



Study on the Closure Time in Healthy Small-Breed Dogs by Platelet Function Analyzer-200

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Abstract Platelet function evaluation by PFA-100 or -200 has been known to be objective and sensitive for assessing platelet function and dysfunction of Von Willebrand Factor in humans and dogs. However, using the C/EPI cartridge in dogs is controversial. This study aimed to establish a reference range for PFA closure time in healthy small breed dogs (body weight < 10 kg) and to evaluate the effectiveness of both C/ADP and C/EPI cartridges for these dogs. Citrated blood samples were collected from 50 clinically healthy small breed dogs that were admitted for presurgical procedures or health checkups, and closure times were measured using the PFA-200. Reference ranges were determined as 42-144 s (median 67 s, mean 71.2 s, SD \pm 21.2 s, 95% RI 43-140 s), for CT-C/ADP and 41-200 s (median 87, mean 91.2 s, SD \pm 31.8 s, 95% RI 44-195 s) for CT-C/EPI. The present study demonstrated that the reference ranges for PFA closure times in small breed dogs are in line with existing reference ranges. The utilization of C/ADP cartridges is the preferred choice for evaluating platelet function in small breed dogs. However, due to variable responses of epinephrine to platelet aggregation in dogs, caution should be exercised when using C/EPI cartridges.

Key words closure time, PFA-200, platelet function, small-breed dog.

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Introduction

Decreased platelet count or function can lead to primary hemostatic disorder, resulting in hemorrhage. If the platelet count, coagulation test such as PT and aPTT and fibrinolytic products are normal in bleeding patients, thrombopathia (platelet function disorder) should be suspected. While thrombocytopenia is accurately evaluated through an automated platelet analyzer, platelet function defects with normal platelet count is difficult to diagnose (4,14). Buccal mucosal bleeding time (BMBT), the time required from cutting the oral mucosa to stopping bleeding, depends, in particular, on the ability to form a primary platelet plug and is used for screening purposes to evaluate platelet function (4). However, this measurement of bleeding time in dogs are not widely available due to the time-consuming and difficulty of standardization. Although it is possible to diagnose thrombopathia through platelet aggregometry using a variety of agonists, it is a labor-intensive and expensive method, so it is only available in larger and specialized laboratories (4).

In 1995, device for automatically measuring platelet function has been developed. Platelet function analyzers (PFA-100 and PFA-200) use whole blood flow through a capillary device to mimic primary hemostasis that occur *in vivo* (8). PFAs provide single endpoint (closure time; CT) results when blood flow through the device is occluded as a result of platelet adhesion and subsequent aggregation after exposure to a membrane-coated platelet agonist in a disposable cartridge device. The PFA-100 has been validated as an objective and sensitive coagulation instrument for evaluating platelet aggregation and the function of Von Willebrand Factor in dogs (4,11,15). Results are obtained within 10 minutes, and the instrument is easy to operate, unlike BMBT or platelet aggregometry. There are two cartridge types; one is coated with C/ADP, and the other with C/EPI (8).

In dogs, studies on the PFA for congenital platelet dysfunction diseases or von Willebrand's disease (3), evaluation of platelet function through PFA in heart (5), kidney (7) or liver disease (23), and the function of PFA as monitoring for drugs that act on platelets were conducted in dogs (10,17,19).

Unlike in human medicine, the role of epinephrine on platelet aggregation in dogs is known to be controversial (8,16). According to Mischke and Schulze, responses of platelets to epinephrine in dogs vary, or no response at all (16). Therefore, it is disputable that C/EPI of PFA is utilized in relation to canine platelet function. On the other hand, in a previous study the clinical sensitivity and specificity of C/ADP for evaluating healthy dogs and dogs with primary hemostatic disorders were 95.7% and 100% respectively (4). Since ADP

is very sensitive to platelet aggregation in dogs, evaluating platelet function using C/ADP cartridge can be useful.

This device has recently been introduced in veterinary medicine in Korea, where most of the dogs are small breed dogs, a reference range study for small breeds has not been performed yet. Hematological reference intervals are affected by several factors, including the genetic factor of breeds (13). The aim of this study is to establish reference ranges for the CT of platelet function analyzer in healthy small breed dogs and to evaluate the applicability of both C/ADP and C/EPI cartridges in small breed dogs using PFA-200.

Materials and Methods

Animals

In this study, reference population consisted of 50 healthy dogs weighing less than 10 kg with available blood test results (CBC, biochemical profile, PFA test) were selected. These dogs were admitted to a local veterinary clinic in South Korea from July to September 2021, for general health checkup, neutering, or teeth scaling, whose owners volunteered to donate blood samples. All dogs had no recent history of illness and clinical signs that would affect blood test before blood sampling or during physical examination.

Procedures

Blood sampling

All blood samples were collected by cephalic or jugular venipuncture using sterile disposable needles into EDTA tubes (Tabung EDTA K3 0.5 mL; Golden Vac, Hermosillo, Mexico) for blood cell count, lithium heparin tubes (Micro Lithium heparin 0.5 mL; Golden Vac, Hermosillo, Mexico) for biochemistry, followed by 1.8 mL tubes containing 0.2 mL 3.2% citric acid sodium citrate buffer solution (VACUETTE Coagulation tube, containing 3.2% (0.109 mol/L) Sodium Citrate, 1.8 mL blood volume) as anticoagulant for platelet function analyses. After blood collection, the tubes contain blood were carefully swaying four times and rolled.

All blood samples within EDTA tubes were used to perform routine CBC (Horiba scil VET abc Plus+, SCIL Animal Care company, Altorf, France), within lithium heparin tubes were centrifuged to obtain plasma. Plasma samples were used to perform biochemical assays (Fuji DryChem NX500, Fujifilm, Tokyo, Japan). Dogs were excluded who were in thrombocytopenia or in anemia whose hematocrit of less than 40%. In cases of thrombocytopenia detected by CBC, manual count by evaluating the blood smear was performed. Thrombocytopenia (PLT <200 / μ L) shown by both tests were excluded in this study. Dogs without thrombocytopenia and anemia

were tested for PFA with blood in sodium citrate tubes.

CBC and chemistry tests were performed immediately within 30 min, and PFA tests were performed between 30 min and 4 h after blood collection. Lipemia, hemolysis, or icteric samples were excluded.

PFA test procedures

Platelet function was measured using the PFA-200 (Siemens Healthcare Diagnostics, Marberg, Germany). The analyzer uses two cartridges. One is coated with collagen and epinephrine (C/EPI), and the other is coated with collagen and adenosine diphosphate (C/ADP). Each cartridge was stored at 4°C and placed at room temperature for 15 min prior to analysis. After increasing the temperature of cartridges, citrate tubes were agitated four times before each analysis and 800 µL of blood was pipetted into each cartridge. The cartridge has a small hole of 150 µm and measures the time it takes for 800 µL citrate-treated whole blood to close due to plug formation as it passes through the hole (CT). The maximum measured time is 300 s. If the measurement was terminated at a specific time

before 300 s and the result value was > 84 , etc., it was excluded from the result.

Statistical analysis

The statistical descriptive analysis such as mean, standard deviation, median, minimum, and maximum values were performed for all CTs data. The analysis was performed using data corresponding to 2.5% and 97.5% quantiles for all data, and out-of-range data were considered as outlier. Before conducting group comparisons and correlation analysis, the Kolmogorov-Smirnov normality test was used to assess the normality of the data. The Kolmogorov-Smirnov normality test is a method that can be applied when the sample size is 30 or more, based on the application of the Central Limit Theorem. In this study, the data consisted of 50 observations, satisfying the condition for the application of this method. The Mann Whitney U-test was used to statistically compare CTs between females and males. All statistical analysis, including descriptive analysis, normality test, and correlation analysis, were performed in a SPSS version 20.0 (IBM Co., Armonk, NY, USA) environment.

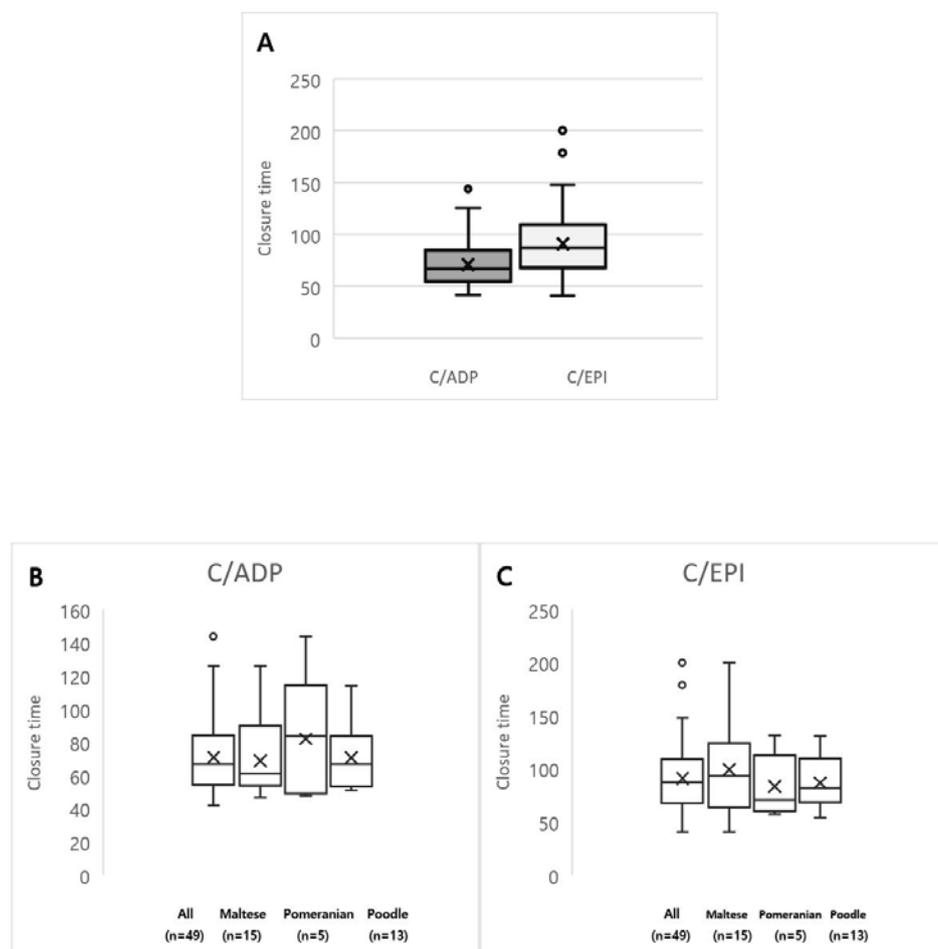


Fig. 1. Ranges of CT measured by C/ADP and C/EPI cartridges. A, indicates the CT ranges for all healthy small breed dogs. B and C indicate the CT range for each small breed (25-75 percentile).

Results

Case characteristics

Fifty dogs (eight intact males, 15 castrated males, 21 intact females and six spayed females ranging in age from one to 13 years were included in the study) were sampled. Breeds represented were 15 Maltese, 14 Poodles, five Pomeranians, two Shiba Inus, nine others (one Bichon Frise, one Yorkshire terrier, one Shih Tzu, one Welsh corgi, one Chihuahua, one French bulldog, one Miniature pincher, one Miniature Schnauzer and one Pekinges) and five dogs were mixed breed. All dogs weighed less than 10 kg, classified small breeds, who were clinically healthy and without thrombocytopenia and anemia. There were no remarkable findings on blood tests including CBC and chemistry.

Platelet function analyzer CT

Among 50 dogs, CT-C/ADP were measured in 49 dogs and CT-C/EPI in 49 dogs (Fig. 1A). Descriptive statistics are presented in Table 1. For CT of C/ADP, the mean was 71.16 s and the SD was 21.2. For CT of C/EPI, the mean was 91.20 s and the SD was 31.8. In healthy dogs, the maximum measured value of 300 s was not exceeded, except when CTs were not measured due to test errors (e.g., >97 s). Two dogs excluded from this study due to bladder stones had CT using collagen/epinephrine cartridges exceeded the measuring range of 300 s (>300 s). The CT of C/ADP reference interval (RI) was 43 to 140 s, and the CT of C/EPI RI was 44 to 195 s (Table 1).

CTs in different 3 dog breeds and others

The median and 25th-75th percentile range of CT for three dog breeds (Maltese, Poodle and Pomeranian) and other breeds group was shown and no significant differences were found between the breeds, with many overlapping ranges of CT using both C/ADP and C/EPI (Fig. 1B, C).

Discussion

The reference ranges of CT-C/ADP and CT-C/EPI measured in healthy small breed dogs were 43 to 140 s and 44 to 195 s, respectively. No significant differences of those ranges were found among Maltese, Poodle and Pomeranian.

These ranges are similar to the those of previous studies

that established the reference range (4,11,15). According to those studies, CT-C/ADP ranged from 47 to 98 s, and CT-C/EPI was 67 to 300 s. As mentioned above, the overall reference ranges were similar when compared with previous studies, but there were higher upper limit of CT-C/ADP and lower limit of CT-C/EPI in this study. Another difference was that the upper limit of the EPI reference range exceeded 300 s in the previous study (4,11,15). However, the breed information was not available in those studies, it is difficult to consider what is attributable to these differences. However, according to other studies on antiplatelet effects of aspirin or clopidogrel in dogs using PFA, the reported ranges of CTs of control group or healthy animals can be used for comparison of the reference ranges depending on breed size. In C/ADP cartridges, the CT ranges of six healthy beagles (small breed) in Saati's study were 53 to 102 s, 50 to 80 s, and 53 to 111 s, similar to this study (19). In Brook's study, the control group (n = 8) included medium-breed dogs such as Doberman pinscher and large-breeds such as German Shepherd and Newfoundland, which ranged from 59.5 to 115 s as well (2). In Fresno's study, the control group consisted of 20 healthy crossbreed animals, the mean \pm SD of C/ADP CT ranged 49 to 162 s and 50 to 143 ss per ten dogs, respectively (10). The reported ranges for the C/EPI cartridges of each control group were 105 to 283 s, 104 to 256 s, and 78 to 295 s in Saati's study (six healthy beagles) and 75 to 122 s and 102 to 136 s in Fresno's study (10,19). In Blois's study, the range of CT-C/EPI ranged from 72 to 179 s in the healthy group (n = 9) which included a Shetland sheepdog (small breed) and large breeds such as Boxer and Golden retrievers (1). Also, in Nielsen's study to investigate platelet function in clinically healthy dogs of four different breeds, longer PFA CT of C/EPI was found in Cairn Terriers (148 to 241 s, n = 12, small breed) compared to Boxers (95 to 138 s, n = 10, large breed) (18). On the other hand, no significant difference between the small breed Cavalier King Charles Spaniel group (n = 12; 118 to 186) and the large breed Labrador Retriever group (n = 11; 117 to 300) (18,22). In this study, no significant differences were found between breeds even when comparing small dogs. The variation of CT for each breed is wide and outlier values are also seen. Based on these results, it seems that breed differences do not affect CTs using both ADP and EPI cartridges. Differences in reference ranges may be attributed

Table 1. Descriptive statistics of CT of healthy small breed dogs

| Cartridge | Mean | Median | SD | Range (min-max) | 95% RI |
|----------------|------|--------|------|-----------------|--------|
| C/ADP (n = 49) | 71.2 | 67 | 21.2 | 42-144 | 43-140 |
| C/EPI (n = 49) | 91.2 | 87 | 31.8 | 41-200 | 44-195 |

to be interindividual rather than breed-specific variance (20). Further studies that determine interindividual differences in the same breeds are needed.

In some of the studies performed in veterinary medicine on PFA, C/EPI cartridge has not been used because C/EPI cartridges are not useful in dogs (4,15). However, in all 49 healthy dogs in this study completely blocked the ring of the C/EPI cartridge, setting the upper limit of 205 s. And there have been some other studies in which CTs for CT-C/EPI in all experimental animals were measured (1,6,10,18,19). It is not clear why in some dogs the ring of the C/EPI cartridges were closed or not. In these rare cases, aspirin-like defect cannot be ruled out as the cause of prolonged CT-C/EPI. Instead, it is possible that epinephrine is a species-specific difference affecting platelet aggregation (9). In dogs, as epinephrine-related platelet response is not clearly known (21), attention should be given to the evaluation of CTs using PFA with CT-C/EPI cartridges.

It has been reported that various reactions may occur depending on the type of adrenoceptor that epinephrine acts on platelets (21). In humans, the density of excitatory α_2 receptor was determined greater than that of inhibitory β_2 receptor (12). As the predominant excitatory receptor, epinephrine acts as the primary platelet agonist. Therefore, in humans, using C/EPI is more sensitive to platelet function evaluation than C/ADP, so they are tested first by screening. Unlike humans, in our knowledge, α/β adrenoceptor ratio of canine platelet has not been studied (12). According to previous Soloviev's study, based on the results of reduced platelet aggregation at high concentrations of epinephrine in dogs, it can be assumed that when epinephrine acts on inhibitory receptors when it is high above a certain concentration (21). It addresses the need for study which adrenaline receptors dominate depending on the concentration of epinephrine as well as α/β adrenoceptor ratio of dogs and use it for platelet function tests with the dose of epinephrine cartridge specific to dogs. Through such further research, it may be possible to identify the reason for the non-closure outcome.

In conclusion, this report demonstrates that the reference ranges of PFA CT in small-breed dogs are similar to pre-existing reference ranges for large or mixed breeds. The use of C/ADP cartridges in the evaluation of platelet function in small breed dogs is the preferred choice. On the other hand, variable response of epinephrine to platelet aggregation in dogs warrants caution using C/EPI cartridges.

Author Contributions

Conceptualization: KY Lee, Choi US; Data curation: KY Lee, Kim YH; Formal analysis: KY Lee, Kim YH; Investigation: KY Lee; Methodology: KY Lee, Kim YH; Project administration: Choi US; Supervision: Choi US; Writing - original draft: KY Lee; Writing - review & editing: Kim YH, Choi US.

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Conflicts of Interest

The authors have no conflicting interests.

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