# *In vitro* effect of Altered Platelet Count on

# Multiple Electrode Impedance Aggregometry in Dogs

by

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Abstract

The objective of this study was to assess platelet aggregation by impedance aggregometry using canine whole blood with adjusted platelet counts. Citrated venous samples from 24 healthy dogs were aliquoted into unmanipulated and manipulated fractions with progressively depleted platelet counts to yield 2-3 samples per subject. All samples were corrected to native hematocrit (Hct) within 2% and cell counts performed before undergoing aggregation using a Multiplate® analyzer. Aggregation was initiated in all samples by addition of the platelet agonist adenosine diphosphate (ADP). Age, weight, initial platelet count (Pct), plateletcrit (Pct), Hct, and white blood cell count (WBCC) were compared between male and female dogs to assess for statistically significant differences ($P$ <0.05). Aggregation area under the curve (AUC), maximum aggregation (A) and velocity (V) results were analyzed using linear mixed models, controlling for Plt, Pct, Hct and WBCC, and including a random interceptor for subject. Age, weight, initial Plt, Pct, Hct and WBCC did not differ significantly between male and female dogs. AUC, V and A were also not significantly different between gender groups. WBCC was significantly positively associated with AUC, V and A, while Plt, Pct and Hct did not have a significant independent effect. WBCC is positively associated with impedance aggregometry induced by ADP in citrated whole blood samples. Plt, Pct and Hct did not significantly affect results. Large interindividual variation was seen in aggregometry results.

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List of Abbreviations

| | |
|---|---|
| A | Maximum Aggregation |
| ADP | Adenosine Diphosphage |
| ATP | Adenosine Triphosphate |
| AUC | Area Under the Curve |
| CI | Confidence Interval |
| FS | Female Spay |
| Hct | Hematocrit |
| MN | Male Neuter |
| Pct | Plateletcrit |
| Plt | Platelet Count |
| SD | Standard Deviation |
| V | Velocity |
| WBCC | White Blood Cell Count |

## Introduction

Platelets play a pivotal role in both primary and secondary hemostasis, including in formation of the initial platelet plug and subsequent roles as a surface for propagation of thrombin generation, a site for fibrin attachment and in clot retraction. Changes in platelet function can lead to significant morbidity and mortality due to thrombosis or hemorrhage. Tests of platelet function have been used in human medicine since the 1960s[1]. Applications are diverse and include: screening certain patient populations, use in diagnosis of congenital or acquired diseases of primary hemostasis, monitoring in patients receiving antiplatelet therapy, and risk stratification in patients receiving drugs that affect platelet function who are to undergo invasive procedures[1]. Many direct and indirect clinically useful tests of function have been developed, including buccal mucosal bleeding time, clot retraction, light transmission aggregometry, whole blood impedance aggregometry, impact cone and plate analysis, flow cytometry, platelet adhesion assays, thromboelastography and measurements of the mean platelet component[1-3].

Whole blood impedance aggregometers such as the Multiplate analyzer have been used extensively in human medicine. This instrument measures platelet aggregation in a continuous manner in response to addition of classic platelet agonists such as adenosine diphosphate (ADP), arachidonic acid, collagen, ristocetin and thrombin receptor activator for peptide 6. As platelets attach to the Multiplate sensors, a change in impedance is detected and transformed into an aggregation over time curve. Arbitrary aggregation units are generated. The most important parameter for expressing overall platelet activity is the total area under the aggregation curve (AUC), and this is the parameter used in clinical medicine. In addition to AUC, the instrument also calculates the height of the curve and the maximum slope attained, generating the overall aggregation (A) and the velocity (V), respectively. These latter measurements are used for research purposes only.

Impedance aggregometers can be used to diagnose a wide range of acquired and inherited platelet disorders. Benefits of this method include the use of whole blood samples with minimal sample processing, small sample volumes, ease of use, and rapid turnaround[3,4]. Use of impedance aggregometry in clinical veterinary medicine is in its infancy, but several papers in the last few years describe its use in veterinary species and provide standardized testing protocols for canine blood samples[5,6,a]. Recent work in veterinary and human medicine provide evidence of its potential as a biomarker in canine and human septic peritonitis[7,8].

In people, impedance aggregometry results have been shown to be significantly decreased at lower platelet counts, as well as being affected by other blood cell counts[9-13]. While recent canine studies provide reference intervals and standardized testing protocols for healthy dogs with normal platelet counts, there is currently no data available on its use in dogs with thrombocytopenia, limiting its use in this subpopulation of clinical patients.

The purpose of this study is to assess the effect of platelet count on platelet aggregation as measured by impedance aggregometry using the Multiplate analyzer. We hypothesized that impedance aggregometry results would be directly affected by platelet count in dogs. The objective of this study is to assess platelet aggregation by impedance aggregometry *in vitro* using canine whole blood with adjusted platelet counts.

## Materials and Methods

### Animals

Twenty-four clinically healthy dogs (12 ovariohysterectomized females and 12 neutered males) owned by staff and students of the Auburn University College of Veterinary Medicine were enrolled in the study. All dogs weighed more than 10 kg (26.4 ± 8.5 kg) and were older than 12 months (5.2 ± 2.6 years). Breeds included mixed breed (n=19), American Pitbull Terrier (2), Pembroke Welsh Corgi (1), Labrador Retriever (1) and Australian Shepherd (1). An *a priori* power analysis determined that 20 samples would be sufficient to detect a 20% change in platelet aggregation parameters. All study protocols were reviewed and approved by the Auburn University Institutional Care and Use Committee and written owner consent was obtained prior to sampling.

All dogs enrolled in the study met the following inclusion criteria: clinically normal on the basis of physical examination, complete blood count[b], serum biochemistry[c], prothrombin time and activated partial thromboplastin time[d]. Dogs with normal to mildly increased fibrinogen[d] were included. Great Pyrenees, Dobermans, and Cavalier King Charles Spaniels were excluded from enrolment due to increased prevalence of Glanzmann thrombasthenia, type 1 von Willebrand disease and inherited macrothrombocytopenia respectively in these breeds[14-16]. Dogs that had received clopidogrel, nonsteroidal anti-inflammatory drugs (including aspirin), calcium channel blockers, phosphodiesterase inhibitors (including pentoxifylline, sildenafil and pimobendan), antibiotics, or other drugs that may affect platelet function in the 6 weeks period prior to sampling were excluded.

### Preparation of platelet-depleted samples

Nine milliliters of whole blood was taken by atraumatic venipuncture from the jugular vein into a 10mL syringe loaded with 1mL of 4% sodium citrate, to yield a 10mL sample with a 1:9 ratio of citrate to blood. The sample was divided into 4 equal aliquots. A small

sample from the first aliquot underwent automated cell count[c] and a manual blood smear, and the sample was then held at room temperature while awaiting further analysis. The remaining 3 aliquots underwent centrifugation at 3000 rotations per minute for 10 minutes to deliver a relative centrifugal force of 1000g[e]. One sample was held for plasma donation as needed. The other two samples had the majority of the plasma component manually removed and held, the platelet rich buffy coat partially removed by manual pipette and discarded, and then the plasma component and red cell mass reconstituted. The total volume of buffy coat removed was recorded for each sample, and one sample from each dog had more aggressive removal of buffy coat than the other to yield samples with decreased, but variable, platelet counts.

We performed automated cell counts and manual blood smears on all platelet-depleted samples and then removed or added plasma as needed to correct the hematocrit to within 2% of the initial sample. Where plasma volume was adjusted to correct hematocrit, a subsequent cell count and manual blood smear was repeated prior to Multiplate analysis. All blood smears were reviewed for clumping and samples that exhibited clumping were excluded from final analysis.

**Measurement of impedance aggregometry**

Platelet aggregation was measured for each sample by impedance aggregometry[f] between 30 and 60 minutes after initial sample collection. 300µL of pre-warmed 0.9% sodium chloride containing 3mM calcium chloride was added to each of two test cells. The blood sample tube was gently inverted 3 times before collection of 300µL of sample for addition to each test cell. A 3-minute incubation period allowed admixture and recalcification of the sample, as per the manufacturer's instructions. Testing was then performed in duplicate for each sample, with either the addition of 30µL sodium chloride as control, or with the addition of 30µL of commercially available 0.2mM adenosine-diphosphate (ADP)[g], giving a final concentration of 9.5µM. Time from collection to Multiplate analysis, AUC, A, and V

were recorded for each sample, and results from samples analyzed more than 60 minutes after collection were excluded from statistical analysis. All results from samples that displayed activation with addition of sodium chloride alone were also excluded.

**Statistical analysis**

Statistical analyses were performed by use of an open-access statistical software program[h,i]. Measures of platelet function were log transformed as necessary to meet normality assumptions, and models adjusted for patient sex and age where necessary to control confounding (based on a 10% difference between adjusted and reduced models). Linear mixed models were used to estimate the effect of Plt, Pct, WBCC, and Hct on measures of platelet function. Models incorporated a random intercept for subject. Results are reported as percentage changes from reference values. F tests are reported for fixed effects. Significance of all analyses was set at a value of $P < 0.05$.

## Results

For all unmanipulated samples, age, weight, initial platelet count (Plt), plateletcrit (Pct), hematocrit (Hct) and white blood cell count (WBCC) were recorded, along with impedance aggregometry area under curve (AUC), aggregation (A) and velocity (V) results (**Table 1**).

***Table 1*** *– Age and weight variables for 24 dogs and hematocrit, white blood cell count, platelet count and impedance aggregometry results for unmanipulated samples from 23 dogs.*

| **Variable** | **Mean ± SD** |
|---|---|
| Age (y) | 5.2 ± 2.6 |
| Weight (kg) | 26.4 ± 8.5 |
| Hct (%) | 46.8 ± 4.2 |
| WBCC (x $10^{-3}$/ml) | 8.0 ± 1.6 |
| Plt (x $10^{-3}$/ml) | 228.7 ± 55.8 |
| AUC | 26.9 ± 11.2 |
| Aggregation | 57.6 ± 16.2 |
| Velocity | 7.0 ± 2.6 |

Results were compared between male and female dogs to assess for statistically significant differences. Mean Plt, Pct, Hct, WBCC, and impedance aggregometry AUC, A and V did not significantly differ between female and male dogs (**Table 2**).

***Table 2*** *– Mean hematocrit, white blood cell count, platelet count and impedance aggregometry results for unmanipulated samples from 23 healthy dogs classified by sex (female spayed [FS] or male castrated [MN]).*

| **Variable** | **FS (n=12)** | **MC (n=11)** | ***P*-value** |
|---|---|---|---|
| **Hct (%)** | **46.9** | **46.6** | **0.86** |
| WBCC (x $10^{-3}$/ml) | 8.2 | 7.8 | 0.50 |
| Plt (x $10^{-3}$/ml) | 242.7 | 213.5 | 0.21 |
| AUC | 25.8 | 28.0 | 0.66 |
| Aggregation | 56.3 | 59.0 | 0.71 |
| Velocity | 6.6 | 7.5 | 0.46 |

Of the 68 samples included in final analysis, Plt ranged from 48 - 392 x $10^3$/μL (median 150 x $10^3$/μL), Pct ranged from 0.04 - 0.5% (median 0.18%), Hct ranged from 38.1 - 53.5% (median 46.9%), and WBCC ranged from 1.47 - 12.23 x $10^3$/μL (median 5.8 x $10^3$/μL). WBCC was significantly positively associated with AUC, A and V, while Plt, Pct and Hct were not significantly associated with any of these outcome variables (**Table 3**). There was large interindividual variation, with some samples having normal aggregation results despite very low platelet counts.

***Table 3*** *– Relative percentage change in area under curve (AUC), aggregation (A) and velocity (V) with each 25 x $10^{-3}$/ml change in platelet count (Plt), 0.05% change in plateletcrit (Pct), 1 x $10^{-3}$/ml change in white blood cell count (WBCC) and 5% change in hematocrit (Hct) for all samples. *Result is statistially significant (P-value <0.05).*

| **Variable** | **Percentage change in AUC (95% CI)** | **P-value** | **Percentage change in A (95% CI)** | **P-value** | **Percentage change in V (95% CI)** | **P-value** |
|---|---|---|---|---|---|---|
| Plt | 3 (-2.9-5.8) | 0.52 | 4 (-1.7-4.8) | 0.35 | -2 (-4.7-2.6) | 0.59 |
| Pct | -2 (-6.7-5.2) | 0.80 | -3 (-5.7-3.3) | 0.60 | 7 (-2.1-8.2) | 0.25 |
| WBCC | 13.2 (6.8-20.0) | 0.0001* | 10.1 (5.4-14.9) | <0.0001* | 5.5 (0.7-10.5) | 0.027* |
| Hct | -3 (-23.5-20.6) | 0.90 | 3 (-15.3-17.5) | 0.90 | -15 (-24.9-11.2) | 0.46 |

One sample from one dog was unable to be run within 30 minutes of collection, and this single dataset was excluded from further analysis. For two different dogs, a single sample from each displayed platelet clumping on manual smear, and these datasets were also excluded.

## Discussion

In our study population, there was no significant difference between male and female dogs with regards to mean hematocrit, leukocyte count or platelet count, nor were there differences in mean platelet aggregation outcomes. For this reason, data from both groups were pooled for analysis. Interestingly, human studies have demonstrated a significant increase in aggregation in women compared to men using both light transmission and impedance aggregometry, independent of the method of anticoagulation[11,17]. The mechanism by which this occurs is unknown, although theories include differences in hematocrit between genders, leading to different plasma anticoagulant concentrations in anticoagulated samples, as well as the possible influence of estrogen[11]. In one study looking at healthy beagle dogs, males had a higher red cell count than females, while females had higher platelet counts[11,18]. The effect of reproductive status and cycle was not investigated. In reproductive age women, platelet aggregation results significantly differed in different stages of the menstrual cycle[19]. As all dogs enrolled in this study were neutered, it was less surprising that there was no significant difference in either cell counts or aggregation responses between genders.

Our results indicated a significant positive association between white blood cell count and aggregation measurements. Previous in vitro studies in people have found a significant positive association between leukocyte count and platelet aggregation using various agonists, while others have found an inhibitory effect[11,12,20-22]. Theories for increased platelet aggregation include reduction of ATP to ADP via leukocyte-derived ATPases, and production of reactive oxygen species including hydrogen peroxide and cathepsin G[12,23]. Conversely, cytokines such as IL-2 produced in lymphocytes have been shown to inhibit aggregation[21]. Neutrophils have variable effects on aggregation *in vivo* depending on experimental conditions[23,24]. A study that assessed platelet aggregometry in healthy dogs and dogs with septic peritonitis did not find a statistically significant association between

neutrophil count and aggregation initiated by arachidonic acid, ADP or collagen, although the effect of total leukocyte count was not assessed[7]. In spite of conflicting findings, the balance of results support a positive association, as was seen in our study. The association seen here was clinically significant, with each 1 x $10^3$/uL change in count associated with a 13.2% relative change in AUC.

The finding that both Plt and Pct did not significantly affect platelet aggregation results was unexpected. While this is the first study to examine the effect of platelet count on impedance aggregometry in dogs, multiple *in vivo*[4,10-12,26] and *in vitro*[9,13,27] studies in people show a significant positive effect. In some studies, this effect was seen with platelet counts within reference limits[12,27]. Another group found aggregation was significantly inhibited at counts less than 150 x $10^3$/μL, although significant interindividual variation was seen, and some samples yielded normal results at platelet counts of 50 x $10^3$/μL[9]. In another study, aggregation was not significantly reduced at all until platelet counts dropped below 50 x $10^3$/μL[26]. Impedance aggregometers measure changes in electrical resistance between two electrodes suspended in whole blood, with increased resistance occurring as platelets accumulate on these electrodes. The extent of aggregation is thought to be partially reliant on platelet mass, as markedly decreased platelet mass provides limited platelets for accumulation[10]. Pct was analyzed separately from Plt because it is thought to be a more accurate indicator of total platelet mass, which may be a more biologically important indicator of primary hemostasis than platelet count alone, and has the advantage of correcting for patients with altered mean platelet volumes[25]. Independent of the physical effect of platelet mass, markedly decreased Plt and Pct may also affect platelet activation due to an overall decrease in concentrations of predominantly platelet-derived agonists such as thromboxane and ADP[9]. Decreased concentrations of these agonists are expected to attenuate the initiation and amplification phases of the aggregation response. The reason for the lack of

independent effect of platelet count or plateletcrit on aggregation variables in the current study is unclear, although it is possible that a significant effect would be seen with more severely platelet depleted samples. In addition, our study was designed to detect a 20% difference in aggregation outcomes; smaller differences may not have been detected.

While Hct was not significantly associated with aggregation response, the study was designed such that all samples had a Hct within reference range. Multiple human studies have reported a clinically significant attenuation of the aggregation response with increasing hematocrit[12,28]. Postulated mechanisms include erythrocyte metabolism of ADP, as well as the effect of red cell mass on the resultant plasma concentration of anticoagulant in anticoagulated whole blood samples[12,17,29]. Because this study was not designed to detect the effect of increased and decreased hematocrit on aggregation results, the lack of a significant association should be interpreted cautiously.

Previous studies have evaluated the impact of anticoagulant and agonist selection on canine impedance aggregometry. In one prospective study, samples of hirudin and citrate anticoagulated blood from 20 healthy dogs were evaluated with different agonists and agonist concentrations and assessed for coefficients of variance for within-run precision[5]. Results from hirudin-anticoagulated blood yielded significantly higher measurement signals than citrated samples, and ADP was found to be a reliable agonist at 10µmol/L. In this study, citrated samples also demonstrated increased analytical variation compared to hirudin and heparin anticoagulated samples. In another prospective study of 20 clinically healthy and 3 sick dogs, the effect of different anticoagulants, agonists and times before sample processing were assessed. Contrary to the results of a previous study, heparin and citrate were both found to be more reliable than hirudin[6]. ADP yielded the strongest aggregation response, with 10µmol/L being the optimal concentration tested. A pilot study at our institution found ADP

to be a reliable agonist, and citrated samples were also found to yield consistent results, despite mixed success with this anticoagulant in previous studies[a].

The 9.5μM concentration of ADP used to initiate platelet aggregation in this study was higher than the 6.5μM recommended by the manufacturer for use in human samples. The amount used was increased in this study in an attempt to amplify platelet response due to concerns about inadequate aggregation in canine samples with markedly decreased platelet counts and is similar to the 10μM concentration recommended as being optimal in a prospective study of different agonists and agonist concentrations for whole blood aggregometry in canine samples[5]. It would be reasonable to repeat testing with different ADP agonist concentrations in both naturally occurring thrombocytopenia and in more aggressively platelet depleted *in vitro* models. Prospective, *in vivo* studies are needed before applying the results of this study to clinical patients.

A major limitation of our study was our inability to reduce platelet count without a concurrent reduction in leukocyte count. We overcame this by using linear regression modelling to examine the independent effect of leukocyte count.

Another limitation was the inability to correct for increased time to processing for samples with progressively depleted Plt, Pct and WBCC. For analysis of human samples, the manufacturer recommends analysis between 30 and 180 minutes of collection. A pilot study using canine samples found results were not affected if run between 30 and 120 minutes after collection[6]. While the effect of time to processing was limited more strictly in this study by ensuring only inclusion of samples run between 30 and 60 minutes of collection, unmanipulated samples with higher platelet and white blood cell counts were still run earlier within this time window than samples with depleted counts. We believe that this is unlikely to have significantly affected results.

Centrifugation and manipulation of samples also had the potential to cause both premature platelet activation and platelet clumping, the latter of which may have interfered with machine cell counts. It is also possible that centrifugation of manipulated samples may have led to loss of subpopulations of large, hyper- or hypoactive platelets[29,30]. We believe that the effect of premature activation was minimal as each sample was tested in duplicate, with one test being run with the addition of sodium chloride rather than ADP to check for endogenous platelet activation as may be seen with handling. This was seen in only one of our samples and this dataset was excluded from further analysis. Each sample was also evaluated by manual blood smear to check for platelet clumping, and samples that demonstrated clumping were excluded from further analysis. We therefore believe that premature activation and platelet clumping were unlikely to have significantly affected our results.

## Conclusion

This is the first study to examine the effect of circulating blood cell counts on impedance aggregometry in dogs. While platelet count and plateletcrit were not associated with platelet aggregation, these results are at odds with findings in humans and further studies looking at more severely platelet depleted samples or at natural severe thrombocytopenia are recommended to confirm this result. The present study shows that leukocyte count is positively associated with ADP-induced whole blood platelet impedance aggregometry in dogs, and this finding was both statistically and clinically significant. The impact of white blood cell count should be considered when assessing platelet function using impedance aggregometry in this species.

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## Footnotes

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c) Cobas c 311 analyzer, Roche Diagnostics GmbH, Mannheim, Germany.

d) Sysmex CA-500 series, Siemans Healthcare, Tarrytown, NY.

e) Dynac III, Becton Dickinson, Franklin Lakes, NJ.

f) Multiplate analyzer, Roche Diagnostic International Ltd, Rotkreuz, Switzerland

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