

**Szent István University**  
**Postgraduate School of Veterinary Science**

**Examination of count, morphology and function of platelets in healthy  
and diseased dogs**

**PhD Thesis**

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# 1. Introduction

Platelets are the smallest cell in the blood, however their function is very complex. They participate in maintenance of the haemostasis, the save of the integrity of the capillary endothelium, the inflammatory and tissue regeneration processes, the tumour metastasis and also have bactericide characteristics.

The alteration in platelet count is common: thrombocytosis or more often thrombocytopenia may coexist with some disorders. However, there are just few references about the platelet morphology. The function of canine thrombocytes has been already studied in many aspects, however, these investigations have some contradictions.

**In the first part of our work** the most common reasons of thrombocytopenia or thrombocytosis in several diseased dogs were studied. The animals were chosen randomly. This was the first survey in Hungary dealing with the alteration of platelet numbers in a large population. We just collected many cases but did not intend to make statistical analysis.

**Secondly** the morphologic characteristics of platelets were studied. The investigation of platelets, as their structure is very poor, is difficult with light microscope when the smear is stained by routine haematological dyes such as May-Grünwald, Pappenheim, Giemsa, Diff Quick etc. Other cytochemical stainings, do not provide too much additional information. Giemsa-staining is widely used in haematology (Szász, 1981; Powers, 1989; Reagan et al., 1998; Cowell et al., 1999). Since it seems to be the simplest and most appropriate method to evaluate the fine structure of the platelets, this technique was chosen in our work. The main energy source of platelets is glycogen spread over the cytoplasm. Fortunately, this compound might be easily visualised by special dyes, such as periodic-acid Schiff's PAS-reaction (Jain, 1993), however, PAS-stained smears are less commonly used for evaluation of platelet morphology.

The platelets in canine blood are light blue and anucleated (Bessis, 1972; Jain, 1986; Bush, 1991; Hoffbrand and Pettit, 1997; Day et al., 2000). Most of them are medium sized (Cowell et al., 1999). They are round or oval (Bessis, 1972; Erslev and Gabudza, 1975; Handagama et al., 1986; Powers, 1989; Jain, 1993). If the smear was not fixed immediately after blood collection, bizarre shaped platelets may appear (Bessis, 1972). During blood collection thrombocytes may become activated and then grow thin cytoplasmic processes (pseudopods) (Cowell et al., 1999; Hoffbrand et Pettit, 1997). Platelets have multiple, fine purple granules in the cytoplasm, grouped in the centre or dispersed (Bessis, 1972; Jain, 1986; Bush, 1991; Hoffbrand et Pettit, 1997; Day et al., 2000). Aged platelets are smaller and have lower density than young ones (Bessis, 1972; Bush, 1991; Hoffbrand

et Pettit, 1997). In normal condition they have no vacuoles but if blood was collected into an EDTA-tube and was stored more than 24 hours, platelets could swell and show vacuolisation (Bessis, 1972). Even on a well-prepared blood smear small platelet clumps may form (Bessis, 1972; Hoffbrand et Pettit, 1997). Large clumps suggest unsuitable blood collection (Jain, 1986).

The staining of blood cells with PAS is usual in haematological diagnostic work (Szász, 1981), but PAS-staining characteristics of thrombocytes has not been intensively studied so far. Glycogen granules are scattered in the hyalomere and chromomere. In the majority of the studies quantitative changes in glycogen granules of thrombocytes were examined by electron microscope and there were just a few that used light microscope evaluation of PAS-staining smears (Bessis, 1972).

As there are only few detailed descriptions about light microscope morphology of the dogs' platelets, our goal was to investigate canine thrombocytes on Giemsa- and PAS-stained smears in healthy animals and in dogs with pathological conditions.

**In the third part** of my work the characteristics of platelet aggregation in dogs were investigated. Platelets participate in creating the primary haemostatic plug (adhesion, aggregation and release-reaction) and providing procoagulant surface for coagulation factors (McConnell 2000). Any of them can be disturbed but the alteration of the aggregation is the most common. The latest involves two processes: the phospholipid- and the eicosanoid- (arachidonic acid) pathways. The main point of the aggregation is the binding of fibrinogen to its platelet-receptor, GPIIb-IIIa. For the expression of the fibrinogen receptor several stimulating agents are necessary (Blaskó 1998, Jain 1993, McConnell 2000, Zhou and Schmaier 2005). In vitro platelet aggregation in animals has been widely studied (Sinakos and Caen 1967, Mason and Read 1967, Donner and Houskova 1972, Dodds 1978, MacMillan and Sin 1970, Clemmons and Meyers 1984, Soloviev et al. 1997).

#### *Optical aggregometry*

Nowadays the optical aggregometry in platelet-rich plasma (PRP) and whole blood aggregometry are spread (Born 1962, Kurata 1995, Soloviev 1997). In optical aggregometry platelet rich plasma (PRP) is used which is obtained by high speed centrifugation of the citrated blood. Platelet poor plasma (PPP) is also prepared by low speed centrifugation.

The aim of our study was to describe the characteristics of the canine platelet aggregation response to ADP, EPI and ristomycin agonists in healthy dogs using firstly CARAT TX4 (Carat Ltd, Budapest, Hungary) optical aggregometer in veterinary practice that applies as low volume as 180 µl PRP. We also intended to investigate the effect of some diseases on platelet aggregation responses to different agonists. Thirdly, the effect of long-term administration of ketoprofen and carprofen treatment was evaluated.

### *Mechanical methods: the PFA-100*

Tests of platelet functions, especially those for evaluation of aggregation, are limited to laboratories equipped with aggregometers. The optical aggregometry not a routinely used method, and it requires also specific sample handling. There has been a demand in aggregometry for less difficult but reliable techniques and instruments: The PFA-100<sup>®</sup> analyser (Dade-Behring) is a relatively new point-of-care device which is claimed to be able to fulfil the criteria that are necessary for appropriate evaluation of platelet function. It was first described in human medicine (Kundu et al., 1995). The PFA-100 is sensitive and accurate for the study of both congenital and acquired platelet defects (Francis et al., 1999). It is also designed to provide an in vitro quantitative measurement of platelet adhesion and aggregation in whole blood under high shear condition (Kottke-Marchant et al., 1999). The method used by the PFA-100 is based on occlusion of the aperture of the device. The first report on the application of PFA-100 in veterinary medicine was in 1998 by Keidel and Mischke who evaluated also the precision of it (1998, 2002, 2003).

The effects of non-steroid anti-inflammatory drugs (NSAIDs) on platelet function are well known both in human and veterinary medicine (Cronberg et al., 1984; Escudero et al., 1986; Drvota et al., 1991). Carprofen (6-chloro-alpha-methyl-carbazole-2-acetic acid) and ketoprofen (2-(3-benzoylphenyl)-propionic acid) are propionic-acid derivates. Both are extensively used as new NSAIDs in veterinary practice.

The present investigation was conducted to examine further the PFA-100 results for healthy, carprofen and ketoprofen-treated dogs. One aim of the study was to establish reference ranges in greater number of healthy dogs than previously was made. For clinicians it might be important to see how these two NSAIDs influence platelet function results provided by PFA-100 as disadvantageous consequences of long-term NSAID-treatment is well-known (Dowling, 2001).

## **2. Materials and methods**

### **Examination of quantitative platelet disorders**

Three hundred and one dogs with different breed, age and gender were involved in our investigation. In each case the platelet number were altered. Animals were divided into two groups: dogs with thrombocytopenia (n=212) and dogs with thrombocytosis (n=89). We described those diseases which coexist with decreasing or increasing platelet count. The ratios were given in percentages. Statistical analysis was not made.

## **Examination of morphologic alteration**

Blood samples of 201 dogs of several breed, age and sex arrived at the Small Animal Clinic of Internal Medicine, Faculty of Veterinary Science, were involved in the investigation. Based on the final clinical diagnosis, 2 main groups were retrospectively formed: Group 1 (n=20 healthy, control dogs ) and Group 2 (n=181 diseased dogs). Among them 84 animals were suffering from diseases affecting directly the haematological parameters or the haematopoietic tissue. In Group 2 further 97 diseased dogs were suffering of other diseases.

For Giemsa-staining venous blood was collected from all patients into a vacutainer tube with 3.8% sodium citrate as anticoagulant followed by preparation of two blood smears stained by Giemsa. Stained blood smears were first scanned with 400x magnification to notice platelet clumps. Details of intracellular morphology required a 1000x magnification with oil immersion objective. Depending on the patient's platelet count, 50 to 100 platelets were evaluated in each case. Aspects of evaluation were staining characteristics of platelets, presence of anisocytosis, polymorphism, activated and non-activated forms, normal and pathologic granulation, vacuolisation of cytoplasm and other findings.

For PAS staining venous blood samples were collected into K-EDTA-coated vacutainer tube. Immediately thin blood smears were made and stained with PAS method (Bessis, 1972; Szász, 1981). In each smear 50-100 platelets were evaluated according to the following aspects:

- PAS-negative platelets (not stained by PAS): cytoplasm is pale with indefinite cell border
- PAS-positive platelets: cytoplasm is pale and acidophil with fine or rough, purple granules

Similarly to the Giemsa-stained smears, 50-100 platelets were studied. The proportion of PAS-positive and –negative platelets was given and the type of granulation was evaluated.

All reagents for Giemsa- and PAS-staining were purchased from Sigma Ltd.

## **Optical aggregometry**

During a two-year period we collected blood samples from 115 dogs of several breed, age and gender at the Small Animal Clinic of Faculty of Veterinary Science, Szent István University. Dogs were divided into three groups: healthy controls (n=43) having had no medication for 2 weeks, diseased dogs (n=47) and NSAIDs-treated, arthropathic dogs (n=21) having been administered carprofen (2-4 mg/bw twice daily) or ketoprofen (1 mg/bw once daily) for at least 10 days before analysis. All dogs had normal haemostasis-profile. Four and half ml venous blood was taken from each dog into a 3.8% sodium-citrate tube (9:1 v/v) for aggregometry and for haemostatic profile, and further 2.5 ml into a K-EDTA-anticoagulated tube for CBC. For aggregation studies PRP was separated from citrated blood using a light spin centrifugation at 150 g for 10 minutes. Platelet poor

plasma (PPP) was prepared by further centrifugation of the remnant blood with high spin (2000 g for 10 minutes).

M200 two-channel (Omron Ltd., Budapest) and Carat TX4 optical aggregometer (Entec GmbH., Ilmenau, Germany) with computer-based aggregation curve analysis were used. ADP, EPI and ristomycin reagents were purchased from Reanal Ltd. (Budapest, Hungary). Only one final concentration of each agents was applied (ADP 10  $\mu\text{mol/L}$ ; ristomycin 1,5 mg/ml; epinephrine 10  $\mu\text{mol/L}$ , respectively).

Carat TX4 is a four-channel optical aggregometer. The central unit measures the optical density of both PPP and PRP by an infrared optical system and gives their ratio as optical density ratio (ODR). The ODR value must be between 250-350. When higher value is displayed PRP should be diluted with PPP. Due to this advantageous function of Carat TX4 it is not necessary to determine the platelet number of PRP. Besides, the analyser shows another unit smaller in correlation with the light transmittance of PPP. If this value is out of 700-900, PPP is not appropriate (i.e. it is lipaemic or contains too many platelets). The response of platelets to stimulating agonists was measured for maximum 10 minutes as recommended by Bennet (1990), Sink (2002), Zhou and Schmaier (2005). Twenty or 30  $\mu\text{l}$  of each agent was added to 180 or 270  $\mu\text{l}$  of PRP, depending on the available volume of blood. The results of aggregation curves were evaluated according to the presence of aggregation-response to the agent (if any), the presence of lag time (i.e. the time necessary to the induction of platelet aggregation after adding of the agent), the presence of shape change before aggregation (causing a temporary decrease in light transmittance on the curve), the aggregation maximum in % of PRP-caused light transmittance (i.e. the maximal amplitude of the aggregation curve), the presence of primary and secondary phase of aggregation and the reversibility or irreversibility of the aggregation.

### **Mechanical method: the PFA-100**

Altogether 96 dogs of differing breed, age and gender were involved in the experiment. Fifty-eight healthy dogs of the hospital-staff and of veterinary students were used as controls. Dogs did not have either historical evidence or physical and laboratory examination of a bleeding tendency. The remaining 38 elderly dogs with arthropathies (chronic degenerative joint disease) represented the treated groups. These dogs did not suffer of other diseases, were not treated with any other medicine and their blood and urinary parameters were normal. NSAID-treatment was either 1 mg/kg ketoprofen (n=7) or 2-4 mg/kg carprofen (n=31) per os daily for a minimum period of 5 days. The PFA-values of dogs receiving ketoprofen or carprofen were compared to those of healthy dogs. Venous blood was drawn into Na-citrated vacutainer tubes (9:1 blood/citrate v/v ratio) for PFA-100 analysis and for the determination of hemostasis parameters. For PFA-100 analysis whole blood



was used. All measurements were performed within 2 hours after blood collection. The PFA-100 platelet function analyser (Dade Behring Inc., USA, CA) was used following the manufacturer's recommendations. Both ADP and EPI cartridges were applied. The platelet count and haematocrit value were determined in K-EDTA-treated blood samples by an Abacus Vet Junior haematology analyser (Diatron GmbH., Austria).

In the first instance 800-800 µl of citrated blood are pipetted into two types of disposable ADP-collagen- or EPI-collagen containing cartridges. The platelet aggregation stimulated by these two reagents is monitored by the time required (closure time, CT) for full occlusion of an aperture and is expressed in seconds (ADP-CT and EPI-CT, respectively). The maximum measurable CT in both cases is 300 sec. Longer CTs are uniformly displayed as >300 sec. Results are expressed as mean ± SD with ranges. Results of the automatic platelet function analysis in the healthy and treated groups were compared by ANOVA test for ADP-CT. Kruskal-Wallis rank sum test was used for EPI-CT because the values longer than 300 sec were not exactly known and it was possible that ANOVA was unable to find existing difference between groups. For the analysis the R 2.1.1 Language and Environment for Statistical Computing programme was used (R Development Core Team, 2005).

### **3. Results**

#### **3. 1. Examination of quantitative platelet disorders**

The most common cause of thrombocytopenia were: haemolysis, myeloproliferative disorders, nephropathy, hepatopathy, pancreatitis, coagulopathy, DIC, splenic haemangiosarcoma. Thrombocytosis was observed most often after haemolysis, in gastrointestinal disorders, in splenic haemangiosarcoma and hepatopathy. We established that in certain diseases both thrombocytopenia and thrombocytosis may occur.

#### **3. 2. Examination of morphologic alteration**

##### *Giemsa-staining*

##### *Staining characteristics of platelets*

In the control dogs platelets uniformly stained pale blue usually with distinguished granulomere (centromer) and hyalomere zone. Polychromasia, with appearance of basophil (hyperchromic) and mildly stained (hypochromic) platelets, occurred in diseased animals with different frequencies. Most often it was found in bleeding (especially thymus apoplexy) and haemolytic disorders

(immunmediated haemolytic anaemia, IMHA), nephropathies, gastrointestinal disorders and endocrinopathies (first of all in diabetes mellitus and Cushing's disease).

#### *Anisocytosis*

In healthy dogs the dominant platelets were medium-sized with some macrocytes. Anisocytosis was found almost in all diseases in Group 2, except immune-mediated thrombocytopenia. The most obvious anisocytosis was noticed in hyperadrenocorticism and diabetes mellitus and was less frequently found in neoplasmas. Predominance of microcytes or giant platelets was rarely observed. Number of microcytes exceeded the number of any other-sized platelets in nephropathies, hepatic neoplasm, Cushing's-disease and diabetes mellitus. Presence of giant platelets characterised especially the bleeding and haemolytic disorders including thymus apoplexy and IMHA.

#### *Polymorphism*

In the control dogs only round- and oval-shaped platelets were found. In diseased animals irregular forms implied usually comma-, ribbon- or butterfly-shaped platelets. In certain haemopoietic disorders such as lymphoma and idiopathic thrombocytopenic purpura we did not find any alterations of platelets' shape at all. In thymus apoplexy we found severe polymorphism involving almost all the platelets.

#### *Presence of activated and non-activated platelets*

Morphological sign of activation is the appearance of fine cytoplasmic processes (spiderleg-like pseudopods) on the surface of the platelet. This phenomenon can be often found in Giemsa-stained smears even in healthy animals. We formed an arbitrary classification and considered the appearance of non-activated platelets pathologic if their number exceeded the 50% of all platelets and simultaneously showed other morphologic abnormalities (e. g. hypochromasia, presence of vacuoles). In control dogs the presence of activated and non-activated platelets was recorded in similar amount. Very high ratio of non-activated platelets was found in dogs in Group 2 with thymus apoplexy, smaller ratio in nephropathies and in some other diseases. Thick cytoplasmic processes - which were different from the well-known fine pseudopods - were found on platelets' surface in certain haemolytic disorders (such as IMHA, babesiosis), hepatopathies, splenic hemangioma and sepsis.

#### *Normal and pathologic granulation*

Control dogs' platelets had fine, diffuse azurophil granules in their cytoplasm. In the diseased group two characteristic, pathological granulations were recorded:

- pseudonuclear formation, i.e. a strong granulation in the centre of the cell that looked like a well-stained nucleus,
- spot-like formation near to the periphery of the platelet: a strongly stained, one or more big granule localised eccentric.

Almost in all dogs in Group 2 mild to intense pathologic granulation was observed. Spot-like formation occurred more frequently than pseudonuclear formation. Dogs in Group 2 with thymus apoplexy, diabetes mellitus or Cushing's-disease had abnormal, granulated platelets in great number. Pseudonuclear formation of granules was found mainly in IMHA and certain cardiac diseases. In dogs with idiopathic thrombocytopenic purpura and skin diseases platelets did not show any pathologic granulations.

#### *Vacuolisation of the cytoplasm*

In healthy dogs we never met this phenomenon. However, one or more vacuolated platelets were often found in Group 2: most frequent in thymus apoplexy and was considerable in pancreatitis, diabetes mellitus and hyperadrenocorticism, as well.

#### *Other findings*

We found a previously unknown, most probable physiological phenomenon in some platelets both in healthy and diseased dogs. It was a fine, ring-like, azurophil formation near the periphery of the cell. It appeared approximately in 1-2% of platelets.

#### *PAS-staining*

On PAS-stained smears PAS-negative and PAS-positive platelets were recorded in both groups with different proportion. These cytoplasmic granules were *concentrated* on the periphery of platelets (e.g. strongly-stained granules near the cell membrane) or *eccentric* (similarly stained granules somewhere in the cytoplasm). In some cases several fine or rough granules were *diffusely* scattered in the cytoplasm providing a strong, acidophil character to the platelet.

In 20 healthy dogs most of platelets (62%) were PAS-negative. Among PAS-positive platelets the number of platelets containing concentrated granules exceeded those that had diffuse granulation. The granulation was usually mild. In dogs with haematological disorders increased percentage (>65%) of PAS-positive platelets was found. In case of haemorrhagic and haemolytic disorders very strong concentrated cytoplasmic granulation was observed while in immunmediated thrombocytopenia the intracellular distribution of PAS-positive granules was diffuse. In dogs suffering of pyometra and diabetes mellitus almost all platelets were PAS-positive, and proportion of PAS-positivity was also substantial in sepsis, nephropathy and neoplasmas. The number of platelets with strongly-stained granules also increased in these disorders. However, while in nephropathy there were mainly peripheral granulations, in sepsis and pyometra the granules situated mainly eccentrically.

### **3. 3. Optical aggregometry**

Among the three agents only ADP induced aggregation responses in all healthy dogs while EPI and ristomycin stimulated the aggregation similarly in less than half of them. Mean aggregation maximum caused by the three agents was similar with wide ranges. In the healthy dogs we always experienced response to ADP while the aggregation response to EPI and ristomycin was variable. The shape change and the feature of the curves did not show significant changes in the effect of any diseases studied. The ristomycin-induced aggregation was often associated with a lag time that was never present in healthy dogs.

Several diseases influenced the aggregation maximum in ADP-induced response. Chronic renal disease caused inconsistent effects as increased and decreased aggregation maximum values were also found. The most substantial increase of aggregation maximum in the effect of ADP was found in a lymphoma and in Cushing's disease. Ristomycin-induced aggregation maximum in diabetes mellitus nearly doubled. The decreasing and increasing effect of mastocytoma to ADP and to EPI is were also observed. Some disorders (gastro-enteritis and prostatitis) did not influence the aggregation responses at all.

Effect of ketoprofen- and carprofen treatment on platelet aggregation was evaluated only in ADP- and EPI-stimulated PRP. Presence of the aggregation response to ADP and EPI in carprofen-treated dogs was similar to those in healthy ones. Aggregation maximum in ketoprofen-treated dogs on ADP and EPI stimulation was only 61% and 41% of the healthy animals, respectively. NSAIDs did not influence the shape change, lag time values and feature of the aggregation curve.

### **3. 4. Mechanical method: the PFA-100**

There were no significant differences in the closure times measured by ADP and EPI cartridges among control, carprofen or ketoprofen treated dogs ( $P=0.1830$  for ADP-CT [ANOVA] and  $P=0.0572$  [Kruskal-Wallis test] for EPI-CT).

## 4. Discussion and conclusions

### Examination of morphologic alteration

The most frequent morphologic alteration in Giemsa-stained platelets was polychromasia with appearance of basophil (hyperchromic) and mildly stained (hypochromic) platelets. Basophilia refers to ineffective thrombopoiesis and the presence of young platelets (Bessis, 1972). In our study polychromasia has been firstly found in connection with some disorders (thymus apoplexy, nephropathy, diabetes mellitus and Cushing's-disease). Hypochromic cells presumably correspond to aged or agranular platelets (Jain, 1993). Anisocytosis is the sign of disturbance of thrombopoiesis (Bessis, 1972). Interestingly, we did not meet anisocytosis in immunmediated thrombocytopenia most probably due to the low platelet count and the few cases (n=4) we studied. The presence of microthrombocytes usually refers to iron deficiency and immunmediated thrombocytopenia (Bush, 1991; Hoffbrand et Pettit, 1997; Day et al., 2000). We found that some other disorders (nephropathy, hepatic neoplasm, diabetes mellitus and Cushing's disease) were also associated with microcytosis. Probably an iron metabolism disorder and a secondary iron deficiency that accompanied these disorders were in the background. Giant platelets are always pathological in canine blood smears (Cowell et al., 1999). Rarely they appear in serious thrombocytopenia but more often in thrombocytosis, thrombasthenia and thrombocytopathy as a sign of ineffective thrombopoiesis or increased demand for platelets at the periphery. It may be a regenerative response of the bone marrow and the sign of forced thrombopoiesis as the healthy tissue is able for fast compensation resulting in massive haemopoiesis, even forced thrombopoiesis (Bessis, 1972, Jain, 1986 Bush, 1991; Cowell, Hoffbrand et Pettit, 1997; 1999; Day et al., 2000). Varied shapes of platelets have been already described (Bessis, 1972; Hoffbrand and Pettit, 1997). The appearance of these irregular forms probably refers to bone marrow disorders or thrombocyte functional defect (Cowell et al., 1999; Hoffbrand and Pettit, 1997). If platelets are large, abnormally-shaped and there is simultaneously thrombocytopenia, it suggests increased platelet destruction at the periphery (Bush, 1991). It may develop also in thymus apoplexy. We supposed that a hypochromic, spherical platelet was functionally impaired. Appearance of platelets with thin cytoplasmic processes is a physiological phenomenon (Cowell et al., 1999; Hoffbrand and Pettit, 1997), these cells might have activated during the blood collection procedure (Jain, 1986). Unfortunately, one can hardly find any directions how to establish „pathologic activation” on smears in the references. We highly recommend executing the blood collection as much precisely and quickly as possible to avoid thromboplastin contamination and consequent arbitrary platelet activation on the smear. In our investigation another new morphological observation has also been described, i.e. presence of one or more thick cytoplasmic process(es) on the platelet's surface. It may be a sign of extreme form of

platelet activation. The appearance of non-activated (supposing non-functioning) platelets on smears occurred never alone but together with other morphologic alterations as found by others (Bessis, 1972). In this study we found two new granule-formations of in Giemsa-stained thrombocytes. The pseudonuclear formation of granules until now has been described only in healthy cats' blood smears (Bessis, 1972; Hoffbrand and Pettit, 1997). In the case of ineffective thrombopoiesis a few or more distinguished granules can be seen in platelets' cytoplasm (Bessis, 1972). Atypical granulation can be found in the functional defect of the bone marrow (Hoffbrand et Pettit, 1997; Cowell et al., 1999). Presence of pseudonuclear and spot-like formed granules supposes dysthrombopoietic background, including forced bone marrow activity and increased platelet turnover. This process might be resulted in impaired cytoplasm maturation with pathologic granule distribution. The reason of the special distribution of granules is unknown. The granules are absent from the cytoplasm in disseminated intravascular coagulopathy (DIC; Bush, 1991). Vacuolisation – which does not necessarily mean the functional defect of platelets – was also described in DIC (Bush, 1991). We found platelet vacuoles in some other diseases (thymus apoplexy, pancreatitis, diabetes mellitus, and Cushing's disease), too. We think that the reason of vacuolisation might be of toxic or immunmediated origin. Both in healthy and diseased animals sometimes we found a ring-like formation near the periphery of the platelets that has not been mentioned before on Giemsa-stained smears. It might be the cell's cytoskeleton consisted of microfilaments and microtubules. In resting (non-activated) platelets they form a ring under the cell membrane in humans (Bessis, 1972). It might be examined only with electron microscope and has not been described on routinely stained blood smears in animals so far.

It is known that the young, functionally and metabolically active platelets contain more glycogen than the resting ones (Bessis, 1972; Hoffbrand and Pettit, 1997). The cyclic adenosine monophosphate (cAMP) of the platelet regulates their activity. During the shape-change, at the beginning of the activation, the metabolism of platelet is growing, with increasing glucose oxidation. Every substance that causes the increase of the intracellular cAMP results the growing of the glycogen content of the cell (Jain, 1986). The increased number of PAS-positive glycogen granules supposes a more active glycogenesis. Certain disorders, such as septic diseases, may accompany by serious thrombocytopenia. As the result of the response of the bone marrow, forced thrombopoiesis occurs and the presence of many PAS-positive platelets is supposed to be the sign of this response: the greater PAS positivity the stronger glycogenesis is supposed. The intracellular distribution of glycogen can be pathological in several disorders. When glycogen granules form large clumps in more than 10% of platelets, it refers to impaired thrombopoiesis (Bessis, 1972). In thrombocytosis and thrombocytæmia increased number of glycogen particles was also observed (Bessis, 1972). The explanation of presence and changes in distribution of PAS-positive granules in

several disorders are unknown. Most probably electronmicroscopic investigations are necessary to explain these phenomena. According to our values the decrease of the amount of glycogen particles in platelets seems to be not common. They may be metabolically less active cells than those giving stronger PAS positivity.

Finally, we concluded that alterations of platelet morphology do not characterise exclusively any of the disorders studied in our case. It is interesting that the most significant platelet abnormalities (polychromasia, polymorphism, and presence of non-activated platelets, pathologic granulation and vacuolisation) were observed in thymus apoplexy. It suggests increased platelet destruction and utilisation. In diabetes mellitus and Cushing's disease also several morphologic alterations were described. In immunmediated thrombocytopenia platelets do not have morphological abnormalities, only microcytosis occurred on Giemsa-stained smears. In the case of forced thrombopoiesis basophil, giant platelets with or without thick cytoplasmic processes were often found. We suppose that their appearance helps to decide whether the earlier thrombocytopenia was regenerative or not. This may be a new diagnostic observation, which, similarly to the evaluation of anaemias, proves the ability of the bone marrow to response. Although platelets' morphologic evaluation by light microscope is very difficult, there are some aspects during Giemsa- or PAS-stained blood smear evaluation that may help our diagnostic work in practice in the future.

### **Optical aggregometry**

The Carat TX4 optical aggregometer proved to be an appropriate device for canine platelet aggregation studies. Its advantages are the low sample volume (down to 180  $\mu$ L), easy PRP sample handling (as ODR is given), the computer-directed test procedure and the possibility of the direct data export to MS Excel<sup>®</sup>.

Studying the aggregation response to the three agents used in our experiment we always established aggregation response to ADP. These experiences were supported by the findings of Sinakos and Caen (1967), Soloviev et al. (1997) and Feingold et al. And (1986). The feature of the curve was always biphasic, most commonly reversible. Mischke and Schulze (2004) established 71-95% values caused by 10  $\mu$ mol/L ADP which were higher than our 41% aggregation maximum. Clemmons et al. (1984) established  $65.1 \pm 13.1$  %, Callan and Gigers (2001) observed 60% or larger aggregation maximum. However, Boudreaux et al. (1994) found the aggregation maximum as  $27 \pm 19$ % that is even smaller than our values.

As EPI acts via excitatory  $\alpha$ -receptors and inhibitory  $\beta$ -receptors of thrombocytes, the response of mammalian platelets to this agent is variable depending on the adrenoreceptor type present on platelets (Soloviev et al. 1997). Mason and Read (1967) and Soloviev et al. (1997) described that canine platelets always had responded to EPI while Sinakos and Caen (1967) found that they did

not. Mischke and Schulze described variable responses of canine platelets to EPI or no response at all (2004). In most of our healthy dogs there was no aggregation response to EPI, which supports the results of Mischke and Schulze who established that 5, 10, 25 and even 50  $\mu\text{mol/L}$  EPI could not cause aggregation.

Chemical characteristics of ristomycin and its effect on in vitro aggregation is similar to those of ristocetin, therefore ristomycin can substitute it in aggregation studies (Howard and Firkin 1971, Pflieger et al. 1985). Ristocetin is an antibiotic, causing the change of the turbidity of PRP due to agglutination of platelets (Howard and Firkin 1971, Soloviev et al. 1997). In humans the platelets respond to 1.2 mg/ml ristocetin producing a monophasic aggregation curve without shape change (Sinakos and Caen 1967). We used ristomycin at 1.5 mg/ml concentration, as 1.2 mg/ml concentration did not cause aggregation response at all. This elevated concentration of ristomycin caused aggregation in 46% of healthy dogs. There are contradictory experiences about the presence of ristocetin-induced platelet aggregation in healthy dogs. Most researchers found no quantitative aggregation response (Jain 1993, Leis et al. 1980, Feingold et al. 1986). However, Soloviev et al. (1997) recognised that platelets in 7 of 19 dogs (36,8%) responded to ristocetin in whole blood using 1 mg/ml concentration.

Appearance of shape change before aggregation was variable. Both in healthy and diseased dogs we detected shape change when ADP was used. EPI did not induce shape change in aggregation response in our study similarly to the results of Boudreaux et al. (1996). We also observed shape change in ristomycin-induced aggregation that had not been mentioned before.

Lag time is not a common finding of aggregation response. In our healthy dogs it was never observed in ADP-induced aggregation. Similarly to our results, Clemmons and Meyers (1984) also experienced that when aggregation response to EPI was present, it was usually not immediate and developed often following a 3-8 sec lag time. As for lag time in ristomycin-induced aggregation, it has not been established in healthy dogs.

In spite of the fact that the effects of only few diseases and in a small number were evaluated some interesting conclusions can be made. One of the most prominent increases in platelet aggregation to ADP and ristomycin but not to EPI was found in diabetic dogs that has not been described in this species so far. In human diabetics the increased platelet aggregation was often described to ADP due to the microvascular complications (Sagel et al. 1975, Jennings et al. 1986). They experienced increased response also to EPI, however, we did not find it in dogs. We established increased aggregation maximum to ADP in dogs with Cushing's disease. Casonato et al. (1999) described increased response to ristocetin in Cushing's disease in men while Ikkala al. (1985) found decreased aggregation response to ADP and EPI. In pancreatic fibrosis we found increased aggregation response to ADP that has not been mentioned in dogs. In chronic renal disease we observed



different responses. In nephrosis syndrome the aggregation maximum to ADP increased which was in good agreement with the results of Green et al. (1985). We found different responses (hypo- or hyperaggregability) using 10  $\mu\text{mol/L}$  ADP depending on the type of the renal disease. Mischke and Schulze experienced that the response depends on the concentration of ADP, too (2004). Similar results in chronic renal diseases other than nephrosis syndrome were mentioned by Wardrop et al. (1989). However, Brassard et al. (1994) in experimentally induced renal failure and Forsythe et al. (1989) in uremic dogs found normal platelet aggregation with impaired adhesion. Dogs with lymphoma and lymphoid leukaemia had increased aggregation maximum to ADP, as it was described by Boudreaux (2000) and by Thomas and Rogers (1999). However, in our patients a decreased response was found to EPI and ristomycin. As in lymphoproliferative disorders platelets are more active and their production is forced in the bone marrow (Jain 1993, McNiel 1997), their hyperresponsiveness might be explained. We found slight increase in response to ristomycin in haemangiosarcoma and to EPI in mastocytoma that has not been mentioned so far also with increased response to ADP. Bishop and Donald (1979) also investigated the platelet aggregation-inducing effect of mastocytoma cells via intravenously applied tumour cells in mice but they could not induce aggregation of platelets. There were some diseases that caused only decreased aggregation of platelets. Sepsis was a condition when we found impaired aggregation response to ADP. Decreased response of platelets to ADP and ristomycin agents in dilated cardiomyopathy was firstly reported here. The background of these changes is not clear.

While lag time has never been found either in healthy or in diseased animal, we firstly established that lag time of 5-30 seconds in ristomycin-induced aggregation may coexist with some disorders such as cystitis, mitral valve insufficiency, haemangiosarcoma, dilated cardiomyopathy and Lyme borreliosis. Lag time before EPI-induced aggregation was established also in healthy, diseased and treated dogs as well.

We did not find alteration in shape change in diseased dogs. Maurer-Spurej and Devine (2001) observed only shape change in humans during ristomycin-induced aggregation without complete aggregation.

In NSAID-treated dogs we demonstrated that therapeutic dose of carprofen for longer than 10 days had no effect at all on aggregation response but ketoprofen caused decreased aggregation to ADP and EPI agents. Carprofen inhibits mainly cyclooxygenase-2 (COX-2) function (Johnston and Budsberg 1997) while ketoprofen inhibits the activity of both COX-1 and COX-2 (Lemke et al. 2002). Since platelets are rich in COX-1, decreased response in our ketoprofen-treated dogs to ADP- and EPI-induced aggregation is logical and has not been described before. There are only few available references regarding the effect of carprofen on platelet function. Clemmons et al. pointed out that therapeutic dose of carprofen in a single dose or continuous application for one week

developed a dose-dependent, minimal reduction in aggregation response to ADP and EPI [unpublished observation, Clemmons et al.]. In another study on dogs treated by 2.2 mg/kg bw carprofen for five days only a minor change in ADP-stimulated aggregation developed (Hickford et al. 2001). All of these results correspond well to our findings that carprofen in therapeutic dose cannot significantly influence platelet aggregation even in long-term (minimum 10 days) administration.

We are sure that the examination of platelet aggregation requires standardisation in veterinary laboratory practice and strong criteria needed how these aggregation studies should be performed. Presentation of the results should also be unified.

### **Mechanical method: the PFA-100**

In overall terms, the results of our investigations in healthy dogs agreed with those previously obtained by Keidel and Mischke (1998), Callan and Giger (2001) or Mischke and Keidel (2003). The upper ADP-CT limits in their studies were 81, 86 and 98, respectively, while two healthy dogs in our experiment showed 142 and 148 sec, which is much higher. The results for ADP-CT and EPI-CT were respectively only 1.15 times and 1.19 times longer than those observed by Callan and Giger (2001). The range of values for EPI-CT was rather wide. Six (10.3 %) of the healthy dogs displayed longer EPI-CT values than the cut-off point (300 sec) of the PFA-100. For dogs, the measuring range which has been optimised for humans should be widened. This idea might be supported through the results reported by Callan and Giger (2001) and Mischke and Keidel (2003) who also found apparently healthy dogs displaying collagen-EPI CT values > 300 sec. Therapeutic doses of both carprofen and ketoprofen caused no substantial alterations in thrombocyte function of dogs evaluated by PFA-100. However, it is important to know that the effect of NSAIDs on platelet function is dose-dependent. It was found that a low-dose aspirin may not prolong the BT at all (Williams et al., 1993). After an intravenous injection of 20 mg/kg acetylsalicylic acid in dogs the ADP-CT on PFA-100 was prolonged some 1.3-fold but the reference range was not exceeded in every case (Keidel and Mischke, 1998). Clemmons et al. (1997) pointed out that incubation of canine platelets from healthy dogs treated with carprofen also developed a dose-dependent reduction in aggregation response to ADP and epinephrine (see above). In another study on dogs, 2.2 mg/kg carprofen was orally administered for five days (Hickford et al., 2001). The onset of ADP-stimulated platelet aggregation, evaluated by electrical impedance, was significantly delayed when compared to pre-treatment values but the change was minor and not clinically important. The weak effect of carprofen on platelet aggregation may be due to its greater activity against ciklooxigenase-2 (COX-2) than COX-1 as a result of an absence of COX-2 in platelets (Mitchell et al., 1993; Donnelly and Hawkey, 1997; Johnston and Budsberg, 1997; Ricketts et al., 1998).

Ketoprofen has stronger side effects than carprofen therefore it is applicable only for short-term use in dogs and cats (Dowling, 2001). In a report on dogs treated with ketoprofen in the perioperative period it was established that there was an immediate and significant decrease in whole blood platelet aggregation in a presence of collagen and for the 24 hours after surgery compared to the preoperative values (Lemke et al., 2002). Grisneaux et al. (1999) found that the BT was significantly longer for ketoprofen-treated dogs during surgery compared to carprofen-treated and control dogs. The effect on platelet aggregation was not evaluated. In summary therefore, it can be concluded from the present and from the previous results of others that analysis by PFA-100 is a simple, useful method for the screening of the platelet function in dogs before operation but not to evaluate specific platelet disorders . It might be established that therapeutic doses of carprofen and ketoprofen NSAIDs did not cause any changes in platelet function evaluated by PFA-100.

## 5. Summary

The investigation of platelets, the smallest blood cells, is difficult with light microscope when the smear is stained by routine haematological dyes. Morphology of canine platelets (changes in size, shape, staining characteristics, degree of activation and clump-formation, distribution of granules, appearance of vacuoles on Giemsa-stained smears) were investigated in 20 healthy control and 181 diseased dogs. In the group of the sick dogs 84 animals suffered from disorders affecting directly the haematological parameters or the haemopoietic organs (i.e. bleeding, thymus haemorrhage, haemolytic disorders, lymphoma, immune-mediated thrombocytopenia). Ninety-seven dogs suffered from other diseases (hepatopathy, nephropathy, hepatic, splenic or intestinal neoplasm, skin diseases, diabetes mellitus, Cushing's-disease, sepsis) were also involved. The alterations found in platelet morphology were not specific to any disorders. Most common platelet abnormalities were polychromasia and presence of giant platelets. These changes occurred in great number in disorders accompanied by bleeding or haemolysis. Anisocytosis was the most frequent finding in hepatic, splenic or intestinal neoplasmas and certain endocrinopathies. Microcytosis was observed in immune-mediated thrombocytopenia, hepatic neoplasmas and endocrine disorders. Extreme platelet activation was characteristic of haemolysis, hepatopathies, neoplastic diseases and sepsis. Vacuolisation was present in thymus haemorrhage, pancreatitis, diabetes mellitus and Cushing's disease. A new morphologic phenomenon i.e. a ring-like formation of granules was described in the cytoplasm of the platelets both in healthy and diseased animals. In addition, two forms of pathologic granulation were also first described in Giemsa-stained blood smears: the pseudonuclear and the spot-like formation of granules, which were observed especially in disorders of affecting platelets.

The granulation and morphological characteristics of platelets in smears stained by periodic acid-Schiff reaction (PAS) were also studied. Three granulation localisation were observed: peripheral, eccentric and diffuse ones. The ratio of PAS-positive and -negative platelets was evaluated in several diseases. Our findings might support the diagnostic value of evaluation of platelets with light microscope and help clinicians to understand why morphologic changes of thrombocytes might be expected in several diseases.

Functional defects can be studied by in vitro aggregation tests with chemical compounds such as ADP, epinephrine and ristomycin. The aim of the present work was to investigate the effect of some diseases and that of non-steroid anti-inflammatory drug treatment in dogs on platelet aggregation. The examination had been carried out on 115 dog patients in M-200 optical aggregometer and in a CARAT TX4 optical aggregometer, that was used first in veterinary practice. Dogs were divided in three groups: healthy (control) dogs (n=43), diseased dogs with normal haemostasis-profile (n=47)

and dogs with arthropathies and long-term treated (n=25) with ketoprofen (1 mg/bw once daily) or carprofen (2-4 mg/bw twice daily) per orally. CARAT TX4 aggregometer proved to be a useful instrument in studying platelet aggregation in platelet-rich plasma of dogs. After determination of normal platelet aggregation curves in healthy dogs we described increased or decreased aggregation maximum in several illnesses. Carprofen treatment had no effect on platelet aggregation while ketoprofen decreased the aggregation maximum. Standardisation in aggregation methodology is highly recommended.

The PFA-100<sup>®</sup> analyzer (Dade-Behring) is a new point-of-care mechanical aggregometer aimed to fulfil the criteria that are necessary for the evaluation of platelet function. The effect of carprofen and ketoprofen on platelet aggregation was evaluated also by the PFA-100 using ADP and EPI cartridges. Aggregation of platelets was evaluated by PFA-100 in fifty-five healthy dogs, in seven dogs treated with ketoprofen and in thirty-one dogs treated with carprofen in a therapeutic dose for minimum 5 days. Analysis by PFA-100 proved to be simple and reliable. Therapeutic doses of carprofen and ketoprofen had only a minimal effect on platelet aggregation as measured by the closure time of PFA-100, which is the mark of platelet function. The closure times for both the healthy (control) and the treated dogs were often longer than the upper cut-off point (300 sec) of the device, similar to that in trials with humans. The PFA-100 could be a useful tool for veterinary use. This includes the evaluation of platelet aggregation in NSAIDs-treated dogs. The aggregation is not altered by therapeutic doses either of carprofen or ketoprofen. The upper cut-off point should be extended to 350 sec.

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## 7. Publications

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#### In domestic journals, in Hungarian language:

- **Halmay D**, Gaál T, Kótai I. (2002): A thrombocytaszám-változással járó kórképek okai és jelentősége kutyában és macskában. Irodalmi áttekintés. 1. rész. Thrombocytopenia. Magy. Áo. Lapja (Hung. Vet. J.). 124. 419-422.
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- **Halmay D**, Gaál T, Kótai I. (2002): A thrombocytáaggregatio vizsgálatának jelentősége és lehetőségei kutyákban. Irodalmi áttekintés. Magy. Áo. Lapja (Hung. Vet. J.). 124. 542-552.

#### In domestic journals, in foreign languages:

- **Halmay D**, Sótonyi P, Vajdovich P, Gaál T (2005): Morphological evaluation of canine platelets on Giemsa- and PAS-stained blood smears. Acta Vet Hung. 53(3):337-50.
- Gaál T, **Halmay D**, Kocsis R, Abonyi-Tóth Zs (2007): Evaluation of the effect of ketoprofen and carprofen on platelet function in dogs studied by PFA-100 point-of-care analyser. Acta Vet Hung. *Accepted*.

#### In foreign journal, in foreign languages:

- **Halmay D**, Gaál T, Kocsis R (2007): Influencing factors of ADP-, epinephrine- and ristomycin-induced platelet aggregation in dogs. Blood Coagulation and Fibrinolysis. *Accepted*.

#### Further full-text professional papers published in non-scientific journal:

- Kótai I, **Halmay D** (1997): Vérkenetek elemzése III. A kutya és a macska kóros vörösvérsejtjei 1. A vörösvérsejtek nagyságbeli, festődési és alaki eltérései. Kisállatorvoslás, 4 (6), 302-305.
- Kótai I, **Halmay D** (1998): Vérkenetek elemzése V. A kutya és a macska kóros vörösvérsejtjei 3/1. Az anaemiák egyes típusainak elkülönítése. Kisállatorvoslás, 5 (3), 161-164.
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- **Halmay D** (2000): A DIC kóroktana, patogenezise, klinikuma és gyógykezelése. Kisállatpraxis, 2000/6, 12-15.

- **Halmay D** (2001): A vérzéssel járó betegségek elkülönítő kórjelzése. *Kisállatpraxis*, 2001/2, 14-21.
- Kótai I, **Halmay D** (2001): Fehérvérsejtek vizsgálata vérkenetekben II. A neutrophil granulocyták mennyiségi viszonyainak változása. *Kisállatpraxis*, 2001/3, 6-8.

### **Abstracts, posters at congresses:**

- **Halmay D**, Gaál T: Az ADP és a ristomycin indukálta thrombocytá-aggregáció vizsgálata kutyában. Abstract. 51th Congress of the Hungarian Society of Clinical Pathology. 28-31 August 2002, Gyula, Hungary. *Clinical and experimental laboratory medicine (J. Hung. Soc. Clin. Path.)* 29. 40.
- **Halmay D**, Kocsis R, Gaál T: Evaluation of PFA-100 platelet function analyser in dogs. Abstract. ISACB XI. Congress (Valdivia, Chile, 2004). *Proceedings of the Congress.*
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- Vajdovich P, Szécsényi D, **Halmay D**: Present of *Dilofilaria repens* larvae in cytologic specimens. Abstract. ESVCP Congress (Cambridge, 2006) *Proceedings of the Congress.*
- **Halmay D**, Gaál T, Kocsis R: Tapasztalatok kutyák ADP-, epinefrin és ristomycin indukálta aggregációjának vizsgálata során. Absztrakt. MLDT konferencia (Szeged, 2006) *Kongresszusi kiadvány.*
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- **Halmay D**, Kocsis R, Gaál T: A PFA-100<sup>®</sup> thrombocyt funkció-analizáló műszer értékelése kutyákban (2004)
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- Kocsis Róbert (2002): Az egészséges kutya thrombocytáinak aggregációs vizsgálata ADP és ristomycin reagensekkel (Társtémavezető: Dr.Kótai István, SZIE ÁOTK Anatómiai és Szövetani Tanszék)
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