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Assessment of Pro-Inflammatory Cytokines Level in Stored Blood for Transfusion in Port Harcourt, Nigeria

Authors
Chukwu, S. U.*[1] Jeremiah, Z. A.[2], Eze, E. M.[1]
[1]Department of Medical Laboratory Science, Rivers State University, Port Harcourt, Nigeria
[2]Department of Medical Laboratory Science, Niger Delta University, Wilberforce Island, Bayelsa State, Nigeria
*Corresponding Author
Chukwu, S. U.
Email: *sebastine.chukwu1@gmail.com*, Phone: +2348039316098

Abstract
Blood storage is a logistical necessity to maintain an adequate blood supply. In resource limited setting especially, Nigeria, whole blood storage is still being practiced as only few tertiary centers have facility for blood component preparations. Storage time of blood components has been reported to play a major role in the accumulation of cytokines causing adverse transfusion reactions. Hence, it became necessary to determine the trend of accumulation levels of pro-inflammatory cytokines such as interleukin-4(IL-4), interleukin 6(IL-6) and tumor necrosis factor alpha (TNF-α) in order to know the extent to which stored blood could become potentially dangerous to recipients. Using 450ml blood bags, whole blood was collected from 5 donor and aliqnoited respectively into 8 sterile plain vacutainer and stored at 2^0 – 6^0C. Cytokine estimation was done on day 1, 5, 10, 15, 20, 25, 30 and 35 using ELISA development kit. Statistical analysis was done using parametric and nonprametric tests. The mean value of interleukin-4 (IL-4) of 38.60±22.68 on day 1 dropped significantly to 7.40±6.98 on day 5. The mean value of interleukin-6 (IL-6) which was 8.24±6.65 on day 1 equally decreased significantly to 4.78±2.75 on day 5. Also, tissue necrosis factor alpha (TNF-α) which had a mean value of 10.38±8.30 on day 1 dropped significantly to 5.32±2.90 on day 5. After day 5, there was a gradual build-up but still significantly lower up till day 35, but not exceeding the concentration at day 1 of storage except TNF-α, which at day 35 was significantly higher than the baseline value on day 1 of storage. The trend in the concentration of the interleukins during storage appeared similar except in TNF-α where the concentration dropped on day 5, increased steadily up to day 15 and dropped from day 30 to a value quite significant than the base value. Hence, it became evident that the concentration of IL-4, IL-6 and TNF-α were down regulated at the blood bank temperature, and with their sustained levels up to day 30, there seems to be no possible risk for febrile non haemolyic transfusion reactions.

Keywords: *Interleukin-4 (IL-4), Interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α) febrile non haemolyic transfusion reactions. (FNHTR), Pro-inflammatory, Cytokine.*

Introduction

Pro-inflammatory cytokines are bioactive substances that are produced by leukocytes during blood storage. They are also small protein molecules that are secreted by cells of the immune system and are important in cell signaling. Its release and complication in blood transfusion are not uncommon[1]. Scientific laboratory

investigations proved that storage time of blood plays a role in the production and accumulation of pro-inflammatory cytokines and when transfused are responsible for the significant changes in the recipient's immune response, either by activating or down-regulating the immune system[2].

They can present as acute reactions occurring within a few hours of transfusion, or as delayed events, presenting within days or possible years later. Pro-inflammatory cytokines such as interleukin 1 beta (IL-1β), interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) released by white blood cells during storage always accumulate in the supernatant and when transfused may result to some complications such as febrile non-haematolytic transfusion reaction (FNHTR-that results from the destruction of leukocytes in the transfused blood by the recipient's antibodies, causing pyrogen generation in vivo, or pyrogenic cytokines such as IL-1β, IL-4, IL-6, IL-8 and TNF-alpha), regulated upon activation, normal T-cells expressed and secreted (RANTES), transfusion associated immunomodulation (TRIM- caused by transfusion of allogeneic blood) and allergic reactions[3][4].

Although, transfusion of blood products especially red cell concentrate (RBC) has been a life-saving therapy, it has also been implicated with various transfusion adverse reactions ranging from transfusion related acute lung injury (TRALI – a non-cardiagenic lung oedema presenting within six hours after transfusion) to platelet transfusion refractoriness (lack of post transfusion clinical management resulting from increasing platelet turn-over caused by anti-HLA class 1 antibodies), transfusion-induced alloimmunization and post transfusion infection, (due to immunosuppressive effect of leukocyte-contains red blood cell transfusion), thereby resulting to increased transfusion morbidity and patients mortality[5].

Besides, pro-inflammatory cytokines in an acute transfusion reaction can lead to haemolysis, FNHTR, TRALI, allergic reactions, anaphylaxis, and metabolic complications, or in delayed conditions can result to allo-imminization, delayed haemolytic reactions, graft-versus-host disease, disease/viral transfusion and post transfusion purpura[6]. Generally, cytokines such as chemokines, interferons, interleukins, lymphokines and TNF-α are involved in autocrine, paracrine and endocrine signaling as immunomodulating agents, and are produced by a broad range of cells, including immune cells like macrophages, B-lymphocytes and mast cells, as well as endothelial cells, fibroblasts and various stromal cells; Note that a given cytokine may be produced by more than one type of cell[7].

However, it has been postulated over the past years that these biological response modifiers (BRM) in some cases may be generated in vitro due to antigen-antibody incompatibilities between the donors and recipient's blood and when transfused may cause a typical signs and symptoms, an evide-nce that BRM in blood products are the leading cause of transfusion associated morbidity [8][9].

Furthermore, due to the eminent complications associated with transfusion of whole blood, separation of whole blood into various components (Apheresis), and leukoreduced transfusion has become the practice in developed countries to reduce these complications to the minimum[10]. However, in Nigeria, even in some part of Africa, whole blood transfusion is still widely used order than blood components. This has not been successful due to lack of equipment responsible for this separation. Hence, during storage period, pro-inflammatory cytokines released by white blood cells (WBC) accumulates in the supernatant[2].

Therefore, since it has become so evident that pro-inflammatory cytokines are the primary cause of transfusion morbidity, it necessitate the need to evaluate its trend of accumulation in whole blood in relation to the storage time in order to decifer the extent to which blood should be stored prior to transfusion in Port Harcourt region of Nigeria.

Materials and Methods

Study Area

The samples for this study were collected and analyzed in Braithwaite Memorial Specialist

Hospital (BMSH) which is a tertiary health facility situated in Port Harcourt, in the South-South zone of Nigeria. The geographical location is longitude 60 (58'45") and latitude 50(54' 58'). The hospital serves the population both within the state capital and other primary/secondary/private hospitals.

Study Population

A total number of 5 donors constituted study subjects, apparently healthy donors between the ages of 25 – 40 years. All the samples were collected in the same environment and only one algorithm of test were used once the donor is negative on screening. Excluded from this study are pregnant and lactating mothers, menstruating women, donors with known high blood pressure (BP), low Hb level, diabetics, positive for hepatitis B specific antigen (HBsAg), human immuno-deficiency virus (HIV). Veneral disease research laboratory (VDRL), hepatitis C virus (HCV), and donors whose last donation history is not up to three months and had recently been transfused.

Methods

The enzyme linked immunosorbent assay (ELISA) development kit was used for the estimation of the trend of accumulation of the interleukins of focus (i.e. IL-4, IL-6, and TNF-α). The Elabscience Biotechnology Company Ltd (ELISA, USA) kit, specifically designed for the analysis of IL-4, IL-6, and TNF-α was used for the study. Its intended use applies to in vitro quantitative determination of interleukin (4, 6 and TNF-α) concentrations in the plasma of the stored blood. In addition to the ELISA kit was the Dia medix Bp-96 (awearness technology) a microplate reader that estimates cytokines wavelength at 450nm.

Sample Collection

Blood samples were collected from 5 apparently healthy donors with the use of four hundred and fifty millitres (450ml) blood bags containing citrate phosphate dextrose adenine (CPDA-1) as the anticoagulant. Immediately, 32ml of blood were collected from each blood bag and 4ml each aliquoted into 8 separate 4ml sterile plain vacutainer bottles arranged in rows of 5, taken the first day of sample collection as day 1 as shown in the table below.

Table1: Sample Processing Protocol

DAYS	DAY1	DAY5	DAY10	DAY15	DAY20	DAY25	DAY30	DAY35
DONOR1	TUBE 1 D1	TUBE 2 D1	TUBE 3 D1	TUBE 4 D1	TUBE 5 D1	TUBE 6 D1	TUBE 7 D1	TUBE 8 D1
DONOR2	TUBE 1 D2	TUBE 2 D2	TUBE 3 D2	TUBE 4 D2	TUBE 5 D2	TUBE 6 D2	TUBE 7 D2	TUBE 8 D2
DONOR3	TUBE 1 D3	TUBE 2 D3	TUBE 3 D3	TUBE 4 D3	TUBE 5 D3	TUBE 6 D3	TUBE 7 D3	TUBE 8 D3
DONOR4	TUBE 1 D4	TUBE 2 D4	TUBE 3 D4	TUBE 4 D4	TUBE 5 D4	TUBE 6 D4	TUBE 7 D4	TUBE 8 D4
DONOR5	TUBE 1 D5	TUBE 2 D5	TUBE 3 D5	TUBE 4 D5	TUBE 5 D5	TUBE 6 D5	TUBE 7 D5	TUBE 8 D5

At the end of sample aliquots into 8 tubes arranged in column and starting from day 1, the 5 tubes marked in the first row were subjected to centrifugation at 3,000rpm for 15 minutes at room temperature and the plasma from each tube aliquoted into their respective cryovial (1.8ml), labeled accordingly and stored as frozen plasma at the temperature of -56^0C in the refrigerator. Then the process been repeated at a regular interval of five successive days for the tubes in the rest columns for 35 days i.e. day 1, 5, 10, 15, 20, 25, 30 and 35 prior to cytokine estimation.

However, the objectives and details of the study were communicated to the donors through written informed consent form, after approval by the research and ethics committee secretariat, Rivers State Hospital Management Board, Port Harcourt.

Statistical Analysis

The data were entered into Excel spread sheet and analyzed using Statistical Analysis System (SAS) software version 9.4 of 2013 (Chicago, USA). Results obtained were presented in tables and box plots. Data description was presented as mean ± standard error of mean (SEM). Pearson correlation matrix was used for the correlation of cytokines concentration and storage time and the statistical significance was set at $p < 0.05$ (95% confidence limit).

Results

A total of 5 donors were bled using 450ml blood bags, and their cytokines concentration determined on a 5 day interval until day 35.Table 2 below shows the mean ± SEM of the three cytokine parameters in the stored blood for the entire duration of storage. The mean value of interleukin-4 (IL-4) of 38.60±22.68 on day 1 dropped significantly to 7.40±6.98 on day 5. Also, the mean value of interleukin-6 (IL-6) which was 8.24±6.65 on day 1 equally decreased significantly to 4.78±2.75 on day 5. Similarly, tissue necrosis factor alpha (TNF-α) which had a mean value of 10.38±8.30 on day 1 crashed significantly to 5.32±2.90 on day 5. Hence, a significant decrease ($p<0.05$) was seen in the value of the three cytokines on day 5.

After day 5, there was a gradual build-up but still significantly lower up till day 35 but not exceeding the concentration at day 1 of storage, except TNF-α which at day 35 was significantly higher than the baseline value on day 1 of storage.

Table 2: Comparison of mean±SEM of cytokine parameters by Duration of Sample Storage

Treatment	Interleukin-4 (IL-4)	Interleukin-6 (IL-6)	Tissue Necrosis Factor Alpha (TNF-α)
Day 1	38.60±22.68^{a}	8.24±6.65^{a}	10.38±8.30^{a}
Day 5	7.40±6.98^{b}	4.78±2.75^{b}	5.32±2.90^{b}
Day 10	10.20±6.50^{ab}	0.64±0.39^{b}	11.48±9.61^{ab}
Day 15	15.98±7.43^{ab}	2.54±1.04^{b}	15.26±12.28^{ab}
Day 20	13.88±8.16^{ab}	1.68±0.89^{b}	4.48±1.75^{ab}
Day 25	9.64±6.27^{b}	2.12±0.88^{b}	3.22±1.56^{b}
Day 30	13.74±7.84^{ab}	1.00±0.84^{b}	2.36±1.02^{b}
Day 35	37.58±26.35^{a}	7.50±6.91^{b}	17.32±3.03^{ab}

Within parameters, mean±SEM with different superscript are significantly different at $p<0.05$.

Table 3 Pearson's correlation analysis of the relationship between IL-4, IL-6, and TNF-α levels

Parameter	p-value	r-value	r^2-value	$R^2\sqrt{}$
IL-4 and IL-6	0.0001*	0.9018	0.8132	0.9018*
IL-4 and TNF-α	0.002*	0.4726	0.2233	0.4726*
IL-6 and IL-4	0.0001*	0.9018	0.8132	0.9018*
IL-6 and TNF-α	0.001*	0.4767	0.2272	0.4767*
TNF-α and IL-4	0.002*	0.4726	0.2233	0.4726*
TNF-α and IL-6	0.001*	0.4767	0.2272	0.4767*

Legend

IL-4: Interleukin-4; IL-6: Interleukin-6; TNF-α: Tissue Necrosis Factor Alpha

*$p<0.05$, indicates positive correlation; r: Correlation values; $R^2\sqrt{}$: Coefficient of correlation

Discussion

Blood storage is a logistical necessity to maintain an adequate blood supply. In resource Limited settings, especially, the sub Saharan Africa, whole blood storage is still being practiced as only few tertiary centers have facility for blood component preparations. Red blood cells (RBCs) are collected in an acidic solution and then stored in a plastic bag filled with sugar at 4^0C. During storage (up to 42 days), RBCs are metabolically active in the absence of any waste disposal system (i.e. no kidney or liver), so the cells are essentially marinated in a bath of ever-increasing waste products (e.g. lactate).

What seems clear is that storage is adistinctly unnatural state to which we expose blood cells for various periods of time[11]. The overall issue breaks down into two major questions:

1. To what extent do stored blood cells maintain their desired function and therefore constitute an efficacious product?
2. Are there onward effects of storage based changes that result in medical sequalae due to toxic substances that accumulate in the blood?

One major objectives of this study is to evaluate the cytokine's values on whole blood stored in blood bank prior to blood transfusion to recipients with a view to finding out how long a whole blood could be stored before it becomes potentially dangerous to recipients. Whole blood as we know consists of three components, the red blood cells (RBC), the white blood cells (WBC) and the platelets (PLTs). This study was set out to provide answers to these pertinent questions in our peculiar environment where whole blood transfusion is still being practiced, and the major finding was that the three cytokines were reduced or down regulated on day 5, and gradually increased from day 10 to day 35 but not exceeding the baseline value of day 1 except TNF-α.

Red blood cell storage lesion has recently been recognized as an important issue facing transfusion medicine[11]. The issue has attracted numerous studies to determine the potential risks associated with transfusion of RBCs stored over a longer period of time and the underlying mechanisms responsible[12][13][14][15][16][17].

Besides, substantial evidence from in vitro studies exists documenting the changes that blood undergo during storage. A human blood (RBC) has a lifespan of approximately 120 days, ranging from 0 to 120 days of age, which is equivalent to a unit of freshly drawn RBCs. Note that young RBCs can survive for a long period of time after transfusion, but senescent RBCs are rapidly eliminated from the circulation. Therefore, to evaluate the survival time of blood-banked RBCs after transfusion, it is important to determine the proportions of young and old RBCs in the banked RBC unit as well as assess how the proportions and the cells properties changes during storage[18].

Red blood cells has been shown to affect immune function and can induce inflammatory responses after transfusion[19]. Furthermore, in a recent study, it was determined that the pro-inflammatory cytokines and enzyme, macrophage migration inhibitory factor (MIF), was present in RBCs at levels 100-fold higher than the typical plasma concentration[19]. As such after blood collection, the plasma level of MIF could be significantly altered by relatively low levels of haemolysis. In addition, RBCs, or soluble factors released by these cells, can also stimulate the secretion of pro-inflammatory markers from lung fibroblasts[19]. These studies demonstrate that RBCs may be an important component of the immune system and are capable of signaling or receiving signal from other cell types. Despite these studies, the role of RBC in signaling remains poorly understood. Nevertheless, from our statistical observations, it seem to be that cytokine down regulation is time dependent, where interleukin-6 showed a decreased trend from day 1 to day 35 in whole blood storage. Similar results were reported by Jacobi *et al.*, who found IL-6 levels in physiological limits in whole blood[5]. A study by[2], reported that IL-6 was under the detection limit of 3.9pg/ml in whole blood and the levels remained low during the storage[2]. Thus, these findings are comparable with the present study, concluding that IL-6 is not accumulated in whole blood stored at 4^0C.

This study also supports the study of[1] where IL-6 levels remained low at any storage time[1]. In another study by[20], IL-6 and TNF-α showed a mean low level plateau which further corroborate the findings in this research work.

Finally,[21] reported that many factors activate WBCs to generate cytokines during storage like activated complement components, throbin or by cytokines released from damaged WBC, or by non biological surfaces of plastic containers[21]. The measure of CD40L, a pro-inflammatory mediator released by leukocytes and platelet upon activation, indicates that WBC is activated, and cytokines are synthesized due to which the levels increases. This study proves that cytokine concentration is directly

related to WBC content and storage time. At least 3% of all transfusions result in either febrile non haemolytic transfusion, reaction (FNHTR) or allergic reaction[22].

Therefore, IL-6, as an endogenous pyrogen, and always present at low levels in whole blood may cause FNHTR [5].

Conclusion

Since the concentration of interleukins were down regulated on storage in the blood bank, it then shows that the banked whole blood demonstrated a drastic reduction in white blood cells and platelet, as proved in this study that the decreased concentration of the interleukins was directly related to the decrease levels in WBCs and platelets content on day 5 of storage.

This indicated for the reason why interleukin-4, interleukin-6 and TNF-alpha were not only down regulated at blood bank temperature, but also due to the decrease in the concentrations of WBCs and Platelets in the plasma during storage. However, stored whole blood may release white cell and platelet-derived cytokines in a time-dependent manner, and may be associated with many transfusion reactions in due course. At this point, comparative analysis of cytokines shows an increase levels in IL-4, IL-6 and TNF-alpha in a time-dependent manner in whole blood.

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