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The effects of prednisone on haemostasis in leishmaniotic dogs treated with meglumine antimoniate and allopurinol

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Abstract

Thirty dogs naturally infected with *Leishmania infantum* were studied in order to determine the effects of treatment on haemostatic function. The animals were divided randomly into two treatment groups: Group 1 received meglumine antimoniate and allopurinol; Group 2 dogs were given the same treatment plus prednisone. Ten healthy animals were used as untreated controls. Clinical examination and determination of platelet aggregation, coagulation factors and biochemical parameters were undertaken before treatment and after 15, 30 and 60 days. A significant improvement in platelet aggregation was detected after 60 days in Group 1, but only after 15 days in Group 2. In both treated groups, platelet aggregation was lower than in the control group at the end of the study. The results suggest that prednisone may be a useful tool in the treatment of haemostatic disorders during canine leishmaniosis. The potential benefits and risks due to the use of corticosteroids in the treatment of leishmaniosis are discussed.

Keywords: Canine Leishmaniasis; Platelet aggregation; Meglumine antimoniate; Allopurinol; Glucocorticoids

Introduction

Several clinical signs of bleeding such as epistaxis, haematuria, haemorrhagic diarrhoea and disseminated intravascular coagulation have been reported in canine leishmaniasis (CL) (Font et al., 1993; Ciaramella et al., 1997; Koutinas et al., 1999; Ciaramella and Corona, 2003). The pathogenesis of the bleeding is uncertain, but it may be caused by alterations in primary and/or secondary haemostasis (Ciaramella et al., 2005).

In our previous studies, thrombocytopenia has been reported in 29.3% of cases of canine leishmaniasis (Ciaramella et al., 1997) and a deficiency in platelet aggregation has been found in all infected dogs (Ciaramella et al., 2005). Thrombocytopenia and thrombocytopathy may result from changes in the vessel wall due to vasculitis, altered thrombocytopoiesis, or increase in platelet destruction following renal and/or hepatic failure (Ferrer, 1992; Slappendel and Ferrer, 1998; Ciaramella et al., 2005). An immunological component has also been suspected in CL associated with the presence of platelet-bound antibodies in kala-azar patients (Kharazmi et al., 1982).

Recently, a pathogenic association between thrombocytopenia and the presence of antibodies against the platelet membrane has been observed in dogs naturally infected with *Leishmania infantum* (Terrazzano et al., 2006). For these reasons, the authors hypothesised that glucocorticoids could be useful in addition to classic chemotherapy for the treatment of CL. The conventional antileishmania drugs used in human therapy (pentavalent antimonials, amphotericin B, pentamidine or miltefosine) have low clin-

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ical efficacy in dogs as they induce only a temporary remission of clinical signs and do not prevent relapses. Pentavalent antimonials alone or in combination with allopurinol remain the most commonly used drugs for the control of canine CL (Baneth and Shaw, 2002). It is reported that the combined treatment is more effective, because allopurinol blocks RNA synthesis of *Leishmania* spp. and the antimonials simultaneously inhibit leishmanial enzymes needed for glycolytic and fatty acid oxidation (Martinez et al., 1988; Denerolle and Bourdoiseau, 1999). In addition, higher parasitological cure rates (53.3–66.7%) have been achieved with the combined use of allopurinol and meglumine (*n*-methylglucamine) antimoniate (Roura et al., 1997).

To the authors' knowledge, no data were available concerning haemostatic abnormalities during the therapy of CL. The aim of the present study was to evaluate haemostatic and platelet function in dogs naturally infected by *Leishmania infantum* treated with a combination of meglumine antimoniate and allopurinol, alone or in combination with prednisone.

Materials and methods

Animals

Thirty dogs of different breeds and sex, aged between 5 and 9 years, and naturally infected with *L. infantum* were included in the clinical trial. A control group of 10 untreated healthy dogs was also studied. All infected animals showed typical clinical signs of leishmaniasis, but without haemorrhagic pathology. The main symptoms seen were systemic lymphadenomegaly, splenomegaly, diffuse dry exfoliative dermatitis and weight loss. The clinical diagnosis of leishmaniasis was confirmed by direct observation of the protozoa in Giemsa-stained fine needle aspirates of bone marrow and/or lymph nodes, and serologically by an immunofluorescent antibody test (IFAT). A threshold titre of 1/160 was considered indicative of infection.

All dogs were serologically negative for *Ehrlichia canis* (IFAT titre <1:100) and had received no previous treatment with specific anti-*Leishmania* drugs within the previous 8 months. The infected dogs were randomly divided in 2 groups of 15 animals each (Groups 1 and 2). Animals from Group 1 were treated with a combination of meglumine antimoniate (Glucantime, Merial) at the standard dose of 100 mg/kg, subdivided into two daily doses of 50 mg/kg subcutaneously for 30 days plus allopurinol (Zyloric, Wellcome) at the dose of 15 mg/kg bid orally for 30 days. The animals from Group 2 were treated with the same therapy as Group 1 with the addition of oral prednisone (Deltacortene, Lepetit) at a dose of 2 mg/kg/day for 7 days, then 1 mg/kg/day for 7 days and finally 0.5 mg/kg/day for a further 7 days.

Blood collection

Blood was collected by jugular venepuncture from all dogs at 0800 h and from dogs of Groups 1 and 2 before (time 0), and 15, 30 and 60 days after the beginning of the therapy. Blood samples were placed in plastic tubes to obtain serum (for clinical chemistry), or in anticoagulant tubes with sodium citrate 3.8% (to measure haemachrome, platelet aggregation and coagulation factors). Samples were stored at room temperature.

Full blood count, haematocrit

A full blood count was performed within 30 min of collection, using a semi-automated cell counter (Genius S; SEAC Radom Group).

Platelet aggregation

Adenosine 5′-diphosphate (ADP; 0.5–10 μM) and collagen type I calf skin (5–200 μg/mL) were used as agonists. Platelet-rich plasma (PRP) was obtained by centrifuging whole blood at 180 *g* for 20 min, at room temperature (20 ± 25 °C). Autologous platelet-poor plasma (PPP) was prepared from PRP by further centrifugation at 2000 *g* for 15 min. Platelets were counted in a haematocytometer chamber, with a phase-contrast microscope. The PRP counts were adjusted to a platelet count of 250,000/mL by dilution with autologous PPP. Aliquots of 225 μL PRP were incubated for 1 min at 37 °C, before 25 μL of agonist, at varying concentrations, was added. The aggregation profile was recorded for 5 min. All determinations were performed within 3 h of sampling. During this time, blood samples were kept at room temperature. PRP aggregation responses were measured with a Chronolog aggregometer (Haverton) coupled to a recorder.

Quantification of aggregation was determined by measuring the percentage difference in light transmission between PRP and autologous PPP at 5 min. Aggregation of 100% was considered to be equivalent to an 80 ± 85% increase in light transmission. The results represent the mean values of aggregation percentage ± SD, with each test being run in duplicate. The reversibility of platelet aggregation induced by each dose of agonist was assessed from the shape of the aggregation tracings, with a monophasic curve taken to indicate irreversible aggregation.

Coagulation factors

Prothrombin time (PT) (Thromborel S; Dade Behring), activated partial thromboplastin time (APTT) (Pathromtin SL; Dade Behring) and fibrinogen (Multifibren; Dade Behring; the Clauss method) were determined using a semi-automatic coagulometer (Labor Fibrintimer; Coa Data 1000).

Clinical chemistry

Serum total plasma protein (TP), serum protein electrophoresis (on cellulose acetate support), alanine transaminase (ALT), creatinine and urea levels were measured for each dog using commercial kits (Reactivos Spinreacter S.A.).

Statistics

The Student–Newman–Keuls multiple comparisons test was used for the statistical evaluation. $P < 0.01$ and $P < 0.05$ were considered significant.

Results

As shown in Table 1, platelet aggregation (medium% ± SD) in Group 1 was markedly reduced in the control group both for ADP and collagen. A significant improvement in platelet aggregation was observed after 60 days from the onset of treatment and these results were accompanied by a better clinical, haematological and biochemical profile (Table 2), although aggregation values were still significantly lower in respect of the control group ($P < 0.01$). Table 3 shows the results for animals in Group 2. The significant improvement ($P < 0.01$) in platelet aggregation, detected only after 15 days of treatment, remained stable up to day 30 and a further improvement was detected at day 60. Again, platelet aggregation after 60 days from the onset of treatment was lower than in healthy control dogs, but this difference was significant only for

Table 1
Percentage (mean ± SD) of adenosine 5′-diphosphate (ADP)–(0.5–10 μM) and collagen–(5–10 μg/mL) induced aggregation in healthy (controls, $n = 10$) and leishmaniotic dogs (Group 1, $n = 15$) treated with meglumine antimoniate and allopurinol before and 15, 30 and 60 days after the onset of therapy

ADP (μM)	0.5	1	2.5	5	7.5	10
Controls	16.0 ± 4.9	23.5 ± 4.7	39.7 ± 5.1	51.5 ± 4.5	66.7 ± 4.7	93.4 ± 3.4
Group 1 (day)						
0	7.7 ± 2.5[b]	14.5 ± 4.4[b]	22 ± 4.9[b]	36.0 ± 5.0[b]	49.8 ± 5.8[b]	71.5 ± 4.3[b]
15	7.8 ± 2.5[b]	15.1 ± 4.4[b]	22.2 ± 4.9[b]	37.2 ± 5.0[b]	53.7 ± 5.0[b]	72.8 ± 4.3[b]
30	9.0 ± 2.1[b]	15.3 ± 4.4[b]	25.0 ± 5.3[b]	40.2 ± 5.6[b]	56.3 ± 4.7[b]	73.8 ± 5.4[b]
60	12.3 ± 2.9[a]	20.5 ± 2.7[a,b]	34.8 ± 4.6[a,b]	48.9 ± 5.7[a,b]	64.9 ± 4.0[a,b]	86.9 ± 4.2[a,b]
Collagen (μg/mL)	5	10	50	100	150	200
Controls	25.0 ± 5.4	36.6 ± 5.1	54.5 ± 3.9	74.9 ± 5.0	85.7 ± 3.9	95.2 ± 3.8
Group 1 (day)						
0	6.0 ± 1.9[b]	12.3 ± 3.3[b]	29.1 ± 5.0[b]	45.2 ± 4.1[b]	55.7 ± 5.1[b]	69.3 ± 5.6[b]
15	6.9 ± 1.5[b]	13.4 ± 3.1[b]	31.7 ± 6.2[b]	46.7 ± 5.8[b]	58.7 ± 6.0[b]	70.6 ± 5.9[b]
30	7.5 ± 2.9[b]	14.2 ± 3.3[b]	33.2 ± 4.2[b]	47.7 ± 5.5[b]	61.4 ± 4.6[b]	74.4 ± 5.1[b]
60	19.3 ± 4.8[a,b]	30.0 ± 4.5[a,b]	49.0 ± 5.0[a,b]	63.0 ± 5.2[a,b]	74.0 ± 4.2[a,b]	89.2 ± 5.2[a,b]

[a] $P < 0.01$ vs. day 0.
[b] $P < 0.01$ vs. controls.

Table 2
Haematological and biochemical profiles in leishmaniotic dogs treated with meglumine antimoniate and allopurinol (Group 1, $n = 15$) and with meglumine antimoniate, allopurinol and prednisone (Group 2, $n = 15$) before and 60 days after the onset of therapy

	Group I (day)		Group II (day)	
	0	60	0	60
RBC (10^6/μL)	5.7 ± 0.7	6.1 ± 1.1	5.2 ± 1.3	5.9 ± 1.1
WBC (10^3/μL)	9.7 ± 3.9	7.9 ± 2.9	11.1 ± 3.9	10.1 ± 2.4
Hgb (g/L)	15.7 ± 3.1	14.9 ± 4.2	14.7 ± 4.2	15.3 ± 3.4
HCT (%)	39.4 ± 3.5	39.7 ± 5.7	37.4 ± 5.8	38.4 ± 5.6
Plt (10^3/μL)	215 ± 91	286 ± 86	224 ± 78	256 ± 111
Urea (mg/dL)	65 ± 2.4	41 ± 9.6	69.3 ± 9.6	43.8 ± 7.8
Creatinine (mg/dL)	1.8 ± 0.3	1.1 ± 0.4	1.7 ± 0.6	0.9 ± 0.2
ALT (UI/L)	42.8 ± 9.3	38 ± 6.4	51.3 ± 8.6	38.6 ± 9.4
PT (s)	7.4 ± 1.1	8.3 ± 1.4	7.6 ± 1.3	6.7 ± 1.9
APTT (s)	13.7 ± 1.0	14.6 ± 1.2	13.9 ± 1.6	13.4 ± 1.2
Fibrinogen (mg/mL)	301.7 ± 42.8	309.4 ± 93.3	300.2 ± 35.7	189.4 ± 89.3

RBC, red blood cells; WBC, white blood cells; HCT, haematocrit; Plt, platelet count; ALT, alanine transaminase; PT, prothrombin time; APTT, activated partial thromboplastin time.

Table 3
Percentage (mean ± SD) of adenosine 5′-diphosphate (ADP) (0.5–10 μM) and collagen-(5–10 μg/mL) induced aggregation in healthy (controls, $n = 10$) and leishmaniotic dogs (Group 2, $n = 15$) treated with meglumine antimoniate, allopurinol and prednisone before and 15, 30 and 60 days after the onset of therapy

ADP (μM)	0.5	1	2.5	5	7.5	10
Controls	16.0 ± 4.9	23.5± 4.7	39.7± 5.1	51.5 ± 4.5	66.7 ± 4.7	93.4 ± 3.4
Group 2 (day)						
0	7.1 ± 2.2[b]	12.7 ± 3.4[b]	19.2 ± 4.1[b]	35.4 ± 6.1[b]	47.4 ± 5.6[b]	70.3 ± 7.3[b]
15	10.5 ± 2.7[a]	19.3 ± 4.9[a,b]	30.2 ± 7.9[a,b]	44.3 ± 5.8[a,b]	61.7 ± 8.1[a,b]	82.4 ± 9.3[a,b]
30	10.4 ± 2.2[a,b]	18.6 ± 5.2[a,b]	31.1 ± 7.3[a,b]	45.2 ± 7.6[a,b]	62.3 ± 8.7[a,b]	83.2 ± 8.1[a,b]
60	13.9 ± 2.6[a,b]	21.7 ± 4.1[a,b]	35.2 ± 8.6[a,b]	49.2 ± 8.2[a,b]	65.8 ± 9.0[a,b]	90.2 ± 8.2[a,b]
Collagen (μg/mL)	5	10	50	100	150	200
Controls	25.0 ± 5.4	36.6 ± 5.1	54.5 ± 3.9	74.9 ± 5.0	85.7 ± 3.9	95.2 ± 3.8
Group 2 (day)						
0	8.1 ± 1.6[b]	12.0 ± 4.9[b]	27.4 ± 8.3[b]	43.1 ± 3.7[b]	57.2 ± 7.1[b]	67.8 ± 6.1[b]
15	17.1 ± 4.5[a,b]	22.7 ± 5.3[a,b]	41.8 ± 5.9[a,b]	56.3 ± 5.0[a,b]	78.2 ± 8.4[a,b]	80.2 ± 7.8[a,b]
30	17.3 ± 6.2[a,b]	24.1 ± 6.2[a,b]	45.6 ± 8.1[a,b]	59.8 ± 6.3[a,b]	81.3 ± 9.5[a,b]	84.3 ± 6.1[a,b]
60	20.5 ± 7.0[a,b]	31.2 ± 8.7[a,b]	50.3 ± 7.9[a,b]	70.0 ± 9.0[a,b]	83.0 ± 7.9[a,b]	88.8 ± 8.7[a,b]

[a] $P < 0.01$ vs. day 0.
[b] $P < 0.01$ vs. controls.

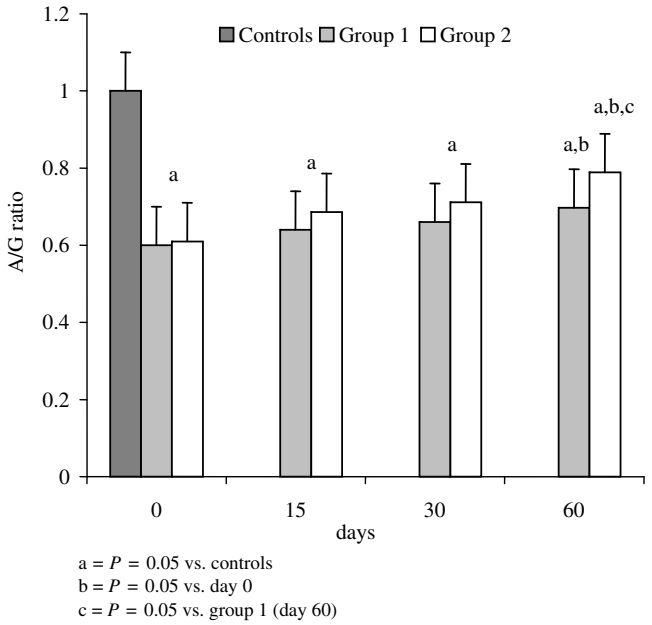

Fig. 1. Albumin/globulin (A/G) ratio (mean ± SD) in healthy (controls, $n = 10$) and treated (Group 1, $n = 15$; Group 2, $n = 15$) dogs before and 15, 30 and 60 days after the onset of therapy.

collagen. A complete remission of platelet aggregation dysfunction was only seen when using ADP at all doses. Clinical improvement, namely reduction of lymphadenomegaly, splenomegaly, diffuse dry exfoliative dermatitis and weight gain (of about 10–15%), was observed after 30 days of therapy, although, at 60 days from the onset of treatment no significant differences were observed in Group 1. Neither group showed any clinical difference after 15 days of therapy.

Fig. 1 shows the albumin/globulin (A/G) ratio during and after therapy; an improvement in the ratio was seen after 60 days for both groups, but was still significantly lower than in the control group. Moreover, a significant difference was detected between Groups 1 and 2 at 60 days. All of the dogs had normal haematological and biochemical findings and it was notable that platelet count and PT, APTT and plasma fibrinogen concentration were all within reference ranges (Table 2).

Discussion

In agreement with previous findings, our results confirmed that platelet aggregation was strongly affected during leishmaniasis in dogs and in the absence of bleeding (Pelagalli et al., 2004; Ciaramella et al., 2005; Cortese et al., 2006). In Group 1, classic therapy with meglumine antimoniate and allopurinol led to a significant improvement in platelet aggregation ($P < 0.01$) after 60 days from the onset of therapy whereas, no significant results were detected at 15 and 30 days of treatment. This suggests that 60 days can be considered the minimum time needed to obtain a significant improvement in platelet function using this treatment option. Such an increase in platelet aggregation is still significantly lower in the healthy dogs ($P < 0.01$) indicating that, despite the improvement in platelet aggregation and clinical condition of the treated dogs, platelet function was still affected by the disease. This suggests that aggregation testing may be a useful tool in monitoring platelet function during and after therapy. However, the test is labour intensive, costly, time consuming and must be performed within 2 h of blood collection. Furthermore, interpretation of results can be difficult and possible platelet activation during PRP preparation has been reported (Harrison, 2005). As a result, other tests are to be preferred for routine analysis.

In Group 2, the addition of prednisone led to a faster improvement in platelet aggregation with a significant increase ($P < 0.01$) only after 15 days of treatment. This increase is also detectable 30 and 60 days from the onset of therapy. After 60 days, a difference between the two agonists was detected: ADP-induced aggregation showed no statistical difference in healthy dogs while platelet aggregation was still significantly lower than in controls when collagen was used as the aggregating agent. The results obtained using ADP suggest that the addition of prednisone not only led to a faster improvement in platelet function, but also to a complete remission of platelet dysfunction. In contrast, collagen demonstrated an alteration of platelet function which can be best explained by the different activity of the two agonists. Pelagalli et al. (2004) showed a decreased ability of platelets to aggregate in CL and a greater sensitivity to collagen than to ADP, possibly due to structural and functional differences in the infected platelet receptors. Since collagen has already been shown to be more sensitive in revealing platelet alteration, we can conclude that platelet dysfunction is still detectable (but to a lesser degree) also in the prednisone treated group compared to the dogs in Group 1.

The improvement in platelet function may be due to prednisone's action on specific anti-platelet antibodies. In a previous work (Terrazzano et al., 2006), we showed the occurrence of platelet-bound IgM as well as IgG isotypes in naturally *Leishmania*-infected dogs. The presence of anti-platelet antibodies in the plasma of infected animals was also found to be significantly associated with the clinical stages of the disease and with the presence of a low platelet count. There was a pathogenic association between leishmaniasis and the presence of antibody against the platelet membrane. Corticosteroids are known to induce immunosuppression and it is possible that prednisone reduces their activity on anti-platelet antibodies by altering platelet receptors involved in the aggregating function of these cells.

It could be argued that the starting dose of prednisone used in the present study (2 mg/kg/day for 7 days, then 1 mg/kg/day for 7 days and finally 0.5 mg/kg/day for a further 7 days) is not immunosuppressive although, it is 204 times higher than doses recommended for an anti-inflammatory action (0.5–1.2 mg/kg/day) during leishmaniasis.

Prednisone is commonly used at sub-immunosuppressive doses in order to control immunopathological events, such as glomerulonephritis, keratitis, uveitis or polyarthritis (Slappendel and Ferrer, 1998), but immunosuppressive doses (2.2 mg/kg every 12 h) are considered dangerous during leishmaniasis, because of adverse effects. In fact, because of their catabolic, hypoalbuminemic and immunosuppressive activities, high doses of corticosteroids should be used with considerable care in CL therapy.

Few data were available regarding the use of corticosteroids during CL. Bergeaud (1988) described a remarkable improvement in clinical symptoms in a group of 43 dogs with severe forms of CL using a dual therapy of corticosteroids (1–2 mg/kg/day) and a classic antimony treatment. Rüfenacht et al. (2005) revealed that the skin lesions in a cat with *Leishmania*-infection combined with pemphigus foliaceus may improve with prednisolone treatment, but the parasite burden can also be increased. In mice with visceral leishmaniasis, Gangneux et al. (1999) showed that there was an increase in the parasite burden in the spleen when the animals were treated with dexamethasone for a prolonged period. Furthermore, in humans, mice and dogs, cellular immunity is important in the control of leishmaniasis (Moreno and Alvar, 2002) and because glucocorticoids can decrease cellular immunity, they influence the host-parasite balance and as a result are unsuitable for use as sole therapy in *Leishmania*-infected animals (Poot et al., 2005). In our study, we found that there was a faster improvement in platelet function and with no adverse effects.

Despite the changes in coagulation and fibrinolysis reported in CL (Valladares et al., 1998; Font et al., 1994; Moreno, 1999), the normal PT, APTT and plasma fibrinogen concentration in all of the dogs in our study indicated that secondary haemostasis in CL was not significantly altered, consistent with the findings of Varela (1992) and Juttner et al. (2001). In previous studies, we have also reported a normal secondary haemostasis in CL, but an increase in APTT plasma concentration (Ciaramella et al., 2005; Cortese et al., 2006).

Conclusions

Our findings suggest that the addition of prednisone to the classic therapy of meglumine antimoniate and allopurinol can be successfully used to improve the responses to therapy leading to an improvement in platelet function. However, since several adverse effects of corticosteroids are known, their use in the treatment of leishmaniosis in dogs should be always accompanied by an accurate evaluation of risks and benefits.

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