Short-term exogenous glucocorticosteroidal effect on iron and copper status in canine leishmaniasis (*Leishmania infantum*)

K.K. Adamama-Moraitou, M.N. Saridomichelakis, Z. Polizopoulou, M. Kritsepi, A. Tsompanakou, A.F. Koutinas

Abstract

Prednisolone was administered as an anti-inflammatory for 7 consecutive days in 11 dogs with leishmaniasis (CL group) and 5 clinically normal dogs (control group). After a 15-day wash-out phase, the same medication was given as an immunosuppressive for another 7-day period. In both animal groups and experimental periods an overall significant increase of serum iron and transferrin saturation was noted. Serum copper showed a significant increase during the anti-inflammatory period in the control group and a significant decrease during the immunosuppressive period in the CL group. No differences or changes of any kind regarding bone marrow hemosiderin were found between the 2 groups either before or after the end of both experimental periods. The only change noticed in the hematocrit values was a significant decrease in the control group after the end of the anti-inflammatory period. Based on these findings the use of prednisolone cannot be recommended and, if contemplated, should be carefully monitored, especially at an immunosuppressive dosage, because it may promote parasite replication through the induction of increased serum iron levels and hypocupremia.

Résumé

De la prednisolone a été administrée comme agent anti-inflammatoire durant 7 j consécutifs à 11 chiens souffrant de leishmaniose (groupe CL) et 5 chiens cliniquement normaux (groupe témoin). Après une période d'élimination de 15 j, la même médication a été donnée à titre d'agent immunosuppresseur pour une autre période de 7 j. Dans les deux groupes d'animaux et durant les deux périodes expérimentales, une augmentation significative de la concentration du fer sérique et de la saturation de la transferrine ont été notées. La concentration de cuivre sérique a augmenté de façon significative durant la période anti-inflammatoire pour le groupe témoin et a diminué de manière significative durant la période immunosuppressive pour le groupe CL. Aucune différence ou changement d'aucune sorte de l'hémosidérine de la moelle osseuse n'a été trouvé entre les 2 groupes soit avant ou après la fin des 2 périodes expérimentales. Le seul changement remarqué dans les valeurs d'hématocrite était une réduction significative pour le groupe témoin à la fin de la période de traitement anti-inflammatoire. À la lumière de ces résultats l'utilisation de prednisolone ne peut être recommandée; si toutefois elle était considérée, elle devrait être étroitement surveillée, surtout si un dosage immunosuppresseur est prescrit, étant donné que cela peut favoriser la réplication du parasite via l'induction d'une augmentation de la concentration du fer sérique et une réduction du cuivre sérique.

(Traduit par Docteur Serge Messier)

Introduction

Glucocorticosteroids are widely used in canine medicine due to their anti-inflammatory and immunosuppressive properties (1). Their use in clinically healthy dogs may result in increased serum iron (SI) and transferrin saturation (SAT) levels, while total iron binding capacity (TIBC) usually remains unchanged (2). However, their effect on SI concentrations in various canine diseases is still unknown (3). Furthermore, an increase in plasma copper (Cu) concentration was noticed after glucocorticosteroidal administration in healthy rats (4) and humans (5).

Canine leishmaniasis (CL), as it occurs in the Mediterranean countries, is a multisystemic progressive disease of zoonotic potential (6,7). *Leishmania* spp. needs an adequate supply of iron (Fe) for the completion of its life cycle (8–10). As many of the harmful effects of CL are considered to be immune-mediated (6), the use of glucocorticosteroids, given in combination with antileishmanial drugs, might help resolve some of the underlying lesions (11).

The purpose of the present study was to evaluate the Fe and Cu status in dogs with CL, before and after the systemic use of glucocorticosteroids, given on a short-term basis.

Materials and methods

Eleven dogs with naturally occurring cases of CL, diagnosed based on history, clinical presentation, and clinicopathological findings,

Clinic of Companion Animal Medicine (Adamama-Moraitou, Saridomichelakis, Koutinas); Laboratory of Clinical Diagnosis and Clinical Pathology (Polizopoulou, Kritsepi), School of Veterinary Medicine, Aristotle University of Thessaloniki, Stavrou Voutyra 11, GR-546 27, Thessaloniki, Greece; Department of Haematology, "George Papanikolaou" General Hospital, GR-570 10, Exokhi, Thessaloniki, Greece (Tsompanakou).

Address all correspondence and reprint requests to Dr. K.K. Adamama-Moraitou; telephone: +30 231 099 4518; fax: +30 231 099 4516; e-mail: kadamama@vet.auth.gr

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and confirmed by the detection of amastigotes on Giemsa — stained lymph node and bone marrow aspiration smears, in association with positive (≥ 1:200) indirect immunofluorescence antibody test (IFAT) serology, were used in this study. Of these various breeds of dogs, 9 (81.8%) were intact males and 2 (18.2%) intact females, with ages ranging from 1.5 to 11 y (median, 4.5 y) and body weights (BW) from 18.2 to 50.7 kg (median, 23.4 kg). After the diagnosis of CL, all of these dogs were adopted out to our clinic by their owners.

The control group was comprised of 5 clinically healthy and sexually intact (4 males, 1 female) dogs with ages ranging from 1.5 to 3 y (median, 1.5 y) and BW from 5.2 to 27.8 kg (median: 18.5 kg). All were negative for CL with the aforementioned tests.

The project underwent ethical review and was given approval by the Animal Care and Use Committee of the School of Veterinary Medicine, Aristotle University of Thessaloniki, Greece. The care and use of the experimental animals complied with the European Community (EC) Guidelines to the Care and Use of the Experimental Animals (12). After the end of the study all these dogs were treated for CL with a combination of meglumine antimonate and allopurinol and subsequently adopted by staff members and students of our clinic.

Care and management of dogs

For 1 mo prior to and during the trial, all animals were fed a commercial dry dog food (Canine Maintenance; Hill's Pet Nutrition, Topeka, Kansas, USA), containing 172.8 μg/g Fe and 17.4 μg/g Cu in dry matter (DM). Dietary intake was adjusted for BW, size, and physical activity of each dog (13). The animals were kept in separate cages or runs during the 2-month period of the investigation and given free access to water. The vaccination status of all dogs was current and anthelminthics were administered regularly.

Study design

The study included the anti-inflammatory and immunosuppressive experimental periods, separated by a 15-day wash-out phase to negate any residual effect. In the first (anti-inflammatory) period, prednisolone tablets (Deltacortril, 5 mg; Pfizer, Athens, Greece) were given orally at an anti-inflammatory dose (0.5 mg/kg BW, q12h) for 7 consecutive days, whereas in the second (immunosuppressive) period the drug was given at an immunosuppressive dose (2 mg/kg BW, q12h) for another 7 d.

Serum iron, TIBC, SAT, and Cu measurements

Venous blood was obtained by jugular venipuncture every 48 h during both experimental periods on days 0, 2, 4, 6, and 8, as well as once after discontinuation of the drug (day 10). Stainless steel hypodermic needles and acid-washed centrifuge tubes were used to avoid any possibility of Cu contamination. Serum was separated and stored at −20°C until analyzed. Serum iron, TIBC, SAT, and Cu were measured by the methods described by Olsen and Hamlin (14), using an atomic absorption spectrophotometer (Perkin Elmer Analyst 100; Buck Scientific, East Norwalk, Connecticut, USA).

Bone marrow iron stores

Bone marrow smears, obtained from the iliac crest under local anesthesia with a 16-gauge Rosenthal needle before (day 0) and 48 h after the end of each experimental period (day 10), were stained by using the Prussian-blue technique (15). Bone marrow hemosiderin stores were evaluated in macrophages (16), and subjectively scored as +, ++, or +++, according to the amount of the blue granules observed, by one of the authors (AT), in a blinded fashion.

Haematology, serum biochemistry, and urinalysis

Hematocrit (PCV) value and hemoglobin (Hb) concentration were measured using an automated veterinary cell counter (Hematologie, Compteur Analyseur MS9; Melet Schloesing Laboratoires, Gergy-Pontoise, France), on the blood samples collected in ethylene diamine tetraacetic acid (EDTA) treated tubes (Sarstedt, Aktlengesellschaft and Company, Nümbrecht, Germany) prior to (day 0) and 48 h after the end (day 10) of each experimental period. Total proteins (TP), blood urea nitrogen (BUN), creatinine (Cr), and inorganic phosphorus (P) concentrations were determined prior to (day 0) administration of prednisolone in both experimental periods. At the same time, a complete urinalysis was performed on fresh urine samples obtained by antepubic cystocentesis.

Statistical analysis

The data were subjected to a two-way analysis of variance (ANOVA), while the differences among means were tested with Duncan-test. The Student's *t*-test was used for the comparison of PCV and Hb between the 2 groups. To determine the overall tendency (days 0 to 8), a Spearman's time correlation analysis was also performed for each parameter, followed by the calculation of correlation coefficients. The level of significance was set at $P < 0.05$.

Results

Serum iron, TIBC, SAT, and Cu measurements

In both the CL and control groups, a significant overall increase of SI and SAT values was noticed during the anti-inflammatory period (days 0 to 8), TIBC decreased in the former group only, and Cu concentration showed a significant increase in the control group only (Tables I to IV). These effects disappeared 2 d after the discontinuation of prednisolone (day 10) for SI in both groups and SAT in the control group (Tables I and III). Again, in the immunosuppressive period, both the CL and control groups showed a significant increase in the overall SI and SAT values, but no changes involving TIBC. By contrast, Cu concentration showed a significant decrease in CL group only. In both groups, the values of all these parameters returned to the baseline level 2 d after the discontinuation of the immunosuppressive dosage (day 10) (Tables I to IV).

No statistical differences were found between CL and control groups for all the aforementioned parameters, either on the anti-inflammatory or the immunosuppressive prednisolone dosage.

Bone marrow iron stores

No statistical differences or changes of any kind regarding bone marrow hemosiderin were found between the 2 groups either prior to or 48 h after the end of the anti-inflammatory and immunosuppressive trials (Table V).

Table I. Serum iron (SI) concentration (μmol/L) in the control and CL groups prior to (day 0), during (days 2, 4, 6, 8), and after (day 10) oral administration of prednisolone at anti-inflammatory and immunosuppressive doses

	Time (days), mean ± $s_{\bar{x}}$					
Groups	0	2	4	6	8	10
Anti-inflammatory period						
Control group (n = 5)	38.52 ± 4.67[a]	55.70 ± 9.32[a]	65.30 ± 3.04[b]	75.75 ± 11.07[b]	56.85 ± 9.05[a]	38.66 ± 3.20[a]
CL group (n = 11)	38.79 ± 2.94[a]	52.92 ± 2.55[b]	58 ± 3.34[b]	97.44 ± 6.03[b]	47.58 ± 4.06[a]	34.11 ± 1.89[a]
Immunosuppressive period						
Control group (n = 5)	34.22 ± 3.36[a]	74.03 ± 9.62[b]	78.33 ± 4.04[b]	84.49 ± 8.45[b]	68.88 ± 8.68[b]	34.51 ± 5.32[a]
CL group (n = 11)	43.87 ± 2.79[a]	76.48 ± 5.22[b]	71.34 ± 5.12[b]	78.37 ± 8[b]	68.41 ± 3.95[b]	30.66 ± 3.46[a]

CL — Canine leishmaniosis

[a,b] Different superscripts represent a significant difference ($P < 0.05$)

Table II. Total iron binding capacity (TIBC) of transferrin concentration (μmol/L) in the control and CL groups prior to (day 0), during (days 2, 4, 6, 8), and after (day 10) oral administration of prednisolone at anti-inflammatory and immunosuppressive doses

	Time (days), mean ± $s_{\bar{x}}$					
Groups	0	2	4	6	8	10
Anti-inflammatory period						
Control group (n = 5)	108.12 ± 7.26[a]	95.80 ± 2.20[a]	97.52 ± 2.81[a]	125.44 ± 6.98[b]	85.06 ± 9.53[c]	82.48 ± 7.64[c]
CL group (n = 11)	110.20 ± 4.09[a]	97.38 ± 1.56[a]	98.16 ± 1.01[a]	141.51 ± 9.24[b]	79.28 ± 3.30[c]	66.52 ± 2.87[c]
Immunosuppressive period						
Control group (n = 5)	99.38 ± 7.34[a]	107.69 ± 13.60[a]	102.53 ± 6.69[a]	118.28 ± 6.20[a]	97.66 ± 5.33[a]	98.81 ± 5.94[a]
CL group (n = 11)	103.10 ± 8.81[a]	101.41 ± 5.51[a]	105.58 ± 17.35[a]	114.56 ± 7.18[a]	93.60 ± 4.48[a]	81.10 ± 3.94[a]

CL — Canine leishmaniosis

[a,b,c] Different superscripts represent a significant difference ($P < 0.05$)

Table III. Saturation of transferrin values (%) in the control and CL groups prior to (day 0), during (days 2, 4, 6, 8), and after (day 10) oral administration of prednisolone at anti-inflammatory and immunosuppressive doses

	Time (days), mean ± $s_{\bar{x}}$					
Groups	0	2	4	6	8	10
Anti-inflammatory period						
Control group (n = 5)	36.13 ± 4.81[a]	58.77 ± 10.56[b]	67.10 ± 3.28[b]	60.58 ± 8.37[b]	66.17 ± 8.08[b]	47.42 ± 3.06[a]
CL group (n = 11)	35.76 ± 3.14[a]	54.70 ± 3.24[b]	59.18 ± 3.49[b]	67.17 ± 3.75[b]	59.95 ± 4.69[b]	51.89 ± 3.01[b]
Immunosuppressive period						
Control group (n = 5)	37.40 ± 3.33[a]	73.17 ± 10.21[b]	74.76 ± 4.75[b]	67.20 ± 3.50[b]	70.73 ± 8.36[b]	33.49 ± 8.02[a]
CL group (n = 11)	44.25 ± 2.80[a]	75.88 ± 3.92[b]	74.80 ± 5.68[b]	68.24 ± 4.65[b]	73.11 ± 2.58[b]	38.23 ± 4.55[a]

CL — Canine leishmaniosis

[a,b] Different superscripts represent a significant difference ($P < 0.05$)

Hematology

Prednisolone did not have a significant overall effect on PCV values or Hb concentrations in CL group. However, PCV decreased significantly in the control group 48 h after the end of the anti-inflammatory period (day 10).

In the CL group, anemia (PCV < 0.37 L/L and/or Hb < 1.2 g/dL) was detected in 8/11 (72.7%) prior to and in 10/11 (90.9%) 48 h after the end of the anti-inflammatory period. When the same group was put on the immunosuppressive dosage, anemia was detected in all of the dogs at the beginning (day 0) and the end (day 10) of the observation period. Anemia was not detected in any of the control dogs (Table V).

Serum biochemistry and urinalysis

No abnormalities were found in serum biochemistry of any of the dogs belonging to either group. Urinalysis revealed moderate to severe glomerular proteinuria in 10/11 (90.9%) dogs in the CL group. Serum biochemistry and urinalysis were normal in the control group.

Discussion

The most rational indication for glucocorticosteroidal treatment in dogs with CL is the presence of life-threatening epistaxis (17). In these cases, an initial intravenous bolus of dexamethasone, is usually

Table IV. Serum copper (Cu) concentration (μmol/L) in the control and CL groups prior to (day 0), during (days 2, 4, 6, 8), and after (day 10) oral administration of prednisolone at anti-inflammatory and immunosuppressive doses

	Time (days), mean ± $s_{\bar{x}}$					
Groups	0	2	4	6	8	10
Anti-inflammatory period						
Control group (n = 5)	15.07 ± 1.12[a]	15.83 ± 1.20[a]	14.32 ± 1.23[a]	16.83 ± 0.87[a]	19.72 ± 1.28[b]	19.22 ± 0.98[b]
CL group (n = 11)	16.38 ± 1.07[a]	17.01 ± 1.09[a]	15.24 ± 0.72[a]	16.10 ± 0.79[a]	18.38 ± 0.46[b]	19.47 ± 0.48[b]
Immunosuppressive period						
Control group (n = 5)	13.94 ± 0.58[a]	16.96 ± 0.99[b]	15.7 ± 0.77[a]	14.44 ± 0.66[a]	14.19 ± 0.83[a]	14.07 ± 0.81[a]
CL group (n = 11)	15.30 ± 0.65[a]	18.67 ± 0.81[b]	16.16 ± 0.59[a]	15.13 ± 0.73[a]	14.44 ± 0.37[a]	16.16 ± 0.74[a]

CL — Canine leishmaniosis

[a,b] Different superscripts represent a significant difference ($P < 0.05$)

Table V. Hematocrit (L/L) value, hemoglobin (g/dL) concentration, and bone marrow iron stores in the control and CL groups prior to (time 0) and on the 10th d after oral administration of prednisolone at anti-inflammatory and immunosuppressive doses

	Hematocrit (mean ± $s_{\bar{x}}$)		Hemoglobin (mean ± $s_{\bar{x}}$)		Bone marrow iron stores	
Groups	Day 0	Day 10	Day 0	Day 10	Day 0	Day 10
Anti-inflammatory period						
Control group (n = 5)	0.54 ± 0.03[ac]	0.45 ± 0.02[bc]	1.42 ± 0.08[ac]	1.29 ± 0.06[ac]	++	++
CL group (n = 11)	0.39 ± 0.03[ad]	0.33 ± 0.02[ad]	1.05 ± 0.06[ad]	0.95 ± 0.06[ad]	++	++
Immunosuppressive period						
Control group (n = 5)	0.45 ± 0.02[ac]	0.43 ± 0.02[ac]	1.29 ± 0.06[ac]	1.28 ± 0.04[ac]	++	++
CL group (n = 11)	0.32 ± 0.02[ad]	0.32 ± 0.02[ad]	0.93 ± 0.05[ad]	0.94 ± 0.04[ad]	++	++

CL — Canine leishmaniosis

[a,b] Different superscripts represent a significant difference ($P < 0.05$)

[c,d] Column of the same experimental period with different superscripts differ significantly ($P < 0.05$)

followed by the oral administration of prednisolone, at an anti-inflammatory or immunosuppressive dosage, for a few days. For this reason, in the present study it was decided that prednisolone be administered at both an anti-inflammatory and an immunosuppressive dosing schedule and for a brief period of time.

In CL, anemia is often the result of chronic blood loss (epistaxis, gastrointestinal bleeding), circulating immune complexes-induced hemolysis, and decreased erythropoiesis due to bone marrow suppression or renal failure (18,19). Anemia was detected in the majority (8/11 [72.7%]) of dogs in the CL group at the beginning of the anti-inflammatory period and in all of the dogs at the same time in the immunosuppressive period. Despite the use of glucocorticosteroids, that theoretically increase the number of circulating erythrocytes and diminish the removal of the aged ones (20), anemia was still present in all of the dogs in the CL group at the end of both experimental periods. Furthermore, anemia appeared in 2 dogs that were not initially anemic after the anti-inflammatory period, although the mean PCV values and Hb concentration remained unchanged. Although gastrointestinal ulceration and bleeding may account for this, there was no gross evidence of melena in any of the CL dogs during either experimental period, and no decline of serum TP or SI, that usually accompanies hemorrhagic anemia, was detected. Nevertheless, glucocorticosteroids do not seem to have any long-lasting beneficial effect in the management of anemia in cases of CL, at least when administered on a short-term basis.

The underlying pathogenesis of CL-induced anemia as seen in the CL group is unclear. Chronic blood loss and erythropoietin deficiency due to chronic renal failure (18,21) could be excluded, since there was no compatible historical, clinical, or laboratory evidence. Further, the theory of circulating immune complexes, binding the complement to erythrocytes, and thus shortening their life span (16,18), may not explain the anemia, since glucocorticosteroids, especially at immunosuppressive doses, inhibit this process (22). Suppressed bone marrow erythropoiesis, frequently seen in chronic infections (3,15,23–25,) may lead to decreased SI concentration and SAT levels (23,26), decreased or normal TIBC values, increased ferritin concentration, and normal or increased bone marrow hemosiderin (25). The anemia in the dogs of the CL group may not be attributed to chronic infection, since SI concentration, SAT values, and bone marrow hemosiderin stores were normal at the beginning of both experimental periods. This is in contrast to the results of a previous study of dogs with CL, where bone marrow stores were more abundant and serum ferritin concentration increased in the infected compared to the control dogs (27). The reasons for this discrepancy are not clear; differences in the clinical manifestations of CL, in the dogs' diets, or both could offer a possible explanation, but more studies are needed to conclusively resolve this issue. Therefore, it is believed that anemia during CL infection is a rather complex process with multiple mechanisms involved during its progression.

At the beginning and 2 d after the end of both experimental periods, PCV values and Hb concentrations were found to be within normal limits in all of the dogs in the control group. The significantly higher PCV values on day 0 of the anti-inflammatory period, compared with the 10th d, could be partially attributed to splenic contraction due to stress during blood collection (16), since at the beginning of this experiment the control group dogs were not accustomed in such procedures. Furthermore, their younger age and smaller body size, compared to the CL group, favour of this explanation, since young and small-sized dogs often exhibit stress-induced polyerythrocythemia (16).

All living organisms, including *Leishmania* spp., require Fe for their growth (8–10,15,25,28). In vitro studies have shown that an Fe-deficient environment does not support the growth of promastigotes (9,10), and the addition of Fe salts to incubation fluids may prevent the killing of intracellular amastigotes by activated macrophages (8). In chronic infections, hypoferremia commonly occurs (23,26) as a defensive mechanism, developed to contain various pathogens as they invade (16,24,28). In contrast to the results of a previous study (27), hypoferremia did not appear to occur in the dogs with CL in this study, since the SI and SAT values were similar in both groups at time 0 in both experimental periods. This finding could partially explain the high resistance of CL to various antileishmanial medications (29).

In clinically normal dogs, the SI and SAT values tend to increase during treatment with glucocorticosteroids (2), probably because of the stabilization of neutrophil lysosomal membranes (20,30). Therefore, the inhibition of transferrin release from the membrane-bound granules of neutrophils may result in an increase in SI and SAT values (15,30). The same process may be responsible when oral prednisolone is administered at anti-inflammatory or immunosuppressive dosage to patients with CL, as the SI and the SAT values increased in both experimental periods of this study. Consequently, as Fe inhibits *Leishmania* destruction and removal by macrophages (8), glucocorticosteroids may enhance parasite survival, thus justifying their avoidance in the treatment of CL.

Total body Fe stores can be reliably accessed with the measurement of serum ferritin (31,32), which was not possible in this study due to technical reasons. Therefore, the evaluation of hemosiderin stores in bone marrow aspiration smears, also an accurate method for estimating total body Fe stores (16), had to be pursued instead. The absence of changes in the amount of bone marrow hemosiderin granules in the CL group, either with the anti-inflammatory or the immunosuppressive dosage, could be attributed to the short experimental period.

In dogs, rats, and ewes with chronic infections, hypercupremia and increased ceruloplasmin levels are often noted (23,24,31,33–35). Further, increased serum ceruloplasmin concentration has been found in dogs with CL when compared to healthy animals (27). These changes are probably due to the production of an exogenous leucocytic mediator, which stimulates the hepatic synthesis and release of ceruloplasmin (23,31). Interestingly, this was not confirmed in the CL group in this study, suggesting that hypercupremia may not be a component of the biochemical profile exhibited by *L. infantum* affected dogs.

The use of glucocorticosteroids in healthy rats and in those with chronic non-septic polyarthritis resulted in a significant increase of Cu plasma levels (4). In healthy humans, the intravenous administration of glucocorticosteroids increased plasma Cu concentration (5), while the opposite occurred when they were given per os (36). In this study, the effect on plasma Cu was somehow erratic, as it was increased during the anti-inflammatory period in the control group and decreased during the immunosuppressive period in the CL group. It has been postulated that hypercupremia has an antibacterial effect (24), and may also contribute to containing *Leishmania* spp. replication (37). In this respect, hypocupremia induced by immunosuppressive doses of prednisolone might enhance parasite survival.

In conclusion, anemia was not found to be the result of chronic infection in CL, since SI, SAT, and TIBC values, and bone marrow hemosiderin stores were similar between the affected dogs and the controls. The use of prednisolone, may promote parasite replication because of the induction of increased SI levels and hypocupremia.

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