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Original article

Evaluation of reticulated platelets in dogs with breed-related thrombocytopenia

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Abstract

The aim of this study was to evaluate the percentage of reticulated platelets in healthy dogs with breed-related thrombocytopenia. Seventy two dogs, clinically healthy, were enrolled in the study. Blood was collected from the patients and anticoagulated with tripotassium ethylenediaminetetraacetic acid (K3-EDTA) and sodium citrate. Platelet count was obtained by an impedance haematology analyser and platelet morphology was evaluated by examination of blood smears. Patients were allocated into two groups. Group 1 consisted of 30 dogs with normal platelet count, whereas group 2 was composed of 42 dogs with thrombocytopenia. Thrombocytopenia was present in both K3-EDTA and citrate blood samples. Patients with thrombocytopenia were divided into two subgroups: the first subgroup included dogs with platelet count in K3-EDTA anticoagulated blood from 100 to 200 $\times 10^9/L$, patients in the second subgroup had a platelet count of less than 100 $\times 10^9/L$.

The percentage of young reticulated platelets (RPs) labelled with thiazole orange, and the percentage of platelets coated with platelet surface-associated IgG, were determined in platelet-rich plasma (PRP) by a flow cytometer.

The mean percentage of RPs in K3-EDTA and citrate PRP was significantly higher in dogs with thrombocytopenia than in dogs with normal platelet count. The mean percentage of RPs was significantly higher in citrate PRP than in K3-EDTA PRP in all groups.

The results suggest that idiopathic, asymptomatic thrombocytopenia is not caused by platelet surface-associated IgG. Dogs with breed-related thrombocytopenia have a competent bone marrow.

Key words: immature platelets, thrombocytopenia, canine, flow cytometry

Introduction

Reticulated platelets (RPs) are young platelets, with residual messenger RNA which degrades within 24 hours after synthesis in dogs and mice, that are present in the peripheral blood (Dale et al. 1995, Harrison 1998). Considering the fact that bone marrow aspiration and biopsy are risky procedures, the use of

flow cytometry to evaluate the percentage of RPs may be crucial in patients with thrombocytopenia and poor clinical condition. Flow cytometry was applied for the first time to measure RPs in dogs after labelling with thiazole orange (TO) in 1990 (Kienast and Schmitz 1990). The same method was also used to evaluate young platelets in horses (Russel et al. 1997). Studies showed that dogs with immune-mediated throm-

bocytopenia (IMT), immune-mediated haemolytic anaemia, systemic lupus erythematosus, lymphosarcoma, and haemangiosarcoma have a higher percentage of RPs in comparison with healthy dogs (Weiss and Townsend 1998, Wilkerson et al. 2001).

Thrombocytopenia (low platelet count) can occur as a result of a megacaryocyte disorder, disturbance of platelet production as well as their premature destruction. Platelet count can be also decreased due to sequestration of platelets in the tissues (Wilkerson et al. 2001). Myeloproliferative bone marrow disorders as well as certain drugs (e.g. oestrogens and chloramphenicol) can alter platelet production in the bone marrow (Matuszkiewicz and Winnicka 2006). The most frequent cause of thrombocytopenia is premature platelet destruction due to an abnormal immune response; when no underlying disease is found and aetiological agent remains undetermined IMT is described as primary. Secondary disease can develop in neoplastic conditions (e.g. mammary gland tumour, haemangiosarcoma, and lymphoma), infectious diseases (e.g. babesiosis, leptospirosis, and other bacterial, viral, and parasitic infections), after vaccination with live attenuated viruses (e.g. canine distemper virus), or after drug treatment (e.g. chloramphenicol, cephalosporin, sulphonamides, and gold salts). Rapid platelet consumption is often observed in acute haemorrhage, disseminated intravascular coagulation, or inflammation. Destruction and consumption of platelets are also enhanced in microangiopathies (Couto et al. 2003). Common causes of platelet sequestration are splenomegaly, splenic torsion, and sepsis (Couto et al. 2003).

Thrombocytopenia is the most common acquired haemostatic disorder in the dog (Weiss and Wardrop 2010). However, in case of inherited and congenital conditions haemostatic defects are usually not observed. Routine haematological analysis performed in certain breeds of dogs, i.e. Cavalier King Charles Spaniels (CKCS), Greyhounds, German Shepherds and Akita Inu, using an automated haematology analyzer, frequently detects thrombocytopenia of variable severity. It was confirmed that thrombocytopenia in CKCS is inherited and congenital (Davis et al. 2008). Cowan et al. and Singh and Lamb demonstrated that thrombocytopenia is present in 51% ($118.7 \times 10^9/L$) and 90% ($87.5 \times 10^9/L$) of enrolled CKCS, respectively (Cowan et al. 2004, Singh and Lamb 2005). The Polish Hound is another breed in which idiopathic, asymptomatic thrombocytopenia occurs; the disorder is mild – the average number of platelets is approximately $167 \times 10^9/L$ (Micuń et al. 2006). Multiple studies were conducted in the population of Greyhounds to establish haematological reference values for that breed. In this breed platelet count between 80 and $120 \times 10^9/L$ is

considered normal (Steiss et al. 2000).

It is supposed that spurious thrombocytopenia is frequently misinterpreted as clinically significant. Pseudothrombocytopenia may be caused by the presence of numerous platelet clumps in the sample (that can be identified on a blood smear), or by the presence of megathrombocytes that can be counted as leucocytes by a haematology analyzer. Anticoagulant-induced pseudothrombocytopenia is observed when platelet count in tripotassium ethylenediaminetetraacetic acid (K3-EDTA) blood is low but is within the reference range in citrate-anticoagulated blood (Bizzaro 1995, Wills and Wardrop 2008).

The basic platelet parameters, i.e. platelet count, platelet haematocrit and mean platelet volume (MPV) are measured and calculated by haematology analyzers. In addition, blood smears should be evaluated to exclude or confirm the presence of platelet clumps and determine platelet size and morphology. Numerous megathrombocytes in a blood smear may indicate regeneration of thrombocytes (Weiss and Wardrop 2010). Evaluating of the percentage of reticulated platelets may be helpful in assessing the efficacy of thrombopoiesis.

Materials and Methods

Patient selection

The material for analyses was collected from clinically healthy dogs – patients of the Small Animal Clinic at the Faculty of Veterinary Medicine in Warsaw, which had not been treated nor vaccinated for 4 weeks before the blood collection. Peripheral blood was collected from the cephalic vein into K3-EDTA tubes (Medlab, Poland) and sodium citrate tubes 0.1 mmol/l (Medlab). Platelet count was determined within 20 minutes after collection using Abacus impedance haematology analyzer (Diatron, Hungary) and blood smears were examined for platelet clumping.

Control group consisted of 30 dogs of various breeds – mostly mixed breed. The platelet count in K3-EDTA blood was between 200 and $580 \times 10^9/L$. The thrombocytopenia group comprised 42 dogs with platelet number less than $200 \times 10^9/L$; these patients were divided into two subgroups. The first subgroup (A) included dogs with a platelet count of $100\text{--}200 \times 10^9/L$, the second subgroup (B) included dogs with platelet count less than $100 \times 10^9/L$. Activity of aspartate transaminase (AST), alanine transaminase (ALT) and urea concentration were measured in serum in all enrolled patients. Prothrombin time (PT) and activated partial thromboplastin time (APTT) in sodium citrate plasma were also assessed.

Sample preparation

After haematological analysis, K3-EDTA and citrate platelet-rich plasma (PRP) were obtained by centrifugation of the K3-EDTA and citrate blood samples, respectively (300 g for 10 minutes, 20°C).

Reticulated platelets

500 µL of TO dye – Retic-Count Reagent (Becton Dickinson, USA) (test sample) and 500 µL of phosphate buffered saline (PBS) (control sample) were added to 50 µL aliquots of K3-EDTA and citrate PRP. Samples were incubated for 20 minutes in the dark. Samples were washed with 1 mL of PBS. Cells after centrifugation were suspended in 500 µL PBS. Samples were analyzed immediately after preparation.

Platelet surface-associated IgG (PSAIgG)

FITC-labelled sheep anti-canine IgG monoclonal antibody (AbD Serotec, UK) was used to identify immunoglobulin G-coated platelets. 1 µL of the antibody was added to 50 µL aliquots of K3-EDTA and citrate PRP and incubated for 20 minutes in the dark. Excess antibody was removed by washing samples with 1 mL of PBS. After centrifugation cells were re-suspended in 500 µL of PBS. Samples were analyzed immediately after preparation.

Flow Cytometry

All samples were analyzed using FACSCalibur flow cytometer (Becton Dickinson) with an argon-ion laser (wavelength 488 nm). Platelet populations were identified on a dot plot. 10,000 events were analyzed.

Statistical analysis

Wilcoxon tests were used for the statistical analysis. Data were considered statistically significant if p values were ≤ 0.05 . Descriptive statistics and non-parametric tests for comparing two groups were performed using the STATISTICA 6.0. software.

Results

Patients were allocated to the control and to two thrombocytopenia groups based on haematology re-

sults in blood anticoagulated with K3-EDTA. Values of haemogram in K3-EDTA and in citrate were within reference values in all groups (Table 1-3). Table 4 presents number of dogs belonging to specific breeds in each group.

The serum clinical chemistry values were in normal range in all the dogs. Mean biochemical variables for dogs with platelet count between 200 and 580 $\times 10^9/L$, for dogs with platelet count 100-200 $\times 10^9/L$ and with platelet count below 100 $\times 10^9/L$, respectively: urea (mg/dl): 34.97 ± 12.53 , 32.43 ± 6.78 , 32.74 ± 8.07 ; serum activity of AST (U/l): 27.18 ± 8.40 , 24.76 ± 7.55 , 29.38 ± 6.74 ; ALT activity (U/l): 36.58 ± 11.64 , 45.10 ± 21.75 , 33.58 ± 8.28 . PT (sec.) (11.30 ± 0.59 , 12.35 ± 1.83 , 11.90 ± 0.65) and APTT (sec.) (20.06 ± 1.30 , 20.82 ± 0.95 , 21.37 ± 1.09) were also within reference range in both, control and thrombocytopenia groups.

Platelets were assayed for the presence of PSAIgG to exclude thrombocytopenia caused by anti-platelet antibodies on the surface of platelets. The mean percentages of platelets coated with PSAIgG in K3-EDTA PRP in dogs with platelet count 200 – 580 $\times 10^9/L$, in dogs with platelet count 100 – 200 $\times 10^9/L$, and below 100 $\times 10^9/L$ were: 2.39 ± 0.90 , 2.97 ± 0.49 , 2.51 ± 0.89 , respectively. The percentages in citrate PRP were: 2.96 ± 0.93 , 2.85 ± 0.94 , 2.94 ± 0.41 , respectively. The results of test ruled out thrombocytopenia due to immune mechanisms.

The mean percentage of RPs in dogs with thrombocytopenia was compared with the mean RPs percentage in healthy dogs with platelet number within the normal limits. The values were measured in K3-EDTA and citrate PRP.

Percentage of RPs labelled by TO are shown in Table 5. All dogs with thrombocytopenia, dogs with platelet count between 100 and 200 $\times 10^9/L$ and dogs with platelet count below 100 $\times 10^9/L$, had a significantly higher percentage of reticulated platelets in K3-EDTA ($P=0.00004$ and $P=0.0002$, respectively) and citrate PRP ($P=0.00009$ and $P=0.00004$, respectively) than dogs with normal platelet number. In all dogs the percentage of RPs in citrate PRP was significantly higher than in K3-EDTA PRP.

Discussion

TO and auramine were used in medicine to identify reticulocytes (immature blood cells, that contain RNA) for the first time in the 1980s (Lee et al. 1986). RPs were primarily described in 1969 by Ingram and Coopersmith (Ingram and Coopersmith 1969). Studies performed during the following years proved that TO staining is a non-invasive and clinically useful method for evaluating platelet production in the bone

Table 1. Results of haematological analysis in K3-EDTA and citrate whole blood in dogs with platelet count within normal limits 200-580 x10⁹/L (n=30).

Examined parameter	Whole blood							
	K3-EDTA				sodium citrate			
	values				values			
	min	max	mean	± SD	min	max	mean	± SD
WBC (10 ⁹ /L)	3.50	15.20	8.86	2.76	4.22	15.50	9.88	2.60
PLT (10 ⁹ /L)	210	518	282	68.70	71	489	155.96*	87
PCT (fL/μL)	1.7	3.7	2.5	0.5	0.7	3.4	1.3*	0.6
MPV (fL)	7.10	9.80	8.77	0.64	6.50	10.30	8.60	0.85

Minimal values – min, maximum – max, mean values, and standard deviation – SD. WBCC – white blood cell count, PLT – platelet count, PCT – platelet haematocrit, MPV – mean platelet volume.

Statistically significant differences in comparison with K3-EDTA whole blood: *p<0.05.

Table 2. Results of haematological analysis in K3-EDTA and citrate whole blood in dogs with platelet count between 100 and 200 x10⁹/L (n=22).

Examined parameter	Whole blood							
	K3-EDTA				sodium citrate			
	values				values			
	min	max	mean	± SD	min	max	mean	± SD
WBCC (10 ⁹ /L)	4.98	13.2	9.86	3.67	4.61	19.10	9.90	4.36
PLT (10 ⁹ /L)	119	185	157.35	25.77	10.00	170.00	105.35***	42.41
PCT (fL/μL)	1.1	2.1	1.5	0.2	0.1	1.5	0.9***	0.4
MPV (fL)	7.80	11.90	9.43	1.20	6.4	10.9	8.54***	1.28

Minimal values – min, maximum – max, mean values, and standard deviation – SD. WBCC – white blood cell count, PLT – platelet count, PCT – platelet haematocrit, MPV – mean platelet volume.

Statistically significant differences in comparison with K3-EDTA whole blood: *p<0.01.

Table 3. Results of haematological analysis in K3-EDTA and citrate whole blood in dogs with platelet count below 100 x10⁹/L (n=20).

Examined parameter	Whole blood							
	K3-EDTA				sodium citrate			
	values				values			
	min	max	mean	± SD	min	max	mean	± SD
WBCC (10 ⁹ /L)	6.50	15.10	13.60	2.89	5.90	14.60	12.18*	3.65
PLT (10 ⁹ /L)	5.00	92.00	53.21	31.51	0.00	85.00	33.37*	27.05
PCT (fL/μL)	0.1	1.4	0.6	0.5	0.00	0.9	0.4*	0.3
MPV (fL)	6.30	12.50	9.06	1.44	0.00	10.7	8.17*	2.30

Minimal values – min, maximum – max, mean values, and standard deviation – SD. WBCC – white blood cell count, PLT – platelet count, PCT – platelet haematocrit, MPV – mean platelet volume.

Statistically significant differences in comparison with K3-EDTA whole blood: *p<0.05.

Table 4. Number of pedigree dogs in each group: normal platelet count 200, – 580, $\times 10^9/L$ (n=30), platelet count between 100 and 200 $\times 10^9/L$ (n=22), and platelet count below 100 $\times 10^9/L$ (n=20) in K3-EDTA whole blood.

Dog breeds	Number of dogs at groups		
	platelet number 200-580 $\times 10^9/L$ (n=30)	platelet number 100-200 $\times 10^9/L$ (n=22)	platelet number less than 100 $\times 10^9/L$ (n=20)
Cavalier King Charles Spaniel	0	15	17
Greyhound	5	4	3
German Shepherd Dog	8	2	0
Schnauzer	1	1	0
Mixed breed dogs	16	0	0

Table 5. The percentage of reticulated platelets (RP) labelled by thiazole orange (TO) ($\bar{x} \pm SD$) in K3-EDTA and citrate platelet-rich plasma (PRP) in dogs with platelet count 200-580 $\times 10^9/L$, in dogs with platelet count 100-200 $\times 10^9/L$, and in dogs with platelet count less than 100 $\times 10^9/L$.

PRP	The percentage of RP (TO positive) in dogs with platelet count of:		
	200-580 $\times 10^9/L$ (n=30)	100-200 $\times 10^9/L$ (n=22)	less than 100 $\times 10^9/L$ (n=20)
K3-EDTA	9.71 \pm 2.12	17.95 \pm 4.94	11.57 \pm 1.60
Citrate	11.89 \pm 3.34***	22.92 \pm 7.33**	12.98 \pm 2.03***

Statistically significant differences in comparison with K3-EDTA PRP: **p<0.01, ***p<0.001.

marrow. From the practical point of view, this technique has multiple advantages including simple sample collection. Therefore, it may become a routine laboratory test for evaluation of thrombopoiesis. The percentage of RPs in healthy dogs in K3-EDTA whole blood and in PRP was determined as 11.9 \pm 9.4% and 11.9 \pm 8.2%, respectively (Maruyama et al. 2009). Another study showed that the percentage of RPs was under 7% in K3-EDTA whole blood of healthy dogs with normal platelet count that was stored for 4 hours; the value for non-stored blood was 0-4.3% (Smith and Thomas 2002). Mean RPs percentage obtained in K3-EDTA PRP of healthy dogs by Weiss and Townsend was 9.3 \pm 2.7%. In our study the percentage of RPs in K3-EDTA PRP of dogs with normal platelet count was 9.71 \pm 2.12, that is in agreement with Maruyama et al. and Weiss and Townsend (Weiss and Townsend 1998, Maruyama et al. 2009). The percentage of RPs in PRP was significantly higher in dogs with thrombocytopenia in comparison with dogs with platelet number within the normal range. The highest RPs percentage was observed in dogs with platelet count between 100 000 and 200 000/ μL in K3-EDTA and citrate PRP. These values were not as high as in dogs with IMT (6-50%) or Evan's syndrome (10- 72%) (Smith and Thomas 2002).

Data collected in dogs with IMT showed that percentage of platelets with PSAIgG varied between 5% and 81% (Wilkerson et al. 2001). In this study low

percentage of platelets coated with PSAIgG ruled out thrombocytopenia caused by immune factors.

Most of the dogs in the study group were CKCS and a high probability of thrombocytopenia in this breed was evidenced by Cowan et al. (2004), and Singh and Lamb (2005). Studies conducted by Davies et al. (2008) showed abnormal structure of the $\beta 1$ tubulin in CKCS, the protein which is accountable for normal platelets formation, and showed correlation between the mutation of the $\beta 1$ tubulin gene and macrothrombocytopenia in this breed (Davis et al. 2008).

Higher percentage of RPs in dogs with platelet number between 100 and 200 $\times 10^9/L$ comparing with dogs with platelet count below 100 $\times 10^9/L$, could be an effect of a lower number of megathrombocytes in the first subgroup (Olsen et al. 2001).

The presence of megathrombocytes is the reason of elevated MPV in dogs with thrombocytopenia. Furthermore, if the platelet count is under 100 $\times 10^9/L$, the total white blood cell count can be falsely elevated – megathrombocytes can be misclassified as leucocytes by an impedance haematology analyzer (Bertozolo et al. 2007). If such a case is suspected blood smears should be examined for detection of megathrombocytes.

A lower percentage of RPs in dogs with thrombocytopenia below 100 $\times 10^9/L$ can be caused by a high number of megathrombocytes in their blood.

Studies conducted by Cowan et al. (2004) showed numerous megathrombocytes in the blood of CKCS with low number of platelets. Their morphology was evaluated using an electron microscopy – differences between megathrombocytes and normal platelets were not noted.

K3-EDTA is an anticoagulant commonly used in haematology; sodium citrate, on the contrary, is mostly used for coagulation analysis. The percentage of TO-positive cells (RPs) was higher in citrate PRP – it could be caused by a non-specific binding of TO with platelet dense granules due to a higher platelet activity in citrate PRP than in K3-EDTA PRP. This theory may be confirmed by the results of the analysis of reticulocytes in K3-EDTA and sodium citrate-anticoagulated blood in humans; differences in the percentage of reticulocytes between anticoagulants were not noted (Lippi et al. 1999).

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