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Research article

The *in vitro* red blood cell microvesiculation exerts procoagulant activity of blood cell storage in Southeast Asian ovalocytosis

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ABSTRACT

Southeast Asian ovalocytosis (SAO) is characterized by the misfolding of band 3 protein in red blood cells (RBC). The abnormal structure of the band 3 protein results in dysmorphic RBC and related functions. Previous data showed that *in vitro* storage under hypothermic conditions alters band 3 protein structure and function. Microvesiculation includes shedding of RBC membranes, called RBC-derived microparticles/extracellular vesicles (RMP/EVs), and storage lesions. Unfortunately, there is no evidence of RBC microvesiculation under *in vitro* storage conditions in heterozygous SAO individuals. This study determined the generation of REVs and procoagulant activity during the storage of SAO blood samples in southern Thailand. Venous blood was collected from eight SAO and seven healthy individuals, preserved in citrate phosphate dextrose-adenine 1 (CPDA-1) at 4 °C for 35 days. The absolute numbers of REVs and PS-expressing RBCs were analyzed using flow cytometry. The procoagulant activity of the produced extracellular vesicles was determined by a clotting time assay. The results showed a significant increase in the number of REVs and PS-expressing RBCs in the SAO blood samples. Significantly correlated PS externalization and procoagulant activity were observed in the SAO blood samples. These lines of evidence indicate that the abnormality of the Band 3 protein is possibly involved in aberrant microvesiculation, exerting procoagulant activity *in vitro*. Increased pools of REV production and abnormal storage lesions in SAO blood samples should be a concern. Notably, the mechanisms underlying membrane vesiculation depend on the extent of blood cell storage under hypothermic conditions.

1. Introduction

Human red blood cells (RBCs) play a pivotal role in delivering oxygen to peripheral tissues. The RBC is a biconcave cell with a

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diameter of 7 μm. The cell lacks a nucleus and has a flexible plasma membrane, characterized by phospholipid bilayers embedded with integral membrane proteins. Band 3 is the major constituent integral membrane protein that accounts for 60%–80% of total RBC membrane proteins [1]. The misfolding of the Band 3 protein facilitates dysmorphic RBC, which has been observed in some hematological disorders such as hereditary spherocytosis (HS), hereditary elliptocytosis (HE), or hereditary pyropoikilocytosis (HPP) [2]. Southeast Asian ovalocytosis (SAO) is a congenital disease caused by the deletion of *SLC4A1* (formerly *AE1*). This gene encodes the Band 3 protein [1,3]. The deletion of 27 base pairs results in the deletion of 9 amino acids at position 400–408, which affects the RBC membrane and cell structure. The dysmorphic shape of RBC in SAO is demonstrated by an oval shape, which accounts for more than 50% of the total RBC population. Dysmorphic RBC in SAO have shown benefits in preventing the entry of *Plasmodium vivax,* with no exact mechanisms [4]. A high prevalence of SAO has been reported in Malaysia, Papua New Guinea, Philippines, and other parts of Southeast Asia. In Thailand, the prevalence is prominent in the lower part of the southern region [5]. Although abnormal RBC shapes were predominantly observed in heterozygous SAO individuals, hemolysis and hyperbilirubinemia were observed during the first three years after birth. Interestingly, the hemolytic crisis resolves without any specific medication. Adult individuals with SAO demonstrate subclinical or no severe clinical signs or symptoms [6–8].

Previous data have shown that several defects in RBC physiology and dysmorphic RBC facilitate membrane vesiculation. The release of small membrane vesicles to the extracellular spaces is generally termed as extracellular vesicle (EV) generation. EV is formerly known as microparticle or exosomes which had been used interchangeably. The revised nomenclature, definitions and categorizations of EVs have been endorsed in the field by the International Society for Extracellular Vesicles (ISEV). Recent guidelines of EV nomenclature following Minimal information for studies of extracellular vesicles 2018 (MISEV 2018) suggested that EV is a general term for particles that are naturally released from the cells. These particles are composed of lipid bilayer without replicability. EV subpopulations are diverse based on selected criteria. For instance, the size of EV delineated EVs into small EVs, medium EVs and large EVs with size ranging from $<$100 nm, $<$200 nm and $>$200 nm, respectively. Furthermore, using biological properties of EVs membranes give rise to more relevant details of EVs. For example, the combination of phosphatidylserine-expressing particles with surrogate biomarkers of cell types enables the characterization of EV heterogeneity.

The implication of EV generation has become increasingly interesting in many fields, such as the role of EVs in the metastasis of some cancers, inflammatory responses and thrombiembolism. However, the distinct mechanisms of EV biogenesis vary among the cell types and disease settings.

RBCs constitute a significant part of the blood components in blood circulation. Outward blebbing and shedding of RBC membranes have been observed during the genesis of RBC-derived extracellular vesicles (REVs), under both normal and pathological conditions [9]. For instance, increased shedding of the small sizes of phospholipid-containing particles has been reported in thalassemia patients [10–12] and RBC membrane disorders, such as hereditary spherocytosis, hereditary elliptocytosis, and G-6PD deficiency [9, 13].

Interestingly, accumulating data have shown that the increased osmotic fragility testing (OF test) in SAO blood samples suggests the fragility and deformability of the RBC membrane among these groups [14]. The aberrant features of membrane vesiculation are possibly associated with intrinsic defects among heterozygous SAO individuals with the misfolded Band 3 protein.

Band 3 is an important membrane protein that is linked to spectrin, which is the cytoskeleton of the RBC membrane. Aggregation of Band 3 protein was observed in stored RBCs, which contributes to the disruption of cytoskeletal proteins and the release of membrane vesicles [15,16].

The *in vitro* storage of RBCs has been associated with increased membrane vesiculation. Although additive solutions have been extensively developed, the longer storage time of RBC in blood banking procedures is still a concern for membrane vesiculation. Increased REV during extended RBC storage has been reported in normal RBCs [17,18]. However, the mechanism of REV generation has been uniquely described, particularly in aged RBCs that are preserved in specific settings. The proposed consequences of storage lesions initiated by the depletion of ATP. Thereafter, the accumulation of reactive oxygen species (ROS) facilitates disruption of the cytoskeleton and PS exposure [19]. In addition, PS exposure and cation alterations coincided with changes in RBC morphology and lesions upon storage. Burger et al. suggested that lower intracellular potassium levels during RBC storage impair flippase activity and facilitate PS exposure [19–22].

The nature of phosphatidylserine (PS)-expressing particles, which potentially act as initiators of thrombus formation, raises the importance of data on EV generation either *in vivo* or *in vitro* [23]. The thrombogenicity of PS-expressing MPs is characterized by an increased negative charge on their surface. Laboratory-based evidence has shown that REV and storage lesions induce coagulopathy and proinflammatory activity. However, transfusion of REV induced thrombotic events in a murine experimental model [24]. Several studies have indicated the risk of elevated accumulation of MPs in blood products, particularly packed RBCs [25]. One study has shown that irreversibly dysmorphic RBCs, upon long preservation, were rapidly cleared after transfusion [26]. Unfortunately, there is no direct evidence of coagulopathy induced by the transfusion of REV or aged RBC in a clinical study.

In Thailand, some individuals with hematologic disorders with normal hematocrit and hemoglobin levels are eligible for blood donation. For example, blood donors with thalassemia or G6-PD deficiency have been studied for MP production from blood products [12]. A higher prevalence of SAO was reported in the lower southern part of the country, particularly in the area of the Thai-Malaysia border [5] (article in Thai). This improves the chance of obtaining blood donations by these donors. Whereas the processing of blood products is routinely performed under hypothermic conditions and specific preservatives, there is a lack of evidence of MP/MV generation following storage of RBCs among these groups. This study investigated the characteristics of *in vitro* RBC microvesiculation and storage lesions in asymptomatic subjects with SAO who are eligible and potential candidates for blood donation.

2. Materials and methods

2.1. Subject recruitment and ethical consideration

This cross-sectional study was conducted from October 2020 to March 2021 in Tha Sala District, Nakhon Si Thammarat Province, Thailand. This study was approved by the Human Research Ethics Committee of Walailak University (WUEC-20-249-01). Informed consent was obtained and recruitment procedures were performed before the commencement of the study.

The volunteers were screened for SAO using a blood smear. Subjects with SAO were recruited based on the presence of ovalocytosis and theta cells (RBCs with two stomas showing a transverse bar/slit in the middle of the cells) on blood smear examination and an age range of 18–60 years. Previous reports showed that some RBC membrane defects (such as spherocytosis and elliptocytosis) and hematological disorders (e.g., thalassemia and hemoglobinopathies) exert hypercoagulable state [27,28]. Therefore, these anomalies were excluded to prevent the interference of procoagulant activity. Finally, eight individuals with confirmed SAO and age- and sex-matched healthy controls were included in the study.

2.2. Complete blood count (CBC) and PCR confirmation of SAO

EDTA blood was collected for CBC testing using Sysmex Xn 1000i (Sysmex Corporation, Kobe, Japan). A blood smear was prepared using the Wright stain at the Hematological Laboratory, School of Allied Health Sciences, Walailak University. The buffy coat was collected for DNA purification using a commercial kit (Gene Aid) (Geneaid Biotech, New Taipei City, Taiwan). PCR was performed as previously described [6] with some modifications.

A deletion of 27 bp in the *SLC4A1* gene (accession number NP_000333.1) was amplified using the forward primer 5' GGG CCC AGA TGA CCC TCT GC 3' and the reverse primer 5' GCC GAA GGT GAT GGC GGG TG 3'. The PCR cycling consisted of an initial denaturation at 95 °C for 15 min, 30 cycles of denaturation at 95 °C for 1 min, annealing at 65 °C for 1 min, extension at 72 °C for 1 min, and a final extension at 72 °C for 9 min. The PCR products were separated by 1% agarose gel electrophoresis and visualized by staining with ethidium bromide. The expected sizes of the amplified products were 175 and 148 bp for the normal and mutant Band 3 genes, respectively.

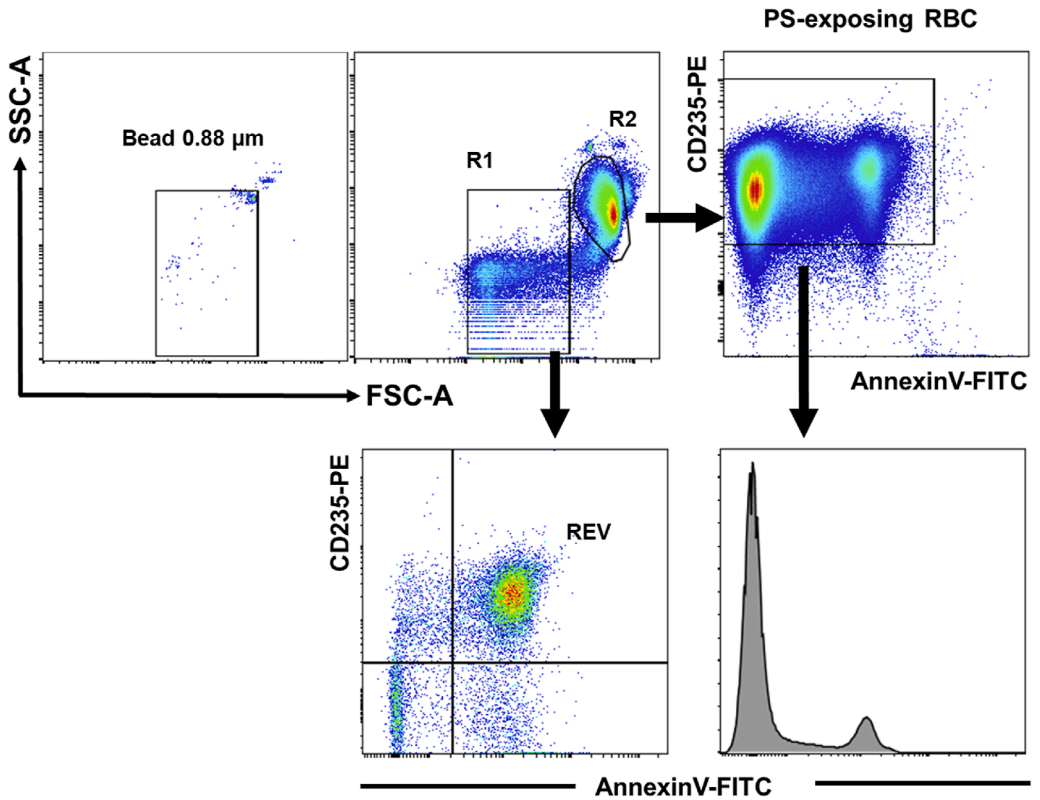

Fig. 1. Gating strategies used for REV enumeration; The boundary of the extracellular vesicles gate (EV gate) was created using standard bead size = 0.88 µm and designated as R1. EV's acquired in R1 were analyzed for RBC-derived EV (REV) by CD235-PE+/AnnexinV-FITC+. The events of REVs were obtained for absolute REV calculation. The PS-bearing RBC was gated as shown in R2 and then gated for the expression of CD235-PE+. The expression of phosphatidylserine on the RBC membrane was determined by mean fluorescence intensities (MFIs).

2.3. Citrate phosphate dextrose-adenine 1 (CPDA-1) preparation, blood collection, and storage conditions

The anticoagulant was prepared as follows: citric acid 188 mg, sodium citrate 1.45 g, sodium phosphate 121.5 mg, dextrose 1.82 g, and adenine at 17.3 mg (Sigma-Aldrich, MO, USA) were dissolved in 63 mL of distilled water. The anticoagulant was kept sterile and filtered with a 0.2 μM filter before use. Blood was drawn to a volume of 10 mL with an aseptic technique and then gently mixed with 1.4 mL of CPDA-1 solution. The CPDA-1-preserved blood was centrifuged at 1200 rpm for 10 min. The buffy coat and plasma were then removed. Packed RBCs were stored at 4 °C and assayed for REV- and PS-externalization.

2.4. Flow cytometry and REV quantification

REVs were determined using flow cytometry at the indicated time points. All monoclonal antibodies and staining buffers were purchased from Biolegend (CA, USA). The staining protocol for REVs was modified from previous studies [29,33]. Five microliters of 1:100 diluted CPDA-1 preserved RBC was stained with 2 μL of anti-CD235-PE and 2 μL of anti-annexin V, as RBC markers and phosphatidylserine staining, respectively. 1X Annexin V binding buffer at 41 μL were added mixed, and incubated for 15 min at 25 °C. The stained REVs were resuspended in 350 μL of 1X annexin V binding buffer to obtain a final volume of 400 μL. The absolute number of REV was measured using Count Bright beads (Molecular Probes, CA, USA). Following the manufacturer's instructions, 50 μL of well-mixed count bright beads was added to the stained mixture using the reverse pipetting technique, mixed, and then subjected to flow cytometry using a BD FACSVerse flow cytometer (BD Biosciences, San Jose, CA, USA).

The EV gate was created using standard beads of 0.88 μm (Spherotech, IL, USA). The characteristics of MP were determined by the expression of phosphatidylserine in the cell membrane stained with anti-annexin V FITC. The EV gate was then analyzed for the expression of CD 235, which is an RBC marker (Fig. 1). The number of AnnexinV+/CD235+ cells was counted. A stopping gate was established for the acquisition of 1000 counting bead events. The absolute number of REV was calculated according to the manufacturer's instructions. Briefly, REV (particle/μL) was obtained by multiplying the number of REV counts per acquired bead count by the pre-calculated bead number per resuspension volume. The REV was enumerated at the indicated time points using the same tube of CPDA-1-preserved blood. Since previous evidence showed that bacterial infection and proinflammatory cytokines associated with increased EVs.

Since the increased EVs have been reported in association with elevated proinflammatory cytokines and bacterial burden [30,31]. Therefore, the blood samples were obtained with aseptic techniques. The sterilization test was checked by cultivation on tryptic soy agar (TSA) (HiMedia, Mumbai, India) to exclude the possibility of bacterial contamination during blood preservation and possibility of false-elevated numbers of extracellular vesicles.

2.5. Analysis of phosphatidylserine-expressing RBC

PS-expressing RBCs obtained at the indicated time points were compared between the SAO and healthy subjects. RBCs were gated by FSC/SSC followed by CD235+. The expression of annexin V on RBCs were determined by comparing the mean fluorescence intensities (MFIs). The absolute number of PS-expressing RBCs was calculated as described in the REV enumeration method for gated RBCs.

2.6. Clotting time assay

Procoagulant activity related to the REV numbers and PS-expressing RBCs was determined by clotting time assay following the study by Hashemi et al. [32] with some modifications. CPDA-1 treated blood was prepared from SAO individuals at a ratio as described above. The platelets were collected by centrifugation at 900 rpm for 10 min at room temperature and the plasma was kept sterilized at 4 °C until analysis. The procogulant activity of the generated REV was determined. REVs obtained by two sources of stored-blood samples including plasma and diluted RBC. The RBC concentrates (500 μL) were centrifuged at 2000 rpm for 10 min. The plasma at 50 μL was mixed with 50 μL normal plasma to constitute REV-derived plasma. While the PS-expressing RBCs were diluted with PBS at a ratio of 1:20. REV-derived plasma and diluted RBC were mix with 50 μL of pooled normal plasma at a ratio of 1:1, incubated in a water bath at 37 °C for 2 min. The clotting time was recorded from the addition of pre-warmed 25 mmol/l Ca_2Cl to the mixture until clot formation was observed. The prothrombotic activity of PS-expressing RBC was investigated in stored RBCs. Pack RBCs (5 μL) were gently mixed with 95 μL autologous plasma. The diluted RBCs were mixed with 50 μL of plasma obtained from healthy volunteers. The clotting time assay was performed as described above and compared with blood samples from stored RBCs obtained from healthy volunteers. The aged sample obtained by storing the blood samples at ambient temperature for 24 h was used as a positive control for procoagulant activity.

2.7. Statistics analysis

The mean of duplicated REV numbers and PS-expressing RBC were compared at the indicated time points using a paired *t*-test. The mean RBC indices and hematologic parameters among SAO blood samples were compared between days 0 and 35 of blood preservation using the paired *t*-test.

The significance differences of mean REV numbers, PS-expressing RBC, and MFI at indicated time points were compared using the Mann-Whitney *U* test. The correlation between REV number, PS-expressing RBC, and procoagulant activity was calculated using the

Spearman rank test. All statistical analyses were performed using the GraphPad Prism software (version 7) (GraphPad Software, San Diego, CA, USA).

3. Result

3.1. Patient demographics and control

The recruitment of SAO patients in the study showed that 8 of the 236 volunteers (3.36%) had ovalocytosis with theta cells in the blood smear preparation. *SLCA41* gene deletion in the screened SAO was confirmed using PCR (Fig. S1). Complete blood count was used to determine the WBC count, WBC differential count, platelet count, and RBC parameters. RBC indices, including mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and red blood cell distribution width (RDW) were compared between patients with SAO and healthy volunteers. The results showed significantly increased MCHC (32.9 ± 1.0 Vs 36.0 ± 0.8), RDW (12.8 ± 1.0 Vs 14.6 ± 0.8), and WBC counts (6.2 ± 0.7 Vs 8.3 ± 2.0) among SAO subjects compared to those in healthy volunteers, respectively. (Table 1).

3.2. Changes in RBC indices and quantification of REV in SAO and healthy control

Under *in vitro* storage conditions, plasma membranes of blood cells undergo degradation, resulting in the generation of MPs. Previous data showed that elevated levels of platelet-derived microparticles (PMPs) were predominantly observed during blood cell storage [33]. However, our study determined the association of the misfolded Band 3 protein in RBCs of individuals with SAO. Therefore, the REV numbers in stored RBCs were investigated using monoclonal antibodies specific to annexin V and CD235, surrogate markers of MPs and RBC. Flow cytometry was used to obtain REV numbers at the indicated time points and to compare the blood samples obtained from SAO and healthy volunteers.

A recent study revealed that *in vitro* storage of RBCs altered RBC indices and morphology [34]. Our results indicate that RBC parameters obtained by day 35 of SAO blood storage showed a significantly decreased RBC count, hemoglobin, and hematocrit. Hemolysis was observed at this time point. Conversely, significant increases in MCV, MCH, MCHC, and RDW were observed (Table 2).

3.2.1. REV generation in vitro of SAO and healthy volunteers

Compared to the initial preservation (day 0 of blood preservation), REV levels increased significantly on day 21 of blood preservation in healthy controls. In contrast, this significance was observed 14 days earlier in preserved-RBC samples obtained from patients with SAO (Fig. 2a–b). The comparison of REV numbers between SAO and healthy blood samples showed a significant increase in REV numbers from day 7 to day 35 of preservation (Fig. 2c). The elevation pattern in REV numbers suggested that *in vitro* storage conditions facilitated increased REV generation in a time-dependent manner.

3.2.2. Baseline REV numbers in SAO and healthy volunteers

The REV numbers were quantified and compared between SAO subjects and healthy controls on the first day of blood preservation. The result showed that the REV numbers were not significantly different between the two groups ($p = 0.396$) (11,220 ± 4804.50 particles/μL and 9676.54 ± 1988.97 particles/μL, respectively).

3.3. The storage lesions on CPDA-1 preserved RBC

The shedding of REV in blood samples facilitates membrane lesions in RBC. These lesions were considered part of the

Table 1
Hematological parameters of the enrolled subjects (mean ± SD).

Parameters	Healthy volunteers	SAO
Sex (M/F)	(3/4)	(3/5)
Age	25 ± 2.1	27 ± 3.4
RBC (10^6/μL)	4.8 ± 0.3	4.8 ± 0.5
Hb (g/dL)	13.5 ± 0.8	14.2 ± 1.4
Hct (%)	41.1 ± 1.4	40.4 ± 3.5
MCV (fL)	84.5 ± 6.4	82.6 ± 3.5
MCH (pg)	28.8 ± 2.4	29.6 ± 1.6
MCHC (%)	32.9 ± 1.0	36.0 ± 0.8**
RDW	12.8 ± 1.0	14.6 ± 0.8**
WBC (10^3/μL)	6.2 ± 0.7	8.3 ± 2.0*
Plt count (10^3/μL)	249 ± 85.1	286 ± 29.8

Note: *, ** = significant difference in SAO compared to healthy individuals (*p = 0.0103, **p = 0.0003).
RBC, RBC count; Hb, hemoglobin; Hct, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red blood cell distribution width; WBC, white blood cell; Plt, platelet count.

Table 2
Comparison of RBC indicies in SAO blood sample on Day0 Vs Day35 of storage (4 °C) (mean ± SD).

RBC indices	Day of blood storage	
	Day 0	Day 35
RBC (10^6/μ)L)	4.8 ± 0.5	3.0 ± 1.8*
Hb (g/dL)	14.2 ± 1.4	9.6 ± 2.5*
Hct (%)	40.4 ± 3.5	32.6 ± 2.3*
MCV (fL)	82.6 ± 3.5	87.2 ± 1.8**
MCH (pg)	29.6 ± 1.6	31.2 ± 3.2*
MCHC (%)	36.0 ± 0.8	38.2 ± 1.8*
RDW	14.6 ± 0.8	16.4 ± 1.5*

Note: *, ** = significant difference between SAO and healthy individuals (*p < 0.005, **p = 0.0003).
RBC, RBC count; Hb, hemoglobin; Hct, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red blood cell distribution width; WBC, white blood cell; Plt, platelet count.

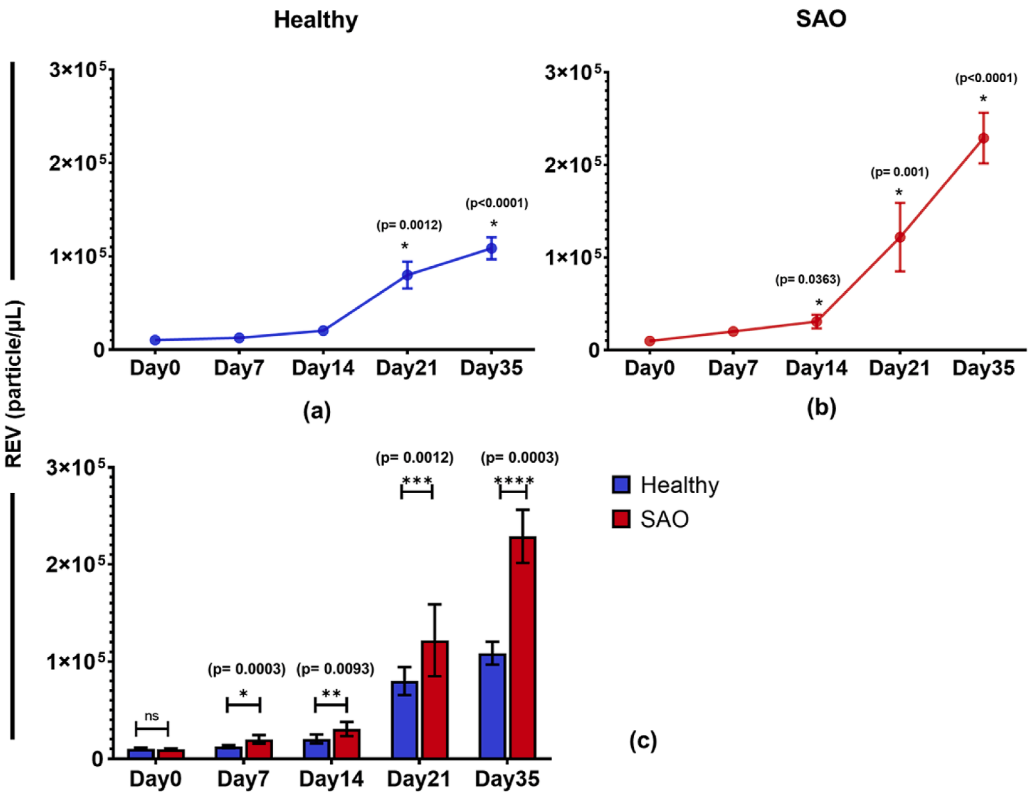

Fig. 2. The absolute REV numbers in SAO preserved-blood samples. The mean of REV numbers in healthy (a) and SAO (b) were obtained at indicated time points. The comparison of the mean of absolute REV numbers between the healthy group and SAO at indicated time points (c). ns = not significantly different.

microvesiculation characterized by PS expression on the surface. We further investigated the externalization of PS in the RBC membrane by annexin V expression using flow cytometry. The expression of annexin V was determined in gated RBC. The results showed that the increase in annexin V MFI-expressing RBC was time dependent in both SAO and healthy individuals. A significant increase in PS-expressing RBCs numbers and annexin V + MFI was observed starting at similar time points (day 14) of blood preservation in both healthy and SAO subjects (Fig. 3a).

Compared to the RBC lesions in SAO and healthy individuals, a significant increase in annexin V MFI expression was observed in SAO RBC on day 7. In contrast, a significant increase in PS-expressing RBC was observed later (day 21 of blood preservation) (Fig. 3b). This suggests that the *in vitro* storage conditions facilitated significant shedding of REV even after 7 days of blood storage. A significant number of PS-expressing RBC indicated storage lesions of stored RBC, which were later observed.

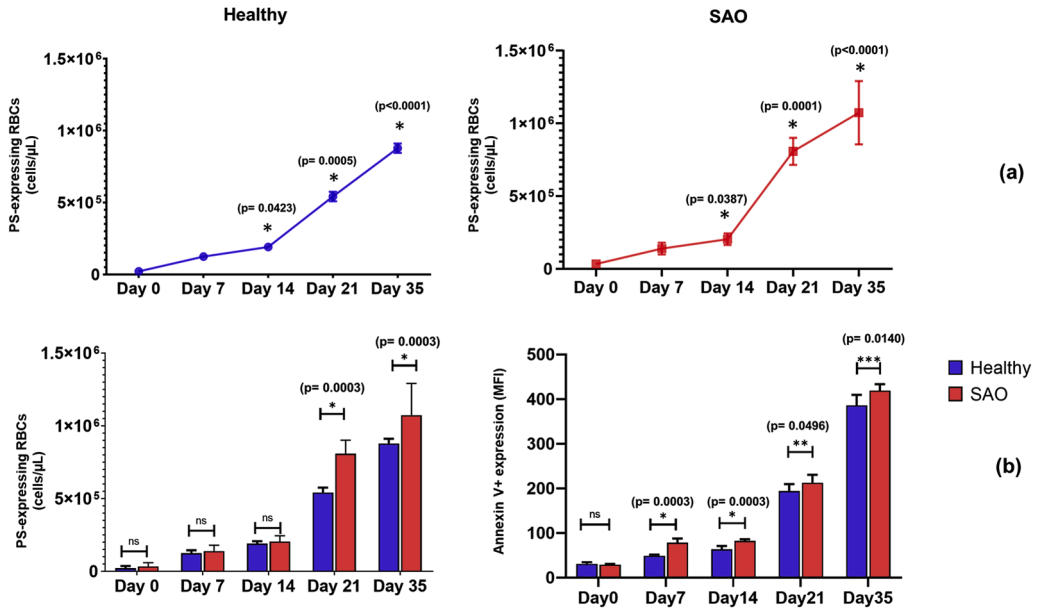

Fig. 3. The expression of phosphatidylserine in preserved RBC. The absolute numbers of PS-expressing RBC at indicated time points (a). Bar plot comparing PS-expressing numbers and PS-expressing intensities of RBC between healthy and SAO individuals (b).

3.4. Correlation of annexin V^+ RBC number, annexin V^+ RBC MFI, and REV production

Given that PS-exposed RBCs are markers of RBC membrane shedding and MP generation, we tested the correlation between PS-bearing RBC numbers and MFI to REV numbers between healthy and SAO subjects during *in vitro* storage conditions. The results demonstrated that the numbers of PS-expressing RBC and MFI were significantly correlated with REV numbers in both groups

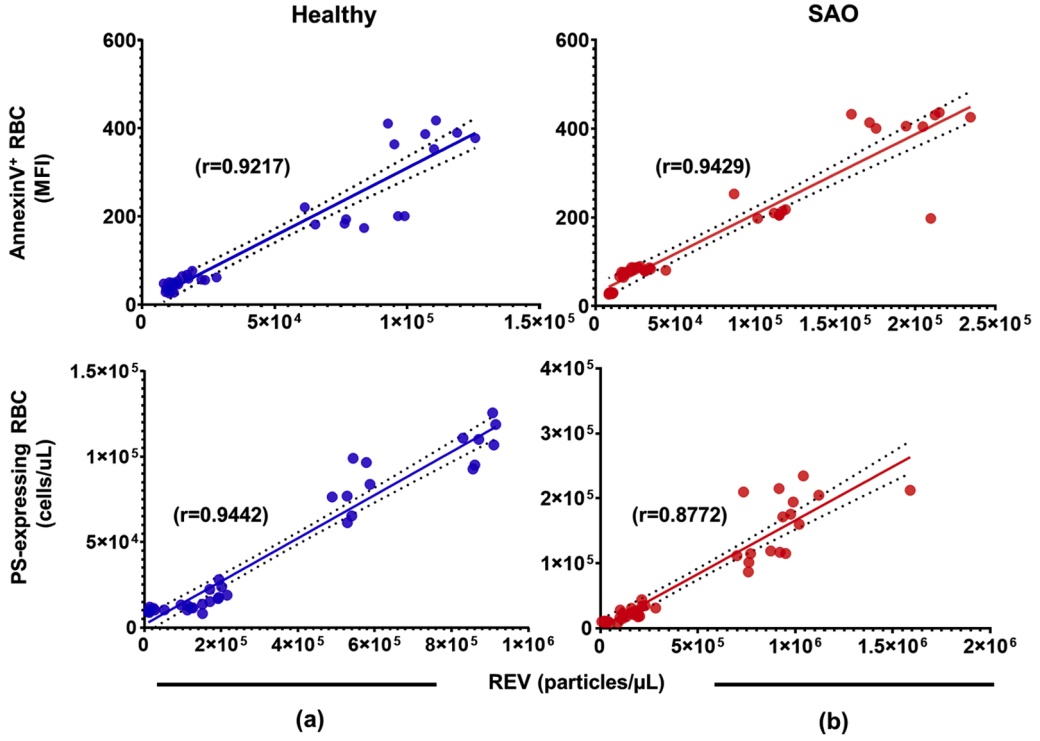

Fig. 4. The correlation between the expression of PS-expressing RBC and REV numbers. Correlation of REV numbers to PS-expressing RBC MFI (a) and absolute numbers. The significant correlations were calculated using the Spearman Rank test.

(Fig. 4a–b). This suggests that the degree of PS externalization significantly correlates with the concentration of REV, irrespective of the presence of the SAO mutation.

3.5. Procoagulant activity of generated REV and stored-RBC in SAO blood samples

The expression of phosphatidylserine on the cell surface is considered the initial factor in the coagulation cascade. The clotting time assay showed that CT in EVs obtained from different sources of SAO blood samples was significantly decreased compared to that in healthy controls (Fig. 5a). Decreased CT of REV obtained from the SAO plasma was observed on day 35 of blood preservation. Interestingly, a shortage of CT values in SAO blood samples was observed earlier in REV obtained using diluted RBC (day 21 of preservation). The correlation test demonstrated that the inverse correlation between CT values and REVs was significant in plasma-derived REV and REV derived from diluted RBC ($p = 0.0006$ and $p < 0.000$, respectively) (Fig. 5b).

4. Discussion

EVs are typically generated during the physiological state, which is controlled by a homeostasis mechanism [35]. However, the pathological effects of increased EVs are usually reported in several disease models, such as autoimmune diseases, inflammation, cancers, and thromboembolisms. In the field of hematological diseases, defects in the Band 3 protein, a major RBC membrane protein, facilitate deformities of RBC and shedding of EVs.

Our study showed aberrant microvesiculation in SAO individuals with defective RBC band 3 protein. The significance of some parameters associated with RBC were observed among recruited SAO samples including MCHC and RDW. These indices indicated the anomalies of RBC in SAO individuals. The increased RDW is possibly associated with the increased number of ovalocyte that affects the measurement by automated cell counter. Whereas higher MCHC represents the higher concentration of hemoglobin contents within RBCs. Interestingly, higher WBC counts have been observed among SAO individuals. This significance was concordance to the studies by Straat et al. and Crawford et al. The higher number of WBC possibly mediated by proinflammatory responses to REVs [36,37]. However, direct evidence of REVs mediated proinflammatory and the alteration in WBC numbers among SAO individuals should be further investigated.

The baseline REV numbers among SAO individuals were not significantly different from those of the healthy volunteers. Suggesting that the abnormality of the Band 3 protein among the recruited participants was not directly associated with REV generation during physiological conditions. However, the significance of REV numbers was observed earlier in the storage of SAO blood samples than in healthy blood samples. Remarkably, the 20-fold increase in REV numbers on day 35 of blood cell storage with unusual conditions. These evidence indicated that the aberrant features of RBC membrane integrity in SAO individuals might be triggered by some stimuli or conditions. Our results are in concordance with the relevant evidence that *in vitro* storage of blood cells facilitates generation of EVs

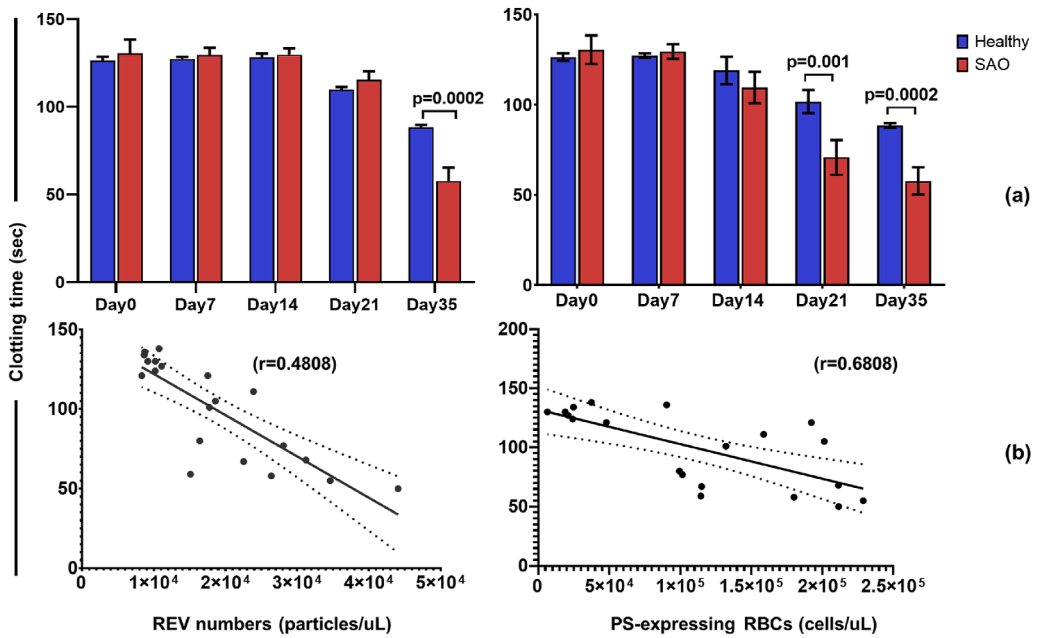

Fig. 5. Clotting time assay of generated REV and PS-expressing RBC. The clotting time assay was compared between SAO blood samples and healthy volunteers at indicated time points. Comparison of clotting time obtained from different sources, 50 μL of REVs-derived plasma was mix with 50 μL of pooled normal plasma (a). While the PS-expressing RBCs were diluted with PBS at a ratio of 1:20 (b) and then measured for clotting time. The correlation of clotting time assays to REV numbers and PS-expressing RBC were calculated using the Spearman Rank test.

[32,35,38,39]. Additionally, our results identify factors that might contribute to the acceleration of the deterioration of cellular integrity in SAO individuals. These factors include lower temperatures and addition of exogenous preservatives. The significant gradual loss of membrane integrity coincides with increased membrane vesiculation, particularly the consistent data observed on day 35 of blood preservation.

Previous evidence has shown that storing blood cells in citrate-containing solutions facilitates cation changes and increases cell size [19, 35]). These results highlight the importance of biochemical changes that could be the underlying mechanisms of microvesiculation of SAO blood cells. Although the given supplement additives provide sufficient energy sources for cellular metabolism, depletion of ATP was observed under *in vitro* conditions. The decrease in ATP levels during *in vitro* storage was hypothesized to be an initial step in membrane vesiculation and band 3 protein rearrangement [19]. Previous data showed that clustering and microaggregation of the Band 3 protein were observed during cold storage [40]. Karon et al. reported that storage at cold temperatures altered Band 3 oligomericity in the RBC membrane [16]. In addition, significantly elevated REVs were observed in hypothermic storage compared to physiological temperature [38]. These findings provide relevant data regarding the effects of temperature on Band 3 protein rearrangement. This suggests that the function of the Band 3 protein is temperature dependent [41], and has highlighted the importance of storage lesions facilitated by lowering the temperature.

Meanwhile, increased PS-expressing EVs or storage lesions on preserved RBC indicate pools of microvesiculation. The externalization of phosphatidylserine provides binding sites for coagulation factors, both intrinsic and extrinsic [42,43]. Our results showed an inverse correlation between clotting time and either REV number or PS-expressing RBCs. This suggests that microvesiculation of RBC in SAO blood samples could facilitate procoagulant activity following RBC preservation.

The observations of increased PS-expressing EVs and their procoagulant activity could be crucial in specific circumstances, particularly during blood banking procedures used for transfusion. Typically, storing blood cells is not a routine procedure for preparing blood samples for clinical laboratory analysis. Hypothermic storage with specific preservation is a practical procedure for blood banking that preserves blood cells for transfusion. Our results shed light on the possibility of unusual generation of REVs accumulated under specific conditions that potentially facilitate thrombogenicity. Considering the time points of RBC storage, the significantly shortened clotting time coincided with the time point of elevation of PS-bearing RBCs instead of REV numbers. Suggesting that the PS-expressing RBC might be a representative biomarker for quality control of thrombogenicity during RBC storage. However, direct evidence from clinical observations should be further studied.

Although the dysfunction of the Band 3 protein has been reported to be temperature-dependent, distinct mechanisms and factors have been proposed, such as the generation of oxidative stress and the leakage of cations during hypothermic storage [19,41]. Another function of Band 3 is associated with bicarbonate transport [44]. The alterations in controlling blood CO_2/HCO_3^- homeostasis should be investigated further, particularly during storage at lower temperatures.

Additionally, prolonged storage of SAO blood cells in CPDA-1 is associated with the unusual phenomenon of REV generation. As CPDA-1 has a high sugar content (a 40-fold increase compared to the normal state), the mechanisms of membrane vesiculation are still elusive. In particular, the association of REV generation in some groups of patients with SAO should be considered, such as those with a hyperglycemic state. Beside the value of using REV numbers in QC of blood banking, the recent case report of SAO with critical COVID-19 pneumonia showed the significance association between SAO and severity of disease [45]. The osmotic gradient ektacytometry analysis revealed that COVID-19 infection showed decreased osmotic fragility. Interestingly, multiple episodes of thrombosis events were observed during the treatment course. Suggesting that, the risk of thrombosis among SAO patients coexisting with some diseases i.e. infections and cardiovascular diseases should be taken into consideration. In Particular, the identification of biomarkers associated with REVs is recommended for the patients at risk of thrombogenesis. Our results gave rise to the use of PS-expressing RBC as a promising biomarker for thrombotic events among SAO patients. However, the underlying mechanisms and pathophysiology of REVs generation should be further investigated in SAO patients with distinct diseases/conditions.

This study has some limitations. Due to strict inclusion criteria and the low prevalence of SAO patients, the number of recruited participants might have been low ($n = 8$ for SAO and $n = 7$ for healthy participants). Moreover, our results demonstrated increased microvesiculation after *in vitro* storage in a test tube. Unfortunately, these conditions are not representative of blood-bag preservation. Distinct environmental factors associated with the deterioration of blood cells should be considered. Previous study showed that vigorous conditions in small tubes result in significant loss of membrane integrity after 14 days of preservation [38]. Vesiculation increases with prolonged storage but increases markedly after day 21 [17,18]. Therefore, the comparison of EV genesis on a larger scale of blood cell preparation is recommended to investigate and fulfill the aspect of transfusion.

In summary, our investigation demonstrated the microvesiculation of the abnormal Band 3 protein of RBC, which is related to elevated EVs and procoagulant activity. Our results shed light on abnormalities in RBC membrane vesiculation, which might potentially facilitate thrombotic events in specific settings. Additional factors, underlying mechanisms, and characterization of Band 3 protein aggregation contributing to aberrant microvesiculation should be further investigated. In particular, there is a high prevalence of SAO individuals who are candidates and eligible for blood donation.

Contribution

IP and DP conceived and designed the experiments. IP and DP performed the experiments. IP, PK, EN and KP analyzed and interpreted the data. IP and PB contributed reagents, materials, analysis tools and data. IP wrote the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2022.e12714.

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