DOI: 10.5965/223811712332024431

Revista de Ciências Agroveterinárias 23 (3): 2024
Universidade do Estado de Santa Catarina

Use of the vortex as dissociator of platelet aggregates in pseudothrombocytopenia in small animals

Uso do vórtex como dissociador de agregados plaquetários na pseudotrombocitopenia em pequenos animais

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Submission: 01/21/2024 | Accepted: 09/05/2024

RESUMO

A pseudotrombocitopenia é a falsa diminuição na contagem plaquetária causada, na maioria das vezes, por erros pré-analíticos, representando um importante problema na clínica de caninos e felinos. Objetivou-se avaliar o uso de agitador vórtex para resolução laboratorial da pseudotrombocitopenia nessas espécies. Executou-se o projeto com amostras de caninos (n=100) e felinos (n=100) oriundas da rotina laboratorial, que apresentavam diminuição na contagem plaquetária, além da presença de agregados plaquetários. As plaquetas foram avaliadas quantitativamente pré e pós-tratamento pela contagem automatizada por bioimpedância e por estimativa em lâmina. O tratamento foi realizado por agitação em vórtex a 3600 rotações por minuto durante três minutos. Paralelamente avaliou-se a frequência de variações na plaquetometria de 100 caninos e 100 felinos. Verificou-se diferença significativa entre as plaquetas pré e pós-tratamento (p<0,001 para caninos e p=0,0097 para felinos), obtendo-se valores maiores em todos os pacientes avaliados após o tratamento com agitação, com médias de plaquetas dentro dos valores de referência para as espécies avaliadas. Felinos apresentaram a maior frequência de pseudotrombocitopenia e agregados plaquetários, e também foi a espécie que teve menor dissociação de agregados, considerando que a dissociação plaquetária se mostrou proporcional à intensidade de agregados pré-agitação nas duas espécies. Conclui-se que o uso de agitador no protocolo proposto é capaz de diminuir a incidência de pseudotrombocitopenia, diminuindo a faixa de contagem subestimada. Porém, a técnica não deve ser usada como único parâmetro para resolução da pseudotrombocitopenia, considerando que a dissociação dos agregados através da agitação não é total, embora minimize os falsos diagnósticos e auxilie o clínico em uma conduta terapêutica mais adequada.

PALAVRAS-CHAVE caninos; felinos; hematologia; patologia clínica; trombocitopenia.

ABSTRACT

Pseudothrombocytopenia is a false decrease in platelet count caused, most of the time, by pre-analytical errors, representing an important problem in canine and feline medicine. The objective was to evaluate the use of a vortex shaker for laboratory resolution of pseudothrombocytopenia in these species. The project was carried out with samples from canines (n=100) and felines (n=100) from the laboratory routine, which showed a decrease in platelet count in addition to the presence of platelet aggregates. Platelets were quantitatively evaluated pre- and post-treatment by automated bioimpedance counting and slide estimation. The treatment was carried out by vortex agitation at 3600 rotations per minute for three minutes. In parallel, the frequency of variations in platelet measurements of 100 canines and 100 felines was evaluated. There was a significant difference between pre- and post-treatment platelets (p<0,001 for canines and p=0,0097 for felines), obtaining higher values in all patients evaluated after treatment with agitation, with mean platelets within the reference values for the species evaluated. Felines had the highest frequency of pseudothrombocytopenia and platelet aggregates, but also the species that had the lowest dissociation of aggregates, considering that platelet dissociation was proportional to the intensity of pre-agitation aggregates in both species. It is concluded that the use of a shaker in the proposed protocol is capable of reducing the incidence of pseudothrombocytopenia, reducing the underestimated counts. However, the technique should not be used as the only parameter for resolving pseudothrombocytopenia, considering that

the dissociation of aggregates through agitation is not complete, although it minimizes false diagnoses and helps the clinician in a more appropriate therapeutic approach.

KEYWORDS: canines; cats; clinical pathology; hematology; thrombocytopenia.

INTRODUCTION

The fundamental part of the evaluation of platelet hemostasis includes platelet count, which is the quantitative platelet assessment, and it should be found in adequate values to perform its hemostatic role (TORRES et al. 2020). A decrease in these values is referred to as thrombocytopenia, while an increase is known as thrombocytosis (KUTER 2019). Platelet aggregation can produce falsely decreased total platelet values, thus being called pseudothrombocytopenia (RIOND et al. 2015).

Pseudothrombocytopenia is an *in vitro* phenomenon resulting in underestimated platelet counts, often associated with challenging blood draws. It can occur due to various factors, with platelet aggregation being the primary cause. Platelet underestimation can also occur due to analytical errors in automated counts, particularly in impedance-based systems, as macrothrombocytes may be mistaken for similarly-sized erythrocytes in feline samples. Less commonly in animals, platelet satellitism is one of the causes of pseudothrombocytopenia (RUSSEL 2010, THOMAS 2010). According to Rivera et al. (2023) Platelet satellitism is observed in blood counts when two or more platelets adhere to the cytoplasmic membrane of a leukocyte.

Feline platelets exhibit a higher tendency for aggregation due to species-specific factors, including their inherent aggregation propensity, larger platelet size, elevated serotonin levels, granule release in response to serotonin exposure, and irreversible aggregation at low adenosine diphosphate (ADP) concentrations (RIOND et al. 2015). In a study conducted by DE MELO et al. (2020) noted that platelet aggregates were the most prevalent pre-analytical error in feline samples, occurring in 35% of routine laboratory specimens. Other studies demonstrate the high frequency of this alteration in felines, reaching 36% (MORITZ & HOFFMAN 1997), 62% (ZELMANOVIC & HETHERINGTON 1998) and up to 71% (NORMAN et al. 2001) of feline samples collected with EDTA.

Vortex agitation is recommended for samples exhibiting pseudothrombocytopenia in human subjects, according to GULATI et al. (1997) to minimize interference. In such instances, the authors recommend reporting post-treatment platelet values in the medical report when the difference exceeds 10% compared to pre-treatment levels. Platelet disaggregation was complete in 44% of human samples with pseudothrombocytopenia, while in most cases, dissociation was incomplete when samples were subjected to 1-2 minutes of agitation at maximum vortex speed (8-10 on a scale of 1-10). The previously described technique for humans was also evaluated in 42 felines with platelet aggregates by TVEDTEN & CORCAL (2001). This method increased the total platelet count in all but one sample when subjected to one minute of agitation at 3000 rpm using an S8223 Vortex Genie mixer (VWR Scientific Products, Chicago, IL, USA). Platelet aggregates were observed in most samples, with complete disaggregation occurring in only 12% of tested patients.

Although Gulati et al. (1997) recommend notification when the discrepancy exceeds 10% in Medicine. TVEDTEN & CORCAL (2001) report that a significant difference in felines would be greater than or equal to 100%, or increases of more than 100,000 platelets/microliter, indicating pseudothrombocytopenia rather than true thrombocytopenia in such cases. Furthermore, the authors suggest that true thrombocytopenia in felines can be strongly suspected when the difference with treatment is less than or equal to 50,000 platelets/microliter. To date, no evaluation of this technique for canines has been reported in the literature.

This study aimed to evaluate the use of a vortex mixer as an alternative for resolving laboratory-induced pseudothrombocytopenia in dogs and cats, utilizing a larger sample group than previously reported in the literature. The research focused on examining dissociation differences between canine and feline samples subjected to higher intensity agitation for extended periods, compared to the study conducted by TVEDTEN & CORCAL (2001). Additionally, the study assessed the frequency of platelet aggregates and variations in platelet counts in these species at a veterinary laboratory in the Videira region of Santa Catarina.

MATERIAL AND METHODS

Experiment location

The project was conducted at the Amigovida Laboratory - Veterinary Analyses (Videira, Santa Catarina) using samples collected by affiliated veterinary clinics from cities in the mid-western region of Santa Catarina (Caçador, Lebon Régis, Rio das Antas, Arroio Trinta, Salto Veloso, Treze Tílias, Fraiburgo, Campos Novos, Tangará) between January and July 2022.

Frequency of Platelet Count Variations and Platelet Aggregates

100 canine and 100 feline samples collected in potassium EDTA tubes that did not contain clots, were in tubes within the expiration date and were collected according to the correct quantity indicated on the tube were evaluated exclusively during the month of May 2022 until 100 samples of each species were obtained in order to establish the frequency of variations in plateletometry and platelet aggregates observed in blood smears, while other samples continued to be selected from January until July 2022 to be used to evaluate the vortex's performance in dissociating aggregates. Four variations were considered: pseudothrombocytopenia, thrombocytopenia, thrombocytosis, and normal platelet count. Pseudothrombocytopenia was defined as a decrease in total platelet count by electrical impedance with the presence of platelet aggregates and platelet satellitism on blood smear. Thrombocytopenia was characterized by decreased platelet counts without aggregates. Thrombocytosis was identified as elevated platelet values. Normal platelet count was defined as values within the reference range. The minimum reference values established in the laboratory were 175,000 platelets/microliter for canines and 200,000 platelets/microliter for felines, and the maximum reference values established in the laboratory were 500,000 platelets/microliter for canines and 800,000 platelets/microliter for felines. Samples with decreased platelet counts and the presence of macroplatelets were not classified as pseudothrombocytopenia. To avoid analytical errors, all samples were required to show agreement within a 20% deviation between microscopic estimation and automated electrical impedance counting.

Sample selection criteria for treatment

Samples were selected from tubes containing potassium ethylenediaminetetraacetic acid (EDTA) anticoagulant that met the sample quality criteria: absence of clots or fibrin upon visual inspection, and exhibiting decreased platelet counts. After the approval of the quality criteria, the animals that presented platelet values below the reference values stipulated by the laboratory in the count by electrical impedance adjacent to the presence of platelet aggregates in the blood smear slide were included in the sample group for evaluation of treatment efficacy. The study included both healthy and diseased animals, without distinguishing between sex, age, or breed.

Graduation of platelet aggregates from pre-treatment samples

The presence of platelet aggregates was assessed in blood smears stained with May-Grunwald Giemsa according to the manufacturer's specifications (Laborclin®, Brazil), with clusters of three or more platelets considered positive. The quantification of platelet aggregates was subjectively determined using a methodology adapted from SILVA (2017). Aggregation was classified as mild when small aggregates (3-10 platelets) were observed in the feathered edge of the blood smear; moderate when small and medium-sized aggregates (10-20 platelets) were present along the lateral edge and feathered edge; and severe when small, medium, and large aggregates (>20 platelets) were found throughout the smear.

Platelet count by electrical impedance

Electrical impedance counting was performed following sample homogenization using an automatic homogenizer (Agrot-1213, Spinlab®) at 18 rotations per minute for a minimum of 10 minutes. Throughout the project, the hematology analyzer (SDH-3-VET, Labtest®) underwent daily internal quality control (Controllab®) using Levey-Jennings charts and Westgard rules, as well as quarterly proficiency testing.

Platelet count by estimate on slide

In addition to impedance-based platelet counting, samples underwent estimation using a blood smear technique adapted from the methodology proposed by SILVA et al. (2007). The platelet count per microliter was determined by averaging 10 immersion fields (1,000x total magnification) evaluated between the body and fringe of the smear where red blood cells were close but not overlapping, and multiplying the result by 15,000. Areas with platelet aggregates were excluded from the slide count estimation.

Treatment protocol for samples using vortex

Following platelet count by electrical impedance, smear estimation, and grading of platelet aggregates, the samples underwent treatment. The treatment involved vortex agitation (Na3600, FORTECIENTÍFICA®) at 3600 revolutions per minute (rpm) for three minutes. The sample was then homogenized for at least 10 minutes using an automatic homogenizer (Agrot-1213, Spinlab®) at a speed of 18 rotations per minute.

Following treatment, a new slide was prepared, stained using the same method, and platelet count was

reassessed through both microscopic estimation and electrical impedance.

Statistical analysis of data

The data underwent descriptive analysis and normality testing (Shapiro-Wilk) using SAS software (2013). To analyze the relationships between platelet count per microliter and the variables of time, platelet counting method, and species, Spearman's correlation analyses were performed (PROC CORR). For parametric variables, Student's t-test was employed at a 5% significance level to compare pre- and post-treatment means. *Odds ratio* analysis with chi-square test was used to assess the likelihood of platelet aggregates in canines and felines, as well as the frequency of variations in platelet count.

RESULTS

Normal platelet counts were the most prevalent among canine platelet variations, observed in 64% of evaluated animals. Thrombocytosis was found in 12% of cases, while 14% exhibited true pseudothrombocytopenia (excluding animals with low platelet counts due to the presence of macroplatelets (Figure 1)). True thrombocytopenia was the least frequent, occurring in 10% of the samples. In the assessment of platelet aggregate frequency, regardless of platelet count, canine samples with aggregates accounted for 34% of the samples evaluated during this period.

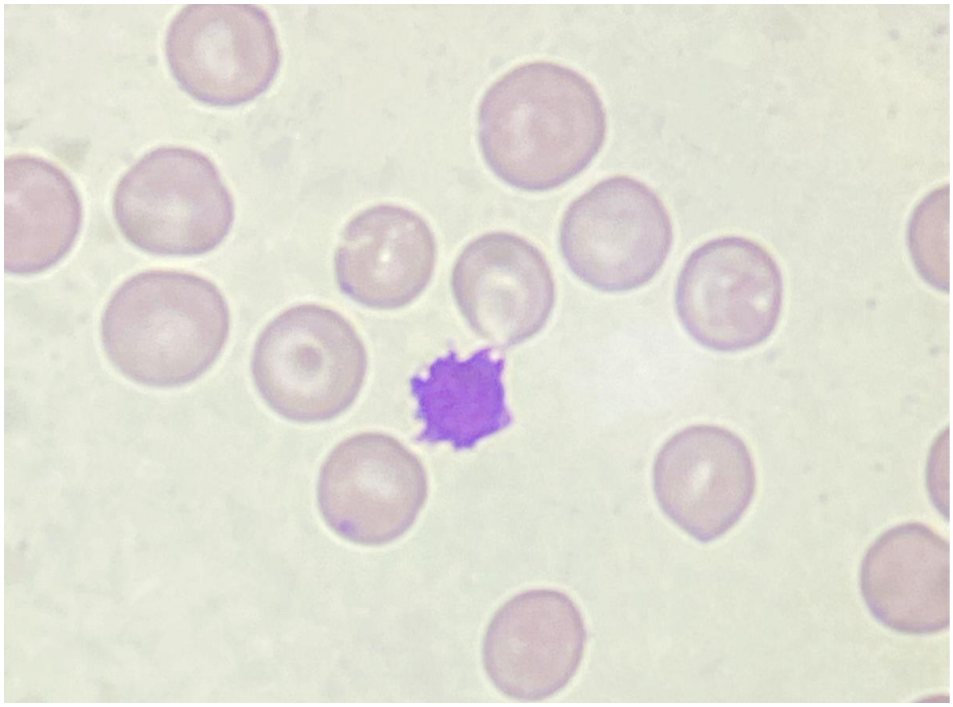

Figure 1. Canine macroplatelets in their activated form presenting pseudopods (100x objective, May-Grunwald Giemsa stain, under painting in mineral oil).

Felines exhibited a higher incidence of pseudothrombocytopenia (Figure 2) compared to canines, accounting for 60% of the analyzed samples. *Odds ratio* analysis revealed that felines are six times more likely to exhibit platelet aggregates compared to canines in this population, with a 95% confidence interval ($p<0.0001$). It was also observed that felines exhibited lower frequencies of thrombocytopenia and thrombocytosis, with only 4% of samples falling into each classification. Normal platelet counts were observed in 32% of feline samples, while 76% of feline samples exhibited platelet aggregates, regardless of platelet count.

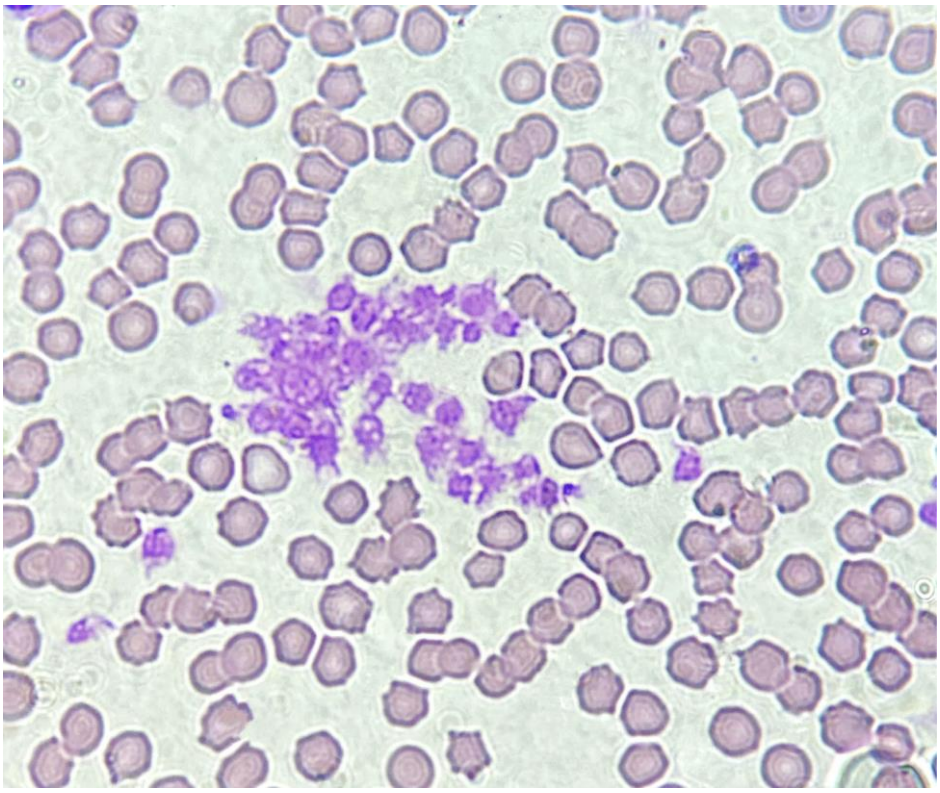

Figure 2. Feline platelets distributed in the form of a platelet aggregate, presenting macroplatelets in an activated state (presenting pseudopods and cytoplasmic granulation) within the aggregate (100x objective, May-Grunwald Giemsa stain, under immersion in mineral oil).

Platelet counts in canines and felines showed statistically significant increases ($p<0.0001$) following treatment, with all animals in the sample group exhibiting higher platelet values post-vortexing compared to pre-treatment levels (Figure 3). However, it was noted that not all animals exhibited values within the reference range following the use of the agitator.

The results demonstrate a more pronounced mean difference between pre- and post-treatment measurements in canines compared to felines, suggesting that the technique provides more effective dissociation in this species, although statistically significant differences were observed in both species before and after treatment ($p<0.0001$).

Platelet aggregate intensity prior to treatment influences dissociation, with higher intensities leading to greater and more gradual dissociation in both canines and felines (Figures 4 and 5).

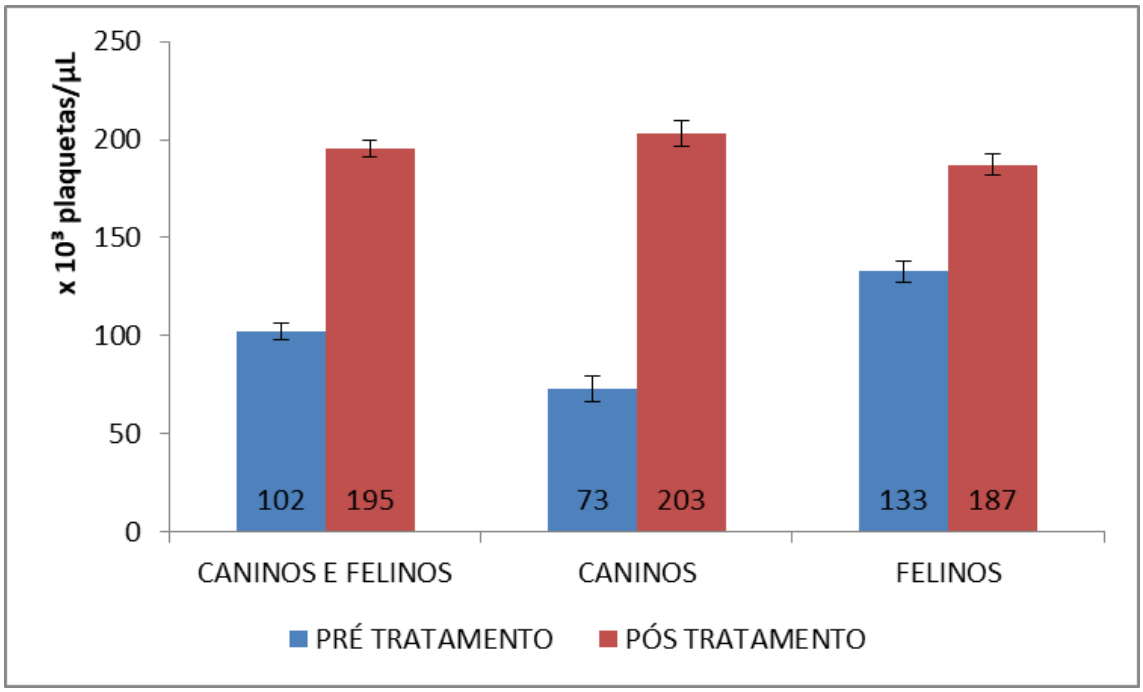

Figure 3. Means±standard deviation (SD) of platelet count per microliter in canines and felines pre-treatment and post-treatment using vortex agitation at 3600 rotations per minute for three minutes using electrical impedance counting and slide estimation.

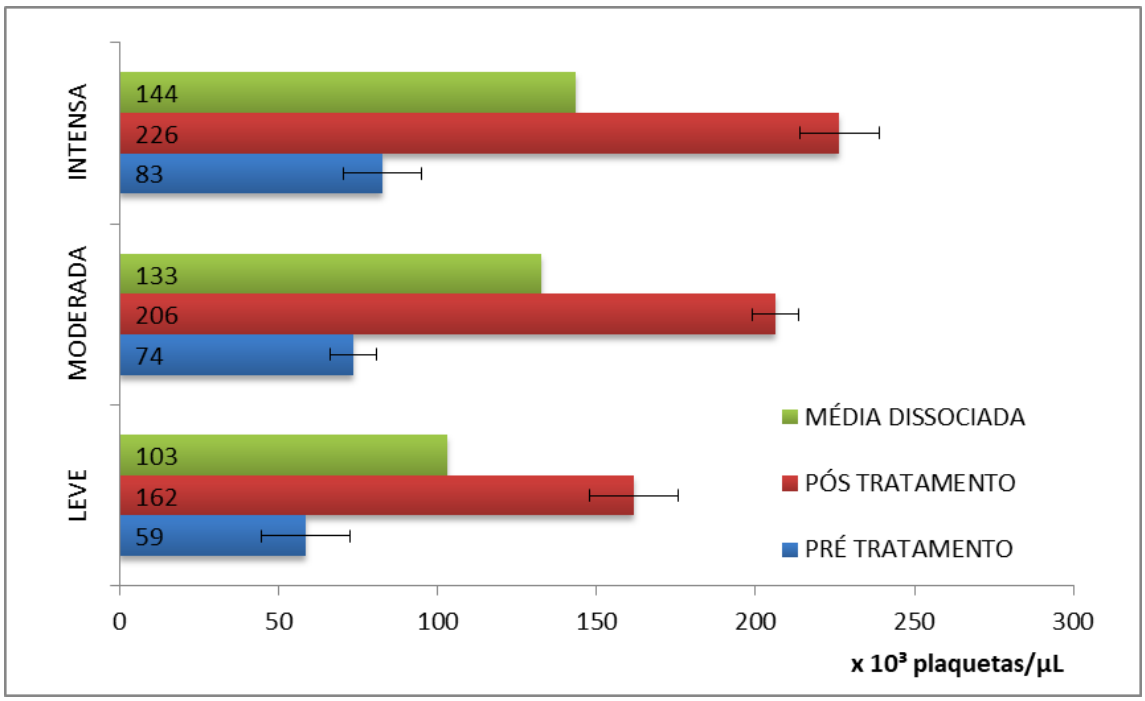

Figure 4. Means±SD of platelets per microliter in canines pre-treatment, post-treatment and difference between pre- and post-treatment means of samples – dissociated platelet mean - categorized into mild, moderate and intense presence of platelet aggregates.

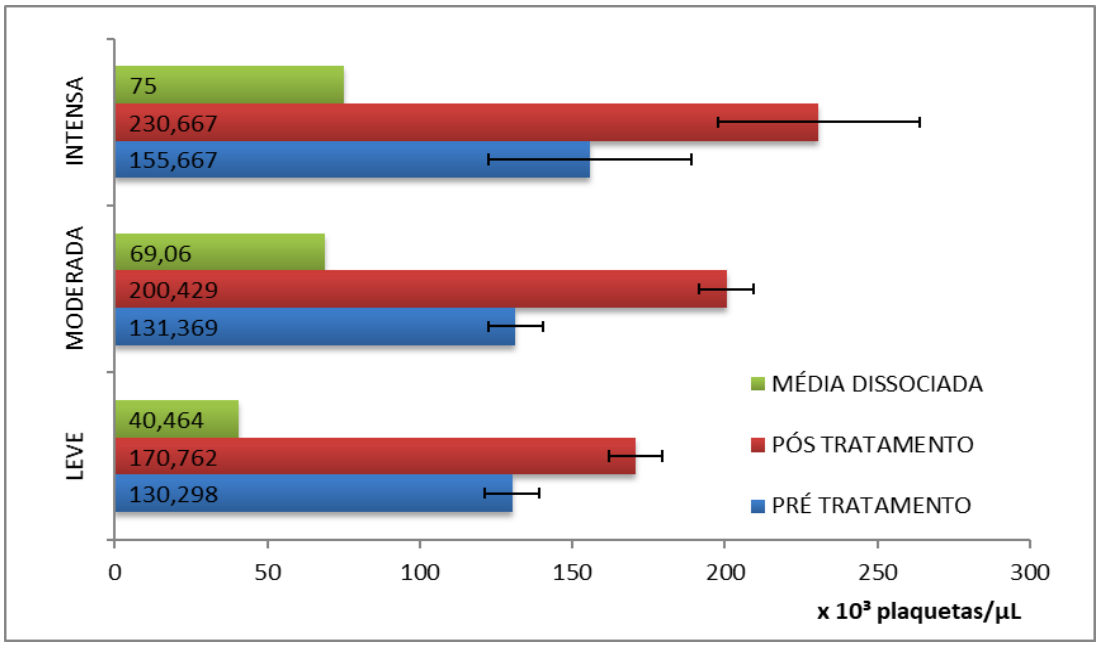

Figure 5. Means±SD of platelets per microliter in felines pre-treatment, post-treatment and difference between pre- and post-treatment means of samples categorized as mild, moderate and intense presence of platelet aggregates.

Results demonstrated that canines, even with mild platelet aggregation, exhibited a mean pre- and post-treatment difference of 103,046 platelets/microliter. Conversely, felines with intense aggregation tended to dissociate an average of 75,000 platelets/microliter, while those with mild aggregation dissociated only 40,464 platelets/microliter on average.

There was no interaction between the method variable (electrical impedance or smear estimation for platelet count) and time (before or after vortex treatment), indicating that the two techniques do not differ in their treatment outcomes (p=0.0537).

Although no statistically significant interaction between time and method was observed, comparison of means revealed elevated platelet counts both pre- and post-treatment when assessed by electrical impedance compared to manual estimation (Figure 2).

DISCUSSION

Pseudothrombocytopenia is a common issue in feline blood tests, and this study supports the findings of DE MELO et al. (2020), obtaining a high frequency of pseudothrombocytopenia in this species. The issue is also observed in human medicine, and when pseudothrombocytopenia occurs with multiple anticoagulants, it is recognized as multiple anticoagulant-induced pseudothrombocytopenia (LARDINOIS et al. 2021). In the feline population evaluated in this study, pseudothrombocytopenia occurred at a frequency of 60%, surpassing the incidence observed in canine examinations. This finding aligns with the 62% prevalence of platelet count variation reported in cats by ZELMANOVIC & HETHERINGTON (1998).

TVEDTEN & CORCAL (2001), when testing the vortex effect on platelet aggregate dissociation in felines, did not achieve dissociation in all samples, unlike this study which used a larger sample group and achieved dissociation in all felines. This finding contradicts the assertion of GULATI et al. (1997) that extending vortex agitation beyond two minutes likely wouldn't increase platelet disaggregation, based on human studies, possibly due to interspecies differences between humans and felines. RIOND et al. (2015) also support the assertion that agitation for over two minutes enhances platelet dissociation in felines, as 24 hours of homogenization without vortex agitation resulted in normal platelet counts in all cats previously exhibiting pseudothrombocytopenia.

As in the study by RIOND et al. (2015), evaluating the effect of 24-hour homogenization, and TVEDTEN & CORCAL (2001), assessing the impact of vortex agitation at 3000 rpm for one to two minutes, demonstrate that platelet dissociation is incomplete in the proposed treatment of this study, which involves vortex agitation at 3600 rpm for three minutes, with platelet aggregates persisting post-treatment.

This study reveals that canines exhibit the highest mean difference between pre- and post-treatment values, suggesting they are the species with the greatest platelet dissociation. Meanwhile, felines, which exhibited lower dissociation, emerge in this study as the species most affected by platelet aggregates. Their reduced dissociation may be attributed to species-specific characteristics, such as irreversible aggregation in response to low adenosine diphosphate (ADP) concentrations, as reported by Riond et al. (2015).

In humans, reporting post-vortex platelet counts in laboratory results is recommended when the difference exceeds 10% of the pre-treatment value, as suggested by GULATI et al. (1997) According to TVEDTEN & CORCAL (2001), a significant difference in felines is observed when platelet counts increase by 100% or more, or by over 100,000 platelets per microliter. However, feline patients with higher initial platelet aggregation intensity exhibited a mean pre- and post-treatment difference of 75,000 platelets/microliter, while those with mild aggregation intensity showed a mean difference of 40,464 platelets/microliter. These findings suggest that reporting only increases of 100,000 platelets/microliter would be insufficient for this study. Instead, adopting a 10% variation threshold, as recommended in human medicine, may be more appropriate. Considering that when post-vortex platelet counts remain below the reference range, true thrombocytopenia cannot be definitively diagnosed, and further evaluations are necessary due to incomplete platelet dissociation. However, if the post-vortex platelet count aligns with the reference range, true thrombocytopenia can be ruled out.

Dogs, despite the presence of mild platelet aggregates, exhibit significant platelet dissociation according to the current study. Reporting an increase of over 100,000 platelets/microliter in laboratory results, as proposed in the study by TVEDTEN & CORCAL (2001) for felines, appears to be applicable to canines. However, utilizing the same standard proposed by GULATI et al. (1997) of reporting variations starting from 10%, as in humans, would be more effective considering that pseudothrombocytopenias may present values close to the lower limit of the reference range.

Although no statistically significant difference was observed between the means of vortex association with smear estimation and electrical impedance platelet counting ($p = 0.0537$), the results suggest that combining vortex agitation with subsequent electrical impedance evaluation may yield superior outcomes based on mean differences. However, it remains crucial to perform smear evaluations to rule out analytical errors, such as low platelet counts due to macroplatelets. This theory aligns with the findings of BARROS et al. (2023) using human samples demonstrating the necessity of slide review and platelet reassessment in clinical laboratories that do not employ fluorescence-based platelet counting but instead rely on techniques susceptible to errors due to variations in typical dimensions, such as electrical impedance.

The lower mean platelet count observed in the field estimation method compared to electrical bioimpedance counting may be attributed to the correction factor used, which varies in recommendations across the literature. In this work, the methodology chosen for correcting estimates in sheets is proposed by SILVA et al. (2007), in which the correction factor is 15,000. However, various correction factors exist in the literature, which depend on factors such as microscope field size, light type, hematocrit, patient hemoglobin levels, and species. According to COMAR et al. (2009) demonstrated that automated platelet counting offers superior accuracy, yet remains susceptible to errors without concurrent morphological assessment, potentially overlooking causes of pseudothrombocytopenia or miscount due to macroplatelets. Additionally, various estimation-based counting methodologies tend to underestimate total platelet values, as platelets may be obscured by erythrocytes or not fully visible.

Not all animals achieved platelet counts within the species-specific reference range following the proposed dissociation methodology. The data suggest that platelet aggregate dissociation is incomplete, as evidenced by the persistence of platelet aggregates observed in blood smear evaluations following treatment. These findings align with recommendations in human medicine by LARDINOIS et al. (2021) The necessity of reporting in the medical report the methodology used for platelet evaluation, the sample used with which type of anticoagulant, and, if an additional dissociation technique is used, it should also be reported.

CONCLUSION

Pseudothrombocytopenia in small animals is highly prevalent in the Midwest region of Santa Catarina, with a higher incidence in felines compared to canines, showing a six-fold increased likelihood of developing platelet aggregates. The use of vortex as a platelet aggregate dissociator, at a speed of 3600 rpm for three minutes, is recommended to reduce pre-analytical interferences in both species, with greater efficacy in

canines.

It is recommended to report post-agitation results in complete blood counts when there is a 10% or greater variation from pre-agitation values, accompanied by a morphological assessment of platelets on a blood smear. This approach aims to rule out false thrombocytopenia due to analytical errors caused by the improper counting of large platelets as erythrocytes through bioimpedance. It is recommended that when post-vortex platelet counts fall within the reference range for platelet measurement, true thrombocytopenia should be ruled out. If these values are not achieved, a repeat sample collection should be considered.

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