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ORIGINAL ARTICLE

Determining the Optimal Short-Term Storage Duration for T Cells Extracted from Peripheral Blood Prior Flow Cytometry Analysis

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SUMMARY

Background: **Researching medical sample storage is crucial for maintaining the integrity of biological specimens and ensuring the accuracy of research investigations and diagnostic tests. Improper storage conditions can lead to sample degradation, compromising the reliability of results. Standardized storage procedures are essential for quality control, particularly in multicenter trials where samples are collected and processed at various locations. Moreover, ethical considerations dictate careful handling of patient samples to uphold privacy and rights.**
Methods: **This study focuses on the surface phenotype of T cells, which is vital for diagnosing immunodeficiency disorders and autoimmune diseases and for monitoring disease activity and treatment efficacy. The effect of storage duration on T cell surface proteins is multifactorial, influenced by factors like protein degradation, cellular metabolism, and cytokine release. Long-term storage can lead to the gradual loss of T cell function, necessitating techniques to preserve cell activity. Changes in surface markers can affect disease diagnosis, emphasizing the importance of accurate sample processing.**
Results: **Findings from this study reveal time-dependent changes in T cell surface markers during storage. CD3 levels declined significantly after the fourth day, with FITC labeling proving superior to APC. CD4 levels remained consistent until the fourth day, contrasting with previous findings on foreskin tissue. HLA-DR levels declined rapidly, indicating unsuitability for storage, consistent with other studies on cryopreserved cells. CD16 and CD8 levels decreased gradually, while CD56 declined rapidly after the third day, consistent with recent research.**
Conclusions: **There were detectable and significant differences after the samples were stored for an improper period, which may have affected the integrity of the results, suggesting that understanding the factors influencing T cell surface protein changes during storage is crucial for maintaining result integrity.**
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KEYWORDS

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INTRODUCTION

The development of gel-filled blood collection tubes, forming a protective barrier during centrifugation, has significantly enhanced serum analyte stability in the primary tube, eliminating the need for serum aliquotation. However, few published studies address short-term storage and pre-centrifugation delays, and specimen expo-

sure conditions often align with the manufacturer's specified limitations. Other investigations have explored analyte stability in the whole blood. Some developing countries, such as in the Middle East region with its extensive terrain and diverse climate, face unique challenges. Healthcare providers in distant or regional areas frequently lack access to centrifuges, which exposes specimens to varying temperatures during transportation, thereby delaying the centrifugation process [1-3]. Blood sample storage conditions and transportation time significantly impact biochemical results. Studies have examined storage duration and environmental effects on human-origin samples in contemporary laboratories, and frequent requests for "add-on" tests for existing samples aim to avoid the need for a second venipuncture. However, the stability of these samples raises concerns, especially with practices such as at Leeds General Hospital, where samples are stored in sample manager modules without caps for up to 16 hours post-analysis, leaving the samples vulnerable. Detailed information about preanalytical conditions, as emphasized in the College of American Pathologists (CAP) checklist or ISO 15189:2012 norm, is crucial. Specific guidelines, like CLSI Guideline H21-A5, recommend assessing most coagulation parameters within 4 hours, except for tests tracking full-dose unfractionated heparin therapy, allowing a maximum 2-hours delay. Economic pressures and the rationalization of biology have led to a concentration of clinical laboratories into core laboratories in recent years [4-7]. Consequently, the elapsed time from specimen collection to delivery to the laboratory has dramatically increased due to longer distances between collection and technical sites. In such conditions, a 4-hours delay could be limiting in some cases, challenging the universal application of these recommendations in routine practice. The careful control of preanalytical conditions, particularly the delay between blood collection and analysis, is essential to avoid sample deterioration that could invalidate analysis results. In our study, we aimed to examine how varying short-term storage durations of peripheral blood samples impact T cell phenotype by assessing surface markers before subjecting them to flow cytometry analysis, which determine the ideal storage period post-collection to ensure precise testing results [8-15].

MATERIALS AND METHODS

The study design is prospective cross-sectional. Data information and samples were collected from the College of Applied Medical Sciences of Taif University. The research cohort comprised 20 healthy adults, evenly split between males and females, drawn from Taif University's College of Applied Medical Sciences. Rigorous selection criteria ensured the inclusion of a homogeneous, healthy population, thereby enabling any alterations in leukocyte protein expression to be ascribed to storage duration rather than individual variances or health factors. Each participant`s blood sample was collected via sterile venipuncture methods, utilizing EDTA tubes to forestall coagulation. Subsequently, the samples were partitioned into smaller aliquots and stored at 4°C. Period of storage is ranging from < 2 hours, 24 hours, 48 hours, 72 hours, and 96 hours. After the designated storage period, leukocytes have been isolated from each blood sample by using standard laboratory techniques called Ficoll-Paque^{TM} PLUS (GE Healthcare, Lot#: 100 91322) blood cell separation. Isolated leukocytes have been washed with phosphate-buffered saline (PBS) (1 x) to remove any residual plasma or anticoagulants. Leukocytes have been resuspended in a staining buffer and incubated with monoclonal antibodies specific for the proteins of interest (20 minutes at 4°C in a dark environment). Following antibody labeling and the incubation period, leukocytes have been analyzed using flow cytometry (FACs Canto) to measure the fluorescence intensity of the bound antibodies. Flow cytometry allows for the quantification of specific protein expression levels on the surface of individual leukocytes. The preferred surface markers were labeled by anti-CD3 to detect the CD3 protein, a marker of T cells (BD LOT#: 9029619), anti-CD4 to detect the CD4 protein, a marker of helper T cells (BD LOT#: 3337661), anti-CD8 to detect the CD8 protein, a marker of cytotoxic T cells (BD LOT#: 28424), anti-CD56 to detects the CD56 protein, a marker of natural killer (NK) cells (BD LOT#: 200 70295), anti-CD16 to detect gamma delta T cells, a unique subset of T cells (BD LOT#: 20070295), and anti-HLA-DR, which is commonly used to detect auto immune diseases and compatibility between the donor and recipient in transplantation (BD LOT#: 9179143). Pearson´s chi-squared test was applied, and the significance level of $p < 0.05$ was evaluated.

RESULTS

Samples were processed each day via separation and staining methods. The histograms of each surface marker were illustrated. Histograms in flow cytometry are essential visual representations of the data collected during an analysis. They display the distribution of cellular characteristics, such as fluorescence intensity or cell size, within a sample. Gating strategies are shown in Figure 1, where lymphocyte population were gated and analyzed by applying histogram to each surface marker.

The overall variation between all the surface markers indicated a decline in the levels of proteins. These were notably significant, when means were evaluated by using Pearson´s chi-squared test. The values are collectively shown in Figure 2. Also, Table 1 can show the effect of short-term storage on T cell surface markers.

Table 1. The effect of short-term storage on T cells.

Marker Days	CD3	CD16	HLA-DR	CD8	CD4	CD56
1						
2						
3						
4						
5						

Green indicates safe short-term storage, red indicates unrecommended short-term storage.

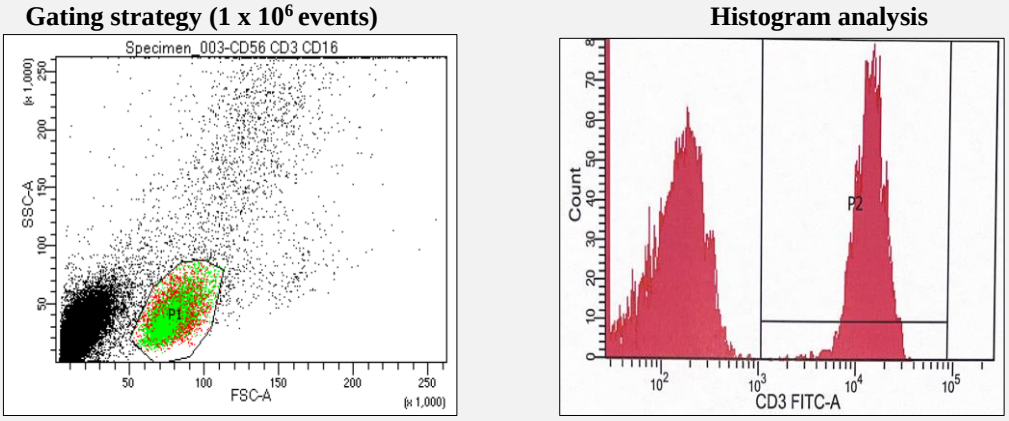

Figure 1. Gating strategies of the lymphocytes; 1 x 10^6 events were analyzed from each sample.

The percentage of the surface marker of each sample was processed by using histograms.

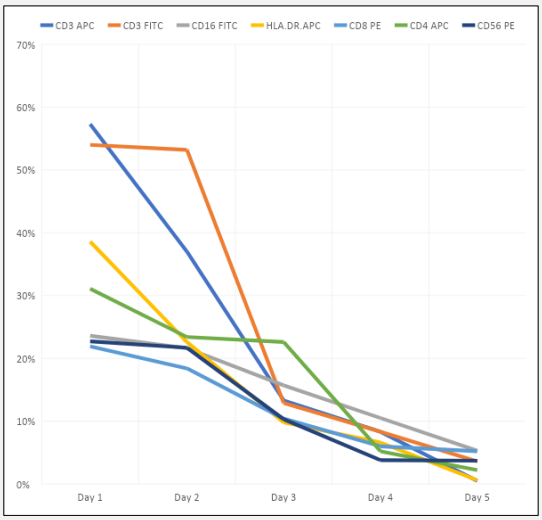

Figure 2. The line diagram indicated a plummet in surface protein values from day 2 onwards.

DISCUSSION

Researching medical sample storage is important for several reasons. Firstly, blood, tissue, urine, and other biological specimens are among the medical samples whose integrity must be preserved under ideal storage conditions [5,6]. The accuracy and dependability of research investigations or diagnostic tests may be jeopardized by sample degradation caused by improper storage conditions, light, humidity, or contamination exposure. Secondly, the outcomes of research analysis or diagnostic tests might be strongly impacted by the storage conditions of medical samples. Thirdly, to guarantee quality control in medical and scientific contexts, storage procedures must be carefully recorded and closely observed. Standardized methods for gathering, processing, and storing samples reduce variation and guarantee uniformity in sample management techniques [5,6,16]. In multicenter clinical trials or longitudinal research, where samples may be collected and processed at several locations, this is very crucial. Fourthly, patients or study participants are frequently asked for their consent to take medical samples, and it is morally required to make sure that these samples are handled with care [12]. This entails upholding patient privacy, following legal guidelines, and putting strong security measures in place to safeguard sample donors' rights and privacy. Our work focused on studying the surface phenotype of T cells at different storage periods. T cells' clinical diagnosis is important regarding the analysis of many diseases [13,14,17]. The effect of different storage periods of T cells can be shown by the changes in surface proteins of T cells [1,2,18]. The difference in value is multifactorial and could be due to several factors, such as protein degradation, impact of cell´s membrane integrity, metabolism activity of the cells, lack of nutrients, lack of blood circulation flow, cellular activation or inhibition, the release of cytokines, and possible contamination. The T cell response was reported by a study that stated that long cryopreservation has led to gradual weakening and losing of T cell functions [2,3,18,20]. This finding is consistent with several other studies that have reported that long storage with different scenarios can lead to loss of activity and function of immune cells [3,14,20,21]. Therefore, other studies have used several techniques to improve the function of these lost cells and reported positive findings. Surface markers of T cells are involved in several diagnoses of diseases, and one of the common methods for measuring both levels and mean fluorescence intensity of these markers is by FACs. Any changes in these markers can lead to different and false diagnoses. And processing fresh samples is not always possible due to conditions involving pre-analytical, analytical, and post-analytical quality conditions. Therefore, reporting false results is fatal and can be evidence of malpractice. Our study led to the following findings: CD3-surface marker has declined to less than 3 folds in the 4th day of storage, and this was detected by using FITC; however, when staining was performed using APC, the surface marker was weakly discovered by the 3rd day. This indicates that FITC is better for labelling than APC. Our findings are inconsistent with a previous study that reported that using APC is better than FITC, when they have studied CD19 surface receptor of B-cells [20]. When we labeled CD4, this surface protein has sustained consistent levels until the fourth day of storage, when the surface marker has declined by 6-folds from 31% to 5%. Which was inconsistent with other studies [2,18,20]; they have, however, used foreskin tissue in their work. On the other hand, HLA-DR gradually declined from the 2nd day of storage, indicating that this surface marker should be quantified freshly and that it is unsuitable for storage. This is consistent with another study that was reported, a study on human bone marrow derived mesenchymal stem cells following cryopreservation [2,18]. However, our finding is inconsistent with a similar study to ours which reported opposite findings [1]. CD16 also declined rapidly by one-fold each day of the storage reaching low levels, and this is consistent with another recent study [1]. Also, CD8 surface marker has gradually reduced to reach minimal levels, and this also was reported by another study [1], even though they still can produce normal levels of cytokines [15]. CD56 has gradually declined by the 3rd day to reach minimal levels, and this was also reported by another study [1].

CONCLUSION

Overall, changes in surface proteins of T cells after storage are likely multifactorial and influenced by a combination of factors related to sample handling, storage conditions, and cellular physiology. Understanding these factors is important for ensuring the integrity of experimental results in studies involving T cell analysis. Our study has reported that short-term storage of T cells can lead to different values, which will greatly impact the results and lead to false reports. Our study has limitations, which include the following: cytokines were not measured in a similar manner, and they influence the levels of surface markers expression; the electrolytes, such as calcium potassium and sodium, were not measured; only surface markers were measured, and other intracellular markers were not included. Our future work will include the effect of cryopreservation effect on T cells and will measure all other markers not included in this study, including a group of patients from this study to measure the effect of different storage conditions on their immune cells, compared to healthy participants.

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Declaration of Interest:
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