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Original article

# Flow cytometric assessment of activation of peripheral blood platelets in dogs with normal platelet count and asymptomatic thrombocytopenia

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## Abstract

Platelets play a crucial role in hemostasis. Their activation has not yet been evaluated in healthy dogs with a normal and low platelet count. The aim of this study was to determine the influence of activators on platelet activation in dogs with a normal platelet count and asymptomatic thrombocytopenia. 72 clinically healthy dogs were enrolled. Patients were allocated into three groups. Group 1 consisted of 30 dogs with a normal platelet count, group 2 included 22 dogs with a platelet count between 100 and 200x10<sup>9</sup>/l and group 3 consisted of 20 dogs with a platelet count lower than 100x10<sup>9</sup>/l. Platelet rich-plasma (PRP) was obtained from peripheral blood samples using tripotassium ethylenediaminetetraacetic acid (K3-EDTA) as anticoagulant. Next, platelets were stimulated using phorbol-12-myristate-13-acetate or thrombin, stabilized using procaine or left unstimulated. The expression of CD51 and CD41/CD61 was evaluated. Co-expression of CD41/CD61 and Annexin V served as a marker of platelet activation. The expression of CD41/CD61 and CD51 did not differ between the 3 groups. Thrombin-stimulated platelets had a significantly higher activity in dogs with a normal platelet count than in dogs with asymptomatic thrombocytopenia. Procaine inhibited platelet activity in all groups. In conclusion, activation of platelets of healthy dogs *in vitro* varied depending on the platelet count and platelet activator.

**Key words:** asymptomatic thrombocytopenia, canine, flow cytometry, platelet activation

## Introduction

Platelet activation in a response to a blood vessel wall damage plays a crucial role in the process of hemostasis. Platelets can become activated after adhesion to extracellular matrix in the site of endothelial

injury and/or in response to the action of secondary mediators such as thrombin, adenosine-5'-diphosphate (ADP) and thromboxane A<sub>2</sub> (Boudreaux et al. 2010). The degree of platelet activation depends on the number and the density of surface receptors, composition of cytoplasmic granules and the concentration of

signaling molecules. Also other biological components such as collagen, serotonin or adrenalin, anticoagulants or phorbol esters stimulate platelet activation (Casonato et al. 1994, Moritz et al. 2003). Procaine, on the contrary, inhibits this process by stabilization of human platelet membrane (Watafa et al. 1999).

Expression of integrin receptors and phosphatidylserine on the surface of platelets are considered as markers of their activation. Integrins are glycoprotein transmembrane receptors which are engaged in platelet adhesion and aggregation during the formation of a platelet plug. GP $\alpha$ Ib $\beta$ 3 (CD41/CD61) and GP $\alpha$ v $\beta$ 3 (CD51), adhesion molecules for fibrinogen, vitronectin, thrombospondin and von Willebrand (vW) factor, are considered as one of the most important platelet antigens (Philips et al. 1988, Boudreaux et al. 2010). Human vitronectin receptor GP $\alpha$ v $\beta$ 3 (CD51), on the other hand, is less abundant and is involved in the clotting cascade and in the process of fibrinolysis (Preissner and Seiffert 1998).

As the location of phosphatidylserine and other phospholipids in the platelet membrane depends on their functional status, they serve as markers of platelet activation (Dachary-Prigent et al. 1993). In non-activated platelets, phosphatidylserine is present only on the inner surface of the membrane. Platelet activation using thrombin, ADP or collagen causes its relocation to the extracellular surface through a protein kinase C-activated phosphatidylserine translocase. Thereby, this increases platelet procoagulant activity and enhances binding of fibrinogen leading to promotion of clot formation (Bode et al. 1991, Briddon et al. 1999). However, procaine has been shown to accelerate the depolarization of the mitochondrial membrane and to activate caspase-3 and caspase-9 leading to an increased phosphatidylserine expression (Alberio and Dale 1999).

Asymptomatic thrombocytopenia is common in several breeds of dogs, including Cavalier King Charles Spaniel (CKCS) and Greyhound (Olsen et al. 2001, Davis et al. 2008). Mild familial thrombocytopenia was also reported in German Shepherd, Shiba Inu and Akita Inu (Dachary-Prigent et al. 1993, Steiss et al. 2000, Singh and Lamb 2005, Bertazzolo et al. 2007, Larcia-Garcia et al. 2008). Interestingly, affected individuals, despite a low platelet count, are not more prone to the development of coagulation disorders (Brown et al. 1994, Cowan et al. 2004, Bertazzolo et al. 2007, Boudreaux et al. 2010).

The aim of the study was to assess the activation of peripheral blood platelets in healthy dogs with normal platelet count and asymptomatic thrombocytopenia. Platelet phenotype was determined by the evaluation of the expression of platelet surface

antigens and phosphatidylserine in K3-EDTA blood samples after incubation with platelet stabilizers, activators and without stimulation.

## Materials and Methods

### Animals and blood samples

Dogs with thrombocytopenia and normal platelet count were recruited in the study between January 2005 and December 2011 among patients undergoing routine blood screening at two veterinary clinics in Warsaw (Small Animal Clinic at the Faculty of Veterinary Medicine, Warsaw University of Life Sciences and Veterinary Clinic Guderskiego 5). The inclusion criteria were: lack of clinical and laboratory signs of disease, including absence of clinical coagulation disorders, no history of treatment or vaccination during the 4 weeks preceding the sampling, absence of platelet clumps in the blood smear. Dogs were assigned to 3 groups according to the platelet count in K3-EDTA-anticoagulated blood. Group 1 included dogs with a normal platelet count (between 200 and 580x10<sup>9</sup>/l). Patients with a platelet count lower than 200x10<sup>9</sup>/l were considered thrombocytopenic and were allocated to either group 2 (dogs with platelet count between 100 and 200x10<sup>9</sup>/l) or group 3 (patients with platelet count lower than 100x10<sup>9</sup>/l).

Peripheral blood samples were obtained by venipuncture from the cephalic vein into K3-EDTA tubes (Medlab, Raszyn, Poland).

### Hematology, clinical biochemistry and coagulation times

Complete blood count was determined within 20 minutes after blood collection using an Abacus hematology analyzer on K3-EDTA blood samples (Diatron, Budapest, Hungary). May-Grunwald Giemsa-stained blood smears were examined under a light microscope. Serum aspartate transaminase (AST) and alanine transaminase (ALT) levels were measured with an automated biochemistry analyzer Pointe-180 (Pointe Scientific, Warsaw, Poland) using a kinetic method. Urea concentration was evaluated on the same analyzer by urease method (indirect measurement). The absorbance was measured at 340 nm. Prothrombin time (PT) and activated partial thromboplastin time (APTT) were assessed in citrate plasma by a K-3002 Optic Blood Coagulometer (Elza-Bis Ksel-med, Grudzi dz, Poland) with nephelometric method using Dia-PT 2, Dia-APTT, Dia-CaCl<sub>2</sub> reagents (Bio-Ksel, Grudzi dz, Poland).

### Preparation of platelet rich-plasma

Platelet-rich plasma (PRP) was obtained using K3-EDTA blood samples by centrifugation (300 g, 10 min, 20°C) (Moritz et al. 2003).

### Identification of platelets / Identification of platelet surface receptors

The expression of CD41/CD61 (integrin  $\beta 3$ ) and CD51 ( $\alpha v\beta 3$  integrin – vitronectin receptor) was evaluated using monoclonal antibodies. Platelets from K3-EDTA PRPs were suspended in a physiological phosphate buffered saline solution containing 5 mM sodium azide, 0.1% glycine and 1% goat serum (PBS) (Watała et al. 1999, Wilkerson and Shuman 2001).

Samples were incubated for 20 min in the dark with a mouse anti-human CD41/CD61 antibody conjugated with fluorescein isothiocyanate (FITC) (clone VIPL2, BD Pharmingen, San Jose, USA), or a mouse anti-human CD51 antibody conjugated with phycoerythrin (PE) (clone LM609, Chemicon, Temecula, USA) (Poncz et al. 1987). Both antibodies were reported to be cross-reactive with canine antigens (Poncz et al. 1987, Schlossman et al. 1995, Lebrec et al. 2012). After washing, samples were resuspended in the buffer and analyzed.

### Evaluation of platelet activation

Annexin V was used to evaluate platelet activation by detection of platelets expressing phosphatidylserine. Before staining, platelets in K3-EDTA PRP were stabilized by addition of 10 mg/ml procaine (Polfa Tarchomin, Warsaw, Poland) and incubation for 20 min in the dark or activated by incubation as before but with 10 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, Saint Louis, USA) or 2 U/ml thrombin (Biomed, Warsaw, Poland) instead of procaine (Watała et al. 1999, Wilkerson and Shuman 2001, Moritz et al. 2003). After spinning (220 g, 8°C for 15 min), samples were suspended in Annexin V Binding Buffer (BD Pharmingen) (Wilkerson and Shuman 2001) and incubated with Annexin V labeled with PE (BD Pharmingen) and a mouse anti-human CD41/CD61 antibody (clone VIPL2, BD Pharmingen) for 20 min in the dark at room temperature. Samples were then washed with buffered saline solution, spun (220 g, 15 min, 8°C), resuspended in the PBS and analyzed within 30 min.

### Flow cytometric analysis

Samples were analyzed using FACSCalibur flow cytometer (BD) and CellQuestPro software (BD). 10,000 events were analyzed in each sample. Platelets and their fractions were identified on the forward scatter height (FSC-H) against side scatter height (SSC-H) plot. The normoplatelet region (R1) was set to include >95% of the platelets. Other platelet fractions, microparticles (R2) and aggregates (R3), were defined as particles smaller or larger than normoplatelets, respectively (Fig. 1) (Moritz et al. 2003).

### Statistical analysis

Results were presented as mean  $\pm$  standard deviation (SD). Statistical analysis was performed using STATISTICA 6.0 software (StatSoft, Tulsa, USA). The distribution of the data was tested by Shapiro-Wilk test. Wilcoxon signed-rank test was used to determine whether there were significant differences between procaine, PMA, thrombin or unstimulated platelets. The statistical significance of differences between patients groups was evaluated by Mann-Whitney U test. Differences were considered significant when  $p < 0.05$  and highly significant when  $p < 0.01$ .

## Results

### Animals

Seventy two dogs met the inclusion criteria and were enrolled in the study. Their age ranged from 1 year to 11 years (median 3.5 years), they were 26 intact males, 3 neutered males, 28 intact females and 15 neutered females. Among thirty dogs included in group 1, sixteen were of mixed-breed, five were Greyhounds, three Standard Schnauzers, three Miniature Schnauzers, two Giant Schnauzers and one was German Shepherd. The age ranged from 1 year to 11 years (median 4 years), they were 8 intact males, 2 neutered males, 8 intact females and 12 neutered females. Group 2 consisted of twenty two dogs, of which fifteen were Cavalier King Charles Spaniels, four Greyhounds, two German Shepherds and one was Miniature Schnauzer. They were 1 year to 9 years old (median 3.5 years) and 10 intact males, 10 intact females and 2 neutered females. Group 3 included twenty dogs, of which seventeen were Cavalier King Charles Spaniels and three were Greyhounds. Their age ranged from 1 year to 8 years old (median 3.5) and they were 8 intact males, 1 neutered male, 10 intact females and 1 neutered female.

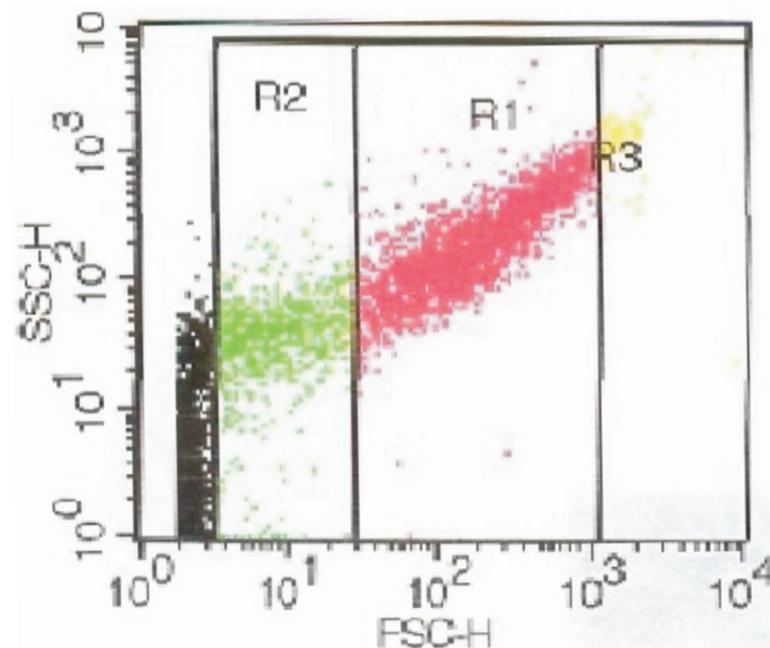


Fig. 1. Representative forward scatter height (FSC-H) versus side scatter height (SSC-H) plots showing the gating strategy of platelet fractions: normoplatelets (R1), platelet microparticles (R2), and aggregates (R3) in tripotassium ethylenediaminetetraacetic acid (K3-EDTA) platelet-rich plasma (PRP).

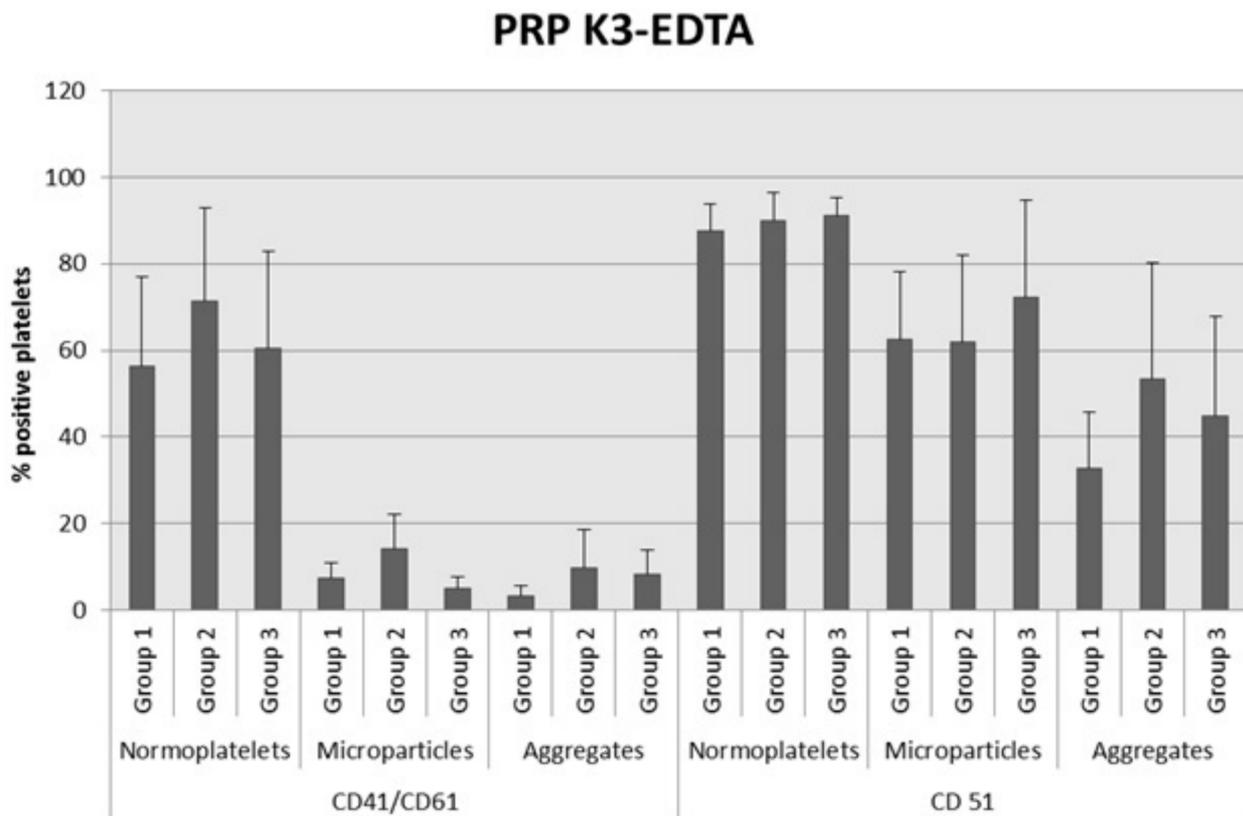


Fig. 2. The percentage of CD41/CD61+ and CD51+ normoplatelets, platelet microparticles, and aggregates in tripotassium ethylenediaminetetraacetic acid (K3-EDTA) platelet-rich plasma (PRP) in dogs with a platelet count between 200 and 580x10<sup>9</sup>/l (group 1) (n=30), between 100 and 200x10<sup>9</sup>/l (group 2) (n=22), and below 100x10<sup>9</sup>/l (group 3) (n=20). Mean ± standard deviation (SD).

## Clinical and clinicopathological findings

Patients' history, physical examination, hematology, clinical chemistry and coagulation times were unremarkable. However, some dogs had asymptomatic thrombocytopenia and they were included in group 2 or group 3. None of the patients had platelet clumps present in the blood smears.

## Expression of platelet surface receptors

It was shown that the percentage of CD51 and CD41/CD61+ platelets in the normoplatelet and microparticles fraction in all 3 groups was not statistically significant in comparison to unstimulated sample (Fig. 2).

## Platelet activation

Percentage of platelets co-expressing CD41/CD61 and Annexin V in the K3-EDTA PRP with or without stimulation is presented in Fig. 3.

In dogs with a normal platelet count (group 1) the K3-EDTA PRP incubated with procaine had a significantly lower percentage of normoplatelets and platelet microparticles co-expressing CD41/CD61 and Annexin V than in unstimulated samples. The numbers of double-positive normoplatelets and microparticles were highly significantly lower in comparison with the unstimulated samples in dogs with thrombocytopenia (group 2 and 3). In dogs with a normal platelet count and with thrombocytopenia the expression of CD41/CD61 in aggregates in PRP incubated with procaine was not statistically significantly different in comparison to unstimulated sample.

The numbers of double-positive normoplatelets after PMA activation in PRP were highly significantly greater in comparison with the unstimulated samples in normal dogs, but in microparticles and aggregates regions there were no statistically significant differences. In dogs with a platelet count between 100 and 200x10<sup>9</sup>/l (group 2) the numbers of CD41/CD61+ Annexin V+ platelets were highly significantly lower in comparison with the control samples only in aggregates region in PMA samples.

In group 3 (dogs with platelet count below 100x10<sup>9</sup>/l) the co-expression of CD41/CD61 and Annexin V in normoplatelet and aggregate subpopulation in the K3-EDTA samples was highly significantly lower after incubation with PMA in comparison to unstimulated sample.

The percentage of normoplatelets, platelet microparticles and platelets aggregates was significantly

lower or highly significantly lower after activation with thrombin in comparison with the unstimulated samples in all dogs (group 1, 2 and 3).

## Discussion

Our study confirmed that the expression of CD51 and CD41/61 on platelets surface does not vary between platelets regions and between dogs with normal platelets count and dogs with thrombocytopenia. Lack of differences in the expression of vitronectin and integrin  $\alpha_{IIb}\beta_3$  receptors between groups suggests that their conformation is stable in the K3-EDTA samples.

The results of this study have also confirmed that procaine is an inhibitor of platelet activity in both, dogs with a normal platelet count and dogs with thrombocytopenia. The percentage of CD41/CD61+ Annexin V+ platelets was lower in the presence of K3-EDTA in all platelet regions in comparison with unstimulated samples.

The anticoagulant properties of K3-EDTA are a result of removal of ions from the platelet environment. Elimination of calcium and magnesium decreases the stability of certain platelet membrane glycoproteins, thus causing transmission of the signal into the cells and initiation of release of alpha-granules. An increase in intracellular concentration of Ca<sup>2+</sup> is one of the factors responsible for platelet microparticles formation (Blockmans et al. 1995, Boudreaux et al. 2010). The percentage of microparticles stabilized by procaine was lower in comparison with unstimulated samples in all dogs groups and highly significantly lower in groups 2 and 3, but in group 1 it was significantly lower. The above finding confirms that procaine is a weak platelet membrane stabilizer. Procaine has been proven to selectively inhibit platelet activation in samples anticoagulated with acid citrate dextrose. Depending on its concentration it stabilizes platelet cell membrane, inhibits the release of calcium and decreases mobilization of calcium from the delta-granules (Watała et al. 1999).

PMA, a protein kinase C stimulator, slowly activates platelets causing their degranulation but not aggregation (Zucker and Nachmins 1985, Moritz et al. 2003). Moritz et al. (2003) have shown that a higher percentage of activated canine platelet microparticles and aggregates is present in the citrate blood after activation with PMA in comparison with the K3-EDTA PRP, by evaluation of selectin P expression. *Ex vivo* stimulation with PMA caused a marked increase in CD62P expression in normoplatelets and a mild increase in the region of platelet microparticles and aggregates (Moritz et al. 2003).

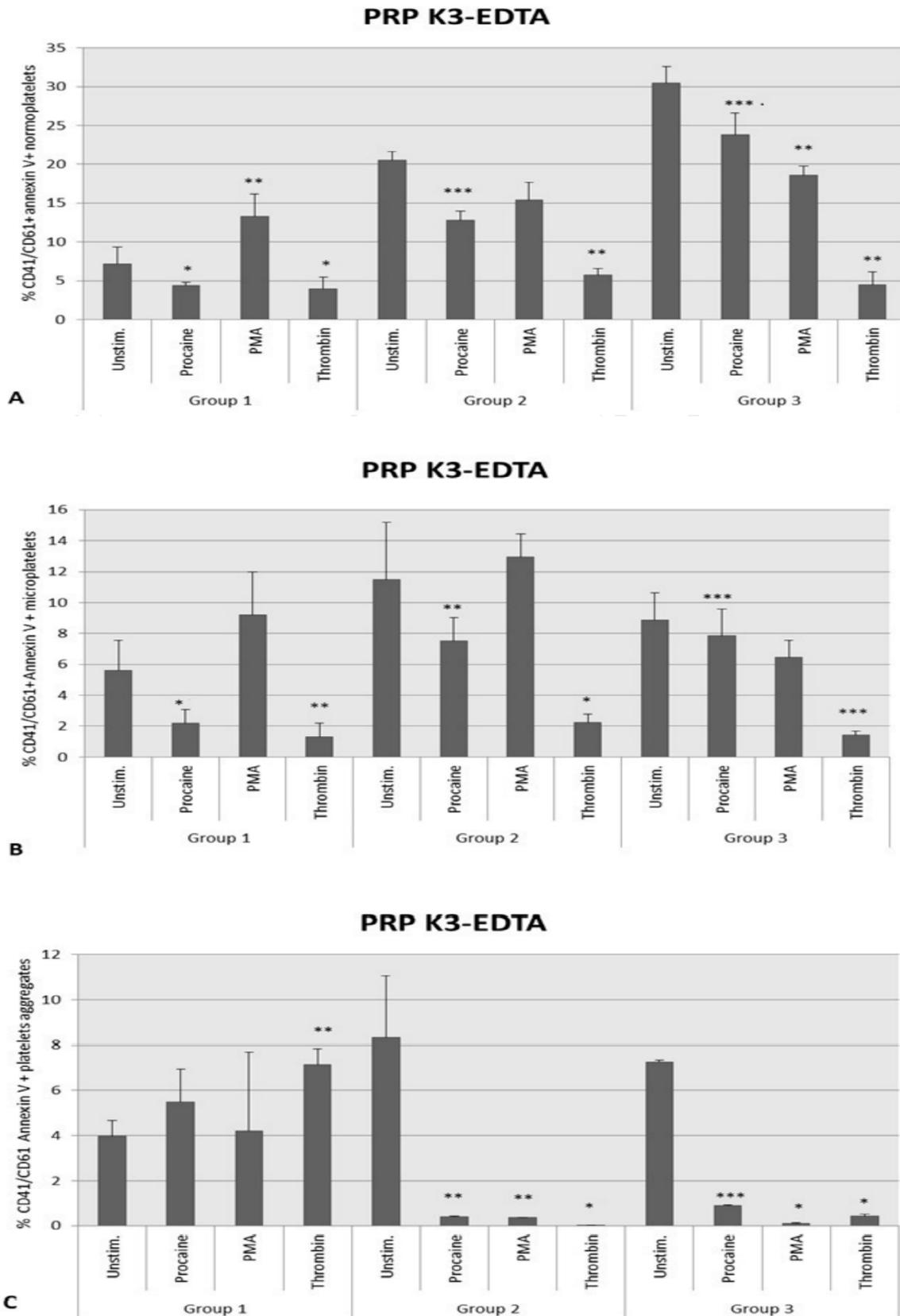


Fig. 3. The percentage of CD41/CD61+ annexin V+ normoplatelets (A), platelet microparticles (B), and aggregates (C) in tripotassium ethylenediaminetetraacetic acid (K3-EDTA) platelet-rich plasma (PRP) after stimulation with procaine, phorbol 12-myristate 13-acetate (PMA) or thrombin or unstimulated in dogs with a platelet count between 200 and 580x10<sup>9</sup>/l (group 1) (n=30), between 100 and 200x10<sup>9</sup>/l (group 2) (n=22), and below 100x10<sup>9</sup>/l (group 3) (n=20). Mean ± standard deviation (SD). Significant differences in comparison to unstimulated platelets \*p<0.05, \*\*p<0.01, \*\*\*p<0.001.

Our study confirmed that PMA is a platelet activator in dogs with a normal platelet count. PMA activation is less prominent in dogs with thrombocytopenia. In addition, the percentage of activated normoplatelets (CD41/CD61+ Annexin V+) in the K3-EDTA samples in dogs with a normal platelet count was higher than in the unstimulated samples. The percentage of activated platelets in group 2 was lower in the region of platelet aggregates, whereas, in group 3 it was lower in the region of normoplatelets and aggregates. This findings are in agreement with observation made by Moritz et al. (2003) and confirm the previous observation suggesting that PMA is a slow platelet activator (Whiss et al. 1998, Moritz et al. 2003).

GP $\alpha$ Ib $\beta$ 3 (CD41/CD61) is the most abundant of all platelet receptors. It is present not only on the cell surface but also in platelet alpha and delta granules and in the open canalicular system. Degranulation of platelet alpha-granules causes externalization of this receptor leading to its increased expression on the cell surface, making it an early marker of platelet activation (Poncz et al. 1987, Blockmans et al. 1995). Activation of human platelets by thrombin or collagen causes changes in the conformation of CD41/CD61 through phosphorylation of cytoskeletal and cytoplasmic proteins, allowing binding of fibrinogen and other cell adhesion ligands in the process of platelet aggregation (Philips et al. 1988, Blockmans et al. 1995, Boudreaux et al. 2010). It has been also shown that its expression is reduced in the later phases of clot formation due to its decreased accessibility to antibodies after binding of fibrinogen (Philips et al. 1988). As shown by this study the percentage of activated platelet aggregates after stimulation with thrombin was higher in dogs with a normal platelet count in comparison with unstimulated cells what confirms that thrombin is a strong physiologic platelet activator. A similar finding was not identified in group 2 or group 3 suggesting that platelets from thrombocytopenic dogs are less prone to thrombin activation. This observation may be explained by a weaker enzymatic action of thrombin on the protease-activated receptor-1 (PAR-1) and protease-activated receptor-4 (PAR-4) in comparison with dogs with a normal platelet count.

A limitation of this study was a risk of obtaining an artifactual platelet count due to the limited accuracy of the currently available diagnostic techniques. It could have led to misallocation of patients into the 3 groups. Since the dogs which had platelet clumps present in the blood smear were not included in the study, the likelihood of incorrect classification should not be considered as high. This eliminates the

risk of EDTA-induced thrombocytopenia and further decreases the danger of enrolling in the study dogs with pseudothrombocytopenia.

In contrast to the protocol of PRP preparation suggested by Moritz et al. (2003), physiological phosphate buffered saline solution used in this study did not contain formaldehyde (Moritz et al. 2003). Authors decided not to use formaldehyde following the literature data suggesting that it may influence the cell size and CD41/CD61 receptor properties (Watafa et al. 1999, Wilkerson and Shuman 2001).

Interestingly, unlike in nucleated cells, phosphatidylserine expression on the surface of peripheral blood platelets indicates their activation and has not been associated with the presence of any metabolic pathways characteristic for apoptosis (Bode et al. 1991, Dachary-Prigent et al. 1993).

Further research should assess the platelet activation in a greater number of dogs, including CKCS, as CKCS have been shown to be predisposed to a mitral valve insufficiency. Nearly all CKCS are affected. Dogs of this breed are a good candidate for an animal model of the human equivalent of this disease (Beadrow and Buchanan 1993). A greater availability of platelet activation markers such as CP62P (indicator of platelet degranulation) and PAC-1 (active fibrinogen receptor), could allow a more in-depth assessment of the course of platelet activation. Further studies of platelet functions should also focus on microplatelets, a particles with proven proinflammatory properties which participate in atherosclerotic plaque formation.

Peripheral blood platelets are an interesting object of research which extends beyond the field of hemostasis. Platelets are involved in a number of pathological processes. Studies evaluating the mechanisms of platelet activation and identifying factors which affect them are important problem of research in medicine. Further studies should also clarify the relationship between platelets and mitral valve insufficiency in CKCS. Studies in this field should be considered particularly important considering findings of Idriss et al. (2014) who demonstrated the role of platelets and microplatelets in the development of the mitral valve insufficiency in humans (Idriss et al. 2014).

In conclusion, the platelet activation was the strongest in dogs with a normal platelet count. Thrombin-stimulated platelets had a higher activity in dogs with a normal platelet count than in dogs with thrombocytopenia. Procaine decreased the number of activated platelets. The expression of CD51 and CD41/CD61 did not differ between the dogs with a normal and low platelet count.

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