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Differential impacts of hemolysis on coagulation parameters of blood samples

A STROBE-compliant article

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Abstract

This study aimed at investigating the impact of hemolysis on different coagulation parameters.

A total of 216 venous blood samples without visible hemolysis were collected from adult patients at a tertiary referral center over six months. The plasma obtained was quantified for six coagulation parameters including prothrombin time, activated partial thromboplastin time, fibrinogen, D-dimer, antithrombin III, and protein C. The rest of the plasma from each blood sample was aliquoted into three tubes, each containing 1 mL of plasma with three different volumes of cell-free hemoglobin (i.e., 2, 4, 8 μL) from lysed RBCs to create simulated hemolyzed blood samples with hemoglobin concentration of approximately 0.1, 0.2, and 0.4 g/dL to mimic mild (1+), moderate (2+), and severe (3+) hemolysis, respectively, before repeating the coagulation tests to determine possible correlation between the simulated degree of hemolysis and the changes in test results of the coagulation parameters.

Spearman correlation analysis showed significant decreases in the values of activated partial thromboplastin time, fibrinogen, D-dimer, and protein C values with an increasing degree of simulated hemolysis (all $P < .01$). Comparison of the percentage bias of biological variance showed significant positive associations of cell-free hemoglobin concentrations with the percentage bias of D-dimer and protein C. However, only the former was still within the range of biological variance under condition of simulated hemolysis. Besides, the presence of cell-free hemoglobin regardless of concentration had a notable impact on the percentage bias of activated partial thromboplastin time, whereas the influence was non-significant for prothrombin time, fibrinogen, and antithrombin III.

The results showed different impacts of simulated hemolysis on six coagulation parameters, highlighting the dependence of clinical reliability on the coagulation parameter to be investigated in hemolytic blood samples.

Abbreviations: APTT = activated partial thromboplastin time, PT = prothrombin time.

Keywords: antithrombin III, coagulation, fibrinogen, hemolysis, protein-C

1. Introduction

Although hemolysis has been reported to occur in over 3% of all blood samples in daily medical practice[1] and account for up to 40% to 70% of all specimens unsuitable for producing reliable results,[2] evidence on the suitability of using hemolyzed blood samples to produce reliable clinical data is limited.[3] Hemolysis may occur as a complication of transfusion,[4] inflammatory conditions with complement activation,[5,6] and can be a result of the technique chosen for venous blood sampling,[7] delay in sample transportation, the choice of collection tube, inappropriate storage conditions [8] as well as a presentation of certain hemolytic diseases such as sickle cell disease.[9] Since recollecting

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blood samples from patients may not be possible because most hemolytic samples are from the emergency department, intensive care units, and pediatric units,[10] there has been much discussion focusing on the effects of hemolysis on the accuracy of laboratory tests including potassium concentration[11] and coagulation profile.[8,12]

The guideline of the Clinical and Laboratory Standard Institute discouraged the use of blood samples with hemolysis for the assessment of coagulation profile on the assumption that hemolysis may cause artifactual interference through coagulation factor activation.[13] On the other hand, due to differences in subject populations, parameters chosen, and the standards for determining the degree of hemolysis in previous studies, the results remain inconclusive. More importantly, whether the data on coagulation profile generated from hemolytic samples are still reliable for clinical interpretation remain unclear.

Using the concept of biological variance, this clinical study aimed at investigating the impact of blood sample hemolysis on coagulation by adopting blood samples at three different levels of simulated hemolysis with known cell-free plasma hemoglobin concentrations for correlation to elucidate:

(1) Whether a significant association exists between the degree of simulated hemolysis (i.e., concentration of cell-free hemoglobin) and the value of a particular coagulation parameter, and
(2) Whether hemolysis-related percentage biases of the coagulation parameters are within clinically acceptable ranges.

2. Materials and methods

2.1. Sample collection

Random venous blood samples were collected from patients over the age of 18 at a tertiary referral center over six months. The sources of patients included outpatient clinics, emergency department, and wards. Blood samples with volume less than 4.5 mL and those with notable hemolysis after centrifugation were excluded from the study. The protocol and procedures of the study were reviewed and approved by the Institutional Review Board (IRB) at Chang Gung Memorial Hospital (IRB No. 97-1658B). All procedures complied with the World Medical Association Declaration of Helsinki regarding ethical conduct of research involving human subjects.

2.2. Preparation of plasma samples and study parameters

Each 4.5 mL venous blood sample in a 5.0 mL BD blood collection tube with 0.5 mL 3.2% sodium citrate (Catalog No. 366415, Becton Dickinson, Franklin Lakes, NJ, USA) was centrifuged at 1,500 g for 10 minutes (KUBOTA 4000 centrifuge, Fujioka Japan). Using the SYSMEX CA-1500 Coagulation Analyzer (Kobe, Japan), the plasma obtained was quantified for six coagulation parameters including prothrombin time (PT) (Dade INNOVIN, Siemens Healthcare Diagnostics Products, GmbH. 35041, Marburg/Germany), activated partial thromboplastin time (APTT) (Dade Actin FSL, Siemens Healthcare Diagnostics Products, GmbH. 35041, Marburg/Germany), and fibrinogen (Dade Fibrinogen Determination Reagents, Siemens Healthcare Diagnostics Products, GmbH. 35041, Marburg/Germany) using the clotting method as well as D-dimer with the immuno-turbidity approach (INNOVANCE D-dimer, Siemens Healthcare Diagnostics Products, GmbH. 35041 Marburg/Germany), antithrombin III (Siemens Antithrombin III assay, Siemens Healthcare Diagnostics Products, GmbH. 35041, Marburg/Germany) and protein C (Dade Behring Protein C Reagent, Siemens Healthcare Diagnostics Products, GmbH. 35041, Marburg/Germany) using the chromogenic method according to the manufacturer's instructions.

2.3. Preparation of hemolytic blood samples

The rest of the plasma from each blood sample was aliquoted into three 5 mL polystyrene test tubes (12 mm x 75 mm, Universal Medical, Catalog No. GS-110409, New Jersey, USA) each of which contained 1 mL of plasma. Hemolyzed RBCs from the same blood sample were prepared by the heat shock method. Briefly, the sedimented RBCs at the bottom of each blood sample after centrifugation were frozen at -20°C for two hours before being thawed at 37°C for 20 minutes. The samples were then subjected to centrifugation at 1,500 g for 10 minutes (KUBOTA 4000 centrifuge, Fujioka Japan). Only the supernatant was used for the subsequent experiments to ensure the absence of intact RBCs. Into the three test tubes with 1 mL of plasma mentioned above, three different volumes of cell-free hemoglobin (i.e., 2, 4, 8 μL) were respectively added into each tube to produce plasma samples with hemoglobin concentration of approximately 0.1, 0.2, and 0.4 g/dL, respectively, analyzed with a Sysmex XE-2100 automated Hematology System analyzer (Sysmex Corporation, Kobe, Japan). These samples were labeled as hemolysis 1+, 2+, and 3+, respectively to simulate different degrees of hemolysis (Fig. 1A). The plasma sample in each tube was then subjected to quantification of the six parameters (i.e., PT, APTT, fibrinogen, D-dimer, antithrombin III, and protein C) as mentioned above. Quantification of all coagulation parameters was performed within two hours after phlebotomy.

2.4. Definitions of reference intervals for study parameters

The study parameters and the reference intervals adopted were in accordance with the criteria previously described as follows: PT (8.0–12.0 sec),[14] APTT (23.9–35.5 sec),[14] fibrinogen (180–350 mg/dL),[14] D-dimer (<0.5 mg/L fibrinogen-equivalent unit,[14] antithrombin III (70%–140%),[14] and protein C (70%–140%).[14] The biological variances of PT, APTT, fibrinogen, D-dimer, antithrombin III, and protein C were 4.0%,[15,16] 2.7%,[15–17] 10.7%,[15,17–20] 23.3%,[21] 5.2% [15,17,20,22] and 5.6%,[23] respectively.

2.5. Computation of percentage bias

Taking into account the intra-individual variations of biological variance, t he percentage biases (% bias) of a coagulation parameter from three plasma samples with known hemoglobin concentrations to mimic three different degrees of hemolysis from each testing subject were calculated by subtracting the value of that parameter in a non-hemolytic sample (x) from that in plasma with hemolysis (y) and divided by x so that: $\% \text{ bias} = [(y - x)/x] \times 100\%$. A change in percentage bias with the degree of simulated hemolysis (i.e., 1+, 2+, and 3+) reflected an impact of cell-free hemoglobin on the value of that parameter. In addition, to assess the clinical validity of reporting the value of a coagulation parameter from a hemolytic blood sample, the absolute value of percentage bias was computed and compared with the biological variance of that parameter.[24]

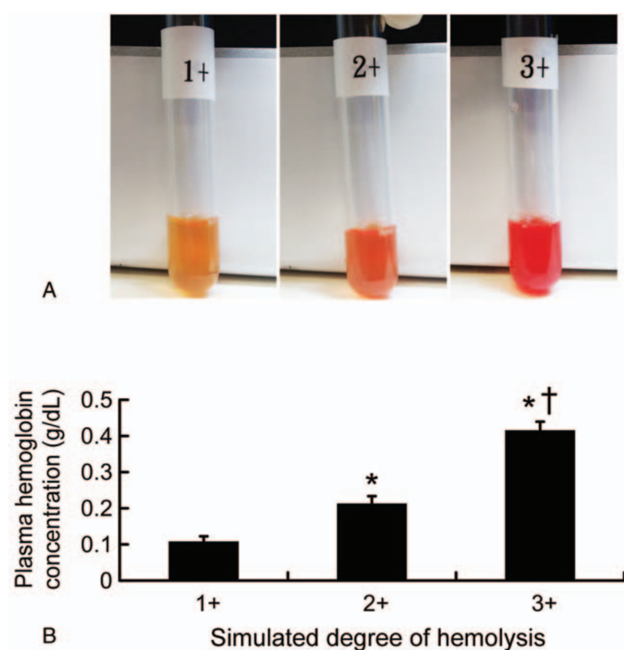

Figure 1. (A) Gross appearances of one milliliter of plasma samples after addition of 2, 4, 8 μL of cell-free hemoglobin into 1 mL of plasma to mimic mild (1+), moderate (2+), and severe (3+) hemolysis, respectively; (B) Hemoglobin concentrations in plasma samples with simulated mild (1+), moderate (2+), and severe (3+) hemolysis for the present study. $^{*}P<.0001$ vs 1+; $^{†}P<.0001$ vs 2+ with independent-samples t-test. Error bars representing standard deviations (SD).

2.6. Statistical analysis

All statistical analyses were performed with SPSS for Windows version 24.0 software (SPSS, Inc., Chicago, IL). Average values are expressed as mean ± standard deviation. Significance of difference in cell-free hemoglobin concentrations among sample with the three degrees of simulated hemolysis was determined with independent-samples t-test. Scatter plot was used for expressing the distribution of the values of a parameter at different degrees of simulated hemolysis, while the significance of association between cell-free hemoglobin concentrations and the corresponding values of each coagulation parameter among the testing subjects was determined using Spearman correlation analysis. The absolute values of percentage bias were used for evaluating the impact of cell-free hemoglobin concentration on the deviation of percentage bias of a parameter from its biological variance. The distributions of deviations are expressed as scatter plots and box plots. The former was utilized to inspect the distribution of percentage biases from three different degrees of simulated hemolysis, while the latter was used to identify the medians and quartiles of hemolysis-related percentage biases of all coagulation parameters. The relationship between deviations and the degrees of simulated hemolysis was assessed with Spearman correlation analysis. A probability value of less than 0.05 was considered statistically significant.

3. Results

3.1. Blood samples

A total of 234 venous blood samples from 234 adult patients were collected within the study period. Of the 234 samples, five samples found to be grossly hemolytic after centrifugation and 13 less than 4.5 mL were excluded from the present study. As a result, 216 venous samples were analyzed.

3.2. Validation of hemolysis model using plasma samples with different hemoglobin concentrations

By adding different amounts (i.e., 2, 4, 8 μL) of cell-free hemoglobin from lysed RBCs into three 1 mL plasma aliquots from the same blood sample, a model mimicking hemolysis of different severity (1+, 2+, and 3+, respectively) was created (Fig. 1A). Quantification of hemoglobin concentrations showed a hemoglobin concentration of 0.10 ± 0.02, 0.21 ± 0.02, and 0.41 ± 0.03 g/dL for 1+, 2+, and 3+, respectively. There were highly significant differences among the three groups ($p<0.0001$) (Fig. 1B).

3.3. Association of cell-free hemoglobin concentrations at different degrees of simulated hemolysis with corresponding values of coagulation parameters

Spearman correlation analysis demonstrated a significant decrease in the values of APTT ($P<.001$), fibrinogen ($P=.007$), D-dimer ($P<.001$), and protein C ($P=.014$) with an increasing degree of simulated hemolysis (Fig. 2). On the other hand, there was no significant association of the degree of simulated hemolysis with the fluctuations in the values of PT and antithrombin III.

3.4. Percentage bias and intra-individual variations of biological variance

Spearman analysis of the correlation between the deviations from non-hemolytic values (i.e., absolute values of percentage biases) of the six coagulation parameters and different degrees of simulated hemolysis demonstrated a negative correlation for APTT but a positive association for D-dimer ($P=.025$) and protein C ($P=.046$) (Fig. 3, left panel). Inspection of the corresponding box plots (Fig. 3, right panel) revealed notable deviations of APTT from its biological variance regardless of the degree of simulated hemolysis, while protein C showed a significant impact of cell-free hemoglobin concentration on its percentage bias with the median shifted out of its biological variance starting from a simulated hemolysis of 2+ onwards. On the other hand, for the other parameters including PT, fibrinogen, D-dimer, and antithrombin III, their medians stayed within their biological variances regardless of the level of simulated hemolysis.

4. Discussion

The validity of clinical data on coagulation from hemolytic blood samples remains controversial.[8,25] While some authors proposed that hemolysis may hasten coagulation,[25,26] others suggested the opposite.[8] The results of previous studies addressing the issue remain inconclusive mainly because of the limited number of subjects recruited, the relatively small number of coagulation parameters included, or the adoption of merely one or two methods for measurement.[8,12,25,27] Utilizing over 200 blood samples, the present study is the first to investigate the impact of different degrees of simulated hemolysis on the laboratory coagulation profile using six parameters that involved

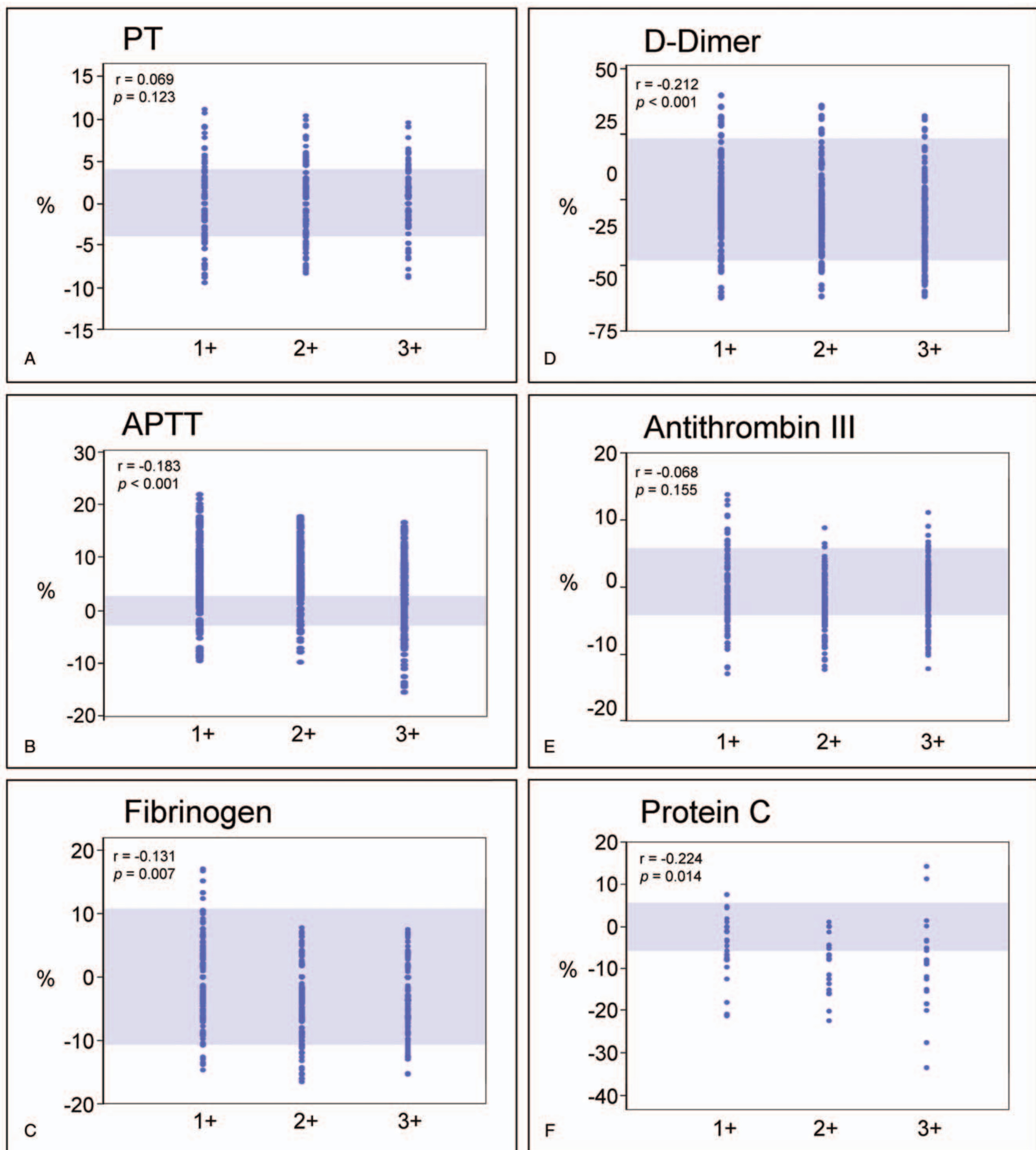

Figure 2. Scatter plots on data distribution of the six coagulation parameters at different levels of simulated hemolysis. (A) Prothrombin time (PT); (B) Activated partial thromboplastin time (APTT); (C) Fibrinogen; (D) D-dimer; (E) Antithrombin III; and (F) Protein C. Range of intra-individual variations of biological variance for each parameter marked as shaded area. Results of Spearman correlation analysis also shown.

three quantitative methods including the clotting, immunoturbidity, and chromogenic approaches. Our findings revealed a significant reduction in the values of APTT, fibrinogen, D-dimer, and protein C with an increasing degree of simulated hemolysis, indicating an impact of cell-free hemoglobin on these coagulation parameters. More importantly, our results demonstrated a notable influence of cell-free hemoglobin on the percentage bias of two of the parameters (i.e., APTT, protein C), whilst the effect was acceptable for others (i.e., PT, fibrinogen, D-dimer, antithrombin III). Therefore, our findings highlighted the importance of correct interpretation of the results of different coagulation parameters in hemolyzed blood samples.

Potential causes of inaccuracy in laboratory interpretation of data from hemolytic blood samples include release of substances

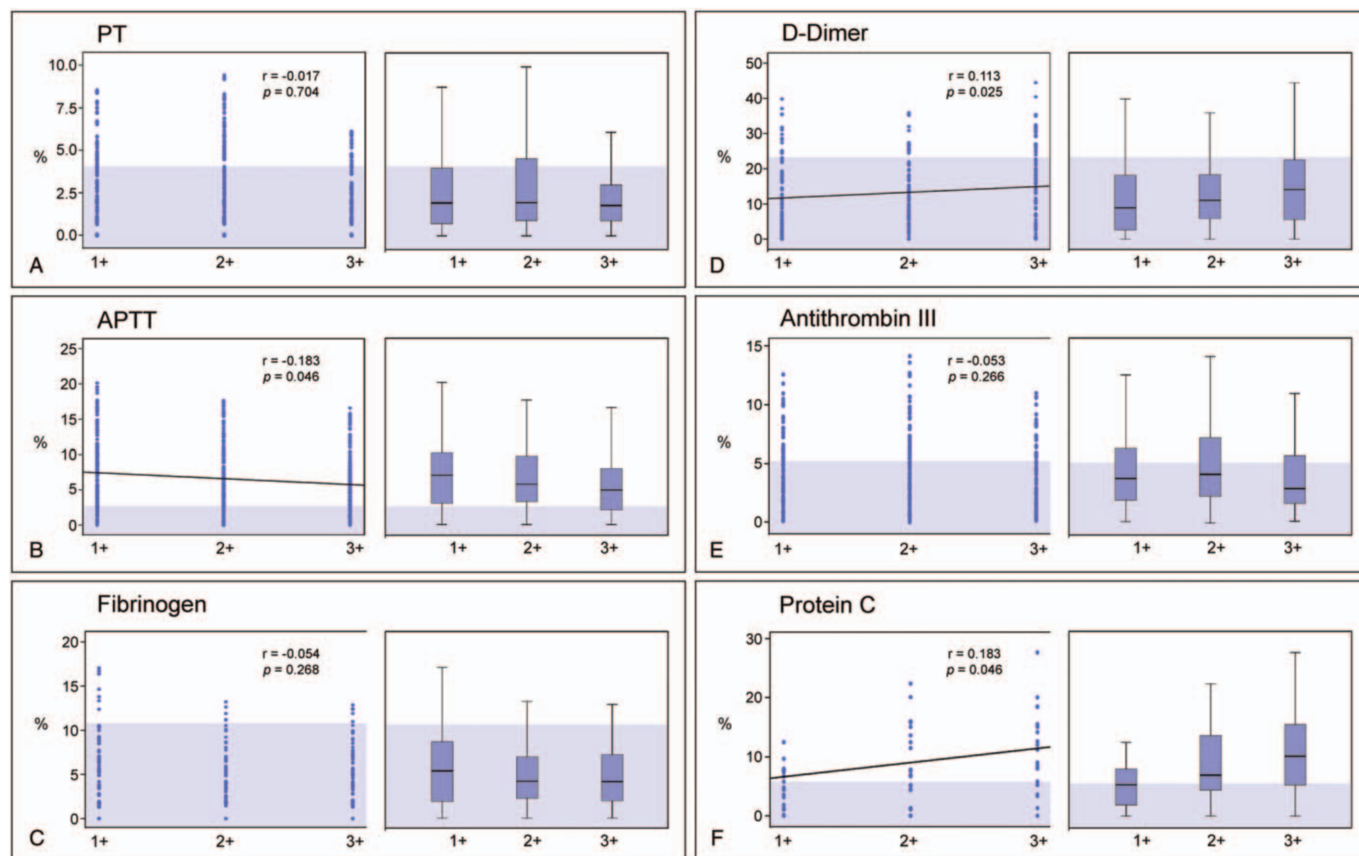

Figure 3. Scatter plots (left panel) showing distribution of percentage bias of the six coagulation parameters at different levels of simulated hemolysis. (A) Prothrombin time (PT); (B) Activated partial thromboplastin time (APTT); (C) Fibrinogen; (D) D-dimer; (E) Antithrombin III; and (F) Protein C with results of Spearman correlation analysis shown. Box plots (right panel) demonstrating data distribution as minimum, maximum, sample median as well as first and third quartiles. Range of intra-individual variations of biological variance for each parameter marked as shaded area.

with high intracellular concentrations causing falsely elevated plasma/serum levels, falsely reduced circulating levels due to dilution from leakage of intracellular water, and interference of hemoglobin with absorbance on spectrophotometric quantification.[12] It has also been speculated that hemolysis-induced anionic membrane phospholipid exposure may accelerate coagulation and cause shortening of test results.[8] In contrast, another speculation postulates that exposed membrane phospholipid may compete with thromboplatin for activated factor VII a, thereby reducing its bioavailability and prolonging the test results.[28] The forms of hemoglobin in hemolytic blood samples also raised some concerns. While hemoglobin contained within microvesicles is believed to compete with thromboplastin for factor VIIa or provide a thrombogenic surface for activation or propagation of the coagulation cascade, stroma-free hemoglobin is free from those actions.[8,12] The present study, which focused on the influence of cell-free hemoglobin on six coagulation parameters, did not investigate the impact of membrane phospholipid on laboratory results. Therefore, the findings of the current investigation suggested a significant negative association between plasma cell-free hemoglobin concentration and the values of APTT, fibrinogen, D-dimer, and protein C. An important concern is that the interference of cell-free hemoglobin with test results may also vary with the quantitative approach. The reduction in values of APTT and fibrinogen, which were quantified with the clotting method, may implicate the clustering of hemoglobin within microvesicles that offer a thrombogenic surface for activating the coagulation cascade. Besides, our findings suggested significant interferences of cell-free hemoglobin with the immuno-turbidity and chromogenic quantifications of D-dimer and protein C, respectively. Nevertheless, except for APTT and protein C, such cell-free hemoglobin-related fluctuations did not notably affect the percentage bias of other coagulation parameters included in this study. Such findings could have significant clinical implications on the interpretation of the test results.

In summary, our findings demonstrated different impacts of simulated hemolysis on the six parameters included in the present investigation. While the influence was acceptable for data interpretation for PT, fibrinogen, D-dimer, and antithrombin III, hemolysis could render the data invalid for APTT and protein C.

4.1. Limitations

The supernatant hemoglobin concentration of non-hemolytic blood samples has been reported to be between 0 and 30 mg/dL, while individual difference in judgment may exist for specimens with supernatant hemoglobin levels between 20 and

30 mg/dL.[8] One of the limitations of the present study was that the supernatant hemoglobin concentrations of the nonhemolytic samples were not determined for studying its possible association with the values of different parameters. Another limitation was the use of plasma hemoglobin concentrations of only up to 0.4 g/dL to simulate severe hemolysis in the current study to be compatible with the clinical situation. Therefore, the impact of a higher degree of hemolysis (i.e., higher concentration of cell-free hemoglobin) on the coagulation profile was not investigated. In addition, the temperature factor was not taken into consideration in creating the model for the present study as cell-free hemoglobin generated from the heat shock approach may have a physiological impact on the coagulation process different from that from purely mechanical means, which better reflects the clinical scenario. Finally, because the blood samples in the present study were delinked from the patients' information due to the Institutional Review Board policy on sample use, possible influences of the patients' diseases and medications on the test results cannot be ruled out. On the other hand, because our blood samples were from patients with abnormal coagulation profiles (e.g., those with decompensated cirrhosis or under anticoagulant treatments) and also from individuals with normal coagulation, one of the merits of the present study is that our findings remain applicable to clinical interpretation regardless of the coagulation profiles of the blood samples taking into account the large sample size. Further studies are warranted to address possible impacts of demographic factors and diseases on the coagulation profile.

5. Conclusions

The results of the present study demonstrated different impacts of hemolysis simulated by different cell-free hemoglobin concentrations on the interpretation of data acceptability of six coagulation parameters with three different approaches to quantification. The significant negative associations between cell-free hemoglobin concentration and the test results of APTT, fibrinogen, D-dimer, and protein C as well as the notable increases in percentage bias for APTT and protein C in samples with simulated hemolysis highlighted that caution has to be taken on clinical interpretation of coagulation parameters from hemolyzed blood samples.

Author contributions

LLP, MCW, and CKS conceived the study. CHL, KCH, and ITT helped in literature search. LLP and CHL were involved in protocol development, gaining ethical approval, and data analysis. MCW and CKS wrote the draft of the article. All authors reviewed and edited the article as well as approved the final version of this work.

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Writing – review & editing: Ming-Chung Wang, Cheuk-Kwan Sun.

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