ORIGINAL ARTICLE

Study of Pre Storage Leucodepleted Effects on Cell Blood Count and Cytokines Level in Whole Blood During Storage

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ABSTRACT

Introduction: The presence of white blood cells (WBCs) and other proteins for example, cytokines in the red blood component during storage may have an effect on cancer patients requiring blood transfusions, and leucodepletion method would have to be used. This study aimed to evaluate the residual WBCs in red cell components following leucodepleted and to determine cytokine levels during storage of red cell components. **Materials and methods:** A total of 350 mL of whole blood was withdrawn from 31 healthy volunteers. Half of this volume (175mL) was leucodepleted (LR) at room temperature, while the other half (175mL), was not leucodepleted and used as a control. Full blood count (FBC) and flow cytometry (FCM) with residual WBCs subsets were performed. Cytokines were measured in plasma samples collected from non LR and LR bags after 0, 10, 20, and 30 days. T-helper (Th) lymphocyte subgroups and gene expression were analysed in the non-LR samples using real-time polymerase chain reaction. **Results:** There was a significant difference in the mean of all FBC parameters and residual WBCs subsets between these two groups ($p < 0.05$). There was a significant difference in TGF-β and IFN-γ between non-LR and LR samples on day 0 to day 10. TGF-β level showed an increase up to day 30 in non-LR samples. T-bet, GATA-3, and Foxp3 gene expression were detected in non-LR samples. **Conclusion:** Leucodepletion demonstrated a significant effect on WBC count, TGF-β and IFN-γ levels, during blood storage.
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Keywords: Leucodepletion, White blood cells, Whole blood, Flow cytometry, Cytokines

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INTRODUCTION

Blood transfusion is the intravenous administration of blood components into a person's circulation. This immediate intervention is critical when dealing with life-threatening situations, while having less significant long-term implications (1). Despite pre-screening for infectious diseases to prevent adverse reactions, a patient may still experience effects or reactions after transfusion.

The leucocytes in blood components can mediate febrile transfusion reactions (2), induce alloimmunisation of human leucocyte antigen (HLA) in transfusion recipients (3), and transmit other cell-associated pathogens like cytomegalovirus (CMV) (4). Simultaneously, the presence of proteins for example, cytokines found in the blood component themselves, may also incite reactions in the patient undergoing transfusion. Several studies have shown higher levels of cytokines like tumour necrosis factor-alpha (TNF-α), interleukin (IL) -1 beta (IL-1β), IL-8, and RANTES in pre leucodepleted (LR) RBCs and platelet concentration during storage compared to post-LR RBCs (2). IL-22 and the transforming growth factor-beta (TGF-β) were also detected from day 21 until day 42 in non-LR RBCs (5). According to a study by Sut et al., the release of cytokines and lipid factors by the donor's leucocytes during RBCs storage can activate neutrophils (6). Previous studies have also revealed that the effect of cytokines in whole blood (WB) was higher than the effect of cytokines in buffy coat depleted RBCs during the storage period up to day 21 (7,8). Meanwhile, the balance of cytokines in tumour microenvironment can have a significant impact on cancer progression. Some cytokines are associated with promoting tumour growth and metastasis, while others have antitumour effects. For example, pro-inflammatory cytokines such as IL-6 and TNF-α have been shown to promote growth by stimulating angiogenesis, suppressing immune

responses, and promoting cancer cell survival (9,10). Therefore, several approaches have been considered to prevent adverse reactions related to transfusion. The transfusion of leucocyte-reduced RBCs concentrates is one of those approaches (11,12); whereby LR would leave a leucocyte count of < 5 x 10^6 per unit (99.9%, or a 3 log; reduction)(13).

In this country, LR blood components are employed for individuals in need of frequent transfusions, such as thalassaemia patients. These individuals face the risk of developing alloimmunisation and transfusion-transmitted infections due to multiple transfusions. The haemovigilance report in 2017 from the National Transfusion Medicine Service in Malaysia suggested the use of leucocyte-reduced blood components to reduce adverse transfusion events associated with WBC. However, there is a recommendation for further research on the cost-effectiveness of incorporating this practice into routine transfusions (14). Meanwhile, pre-storage LR is mandatory in Canada and most of Europe, while in the USA, it is not mandatory but recommended by the FDA due to its high cost (15).

There is a lack of studies comparing the reduction of white blood cell (WBC) subsets in whole blood between genders in this country. Researchers in Japan, used autologous blood for certain parameters only, such as WBCs and platelet count (16). Similarly, study by Ghezelbash et al. (2018) explored the effects of LR on packed red cells, noted a reduction in leucocytes in platelet concentrations as well (17). Hence, by considering gender disparities, the aim of this study was to gather insights into how LR techniques could impact whole blood differently based on gender.

Furthermore, the transcription factor that starts and controls the formation and differentiation of white blood cells, especially T-cells, is commonly linked to the T-cell receptor (TCR) complex. It involves different factors and genes that are important for T-cell growth. Some of the factors for T-cell development are as follows: GATA-3, which helps T-cells turn into a certain type called T-helper 2 (Th2) cells (18); T-bet, which helps make T-helper 1 (Th1) cells and responds to certain infections (19); and FOXP3, which is needed for regulatory T cells (Tregs) that prevent the immune system from attacking the body (20). In this study, the detection of transcription genes was performed to verify their presence in WBCs. This step can determine the presence of cytokines, that were not originating from the WBCs but from other sources. Therefore, this study was designed to evaluate the properties of the Macopharma LEUCOLAB filter system, and to assess the effect of buffy coat removal on the efficiency of LR and FBC parameters in WB. This study also aimed to examine cytokines level in RBCs during storage.

MATERIALS AND METHODS

Study Design and Participants

This case-control study was conducted at the Transfusion Medicine Unit of the Advanced Medical and Dental Institute (AMDI), Universiti Sains Malaysia, in Penang from August 2019 till December 2020. This study enrolled healthy Malaysian blood donors aged between 18 and 60 years old. Exclusion criteria included non-Malaysian, individuals younger than 18 years old or older than 60 years old, and those ineligible for blood donation. Participation in this study was contingent upon obtaining written informed consent from the subjects.

Leucoreduced blood (LR) and non LR blood preparation

Written agreed consents were obtained from 31 healthy blood donors, whom each donated 350 mL of WB at the Transfusion Medicine Unit, Advanced Diagnostic Laboratory, USM. In brief, whole blood; that has been anticoagulated with 49 mL of CPD (comprising 3.06 g of sodium chloride, 1.83 g of mannitol, 3.15 g of dextrose monohydrate, and 0.05 g of adenine) was collected using a CPD/SAGM double blood bag system (JMS, Singapore).

Half of the donated blood passed through the Macopharma LEUCOLAB LCG2b/LCG4 leucodepleted filter at room temperature, following the manufacturer's recommendations. The remaining half was transferred into the transfer bag (JMS, Singapore) and used as a control. Both blood bags were stored at 4°C in the standard blood bank refrigerator for 42 days.

This process was repeated for 31 sets of RBCs, resulting in a total of 62 individual units of RBCs.

Haematological parameters

Approximately 3 mL of blood sample was collected from each blood segment before storage and after the filtration process for every bag. Prior to conducting the full blood count analysis, the haematology analyser (Sysmex KX-21) was calibrated, and quality assurance procedures were performed.

Residual WBC enumeration of non-LR and LR bags using standard flow cytometry

Monoclonal antibodies to CD3, CD4, CD8, CD14, CD16, and CD66b, as well as non-immunised isotype-matched antibodies (BD Biosciences, US), were used to determine the percentage and absolute count of lymphocyte subsets and granulocytes in the donated blood, as well as in the non-LR and LR bags. Surface markers were stained using the lyse-wash method for 100 µL of whole blood samples. A four-colour combination for antibody staining was performed. RBCs were lysed with BD FACS lysing solution and each sample was analysed using a flow cytometry (FACS Canto, Becton-

Dickinson).

Measurement of cytokines level

Supernatants from non-LR and LR samples were obtained for cytokine analysis by centrifuging the blood-filled test tubes at 3,500 g for 10 minutes. The plasma samples were transferred into clean test tubes and stored in a freezer (Revco, St. Louis, MO, USA) at 80°C. Interleukin IL-2, IL-6, transforming-growth factor (TGF)-β and interferon (IFN)-γ levels were measured in these samples using commercial enzyme-linked immunosorbent assay (ELISA) kits (Invitrogen) according to the manufacturer's recommendations. Minimum detectable levels of the ELISA were as follows: 2.0 pg/mL for IL-2, 2.0 pg/mL for IL-6, 1.96 pg/mL for TGF-β, and 1.96 pg/mL for IFN-γ.

Evaluation of transcription factors in non-LR samples using Real-time Polymerase Chain Reaction (RT-PCR)

The expression of specific transcription factors T-bet, GATA3, and Forkhead BoxP3 (FOXP3) of Th cell subgroups was evaluated among three non-LR samples. Peripheral blood mononuclear cells (PBMCs), RNA extraction, complementary DNA (cDNA) synthesis, and RT-PCR were used in this process. Ficoll® was used to separate PBMCs using a density gradient (Histopaque-1077, Sigma-Aldrich, St. Louis, MO, USA). Total RNA was extracted using a commercial kit (MO BIO Laboratories Inc., Carlsbad, CA, USA), which was then used to produce cDNA using a commercial First Strand cDNA Synthesis Kit (New England BioLabs Inc, Ipswich, MA, USA). The obtained cDNA samples were stored in a freezer at −20 °C prior to the RT-PCR analysis. Real-Time Ready kit (Roche, Mannheim, Germany) that was designed for this study was used to measure the expression of specific transcription factors by RT-PCR. Panels consisted of three genes related to the transcription factors (Tbet, GATA3, FOXP3) and glyceraldehyde 3- phosphate dehydrogenase (GAPDH) were used as reference genes, and positive and negative controls, respectively. The expression of specific transcription factors was measured using the Light-Cycler 480 RT-PCR (Roche). Following the RT-PCR analysis, relative quantification of target gene expression was performed. All data were analysed using the ΔΔCt method.

Statistical Analysis

All patients' data were analysed using a statistical software package, SPSS Statistics, version 24. The cytokines level was compared using repeated ANOVA. In this study, a *p*-value of < 0.05 was considered significant.

Ethical Approval

Human ethics approval was obtained from the Human Research Ethics Committee, Universiti Sains Malaysia, USM/JEPeM/1810079. Written consents have been obtained from all volunteers.

RESULTS

The donor's age ranged between 19 and 38 years old. Among 31 donors, 24 were males and seven were females. Following the donation process, blood components were divided equally into 31 LR bags and 31 non-LR bags.

1.Haematological Parameters

i.Leucocyte subsets in healthy blood donors

Full blood counts for lymphocytes and granulocytes are higher in the blood of female donors, as shown in Table I.

Table I: Comparison of leucocyte counts in the blood of female and male donors using a haematology analyser.

Leucocytes count	Male (n=24) Mean (SD)	Female (n=7) Mean (SD)	Overall Total (n = 31) Mean (SD)	*p* -value
Total WBC (x 10^9)	6.88 (1.62)	6.87 (1.35)	6.87 (1.49)	0.99
Lymphocytes (%)	30.26 (16.5)	40.08 (21.6)	35.17 (19.1)	0.29
Neutrophils (%)	41.08 (20.7)	44.9 (12.1)	42.99 (16.4)	0.55
Mixed Neutrophil (%)	30.31 (12.73)	26.11 (16.61)	28.21 (14.67)	0.42

*p-value > 0.05 not significant

In this study, 31 healthy blood donors, consisting of 24 males, and 7 females, have volunteered for this study. Table I shows a comparison of the mean values of total WBCs counts between samples from female and male donors, indicating minimal difference between the two groups. The results have also revealed that samples from female donors exhibited a higher leucocyte count at 40.08% compared to from males at 30.26%, while the neutrophils count was also higher in sample from females at 44.9% compared to from males at 41.08%. The mixed neutrophils parameter included other granulocytes. Although there was a gender-based difference in these results, no significant difference was identified upon conducting a paired t-test, $p = 0.42$.

ii.Full blood counts between non-LR and LR bags (n = 62)

A total of 62 samples were analysed using the automated haematology analyser. Table II shows the mean values of the FBC between non-LR and LR samples. WBC, RBC, haemoglobin, and platelet count in non-LR samples showed significant differences compared to in LR samples with p-value of less than 0.05.

Table II: Comparison of mean total WBCs count, RBC count, haemoglobin level, and platelet count using a haematology analyser between non-LR and LR bags (n=62).

Parameters	Mean (SD)		Mean difference (95% C1)	t- statistic (df)	*p*-value
	Non-LR (n=31)	LR (n=31)			
WBC x10^9 /L	6.88 (1.54)	0 (0)	6.88 (6.31, 7.45)	24.9 (30.0)	0.00*
RBC x 10^{12}/L	5.26 (0.48)	4.54 (0.53)	0.72 (0.46, 0.98)	5.51 (60.0)	0.00*
Haemoglobin g/dL	14.5 (1.18)	12.6 (1.09)	1.84 (1.23, 2.44)	6.09 (56.0)	0.00*
Platelet x 10^9 /L	250.9 (48.3)	1.06 (2.36)	249.9 (232.2, 257.6)	28.7 (30.1)	0.00*

*p-value < 0.05 is significant
Abbreviation: Non-LR; = non leucoreduced, LR;= leucoreduced

A total of 62 samples were analysed using an automated haematology analyser. Table II shows the mean values for FBC between non-LR and LR samples. WBC, RBC, haemoglobin, and platelet counts in non-LR samples showed significant differences compared to in LR samples with p-value <0.05.

iii. Lymphocyte subsets in healthy blood donors

The average absolute counts and percentages of lymphocyte subsets using standard flow cytometry methods showed higher levels of absolute count for total T cells (CD3+) in the blood of the female's donors. Nonetheless, other percentages appear to be similar, as shown in Table III.

Table III: Comparison of mean lymphocyte subsets using flow cytometry in the blood of male and female donors.

Lymphocytes (%)	Male Mean (SD)	Female Mean (SD)	Overall Mean (SD)	*p*-value
CD3+	30.26 (16.48)	34.41 (21.58)	32.35 (19.03)	0.65
CD3+ CD4+	9.26 (5.09)	10.49 (1.99)	9.88 (3.54)	0.35
CD3+ CD8+	8.39 (4.72)	8.47 (4.20)	8.43 (4.46)	0.96
CD3+ CD16+	3.37 (3.13)	3.77 (5.82)	3.57 (4.48)	0.87

*p-value >0.05 not significant
Abbreviation: Non-LR; = non leucoreduced, LR; = leucoreduced

Table III shows the percentages of lymphocyte subsets using standard flow cytometry methods. CD3+ results showed a higher value in the samples from females at 34.41% compared to from males at 30.26%. The results for CD3+CD4+ revealed 10.49% in the samples from females compared to from males at 9.26%. There was a slight difference in CD3+CD8+ percentages with 8.47% in the samples from females and 8.39% in the samples from males. CD3+CD16+ percentages also showed a slightly higher value in the samples from female at 3.77% when compared to 3.37% from males.

iv. CD markers between non-LR and LR groups

Table IV shows the comparison of mean CD markers of lymphocytes results between non-LR and LR samples. This study used a panel of WBCs subset markers CD3, CD4, CD8, CD16, CD14, and CD66b was observed that all CD markers in non-LR samples showed significantly higher results compared in LR samples with p-value < 0.05.

Table IV: Comparison of mean CD markers using flow cytometry between non - LR and LR bags (n=62).

CD marker	Mean (SD)		Mean difference (95% C1)	t-statistic (df)	*p*-value
	Non-LR (n=31)	LR (n=31)			
CD3	31.1 (17.5)	0.40 (0.56)	30.74 (24.4 , 37.2)	9.77 (30.1)	0.00*
CD4	9.54 (4.57)	0.39 (0.11)	9.50 (7.82 , 11.2)	11.5 (30.0)	0.00*
CD8	8.40 (4.53)	0.04 (0.11)	8.36 (6.73 , 9.98)	10.3 (30.0)	0.00*
CD16	3.33 (3.73)	0.616 (0.17)	3.27 (1.90 , 4.64)	4.88 (30.1)	0.00*
CD14	1.22 (1.32)	0.087 (0.25)	1.13 (0.64 , 1.62)	4.69 (32.3)	0.00*
CD66b	40.74 (19.2)	1.40 (6.13)	39.35 (32.01, 46.7)	10.88 (36.08)	0.00*

*p-value < 0.05 is significant
Abbreviation: Non-LR; = non leucoreduced, LR; = leucoreduced

Table IV shows the comparison of the mean CD markers of lymphocytes in non-LR and LR samples. A panel of WBCs subset markers CD3, CD4, CD8, CD16, CD14, and CD66b were used in this study. All CD markers results showed significantly higher values in non-LR samples compared to LR samples with p-value < 0.05.

2.Cytokines level

An increasing trend in the mean cytokines level can be observed in RBCs of non-LR samples during storage at 4˚C. In the non-LR sample, TGF-β displayed an increasing trend in cytokines level until day 30, while others showed decreasing trends until day 30. In LR samples, no increase in the level of cytokines was observed. The results of the selected cytokines are summarised in Table V. The levels of cytokines in non-LR RBCs were significantly higher than in LR RBCs, except for IL-6 and IL-2, where IL-6 level was almost not detected in any sample of both bags.

Table V: Comparison of mean differencse and CI of cytokine levels in non-LR and LR of RBCs during storage time at 4 °C.

Cytokines	Storage of RBC	Non-LR			LR		
		Mean Diff	***p***	**95% CI**	**Mean Diff**	***p***	**95% CI**
TGF-β	Day 0– Day 10	-17.0	0.12	(-2.78, -24.47)	0.13	1.00	(-0.50, 0.77)
	Day 0– Day 20	-53.14	0.00*	(-81.79, -24.47)	-0.35	1.00	(-1.60, 0.90)

CONTINUE

Table V: Comparison of mean differencse and CI of cytokine levels in non-LR and LR of RBCs during storage time at 4 °C. (CONT.)

Cyto-kines	Stor-age of RBC	Non-LR			LR		
		Mean Diff	***p***	**95% CI**	**Mean Diff**	***p***	**95% CI**
TGF-β	Day 0–Day 30	-61.66	0.00*	(-86.05, -37.27)	-1.46	0.15	(-3.21, 0.29)
	Day 10 –Day 20	-36.13	0.00*	(-60.51, -11.74)	-0.48	1.00	(-1.67, 0.71)
	Day 10 –Day 30	-44.65	0.00*	(-68.64, -20.67)	-1.59	0.41	(-3.15, 0.04)
IFN-γ	Day 0 –Day 10	1.58	1.00	(-2.430, 5.620)	2.40	0.21	(-1.39, 6.19)
	Day 0 –Day 20	3.75	0.04*	(0.141, 7.348)	3.52	0.81	(-0.47, 7.50)
	Day 0 –Day 30	2.47	0.74	(-1.842, 6.781)	2.16	0.30	(-2.10, 6.37)
	Day 10 –Day 20	2.15	0.79	(-1.683, 5.982)	1.12	0.79	(-0.14, 2.37)
	Day 10 –Day 30	0.87	1.00	(-2.758, 4.507)	-0.24	0.74	(-1.72, 1.23)
IL-2	Day 0 –Day 10	0.58	0.46	(-0.99, 2.16)	1.76	0.37	(-2.16, 5.67)
	Day 0 –Day 20	3.09	0.29	(-2.74, 8.92)	2.38	0.37	(-2.97, 7.74)
	Day 0 –Day 30	2.15	0.46	(-3.65, 7.94)	1.23	0.66	(-4.47, 6.92)
	Day 10 –Day 20	2.51	0.35	(-2.92, 7.94)	0.63	0.44	(-1.00, 2.25)
	Day 10 –Day 30	1.56	0.56	(-3.81, 6.94)	-0.53	0.65	(-2.91, 1.85)
IL-6	Day 0 –Day 10	0.58	0.46	(-0.99, 2.16)	1.76	0.21	(-2.16, 5.67)
	Day 0 –Day 20	3.09	0.29	(-2.74, 8.92)	2.39	0.81	(-2.97, 7.74)
	Day 0 –Day 30	2.15	0.46	(-3.65, 7.94)	1.23	0.30	(-4.47, 6.92)

CONTINUE

Table V: Comparison of mean differencse and CI of cytokine levels in non-LR and LR of RBCs during storage time at 4°C. (CONT.)

Cyto-kines	Stor-age of RBC	Non-LR			LR		
		Mean Diff	***p***	**95% CI**	**Mean Diff**	***p***	**95% CI**
IL-6	Day 10 –Day 20	2.51	0.35	(-2.92, 7.94)	0.62	0.79	(-1.00, 2.25)
	Day 10 –Day 30	1.56	0.56	(-3.81, 6.94)	-0.53	0.74	(-2.91, 1.85)

*Significant if *p*-value < 0.05
Abbreviation: Non-LR; = Non leucoreduced, LR; = Leucoreduced

Table V summarises the differences in cytokines levels during storage using the one-way repeated measures ANOVA. The cytokines levels in non-LR samples were significantly higher than LR samples, *p*- value < 0.05. An exception was observed for IL-6, whereby IL-6 level was almost not detected in any sample in both bags. The results of the one-way repeated measures ANOVA for IFN-γ levels, showed significant effect on RBCs storage ($F(2.65, 161.95) = 2.67$, $p < 0.05$) in the non-LR bags. Bonferroni post hoc tests showed that IFN-γ level in RBCs was higher on day 10 of storage (mean = 5.15; SD = 1.44) compared to on day 20 of storage (mean = 3.00; SD = 1.27; $p < 0.05$). However, IFN-γ level in RBCs was not significantly different between day 0 (mean = 6.75; SD = 1.75) and day 30 of storage (mean = 4.28; SD = 1.27). These results showed that IFN-γ level was reduced after day 20 of storage in the non-LR bag. Although there was a slight mean difference between day 0 (mean = 3.99; SD = 11.17) and day 20 (mean = 0.48; SD = 1.19), statistical analysis proved that it was not significant ($p > 0.05$) in the LR samples.

Meanwhile TGF-β results showed a statistically significant ($p<0.05$) value in the non-LR bag ($F(2.56, 76.85) = 22.39$, $p<0.05$). Bonferroni post hoc tests showed that the TGF-β level kept increasing from day 10 until day 30 of RBCs storage and was statistically significant ($p<0.05$). No significant differences were observed in LR samples throughout RBCs storage.

3.Analysis of Th subtypes in non-LR samples by Real-Time PCR

Using RNA extracted from PBMCs of non-LR and LR samples, qPCR was performed to quantify the Th subgroup expression level. However, Th subgroups and specific transcription factors in LR samples were not measured because the number of leucocytes within these products following 4 log reduction is theoretically expected to be insufficient. The results were analysed using comparative ΔΔCT analysis. GAPDH was used as a housekeeping gene to normalise the gene expression

value of the target gene. A bar graph of the standard error of gene expression is shown in Fig.1, while the Relative Quantification (RQ) values for three different types of gene expression (Tbet, GATA3, and FOXP3) in three samples of non-LR samples are shown in Fig. 2. These findings indicated the presence of these types of gene expression in all samples. However, the Kruskall-Wallis's analysis, result showed that the difference in gene expression across samples was not significant (p>0.05).

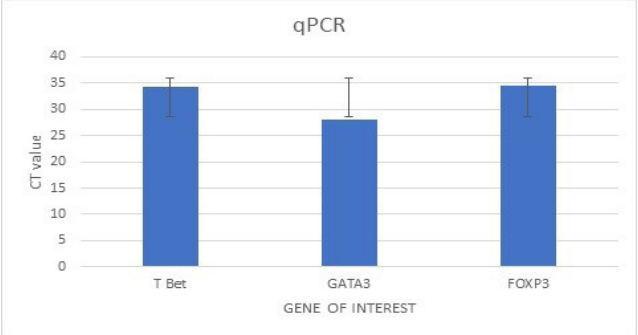

Fig. 1: Differences of CT values between 3 genes of interest in non-LR samples

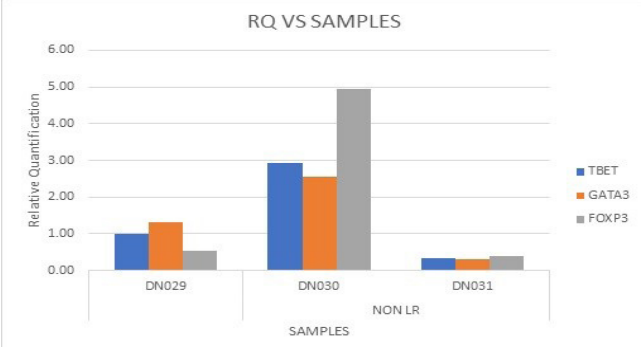

Fig. 2: Relative quantification of gene expression of Th subtypes in non-LR samples.

DISCUSSION

The levels of lymphocyte subsets in the blood of healthy male and female donors were compared in this study. The female donors have higher percentages in T cells than the males, which was consistent with the findings of a previous study (21). Furthermore, a similar pattern of higher CD8+ T cell distribution was observed in the female donors, as similarly reported in another study (22). However, these findings contradicted the elevated levels of CD8+ T cells found in males, as reported by other study (23). A prior study, noted that female immune responses produced stronger cellular and humoral immune reactions compared to male immune responses, which provided a better defence against bacterial infection (24). Another study involving 1,076 healthy females discovered that those who received smallpox vaccination showed significantly higher neutralising antibody titres compared to males (25). Meanwhile, research on mice demonstrated that young proestrus female mice survived trauma haemorrhage and subsequent sepsis better than young males (26). This study noted a discrepancy because the number of female blood donors (n = 7) was lower than the number of male blood donors (n = 24). Thus, the results of this study could have been indirectly influenced. Age could have also influenced the study outcomes, whereby the age of the blood donors involved varied from 19 to 33 years old. Numerous studies have investigated peripheral lymphocytes reference range, highlighting that normal range of each subset may differ depending on age, gender, and races (21,27).

In this study, the WBCs and platelet counts of the donated blood were significantly reduced after undergoing leucodepletion. A similar finding was reported, wherein WBCs and platelet counts were not detected following leucodepletion (28). The loss of RBCs during leucodepletion using in-line filters has been reported with a mean red cell loss approximately 13.1%. In a study conducted in Singapore, the authors reported 15% red cell loss and 99.99% reduction in WBCs (29), which were almost similar to the results of this study. There were also minor differences in the amount of haemoglobin (approximately 2g) before and after filtration, which indicated that haemoglobin lost due to filtration. This study opined that this lost was due to the buffy-coat elimination, which resulted in lower mean RBCs and haemoglobin counts per bag. However, this observation was in contrast to the one made by previous study, where no significant difference was observed between the haemoglobin level and RBCs counts in non-LR and LR bags (30). Meanwhile, this study found significant reduction in haemoglobin level and RBCs count in LR samples. Despite this reduction, the whole blood (WB) remained useful for transfusion purposes. This observation was supported by the levels of RBCs and haemoglobin remained within the acceptable range, as refer to Transfusion Practice Guidelines for Clinical and Laboratory Personnel in Blood Bank (31).

The pre-storage leucodepletion method allowed approximately 4 log10 leucocytes to be removed from a single unit of red cells. According to the American Association Blood Bank (AABB) requirements, a unit must have leucocyte content of less than 5 x 10^6, and leucocyte reduction due to filtration of RBCs must not result in red cell loss of more than 15% (32). Meanwhile, the Council of Europe guideline, states that residual leucocytes must be less than 1 x 10^6 per unit; and each unit must contain at least 40 g of haemoglobin after LR (33). In this study, all units that were prepared using the filter met all of these requirements. Other parameters, such as mean cell volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were also within

the reference range similar to other studies (34,35).

In terms of residual leucocytes, a previous study showed that buffy-coat removal improved filtration efficiency (36). WBCs subset removal in whole blood was compared in this study, before and after undergoing leucodepletion by using flow cytometry. The overall residual of WBCs subset removal was found to be successful. In this study, the pattern of WBCs removal via whole blood leucodepletion was similar to platelet filters, in which the removal of monocytes and T-lymphocytes was marginally greater than the removal of granulocytes and B-lymphocytes. These findings were consistent with those reported by Ledent et al.; (2000), whereby after filtration, the percentage of T-lymphocytes in WBCs subsets in buffy coat-derived pack cells was decreased (37).

The various WBCs have different functions in the body's defence system. The extent to which functionally distinct WBCs populations are removed is an important issue concerning WBCs reduction. Moreover, lymphocytes, monocytes, and granulocytes subsets are involved as carriers of other proteins, e.g., cytokines and other immunoglobulins that can also cause transfusion reactions in certain patients. Therefore, the level of cytokines in RBCs was compared in this study, before and after leucodepletion during the storage period at 4˚C.

The ELISA method was used to measure IL-2, IL-6, IFN-γ, and TGF-β levels. The results showed that IL-2 levels were detected in only eight out of 31 non-LR samples, and in 11 out of 31 LR samples. However, when compared to the range provided by the manufacturer, these IL-2 levels were not within the acceptable range, with the value below the detectable range and not statistically significant. These results aligned with the results reported by a previous study (2). Conversely, Huang et al.; (2016) reported that IL-2 was detected in cervical cancer specimens using immunohistochemistry (38). Interestingly, among the cytokines listed, only IL-6 levels were not found in any of the samples. This could be due to their rapid degradation, the ELISA's low detection sensitivity, their short half-life, or consumption within the product during storage.

Moreover, IFN-γ and TGF-β levels were only detected in non-LR samples. However, a decrease in the IFN-γ level and an increase in TGF-β levels were statistically significant at day 30 of RBCs storage. In non-LR samples, TGF-β levels have consistently increased across all 31 samples until the end of RBCs storage. Increasing TGF-β levels during storage were also reported by previous research by Baumgartner et al. (39). Another study reported similar findings as the present study, whereby TGF-β levels can be detected up to day 42 of the blood storage period (2). Generally, TGF-β cytokines are produced by CD4+ cells in lymphocytes and can be generated from other sources and cells. Therefore, TGF-β was more likely to be detected in these blood samples.

When TGF-β cytokines are transferred to the patient via allogeneic blood transfusion, their levels can be elevated and inhibit leucocyte activation, or enhance the immunosuppressive properties of TRIM by inducing differentiation of naive Th cells into Treg cells, especially in patients who have received multiple transfusions. The presence of elevated TGF-β cytokine required activator protein observed in prostate cancer patients (40). Meanwhile, TGF-β levels were correlated to the presence of VEGF (Vascular Endothelial Growth Factor) expression in gastric cancer patients (41). Thus, when there was an increase of TGF-β levels in non-LR supernatants but an absence in LR samples, these finding became an indication of the effectiveness of leucodepletion in avoiding TRIM and elevated TGF-β levels in certain patients.

Expression of IFN-γ was observed in the non-LR sample from day 0 to day 30, but this trend began to decrease from day 20 to day 30 during the storage period. This decrement could be due to the low survival rate of WBCs in the lymphatic system, which is around 13 to 20 days. Meanwhile, IFN-γ expression was detected in the LR samples but it was not statistically significant. These findings were consistent with the finding of previous studies (2,5,16). IFN-γ is produced by NK cells, while Th1 is produced by CD4 and CD8 cells (42,43). IFN-γ acts as an antiviral in the immunoenvironment while in the tumour environment, it acts as an antitumour that prevents tumour cells from multiplying (44). Th lymphocyte subsets; and specific transcription factors, were also analysed in the non-LR samples. Since the number of leucocytes in the product following the 4log leucodepletion is theoretically expected to be insufficient, no Th subgroups or specific transcription factors were evaluated in the LR samples. T-bet and GATA-3 are transcription factors involved in the formation of WBCs. This study detected genes expression in three non-LR samples, with the presence of 95% of GATA-3 and FOXP3, while 75.6% of T-bet in these non-LR samples. These findings were similar to the findings of another study (5). As detailed in a review article from China, Th1 cells were differentiate from naive Th cells in the presence of a specific transcription factor TBX21 (T-bet), which was expressed following IFN-γ stimulation; and can produce copious amounts of IFN-γ (45). In this study, the presence of CD3+ cells, CD4+ cells and IFN-γ in non-LR, suggested that Th1-type cells were identified from T-bet gene expression in these samples. The significant reductions of IFN-γ levels in non-LR samples were observed after being stored past days 20, could indicate a decrease in the Th1 type response. This finding was consistent with findings of previous studies that found reductions in Th1 type cytokine levels in recipients after undergoing allogeneic

blood transfusion(46,47).

Whole blood that has been stored may release cytokines from WBCs in a time-dependent manner and may be involved in several transfusion reactions. The use of buffy coat-depleted RBCs may help avoid many of the adverse effects caused by leucocytes and cytokines. Some researchers have also demonstrated that the pre-storage leucodepletion was able to minimise unadjusted in-hospital mortality rates, post-transfusion fever, and antibiotic use in these patients (48). Other studies have also revealed that reduction in CMV transmission was also helpful as it also reduced febrile non-haemolytic transfusion reactions, (49).

CONCLUSION

Storage of whole blood may cause a time-dependent release of cytokines from WBCs and may induced many transfusion reactions in cancer patients. IFN-γ and TGF-β levels began to increase in a time-dependent manner in non-LR versus LR samples, implying that stored LR blood should be used instead of non-LR for transfusion.

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