Influence of domperidone supplementation on short-term changes in C-reactive protein and paraoxonase-1 in dogs with leishmaniasis undergoing meglumine antimoniate and allopurinol therapy

Running header: CRP and PON-1 in dogs treated with domperidone

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Abstract

Background: C-reactive protein (CRP) and paraoxonase 1 (PON1) might increase and decrease in canine leishmaniasis (CanL), retrospectively, and both can rapidly normalize after therapy. Recently, supplementation of conventional therapy with domperidone, which increases the activity of cells involved in acute phase responses in vitro, has been recommended for mild forms of CanL. However, no studies have investigated the effects of domperidone supplementation on early CRP or PON1 changes in dogs being treated for CanL.

Objectives: The aim of this study was to evaluate whether domperidone, added to conventional treatments, modifies CRP concentration and PON1 activity kinetics in CanL dogs responsive to conventional therapy.

Methods: Serum CRP concentrations and PON1 activities were measured in dogs with mild CanL before (t0) and 3 (t1), 7 (t2), 14 (t3), and 21 (t4) days after treatment with N-methylglucamine antimoniate and allopurinol alone (n = 18) or combined with domperidone (n = 18).

Results: CRP concentrations increased at t1 in the domperidone group, especially when the CRP concentration at t0 was normal. However, the concentrations normalized at t-4 in 18/18 dogs compared with 14/18 dogs not receiving domperidone. The median PON1 activity decreased at t-1 in the domperidone group, and this decrease was more significant in dogs with normal PON1 activity at t-0.

Conclusions: Based on these results, transient increases in CRP concentrations or decreases in PON1 activities after domperidone administration should not be erroneously interpreted as signs of a worsening disease process.

Keywords: Acute phase protein, Canine leishmaniasis, Monitoring, Prognosis, Treatment

Introduction

Canine Leishmaniasis (CanL) is a protozoal disease caused by *Leishmania infantum* and characterized by broad-spectrum clinical signs and laboratory findings [1,2] The inflammatory response that characterizes CanL dogs is composed of increases in serum acute-phase protein concentrations (APPs), such as C-reactive protein (CRP),[3] and oxidative stress.[4] Decreases in serum paraoxonase 1 (PON1) activity (an antioxidant enzyme) and increases in serum APP concentrations have been demonstrated to be markers of CanL severity.[5-7]

Laboratory tests can be used, along with clinical monitoring, to assess responses to anti-leishmania treatments during the follow-up.[8] However, supportive treatments could ameliorate laboratory profiles even if leishmania-induced inflammation persists. Conversely, clinicopathologic changes could take a longer time to normalize in dogs responsive to therapy if the initial disease was severe. Therefore, markers of inflammation are preferred to investigate the early treatment responses. However, α_2- and γ-globulins tend to normalize in 2-3 or 4-6 weeks after antimonial treatments, respectively. Electrophoretograms could take 90-120 days to normalize.[5] Conversely, CRP concentrations return to within the reference intervals (RIs) in less than one month, whereas PON-1 activity returns in less than two weeks and both remain within RIs for up to 90 days after treatment[5,9]

Domperidone is an anti-dopaminergic drug that induces the release of prolactin, which, in turn, potentiates innate/cell-mediated responses. Domperidone enhances the phagocytic and oxidative responses of canine neutrophils in vitro.[10] In a clinical trial, domperidone administration decreased the seroprevalence of CanL and the rate of clinical cases. It also activated cell-mediated immunity, as evidenced by the leishmanin skin and lymphocyte blastogenesis tests.[11] Based on this mechanism of action, domperidone might influence inflammatory or oxidative responses. Therefore, the markers routinely used to monitor early therapeutic responses could provide misleading information, as anecdotally observed during routine activity at our diagnostic laboratory. However, no studies have focused on the effects of domperidone on early CRP or PON1 changes are available. Thus, the

aim of this study was to evaluate whether domperidone supplementation modifies serum CRP and PON1 kinetics in dogs that show therapeutic responses during the first days or weeks of therapy. To this aim, dogs routinely admitted to our institutions for the diagnosis and treatment of CanL were enrolled in this study based on the following inclusion criteria:

- Sick dogs with clinically evident leishmaniasis that corresponded to stage C of the canine leishmaniasis working group (CLWG)[2] defined as dogs with (1) mild clinical (ie, skin lesions, lymphadenopathy) or clinicopathologic abnormalities (ie, electrophoretic changes and blood or urinary changes consistent with chronic kidney disease stage I or II of the international renal interest society [IRIS] staging system)[12] and (2) positive molecular or cytologic parasitic detection within typical lesions (eg, pyogranulomatous dermatitis, hyperplastic lymph nodes, and plasmacytic bone marrow infiltration) and/or (3) IFAT antibody titers > 4-fold higher than the cut-off set in our laboratory to discriminate positive vs negative samples (1:80).
- The presence of written informed consent from the owners about study inclusion. Therefore, according to our Institutional regulations (Decision no. 2/16), since the drugs used in this study were standard for CanL treatments and blood samples were used for diagnostic purposes or to monitor the health statuses after therapy, formal approval from the Institutional Ethical Committee was not needed.

The exclusion criteria were:

- Dogs in stage D (severe CanL) of the CLWG classification.[2] These dogs were excluded since domperidone is not licensed for severe CanL.
- Dogs not showing therapeutic responses. Those dogs with clinical signs and/or laboratory changes consistent with renal disease that did not improve in the first two weeks of therapy.
- Previous or current administration of leishmanicidal or leishmaniostatic drugs or anti-inflammatory, antibiotic, or immunomodulatory drugs.

- The presence of clinical or laboratory changes consistent with metabolic or endocrine diseases potentially interfering with the results (eg, diabetes mellitus, hyperadrenocorticism).
- The presence of other vector-borne diseases.

Thirty-six dogs fulfilled the inclusion criteria (Table 1). Among these, 18 received only antimonial and allopurinol (ND) treatments, while 18 also received domperidone (PD). All the dogs were treated with N-methylglucamine antimoniate (Glucantime, Merial Italia S.p.a., Assago, Milan, Italy; 100 mg/kg, SC once a day for 30 days) and allopurinol (Zyloric, Teofarma, Pavia, Italy; 10 mg/kg, PO, twice a day for 30 days), following the CLWG guidelines for leishmaniasis therapy.[13] Dogs were randomly allocated into the ND and PD groups after assessing owner compliance. In the PD group, domperidone (Leisguard, Ecuphar Italia S.r.l., Milan, Italy) was administered at the dose recommended in the literature (0.5 mg/kg, PO, once a day for 1 month).[11]

Dogs were physically examined before the first treatment was administered (t-0), and 3 (t-1), 7 (t-2), 14 (t-3), and 21 (t-4) days after treatments were administered. Although dogs were treated for 30 days, sampling schedules were restricted to day 21, since in a previous study, CRP and PON1 levels tended to normalize within three weeks.[5,] At each visit, blood was collected from the cephalic vein and transferred and divided into tubes containing EDTA and tubes without an anticoagulant (Venoject, Terumo Italia S.r.l., Rome, Italy). Routine hematology was performed at the sampling site with an automated impedance hematology analyzer (Mindray BC-2800, Shenzen Mindray Bio-medical Electronics, Shenzen, China) or a laser-based counter (Sysmex XT2000iV, Sysmex Co., Kobe, Japan) on blood collected in EDTA. Results were compared with the RIs of each sampling site to check for the presence of hematologic changes consistent with CanL (eg, non-regenerative anemia, leukocytosis). Serum was obtained by centrifugation (1,100*g*, 8 min) of blood collected in plain tubes and used to assess the presence of biochemical changes consistent with leishmaniasis (azotemia, hyperproteinemia, changes in serum protein electrophoresis) on admission or after treatments. A clinical chemistry panel (total bilirubin, BUN, creatinine, glucose, total protein, ALP, ALT, AST, GGT, calcium, cholesterol, triglycerides, amylase, phosphorus) was performed using

automated analyzers (Mindray BC-120, Shenzen Mindray Bio-medical Electronics or Cobas Mira, Roche diagnostic, Basel, Switzerland), and the results were compared with RIs at each sampling site. Urine samples were collected at each visit to assess and quantify possible proteinuria, and agarose gel serum protein electrophoresis was performed at t-0 and t-4.

Cytologic examinations were performed on bone marrow or lymph node aspirates and cutaneous lesion scrapings after the prepared slides were stained with a May Grünwald Giemsa stain. PCR was performed in an external laboratory (IDEXX Laboratories Italia Srl, Milan, Italy). IFAT was performed using a commercially available kit (FLUO leishmania, Agrolabo Spa, Scarmagno, TO, Italy). The presence of concurrent vector-borne diseases was excluded using a pet-side ELISA kit (SNAP 4Dx Plus, IDEXX Laboratories, Inc, Westbrook, ME, USA)

The remaining serum was stored at -20°C for the measurement of serum CRP concentrations and PON1 activities on the Cobas Mira analyzer using a canine-specific immunoturbidimetric kit (Randox Laboratories, Crumlin, United Kingdom) and paraoxon-based method, respectively. The paraoxon-based method has been previously validated.[5] For these measurements, samples collected at the other sites were periodically sent to the University of Milan on ice and processed within 6 months.

Statistics were analyzed using Analyse-it v 5.01 (Analyse-it Software Ltd, Leeds, United Kingdom) with statistical add-in software for Excel (Microsoft, Redmond, WA, USA). Since the Shapiro-Wilk test demonstrated that data were not normally distributed, the Mann-Whitney U test was used to compare the results obtained in the ND and PD groups at each sampling time. The Friedmann test, followed by the Bonferroni post-hoc test, was used to compare the results obtained during the sequential samplings within each treatment group. This comparison was initially performed on the whole caseload and then repeated on dogs with normal CRP or PON1 values and those with values outside the RIs at t-0. Finally, the power of the study was calculated in terms of the probability of type II error (beta error, ie, the probability of not rejecting the null hypothesis when the null hypothesis is false) given the sample size for each time after the treatment. It measured the power in

being able to identify the probability of failing to detect a real treatment effect as significant due to the small sample size.

CanL was diagnosed on cytology or PCR and serology in 28 and 8 dogs (5 in the PD and 3 in the ND group), respectively. Sex and median age were not significantly different between the two groups (P=0.631 and P=0.351, respectively). The most common clinical or laboratory abnormalities were skin lesions and enlarged lymph nodes. Skin lesions and lymph node enlargement were present individually or together and with or without clinicopathologic changes that mostly included polyclonal gammopathies and proteinuric nephropathies. A few dogs had mild normocytic, normochromic non-regenerative anemias.

Although the clinical condition of all the dogs improved in two weeks, according to the inclusion criteria, electrophoretic changes and proteinuria were still present at the end of the study period, as expected in treated CanL dogs.[8]

CRP concentrations and PON1 activities were not significantly different between the groups at t-0, although individual variability was higher in dogs receiving domperidone (Figure 1). In both groups, CRP values were higher than the RIs in more than half of the dogs at t-0 and tended to increase at t-1. However, CRP increases were only significant in dogs receiving domperidone; the CRP concentrations increased at t-1 were significantly higher than those in the ND group. CRP concentrations gradually decreased over time and were within the RIs of all PD dogs and all but four dogs in the ND group. The probability of type II error was 26.7%, 74.4%, 49%, and 29.9% at T1, T2, T3, and T4.

No significant differences in PON1 activity were found between groups. However, PON1 activity at t-0 was within the RIs in most dogs of both groups. PON1 activity was significantly decreased at t-1 compared with t-0 in the PD dogs only; the median value at t-1 was lower than the lower reference limit.

The sample results with normal or abnormal values at t-0 were analyzed separately (Table 2). In the PD dogs with normal values at admission, the CRP concentrations and PON1 activities at t-1 were

significantly different than those values at t-0, and CRP concentrations exceeded RIs. In contrast, in dogs with abnormal CRP or PON1 values at admission, no significant differences were found at t-1. The t-2 to t-4 data revealed a decreasing trend in both groups, although, different statistically significant levels were found between the two groups over time.

The results of the current study demonstrated that domperidone supplementation induces transient increases in CRP concentrations and corresponding decreases in PON-1 activity. These changes were more significant in dogs with values within the RIs at t-0 than in the entire caseload, including dogs with already altered CRP concentration or PON activity values, thus masking further changes induced by the treatments. In other words, the pre-treatment CRP or PON1 values influenced the magnitude and statistical significance of the changes.

The design of this study does not provide evidence of whether the observed effects depend on the domperidone administration or the three-drug combination. However, this would require an additional group treated with domperidone alone, which would be unethical and is in conflict with the current CanL therapeutic guidelines.[10] Although phagocyte function has also been shown to be activated with antimonials,[14] domperidone was shown to activate phagocytic oxidative responses when used as a sole agent.[10] Moreover, although CRP and PON1 tended to normalize in both groups, only the PD group had normal CRP values at t-4 in all dogs, suggesting that the transient activation at t-1 might accelerate the return to normal CRP levels. A two-way ANOVA would have provided more detailed information on the combined effect of time and treatment; however, the data distribution analysis revealed that three main assumptions were not present to run a two-way ANOVA (ie, outliers were present, data were not normally distributed, and variance was not homogeneous based on Lavene's test). Therefore, this approach was not followed in this study.

PON1 activities suggested that domperidone amplifies oxidative responses as decreased PON1 activity has been shown in many diseases characterized by oxidative stress.[15] Therefore, although no oxidants were measured in the current study, the decreased PON1 activity seen after

domperidone administration suggested that treatment-induced oxidant release and the consumption of antioxidants, such as PON1.

Independent of these speculations on the pathogenesis of changes in CRP concentrations and PON1 activities in treated CanL dogs, this study demonstrated that the increased CRP and decreased PON1 values during the first days of treatment should not be interpreted as worsening signs of CanL infections when domperidone is added to conventional therapy, especially if the two analytes are within the RIs before treatment.

This study has three main limitations. First, the number of dogs included in the study was low, due to the application of strict inclusion criteria. However, these criteria increased the reliability of the results. The probability of type II error was relatively low at t-1, thus reinforcing the results. Similarly, the probability was quite low at t-4; therefore, we are confident that the lack of significant differences at that time point reflected an end to the domperidone CRP effect. A bigger sample size could have shown a significant difference at t-2 and t-3 compared with t-4. Second, the analysis did not include other APPs or oxidation markers used in previous studies;[4] however, we selected CRP and PON1 because these analytes could provide the earliest prognostic information during treatment.[5] Third, the study was not focused on the treatment effects of clinical or laboratory findings or parasite loads, and the details about clinical and laboratory data were not recorded during the follow-up sampling times. Moreover, only dogs that were clinically normal for two weeks before inclusion into the study were included, which further hampered the ability to evaluate the possible effects of domperidone on the peak time of clinical recovery

In conclusion, this is the first study investigating the kinetics of CRP and PON1 analytes after domperidone administration combined with allopurinol and meglumine antimoniate administration compared with allopurinol and meglumine antimoniate administration alone. This study also demonstrated that the respective transient increases and decreases in serum CRP concentrations and PON1 activities support the hypothesis that respiratory bursts are activated in vivo, as has already been demonstrated, in vitro. In addition, these results are important in preventing the

misinterpretation of transient CRP increases and/or PON1 decreases in the first days after domperidone administration.

Conflict of interest

The authors do not have conflicts of interest potentially interfering with the results of this study.

Acknowledgments

This research did not receive any specific grant.

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296 **Table 1:** Signalments and clinical and laboratory findings of the dogs with canine leischmaniasis in
297 the study.

	Domperidone (n=18)	**No Domperidone (n=18)**
Breed	Crossbred (n=8); Pointer (n=2); Akita Inu, Border Collie, Boxer, Bracco Italiano, Epagneul Breton, Cocker, Labrador Retriever, Sharpei (n= 1)	Crossbred (n=5), German Shepherd dog (n=3), Boxer, Golden Retriever (n=2), Greyhound, Labrador Retriever, Pointer, Rottweiler, Segugio Italiano, Springer Spaniel (n=1)
Sex	12 males, 6 females	10 males, 8 females
Age	2-13 years (median: 6 years)	3-12 years (median: 7 years)
Skin lesions	n=6 (2 with SPE changes, 2 with SPE changes and in IRIS stage II BP, 1 in IRIS stage I P, 1 anemic)	n=4 (2 with SPE changes, 1 in IRIS stage II NP, 1 in IRIS stage II BP)
Skin lesions and enlarged lymph nodes	n=4 (1 with SPE changes, 1 in IRIS stage II BP, 1 anemic)	n=6 (1 in IRIS stage II P, 1 in IRIS stage I P, 1 with SPE changes and in IRIS stage II P, 1 anemic)
Enlarged lymph nodes	n=4 (1 with SPE changes, 1 in IRIS stage II P, 1 anemic)	n=6 (1 with SPE changes, 1 with SPE changes and in IRIS stage II NP, 1 in IRIS stage II BP, 1 in IRIS stage II P)
Proteinuric nephropathy without other physical abnormalities	n=3 (2 in IRIS stage II P, 1 in IRIS stage I P)	n=2 (2 in IRIS stage II P)

Epistaxis	n=1 (with SPE changes in IRIS stage II NP)	n=0

SPE = serum protein electrophoresis; IRIS stage I =serum creatinine <1.4 mg/dL; IRIS stage II = serum creatinine 1.4-2.8 mg/dL; NP = non-proteinuric, ie, urinary protein-to-creatinine (UPC) ratio <0.20; BP = borderline proteinuric, ie, UPC ratio 0.21-0.50; P = proteinuric, ie, UPC ratio >0.50.

Table 2: C-reactive protein (CRP) concentrations and paroaxonase-1(PON1) activities recorded over time in the serum of dogs with canine leishmaniasis (CanL) who received conventional therapy with or without domperidone supplementation. Results were recorded before the first (t-0), and 3 (t-1), 7 (t-2), 14 (t-3), and 21 (t-4) days after therapy and are presented as the mean ± standard deviation (SD) and as the median value (in brackets).

		CRP (mg/L)		**PON1 activity (U/mL)**	
		PD	ND	PD	ND
		(N=7; A=11)	(N=7; A=11)	(N=10; A=8)	(N n=11; A=7)
N	t-0	5.23 ± 4.04	5.88 ± 2.69	170.4 ± 47.4	167.8 ± 38.6
		(4.10)	(6.09)	(165.1)	(165.9)
	t-1	24.21 ± 16.59*	6.82 ± 3.14	139.4 ± 33.6**	160.5 ± 37.1
		(19.40)	(6.93)	(125.5)	(155.9)
	t-2	10.03 ± 6.47	6.52 ± 3.46	152.9 ± 25.4	165.5 ± 42.3
		(11.90)	(7.77)	(148.4)	(151.1)
	t-3	7.10 ± 6.00	5.55 ± 2.97	168.3 ± 41.0†	164.9 ± 40.4
		(7.30)	(6.52)	(151.9)	(152.3)
	t-4	3.81 ± 4.99$^{\ddagger\ddagger,\circ}$	3.68 ± 2.70†	161.4 ± 41.5	182.6 ± 49.6$^{\dagger,\circ}$
		(1.00)	(3.78)	(152.4)	(172.1)
A	t-0	30.40 ± 11.56	21.67 ± 11.51	100.4 ± 17.5	105.9 ± 18.0
		(33.00)	(16.80)	(104.4)	(113.4)

t-1	39.71 ± 29.02 (33.70)	22.47 ± 8.74 (19.11)	113.9 ± 37.3 (103.2)	104.4 ± 15.5 (99.7)
t-2	25.40 ± 22.18 (16.10)	16.85 ± 8.39†† (16.50)	119.9 ± 23.2 (117.3)	109.6 ± 19.2 (110.7)
t-3	12.66 ± 9.59*.†.‡ (10.90)	14.02 ± 6.31**.††† (14.57)	123.9 ± 27.2* (125.0)	116.6 ± 12.8† (124.0)
t-4	3.97 ± 2.92**.††.‡‡‡.° (3.60)	9.86 ± 3.80**.†††.‡‡.° (8.40)	149.3 ± 40.9*.‡.° (138.1)	116.4 ± 21.2 (119.8)

CRP and PON1 activity results in dogs during conventional CanL treatments with (PD) and without domperidone (ND), grouped based on normal (N) or abnormal (A) CRP or PON1 activity values at admission. * = P <0.05 vs t-0; ** = P <0.01 vs t-0; *** = P <0.001 vs t-0; † = P <0.05 vs t-1; †† = P <0.01 vs t-1; ††† = P <0.001 vs t-1; ‡ = P <0.05 vs t-2; ‡‡ = P <0.01 vs t-2; ‡‡‡ = P <0.001 vs t-2; °° = P <0.01 vs t-3; °°° = P <0.001 vs t-3.

Figure legends

Figure 1. Fluctuations in C-reactive protein (CRP) concentrations and paraoxonase 1 (PON1) activities in dogs with canine leishmaniasis receiving conventional therapy and domperidone (left graphs) or in dogs receiving only conventional therapy (right graphs). The box plots indicate the median as a line across, a box from the 1st to 3rd quartiles (interquartile range or IQR) and whiskers extending to further observation within the I quartile minus 1.5 * IQR or to further observation within the III quartile plus1.5*IQR. Black dots indicate each single value. Near outliers are indicated with the open circles. The shaded area indicates the reference interval (<11.4 mg/L for CRP, 116-250 U/mL for PON1) * = P<0.05 vs t-0; ** = P<0.01 vs t-0; *** = P<0.001 vs t-0; † = P<0.05 vs t-1; †† = P<0.01 vs t-1; ††† = P<0.001 vs t-1; ‡ = P<0.05 vs t-2; ‡‡ = P<0.01 vs t-2; ‡‡‡ = P<0.001 vs t-2; °° = P<0.01 vs t-3; °°° = P<0.001 vs t-3. Data that are significantly different between treatment groups are included in a black rectangle on the X-axis.