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A Prospective Study of Clopidogrel Therapy in Dogs with Primary Immune-Mediated Hemolytic Anemia

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Background: A major cause of death in dogs with primary immune-mediated hemolytic anemia (pIMHA) is thrombotic disease. Ultralow-dose aspirin (ULDA) is commonly used to prevent thrombosis in dogs with pIMHA; however, the efficacy of antiplatelet agents in dogs with pIMHA is unknown.

Hypothesis: The use of clopidogrel (CL), alone or in combination with ULDA, would improve survival to discharge and at 90 days without important adverse effects compared with ULDA alone in dogs with pIMHA treated with standard immunosuppressive therapy.

Animals: Twenty-four client-owned dogs with pIMHA.

Methods: Prospective, positive-controlled, unmasked clinical trial with dogs randomized in 3 treatment groups to receive PO ULDA or CL or both.

Results: There was no identifiable adverse reaction, evidence of hemorrhage, or increase in transfusion requirements associated with CL therapy, either alone or combined with ULDA, compared with ULDA alone. There was no significant difference between treatment groups with respect to survival to discharge and at 90 days.

Conclusions and Clinical Importance: This study suggests that CL therapy, alone or in combination with ULDA, was safe and had similar short-term survival compared with ULDA alone in a small group of dogs with pIMHA able to tolerate oral medications and treated with standard immunosuppressive treatment.

Key words: Aspirin; Heparin; Thromboembolism; Thrombosis.

Immune-mediated hemolytic anemia (IMHA) is a common hematological disease of dogs. IMHA could be primary or secondary in nature. The majority of dogs with IMHA have primary or idiopathic disease.[1] Treatment typically involves administration of corticosteroids in conjunction with other immunosuppressive agents.[2] The case fatality associated with primary IMHA (pIMHA) in dogs is high as 50–70% in some studies, particularly during the first 2 weeks of therapy.[1,3] Thromboembolic disease is a common complication of pIMHA in dogs, and venous thrombosis and pulmonary thromboembolism (PTE) are thought to account for up to 80% of the deaths in dogs with pIMHA.[4–7] Most dogs with pIMHA are hypercoagulable during hospitalization as demonstrated by evaluation of coagulation parameters or thromboelastography (TEG).[8,9] As suggested in a recent prospective study, development of an effective prophylactic approach for hypercoagulability could markedly reduce the case fatality rate in dogs with pIMHA.[10]

A retrospective study reported improved survival rates in dogs with IMHA treated with ultralow-dose aspirin (ULDA).[11] Another study indicated that most dogs with IMHA had increased levels of P-selectin, a marker of platelet activation, compared with healthy control dogs.[12] On the other hand, recent studies showed that the currently recommended antithrombotic dosing of aspirin (0.5 mg/kg/d) was ineffective in healthy dogs.[a,b] However, platelet inhibitors in dogs with pIMHA have not been evaluated in prospective controlled clinical trials.[10,11,13,c,d] Clopidogrel[e] (CL) is a newer antiplatelet drug that irreversibly inhibits the ADP receptors P2Y12 on the platelet membrane and thus has a unique mechanism from the cyclooxygenase-inhibiting effect of aspirin.[14] A single CL dose of 10 mg/kg effectively inhibits platelet aggregation within 24 hours of administration, and a subsequent daily dose of 2 mg/kg maintained adequate platelet inhibition in healthy dogs.[f] The use of CL in 12 dogs with pIMHA appears safe.[d]

We hypothesized that the use of CL, alone or in combination with ULDA, would improve survival to discharge and at 90 days without important adverse effects compared with ULDA alone in client-owned dogs with pIMHA treated with standard immunosuppressive therapy. The purpose of this study was to compare the survival to discharge and the 90-day survival rate between dogs with pIMHA treated with CL, alone or in combination with ULDA, compared with dogs treated

Abbreviations:

CL	clopidogrel
IMHA	immune-mediated hemolytic anemia
pIMHA	primary IMHA
PTE	pulmonary thromboembolism
RBVH	Red Bank Veterinary Hospital
TEG	thromboelastography
ULDA	ultralow-dose aspirin

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with ULDA alone in conjunction to standard immunosuppressive therapy. In addition, the frequency of fatal and nonfatal thrombotic episodes, transfusion requirements, and the clinical evidence of hemorrhage among the 3 treatment groups were compared.

Materials and Methods

Study Design

The study was a prospective, single-center, positive-controlled, unmasked clinical trial with dogs randomized in 3 treatment groups to receive PO ULDA or CL or both. Randomization was based on blocks of 3 using a table of random numbers. Block randomization was used with a 1:1 allocation ratio to maintain similar sample sizes in the 3 treatment groups.[15] Allotment to the treatment groups was based on sequentially numbered cards to be seen at the time of case enrollment.

Study Population

Client-owned dogs were enrolled at Red Bank Veterinary Hospital (RBVH) between September 2008 and November 2009. The study protocol was approved by the Institutional Animal Care and Use Committee. All dogs were evaluated on a referral basis.

Inclusion Criteria

For inclusion into the study, dogs had to be diagnosed with IMHA based on the presence of regenerative anemia (hematocrit <30% with >60,000 reticulocytes/μL), evidence of hemolysis (hyperbilirubinemia, hemoglobinemia, bilirubinuria, or hemoglobinuria), and at least one or more of the following criteria: presence of spontaneous persistent agglutination (that persisted after dilution with saline); at least 2+ spherocytes noted on blood smear; or a positive direct Coombs' test. Spherocytes were reported as 1+ if 1–10 spherocytes were noted in the monolayer of the blood smear per 100× objective field, 2+ if 11–50 spherocytes were noted per 100× objective field, and 3+ if 51–150 spherocytes were noted per 100× objective field.[16] Spherocytes were reported as occasional if they were only seen in some 100× objective fields. At least 10 high-power fields were evaluated for each count. The direct Coombs' test was performed at 37°C with a combined Coombs' reagent containing goat anticanine immunoglobulin G, immunoglobulin M, and complement C3.[g] Erythrocytes were washed 4 times before the addition of the Coombs' reagent. Dogs were eligible for inclusion in the study only if their owner gave informed consent.

Exclusion Criteria

Dogs were excluded from the study if they had received any corticosteroids for more than 48 hours before presentation, any anticoagulants (unfractionated heparin or low-molecular-weight heparin for longer than 24 hours before enrollment, and aspirin within 7 days of admission), any additional immunosuppressive therapy, hemoglobin based on oxygen carrying solution, previously undergone splenectomy, had severe thrombocytopenia (platelet count <40,000/μL), had relapsing IMHA, were exposed to any drugs or toxins within 4 weeks before presentation, or were diagnosed with neoplasia or vector-borne disease based on the diagnostic investigation outlined below.

Diagnostic Investigation

Each dog was evaluated with a complete history, including any known exposure to vaccinations, drugs or toxins, physical examination, CBC, slide agglutination test, direct Coombs' test, serum chemistry, urinalysis, coagulation profile (prothrombin time, activated partial thromboplastin time), 3-view thoracic radiography, abdominal ultrasonography, heartworm antigen testing, and polymerase chain reaction testing for vector-borne diseases (*Anaplasma phagocytophilum*, *Anaplasma platys*, *Babesia canis*, *Babesia gibsoni*, *Bartonella henselae*, *Bartonella vinsonii*, *Borrelia burgdorferi*, *Ehrlichia canis*, *Ehrlichia chaffeensis*, *Ehrlichia ewingii*, *Mycoplasma haemocanis*, *Mycoplasma haematoparvum*, *Neorickettsia risticii*, *Rickettsia rickettsii*). Coagulation assays were performed in singlet with commercial reagents and an automated coagulation instrument.[h] Complete necropsy was offered for each enrolled dog who did not survive to the end of the study period.

Test Treatment

Within 24 hours of admission, enrolled dogs were randomized to receive either CL using a loading dose of 10 mg/kg PO on day 1, followed by a maintenance dose 2–3 mg/kg q24h for 89 days, or ULDA at a dose of 0.5 mg/kg PO q24h for 90 days, or both (CL-ULDA) with the above drug protocols and starting at the same time on day 1. During the 3-month study period, any sign possibly attributed to an adverse drug reaction was recorded, including any evidence of hemorrhage, vomiting, diarrhea, weight loss, pruritus, cutaneous rash, thrombocytopenia, neutropenia, and increased hepatic enzymes.[17–20]

Concomitant Treatments and Monitoring

All dogs were hospitalized in the critical care unit at RBVH for 24 hour monitoring and care. Starting on day 1, all dogs were treated with doxycycline[i] (5 mg/kg PO or IV q12h), prednisone[j] (1.5 mg/kg PO or IV q12h), azathioprine[k] (2 mg/kg PO q24h), famotidine[l] (0.5 mg/kg PO or IV q24h), and sucralfate[m] (50 mg/kg PO q8h). Packed red blood cells, colloid or crystalloid or both fluid with electrolyte supplementation was administered at the attending clinician's discretion. For each case, hematocrit and physical examination was repeated at least every 12 hours from admission until discharge. On day 14, the attending clinician was allowed to use any additional immunosuppressive drugs if indicated for medical reasons, such as azathioprine intolerance or persistent anemia and spherocytosis. The use of oral famotidine and sucralfate was recommended for all dogs during the 90-day study period. In all dogs, physical examination, CBC, and serum chemistry were performed at least on days 7, 14, and 90. Prednisone dosage was tapered at the attending clinician's discretion. CBCs were evaluated in ethylenediaminetetraacetate-anticoagulated blood by use of automated cell counters.[n]

Study Endpoints

The primary endpoints of the study were survival to discharge and survival rate at 90 days. All dogs were monitored for 90 days from the enrollment in the study. Secondary endpoints were the occurrence of fatal or nonfatal thrombotic episodes based on clinical, diagnostic imaging, necropsy findings or both. Clinical findings considered to be consistent with PTE were acute onset of hypoxemia with hypocapnia, otherwise unexplained respiratory distress, or a supportive pulmonary angiographic imaging study. Findings considered to be consistent with thrombosis of other sites were ascites, otherwise unexplained organ failure, or acute onset of neurologic signs. Other secondary endpoints included acute or delayed adverse reactions to study drug (hemorrhagic, gastrointestinal, and dermatologic adverse effects), duration of hospitalization, transfusion requirements, days to complete hematologic response to therapy (defined as the time required for the hematocrit to return to laboratory reference range, 39–55%), and rate of relapse (defined as a hematocrit decrease by 50% compared with previous measurement or any hematocrit of <25% after initial response).

Statistical Analysis

Power calculations were based on previous available studies of IMHA in dogs.[1–3,5,7,11] A study population of 8 dogs for each treat-

ment group was estimated to provide a power of approximately 80% at 0.05 significance level to detect a 50% difference in survival rates at discharge and 90 days among the 3 treatment groups. Data distribution were assessed for normality by the D'Agostino-Pearson test. Data from dogs in 3 treatment groups were assessed by multifactor analysis of variance (ANOVA). Where data were not normally distributed, the Kruskall-Wallis test was used instead of ANOVA. The Kaplan-Meier estimates of the distribution of times from diagnosis to death were computed, and the Mantel-Cox log-rank analysis was performed to compare the survival curves among the 3 treatment groups. Results are presented as median and range unless indicated otherwise. Statistical analyses were performed by a standard statistical software package.[o] For all analyses, P value $<.05$ was considered statistically significant.

Results

Characteristics of the Study Population

Thirty-one dogs met the inclusion criteria; 7 dogs were excluded because of inability to tolerate oral medications (n = 4) or because of the owner's financial constraints (n = 3). Eight dogs were enrolled into the CL group, 8 into the ULDA group, and 8 into the CL-ULDA group. Breeds represented were Cocker Spaniel (n = 3), mixed breed dog (n = 3), Pitbull (n = 3), Shih Tzu (n = 2), Rottweiler (n = 2), Maltese (n = 2), and one each of the following breeds: Airedale Terrier, Miniature Schnauzer, Beagle, Standard Poodle, English Springer Spaniel, Pomeranian, Toy Poodle, Collie, and Miniature Dachshund. On presentation, there was no statistical difference between the treatment groups with regard to age, sex, body weight, rectal temperature, and clinicopathologic findings (Table 1). All dogs received corticosteroids for <48 hours before enrollment in the study. Corticosteroids administration was started on admission in 14 dogs (5/8 in the ULDA group, 6/8 in the CL group, and 4/8 in CL-ULDA group). The other dogs received corticosteroids for 24 hours before presentation, and there was no significant difference among the 3 treatment groups regarding when corticosteroid therapy was initiated.

Primary Endpoint

Overall, 5 dogs did not survive the study period and necropsy was performed in 2 of them. No secondary cause for IMHA was identified in these 2 dogs at necropsy. One dog in each treatment groups did not survive to discharge from the hospital. Suspected thrombotic events occurred in 1 dog of the CL with fatal acute onset of neurologic signs, and in 1 dog of the ULDA group with fatal PTE based on arterial blood gas analysis, clinical, and radiographic findings. The dog in the CL-ULDA group was euthanized on day 9 because of refractory anemia. Necropsy did not reveal any thrombi. Six out of 8 dogs in each of the CL and the ULDA group were alive at day 90, and 7 out of 8 dogs in the CL-ULDA group were alive at day 90. The additional fatality on the CL group was because of humane euthanasia secondary to intolerable adverse effects of chronic corticosteroid administration on day 56, and the additional fatality in the ULDA group was because of sepsis on day 28. The latter dog necropsy revealed multiple organ microthrombi. Thrombotic events were not suspected in any dogs in the CL-ULDA group. There was no significant difference between groups with regard to occurrence of thrombosis. Survival analysis did not indicate any differences between the treatment groups at discharge and 90 days (Table 2).

Secondary Endpoints

There were no identifiable immediate or delayed adverse drug reactions detected over the 90-day period: no evidence of hemorrhagic and dermatologic adverse effects was noted in any dogs based on clinical signs. A self-limiting diarrhea attributed to the CL loading dose administration was observed in 2 dogs (1 dog in the CL-ULDA group and 1 dog in the CL group), but the frequency of diarrhea was not significantly higher compared with the ULDA group. There was no difference between the treatment groups as to duration of initial

Table 1. Comparison of dog characteristics and clinicopathologic findings at admission between groups of dogs with pIMHA receiving different antithrombotic therapy.

Parameter	CL (n = 8)	ULDA (n = 8)	CL-ULDA (n = 8)	Test	*P*-Value
Age (years)	6.1 (1.9–10.2)	6.5 (2.8–11.1)	5.4 (1.4–9.1)	AN	.49
Sex	6 SF, 2 NM	5 SF, 3 NM	7 SF, 1 NM	AN	.38
Body weight (kg)	19.8 (4.2–32.1)	22.7 (3.1–29.7)	20.5 (4.8–35.7)	AN	.68
Rectal temperature (°F)	101.3 (99.7–103.2)	102.1(99.9–103.8)	101.6 (100.7–104.2)	AN	.82
Neutrophil segs ($10^3/\mu L$)	18.9 (10.4–37.4)	16.3 (8.7–32.1)	21.8 (7.4–33.8)	AN	.27
Neutrophil bands ($10^3/\mu L$)	0.45 (0.12–1.87)	0.32 (0.22–2.38)	0.41 (0.32–3.73)	KW	.18
Hematocrit (%)	13.3 (9.8–21.7)	14.7 (8.7–19.3)	12.9 (9.1–22.9)	AN	.46
Serum bilirubin (mg/dL)	3.1 (0.6–12.6)	2.2 (0.7–4.5)	2.2 (0.9–10.4)	KW	.55
Platelet count ($10^3/\mu L$)	139 (78–367)	174 (99–412)	163 (115–395)	KW	.23
aPTT (s)	13.1 (12.2–14.7)	12.3 (10.8–15.4)	11.9 (10.7–14.6)	AN	.41
PT (s)	6.3 (6.1–7.4)	6.9 (6.3–7.9)	7.5 (6.8–8.2)	AN	.16
Auto-agglutination+	7 dogs	7 dogs	8 dogs	AN	.91

Data are expressed as median (range).

pIMHA, primary IMHA; CL, clopidogrel group; ULDA, ultralow-dose aspirin group; CL-ULDA, clopidogrel and ultralow-dose aspirin group; SF, spayed female; NM, neutered male; aPTT, activated partial thromboplastin time; PT, prothrombin time; AN, ANOVA; KW, Kruskall-Wallis.

Table 2. Comparison of study endpoints between groups of dogs with pIMHA receiving different antithrombotic therapy.

Study Endpoint	CL (n = 8)	ULDA (n = 8)	CL-ULDA (n = 8)	Test	*P*-Value
Survival at discharge	7/8	7/8	8/8	AN	.81
90-day survival	6/8	6/8	7/8	AN	.56
Hospitalization time (day)	4 (2–8)	6 (3–10)	5 (2–9)	AN	.32
PRBC transfusion (mL/kg)	13 (0–28)	17 (12–37)	14 (11–31)	AN	.29
Hematologic response (day)	18 (7–28)	16 (6–35)	22 (7–42)	AN	.27
Evidence of hemorrhage	0/8	0/8	0/8	AN	1.0
Evidence of thrombosis	1/8	1/8	0/8	AN	.83
Vomiting	0/8	0/8	0/8	AN	1.0
Diarrhea	1/8	0/8	2/8	AN	.61
Relapse	0/8	0/8	0/8	AN	1.0

Data are expressed as median (range).

PRBC, packed red blood cell; AN, ANOVA; pIMHA, primary IMHA; CL, clopidogrel group; ULDA, ultralow-dose aspirin group; CL-ULDA, clopidogrel and ultralow-dose aspirin group; KW, Kruskall-Wallis.

hospitalization, transfusion requirements, time of complete hematologic response to therapy, or rate of relapse (Table 2). Cyclosporine was used in 6 dogs (n = 2 in each group) after 14 days of therapy because of persistent anemia and spherocytosis in 4 dogs, and adverse effects of azathioprine in 2 dogs.

Discussion

The results of this prospective study revealed similar short-term survival rates in dogs with pIMHA receiving standard immunosuppressive therapy and CL, or ULDA, or both. A previous retrospective study reported improved survival rates in dogs with pIMHA treated with ULDA.[11] Because of its availability, lack of need for monitoring, ease of administration, and minimal cost, ULDA is commonly used as standard antithrombotic therapy for dogs with pIMHA. Although the high survival in the ULDA group was unexpected and it may have prevented the detection of a potentially beneficial effect of CL, our study failed to identify any significant difference in survival of dogs with pIMHA and treated with CL, ULDA, or both, and ULDA remains a less expensive means of anticoagulation.

Interest in CL for thromboprophylaxis in humans began with the CL versus aspirin in patients at risk of ischemic events trial.[20] CL alone was found to be superior to aspirin alone in reducing the risk of ischemic stroke, myocardial infarction, or death in humans from vascular causes.[20] As such, CL was further evaluated in 4 large human clinical trials which supported the use of combined aspirin and CL therapy to reduce ischemic events in human patients with unstable angina, myocardial infarction without ST-segment increase, or myocardial infarction with ST-segment increase, as well as those undergoing angioplasty and stenting.[21–24] However, a later study found CL plus aspirin was not significantly more effective than aspirin alone in reducing the rate of myocardial infarction, stroke, or death from cardiovascular causes in human. In addition, the use of CL was associated with an increased risk of significant bleeding defined as requiring a blood transfusion.[25] In the present study, dogs receiving CL either alone or in combination with ULDA also did not demonstrate an increase in transfusion requirements. Therefore, CL may represent a safe and equally effective alternative means of anticoagulation in dogs unable to tolerate aspirin therapy.

Although antemortem diagnosis of thrombosis is a diagnostic challenge, no thrombotic events were suspected in any dogs in the CL-ULDA group. Necropsy of the only dog in the CL-ULDA group who was euthanized did not reveal any evidence of thrombosis. Because rapid postmortem lysis of microthrombi is common, pathological examination of tissues is not a sensitive method for detection of thrombosis, especially if necropsy examination is delayed. It is therefore possible that thromboembolic disease contributed to the death of this dog in which there were no significant findings at necropsy. Efficacy and safety of combined CL and ULDA in dogs with pIMHA warrants further investigation.

Limitations of this study include small sample size, lack of blinding, lack of placebo controls, and long-term follow-up data. The lack of masking of the clinicians may introduce potential bias, such as subjective drug selection and perception of prognosis, which can affect the results of the study. This potential risk was minimized by randomization, standardized treatment protocol, and very similar groups at study entry. In addition, as the inclusion criteria for the study required dogs to be able to tolerate oral medications, this may have biased the selected population toward less severely affected dogs with pIMHA. This is reflected in the relatively high survival rates in all study groups compared with previous studies.[1–3,5,7,11] Consequently the increased survival rates of all treatment groups may have compromised the ability to identify a statistically significant survival benefit of CL therapy. Despite power calculations, it is impossible to rule out the possibility of type 1 error. Finally, TEG and other testing to assess platelet function have been reported as sensitive tools for monitoring CL and ULDA therapy in healthy dogs,[26,b] but unfortunately they were not available at the authors' institution at the time of this study.

In conclusion, this study reported that CL therapy, alone or in combination with ULDA, was safe and had similar short-term survival compared with ULDA alone in a small group of dogs with pIMHA, able to tolerate

oral medications, and treated with standard immunosuppressive treatment. Larger prospective, double-blinded, placebo-control studies are warranted to evaluate and verify these preliminary results regarding efficacy and safety of CL in dogs with pIMHA.

Footnotes

[a] Hoh CM, Smith SA, McMichealM, et al. Urinary thromboxane metabolites are affected by aspirin administration to healthy dogs. SAIM Research Focus, Proceedings ACVIM Forum, Anaheim, CA, 2010 (abstract)

[b] Shearer L, Kruth SA, Wood D. Effects of aspirin and clopidogrel on platelet function in healthy dogs. J Vet Intern Med 2009;23:745 (abstract)

[c] Orcutt ES, Polzin DJ, Armstrong PJ, et al. Comparison of individually monitored versus low-dose aspirin on survival of dogs with immune-mediated hemolytic anemia. J Vet Intern Med 2009;23:693 (abstract)

[d] Haviland RL, Pacifico N, Bianco D. Clopidogrel therapy in dogs with immune-mediated hemolytic anemia. J Vet Intern Med 2009;23:745 (abstract)

[e] Clopidogrel bisulfate, Plavix, Bristol-Myers Squibb/Sanofi Pharmaceuticals Partnership, New York, NY

[f] Goodwin JC, Hogan DF, Green HW. The pharmacodynamics of clopidogrel in the dog. J Vet Intern Med 2007;21:609 (abstract)

[g] Canine Coombs' Reagent, VMRD Inc, Diagnostics, Pullman, WA

[h] STA Compact, Diagnostica Stago, Parsippany, NJ

[i] Doxycycline tablets, IVAX Pharmaceuticals Inc, Miami, FL

[j] Prednisone tablets, Watson Labs, Corona, CA

[k] Azathioprine, Roxane Labs, Columbus, OH

[l] Famotidine, Pepcid AC tablets, distributed by Goldline Labs, manufactured by IVAX Pharmaceuticals Inc

[m] Sucralfate tablets, Major Pharmaceuticals, Livonia, MI

[n] Cell-Dyne System 3500, Diagnostics Division, Abbott Laboratories, Santa Clara, CA

[o] SPSS 14.0 for Windows, Microsoft, Redmond, WA

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