Effects of Freeze-Thaw Times on Screening Coagulation Tests and Factors VIII and IX Activities in Citrate-Anticoagulated Plasma at -20°C and -80°C

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SUMMARY

Background: Accurate determination of screening coagulation tests and factors VIII and IX activities (FVIII:C and FIX:C) in fresh plasma is very important for diagnosing abnormalities in the intrinsic or extrinsic coagulation pathways and factor deficiencies. If thawed samples cannot be detected for all required items at the same time, or need to be re-tested or re-stored, the thawed samples need to be re-frozen. We planned to perform in-house validation studies on freeze-thawed samples for screening coagulation tests, FVIII:C and FIX:C.

Methods: Mean percent changes, numbers of samples with > 10% changes, and difference plots were evaluated to determine clinically relevant differences between results for fresh and freeze-thawed samples. The statistical significance of differences between repeated-measure multiple groups and baseline values were evaluated by repeated-measures analysis of variance.

Results: The acceptable freeze-thaw cycles for activated partial thromboplastin time, fibrinogen, prothrombin time/international normalized ratio, thrombin time, and FIX:C were three times at -20°C/-80°C, while the acceptable freeze-thaw cycles for FVIII:C were three times at -80°C and once at -20°C.

Conclusions: The freeze-thaw results on stabilities were affected by time and temperature, with lower temperature and fewer times associated with more stable activity.

KEY WORDS

screening coagulation tests, factor VIII, factor IX, clinically relevant differences, freeze-thaw

INTRODUCTION

Screening coagulation tests including activated partial thromboplastin time (APTT), fibrinogen (Fbg), prothrombin time (PT), international normalized ratio (INR), and thrombin time (TT) are quite useful for detecting abnormalities in the intrinsic or extrinsic coagulation pathways, monitoring anticoagulant therapy, managing the risk of bleeding or thrombosis in patients with liver failure, and evaluating therapy in patients with hemophilia [1-6]. Factors VIII and IX activities (FVIII:C and FIX:C) are useful for diagnosis and treatment of hemophilia A (FVIII:C) and B (FIX:C) [5].
Pre-analytical conditions are very important for laboratory assessments of these tests [7,8]. Unqualified specimen preservation conditions can lead to unreliable results and interfere with clinical decisions. With the development of a hierarchical medical system in China, coagulation tests, FVIII:C, and FIX:C in some samples cannot be detected in community hospitals and clinics, and thus the collected samples are sent to independent clinical laboratories for analysis [9-11]. Moreover, the establishment of biobanks to facilitate the development of drugs and diagnostic tests has led to the development of biobank networks that collect and preserve large numbers of high-quality clinical biospecimens [12,13]. Although our preliminary tests established the short-term/long-term storage times and optimal temperatures for screening coagulation tests on FVIII:C and FIX:C, freeze-thaw cycles and temperatures are lacking for these items in citrate-anticoagulated plasma samples [14-16]. Previous studies have focused on freeze-thaw studies of coagulation factor assays in fresh-frozen plasma (FFP) for transfusion [17-20]. The Clinical and Laboratory Standards Institute H21-A5 recommendation states that individual laboratories should perform in-house validation studies [21]. Therefore, we planned to perform in-house validation studies on freeze-thawed samples for screening coagulation tests on FVIII:C and FIX:C in citrate-anticoagulated plasma in our laboratory.

MATERIALS AND METHODS

Methods
The sample population consisted of 76 asymptomatic individuals (38 men and 38 women; mean age: 40 years; age range: 20 - 59 years) recruited from the First Affiliated Hospital of Zhejiang University for physical examination in October 2016. A blood sample was collected from each subject in the morning after a 12-hours fast. A 2.7 mL venous whole-blood sample was collected into one tube containing 0.109 M sodium citrate as an anticoagulant (Becton Dickinson, Franklin Lakes, NJ, USA) at a blood-to-anticoagulant ratio of 9:1. The 76 samples were divided into equal aliquots and divided into two groups to be used to detect the freeze-thaw stability of PT/INR, APTT, Fbg, TT, FVIII:C, and FIX:C at -80°C and -20°C. All samples were centrifuged (10 minutes, 3,000 x g) to obtain fresh platelet-poor plasma. The 76 samples were then quickly divided into four Eppendorf tubes (numbered 0, 1, 2, 3) and capped. The Eppendorf tubes used for the aliquots were composed of a non-activating plastic. The No. 0 aliquots (freeze-thawing 0 times) were tested immediately as baseline (0 hour), and all testing was completed within 30 minutes after sample collection. The No. 1-3 aliquots were snap-frozen at -20°C (38 samples)/-80°C (38 samples). After 24 hours, all aliquots were thawed in a 37°C water bath for 6 minutes and mixed by 10 end-over-end inversions. The No. 1 aliquots (freeze-thawing once) were tested using the same lots of reagents and same instrument as the fresh sample testing above, while the No. 2 and 3 aliquots were re-frozen at -20°C/-80°C. After another 24 hours, the No. 2 and 3 aliquots were again thawed in a 37°C water bath for 6 minutes and mixed. The No. 2 aliquots (freeze-thawing twice) were tested as described above, and the No. 3 aliquots were re-frozen at -20°C/-80°C. After another 24 hours, the No. 3 aliquots (freeze-thawing three times) were thawed in a 37°C water bath for 6 minutes, mixed, and tested as described above.

Ethics statement
This study was approved by the Ethics Committees of the First Affiliated Hospital of Zhejiang University (Ethical Application Ref: 2015-15). All subjects provided written informed consent for their samples to be used in the study.

Laboratory assays
The aliquots were tested for PT/INR, APTT, Fbg, TT, FVIII:C, and FIX:C by coagulation assays using a Sysmex CS5100 system (Sysmex, Kobe, Japan) and Siemens reagents (Siemens, Marburg, Germany). The results were expressed as %.

Statistical analyses
The coagulation factor activity results were reported as mean ± standard deviation. The statistical significance of differences in values for repeated-measure multiple groups or frozen samples compared with baseline values was evaluated by repeated-measures analysis of variance (ANOVA). Differences between the values for PT/INR, APTT, Fbg, TT, FVIII:C, and FIX:C before and after freeze-thawing were calculated by the following equation: (value after freeze-thawing X times - value at baseline) x 100% / value at baseline. In accordance with our previous studies [14,15] and that by van Geest-Daaldero et al. [7], a clinically relevant difference was defined as a mean change of > 10%. If the number of individuals with > 10% changes was less than 25% of the total 38 samples (i.e., less than 10 samples), the effect was termed moderate. If more than 25% of the total 38 samples (i.e., more than 10 samples) had > 10% changes, the effect was deemed large. Difference scatter plots of the percent changes were drawn with the percent changes of all samples on the Y-axis and the tested items after storage at -20°C/-80°C on the X-axis. Two dotted lines were drawn in these plots to indicate plus or minus 10% changes. In Figure 1, the individual samples and the numbers of samples with changes exceeding ± 10% can be easily visualized. The trends for mean percent differences in PT/INR, APTT, Fbg, TT, FVIII:C, and FIX:C in samples after freeze-thawing three times were evaluated. Values of p < 0.05 were considered statistically significant. All statistical analyses were performed using IBM SPSS Statistics for Windows version 20 (IBM Corp. Armonk, NY, USA).
### Table 1. Values for screening coagulation tests, FVIII:C, and FIX:C in samples subjected to freeze-thawing at -80°C and -20°C.

<table>
<thead>
<tr>
<th>Cycles of freeze-thaw</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>-20°C</strong></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>PT (s)</td>
<td>11.9 ± 0.68</td>
<td>11.7 ± 0.68 *</td>
<td>12.0 ± 0.74 *</td>
<td>11.8 ± 0.76</td>
</tr>
<tr>
<td>INR</td>
<td>1.04 ± 0.06</td>
<td>1.02 ± 0.06 *</td>
<td>1.04 ± 0.07 *</td>
<td>1.03 ± 0.07</td>
</tr>
<tr>
<td>APTT (s)</td>
<td>30.2 ± 3.43</td>
<td>31.2 ± 3.90 *</td>
<td>31.7 ± 4.03 *</td>
<td>32.2 ± 4.40 *</td>
</tr>
<tr>
<td>Fbg (g/L)</td>
<td>2.87 ± 0.94</td>
<td>2.89 ± 0.93</td>
<td>2.93 ± 0.95 *</td>
<td>2.89 ± 0.94 *</td>
</tr>
<tr>
<td>TT (s)</td>
<td>18.1 ± 1.12</td>
<td>18.1 ± 1.24</td>
<td>17.9 ± 1.24 *</td>
<td>17.9 ± 1.22 *</td>
</tr>
<tr>
<td>FVIII:C (%)</td>
<td>138.7 ± 33.42</td>
<td>132.6 ± 30.26 *</td>
<td>121.4 ± 30.49 *</td>
<td>120.0 ± 30.45 *</td>
</tr>
<tr>
<td>FIX:C (%)</td>
<td>77.9 ± 13.72</td>
<td>75.9 ± 12.39 *</td>
<td>72.3 ± 12.24 *</td>
<td>74.1 ± 12.66 *</td>
</tr>
<tr>
<td><strong>-80°C</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PT (s)</td>
<td>11.6 ± 0.72</td>
<td>11.6 ± 0.70</td>
<td>11.5 ± 0.65 *</td>
<td>11.3 ± 0.69 *</td>
</tr>
<tr>
<td>INR</td>
<td>1.01 ± 0.07</td>
<td>1.01 ± 0.07</td>
<td>1.00 ± 0.06 *</td>
<td>0.98 ± 0.06 *</td>
</tr>
<tr>
<td>APTT (s)</td>
<td>32.4 ± 5.60</td>
<td>32.3 ± 5.98 *</td>
<td>32.6 ± 5.92</td>
<td>33.03 ± 6.15 *</td>
</tr>
<tr>
<td>Fbg (g/L)</td>
<td>2.91 ± 0.89</td>
<td>2.88 ± 0.88 *</td>
<td>2.94 ± 0.88</td>
<td>2.90 ± 0.88</td>
</tr>
<tr>
<td>TT (s)</td>
<td>18.6 ± 1.03</td>
<td>18.6 ± 1.04</td>
<td>18.4 ± 1.02 *</td>
<td>18.4 ± 1.04 *</td>
</tr>
<tr>
<td>FVIII:C (%)</td>
<td>119.2 ± 32.82</td>
<td>118.3 ± 32.47</td>
<td>119.6 ± 33.79</td>
<td>114.9 ± 32.83 *</td>
</tr>
<tr>
<td>FIX:C (%)</td>
<td>71.7 ± 14.67</td>
<td>71.5 ± 14.09</td>
<td>71.5 ± 14.32</td>
<td>71.2 ± 13.99 *</td>
</tr>
</tbody>
</table>

*0 - freeze-thawing 0 times, 1 - freeze-thawing one time, 2 - freeze-thawing two times, 3 - freeze-thawing three times. * - p < 0.05 vs. baseline results.

### Table 2. Acceptable freeze-thawing times for coagulation tests for FVIII:C and FIX:C.

<table>
<thead>
<tr>
<th>Terms</th>
<th>d1</th>
<th>d2</th>
<th>d3</th>
<th>Accept</th>
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</thead>
<tbody>
<tr>
<td><strong>-20°C</strong></td>
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<tr>
<td>PT (s)</td>
<td>-0.92 (-3.23 - 0.0)</td>
<td>0.82 (-1.72 - 3.70)</td>
<td>0.0 (-3.36 - 5.15)</td>
<td>three times</td>
</tr>
<tr>
<td>INR</td>
<td>-1.05 (-3.06 - 0.0)</td>
<td>0.94 (-1.98 - 3.92)</td>
<td>0.0 (-3.85 - 5.0)</td>
<td>three times</td>
</tr>
<tr>
<td>APTT (s)</td>
<td>3.16 (-1.89 - 8.57)</td>
<td>-5.13 (-1.10 - 11.43)</td>
<td>7.20 (-3.03 - 14.57)</td>
<td>three times</td>
</tr>
<tr>
<td>Fbg (g/L)</td>
<td>0.0 (-4.98 - 4.48)</td>
<td>2.01 (-1.92 - 6.04)</td>
<td>0.0 (-2.66 - 5.69)</td>
<td>three times</td>
</tr>
<tr>
<td>TT (s)</td>
<td>0.0 (-3.82 - 2.34)</td>
<td>-1.88 (-5.10 - 1.58)</td>
<td>-1.10 (-4.19 - 1.68)</td>
<td>three times</td>
</tr>
<tr>
<td>FVIII:C (%)</td>
<td>-4.41 (-10.49 - -0.94)</td>
<td>-12.33 (-19.65 - -5.57) *</td>
<td>-13.60 (-18.92 ~ -8.95) *</td>
<td>once</td>
</tr>
<tr>
<td>FIX:C (%)</td>
<td>-2.48 (-5.88 - 1.49)</td>
<td>-7.09 (-10.43 - -1.70)</td>
<td>-4.96 (-10.43 ~ -0.81)</td>
<td>three times</td>
</tr>
<tr>
<td><strong>-80°C</strong></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>PT (s)</td>
<td>0.40 (-4.92 - 3.81)</td>
<td>-0.40 (-5.74 - 1.90)</td>
<td>-2.39 (-6.56 - 0.95)</td>
<td>three times</td>
</tr>
<tr>
<td>INR</td>
<td>0.45 (-5.61 - 3.30)</td>
<td>-0.41 (-6.54 - 2.20)</td>
<td>-2.36 (-7.48 - 1.10)</td>
<td>three times</td>
</tr>
<tr>
<td>APTT (s)</td>
<td>1.40 (-2.8 - 6.0)</td>
<td>0.32 (-2.45 - 0.86)</td>
<td>1.86 (-3.27 - 6.86)</td>
<td>three times</td>
</tr>
<tr>
<td>Fbg (g/L)</td>
<td>-0.53 (-5.38 - 2.26)</td>
<td>0.0 (-2.96 - 5.66)</td>
<td>0.0 (-4.04 - 4.60)</td>
<td>three times</td>
</tr>
<tr>
<td>TT (s)</td>
<td>-0.26 (-3.59 - 2.70)</td>
<td>-1.10 (-4.19 - 1.68)</td>
<td>-1.02 (-5.35 - 1.60)</td>
<td>three times</td>
</tr>
<tr>
<td>FVIII:C (%)</td>
<td>0.0 (-5.49 - 3.63)</td>
<td>0.0 (-3.75 - 4.36)</td>
<td>-3.63 (-11.61 ~ 4.56)</td>
<td>three times</td>
</tr>
<tr>
<td>FIX:C (%)</td>
<td>0.0 (-3.32 - 3.38)</td>
<td>0.0 (-3.53 - 2.62)</td>
<td>-0.88 (-4.21 - 3.29)</td>
<td>three times</td>
</tr>
</tbody>
</table>

d1 - difference 1 = (value after first freeze-thawing - value at baseline) x 100%/value at baseline, d2 - difference 2 = (value after second freeze-thawing - value at baseline) x 100%/value at baseline, d3 - difference 3 = (value after third freeze-thawing - value at baseline) x 100%/value at baseline, Accept - acceptable freeze-thawing times. * - More than 25% of samples had > 10% changes.
RESULTS

The baseline results and all freeze-thaw results for PT/INR, APTT, Fbg, TT, FVIII:C, and FIX:C, and the differences between the baseline and freeze-thaw values are shown in Table 1. Figure 1 shows the individual percent differences in PT/INR, APTT, Fbg, TT, FVIII:C, and FIX:C after freeze-thawing with storage at -20°C/-80°C, and the two dotted lines represent plus or minus 10% changes.

As shown in Figure 1a and 1b, we found that the third percent differences of PT/INR, Fbg, and TT of all samples at -20°C/-80°C had ± 10% changes, and that the first percent differences of APTT at -20°C, first and second percent differences of FVIII:C at -80°C, and third percent differences of APTT and FIX:C at -80°C of all samples had ± 10% changes. At -20°C, we found one percent difference of FVIII:C that exceeded 10% change after freeze-thawing once, one percent difference of FIX:C, 32 percent differences of FVIII:C, and one percent difference of APTT that exceeded 10% changes after freeze-thawing twice, and one percent difference of FIX:C, 32 percent differences of FVIII:C, and 6 percent differences of APTT that exceeded 10% changes after freeze-thawing three times. At -80°C, we also found one sample percent difference of FVIII:C that had less than 10% change after freeze-thawing three times. Although there were some samples with changes exceeding ± 10%, the numbers of these points for APTT and FIX:C were all less than 10, except that of FVIII:C. Therefore, we propose that the acceptable freeze-thaw times for PT/INR, APTT, Fbg, TT, and FIX:C were three times at -20°C/-80°C, while the acceptable freeze-thaw times for FVIII:C were three times at -80°C and once at -20°C (Table 2).

DISCUSSION

True activity and level determination of factors and coagulation tests in fresh plasma samples is very important for diagnosing abnormalities in the intrinsic or extrinsic coagulation pathways and factor deficiencies and for procedures such as monitoring of anticoagulant therapy [2-5,22]. Accurate detection of screening coagulation tests, FVIII:C, and FIX:C can be affected by sample collection, transportation, storage temperature, and time [1,7,8]. In our previous studies [14,15], we found that along with prolonged time from sample collection to testing, screening coagulation test results increased or decreased and FVIII:C and FIX:C gradually decreased after both short-term storage (24 hours, 4°C or 25°C).

Figure 1. Difference scatter plots between fresh samples and freeze-thawed samples stored at -20°C/-80°C. 1a: at -20°C, 1b: at -80°C.
and long-term storage (1 year, -80°C or -20°C). If the required test items cannot be detected in samples at the same time or need to be re-tested, or if precious samples need to be re-stored in biobanks, the thawed samples need to be re-frozen. Our study is the first to investigate how the numbers of freeze-thaw (snap-freezing at -20°C/-80°C and thawing in a 37°C water bath) times influence accurate detection of screening coagulation tests, FVIII:C, and FIX:C. We determined the levels of screening coagulation tests on FVIII:C and FIX:C and compared the baseline results and all freeze-thaw results. We found that the acceptable freeze-thaw cycles for screening coagulation tests and FIX:C were three times at -20°C/-80°C, while the acceptable freeze-thaw times for FVIII:C were three times at -80°C and once at -20°C, with FVIII:C being especially unstable at -20°C. Therefore, the freeze-thaw stabilities results were affected by time and temperature, with lower temperature and fewer times associated with more stable activity. When the factor activities and coagulation tests cannot be determined within the acceptable time frames proposed in our earlier study, plasma can be frozen and thawed at appropriate times for analysis, and even frozen and thawed multiple times. No studies have analyzed the freeze-thaw stabilities of screening coagulation tests for FVIII:C and FIX:C in fresh citrate-anticoagulated plasma, besides FFP samples. Many studies have examined the effects of freeze-thaw cycles on the coagulation properties of FFP units that were re-frozen and re-thawed to avoid wastage of blood components and were proven useful for rare donor, autologous, and massive transfusion programs [17-20,23]. As early as 1989, Dzik et al. [19] found a slight, but clinically unimportant, prolongation of PT and APTT and a decrease in factor V and VIII:C levels in twice freeze-thawed FFP compared with control plasma. Ben-Tal et al. [17] found that FVII:C, FIX:C, FX:C, PT, and Fbg remained stable and adequate for transfusion in twice freeze-thawed FFP. Philip et al. [18] found that FII, FVII, FIX, and FX activities and Fbg were stable and adequate for transfusion in twice freeze-thawed FFP and that the FFP could be safely used for transfusion. Gosselin et al. [23] evaluated the effect of freezing on coagulation tests (PT, APTT, Fbg, D-dimer, anti-thrombin) and factor activities (FV, FVII, FVIII, FIX, lupus anticoagulant, anti-Xa) by comparing results between fresh and once freeze-thawed plasma and found that significant differences existed between fresh and frozen plasma samples tested for PT, APTT, and FVIII, albeit without clinical significance. In the study by Gosselin et al., plasma samples were aliquoted into capped storage vials and snap-frozen at -70°C for at least 1 week after completion of baseline coagulation testing. This was different from our study, in which the collected plasma was quickly divided into four Eppendorf tubes and capped, and baseline results were tested immediately. In doing this, we reduced any interference of storage time at room temperature with the coagulation results. In addition, Gosselin et al. only examined once freeze-thawed samples, while we examined three times freeze-thawed samples.

This study has some limitations. First, we used only one type of analytical instrument (CS5100) and reagents from one manufacturer (Siemens). Second, our study was only based on asymptomatic individuals, and did not contain patients with oral anticoagulant therapy, hepatitis, liver failure, and hemophilia. Therefore, our results cannot be used in these situations. Third, our study was a single-center study and based solely on the Han population in Zhejiang province. Fourth, we only froze samples in a -80°C freezer, not in dry ice, and we only thawed samples in a warm water bath at 37°C, not with a microwave warmer at 37°C/22°C or at other temperatures. Different freeze-thawing conditions may affect the results of coagulation tests and factor activities [24-26].

CONCLUSION

Our results need to be validated by further multicenter studies with different study populations, races, reagents, instruments, temperatures, and determination methodologies.

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Author Contributions:
L.M.F. designed the experiments. Y.Z, G.F.F., and J.Z. performed the experiments. G.F.F. wrote the main manuscript. All authors reviewed the manuscript.

Declaration of Interest:
The authors declare no competing financial interests.

References:


