



Effects of donor age, donor sex, blood-component processing, and storage on cell-derived microparticle concentrations in routine blood-component preparation



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ABSTRACT

Background: A number of factors cause increases in the number of cell-derived microparticles (MPs) in blood components. However, the overall effects of these factors on the concentration of MPs during routine blood-component preparation have not fully been elucidated.

Aim: To evaluate the effects of donor age, donor sex, blood-component preparation, and storage on MP concentrations.

Methods: Flow cytometry was used to quantitate the number of whole blood-derived MPs.

Results: The total MP concentration was similar in male and female donors ($26,044 \pm 1254$ particles/ μL vs. $27,696 \pm 1584$ particles/ μL). The total MP concentration did not differ significantly among the different age groups: 18–30 years ($28,730 \pm 1600$ particles/ μL), 31–40 years ($24,972 \pm 5947$ particles/ μL), and 41–58 years ($25,195 \pm 1727$ particles/ μL). However, the total number of MPs in fresh plasma ($152,110 \pm 46,716$ particles/ μL) was significantly higher ($p < 0.05$) than that in unprocessed whole blood ($26,752 \pm 985$ particles/ μL), fresh packed red blood cells (PRBCs) ($28,574 \pm 1028$ particles/ μL), and platelet concentrate (PC) ($33,072 \pm 1858$ particles/ μL). Furthermore, the total numbers of MPs in stored PRBCs and fresh-frozen plasma (FFP) were significantly higher ($p < 0.05$) than those in fresh PRBCs and fresh plasma, respectively.

Conclusions: The study suggests that donor factors, blood-component processing and storage contribute to the MP concentration in routine blood-product preparation. The findings can improve quality control and management of blood-product manufacturing in routine transfusion laboratories.

1. Introduction

The formation of cell-derived microparticles (MPs) disrupts the interaction between the cytoskeleton and lipid bilayer [1]. Upon apoptosis or cellular activation, MPs can be generated from various cells, including red blood cell-derived (RMPs), platelet-derived MPs (PMPs), leukocyte-derived MPs (LMPs), and endothelial cell-derived MPs (EMPs). Accumulated evidence has demonstrated elevated numbers of MPs in several diseases and pathological conditions [2,3]. Apart from the roles of MPs in disease, studies have documented the clinical importance of MPs in transfusion medicine [4,5]. The procoagulant activity of MPs in stored blood components has also been demonstrated [6]. In addition, recent studies suggested that elevated numbers of RMPs in blood products may be responsible for complications in blood transfusion recipients by modulating the function of immune cells [7,8]

and that transfusions containing high doses of RMPs may be associated with an inflammatory response [9]. Studies also indicated that an interaction between PMPs and leukocytes may be responsible for allergic transfusion reactions [10,11]. Furthermore, research demonstrated that LMPs played a role in endothelial dysfunction by recruiting inflammatory cells to the vascular system, leading to the progression of atherosclerotic lesions [12]. Given the potential effects of MPs in post-transfusion reactions, determining the factors that contribute to MP concentrations in blood products is important to minimize MP concentrations and reduce the risks of transfusion reactions associated with MPs.

Recent studies of factors contributing to increased numbers of MPs in blood components pointed to an association between donor variability and increased numbers of MPs in blood components [13,14]. Different blood components are prepared using different procedures,

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which influence the concentration of MPs in blood products [15–17]. After preparation, some blood components may be stored before being transfused into recipients. The effect of this storage on MP concentrations has been documented. One study found a higher number of MPs in older RBCs than in fresh ones [18]. In a study of MPs in fresh-frozen plasma (FFP), both the preparation protocol and storage time were associated with increased numbers of MPs in FFP [15]. In addition to the aforementioned factors, studies demonstrated that the rate of MP accumulation in RBCs stored in citrate phosphate dextrose (CPD)/saline, adenine, glucose, and mannitol was slower than that in RBCs stored in citrate phosphate dextrose adenine (CPDA) [19]. Despite knowledge of the contribution of these factors to increased numbers of MPs in blood components, the overall effect of these factors on MP concentrations in routine blood-component preparation remains unclear.

In the current study, we compared MP levels in 1) donors of different ages and sex; 2) fresh packed RBC (PRBCs), platelet concentrate (PC), and fresh plasma versus those in unprocessed whole blood; 3) fresh PRBCs versus those in stored PRBCs; and 4) fresh plasma versus those in FFP.

2. Material and methods

Fluorescein-isothiocyanate conjugated annexin V (Annexin V-FITC), phycoerythrin-conjugated CD235a (CD235a-PE), peridinin chlorophyll-conjugated CD45 (CD45-PerCP), allophycocyanin-conjugated CD41a (CD41a-APC), and $10 \times$ annexin V binding buffer were purchased from ImmunoTools (Friesoythe, Germany). CountBright™ counting beads were obtained from Invitrogen (Carlsbad, CA, USA). Blank calibration particles of $1.09 \mu\text{m}$ were purchased from Spherotech (Lake Forest, IL, USA).

2.1. Blood-component preparation and sample collection

The present study was approved by the Institutional Review Board of Siriraj Hospital, Mahidol University School of Medicine, Bangkok, Thailand (COA no.395/2016). Written consent was obtained from blood donors after the procedure had been explained to them in detail, including its benefits and possible hazards. Blood samples were then collected and processed using standard procedures of the Department of Transfusion Medicine, Faculty of Medicine, Siriraj Hospital, Mahidol University. Whole blood samples were collected in tubes containing tripotassium ethylenediaminetetraacetic acid, and complete blood counts (CBCs) were then analyzed using a Coulter AcT 5-part differential (5 diff) (Beckman Coulter, Fullerton, CA, USA). These samples were also used for MP analysis and defined as unprocessed whole blood.

The overall processes of blood component manufacturing and sample collection are summarized in Fig. 1. Units of whole blood were collected into triple blood collecting systems (JMS Triple Blood Bag; CPD-SAGM Solution; JMS Singapore Pte. Ltd., Singapore). Each system included a 450-ml collection bag containing 63 ml of CPD, a 400-ml bag containing 100 ml of SAGM red cell preservative solution, and a 400-ml bag for 5-day platelet storage. All units of whole blood were stored at room temperature ($22 \pm 2^\circ\text{C}$) for up to 8 h before the blood components were prepared. The units of whole blood were centrifuged at $3100 \times g$ for 5 min at $22 \pm 2^\circ\text{C}$ in a centrifuge (Heraeus™ Cryofuge™ 6000i; Thermo Electron LED GmbH, Langenselbold, Germany) and separated into PRBCs and platelet-rich plasma using a manual extractor. The platelet-rich plasma was transferred into a 400-ml bag for platelet storage, centrifuged at $3800 \times g$ for 5 min at $22 \pm 2^\circ\text{C}$, and separated into platelet-poor plasma and PC. After the addition of 100 ml of SAGM, the units of PRBCs were stored at $4 \pm 2^\circ\text{C}$. The plasma units were then transferred into a 400-ml bag, rapid-frozen to -30°C using a shock freezer (TPSU 40; Thalheimer, Ellwangen, Germany or MBF 12; Dometic, Hosingen, Luxembourg) and stored at $-30 \pm 10^\circ\text{C}$. The FFP was thawed in a plastic bag in a water bath (Memmert Waterbath WPE45;

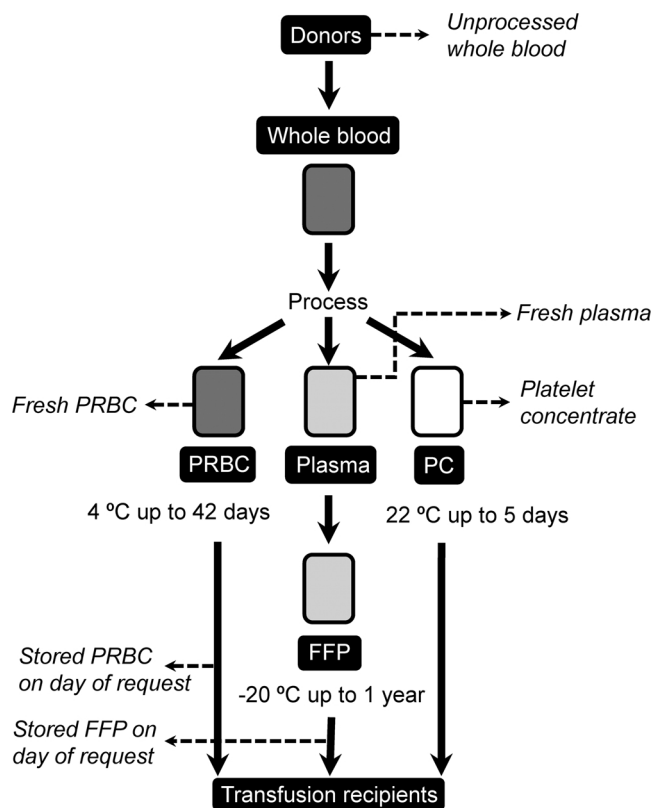


Fig. 1. Schematic diagram showing blood component processing (solid arrows) and sample collection (dotted arrows).

Memmert GmbH + Co. KG, Germany) or a plasma thawer (W-PFD® Plasma Fast Thawer; KW Apparecchi Scientifici, Italy) for 20–30 min at 37°C . The samples were processed to quantitate MPs within 2 h after finishing the preparation process and before sending each blood component to its recipient for transfusion.

PRBC, plasma, platelet concentrate, and FFP samples were taken from heat-sealed segments after thorough mixing of the bag, followed by stripping of the sampling pipe. The content of each segment was immediately transferred into a 0.5 mL microcentrifuge tube and carefully mixed before determination of the MP concentration.

2.2. Flow cytometry quantitation of MPs

The whole blood samples, PRBCs, and PC were diluted with phosphate-buffered saline (1:100). Undiluted FFP samples were used for MP analysis. For analysis, $5 \mu\text{L}$ of each sample was incubated with $3 \mu\text{L}$ each of annexin V-FITC, CD45-PerCP, and CD41a-APC; $2 \mu\text{L}$ of CD235a-PE; and $20 \mu\text{L}$ of $1 \times$ annexin V binding buffer. After 15 min, $300 \mu\text{L}$ of $1 \times$ annexin V binding buffer was added to the tubes. The samples were analyzed immediately using an FACSCalibur flow cytometer (BD, San Jose, CA, USA) equipped with two lasers: a 488 nm (blue) and 635 nm (red).

The MP gate was set on the dot-plot of forward scatter versus the side scatter (SSC) according to $1\text{-}\mu\text{m}$ standard beads. The SSC versus the annexin dot-plot was used to identify the total number of annexin V-positive MPs (Fig. 2). Then, the events of RMPs, PMPs and LMPs from the previous dated annexin V-positive MPs were determined on a histogram plot of CD235a-PE, CD41a-APC, and CD45-PerCP, respectively. The MP concentrations were quantitated as described previously [20]. The consistency of the flow rate was monitored throughout the acquisition period. Prior to a dilution experiment, the performance of the flow cytometer was optimized using CaliBRITE beads (BD, San Jose, CA, USA) and FACSComp software, version 5.1. The dilution

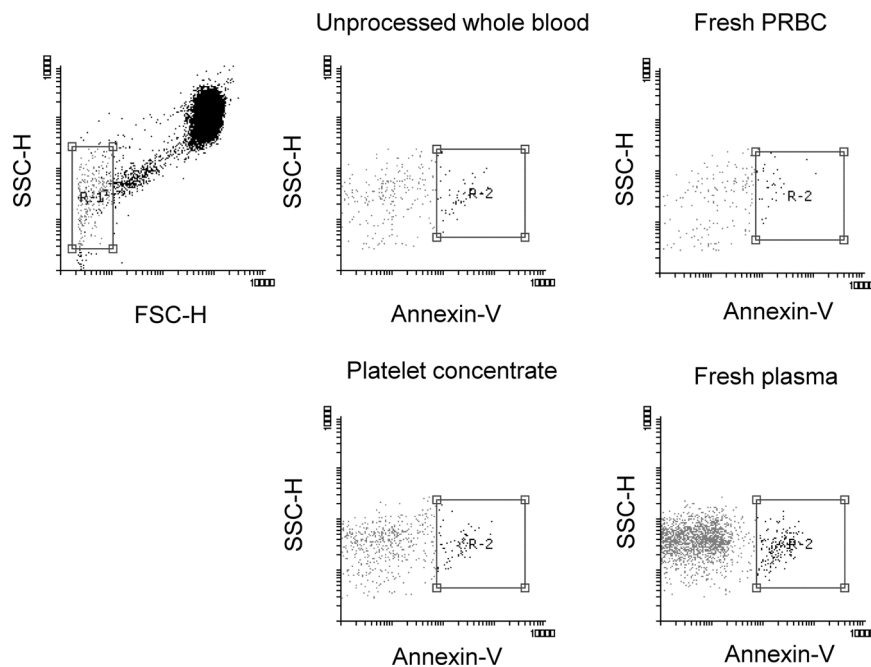


Fig. 2. MP gating in unprocessed whole blood, PRBC, platelet concentrate and fresh plasma.

experiment showed linearity in the range of $225\text{--}2.4 \times 10^6$ particles/ μL .

2.3. Heterogeneity analysis

The averages of total MPs, RMPs, PMPs, and LMPs in unprocessed whole blood were quantified. The fold-change of each parameter was then calculated by dividing each measurement by the group's mean and measurements with fold-changes of more than 2 were identified.

2.4. Statistical analysis

The data analysis and graphing were performed using GraphPad Prism software, version 5.0 (GraphPad, San Diego, CA, USA). The results of MP quantitation were expressed as mean and standard error. Linear regression was used to calculate the correlation coefficients (r^2). The Mann–Whitney U test was conducted to determine the mean difference between the groups. A value of $p \leq 0.05$ was considered statistically significant.

3. Results

3.1. Donor characteristics and hematologic data

Blood samples were collected from 150 donors. After blood collection, blood products, including PRBC, PC, plasma, and FFP were prepared. Blood products from 24 donors did not pass the quality control criteria after preparation and were not therefore sent to the intended recipients. Thus, 126 samples were included in the final analysis. The mean age of the 126 donors was 34 years (range: 18–58 years), and the ratio of males to females was 1.3:1 (72/54). The mean and standard deviation of RBCs, white blood cells, and platelets were $5 \times 10^6 \pm 5 \times 10^5$ cells/ μL , 7143 ± 1491 cells/ μL , and $253,000 \pm 65,180$ cells/ μL , respectively, and the value for the mean platelet volume was 9 ± 0.9 fL. The RBC indices, including hemoglobin (g/dL), hematocrit (%), mean corpuscular volume (fL), mean corpuscular hemoglobin (pg), mean corpuscular hemoglobin concentration (g/dL), and RBC distribution width, were 13.8 ± 1.2 , 42.4 ± 3.3 , 84.8 ± 7.1 , 27.7 ± 2.6 , 32.6 ± 1 , and 13.8 ± 1.1 ,

respectively.

3.2. Effects of donor sex and age on MP concentrations and heterogeneity in unprocessed whole blood

First, we determined whether donor sex and age affected MP concentrations. The results revealed no significant difference in total MPs between male and female donors ($26,044 \pm 1254$ vs. $27,696 \pm 1584$ particles/ μL) and no significant differences in total MPs among donor age groups: 18–30 ($n = 57$; $28,730 \pm 1600$ particles/ μL), 31–40 ($n = 24$; $24,972 \pm 1214$ particles/ μL), and 41–58 ($n = 45$; $25,195 \pm 1727$ particles/ μL).

We next examined MP heterogeneity. The total concentrations of MPs, RMPs, PMPs and LMPs in unprocessed whole blood and other blood products are summarized in Table 1. For unprocessed whole blood, the fold-changes of each parameter were then calculated, as described in the Materials and methods. The results are shown in Fig. 3. Regarding total MPs, 77 measurements showed a fold-change of less than 1, and 4 showed fold-changes greater than 2. Regarding RMPs, 50 measurements showed a fold-change of less than 1, and 1 showed a fold-change greater than 2. For PMPs, 81 measurements showed a fold-change of less than 1, and 7 showed fold-changes greater than 2. For LMPs, 64 measurements showed a fold-change of less than 1, and 27 showed fold-changes greater than 2. Our regression analysis of the RMP concentration in unprocessed whole blood and in PRBC showed r^2 of 0.01, $p = 0.13$ (Fig. 4). However, a trend of positive association was demonstrated when compared the PMP concentrations in unprocessed whole blood with those in platelet concentrate with r^2 of 0.06, $p = 0.0049$.

3.3. MP levels in fresh PRBCs, PC, fresh plasma, and unprocessed whole blood

To examine the impact of blood-component preparation procedures on MP concentrations, we compared the total concentrations of MPs, RMPs, PMPs, and LMPs in fresh PRBCs, PC, and fresh plasma with those in unprocessed whole blood. The results demonstrated that the total concentration of MPs in fresh plasma was significantly higher than that in unprocessed whole blood, fresh PRBCs, and PC, as shown in Table 1.

Table 1
Total MP, RMP, PMP and LMP concentration in different blood components.

	Total MPs (particles/μL)	RMPs (particles/μL)	PMPs (particles/μL)	LMPs (particles/μL)
Unprocessed whole blood	26,752 ± 985 (6,930–86,667)	12,291 ± 385 (3,689–29,190)	11,086 ± 518 (640–36,960)	276 ± 32 (0–1,680)
Fresh PRBC	28,574 ± 1028 (2,520–107,169)	18,049 ± 597 (1,890–42,004)	6790 ± 357 (610–27,907)	209 ± 28 (0–2,014)
Platelet concentrate	33,072 ± 1858 (5,460–139,153)	9546 ± 264 (3,081–19,855)	14,065 ± 695 (3,840–56,324)	278 ± 28 (0–1,561)
Fresh plasma	152,110 ± 46,710 (18,900–5.9 × 10 ⁶)	11,104 ± 488 (3,462–31,342)	91,839 ± 29,375 (10,827–3.7 × 10 ⁶)	243 ± 32 (0–2,033)
Stored PRBC	72,110 ± 2973 (22,767–165,326)*	49,627 ± 2375 (13,033–127,785)	16,842 ± 1118 (3,6–5-75,106)*	319 ± 48 (0–3,574)
FFP	2.3 × 10 ⁶ ± 147,945 (281,820–1.4 × 10 ⁷)	56,344 ± 17,826 (3,359–1.5 × 10 ⁶)	2.1 × 10 ⁶ ± 117,953 (233,520–8.9 × 10 ⁶)	489 ± 49 (0–3,024)

Data represent mean ± standard error (min – max).

* indicates significant difference of MP concentration between unprocessed whole blood and stored PRBC unit.

The RMP concentration in fresh PRBCs was significantly higher than that in unprocessed whole blood, PC, and fresh plasma. For PMPs, the concentrations in fresh plasma were significantly higher than those in unprocessed whole blood, PRBCs, and PC. In contrast, there was no significant difference in the concentration of LMPs in unprocessed whole blood, fresh PRBCs, PC, and plasma.

3.4. MP levels in stored PRBCs and FFP

The PRBCs and FFP used in this study had been previously prepared and stored according to routine procedures at the Department of Transfusion Medicine, the Faculty of Medicine, Siriraj Hospital, Mahidol University. The number and origin of MPs in the stored PRBCs and FFP at various time points were quantitated and then compared with those in fresh PRBCs and fresh plasma.

The results demonstrated that the total MP concentration in stored PRBCs was significantly higher than that in fresh PRBC (Table 1). The concentrations of RMPs and PMPs in stored PRBCs were significantly

higher than those in fresh PRBCs. The number of LMPs in stored PRBCs was higher than that in fresh PRBCs, but the difference was not statistically significant. For stored PRBCs, the data were categorized according to the storage duration to determine whether the increase in MPs in stored PRBCs correlated with the length of storage. After the first 10 days of storage, the total MP concentration was 46,242 ± 3783 particles/μL (Fig. 5). Over the next 10 days, the total MP concentration increased to 52,823 ± 3569 particles/μL. The highest concentration of MPs was recorded after 41–42 days of storage, with the average being 115,089 ± 10,438 particles/μL, suggesting that MP concentrations in PRBCs increased over time.

The results demonstrated that the total concentration of MPs in stored FFP was significantly higher than that in fresh plasma, as shown in Table 1. The concentrations of RMPs, PMPs, and LMPs were significantly higher in stored FFP than in fresh plasma. Finally, comparison of the total MP concentration in stored FFP after various storage lengths showed that it was 2.4 × 10⁶ ± 1.8 × 10⁵ particles/μL after the first 10 days of storage (Fig. 6), 2.9 × 10⁶ ± 882,913 particles/μL after

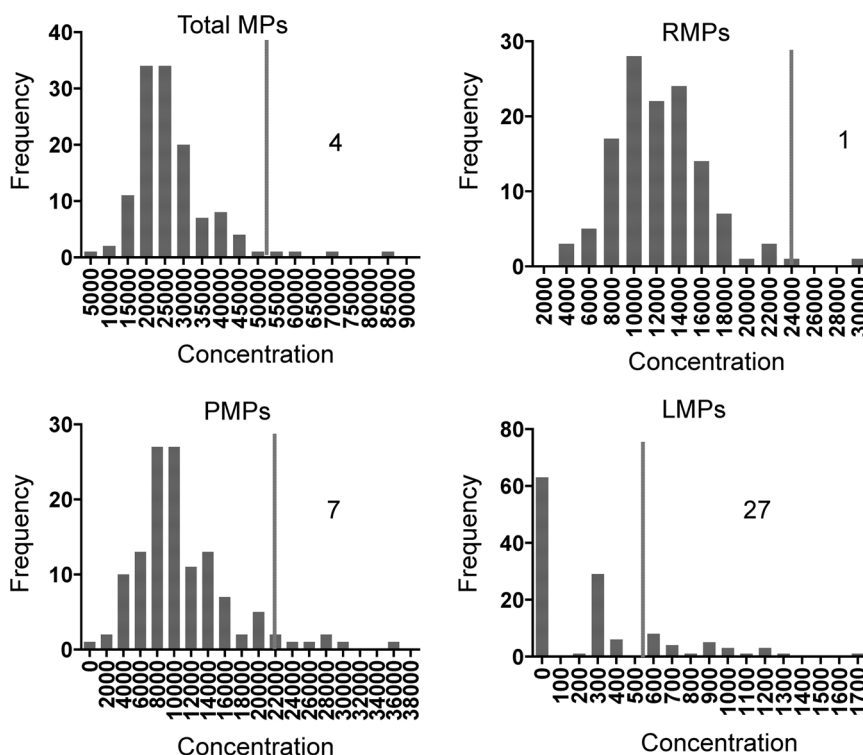


Fig. 3. Frequency plots demonstrating the total MP, RMP, PMP, and LMP concentrations in unprocessed whole blood. The dotted line indicates fold-changes of 2.

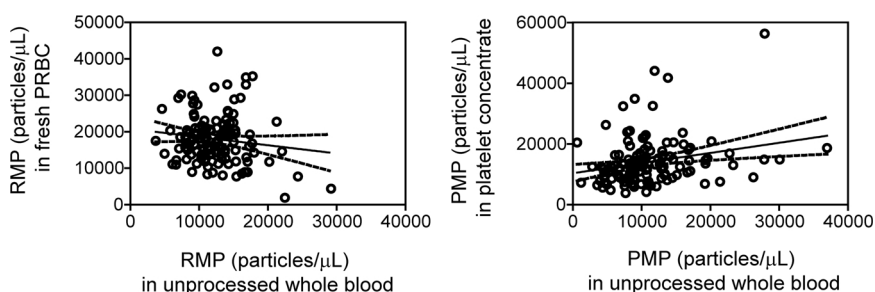


Fig. 4. Linear regression analysis of RMP concentration in unprocessed whole blood vs. in PRBC, and PMP concentration in unprocessed whole blood vs. in PC.

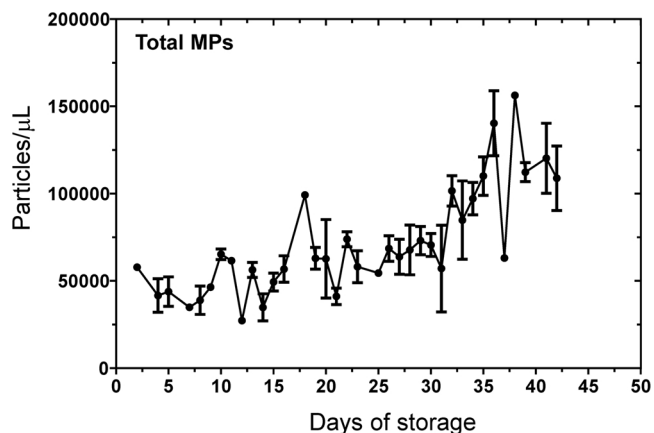


Fig. 5. A line plot showing the total MP concentration in PRBC products after various days of storage.

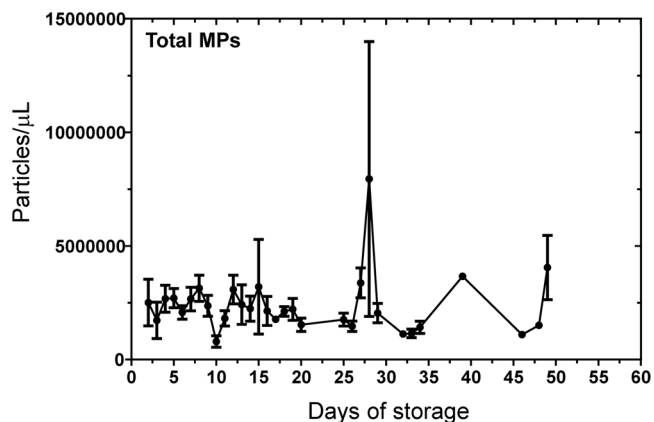


Fig. 6. A line plot showing the total MP concentration in FFP products after various days of storage.

the next 10 days of storage, $1.5 \times 10^6 \pm 293,805$ particles/ μL after 31–40 days of storage, and $2.9 \times 10^6 \pm 1 \times 10^6$ particles/ μL after 41–50 days of storage. This finding suggested that the concentration of MPs in FFP was time independent.

4. Discussion

Accumulated evidence suggests that a number of factors are associated with increases in the number of MPs in blood components [15–17]. However, no investigation has elucidated the effects of these factors on the concentration of MPs in routine laboratories. Thus, the current study aimed to evaluate the overall effects of donor age and sex, blood-component processing, and storage on the concentration of MPs during routine blood-product preparation. The results suggested that donor factors, blood processing and storage in routine laboratories had

an effect on the MP concentration in routine blood component preparation.

Although recent investigations demonstrated effects of donor sex and age on the recovery and biochemistry of RBCs and on transfusion outcomes [13,14,21–23], the results of the present study revealed no difference in the total MP concentration of male and female donors and no difference related to the age of the donors. The difference in these findings may be caused by differences in donors’ activity levels and genetic backgrounds. For example, a previous study reported that MP concentrations increased in donors following moderate exercise [24]. The quality of the blood product was also associated with the donor’s genetic background, including a mutation affecting the RBC membrane protein and glutathione content [14]. Although, we found no impact of donor age and donor sex on MP concentration, our study suggests the effects of other donor factors or donor variability on the MP concentration. This suggestion was supported by our findings of the different MP concentration in unprocessed whole blood and a trend of positive association of the PMP concentration in unprocessed whole blood and the platelet concentrate. These donor factors may be the presence of underlying conditions or level of regular activity. Long-term monitoring of these donors is required to determine whether the increase in MP concentrations is a transient phenomenon.

In the present study, the total numbers of MPs and PMPs were increased in fresh plasma as compared with those in unprocessed whole blood and other components, pointing to an effect of blood-component processing (i.e., centrifugation) on the plasma MP concentration [16]. Upon activation, platelets show increased expression of CD62P and phospholipid phosphatidylserine on their surfaces, and PMPs are released into plasma. Increased numbers of PMPs remain in plasma due to the low speed of centrifugation used in routine processing. This idea is supported by a recent study, which showed that centrifugation at a speed of 20,000 g precipitated PMPs in whole blood samples [25].

Contamination is an important factor to consider when manufacturing plasma products [26]. The increase in the number of MPs in plasma in the present study may be linked to blood product preparation processes. A previous study showing the presence of procoagulant MPs in leukoreduced plasma units provides support for this idea and these MPs could be removed by filtration [27]. Therefore, specific processes may be needed to decrease the MP levels during routine preparation of plasma in transfusion laboratories.

The results of the present study also demonstrated that the MP concentration in stored PRBCs was time dependent, presumably due to changes in biochemical properties, including energy metabolism, redox status, and membrane integrity, in stored PRBCs [28]. Furthermore, recent studies demonstrated that transfusion-induced inflammation and oxidative stress were correlated with long, as opposed to short, storage of PRBCs [9]. Another study demonstrated that stored RBCs were susceptible to generating RMPs both *in vivo* and *in vitro* [29]. In addition, transfusion with PRBCs containing increased numbers of MPs was associated with post-transfusion complications [7,8].

The time dependence of the MP concentration raises several points regarding PRBCs in the transfusion laboratory. For example, in addition to measuring traditional hematologic parameters, quantifying the levels

of MPs in stored PRBC blood products may be essential to maintain the integrity of PRBC components. Given the effect of storage on MP concentrations, optimal preservation strategies and additive solutions are needed to minimize MP concentrations in stored PRBCs [19].

In addition, data on MP levels may be used to evaluate the integrity of RBCs in stored PRBCs. After releasing MPs, RBCs lose their surface membranes, and their integrity is reduced [29], decreasing their chance of survival [30]. Hence, transfusing PRBCs that contain an increased number of MPs may affect a patient's recovery. Further studies should be conducted to examine this possibility, as few studies establishing *in vivo* activity of RMPs have been conducted.

Considering that the levels of MPs present in FFP can affect the product's therapeutic efficacy, quantitation of MPs in FFP is necessary. The results of the current study demonstrated that the magnitude of the increase in MPs was higher in stored FFP than in stored PRBCs. Another interesting finding was that MP concentrations in stored FFP were augmented more than 10 times as compared with those in fresh plasma. This finding could be due to the higher baseline number of MPs in plasma than in PRBCs, which has been documented in previous research [27]. The process of freezing and thawing FFP may further increase the number of MPs in stored FFP [16,31]. In the present study, the finding that the increased number of MPs in FFP was time independent was unexpected. Previous studies of the number of MPs in stored FFP reported greater increases after long-term than short-term storage [15]. The discord in the results may be explained by differences in the freezing and thawing procedures, baseline levels of MPs in the plasma, or short storage times used in the present study.

Overall, the results of the present study suggested that donor factors, blood-component processing and storage contributed to the MP concentration during blood-component preparation in routine transfusion laboratories. Understanding the relationship between these factors is important for quality control during blood-component preparation in these laboratories.

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