Stability of hemostatic proteins in canine fresh-frozen plasma thawed with a modified commercial microwave warmer or warm water bath

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OBJECTIVE
To compare stability of hemostatic proteins in canine fresh-frozen plasma (FFP) thawed with a modified commercial microwave warmer (MCM) or warm water bath (37°C; WWB) or at room temperature (22°C).

SAMPLE
Fresh-frozen plasma obtained from 8 canine donors of a commercial blood bank.

PROCEDURES
A commercial microwave warmer was modified with a thermocouple to measure surface temperature of bags containing plasma. The MCM and a WWB were each used to concurrently thaw a 60-mL bag of plasma obtained from the same donor. Two 3-mL control aliquots of FFP from each donor were thawed to room temperature without use of a heating device. Concentrations of hemostatic proteins, albumin, and D-dimers; prothrombin time (PT); and activated partial thromboplastin time (aPTT) were determined for all samples.

RESULTS
Significant decreases in concentrations of factors II, IX, X, XI, fibrinogen, von Willebrand factor, antithrombin, protein C, and albumin and significant increases in PT and aPTT were detected for plasma thawed with the MCM, compared with results for samples thawed with the WWB. Concentrations of factors VII, VIII, and XII were not significantly different between plasma thawed with the MCM and WWB. Concentrations of D-dimers were above the reference range for all thawed samples regardless of thawing method. No significant differences in factor concentrations were detected between control and WWB-thawed samples.

CONCLUSIONS AND CLINICAL RELEVANCE
Significant differences in hemostatic protein concentrations and coagulation times were detected for plasma thawed with an MCM but not between control and WWB-thawed samples. Clinical importance of these changes should be investigated. (Am J Vet Res 2015;76:420–425)

ABBREVIATIONS
aPTT Activated partial thromboplastin time
AT Antithrombin
FFP Fresh-frozen plasma
MCM Modified commercial microwave warmer
PT Prothrombin time
vWF von Willebrand factor
vWF:Ag von Willebrand factor antigen
vWF:CBA von Willebrand factor collagen-binding activity
WWB Warm water bath

Frozen-thawed plasma products are the primary means of replacing coagulation factors in hypocoagulable veterinary patients. In an emergency setting, rapidly available thawed products are often necessary to control hemorrhage associated with coagulopathies. A major limiting factor is the amount of time required to thaw plasma products, which typically are stored at −20°C or colder. Benefits of rapid thawing of plasma include faster availability of transfusable hemostatic proteins, decreased risk of contamination from contact with water, and lower risk of hypothermia for massive transfusions.

Several methods exist for thawing plasma. Most commonly, bags of frozen plasma are submerged in a WWB until liquefied. This process is frequently unregulated with respect to water bath temperature or final plasma temperature. Depending on size of the bag of plasma, the thawing process may require up to 30 minutes. A device for dry thawing of plasma has received FDA approval; this device offers the benefit of no contact with water and thus a decreased risk of microbial contamination. The fastest method requires the use of commercial plasma-thawing microwave warmers; however, expense associated with these human-grade medical appliances is cost-prohibitive for the veterinary market. Investigators of studies conducted to evaluate hemostatic protein stability for plasma thawed with a microwave warmer have reported discordant results, although the various modifications, thawing protocols, and proteins measured in these reports make it difficult to compare the data.
The authors are aware of only 1 study\(^7\) of canine plasma thawed by use of a microwave warmer. In that study,\(^7\) a small number of hemostatic tests were evaluated, and results were deemed to be unaffected by the microwave thawing process. The study involved use of numerous brief thawing periods and manual agitation until the product was liquefied. A more practical study design and comprehensive hemostatic tests have not been used for microwave-thawed canine plasma.

Therefore, there were 2 objectives for the study reported here. The first objective was to evaluate hemostatic protein concentrations and clotting times for canine FFP thawed by use of an MCM and a WWB. The second objective was to compare hemostatic protein concentrations and clotting times for canine FFP thawed by either of those methods with results for a control FFP aliquot from the same donor that was thawed to room temperature without use of a heating device. We hypothesized that there would be no difference in results among the 3 thawing methods.

**Materials and Methods**

**Sample**

Plasma was obtained once from each of 8 dogs that were donors at a commercial blood bank. Sample collection and blood processing were performed in accordance with standard methods.\(^8\) Plasma from each dog was allocated into two 60-mL bags and two 3-mL aliquots, all of which were frozen at \(-20^\circ\)C. Frozen plasma samples were shipped on dry ice via overnight courier to the investigators’ laboratory at Texas A&M University, where they were immediately transferred to a \(-20^\circ\)C freezer until the time of testing. Blood products were purchased from a commercial blood bank; consultation with our institution’s Office of Research Compliance revealed that it was not necessary for the study to be reviewed by an animal care and use committee.

**MCM**

As described elsewhere,\(^8\) a commercial microwave warmer (700W) was modified with a thermocouple to enable measurement of the surface temperature of bags of plasma (Figure 1). The device was set so that power to the MCM would be interrupted when surface temperature of the plasma bag reached \(19^\circ\)C.

As a modification to absorb direct microwave energy, a plastic beaker filled with water was placed on the turntable; the water-filled beaker was interposed between the magnetron (microwave source) and bag of plasma. A single bag of plasma was mounted against the thermocouple on the wall opposite the magnetron; it was secured so that the surface of the bag was in constant contact with the thermocouple. Surface temperature of the plasma bag was visible at all times on an external light-emitting diode connected to the thermocouple.

**Experimental procedures**

Both 60-mL bags of plasma from a donor were thawed concurrently (one in the MCM and the other in a WWB \([\text{approx} \ 37^\circ\)C]). Thawing by use of the MCM was performed until the surface of the plasma bag reached the predetermined temperature (\(19^\circ\)C) and power to the magnetron was interrupted. If large pieces of frozen plasma were still evident at that time, the bag was removed from the MCM, manually agitated for 5 seconds, and then returned to the MCM for additional microwave thawing until the preset surface temperature of \(19^\circ\)C was again reached. This process was repeated until all plasma in the bag was liquefied. Between 1 and 3 agitations were needed for complete thawing of all bags. Bags thawed in the WWB were fully submerged until the plasma was liquefied. Total thaw time, visual opacity of plasma, and final surface temperature were recorded for each bag. Three milliliters of plasma was obtained from each bag, placed in a serum separator (ie, red top) tube, and immediately refrozen at \(-20^\circ\)C for use in hemostatic protein testing. An additional 3 mL of plasma was obtained from each bag and used for determination of albumin, AT, and fibrinogen concentrations as well as measurement of PT and aPTT. One of the 3-mL aliquots obtained from each donor at the time of blood collection was kept frozen and used for hemostatic protein testing, whereas the other 3-mL aliquot obtained at the time of blood collection was thawed to room temperature (approx \(22^\circ\)C) without use of a heating device and used for determination of albumin, AT, and fibrinogen concentrations and measurement of PT and aPTT.

**Hemostatic tests**

Albumin, AT, D-dimer, and fibrinogen concentrations as well as PT and aPTT were measured on the same day that the samples were thawed. These tests were performed as a paid diagnostic service by personnel at the Texas A&M University clinical pathology laboratory.

Frozen samples were shipped on dry ice via overnight courier to the Comparative Coagulation Laboratory in the Department of Population Medicine and Diagnostic Science at Cornell University. Samples were stored at \(-70^\circ\)C for 1 week. Immediately before samples were assayed, they were removed from the freezer, allowed to equilibrate at room temperature (approx \(22^\circ\)C) for 10 minutes, and then thawed in a water bath (\(37^\circ\)C). Hemostatic proteins measured included coagulation factors (II, VII, VIII, IX, X, XI, and XII), protein C, vWF:Ag, and vWF:CBA.

All samples were measured concurrently to identify differences attributable to the thawing process. Analysis of factors II, VII, VIII, IX, X, XI, and XII; protein C; and vWF:Ag was performed in accordance with methods used at the Comparative Coagulation Laboratory at Cornell University.\(^9\) Assays for intrinsic coagulant factors (factors VIII, IX, XI, and XII) were performed by use of a 1-stage aPTT technique with a commercial aPTT reagent.\(^4\) Coagulant activity of fac-
tors II, VII, and X was measured by use of a 1-stage PT technique and rabbit brain thromboplastin reagent. Plasma AT activity was measured by use of a chromogenic substrate kit and on the basis of inactivation of bovine factor Xa. Clottable fibrinogen concentration was determined via the Clauss method by use of 35 National Institutes of Health units of bovine thrombin reagent/mL. Concentrations of D-dimers were determined by use of a latex-enhanced immunoassay kit.

Statistical analysis

Data were tested for normality with a D’Agostino and Pearson omnibus test. A repeated-measures ANOVA for parametric data and a Friedman test were performed for nonparametric data when comparing variables among thawing methods. Post hoc testing for parametric data was performed with a Tukey multiple comparison test, whereas a Dunn multiple comparison test was used for nonparametric data. A Wilcoxon matched-pairs signed rank test was used to compare thaw time between the WWB and MCM. Values were considered significant at $P < 0.05$.

Results

Significant decreases in concentrations of hemostatic proteins (factors II, IX, X, XI, fibrinogen, vWF:Ag, vWF:CBA, AT, and protein C) were detected for plasma thawed with the MCM, compared with results for plasma thawed with the WWB (Table 1). Both PT and aPTT were significantly prolonged and the albumin concentration significantly decreased for MCM-thawed plasma. There was no significant difference in concentrations of factors VII, VIII, and XII between plasma thawed with the MCM and that thawed with the WWB; however, there was a significant difference in concentrations of these factors between room-thawed control samples and MCM-thawed samples (Figure 2). Concentrations of D-dimers were increased in all plasma samples, including room-thawed control samples, but were not significantly different among thawing methods. Finally, there was no significant difference in the mean concentrations of any analyte between the WWB-thawed and room-thawed control samples. Thawing by use of the MCM required a median of 184 seconds (interquartile range, 156 to 210 seconds), which was significantly ($P = 0.014$) less than the median of 348 seconds (interquartile range, 305 to 395 seconds) for thawing by use of the WWB.

Table 1—Mean ± SD values for hemostatic factors evaluated in FFP obtained from 8 blood donor dogs and thawed at room temperature (22°C), by use of a WWB at 37°C, or by use of an MCM.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Room temperature</th>
<th>WWB</th>
<th>MCM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor II (%)</td>
<td>126.9 ± 41.5*</td>
<td>124.5 ± 35.5*</td>
<td>81.6 ± 34.2</td>
</tr>
<tr>
<td>Factor VII (%)</td>
<td>126.0 ± 31.1*</td>
<td>111.1 ± 21.6</td>
<td>94.8 ± 17.7</td>
</tr>
<tr>
<td>Factor VIII (%)</td>
<td>160.5 ± 124.8*</td>
<td>134.3 ± 85.1</td>
<td>94.8 ± 80.8</td>
</tr>
<tr>
<td>Factor IX (%)</td>
<td>133.5 ± 43.9*</td>
<td>122.4 ± 33.8*</td>
<td>81.6 ± 31.0</td>
</tr>
<tr>
<td>Factor X (%)</td>
<td>114.4 ± 23.4*</td>
<td>113.6 ± 21.8*</td>
<td>75.0 ± 15.6</td>
</tr>
<tr>
<td>Factor XI (%)</td>
<td>82.1 ± 23.4*</td>
<td>77.9 ± 21.4*</td>
<td>63.5 ± 26.7</td>
</tr>
<tr>
<td>Factor XII (%)</td>
<td>102.3 ± 31.1*</td>
<td>100.5 ± 27.8</td>
<td>88.5 ± 30.5</td>
</tr>
<tr>
<td>vWF:Ag (%)</td>
<td>120.1 ± 39.9*</td>
<td>119.0 ± 37.0*</td>
<td>87.0 ± 46.4</td>
</tr>
<tr>
<td>vWF:CBA (%)</td>
<td>81.9 ± 39.7*</td>
<td>74.8 ± 38.9*</td>
<td>56.1 ± 30.7</td>
</tr>
<tr>
<td>Protein C (%)</td>
<td>113.3 ± 16.1*</td>
<td>114.4 ± 16.2*</td>
<td>79.1 ± 34.8</td>
</tr>
<tr>
<td>Albumin (g/dL)</td>
<td>2.7 ± 0.1*</td>
<td>2.7 ± 0.1*</td>
<td>2.8 ± 0.1</td>
</tr>
<tr>
<td>PT (s)</td>
<td>6.9 (6.7–7.1)</td>
<td>6.6 (6.5–6.9)*</td>
<td>7.8 (7.2–8.8)</td>
</tr>
<tr>
<td>aPTT (s)</td>
<td>8.9 ± 0.5*</td>
<td>8.9 ± 0.3*</td>
<td>11.8 ± 1.7</td>
</tr>
<tr>
<td>Fibrinogen (mg/dL)</td>
<td>209.0 ± 63.0*</td>
<td>204.5 ± 63.0*</td>
<td>113.4 ± 48.0</td>
</tr>
<tr>
<td>D-dimers (ng/mL)</td>
<td>714.9 ± 394.7</td>
<td>633.6 ± 285.0</td>
<td>639.3 ± 150.4</td>
</tr>
<tr>
<td>AT (% of NHP)</td>
<td>104.5 ± 15.8*</td>
<td>104.3 ± 16.1*</td>
<td>84.1 ± 14.1</td>
</tr>
</tbody>
</table>

*Within a factor, value differs significantly ($P < 0.05$; overall ANOVA or Friedman test with post hoc testing by use of a Tukey multiple comparison test or Dunn multiple comparison test) from the value for the MCM.
†Value reported is median (interquartile range).
NHP = Normal human plasma.
Differing amounts of gross turbidity were observed in half the MCM-thawed bags (Figure 3). Two bags had evidence of precipitation (white strands) at the bag edges.

**Discussion**

Significant differences in concentrations of several hemostatic proteins and albumin as well as clotting times were detected for FFP thawed with an MCM, compared with results for FFP thawed with a WWB or room-thawed control samples. Although use of the MCM resulted in a more rapid thawing of plasma than did use of the WWB, it did not result in a product that was hemostatically equivalent to plasma thawed by the commonly accepted method of a WWB.

The decrease in hemostatic factor concentrations likely resulted from consumption secondary to activation of coagulation, which was suspected on the basis of grossly evident turbidity and precipitation of white strands (presumed to be fibrin) in several bags. Bags that were subjectively the most turbid also had the greatest decrease in factor concentrations, which suggested that consumption of factors was related to precipitation of proteins as determined by use of visual inspection. However, the finding that the decrease in concentrations of factors VII, VIII, and XII, compared with concentrations for room-thawed control samples, was not significant likely was attributable to the small number of samples and the small numeric change. This could have resulted in failure to detect a difference (type II error). Factor VIII is a labile factor, and factors VII and XII are involved in the initiation of coagulation (VII in the tissue factor pathway and XII in the contact pathway)\(^\text{10}\); therefore, it is likely that a difference in the concentrations of these factors would have been detected in a larger sample population because these factors are likely to be consumed if there is fibrin production in the samples.

The decrease in fibrinogen concentration in the MCM-thawed samples was likely the result of multiple factors. Formation of fibrin strands from activation of coagulation as well as heat precipitation of this protein were the most likely explanations. Presumed fibrin strand formation was especially prominent around the edges and corners of bags where the plasma sample would have been thinnest. Fibrinogen precipitates at 56°C, which suggested that localized overheating (hot spots) existed that could not be detected by the thermocouple of the MCM.\(^\text{11}\) Such localized overheating was evaluated in another study\(^\text{12}\) by the use of multiple temperature sensors in contact with the plasma bag in a commercial microwave blood warmer equipped with metal shields to protect thinner peripheral areas of plasma; no localized overheating was reported for that design. Use of an infrared thermometer to scan the entire surface of a plasma bag may be a more precise way to identify hot spots than is reliance on placement of surface sensors.\(^\text{13}\)

An interesting finding was the presence of increased concentrations of D-dimers, particularly in the control samples for which no external heat source was used for thawing. This has been reported for human plasma thawed in WWBs of various temperatures.
D-dimers are a degradation product of cross-linked fibrin, which forms when factor XIII is activated by thrombin. Increased D-dimer concentrations have been detected in dogs with a variety of disorders, including thromboembolic disease, cancer, and organ dysfunction, as well as after surgery. In addition, D-dimers currently are included in the criteria for diagnosing disseminated intravascular coagulation in dogs and are a screening test for pulmonary thromboembolism. Whether this abnormality in the present study was a result of improper sample collection, storage, shipment, or thawing, iatrogenic increases in patient D-dimer concentrations may affect their diagnostic utility.

Several modifications of microwave warmers for thawing of human plasma have been evaluated during the past several decades. These include metal shielding of the edges and corners of bags, rotation and agitation of bags, and inclusion of a temperature sensor in contact with the bag) designed to interrupt power at a predetermined temperature. Results of these modifications indicate no difference in plasma integrity between the MCM and WWB; however, the particular hemostatic tests performed were not specified. Earlier studies also included use of a regular microwave oven; instead of modifications to the microwave oven, the study design provided brief periods of microwaving for thawing with manual agitation between microwave-thawing periods. In those studies, there was a modest decrease in concentrations of factors V and VIII after microwave thawing, whereas concentrations of factors II, VII, IX, and X remained unchanged. The only veterinary study conducted to evaluate microwave-thawed plasma reported that clotting times and concentrations of fibrinogen, factor VIII, and vWF:Ag were similar to those for FFP thawed in a WWB. No other factors were evaluated, presumably because of a lack of available tests. In that study," plasma was thawed for 20-second periods for a mean of 15 thawing periods with manual agitation between subsequent thawing periods. Because of conflicting results, differences in study designs, and a limited ability for hemostatic factor analysis, the available studies in human and veterinary medicine offer only partial results, differences in study designs, and a limited ability for hemostatic factor analysis, the available studies in human and veterinary medicine offer only partial information about the benefits and disadvantages for microwave thawing of FFP.

Plasma-thawing microwave warmers first received FDA approval in 1989. Current guidelines advise caution in use of these devices because of concerns about creation of hot spots and the possibility that parts of a bag could act as an aerial and cause arcing. Newer models of plasma-thawing microwave warmers operate on a lower wattage (200W), compared with the wattage for earlier models and unmodified commercial microwave ovens (600 to 700W). The new devices are relatively expensive ($14,000); however, they can thaw a 240-mL unit in 2 to 3 minutes. A device for dry thawing of plasma received FDA approval in 2011 and is gaining popularity. This device maintains a contained sealed system at 37°C with no direct water contact, which prevents microbial contamination. Thawing time is approximately 10 to 15 minutes for 2 units, and these dry incubators are easier to maintain than is a WWB.

Thawing times in the present study were almost twice as fast for the MCM as for the WWB. Because thawing time depends on the surface area-to-volume ratio, there is likely to be an even greater difference in thawing times for larger plasma bags. Sixty-milliliter bags were specifically used in the study reported here to minimize wasted product. The other major disadvantage of WWBs, namely the propensity for bacterial contamination as a result of contact with water, was not investigated in the present study because all samples were immediately obtained after thawing and bacterial contamination would not have affected plasma integrity in that time frame. However, when plasma is transfused hours after thawing or is refrozen and thawed later, bacterial contamination would be a relevant concern that should be investigated.

Importantly, clinical implications of the decrease in concentrations of hemostatic factors were not evaluated in the present study. There must be a decrease of 70% in the concentration of any 1 factor before the PT or aPTT is significantly prolonged because these are nonspecific tests for an individual factor concentration. It is also commonly recognized that a prolongation of 25% for the PT and aPTT is considered clinically important. Although both PT and aPTT were significantly prolonged, compared with the baseline value for MCM-thawed plasma, the amounts of individual factors remaining may still have been of short-term therapeutic benefit until additional plasma products could be obtained. Such cost-benefit analysis was not the intent of this study; but it may be a consideration for future studies in which the use of MCM-thawed plasma is evaluated.

It has been reported in a previous study and was confirmed in the present study that intradonor and interdonor variability exists with regard to composition of individual factors in a unit of plasma. A typical healthy donor may have concentrations of 50% to 150% for a particular factor, compared with the reference range obtained for samples collected from 20 clinically normal dogs. This is important because it can be difficult to predict the volume of donor plasma required to replace a particular factor or set of factors in a recipient. For this reason, repeated monitoring of clinical signs (ie, resolution of hemorrhage) and evaluation of results of hemostatic tests should continue to guide transfusion therapy with plasma products. A starting point for transfusion with plasma products is 10 to 20 mL/kg, which is in accordance with previously published volume guidelines.

The present study underscored the variability in concentrations of component factors, particularly with respect to the baseline vWF concentration in 1 donor. Both vWF:Ag and vWF:CBA concentra-
tions were significantly below the reference range for the laboratory, which warrants further investigation. Because each donor served as its own control animal in the study, this did not influence the results. However, it does raise concerns about instituting hemostatic testing for donor dogs. Current standards for human and veterinary blood banks do not require hemostatic protein testing of donors; however, because of geographic and possibly genetic differences, such testing may be considered. This information would be particularly useful for situations in which a clinician must replace a specific factor (such as factors VIII, IX, and VWF in dogs).

Limitations of the present study were related to the experimental nature of the MCM modifications and the small sample size. Physical principles for the use of microwave energy to thaw FFP are realistic; however, further device modification is needed. That includes additional shielding of the edges of plasma bags, use of an agitating platform, more extensive monitoring of surface temperature, and decreasing the microwave wattage. Hemostatic tests would be necessary as a measure of the success of design modifications. Although a larger sample size is almost always of benefit, judicious use of FFP for experimental purposes must also be considered.

In the present study, we performed comprehensive hemostatic testing on plasma thawed by use of a microwave warmer with design modifications, which should be affordable to general practitioners. The study was also designed to be practical and to offer a solution for the common clinical problem of the need for rapidly available thawed plasma. Analysis of the results indicated that the version of the MCM reported here did not provide a thawed product that was hemostatically comparable to the product provided when FFP was thawed with a commonly used WWB. The clinical importance of these differences is unknown; however, additional design modifications and testing are needed before the clinical use of microwave-thawed FFP can be assessed in canine patients.

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Footnotes


References


