIII. This study underscores the contributory role of *L. infantum* infection in the development of joint lesions in CanL. Additionally, alterations in the expression of immune markers TLR2, FOXP3, and COX2 within the synovial membrane imply the perpetuation and exacerbation of the inflammatory processes, shedding light on the intricate pathogenesis of CanL-induced arthritis.

INDEX TERMS: Canine leishmaniasis, synovial fluid, synovial membrane, TLR2, FOXP3, dogs.

RESUMO.- [Expressão de TLR-2, FOXP3 e COX-2 na membrana sinovial de cães com artrite induzida pela leishmaniose canina.] A leishmaniose canina (LC) é uma doença causada pelo protozoário *Leishmania infantum* com diversas manifestações clínicas, incluindo alterações osteoarticulares. A membrana sinovial é o local inicial de eventos patogênicos em todos os

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tipos de doenças articulares mediadas pelo sistema imunológico. Devido a essa relação, o objetivo foi avaliar a influência da infecção natural por *L. infantum* nas características do líquido sinovial e na expressão de TLR-2, FOXP3 e COX-2 na membrana sinovial. Cães naturalmente infectados por *L. infantum* (NID, N=20) foram obtidos na Unidade de Vigilância de Zoonoses (UVZ). Critérios clínicos ortopédicos foram avaliados, assim como foram obtidas amostras de líquido sinovial por artrocentese. O volume, aspecto, viscosidade, contagem total de células nucleadas (TNC) e contagem de neutrófilos e proteína total (PT) no líquido sinovial (LS) foram determinados. Os cães foram anestesiados e posteriormente eutanasiados para obtenção da membrana sinovial. Os animais do estudo apresentaram claudicação (55%), edema (25%), crepitação na articulação do joelho (30%) e luxação patelar medial (20%) e foram positivos no teste da gaveta (25%). Trinta por cento (30%) dos cães apresentaram erosão bilateral do osso subcondral, menisco e tróclea. Amastigotas de *L. infantum* foram visualizadas extracelularmente e dentro de macrófagos (60% dos animais). Os cães foram ainda divididos em três grupos de acordo com a concentração de proteína no líquido sinovial: GI: 2 a 2,5g/dL (valor de referência normal), GII: 2,5 a 6,0g/dL, GIII: >6g/dL. Infiltrado celular inflamatório, bem como aumento da camada da membrana sinovial, foram identificados em 70% (14/20) dos cães. O infiltrado inflamatório estava ausente em membranas sinoviais originadas de GI, presente em GII com intensidade moderada e presente em GIII com intensidade alta. O infiltrado inflamatório era predominantemente composto por macrófagos, linfócitos e neutrófilos. Houve um aumento na expressão dos marcadores TLR2 e COX2 em GIII em comparação com os grupos GII e GI. Contudo, em relação à expressão de FOXP3, houve uma marcação moderada em GI e apenas uma marcação leve em GII e GIII. Este trabalho reforça o papel da infecção por *L. infantum* no desenvolvimento de lesões articulares em cães. Além disso, nosso estudo indica que a LC modifica a expressão de TLR2, FOXP3 e COX2 na membrana sinovial, o que implica a manutenção e progressão do processo inflamatório.

TERMOS DE INDEXAÇÃO: Leishmaniose canina, líquido sinovial, membrana sinovial, TLR2, FOXP3, caninos.

INTRODUCTION

Leishmaniasis, a complex zoonotic disease caused by protozoans of the genus *Leishmania*, significantly impacts human and canine populations (Rodrigues et al. 2017). In urban areas, dogs are the primary reservoir for this parasitic infection (Dantas-Torres et al. 2012). Canine leishmaniasis (CanL) is a multi-faceted parasitic disease known for its variable clinical course, encompassing acute and chronic phases. This condition can potentially affect a wide array of organs and tissues within the canine host, making it a multifarious challenge for veterinarians and researchers (Solano-Gallego et al. 2009).

The clinical manifestations of CanL encompass a spectrum of locomotor problems, with a prevalence reaching up to 30% in affected individuals. These issues include an array of clinical complaints, including muscle atrophy, reduced physical activity, lameness, and pain localized to the distal limbs (Solano-Gallego et al. 2009, Freitas et al. 2012). The apparent clinical signs often correlate with underlying conditions such as synovitis, polymyositis, osteoarthritis, proliferative periostitis, osteolytic lesions, interdigital ulcers, plantar pad lesions, and neuralgia (Spreng 1993, McConkey et al. 2002, Sbrana et al. 2014). Such conditions arise from diverse immunopathological mechanisms, shedding light on the intricate interplay between the parasite and the host's immune system (Koutinas & Koutinas 2014).

Within the scope of immune-mediated joint diseases, the synovial membrane is the pivotal site for initiating pathogenic events. This critical tissue layer plays a central role in orchestrating immune responses, making it a prime location for the convergence of various pathological processes (De Lange-Brokaar et al. 2012, Edilova et al. 2021). It is crucial to explore the possibility of *L. infantum* presence within or passage through the synovial membrane (Silva et al. 2022). Such interactions can trigger complex inflammatory cascades, profoundly disturbing the delicate balance of the joint microenvironment.

Moreover, this disruption may involve the engagement of cell membrane receptors, most notably toll-like receptors (TLRs). TLRs belong to the class of pattern recognition receptors (PRRs) and have the ability to recognize pathogen-associated molecules, setting off intricate intracellular signaling pathways, which, in turn, leads to the activation of transcription factors such as NF-kB, culminating in the production of pro-inflammatory cytokines. In the context of joint health, these cytokines can significantly contribute to the progression of tissue damage and clinical symptoms (De Lange-Brokaar et al. 2012). Additionally, the enzyme cyclooxygenase (COX-2) plays a pivotal role in catalyzing the production of prostanoids, further exacerbating the inflammatory response within the joint (Matte et al. 2001). Conversely, FOXP3, a crucial regulatory protein, controls immune responses, particularly in T cell development and function (Suri-Payer & Fritzsching 2006).

Despite the well-established roles of these molecules, the influence of CanL on their expression within the synovial microenvironment remains largely uncharted. Consequently, this study aimed to assess the impact of natural *L. infantum* infection on synovial fluid characteristics and the expression of TLR-2, FOXP3, and COX-2 in the synovial membrane of dogs.

MATERIALS AND METHODS

Animal Ethics. This research underwent review and approval by the Ethics Committee on Animal Use (CEUA-UECE) under protocol number 6492302/2016.

Study population. Twenty adult mongrel dogs (N=20), comprising both genders and aged between two and six years, were enrolled in this study. Dogs naturally infected with *Leishmania infantum* (NID) were selected from the Zoonosis Surveillance Unit (ZSU) in Fortaleza, Ceará, Brazil, based on their positive serology results for CanL, determined through rapid DPP® and ELISA tests conducted by the ZSU. The animals were submitted to a clinical-orthopedic evaluation, and fluid and synovial membrane material were collected for analysis.

Clinical-orthopedic evaluation. A comprehensive clinical-orthopedic evaluation was conducted for NID dogs. This assessment included the examination of various joint parameters, namely lameness, limb edema, knee joint crepitus, and patellar dislocation. Lameness was assessed through gait analysis. Subsequently, the animals were anesthetized intramuscularly with Thiopentax® to facilitate the evaluation of limb edema and joint crepitus, performed with the animal in lateral decubitus. Additionally, a test for patellar dislocation was carried out. An examination of the integrity of the cruciate ligaments of the knees was also performed. To conclude the evaluation, the animals were euthanized using 10% potassium

chloride intravenously. *Post mortem* examinations included an assessment of the bone and cartilaginous structures of the femorotibial joint, as well as an examination for the presence of erosion in the subchondral bone, meniscus, and trochlea.

Synovial fluid sampling and analysis. After anesthesia, synovial fluid (SF) samples were collected from the animals' knees (N=40). To ensure sterility, trichotomy and joint antisepsis procedures were conducted. SF collection was performed via arthrocentesis in the femorotibial joint, with samples placed in tubes containing anticoagulant (EDTA) for subsequent processing and analysis. The samples were subjected to comprehensive evaluation based on specific physical parameters, encompassing volume, color, turbidity, and viscosity. Synovial fluid of standard composition is characterized by attributes such as transparency, clarity, elevated viscosity, and the absence of flocculent constituents (MacWilliams & Friedrichs 2003, Brombini et al. 2017). The accepted volume range for synovial fluid is observed between 0.05mL and 0.24mL (Boon 1997, De Biasi et al. 2001). Viscosity determination was executed during the transference of synovial fluid from the collection syringe to a glass slide, involving the precise measurement of filament length, expressed in centimeters, formed by the droplet prior to detachment from the syringe's terminus. Categorization criteria included the absence of filament formation, decreased viscosity when filament length was less than 5cm, and normal viscosity when exceeding 5cm (Boon 1997, De Biasi et al. 2001). The biochemical analysis involved the methodology prescribed by the Labtest® commercial kit for total protein quantification. Consideration of protein concentration in synovial fluid deemed it normal within the range of 2 to 2.5g/dL, moderate within the interval of 2.5 to 3g/dL, and elevated when surpassing 3g/dL (Boon 1997, De Biasi et al. 2001).

Cytological analysis of synovial fluid. The SF was diluted (1:10) in a 0.3% buffered saline solution in order to perform a total nucleated cell (TNC) count in a hemocytometer (Neubauer chamber®, Laboroptik, Lancing, UK). Reading was carried out in an optical microscope (Nikon Eclipse E200®) at 400x magnification. The TNC of the SF sample was considered normal with a counting below 1.000 cells/mm^3, moderate counting between 1.000 and 3.000 cells/mm^3, and elevated counting above 3.000 cells/mm^3 (Goldstein 2010).

The morphological evaluation, differential count and parasite search in SF smears were made after May-Grunwald-Giemsa staining. Differential counting was performed using the count of 100 nucleated cells under an optical microscope (Nikon Eclipse E200®) at 1000x magnification. The nucleated cells were classified as neutrophils, lymphocytes, and phagocytic mononuclear cells (MacWilliams & Friedrichs 2003).

Synovial membrane obtaining and histological analysis. Following euthanasia, a latero-lateral incision was made in the femorotibial joint, giving access to the joint capsule, and three fragments of the synovial membrane were collected from each joint. The samples were fixed in 10% formalin for 24 hours and further processed using conventional histological techniques. Histological sections were stained with hematoxylin and eosin (HE) to evaluate synovial membrane structure and inflammatory infiltrate cells.

Immunohistochemistry for TLR-2, FOXP3, and COX-2 in synovial membrane. Sections (5μm) were mounted on silanized glass slides and incubated at 36°C. Antigen recovery was performed using pH 9.0 citrate buffer for 30 min at 97°C. Endogenous peroxidase activity was inhibited by 3% hydrogen peroxide for 10 min, and slides were subjected to TLR-2 anti-rabbit monoclonal antibody (IM-0071 Lot 14071), mouse IG2a FOXP3 monoclonal antibody (sc-53876 lot#F0713) and COX-2 monoclonal antibody, incubated for one hour at room temperature. Slides were washed twice in PBS and then incubated with EnVision polymer reagent (EnVision TM, Dental Connection System/HRP) for 30 min at room temperature. Finally, diaminebenzidine (DAB) was applied for 10 min. Sections were counterstained with Mayer's hematoxylin for 5 min. Staining intensity was analyzed by light microscopy at 400x magnification. Marker expression was classified as none (-), mild (+), moderate (++) and intense (+++), according to the average subjective perception of two observers.

Statistical methods. Data analysis was performed using GraphPad Prism 5.0®. Initially, Grubbs and Kolmogorov tests were employed to detect outliers and assess data homoscedasticity. Total volume, total protein concentration, total nucleated cells (TNC), and neutrophils were analyzed using the Mann-Whitney test. Data were presented as mean and standard deviation, with statistical significance at $P<0.05$.

RESULTS

In the clinical assessment of joints in NID, several observations were made. Among the infected dogs, 55% (N=11/20) exhibited lameness, 25% (N=5/20) displayed hind limb edema, and 30% (N=6/20) showed signs of crepitus in the knee joint. Regarding patellar dislocation, 20% (N=4/20) of the dogs exhibited medial dislocation. Additionally, 25% (N=5/20) of the dogs displayed cranial movement of the tibial plateau relative to the femur, indicative of cranial cruciate ligament damage. During *post mortem* evaluation, 30% (N=6/20) of the dogs exhibited marked bilateral erosion of the subchondral bone, meniscus, and trochlea in the femorotibial joint (Fig.1-2).

SF samples were collected from both knees of 20 dogs (N=40) naturally infected with *Leishmania infantum*. The results of the physical and biochemical characteristics of the SF are summarized in Table 1. Notably, SF was successfully obtained in 100% of the punctures performed. A substantial 72.5% (N=29/40) of the samples had a volume exceeding the reference range of 0.05-0.24mL, with a mean volume of 0.45mL (range: 0.05-1.5mL). In terms of SF color, 62.5% (N=25/40) of the samples appeared yellow, 10% (N=4/40) exhibited a reddish hue, and 27.5% (N=11/40) lacked a distinct color. Anomalies in SF appearance (turbidity) were noted in 82.5% (N=33/40) of the samples, while 17.5% (N=7/40) maintained translucidity.

Regarding viscosity, 17.5% (N=7/40) of the samples displayed no viscosity, 67.5% (N=27/40) showed mild viscosity, and 15% (N=6/40) presented high viscosity. Total protein concentration in the SF from NID dogs revealed that 80% (N=32/40) of the samples had high concentrations, 10% (N=4/40) had moderate concentrations, and 10% (N=4/40) fell within the normal concentration range for the species. Subsequently, NID samples were categorized into three groups based on total protein concentration: GI (2 to 2.5g/dL, N=5), GII (2.5 to 6.0g/dL, N=5), and GIII (>6g/dL, N=10) (De Biasi et al. 2001).

The cytological analysis results are summarized in Table 1. Concerning the total nucleated cell count in SF, 55% of the samples exhibited a high leukocyte count, with a maximum count of 13,860 cells/mm^3 and an average of 5,845 cells/mm^3.

Regarding the differential count of nucleated cells, 30% (N=12/40) of the samples showed a predominance of mononuclear cells, primarily macrophages/synoviocytes and lymphocytes, while 70% (N=28/40) exhibited a preponderance of neutrophils. Notably, some samples displayed polymorphonuclear cell counts exceeding 10% of cellularity, reaching up to 80%. Among the SF mononuclear cells, there was a notable presence of phagocytic macrophages, with

some cells showing kinetoplast-compatible microorganisms suggestive of *Leishmania* spp. These findings were observed in 60% (N=24/40) of the SF samples.

Histological examination of the synovial membrane revealed marked findings. Inflammatory cell infiltrate and an increase in the synovial membrane layer were observed in 70% (N=14/20) of dogs naturally infected with *L. infantum*. The intensity of inflammatory infiltrate varied, with an absence of infiltrate in synovial membranes from GI, moderate intensity in GII, and high intensity in GIII (Fig.3-5). The infiltrate primarily consisted of mononuclear cells, including macrophages, lymphocytes, and limited neutrophils. This cellular infiltrate spanned from the synovial membrane's most superficial layer to the joint capsule's deepest part (Fig.3-14).

Immunohistochemical analysis (Fig.6-14, Table 2) revealed differences in marker expression. TLR-2 and COX-2 markers exhibited higher expression in GIII than in Groups GII and GI. Conversely, FOXP3 expression was moderate in GI and slight in GII and GIII.

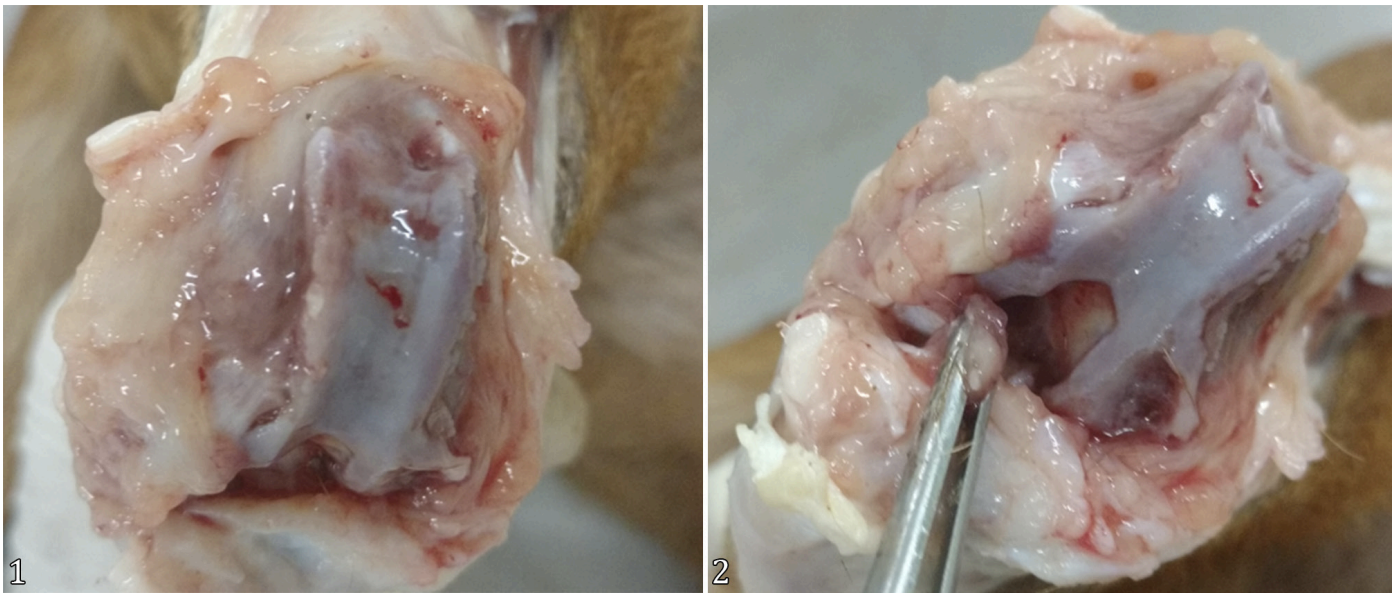

Fig.1-2. Clinical and pathological aspects of the synovial membrane of dogs naturally infected with *Leishmania infantum*. (**1**) Trochlear erosion, cartilage and subchondral bone erosion. (**2**) Cruciate ligament rupture.

Table 1. Physical, biochemical and cellular properties of synovial fluid obtained from knee joints of dogs naturally infected by *Leishmania infantum*

	Category	NID	
		N	%
Total volume	Normal	11	27.5
	Elevated	29	72.5
Viscosity	Absent	7	17.5
	Low	27	67.5
	Elevated	6	15
Color	Incolor	11	27.5
	Yellow	25	62.5
	Red	4	10
Aspect	Translucid	7	17.5
	Turbid	33	82.5
Total protein	Normal	4	10
	Moderate	4	10
	Elevated	32	80
Total nucleated cell	Normal	10	25
	Moderate	8	20
	Elevated	22	55
Neutrophil	<10%	12	30
	>10%	28	70
Leishmania spp. amastigote forms	Absent	16	40
	Present	24	60

NID = naturally infected dogs.

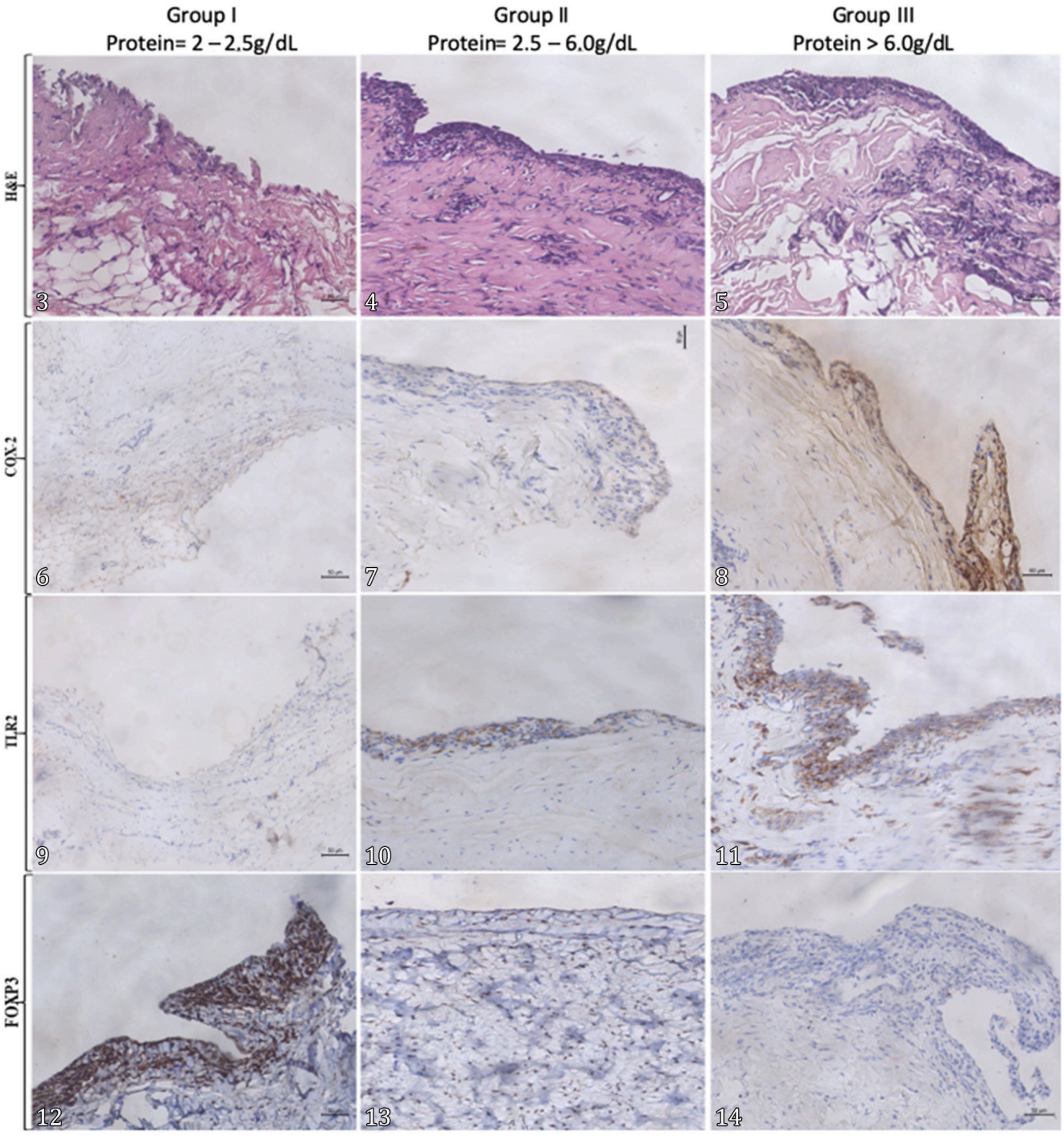

Fig.3-14. Histopathology and immunohistochemistry of the synovial membrane in dogs naturally infected with *Leishmania* spp. The animals were categorized based on total protein concentration in the synovial fluid. COX2 expression was classified as (**6**) mild, (**7**) moderate, and (**8**) intense. TLR2 expression was categorized as (**9**) mild, (**10**) moderate, and (**11**) intense. FOXP3 expression was classified as (**12**) intense and (**13** and **14**) mild. (**3**-**5**) HE, obj.200x, bar = 50µm. (**6**-**14**) IHC, obj.200x, bar = 50µm.

Table 2. TLR2, COX2, and FOXP3 expression in the synovial membrane of dogs naturally infected by *Leishmania* spp.

Immunolabeling	GI	GII	GIII
TLR2	+	++	+++
COX2	+	++	+++
FOXP3	+++	+	+

Expression scores: (+) mild; (++) moderate and (+++) intense; GI = protein concentration ranging from 2.0 to 2.5g/dL, GII = protein concentration ranging from 2.5 to 6.0g/dL, GIII = protein concentration >6g/dL.

DISCUSSION

Immune-mediated arthritis secondary to canine leishmaniasis (CanL) significantly contributes to inflammatory joint damage in dogs (Silva et al. 2022). The underlying mechanisms for this phenomenon involve amastigote forms of *Leishmania infantum* and the activation of type III hypersensitivity mechanisms, which lead to the deposition of immune complexes in the joints (Koutinas & Koutinas 2014). Our study aimed to investigate these mechanisms by performing comprehensive analyses of synovial fluid (SF) and synovial membrane samples.

SF analysis is a valuable tool for diagnosing joint disorders due to its minimally invasive nature, simplicity, and cost-effectiveness (MacWilliams & Friedrichs 2003, Filippo et al. 2014). In our study, SF analysis provided critical insights into the pathophysiology of CanL-induced arthritis. Notably, we detected amastigote forms of *Leishmania* spp. in 60% of SF samples, underscoring the presence of the parasite within the joint microenvironment. Additionally, a spectrum of alterations was observed in SF characteristics, including volume, viscosity, color, and total protein concentration changes. These changes were concomitant with a pronounced increase in cellular infiltrates in samples from dogs naturally infected with *L. infantum*.

Within the inflammatory infiltrate, neutrophils and macrophages emerged as the predominant cell types. Neutrophils play a central role in the pathogenesis of joint inflammation and subsequent tissue destruction. Upon activation, they unleash a cascade of inflammatory responses involving producing oxygen metabolites and releasing granule contents, such as matrix metalloproteinases and chemokines. These factors contribute to bone resorption, cellular damage, and further recruitment of polymorphonuclear cells (Falgarone et al. 2005, Zhang et al. 2005, Morales et al. 2007). Conversely, macrophages are pivotal in phagocytosing *Leishmania* parasites, triggering the production and release of reactive oxygen species (Scorza et al. 2017). The persistence of macrophages and neutrophils in the synovial environment contributes to maintaining a localized pro-inflammatory state, exacerbating joint damage.

Increased turbidity was observed in 82.5% of SF samples, likely associated with suspended particles, such as red and white blood cells or fibrin fragments (MacWilliams & Friedrichs 2003). Viscosity reductions in 67.5% of samples may be attributed to the inflammatory response initiated by *L. infantum*. Normal SF viscosity depends on the amount and polymerization of hyaluronic acid, which can decrease due to factors like synovial membrane damage, plasma influx, or leukocyte-mediated degradation (Boon 1997).

Moreover, the concentration of total proteins in SF was elevated in 90% of the samples, indicating potential inflammatory damage to the synovial membrane. This elevation could result from the involvement of cytokines, chemokines, complement proteins, and immune complexes (Komatsu & Takayanagi 2018). These findings align with our observations of cellular infiltrates and synovial membrane hyperplasia in 70% of samples.

In the *post mortem* assessment, 30% of dogs displayed erosion in the bony and cartilaginous structures of the femorotibial joint, correlating with the predominance of phagocytic cells in SF. Reports suggest that macrophage synovial infiltration can precede bone and cartilage destruction (Choy & Panayi 2001), emphasizing the critical role of these cells in mediating joint injuries, which is consistent with our study's findings.

Immunohistochemical analysis of the synovial membrane revealed significant alterations in marker expression. The increased COX-2 and TLR-2 and decreased expression of FOXP3 were associated with variations in synovial fluid protein concentrations.

COX-2, an enzyme that produces inflammatory mediators, can lead to matrix metalloproteinase activation, collagen synthesis inhibition, and chondrocyte apoptosis (Sokolove & Lepus 2013). In our study, increased COX-2 expression may result from macrophage interactions with *L. infantum*, triggering signaling pathways and culminating in COX-2 expression, further advancing the inflammatory process (Matte et al. 2001). This inflammation directly contributes to joint damage, observed on *post mortem* examination and clinically manifested as lameness.

The upregulation of TLR-2, a cell membrane receptor, may induce the production of inflammatory cytokines, particularly TNF-α and IL-1, known to affect cartilage degradation and chondrocyte apoptosis via caspase activation (Caramés et al. 2007). IL-1 also plays a crucial role in inducing proteolytic enzyme synthesis, such as metalloproteinases, which directly impact joint integrity (Sokolove & Lepus 2013). Although we did not measure cytokine levels in the tissue, the activation of TLR-2 likely leads to their high concentration in joint fluid. Moreover, TLR-2 has been shown to induce cell migration and proliferation in the synovial membrane (McGarry et al. 2015), consistent with our findings of elevated TLR-2 expression alongside increased cellular infiltration and synovial membrane proliferation.

In contrast, the reduced expression of FOXP3, a transcription factor linked to Treg lymphocytes, suggests an immune imbalance in the joints. Activated Treg cells control inflammation through cellular interactions and cytokine production, such as IL-10 and TGF-β (Rosshirt et al. 2021). Our data indicate a reduced Treg lymphocyte population in joints, exacerbating inflammation.

We acknowledge several limitations in our study, including the inability to assess previous joint exposure to other pathogens, such as *Ehrlichia canis*, which may also contribute to joint injuries. Due to financial constraints, the serological and molecular tests for such analyses were not performed.

CONCLUSION

Our study sheds light on the complex interplay of immune mechanisms and inflammatory processes in canine leishmaniasis (CanL) induced arthritis. The presence of *Leishmania* spp. amastigotes in synovial fluid (SF), alterations in SF characteristics, inflammatory cell infiltrates, and immunohistochemical marker expression collectively contribute to understanding the pathophysiology of joint damage in CanL. Further research is warranted to explore potential therapeutic interventions that target these mechanisms and alleviate the clinical impact of CanL-associated arthritis.

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Conflict of interest statement.- The authors declare that there are no conflicts of interest.

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