J. Vet. Sci. (2010), **11**(2), 121−124
DOI: 10.4142/jvs.2010.11.2.121

JOURNAL OF
Veterinary
Science

Effect of storage conditions on prothrombin time, activated partial thromboplastin time and fibrinogen concentration on canine plasma samples

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The present study was to assess the effect of storage conditions on prothrombin time (PT), activated partial thromboplastin time (aPTT) and fibrinogen concentration in blood samples of healthy dogs. Thirty-five dogs of various breeds were included in the study. Citrated blood samples were obtained and plasma was divided into four aliquots to assess selected clotting parameters by means of a coagulometer. The first aliquot was analysed within 1 h after collection, while the remaining 3 were stored at 8°C for 4, 8 and 24 h, respectively. One-way repeated measures analysis of variance documented a significant decreasing effect on PT at 24 h compared to 8 h and on fibrinogen concentration after 8 and 24 h compared to sampling time and at 4 and 24 h compared to 8 h post sampling. In conclusion, the results of this study indicate that only fibrinogen appears prone to significant decrease. In fact, aPTT is not substantially affected by refrigeration for at least 24 h post sampling and PT showed a statistical difference that does not necessary indicate biological significance as the results obtained were within reference intervals for the dog.

Keywords: thromboplastin time, dog, fibrinogen, prothrombin time, storage conditions

Introduction

Prothrombin time (PT), activated partial thromboplastin time (aPTT) and fibrinogen concentration are widely used screening tests in the clinical setting for the evaluation of the canine coagulation profile. Although modern coagulation diagnostics is becoming increasingly complex, PT, aPTT and fibrinogen concentration are still important and reflect the activity of several coagulation factors of the extrinsic and/or the intrinsic system [10]. Clotting tests are useful in the diagnosis of coagulation disorders and the monitoring of anticoagulant therapy [3,5,9,12,15]. Measurements of these parameters are performed by commercial kits and should be performed within 2～3 h of blood collection [4]. In clinical practice, recommended time allowances between collection and plasma analysis are frequently exceeded. Therefore, the potential impact of pre- analytic factors such as storage temperature and time in the measurements need to be taken into account. Previously, the effect of anticoagulant and storage conditions on platelet clumping has been evaluated in dogs [11] and the stability of canine plasma stored at room temperature or at 4°C for hemostasis testing has been investigated at 24, 48, 72 and 96 h post sampling [6]. Additionally, the effect of long-term storage at −20°C [16] and that of 6-month storage on hemostatic function testing [2] in the dog have been reported. The possible impact of 24-h storage on PT, aPTT and fibrinogen concentration, however, has rarely been assessed.

Since it is not always possible for measurement of clotting parameters to be completed within 3 h of blood collection, a good storage procedure of blood samples is necessary in order to obtain reliable results. For this reason, the aim of this study was to evaluate the potential effect of storage at 8°C for 4, 8 and 24 h post-sampling on PT, aPTT and fibrinogen concentration in the dog.

Materials and Methods

Samples

Blood samples were obtained from 35 clinically healthy dogs (1～6 years old) of various breeds, including 22 Mongrels, 6 Labrador Retrievers, 3 Canecorso, 2 Golden

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Retrievers and 2 Boxers. Dogs were deemed healthy if they did not have a history of haemostatic and haematological disorders and if no abnormalities were found on physical examination and complete blood count. No pharmacological treatment was administered for one month prior to the study.

Blood was collected from the cephalic vein and within 20 sec it was transferred into two tubes containing K_3-ethylenediaminetetraacetic acid (K_3-EDTA) and 3.8% sodium citrate, respectively. On EDTA-anticoagulated samples, a complete blood count was performed in an automatic haematology analyzer (HecoVet; SEAC, Italy) within 30 min. Citrated-anticoagulated blood samples were immediately centrifuged (Thermo Scientific CL10 centrifuge) at 3,000 g × 15 min, plasma was removed with a plastic pipette and transferred into Eppendorf microtubes. Plasma samples were divided into four aliquots to assess PT, aPTT and fibrinogen concentration by means of an automatic coagulometer (Clot 2; SEAC, Italy) according to the manufacturer's instructions and to a standard protocol to exclude differences that result from dissimilar test procedures. The first aliquot was analysed within 1 h of blood collection, the second after refrigeration at 8°C for 4 h, the third after refrigeration at 8°C for 8 h and the fourth after refrigeration at 8°C for 24 h.

PT test

The PT was assessed by means of a standard kit suitable for the SEAC Clot 2 coagulometer. The assay procedure consisted of placing 200 µL of tissue factor (PT reagent) in a test tube preheated to 37°C, and subsequently adding 100 µL of citrated plasma. Upon the addition of test plasma, a stopwatch was started and the clotting time was measured. The time, expressed in seconds, from the plasma-reagent mixing to a visually detected clot formation was defined as the PT.

aPTT test

The aPTT was assessed by means of a standard kit suitable for the SEAC Clot 2 coagulometer. The assay procedure consisted of placing 100 µL of citrated plasma and 100 µL of aPTT reagent (preheated to 37°C) in a test tube preheated to 37°C, followed by incubation for 3 min at 37°C, followed by the addition of 100 µL of calcium chloride (preheated to 37°C). Upon the addition of calcium chloride, a stopwatch was started and the clotting time was measured. The time, expressed in seconds, from this addition to a visually detected clot formation was defined as the aPTT.

Fibrinogen determination

Fibrinogen concentration was determined by means of a standard kit suitable for the SEAC Clot 2 coagulometer. The assay procedure consisted of placing 200 µL of 1 : 10 (100 µL of plasma + 900 µL of buffer) prediluted plasma in a test tube preheated to 37°C, followed by incubation for 2 min at 37°C, followed by addition of 100 µL of the fibrinogen reagent. Upon the addition of fibrinogen reagent, a stopwatch was started and the clotting time was measured. For each assay, the results in seconds were automatically converted into mg/dL by an automated mechanical endpoint coagulation instrument.

Statistical analyses

All results were expressed as mean ± SD. One-way repeated measures analysis of variance (ANOVA) was used to determine significant differences. p values < 0.05 were considered statistically significant. Bonferroni's multiple comparison test was applied for post hoc comparison. Data were analyzed using the software (Statistica 7.0; StastSoft, USA).

Table 1. Average values of prothrombin time (PT), activated partial thromboplastin time (aPTT) and fibrinogen concentration, expressed in their conventional units of measurement, obtained during different experimental conditions in 35 healthy dogs

Parameters		Experimental conditions			
		After collection (within 1 h)	After 4 h at 8°C	After 8 h at 8°C	After 24 h at 8°C
PT (sec)	Mean ± SD	7.23 ± 0.68	7.18 ± 0.70	7.57 ± 0.57	6.93 ± 0.64[‡]
	Median	7.20	7.00	7.60	6.91
	Min ~ Max	6.00 ~ 8.40	6.20 ~ 8.80	6.60 ~ 8.50	5.90 ~ 8.00
aPTT (sec)	Mean ± SD	12.45 ± 0.86	12.42 ± 0.80	12.73 ± 0.99	12.51 ± 0.55
	Median	12.70	12.40	12.90	12.70
	Min ~ Max	10.00 ~ 13.60	10.90 ~ 13.80	11.00 ~ 14.00	11.60 ~ 13.10
Fibrinogen (mg/dL)	Mean ± SD	321.50 ± 31.19	336.40 ± 37.28	287.90 ± 39.76[*,†]	228.20 ± 22.90[*,†,‡]
	Median	320.00	334.30	287.00	228.00
	Min ~ Max	260.00 ~ 380.00	260.00 ~ 402.80	220.00 ~ 369.40	181.00 ~ 270.00

[*] $p < 0.001$ compared to after collection, [†] $p < 0.001$ compared to after 4 h, [‡] $p < 0.001$ compared to after 8 h.

Results

Table 1 shows the mean values of PT, aPTT and fibrinogen concentration obtained in the different experimental conditions in 35 healthy dogs, together with standard deviations and statistical significances.

One-way repeated measures ANOVA showed a statistical significant effect of the storage conditions on PT ($F_{(3,102)} = 6.51$; $p = 0.0004$) and fibrinogen concentration ($F_{(3,102)} = 75.59$; $p < 0.0001$). Bonferroni's multiple comparison test showed that there was a statistically significant effect of the storage condition as follows: PT decreased after 24 h *vs*. after 8 h ($p < 0.001$) and fibrinogen concentration decreased after 8 and 24 h *vs*. after collection ($p < 0.001$), decreased after 8 and 24 h *vs*. after 4 h ($p < 0.001$) and decreased after 24 h *vs*. after 8 h ($p < 0.001$).

Discussion

The results of this study suggest that storage of canine plasma at 8°C has a significant effect on certain hemostatic parameters of canine plasma, including PT and fibrinogen concentration, while storage at 8°C for up to 24 h has an insignificant effect on aPPT.

In contrast to another study which demonstrated no significant differences for clotting parameters when samples were stored at room temperature [15], the present study suggested instability of clotting factors when stored at 8°C. In our study, PT decreased 24 h after storage at 8°C in accordance with several studies in humans [7,13] and in dogs [6]. This instability at low temperatures may be the result of clot-induced activation of proteolytic enzymes that are responsible for the slow degradation of factors VIII, IX and XI [8]. PT, in fact, reflects the activities of multiple factors and it has been shown that a significant decrease in any one factor must occur before PT becomes significantly prolonged [6].

In our study, fibrinogen concentration decreased 8 and 24 h after storage at 8°C. In contrast to one study that assessed the effect of freezing on fibrinogen levels [14], refrigeration in this study apparently had a decreasing effect on fibrinogen concentration similar to that of storage at room temperature observed previously [15]. The decrease induced by the storage was minimal but statistically significant. This variation is due to conformational changes of fibrinogen triggered by refrigeration resulting in an altered precipitation tendency. The final turbidity of a fibrin clot generated from previously refrigerated fibrinogen appears to be greater than the turbidity of a fibrin clot formed from fresh plasma [1]; this difference can be demonstrated by kinetic assay, indicating that the changes induced by refrigeration affect the fibrinogen concentration.

We can conclude that samples are quite stable for a few hours post sampling if stored at 8°C. In fact, the results of this study indicate that only fibrinogen appears prone to significant decrease. aPTT was not substantially affected by refrigeration for at least 24 h post sampling and PT showed a statistical difference that does not necessarily indicate biological significance as the results obtained were within reference intervals for the dog.

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