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ORIGINAL RESEARCH

Effect of hemolysis on canine kaolin-activated thromboelastography values and ADVIA 2120 platelet activation indices

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Key Words
Clot strength, coagulation time, dog, hemostasis, mean platelet component concentration, thromboelastogram

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Background: The impact of hemolysis on thromboelastography (TEG) and platelet activation indices has not been evaluated.
Objective: The aim of this study was to investigate the influence of hemolysis induced mechanically (HM) and hemolysis induced by freezing (HF) on TEG, platelet counts (PLT), and platelet activation indicators.
Methods: Blood from 17 dogs was divided into the following samples: controls, HM, and HF. HM was induced by 20 repetitions of expulsion of blood through a 23 g needle. Freezing was at -80°C, followed by warming to 37° and dilution with equal parts room temperature blood at 22°C. TEG variables that were examined included reaction time (R), coagulation time (K), angle (α), maximum amplitude (MA), and clot rigidity (G). Platelet indices were measured with the ADVIA 2120 hematology analyzer.
Results: Hematocrit (HCT) (mean $\pm$ SD) for controls, HM, and HF were 0.41 ± 0.02, 0.39 ± 0.03, and 0.25 ± 0.02 L/L, respectively, consistent with decreases in HCT of 4.8% (HM) and 39.0% (HF). HM resulted in decreased R (2.5 ± 0.9 minutes compared with 5.2 ± 1.9 minutes for controls; $P < 0.001$), and HF resulted in increased K (15.2 ± 8.6 minutes compared with 5.3 ± 4.0 minutes in controls; $P < 0.01$) and decreased α (20 ± 11° compared with 46 ± 17° in controls; $P < 0.001$). MA was decreased more in HF samples (26 ± 2 mm) than in HM (38 ± 8 mm) or control samples (49 ± 9 mm; $P < 0.0001$). The same applied to G values. PLT decreased after HM but not after HF. Hemolysis of both types resulted in decreased mean platelet component (MPC) concentration: control, 19.3 ± 2.0, HM 15.5 ± 3.4, and HF 14.3 ± 0.7 g/dL ($P < 0.0001$).
Conclusion: In hemolyzed samples decreased MPC and R suggested activated primary and secondary hemostasis, respectively, but decreased MA and G indicated reduced clot firmness, possibly due to hyporeactive platelets. TEG and platelet activation indices should be interpreted cautiously after hemolysis.

Introduction

Thromboelastography (TEG) is a global coagulation test to assess primary, secondary, and tertiary hemostasis. Five TEG variables are usually evaluated. Reaction time (R) is primarily influenced by plasma clotting factors and endogenous inhibitors of coagulation.[1] Coagulation time (K) is the time required for a clot, once initiated, to achieve predetermined clot strength. Angle (α) indicates the rapidity of fibrin cross linking.[1] Maximal amplitude (MA) is a measure of peak rigidity manifested by the clot and is mainly dependent on platelet number and function.[1] G is the calculated measure of clot rigidity and thus overall coagulation ability (in dynes per square mm). G is calculated as follows: $G = (5000 \times \mathrm{MA})/(96 - \mathrm{MA})$.[1]

TEG analysis is a useful point-of-care coagulation assay; however, as it is a global test, components such as platelet function that influence the coagulation process are not specifically addressed. Unfortunately, most traditional measures of platelet function or activation, eg, platelet aggregation (light transmission method or

impedance based whole blood aggregometry), are technically difficult and labor intensive.[2,3] An exception is analysis with a platelet function analyzer, which, however, cannot be used reliably in anemic specimens.[4] Consequently, there is increasing interest in automated methods for assessment of platelet activation. Sphering of platelets upon exposure to EDTA and 2-dimensional laser light scattering allow the ADVIA 2120 hematology analyzer to specifically assess platelets. During each analysis platelet indices that are becoming recognized as surrogate markers of platelet activation are routinely provided. These include mean platelet volume (MPV), mean platelet mass (MPM), mean platelet component (MPC), and platelet component distribution width (PCDW).[5–7] The MPC is a measure of platelet refractive index reflecting platelet granularity and thus activation status[8] and in people and dogs correlates well with P-selectin expression.[9–11]

It is known that routine coagulation testing can be affected by hemolysis.[12] In human laboratories, a 3.3% relative prevalence of hemolyzed samples submitted to a clinical laboratory has been reported.[13] Despite many clinical studies evaluating hemostasis in patients and animals, there are only rare investigations assessing the impact of hemolysis on coagulation tests. To the authors' knowledge, the influence of hemolysis on ADVIA 2120 platelet activation indices has not been specifically elucidated in humans or animals. There is a single study that examined the impact of mechanical hemolysis of equine blood on rotation thromboelastometry, a method comparable to TEG, using a ROTEM Gamma thrombelastometer (Pentapharm GmbH, Munich, Germany).[14]

The aim of our study was to investigate ADVIA 2120 platelet activation indices and kaolin-activated TEG analysis after induction of in vitro hemolysis by 2 methods: first, evaluation of the effect of severe shear stress on blood cells by induction of mechanical hemolysis (HM); second, the investigation of the impact of destruction of the cell membranes on coagulation by induction of hemolysis by freeze–thawing.

Materials and Methods

The prospective investigation was approved by the Ethics Committee for Animal Welfare, Giessen, Germany (No. V54-19c20/15cGi18/17), January 2008. TEG and hematologic analysis were always performed by 1 of the authors (O.E.).

Seventeen dogs (15 Beagle dogs, 1 German Shepherd Dog, 1 Golden Retriever) with a mean age of 4 years (range, 3–5 years) were included: 6 intact males, 5 neutered males, and 6 spayed females. The dogs were obtained from 2 breeders of animals for biomedical research (Harlan Winkelmann GmbH, Borchen, Germany and Marshall Bioresources Europe, France). They were fasted for 12 hours before blood sampling. Inclusion criteria were unremarkable physical, hematological, and clinical chemical examinations, unremarkable coagulation profile, no history of increased bleeding tendency, and no medications for at least 2 weeks. Blood was collected from the cephalic vein into siliconized 1.2 mL vacutainer tubes containing 3.18% trisodium citrate (Sarstedt, Nümbrecht, Germany) with an 18 g venous catheter. Samples were carefully checked for proper filling. Only specimens with an exact ratio of 9:1 blood to citrate anticoagulant were included.

Induction of hemolysis

Approximately 6 mL citrated whole blood from each dog was divided into 4 aliquots of 1.3 mL. One aliquot served as a control, and 1 was used for HM sample by rapid aspiration of 1.3 mL into a syringe with a 23 g needle followed by strong expulsion back into the test tube, repeated 20 times. Hemolyzed specimens were prepared from the remaining 2 aliquots by freezing followed by thawing (HF samples): 1 aliquot was frozen at -80°C for 1 hour; the other 1 was stored at room temperature (22°C) for the same period of time and was mixed with the first aliquot in equal parts after thawing at 37°C.

ADVIA 2120 analysis

ADVIA 2120 platelet indices were assessed within 10 minutes of blood collection for control samples and after preparation of hemolyzed specimens (approximately 20–30 minutes after sample acquisition for HM; 60–70 minutes for HF). The ADVIA 2120 system (Siemens Healthcare Diagnostics GmbH, Eschborn, Germany) was equipped with veterinary software version 5.3.1.-MS. ADVIA 2120 analyses included hematocrit (HCT), hemoglobin concentration, platelet count (PLT), platelet activation indices, number of free erythrocyte membranes ("erythrocyte ghosts"), number of erythrocyte fragments (reflecting destruction of erythrocytes), and number of "platelet clumps" (ie, platelet aggregates). Platelet clumps represent the absolute number of events detected in the platelet clump area of the peroxidase scattergram and were reported as unitless numbers. ADVIA 2120 platelet parameters as well as erythrocyte ghosts and fragments were

measured flow cytometrically in the ADVIA 2120 platelet channel.

Platelet activation indices were derived from measurements of intensity of 2-dimensional laser light scatter by the ADVIA 2120 and included MPV, MPC (measurement of platelet density calculated directly from refractive index), MPM (calculated from the platelet dry mass histogram [mean of platelet volume × platelet content/100]), and PCDW (measure of the variation in platelet shape change [MPC × 100/SD of MPC]).

Hemoglobin was detected colorimetrically by the ADVIA 2120 with a cyanide-free method.[15] Free hemoglobin concentration was indirectly assessed by calculating the difference between MCHC (indicative of cellular and free hemoglobin) and flow cytometrically measured corpuscular hemoglobin concentration mean (CHCM) reflecting cellular hemoglobin not affected by hemolysis.[16] A difference between MCHC and CHCM of > 5.78 mmol/L was considered to be an index of hemolysis. This calculation was based on the difference between MCHC and CHCM indicative of hemolysis (1.18 mmol/L) plus the mean bias of 4.6 mmol/L for MCHC using the cyanide free-method of hemoglobin detection. The HCT before and after lysis was compared to calculate the percentage of hemolysis. The hemolysis-induced changes noted in the ADVIA 2120 results (ie, the presence of platelet aggregates, erythrocyte ghosts, and erythrocyte fragments) were evaluated by microscopic evaluation of a blood smear. ADVIA 2120 results were confirmed by microscopic evaluation of a blood smear.

TEG analysis

TEG analyses were performed for control specimens approximately 1 hour after blood collection. HM was induced approximately 20–30 minutes after blood collection and samples were analyzed 30–40 minutes after induction of hemolysis. HF samples were analyzed 60–70 minutes after collection as 1 aliquot was frozen for 1 hour. TEG analyses were performed with recalcified citrated whole blood according to manufacturer recommendations using a TEG 5000 analyzer (Thrombelastograph, Haemonetics Corporation, Braintree, MA, USA). Briefly, 1 mL of citrated whole blood was placed in a silicated vial containing kaolin, buffered stabilizers, and a blend of phospholipids (TEG Hemostasis System Kaolin, Haemonetics Corporation). Mixing was ensured by gently inverting the kaolin-containing vials 5 times. Pins and cups (TEG Hemostasis System Pins and Cups, Haemonetics Corporation) were placed in the TEG analyzer in accordance with standard operating procedures recommended by the manufacturer. Each standard TEG cup was placed in the 37°C prewarmed instrument holder and filled with 20 μL 0.2 M calcium chloride. Then, 340 μL kaolin-activated citrated whole blood was added so that a total volume of 360 μL was reached. Internal quality control materials at 2 levels were run each day during the study (TEG Coagulation Control Level I and II, Haemonetics Corporation). An electrical internal quality control (*e*-test) was also performed.

Statistical analysis

Results were analyzed with GraphPad Prism (GraphPad Software, San Diego, CA, USA), Medcalc version 10.1.2.0 for Windows (Medcalc Software, Mariakerke, Belgium), and BMDP statistical software (BMDP Statistical Software Inc., Los Angeles, CA, USA). A Kolmogorov–Smirnov test was performed to verify the assumption of normality.

Differences between results obtained after HM and HF were assessed with 1-way ANOVA test and Tukey's multiple comparison posttest, respectively. In case of nonnormal distribution or missing values, a Friedman test with Dunn's multiple comparison posttest or a Wald test with Student–Newman–Keuls test were performed, respectively. The mean bias between hemolyzed and nonhemolyzed specimens was calculated with a Bland–Altman plot. The level of significance was set at $P < 0.01$ after Bonferroni's correction.

Results

Effect of hemolysis on ADVIA 2120 results

Data were normally distributed except for counts of erythrocyte fragments, erythrocyte ghosts, and platelet clumps and the hemolysis index (MCHC − CHCM). Mechanical stress induced only a slight decrease in mean HCT compared with baseline values (Table 1). HM was associated with a slight increase in MCHC (median 29.5 mmol/L compared with 27.1 mmol/L in control samples; $P < 0.05$) and a slight but significant increase in median CHCM (22.5 mmol/L compared with 22.0 mmol/L in control samples; $P < 0.001$). By contrast, freezing and thawing resulted in significantly higher degrees of hemolysis than mechanical stress ($P < 0.0001$) reflected by a marked decrease in mean HCT (Table 1). There was a marked increase in MCHC (median 44.6 mmol/L compared with 27.6 mmol/L in control samples; $P < 0.001$) with an unchanged CHCM (median 22.0 mmol/L in both HF and control samples). The increase in MCHC together with

Table 1 ADVIA 2120 RBC and platelet measurements and parameters and TEG variables in control samples and after induction of in vitro hemolysis by either mechanical stress (HM) or freeze-thawing (HF) ($n = 17$ healthy dogs).

	Groups		
Parameter/Measurement (units)	Control	HM	HF
HCT (L/L)	0.41 ± 0.02	$0.39 \pm 0.03^{**,+++}$	$0.25 \pm 0.02^{***,+++}$
MCHC − CHCM (mmol/L)	5.4 (4.9–6.4)	6.8 (5.7–9.2)	22.7 (17.2–28.2)**
RBC ghosts (cells/μL)	0.06 (0.02–0.119)	0.06 (0.01–0.10)$^{++}$	0.92 (0.67–1.02)$^{**,++}$
RBC fragments (cells/μL)	0.02 (0.0–0.03)	0.01 (0.01–0.02)*	0.01 (0.01–0.29)
PLT ($\times 10^9$/L)	281 ± 71	$181 \pm 69^{**,++}$	$261 \pm 56^{++}$
PLT clumps†	44 (27–395)	80 (42–224)$^{+}$	725 (258–1938)$^{**,+}$
MPV (fL)	12.8 ± 2.1	$17.7 \pm 4.0^{***,+++}$	$23.7 \pm 3.1^{***,+++}$
MPC (g/dL)	19.2 ± 2.0	$15.5 \pm 3.4^{**}$	$14.3 \pm 0.7^{**}$
PCDW (g/dL)	7.2 ± 0.7	$7.0 \pm 1.0^{++}$	$5.8 \pm 0.4^{**,++}$
MPM (pg)	2.08 ± 0.17	$2.08 \pm 0.17^{++}$	$2.6 \pm 0.26^{**,++}$
R (min)	5.2 ± 1.9	$2.5 \pm 0.9^{++}$	$5.6 \pm 3.7^{**,++}$
K (min)	5.5 (1.7–16.9)	9.0 (2.3–20.7)$^{++}$	9.9 (5.6–33.2)$^{**,++}$
α (degrees)	46.3 ± 16.8	$41.6 \pm 13.0^{++}$	$20.2 \pm 11.2^{**,++}$
MA (mm)	49.0 ± 9.3	$38.2 \pm 8.5^{***,+++}$	$27.0 \pm 11.6^{***,+++}$
G (Kdyn/cm^2)	5.1 ± 1.8	$3.2 \pm 1.2^{***,+++}$	$2.0 \pm 1.1^{***,+++}$

Results are the mean ± SD for measurements and parameters with a normal distribution (HCT, PLT, MPV, *R*, *K*, α, MA, *G*, MPC, PCDW, MPM) and median (range) if data were not normally distributed (erythrocyte fragments, erythrocyte ghosts, platelet clumps, hemolysis index [MCHC − CHCM]).

†Platelet clumps represent the absolute number of events detected in the platelet clump area of the peroxidase scattergram and are reported as unitless numbers.

Significant difference compared with the control: $^{*}P < 0.01$; $^{**}P < 0.001$; $^{***}P < 0.0001$.

Significant difference between HM and HF: $^{+}P < 0.01$; $^{++}P < 0.001$; $^{+++}P < 0.0001$.

The level of significance was set at $P < 0.01$ after Bonferroni's correction.

HF, hemolysis induced by freezing and thawing; HM, hemolysis induced by mechanical stress; *G*, clot rigidity; *K*, coagulation time; min, minutes; MA, maximum amplitude; mm, millimeter; *R*, reaction time; α, angle.

an unchanged CHCM resulted in significantly increased differences between MCHC and CHCM suggesting markedly greater hemolysis compared with HM (Table 1). A difference > 5.78 mmol/L between MCHC and CHCM, indicative of hemolysis, was observed in all samples after HF and in 16/17 specimens after HM. The difference between MCHC and CHCM slightly exceeded the cut-off value of 5.78 mmol/L in 3/17 samples in control samples (Table 1).

The median number of erythrocyte ghosts was significantly higher in samples after HF than in control samples (Table 1, Figure 1A–C; $P < 0.001$) whereas HM had no significant impact on results (Table 1; $P > 0.05$). Microscopic evaluation of blood smears prepared after HF revealed several strands and fragments of nuclear protein; however, erythrocyte ghosts were not visible. There was a significant difference ($P < 0.01$) between the median numbers of erythrocyte fragments in control samples and after HM, although the absolute difference was not clinically relevant.

Following HF, significant increases in the number of platelet clumps (Table 1, Figures 1D, 1E, and 2A; control samples compared with HM, $P < 0.001$; HM compared with HF, $P < 0.01$) and in MPV (Figure 2B; control samples compared with HM and HF, $P < 0.001$) were noted. There was a marked proportional bias that increased with increasing number of platelet aggregates (Figure 2A). After hemolysis, a significant increase in MPV was observed that was highest after HF as reflected by a mean constant bias of -10.8 fL (Figure 2B). The presence of many large platelets was also confirmed on the blood smear (Figure 1F). There was a significant difference in MPV between all groups (Figure 2B; $P < 0.001$). HM was associated with a decrease in mean PLT compared with control samples (Table 1; $P < 0.001$) and HF (Table 1; $P < 0.001$). After HM, the PLT was below the reference interval of 150×10^9/L in 6/17 specimens with results ranging between 85 and 135 platelets $\times 10^9$/L for those 6 specimens. Induction of hemolysis resulted in a significant ($P < 0.001$) decrease in MPC. Interindividual variation of MPC was markedly higher after HM than in control samples, whereas it was particularly low after HF (Figure 2C). There was a mean proportional bias of 3.8 g/dL in mechanically hemolyzed samples and of 5.0 g/dL after freeze–thawing. The decrease in MPC was associated with a significantly lower PCDW after HF than in control samples or after HM (Table 1; Figure 2D; $P < 0.001$) indicating the dominance of activated

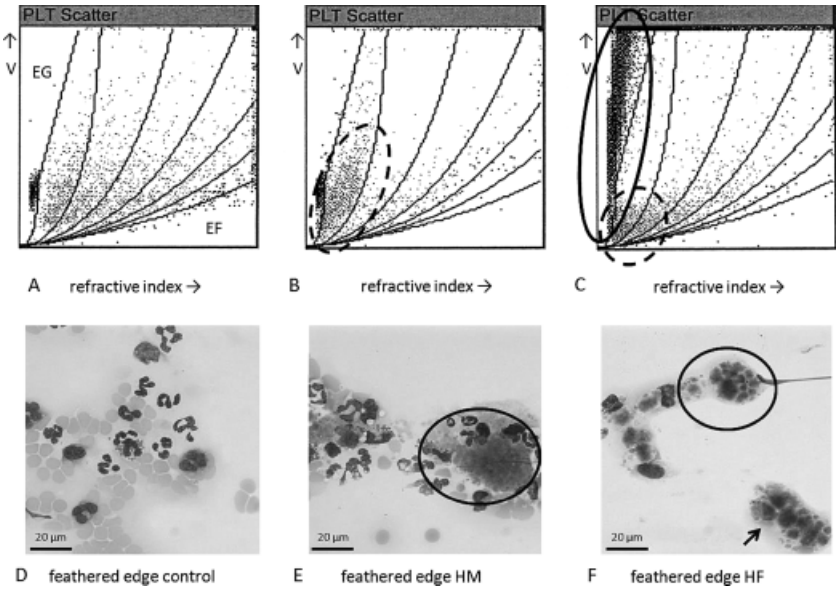


Figure 1. (**A–C**) Changes in ADVIA 2120 platelet scattergrams before and after induction of hemolysis by mechanical stress and freeze-thawing. Platelets were differentiated by 2-dimensional laser light-scattering according to cellular volume (V) and refractive index. Because of this unique laser-based technology, erythrocyte ghosts (EG) and erythrocyte fragments (EF) could be differentiated from platelets and were shown in separate areas of the scattergram. (**A**) Before induction of hemolysis. (**B**) After mechanical hemolysis. A population of platelets with low refractive index (dotted line) is present suggestive of platelet activation. (**C**) After freeze–thawing. Apart from activated platelets (dotted line), numerous erythrocyte ghosts are observed (solid line). (**D–E**) Feathered edge of blood smears. May–Grünwald–Giemsa. (**D**) Before induction of hemolysis. Note absence of platelet aggregates. (**E**) After mechanical hemolysis (HM). Several aggregates of small platelets (circle) are present. (**F**) After freeze-thawing (HF) and addition of an equal volume of nonhemolyzed blood. Several large platelet aggregates (circle) are seen. In contrast to HM, platelets appear significantly larger (arrow).

platelets. HF was associated with a significant increase in MPM compared with control results and HM (Table 1, Figure 2E; $P < 0.001$) with a mean constant bias of -0.54 pg.

Effect of hemolysis on TEG results

R was significantly shorter after HM than in control samples and after HF (Table 1, Figures 3 and 4A, $P < 0.001$). Following HM, a mean proportional bias of 2.7 minutes compared with control samples was present. *K* tended to be higher after HM compared with the control group (Table 1, Figures 3 and 4B); however, this trend was not statistically significant ($P > 0.05$). Following HF, a significantly reduced rapidity of fibrin-cross linking was noted reflected by increased *K* time in comparison with control samples ($P < 0.01$). After HF, TEG *K* values were not reported by the analyzer in 4/17 samples (23.5%) because MA never reached the required 20 mm. In HM and HF samples, mean constant biases of -4.0 and -9.8 minutes, respectively, were observed (Figure 4B). Hemolysis resulted in a decrease in α also indicating reduced rapidity of fibrin cross-linking. Differences between the controls and hemolyzed samples were highly significant for HF ($P < 0.001$) but insignificant for HM ($P > 0.05$). There was a mean constant bias of 4.7° after HM; however, in individual dogs a negative bias was present (Figure 4C). After HF, a marked constant bias of 26.1° was observed. Hemolysis generally resulted in a decreased clot stability and overall coagulation ability indicated by significantly lower MA and *G* values (Table 1, Figure 4D and E; between all groups: $P < 0.0001$). A mean constant bias of 10.8 and 22.0 mm was present after HM and HF, respectively. Similarly, hemolysis resulted in a highly significant decrease in *G* value reflected by a mean constant bias of 1.9 kdyn/cm^2 after HM and 3.1 ± 1.9 kdyn/cm^2 after HF. Except for *R*, hemolysis-induced changes of the variables were more severe when a high degree of hemolysis was present, ie, after HF.

Discussion

In vivo hemolysis may be caused by immune-mediated mechanisms, infectious agents (eg, *Babesia* spp.), drugs (eg, α-lactam antibiotics), toxins (eg, zinc), hereditary erythrocyte enzymopathies, RBC shearing (eg, in micorangiopathy and dissemincated intravascular

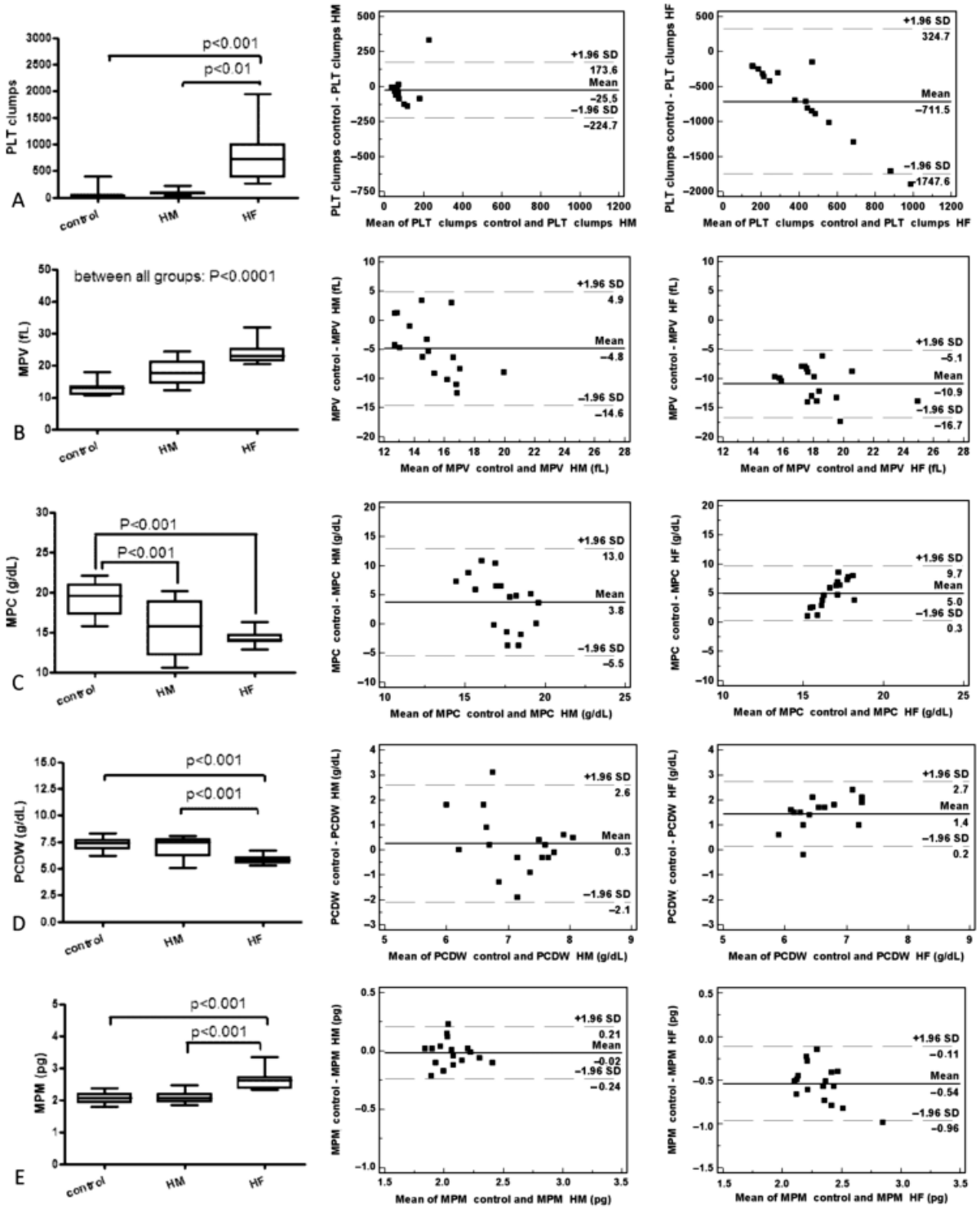


Figure 2. Effect of hemolysis on ADVIA 2120 (**A**) platelet (PLT) clumps (aggregates), (**B**) mean platelet volume (MPV), and platelet activation indices: (**C**) mean platelet component (MPC), (**D**) platelet component distribution width (PCDW), and (**E**) mean platelet mass (MPM) ($n = 17$ healthy dogs). (**Left**) Box and whisker diagrams depicting ADVIA 2120 measurements in control samples and after induction of hemolysis by mechanical stress (HM) and freeze–thawing (HF). The central box represents values from the lower to upper quartile. The middle line is the median. The vertical line extends from minimum to maximum values. The level of significance was set at $P < 0.01$ after Bonferroni's correction. (**Right**) Bland–Altman diagrams plotting the differences between values obtained in samples with hemolysis induced by mechanical stress (HM) or freeze–thawing (HF) and nonhemolyzed samples against means of results obtained in both types of specimens. The mean difference (bias, solid line) between hemolyzed and control samples $\pm$ 1.96 SD (dashed lines) are depicted.

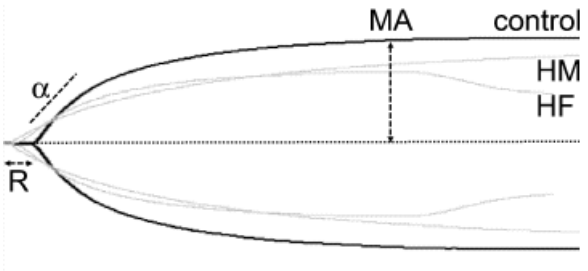


Figure 3. Thromboelastogram of a sample from 1 dog before hemolysis (black line) and after induction of hemolysis (gray lines) by mechanical stress (HM) or freeze–thawing (HF). *R*, reaction time; α, angle (indicating fibrin cross-linking); and MA, maximal amplitude (reflecting clot firmness and platelet function). See text, Table 1, and Figure 4 for comparison of results.

coagulation), and oxidative stress. In vitro causes of hemolysis include traumatic blood collection, transfer of alcohol to the blood specimens, use of needles with a small lumen, vigorous shaking of the sample after collection, exposure to extreme temperatures, or centrifugation at excessive speed for a prolonged time.[17] In the current study, in vitro hemolysis was induced both mechanically, to represent shear stress on RBCs, and by freeze–thawing. HM was designed to reflect the effect of difficult phlebotomy or excessive shaking after sampling; however, the shear stress induced in this in vitro model was greater than expected under normal practice conditions. A lesser degree of HM could occur in vivo. HF does not represent an in vivo situation; however, hemolyzed samples processed in the laboratory often contain lysed leukocytes and fragmented platelets in addition to lysed erythrocytes.[12] Thus, the model of HF appears to be a suitable surrogate, although it does not reflect all possible mechanisms for in vitro whole blood lysis in veterinary laboratories. Dilution of HF samples with an aliquot of untreated whole blood was necessary as freeze–thawing alone results in a HCT value of 0.00 L/L, which does not represent an in vivo situation and would have made TEG analysis impossible. However, this procedure had the drawback that results obtained after HF were not directly comparable with those after HM.

The difference between CHCM and MCHC may be used as an indicator of hemolysis. After induction of hemolysis, most samples exceeded the cut-off value of 5.78 mmol/L; however, there was an overlap with the control group in which a low number of samples had slightly higher differences between CHCM and MCHC. Regarding the cut-off value, the calculation was based on the difference between MCHC and CHCM indicative of hemolysis using the traditional cyanide-based method of hemoglobin detection plus the mean bias between MCHC determined with the cyanide-free method. Further studies are required to calculate the sensitivity and specificity of detection of hemolysis at different cut-off values for the difference between CHCM and MCHC using the cyanide-free method. The current study clearly demonstrated that the majority of hemolysis-induced changes were greater in samples with a greater degree of hemolysis, ie, after HF. However, probable dose-dependent effects were not specifically addressed by producing samples with increasing degrees of hemolysis. Analysis of multiple subgroups for both HM and HF would have required consecutive measurements or >4 TEG analyzers, which were not available in the authors' laboratory. As TEG results have been demonstrated to be highly dependent on storage time after sampling,[1] the number of assays was limited to those that could be performed within 1 hour of sampling. Despite the large number of erythrocyte ghosts detected by the ADVIA 2120 analyzer after induction of HF, erythrocyte membranes were not visible on the blood smear. This was most likely because erythrocyte membranes were disrupted during the process of freeze–thawing resulting in loss of hemoglobin into blood plasma. As staining intensity of erythrocytes is dependent on hemoglobin content it can be hypothesized that totally hemoglobin-depleted lysed erythrocytes become transparent on May–Grünwald–Giemsa-stained blood smear but still produce a signal during flow cytometric measurement.

Decreases in MPC after induction of hemolysis indicated platelet activation by either degranulation[18,19] or fluid uptake.[20] The latter mechanism has been considered to be the major cause of decreased MPC,[20] and is also likely here as large platelets cannot have been released from bone marrow in an in vitro experiment. An increase in MPM has been associated with decreases in MPC and PCDW as well as with increases in MPV in human patients.[6] Based on that study, the increase in MPM after HF reported here could be considered to be indicative of platelet activation, although further studies are necessary to elucidate the diagnostic use of MPM as an indicator of platelet activation in animals and people. The current study clearly demonstrated that hemolysis-induced changes in ADVIA 2120 platelet indices were consistent with platelet activation. Several mechanisms for prohemostatic platelet effects might be operant, including adenosine diphosphate efflux from lysed erythrocytes[21] and exposure of phosphatidylserine on erythrocyte membranes acting as platelet activator, as reviewed elsewhere.[22] Although hemolysis was associated with

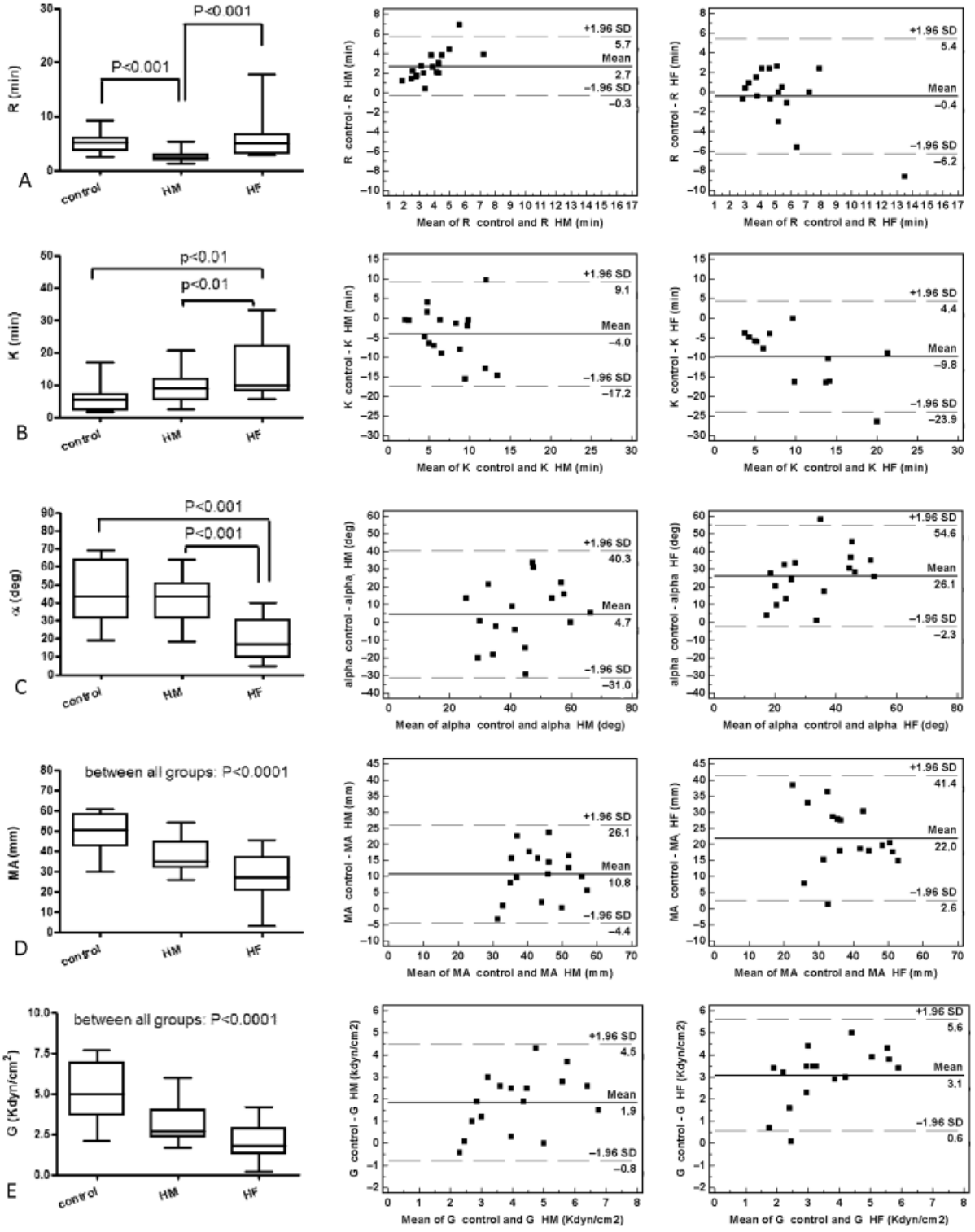


Figure 4. Effect of hemolysis on thromboelastography results: (**A**) reaction time (*R*), (**B**) coagulation time (*K*), (**C**) angle (α), (**D**) maximum amplitude (MA), and (**E**) clot rigidity (*G*) ($n = 17$ healthy dogs). (**Left**) Box and whisker diagram depicting measurements performed either in nonhemolytic recalcified citrated whole blood 1 hour after sample collection or after induction of hemolysis by mechanical stress (HM) or freeze–thawing (HF). The level of significance was set at $P < 0.01$ after Bonferroni's correction. (**Right**) Bland–Altman diagrams plotting the differences between values obtained in samples with hemolysis induced by mechanical stress (HM) or freeze–thawing (HF) and nonhemolyzed samples against means of results obtained in both types of specimens. For explanation of the diagram refer to Figure 2.

platelet activation and activation of secondary hemostasis reflected by decreased *R*-values for HM, the current investigation demonstrated that the overall effect was decreased coagulation ability as shown by decreases in MA and *G* values. As previously reported in people,[23] this paradoxical finding might be explained by the presence of activated, and thus refractory, platelets.

A limitation of the current study (and all other investigations using either the ADVIA 120 or 2120 analyzer for canine specimens) is that MPC can only be assessed if platelets are at least partially rounded, and this is not routinely achieved in canine whole blood samples.[20] Nevertheless, in this study hemolysis-induced changes in MPC were marked and highly significant and, in the authors' opinion, still relevant. A second limitation of the study is the choice of citrate as the anticoagulant. It is well known that the ADVIA platelet-sphering in EDTA-anticoagulated blood is considered advantageous for obtaining accurate MPV and MPC values, because calculations are based on the assumption that scattering is by spherical particles.[24] However, the platelet-activating property of EDTA makes its use as an anticoagulant for measuring platelet activation status questionable, and blood collected on EDTA cannot be used for TEG analysis. Thus, citrated whole blood was investigated in the current study.

In horses, a negative correlation between RBC count and HCT, and maximum rate of clot formation (maximum velocity; V_{max}) derived from the thromboelastometer curve was observed, and thus the authors concluded that the impact of RBCs on coagulation was greater than the presence of lysed erythrocyte membranes.[14] Erythrocytes are known to play an important role during the process of coagulation as they contribute to hemostasis not only through a mechanical effect (ie, margination of platelets to the periphery of the blood vessel),[25] but also through biological effects (ie, release of intracellular adenosine diphosphate and anionic phospholipid exposure resulting in promotion of platelet activation). However, low numbers of erythrocytes cannot be the only explanation for low MA observed here and in the previous study in horses as increased MA, shortened *R*, and increased α were observed in anemic human samples.[26] Therefore, the presence of already activated, and thus hyporeactive (depleted), platelets appears to be the most likely explanation for decreased MA and *G* values despite evidence of increased secondary hemostasis. However, aggregometry was not performed here to further substantiate this hypothesis.

Based on results of the current study, it can be concluded that blood cell lysis has a significant impact on ADVIA 2120 platelet activation indices and TEG parameters. Results should be interpreted cautiously if the difference between CHCM and MCHC is > 5.78 mmol/L.

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