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Effect of prolonged freezing of plasma samples on prothrombin time and activated Partial Thromboplastin Time tests at -20^{o}C in a low sample inflow region in Ghana

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Abstract
The study examines how prolonged storage of PPP at -20^{o}C could have on aPTT and PT. A total of 30 healthy subjects were enrolled in the research. Platelet-poor plasma obtained from blood samples in 3.2% sodium citrate tubes were tested for PT and aPTT on day 1. Subsequently, samples were stored at -200C and tested at a 14-day interval up to 98 days. APTT and PT tests revealed a significant change in values beginning from day 56 (p=0.029) and day 70 (p=0.019) respectively. A significant difference was detected for PT (p=0.0004) and aPTT (p=0.0078) between participants aged 18-20 years and participants aged 21-49 years. Both PT and aPTT showed no significant gender-related difference. This research revealed significant changes in the PT and aPTT results after 70 and 56 days respectively. Therefore, it is recommended that samples for PT and aPPT stored beyond 70 and 56 days respectively at -20^{o}C prior to analysis.

Keywords
Freezing, Prothrombin Time, Activated Partial Thromboplastin Time, Plasma

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Contents

1. Introduction

During the process of hemostasis, the intricate interplay among platelets, vessel walls, and adhesive proteins leads to the formation of a "platelet plug". The normal

hemostatic reaction to vascular injury relies on the intricate coordination among blood coagulation factors, the endothelium of blood vessels, and circulating platelets (Marieb and Hoehn, 2010). Procoagulant and anticoagulant mechanisms in conjunction with a fibrinolysis process form the delicately balanced haemostatic system (Ofosu, Addai-mensah, Boateng, and Anane, 2019). Intrinsic and extrinsic pathways make up the traditional coagulation system. The haemostatic mechanisms serve a number of crucial roles, including keeping blood in a fluid state during circulation within the blood vessels, avoiding excessive bleeding or blood loss at an injury site by the formation of a haemostatic plug. This process is limited to the area around the site of the injury, and ensuring that the plug is eventually removed when healing is complete (Bhatnagar, Bogner, and Pikal, 2007). The generation of thrombin, which interacts with fibrinogen to generate fibrin and ultimately form the fibrin clot, represents the pivotal event in the coagulation pathways. Activated partial thromboplastin time (aPTT) and prothrombin time (PT) are two commonly used tests for coagulation analysis, used to define the intrinsic and extrinsic pathways respectively (Bhatnagar, Bogner, and Pikal, 2007). Plasma proteins, phospholipids, and calcium ions all participate in a series of chemical reactions that cause blood to coagulate. Coagulation factor analysis is done to investigate clotting defects, monitor the effectiveness of anticoagulant therapy, check the effectiveness of factor replacement therapies, or check for both specific and non-specific coagulation inhibitors, such as factor VIII and lupus anticoagulants (Gosselin, 2015). Either citrated plasma separation from whole blood or plasmapheresis collection methods are both viable options for obtaining the sample. Most of the known coagulation factors are present in citrated plasma (Simon, 2015). Prolonged freezing refers to how long the citrated plasma can be preserved in the fridge (Bhatnagar, Bogner, and Pikal, 2007). These tests are conducted using citrated plasma samples and are very crucial in coagulation analysis but however, these tests are not routinely carried out in many laboratories in Ghana, particularly in the district laboratories despite the high demand for these tests by clinicians (Ofosu, Addai-mensah, Boateng, and Anane, 2019) due to the high cost of reagents and equipment setup for these tests. Therefore, many laboratories are forced to store these samples to be run at a later time. Most other laboratories collect multiple samples for coagulation profile over a period before sending them out to be tested. There is however paucity of information on the stability of the coagulation factors and how long they can be stored to remain viable for testing in these low-resourced laboratories in Ghana. This study aimed at evaluating the impact of prolonged freezing on citrated plasma when testing for prothrombin time (PT) and activated partial thromboplastin time (aPTT) parameters in low-resourced countries.

2. Materials and Methods

2.1 Study Design and Site

This was an experimental study conducted at the University of Energy and Natural Resources in Fiapre, Sunyani, Ghana. Participants were recruited from the Fiapre community purposively for conveniency sampling reasons.

2.2 Study Population

Thirty (30) voluntary participants were recruited for the study from the Sunyani West Municipality. The number of participants were selected based on previous literature which selected 16 to 144 cases for experimental studies such as this (Alesci et 2009, Zhao et al., 2017).

2.3 Inclusion and Exclusion Criteria

Both male and female participants between the ages of 18 and 49 who were apparently healthy and consented to the study were recruited. Pregnant women, people with a record of coagulation disorder, on coagulation therapy or had any liver related disease were not included in this study.

2.4 Ethical Consideration

All individuals participating in the study granted their consent. The research received ethical clearance from the Committee on Human Research and Ethics (CHRE) at the University of Energy and Natural Resources in Sunyani, Ghana, under reference number CHRE/AP/013/22.

2.5 Blood Sample Collection and Preparation

Participants' whole blood was collected via phlebotomy, with four (4) milliliters drawn into a 3.2% tri-sodium citrate tube. The samples underwent immediate centrifugation at 4000 RPM for 15 minutes to obtain platelet-poor plasma necessary for coagulation testing.

2.6 Reagents

The study made use of the following reagents: Ozostat Prothrombin Time (PT) reagent, Ozostat activated Partial Thromboplastin Time (aPTT) reagent, and calcium chloride (CaCl2) manufactured by Med Source Ozone Biomedical P.V.T. Limited in India.

2.7 Storage

The citrated plasma was separated and aliquoted into 7 cryotubes for later testing at the specified time points. The samples were stored at -20oC in a digital temperature monitoring refrigerator at the Centre for Research in Applied Biology (CeRAB), University of Energy and Natural Resources. Regular temperature checks were done at routine intervals and random opening of the refrigerator was avoided unless samples were going to be used to run a test for the study.

2.8 PT and aPTT Tests

All tests were run in duplicate, and control plasma was reconstituted in accordance with the instructions on the packaging from the manufacturer under controlled conditions. PT and aPTT reagents were pre-warmed for at least 10 minutes and the test was conducted as per the instructions of the manufacturer. A PT reference range of 11–16 seconds and aPTT reference range of 36–60 seconds were used, as established in a study by Ofosu, Addai-mensah, Boateng, and Anane (2019).

2.9 Data Handling and Analysis

Raw data was collected, edited to remove errors, reorganized, coded, and manipulated with appropriate software for efficient analysis. Access to the data was restricted to the researchers and the supervisor from the start of the study until it's completion. The figures obtained were analyzed using a Microsoft Excel. Controls and all test results were recorded. The analysis of research data was conducted using GraphPad Prism version 9.3.1, and the outcomes were visualized through tables and graphs. A P-value less than 0.05 was deemed clinically and statistically significant in all cases.

3. Results

3.1 Demographics of Study Participants

The study recruited a total of 30 participants, evenly split between 15 males and 15 females. Most participants were above 20 years of age (n=18, 60.0%). This is represented in Figure 1 below.

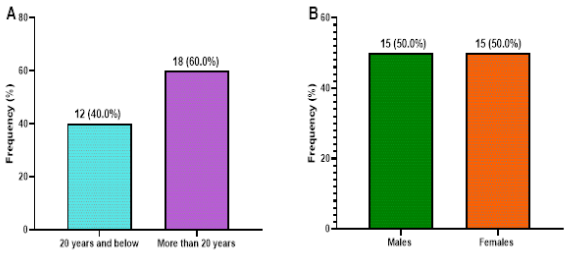

Figure 1. Diagram showing the age(A) and gender(B) distribution of study participants

3.2 Prothrombin Time (PT) and Activated Partial Thromboplastin Time (aPTT) at Baseline.

Day zero or baseline determination of PT and aPTT of the study participants were assessed. The baseline averages for study participants were 13.65 seconds and 34.70 seconds, for PT and aPTT respectively. Prothrombin time was shorter and had a narrower distribution than aPTT. Activated partial thromboplastin time was longer but had a wider distribution. This is shown in Figure 2 below.

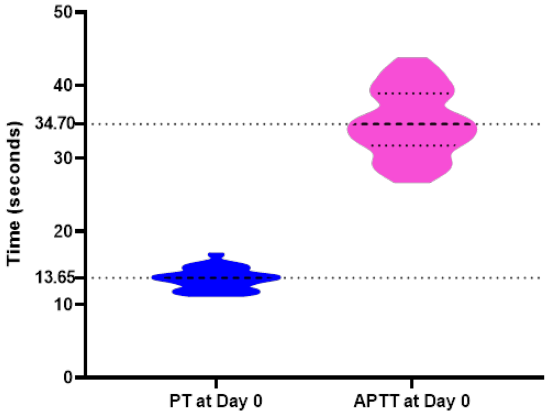

Figure 2. Diagram showing the results for PT and aPTT at day 0 or baseline

3.3 Difference in Prothrombin Time (PT) and Activated Partial Thromboplastin Time (aPTT) at Baseline according to Gender and Age

The average PT was 13.70 seconds for men and 13.30 seconds for women, respectively with no significant difference between them (p=0.908). However, participants between 21-49 years had significantly higher PT than the participants between 18-20 years (13.83s vs. 13.73s; p = 0.0078). This is represented in Fig 3. The aPTT showed no significant variation between sexes (males=34.9s and females =34.5s, p = 0.6469). On the contrary, aPTT was significantly higher among participants aged more than 20 years (36.55s) as compared to those who were 20 years and below (31.25s; p = 0.0004) This is represented in Fig 4.

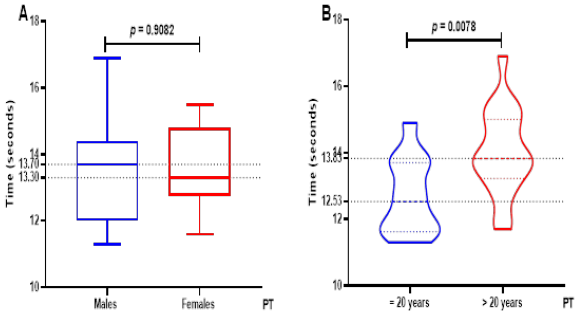

Figure 3. Diagram showing the difference in PT at day zero according to gender(A) and age(B)

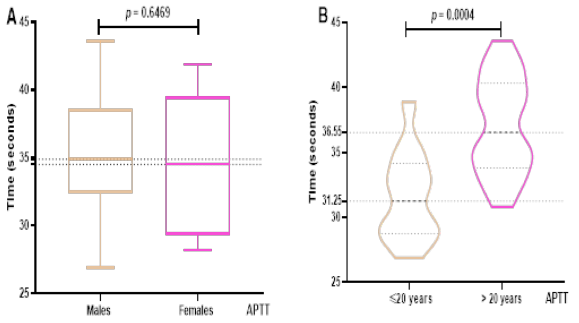

Figure 4. Diagram showing the difference in aPTT at day zero according to gender(A) and age(B)

3.4 The Trend in Average Prothrombin Time (Pt) and Activated Partial Thromboplastin Time (aPTT) For Test Duration

Figure 5 below demonstrates the trend in the mean PT and aPTT levels from the baseline to day 98. PT showed an upward trend from the baseline value (zero) to day 98 of the experiment. Similar to this, the aPTT increased from the start of the experiment (baseline) to end of the testing duration (day 98). However, they were all within the reference range for normal PT and aPTT.

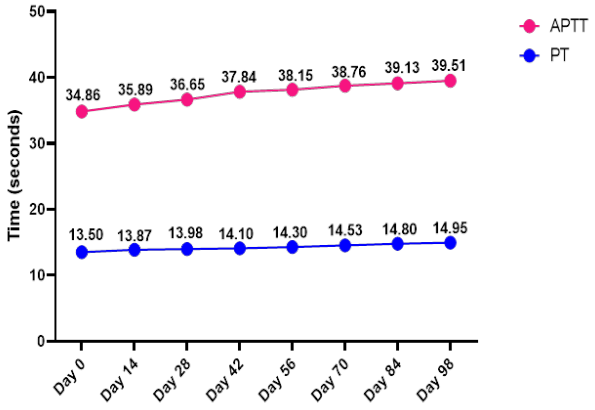

Figure 5. Diagram showing the trend in average PT and aPTT for the duration of the experiment

3.5 Variations in Prothrombin Time (PT) Results Across the days of experiments

Figure 6 below shows a bar chart representation showing PT levels against days. The baseline represents "*fresh plasma*" whiles the remaining days represent "*frozen plasma*". PT levels show a significant increase from the actual value recorded on day zero on the 70th day through to the 98th day.

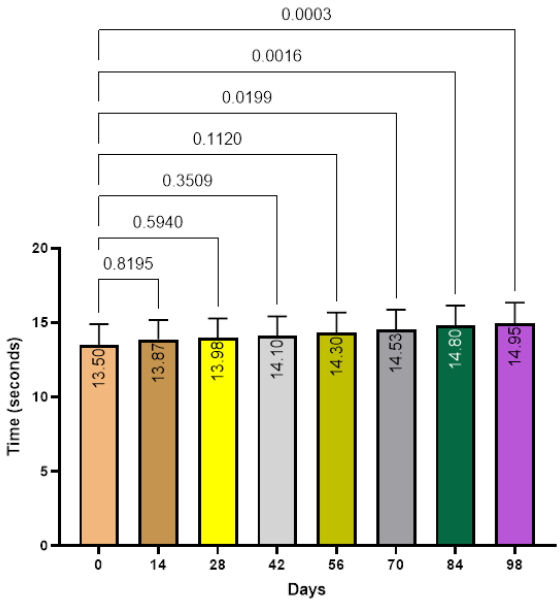

Figure 6. A diagram showing the variations in Prothrombin Time (PT) results across the days of experiments

3.6 Variations in Activated Partial Thromboplastin Time (aPTT) Results across the days of experiments

Figure 7 below shows a bar chart representation showing aPTT levels against days of testing. The PT values recorded began showing a significant variation from the baseline value (34.86s) on day 56 (38.15>34.86, p=0.029).

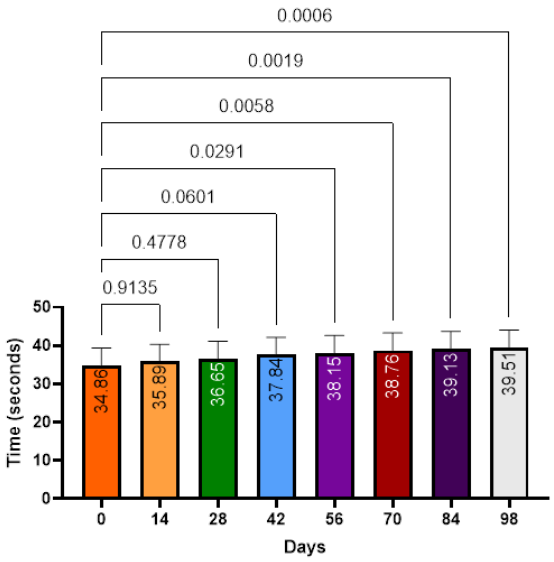

Figure 7. A diagram showing the variations in Activated Partial Thromboplastin Time (aPTT) results across the days of experiments

4. Discussion

The levels (quantity) and effectiveness (quality) of coagulation factors is influenced by sample storage, storage temperature, and the time between sample collection and testing. As advised, "samples that cannot be promptly evaluated should be frozen for later analysis", the Clinical and Laboratory Standards Institute (CLSI) does not, however, outline the ideal times to store the samples (Wayne, PA 2008). This study assessed the effects of prolonged storage of Platelet Poor Plasma at -20^{o}C. The p-value for the comparison between people aged 18-20 years and those aged 21-49 years. However, indicated a (p=0.0004) suggesting substantial change. There were variations among the study participants as storage time increases, according to a statistical comparison of baseline PT (day 0) and PT after freezing (day 14 to day 98) of all the study participants (n=30) (from day 14 to day 98). PT increased gradually but unevenly from baseline to day 98. The PT result from baseline (day 0) to day 56 did not alter in a clinically meaningful way (p=0.1120). On day 70, the PT was significantly different from baseline (p=0.0199). Finally, day 98 displayed a PT of 14.8 (p=0.0003). aPTT assay sample analysis revealed a similar pattern. aPTT test result showed an incremental but uneven increase from baseline to day 98. The stability time of PT was longer than that aPTT. Between baseline (day 0) and day 42, there was no significant difference in the aPTT values (p=0.0601). However, after day 42, the difference in the aPTT values were found to be significant. Activated partial thromboplastin time variation from baseline was significantly different on day 56 (p=0.0291), significantly different on day 70 (p=0.0058), significantly different on day 84 (p=0.0019), and significantly different on day 98 (p=0.0006). This study's results align with those of a prior investigation conducted by Gosselin, Honeychurh, Kang, Dwyre, (2015) which revealed that freezing and thawing conditions have an impact on coagulation testing, though depending on how long the sample was stored, these changes may not be clinically significant. Additionally, they noted that the effect of freezing on the sample's PT stability time is marginally greater than the stability time aPTT. Freezing and storage conditions have a significant impact on PT and aPTT assays, according to (Alesci et al., 2009). Furthermore, they added that samples stored at -20^{o}C, are less stable over the long term than those kept at -70^{o}C. The reason is that the freezing temperature (^{o}C) affects protein preservation because the effect of freezing is concentrated on particular coagulation proteins (factors).

5. Conclusion

The study revealed that prolonged freezing of plasma samples significantly affects PT and aPTT. The research participants' ages ranged over a wide range as well. From the baseline to day 98 of storage, PT and aPTT results varied unevenly. It is advised to evaluate at least labile variables before dispensing platelet poor plasma that has been kept for longer than three months. The PT and aPTT may be included in the pre-transfusion test on samples that have been stored at -20^{o}C for longer than 3 months to evaluate the activity of intrinsic and extrinsic coagulation factors before use. However, concluding from the findings of this experiment, PPP may be stored at a temperature of minus -20^{o}C for PT tests should not exceed day 56 of storage and aPTT tests should not exceed 42 days of storage in order to more effectively manage bleeding disorders that necessitate the transfusion of fresh frozen plasma. There are some shortcomings in our research. PT and aPTT measurements were initially performed using only one type of reagent, Ozostat, which was obtained from Med Source in India. The samples were frozen and stored at a single temperature (-20^{o}C). The study was restricted to the Sunyani Municipality in the Bono region. Since more coagulation reagents are now readily available and vary in composition and efficacy, we advise using them for prothrombin time and activated partial thromboplastin time tests. Additional research can be done in other areas.

6. Acknowledgement

We wish to acknowledge the entire staff of the Department of Medical Laboratory Science for their support during this work. Special thanks go to the Laboratory technicians and the Head of the UENR clinic lab and SDA hospital lab whose guidance and availability led to the success of

this work.

7. Authors' contribution

DNO, AA and RMT conceived and designed the study. RMT, AAJ, BS, ANJ and GOA collected and transferred all data from the field and did the laboratory work. DNO, AA and RMT drafted the manuscript. DNO and AA reviewed the study design and methods used in the study and accordingly, reviewed the manuscript critically.

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