SPANDIDOS PUBLICATIONS

Elevated red blood cell-derived extracellular vesicles under hyperglycemic conditions are associated with CD47 expression and production of intracellular reactive oxygen species

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Abstract. Diabetes mellitus (DM), particularly type 2 DM, is characterized by chronic hyperglycemia, which has notable effects on red blood cells (RBCs), including alterations in membrane integrity, morphology and function. The present study aimed to assess the effect of oxidative stress under hyperglycemic conditions on RBC-derived extracellular vesicle (REV) production using an *in vitro* model. RBCs from healthy participants were cultured in several glucose concentrations (5, 45 and 100 mM) to mimic euglycemic, intermediate and severe hyperglycemic conditions. The levels of intracellular reactive oxygen species (ROS) and CD47-expressing RBCs were measured using flow cytometry. REVs were purified using centrifugation and then their levels were measured using CountBright and flow cytometry. The results revealed a significantly increased level of intracellular ROS and REV production under hyperglycemic conditions. Additionally, hyperglycemia was demonstrated to reduce CD47 expression in RBCs, particularly at higher glucose concentrations. Elevated ROS production was also significantly associated with REV production in the early phase of hyperglycemic conditions, whereas the significant decrease in CD47 expression was observed later. Furthermore, pretreatment with N-acetylcysteine effectively reduced ROS production and REV formation, highlighting the role of oxidative stress in hyperglycemia-induced RBC alterations and REV production. The findings of the present study suggest that prolonged hyperglycemia facilitates intracellular oxidative stress and RBC membrane vesiculation, which markedly contributed to the understanding of the pathophysiology of diabetes-related complications.

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Introduction

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by persistent hyperglycemia, which arises due to either insufficient insulin production (type 1 DM) or insulin resistance [type 2 DM (T2DM)]. T2DM is the most prevalent form of diabetes, accounting for ~90% to 96% of all diabetes cases worldwide. In 2021, an estimated 529 million individuals globally were living with diabetes, with 96% of these cases attributed to T2DM (1). T2DM is identified by the resistance to insulin, leading to persistently increased blood glucose levels. The impact of elevated glucose concentrations on cellular membranes, signaling and function has been extensively studied using *in vitro* models, particularly in the context of DM (2-4).

Several previous studies have reported that prolonged exposure to hyperglycemic conditions affects the structural and functional integrity of cells. These effects influence the shedding of small, membrane-bound particles called extracellular vesicles (EVs) (5-7). EVs [formerly called microparticles (MPs)] are particles that are released from cells and delimited by a lipid bilayer that cannot replicate on their own. The size of EVs range from 100-1,000 nm in diameter. EVs can be classified by distinct properties such as molecular markers, size and their functions (8). Moreover, they have gained increasing attention as key mediators of intercellular communication and as critical players in several physiological and pathological processes, including DM (9,10). These vesicles are involved in regulating immune responses, inflammation and cellular metabolism, all of which are highly relevant to the pathogenesis of DM (11).

Studies have reported that platelet-derived EVs (PEVs) notably contribute to the inflammatory processes associated with DM. For example, studies have reported that PEVs

carry pro-inflammatory molecules, such as cytokines and chemokines, which can modulate endothelial cell function and promote vascular inflammation (11,12). In patients with T2DM, PEVs have been reported to be associated with an increased level of pro-inflammatory markers, such as interleukin-1β and tumor necrosis factor-α (TNF-α), which contribute to insulin resistance and the development of atherosclerosis (13). This inflammatory environment is critical for the development of diabetic complications, such as cardiovascular disease, which is more prevalent in individuals with DM (12).

However, whilst several studies have assessed the role of PEVs in DM research, few studies have evaluated the role of red blood cell (RBC)-derived EVs (REVs) in this field. In diabetes milieu, RBCs undergo several alterations, including changes in membrane integrity, membrane glycation, size and overall morphology. It has been proposed that these disruptions lead to the generation of REVs, which have marked implications for cellular communication and disease progression (13).

On RBCs, there are several surface receptors that control cell physiology. CD47 is one of these crucial membrane proteins which is involved in preventing the phagocytosis of RBCs by macrophages (14). CD47 expression confers prevention RBC from phagocytosis, whereas the decreased CD47 levels on aged RBCs mark them for clearance by hepatic and splenic macrophage (15). The reduction in CD47 expression and increase in phosphatidylserine (PS) expression are associated with RBC clearance in mechanical stress condition. However, previous research reported no significant decreases in CD47 expression among aged RBCs (16). Moreover, in inflammatory-induced conditions, an increase in PS expression on RBC membrane has been reported to be altered (17). However, there is no evidence of an association between CD47 expression and membrane vesiculation under inflammatory-induced conditions such as DM.

Recent studies have highlighted the role of EVs in the progression of DM and its associated complications (5-7,11). However, the specific populations of EVs and their precise impacts on DM remain underexplored. Therefore, the aim of the present study was to assess the effects of hyperglycemia on the integrity and characteristics of RBC membranes under hyperglycemic conditions. Additionally, the study aimed to evaluate the potential mechanisms driving membrane vesiculation and the shedding of REVs using an *in vitro* model.

Materials and methods

Participant recruitment and ethical considerations. The participants were recruited at hematology laboratory, School of Allied Health Sciences, Walailak University, between 6th June 2023 to 5th June 2024. The protocol was approved (approval no. WUEC-23-143-01) by the Walailak University Institutional Review Board (Tha Sala, Thailand). Written informed consents were obtained from all participants prior to the commencement of the study. Participants with no history of hyperglycemia or underlying medical conditions were recruited for the study. Individuals who were pregnant, had recently recovered from a febrile illness, or were taking therapeutic medications were excluded. A total of 6 ml whole blood was collected from 10 healthy participants in 3.2% sodium citrate at ratio of 9:1 following a 12-h fasting period. Clinicopathological characteristics including age and sex distribution are mentioned in Table I.

RBC preparation and culture. RBCs were prepared for *in vitro* culture as previously described by Viskupicova *et al* (17) and Loyola-Leyva *et al* (18), with certain modifications. Briefly, whole bloods were centrifuged at 1,500 x g for 10 min at 4˚C. The plasma was collected for preparing the complete media culture. Packed RBC pellets were washed three times with sterilized 0.9% NaCl. Washed RBC pellets (1 ml) were then resuspended in 1 ml Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich; Merck KGaA) with 10% v/v autologous plasma (4,500 μl DMEM with 500 μl of filtered plasma). Subsequently, washed RBCs were seeded in T25 tissue culture flasks and the glucose-DMEM was replenished at several concentrations (5, 45 and 100 mM) to generate a 30% v/v culture (900 μl washed RBC with 3 ml medium). This *in vitro* hyperglycemic condition ensured consistency with previous studies by Batista da Silva *et al* (19) which demonstrated that *in vitro* glucose treatment induces phenotypic and biochemical changes resembling those in patients with diabetes. The conditioned medium and treated RBCs were collected at 24 and 48 h after treatment for further experiments. Each culture was performed in duplicate.

Quantification of REVs. Glucose-treated RBCs were collected at specific time points and stained, followed by the quantification of REVs using flow cytometry. The staining protocol for REVs was performed as previously described (19,20), with certain modifications. Briefly, the treated RBCs were centrifuged at 500 x g for 10 min at 4˚C and RBC pellets were collected for PS expression analysis. The supernatant was collected, centrifuged further at 14,000 x g for 2 min at 4˚C to obtain platelet-free plasma, then centrifuged further at 14,000 x g for 45 min at 4˚C to obtain non-purified MP pellets (including EVs, apoptotic bodies, or other extracellular structures) for REV quantification. All monoclonal antibodies and staining buffers were purchased from BioLegend, Inc. A total of 10-μl supernatant was stained with 2 μl anti-CD235-PE (cat. no. 349106; BioLegend, Inc.), 2 μl Annexin V FITC and 41 μl 1X annexin V binding buffer (cat. no. 42220; BioLegend, Inc.), which was then mixed and incubated for 15 min at room temperature. The stained REVs were resuspended with 350 μl annexin V binding buffer to make up a final volume of 400 μl. The absolute numbers of REV were measured using CountBright beads (Molecular Probes; Thermo Fisher Scientific, Inc.). Following the manufacturer's instructions, 50-μl well-mixed CountBright beads were added to the stained mixture, mixed and then subjected to flow cytometry (BD FACSVerse™ Flow Cytometer; BD Biosciences). The EV gate was created using standard beads of 0.88 μm (Spherotech, Inc.), positive for annexin V^+ (Fig. 1A). Subsequently, the events (number of acquisition signals) $CD235^+$/AnnexinV^+ were counted and acquired for 1,000 events of counting beads in CountBright gate (Fig. 1B and C). The absolute number of REVs was calculated as follows: REV (particle/μl) which was obtained using the number of REV counts per acquired count beads, was multiplied by the pre-calculated bead number per resuspension volume (Fig. 1D).

Table I. Characteristics and hematological parameters of the enrolled subjects (n=9).

Parameter	Value	Reference values
Sex		
Male	3 (0.3)	-
Female	6 (0.7)	-
Age, years	20±0.24	-
Fasting blood glucose, mg/dl	82.4±5.7	70-99 mg/dl
RBC, $10^6/\mu$l	4.6±0.5	Male: 4.7-6.1, Female: 4.2-5.4
Hb, g/dl	13.6±0.8	Male: 13.8-17.2; Female: 12.1-15.1
Hematocrit, %	39.4±1.4	Male: 40-54; Female: 36-48
Mean corpuscular volume, fl	86.7±6.4	80-100
Mean corpuscular Hb, pg	28.8±2.5	27-33 pg
Mean corpuscular Hb concentration, %	33.9±2.0	32-36%
RBC distribution width-CV (%)	13.8±1.3	11.5-14.5%
White blood cells, $10^3/\mu$l	5.4±0.7	4-11x$10^3/\mu$l
Platelet count, $10^3/\mu$l	211±86.1	150-450x$10^3/\mu$l

Data are presented as n (%) or the mean ± standard deviation. RBC, red blood cell; Hb, hemoglobin.

Measurement of intracellular reactive oxygen species (ROS) in cultured-RBCs. The levels of ROS production were measured intracellularly using a 2',7'-dichlorofluorescin diacetate (DCFH-DA) assay as previously described, with certain modifications (21). Briefly, the cultured RBCs under several hyperglycemic conditions were washed twice with 1X PBS, then cultured with 2 μM working DCFH-DA. RBCs were washed twice with 1X PBS to remove the excess DCFH-DA. A minimum of 10,000 CD235-positive events were recorded per sample then DCF fluorescence expression was assessed using flow cytometry (Fig. 1E-G). Untreated RBCs and RBCs treated with H_2O_2 (100 μM) were used as negative and positive controls, respectively.

Expression of CD47 in RBCs. The expression of CD47 in cultured-RBCs were determined using flow cytometry. A total of 50 μl treated RBCs was mixed with 5 μl anti-CD47 FITC (cat. no. 323106) and 2 μl CD235 PE (cat. no. 349106; both from BioLegend, Inc.), and incubated for 15 min at 25˚C. The cells were washed twice with 1X PBS and resuspended in 1% paraformaldehyde prior to flow cytometry (BD FACS Verse™ Flow Cytometer; BD Biosciences). The proportions of $CD47^+$ RBCs were gated from $CD235^+$ (Fig. 1H and I). Mean fluorescence intensities (MFI) of CD47 expressing RBCs were compared among the treatment conditions (Fig. 1J).

N-acetylcysteine (NAC) treatment. NAC is a precursor for glutathione synthesis, a potent cellular antioxidant (22). In the present study, RBCs pretreated with NAC were used to assess the effect of ROS production on REV shedding during hyperglycemia. NAC was purchased from Sigma-Aldrich; Merck KGaA and a stock solution of 100 mM was prepared by dissolving NAC in sterile PBS, which was then aliquoted and stored at -20˚C until use. RBCs were then resuspended in DMEM supplemented with 10% fetal bovine serum (Hyclone; Cytiva) at a hematocrit of 2%. RBCs were treated with NAC at a final concentration of 1 mM, then incubated at 37˚C in a 5% CO_2 incubator. Subsequently, the cells were incubated with NAC for 24 h. RBCs were then washed three times with PBS to remove residual NAC and resuspended in conditioned medium that was formulated with two concentrations of glucose, 5 and 100 mM, as euglycemia and severe hyperglycemia, respectively. Oxidative stress levels were evaluated using flow cytometry. Control cells were treated with an equal volume of PBS without NAC.

Statistical analysis. In the present study, the statistics were calculated using GraphPad Prism version 7 (Dotmatics). REV numbers, ROS production and CD47 expression on the RBCs under hyperglycemic conditions were compared with the euglycemia control (5 mM). REV numbers and ROS generation in RBCs under NAC-treated conditions were compared with untreated groups. The differences in glucose concentrations between 24- and 48-h cultures were assessed using the Wilcoxon signed-rank test. The dose-dependent effects of glucose on ROS production, CD47 expression, and REV numbers were evaluated by Kruskal-Wallis test, followed by Dunn's multiple comparisons test for post hoc analysis. The correlation between REV number and ROS production were analyzed using the Spearman rank test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Participants demographics. A total of 10 healthy volunteers were recruited in the present study. The screening of blood glucose after fasting was measured using the Accu-check® Performa II (Roche Diagnostics GmbH). A total of 1 volunteer, who showed an abnormal lipid profile and blood glucose levels, was excluded from the study. Finally, 9 recruited participants (6 women and 3 men) were included in the study. The mean age of participants was 20±0.24 years, and the median

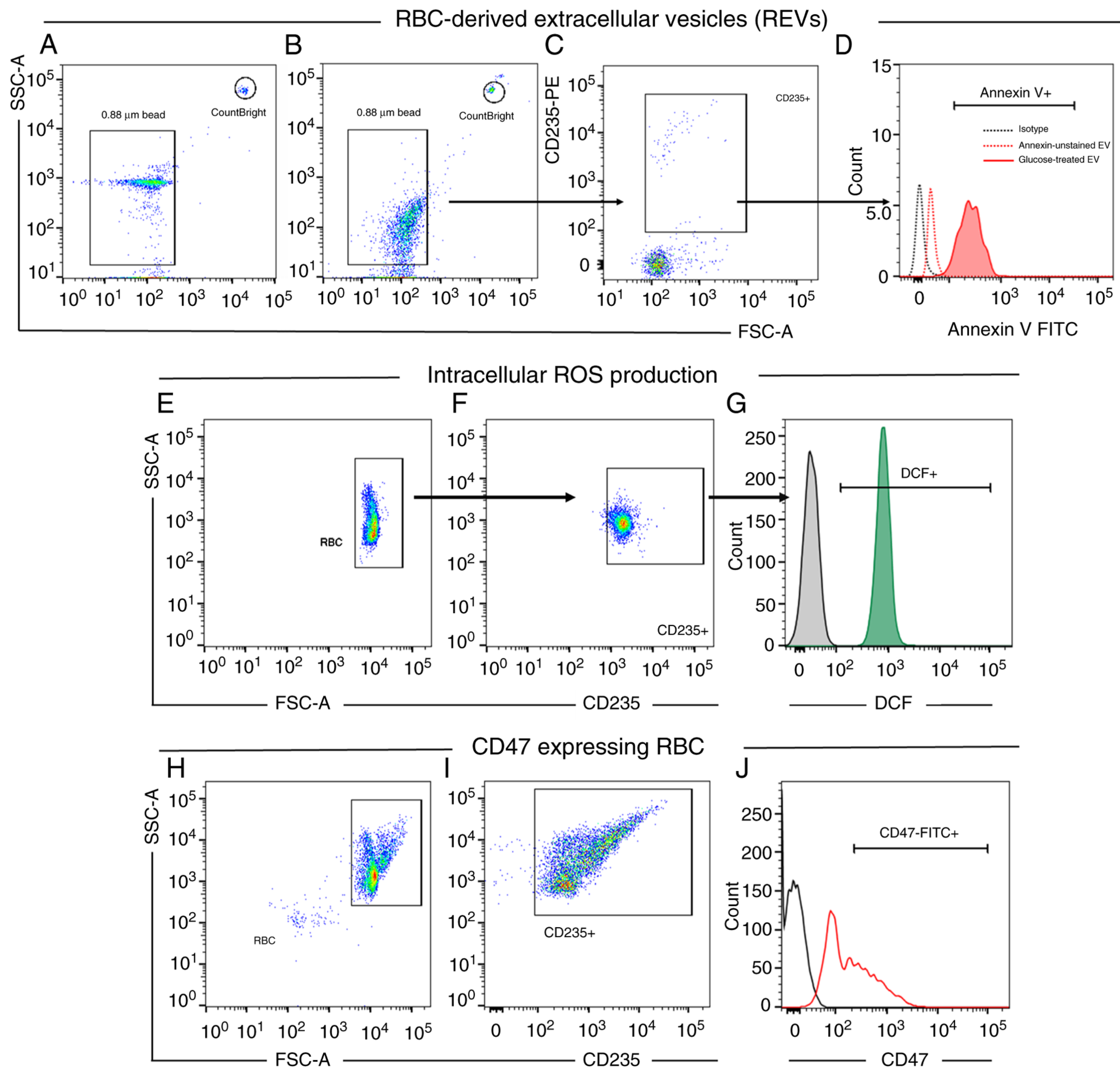

Figure 1. Gating strategies used for flow cytometric analysis. Gating strategies for REV quantification. (A) Dot plot representing size-calibrated beads (0.88 μm) and CountBright bead used in the study. (B) The EV gate was defined using size-calibrated standard beads (0.88 μm). (C) EV events within the EV gate were analyzed to identify REVs, characterized as CD235-PE$^+$. (D) CD235$^+$ RBCs were gated for Annexin-V expression. The number of Annexin V$^+$ events was assessed for REV calculation as follows: (Number of REV events/number of bead events) x number of beads counted per volume. Gating strategies for intracellular ROS production in cultured RBCs. (E) Dot plot demonstrated gating of RBCs from FSC/SSC. (F) RBCs were selected based on CD235+ expression. (G) A histogram was generated to calculate the MFI of DCF fluorescence, which indicates ROS production. The isotype control, shown as a black histogram, served as a negative control. (H and I) A dot plot represents the RBC population gated based on (H) FSC/SSC and (I) CD235$^+$ expression. (J) A histogram was generated to determine the MFI of CD47-FITC. The isotype control, shown as a black histogram, served as a negative control. EV, extracellular vesicle; RBC, red blood cell; REV, RBC-derived EV; MFI, mean fluorescence intensity; DCF, dichlorofluorescein; ROS, reactive oxygen species; FSC, forward side scatter; SSC, side scatter.

fasting blood glucose level was 85.21±2.52 mg/dl. Complete blood count parameters among the recruited participants were within the normal reference ranges (Table I).

RBCs demonstrate elevated ROS levels under hyperglycemic conditions. Prolonged hyperglycemia is considered as a factor driving ROS production (23,24). Therefore, RBCs were treated with several concentrations of glucose. Treated RBCs, which were gated from CD235$^+$, were measured for ROS production by the expression of DCF using flow cytometry (Fig. 1E-G). The results showed a significant increase in ROS production under hyperglycemic conditions. Elevated ROS production was observed at 100 mM glucose (P=0.0004) after 24 h of culture, as well as at 45 mM (P=0.0426) and 100 mM (P=0.0195) after 48 h of culture (Fig. 2A). Mean fluorescence intensities (MFIs) of DCF fluorescence were compared among glucose concentrations at 24 h and 48 h of culture (Fig. 2B and C). The current findings are consistent with previous study, demonstrating dosage-dependent effects on ROS production under hyperglycemic conditions (23) and our supplementary

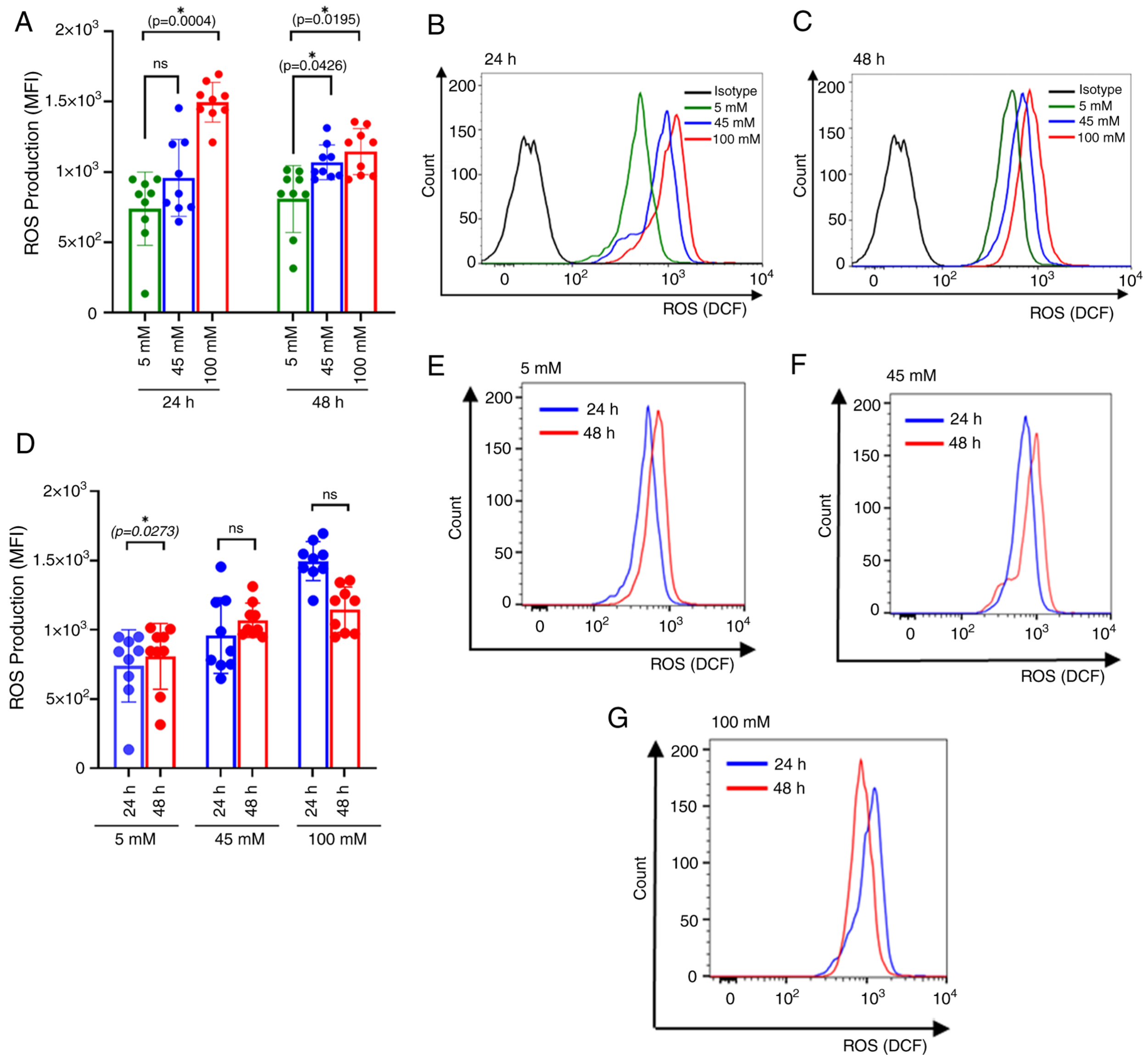

Figure 2. Intracellular ROS production in glucose-treated RBCs. (A) Bar plot comparing intracellular ROS production among different glucose concentrations at 24 and 48 h of incubation. The colors, green, blue and red, correspond with the different treatment groups: Euglycemic control (5 mM), intermediate level (45 mM) and severe hyperglycemia (100 mM), respectively. The significantly increased ROS production was observed at 100 mM glucose concentrations compared with 5 mM after 24 h of treatment (P=0.0004). Similarly, ROS production was significantly higher at 45 mM and 100 mM compared with 5 mM after 48 h of treatment (P=0.0426 and P=0.0034, respectively). (B) Representative histogram comparing the ROS production of glucose-treated RBC. MFIs of DCF in 5, 45 and 100 mM were compared at 24 h of incubation. (C) Representative histogram comparing the ROS production of glucose-treated RBC. MFIs of DCF in 5, 45 and 100 mM were compared at 48 h of incubation. (D) Bar plots represent the levels of ROS production among different treatment groups: Euglycemic control (5 mM), intermediate level (45 mM) and severe hyperglycemia (100 mM) The colors, blue and red, correspond with 24 and 48 h of incubation, respectively. The significance increased ROS production was observed at 5 mM glucose concentration between 24 h and 48 h of culture (P=0.0273), whereas no significance differences of ROS production between 24 and 48 h of glucose culture at 45 and 100 mM. (E-G) Representative histogram comparing ROS production at (E) 5 mM, (F) 45 mM and (G) 100 mM between glucose culture at 24 and 48 h of incubation. Effect of incubation time and glucose concentrations on ROS production at distinct glucose concentrations, compared using the Wilcoxon sign-rank test. Data are presented as the mean ± standard error of the mean, with individual dots indicating measurements from separate samples. *Indicates statistical significance. ROS, reactive oxygen species; RBC, red blood cell; MFI, mean fluorescence intensity; M, molar.

data (Fig. S1G-I). However, the comparison of the time of glucose exposure demonstrated a significant elevation of ROS production specifically in the euglycemic stage (P=0.0273) (Fig. 2D-G).

Hyperglycemic conditions alter CD47 expression on RBCs. CD47 is an integral membrane protein that is a member of the Rh complex. The major role of CD47 on RBCs is the molecular switch that controls the phagocytosis of RBCs. The binding of CD47 with signal-regulatory protein (SIRP)-α that is expressed on macrophages prevents RBCs from phagocytosis by macrophages. A previous study reported a decreased CD47 expression in long-term storage RBCs (15). However, the impact of varying glucose concentrations has not been fully elucidated.

In the present study, glucose-treated RBCs were collected and stained for CD47 expression, represented as the MFI (Fig. 1H-J). The results demonstrated that there was a significant decrease in

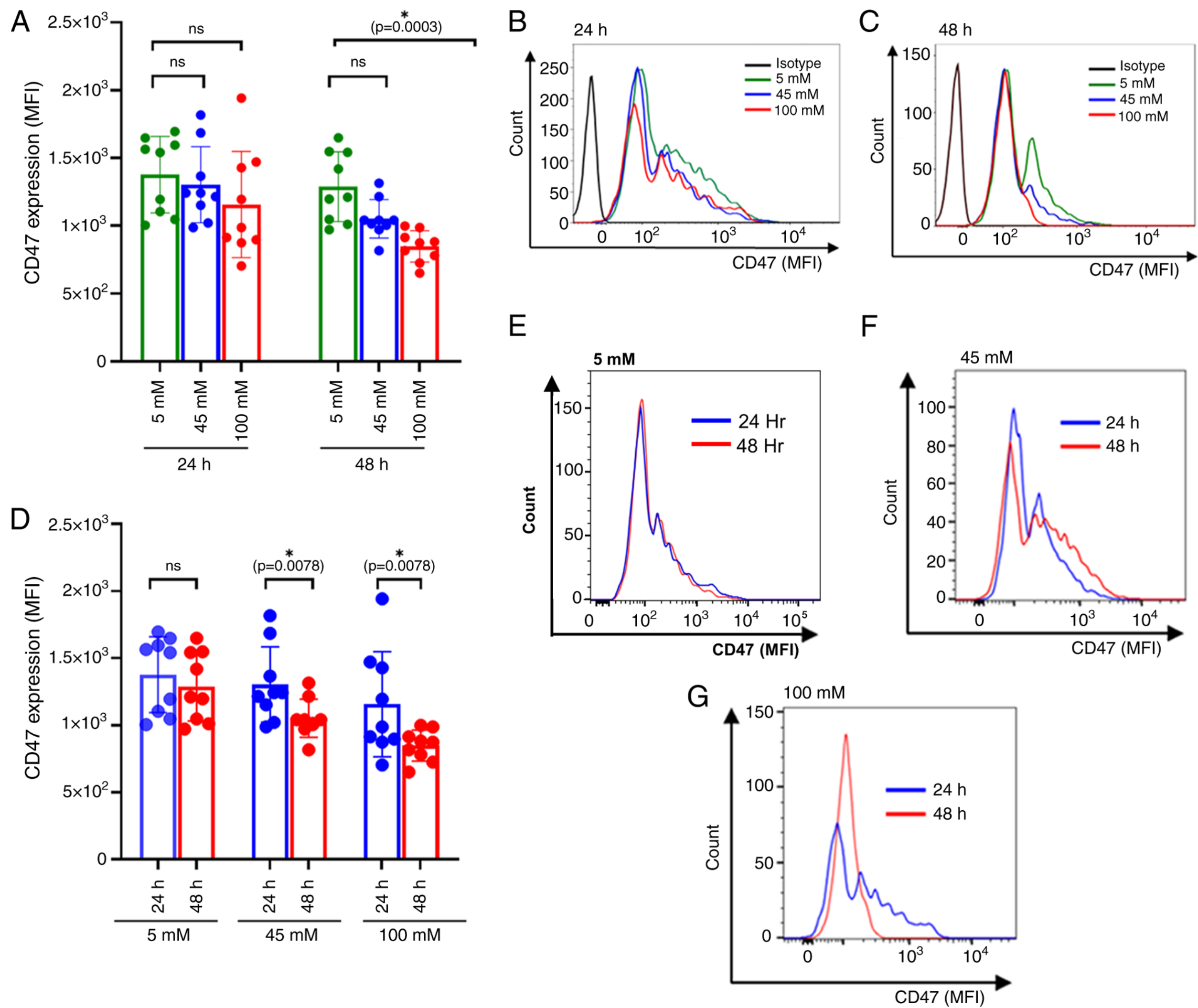

Figure 3. CD47 expression of glucose-treated RBCs. (A) Bar plot comparing the dosage effect of glucose on CD47 expression at 24 and 48 h of incubation. The colors, green, blue and red, correspond with the different treatment groups: Euglycemic control (5 mM), intermediate level (45 mM) and severe hyperglycemia (100 mM), respectively. The significantly decreased CD47 expression was observed at 100 mM glucose concentrations compared with 5 mM after 48 h of treatment (P=0.0003). However, no statistically significant differences of CD47 expressions were observed at 45 and 100 mM compared with 5 mM at 24 h. (B) Representative histogram comparing CD47 expression of glucose-treated RBC. MFIs of CD47 in 5-, 45- and 100-mM glucose treatment were compared at 24 h of incubation. (C) Representative histogram comparing CD47 expression of glucose-treated RBC. MFIs of CD47 in 5-, 45- and 100-mM glucose treatment were compared at 48 h of incubation. (D) Bar plots represent CD47 expression among different treatment groups: Euglycemic control (5 mM), intermediate level (45 mM) and severe hyperglycemia (100 mM). The colors, blue and red, correspond with 24 and 48 h of incubation, respectively. CD47 expression significantly downregulated at 45 and 100 mM of glucose treatment, (P=0.0078 and P=0.0078, respectively). However, no significant differences in CD47 expression were observed at 5 mM glucose concentration between 24 and 48 h of culture. (E-G) Representative histograms comparing CD47 expression at (E) 5 mM, (F) 45 mM and (G) 100 mM glucose concentrations after 24 and 48 h of incubation. Effect of incubation time on and glucose concentrations on CD47 expression at distinct glucose concentrations, compared using the Wilcoxon sign-rank test. Data are presented as the mean ± standard error of the mean, with individual dots indicating measurements from separate samples. *Indicates statistical significance. ROS, reactive oxygen species; RBC, red blood cell; MFI, mean fluorescence intensity; M, molar.

the CD47 expression under different glycemic conditions. The significant were observed at 48 h of incubation at 100 mM glucose culture compared with 5 mM, (P=0.0003) (Fig. 3A). Mean fluorescence intensities (MFIs) of CD47expression on RBCs were compared among glucose concentrations at 24 h and 48 h of culture (Fig. 3B and C) Moreover, the significant decrease in CD47 expression was observed for both intermediate (P=0.0078) and severe hyperglycemic treatment (P=0.0078) conditions at 24 and 48 h after glucose treatment (Fig. 3D-G). This suggests that in vitro hyperglycemic conditions altered CD47 expression on the RBC membrane. Interestingly, the decreased CD47 expression was coincided with the downregulation of CD235 which is RBC membrane biomarkers (Fig. S2).

Increased REV production in hyperglycemic treated conditions. RBCs obtained from healthy participants were treated with different concentrations of glucose. At 5 mM, the *in vitro* model mimicked euglycemia, whereas treatment with 100 mM represented severe hyperglycemic milieu. To assess the effect of glucose concentrations on the duration of exposure, RBCs were treated for 24 and 48 h. The cultured RBCs were collected at specific time points and quantified for REV production. Compared with the euglycemic state, the results revealed a significant increase in REV numbers under 100 mM (P<0.0001) glucose treatment conditions after 24 h of culture, with similar findings at 48 h (P<0.0001) (Fig. 4A-D). This finding indicates a possible dose-dependent

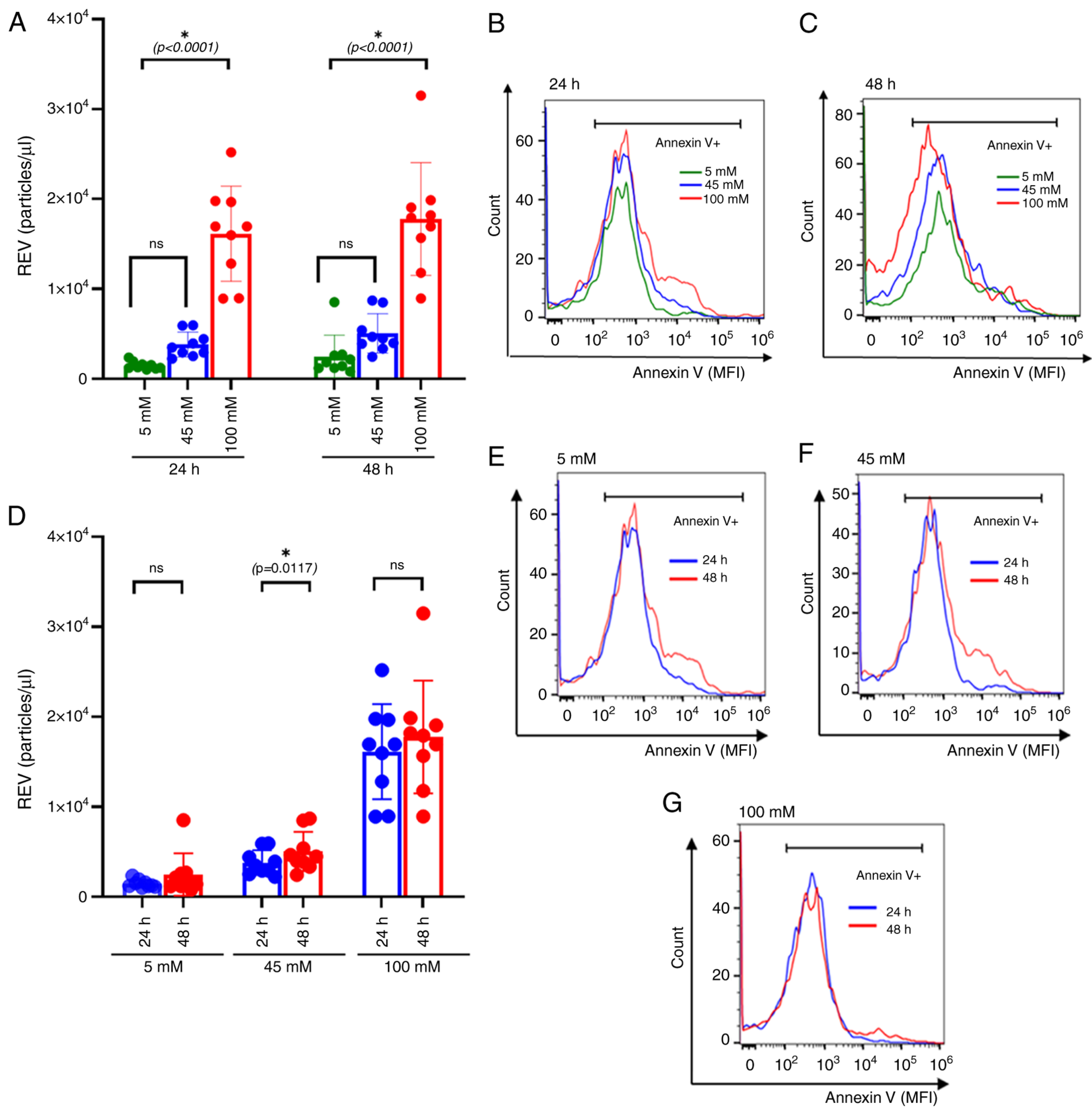

Figure 4. REV formation under different glucose treatments. (A) Bar plot compares REV production at different concentrations. The colors, green, blue and red, correspond with the different treatment groups: Euglycemic control (5 mM), intermediate level (45 mM) and severe hyperglycemia (100 mM), respectively. The significantly elevated REV production was observed at 100 mM glucose concentrations compared with 5 mM either 24 h of treatment (P<0.0001) or 48 h of treatment (P<0.0001). (B and C) Representative histogram compares annexin V expressing EVs that are used for REV calculations. MFIs of annexin V in 5, 45 and 100 mM of glucose were compared at (B) 24 h and (C) 48 h of incubation. (D) Bar plots compared REV production among different treatment groups: Euglycemic control (5 mM), intermediate level (45 mM) and severe hyperglycemia (100 mM). The colors, blue and red, correspond with 24 and 48 h of incubation, respectively. REV significantly increased at 45 mM of glucose treatment, (P=0.0117). However, no significant differences in REV production were observed between 24 and 48 h of culture at 5 and 100 mM glucose concentration. (E-G) Representative histograms comparing annexin V expressing EVs at (E) 5 mM, (F) 45 mM and (G) 100 mM glucose concentrations after 24 and 48 h of incubation. Effect of the incubation time and glucose concentrations on REV production were compared using the Wilcoxon sign-rank test. Data are presented as mean ± standard error of the mean, with individual dots indicating measurements from separate samples. *Indicates statistical significance. REV, red blood cell-derived extracellular vesicle; MFI, mean fluorescence intensity; M, molar.

effect that becomes apparent at higher glucose levels (Fig. S1A-C).

NAC reduces intracellular ROS production and REV formation in RBCs under hyperglycemic conditions. To assess the impact of the inhibition of intracellular ROS and REV production, cultured RBCs were pretreated with 1 mM NAC for 24 h. Subsequently, NAC-pretreated RBCs were cultured in glucose concentrations of 5, 45 and 100 mM, and incubated for 24 h.

The significant differences of ROS production were observed among the NAC-treated group in the intermediate (P=0.0039) and severe hyperglycemic conditions (P=0.0039) (45- and 100-mM glucose; Fig. 5A-D). This

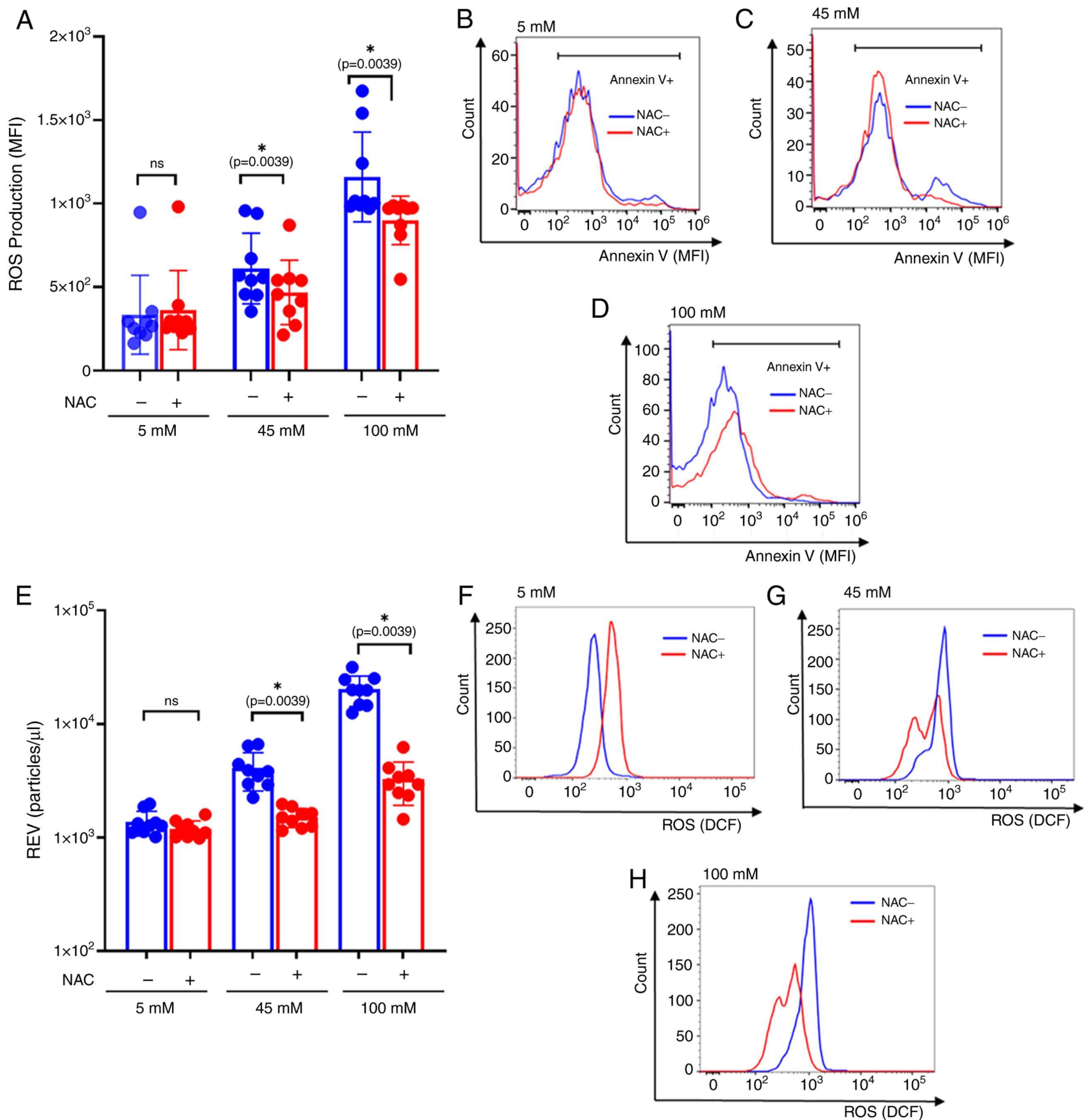

Figure 5. Comparison of REV and ROS production between NAC-pretreated RBCs and the untreated group. (A) Bar plots represent REV production comparing between untreated and NAC-pretreated RBCs after 24 h of incubation. The colors, blue and red and correspond with the different treatment groups: untreated and NAC-pretreated RBC, respectively. NAC-pretreated significantly decreased REV production at 45 mM and 100 mM of glucose concentrations, (P=0.0039 and P=0.0039, respectively). However, no significant differences of REV production was observed between untreated and pretreating NAC condition at 5 mM of glucose culture condition. (B-D) Representative histogram compares annexin V expressing EVs that are used for REV calculations. MFIs of annexin V were compared between untreated and NAC-pretreated conditions at (B) 5 mM, (C) 45 mM and (D) 100 mM of glucose culture. (E) Bar plots represent ROS production comparing between untreated and NAC-pretreated RBCs after 24 h of incubation. The colors, blue and red and correspond with the different treatment groups: untreated and NAC-pretreated RBC, respectively. NAC-pretreated significantly decreased ROS production at 45 mM and 100 mM of glucose concentrations, (P=0.0039 and P=0.0039, respectively). However, no significant differences of REV production were observed between untreated and pretreating NAC condition at 5 mM of glucose culture condition. (F-H) Representative histograms comparing ROS production between untreated and NAC-pretreated under (F) 5 mM, (G) 45 mM and (H) 100 mM glucose concentrations. The effect of the incubation time and glucose concentration on REV and ROS production, were compared using the Wilcoxon sign-rank test. Data are presented as the mean ± standard error of the mean, with individual dots indicating measurements from separate samples. *Indicates statistical significance. ROS, reactive oxygen species; RBC, red blood cell; REV, RBC-derived extracellular vesicle; NAC, N-acetylcysteine, MFI, mean fluorescence intensity; M, molar.

suggests that the intracellular oxidative stress augmented by hyperglycemic conditions may have been involved in REV production.

REV productions were measured using flow cytometry, comparing between the NAC-pretreated RBCs and untreated conditions. The results revealed a significant decrease in

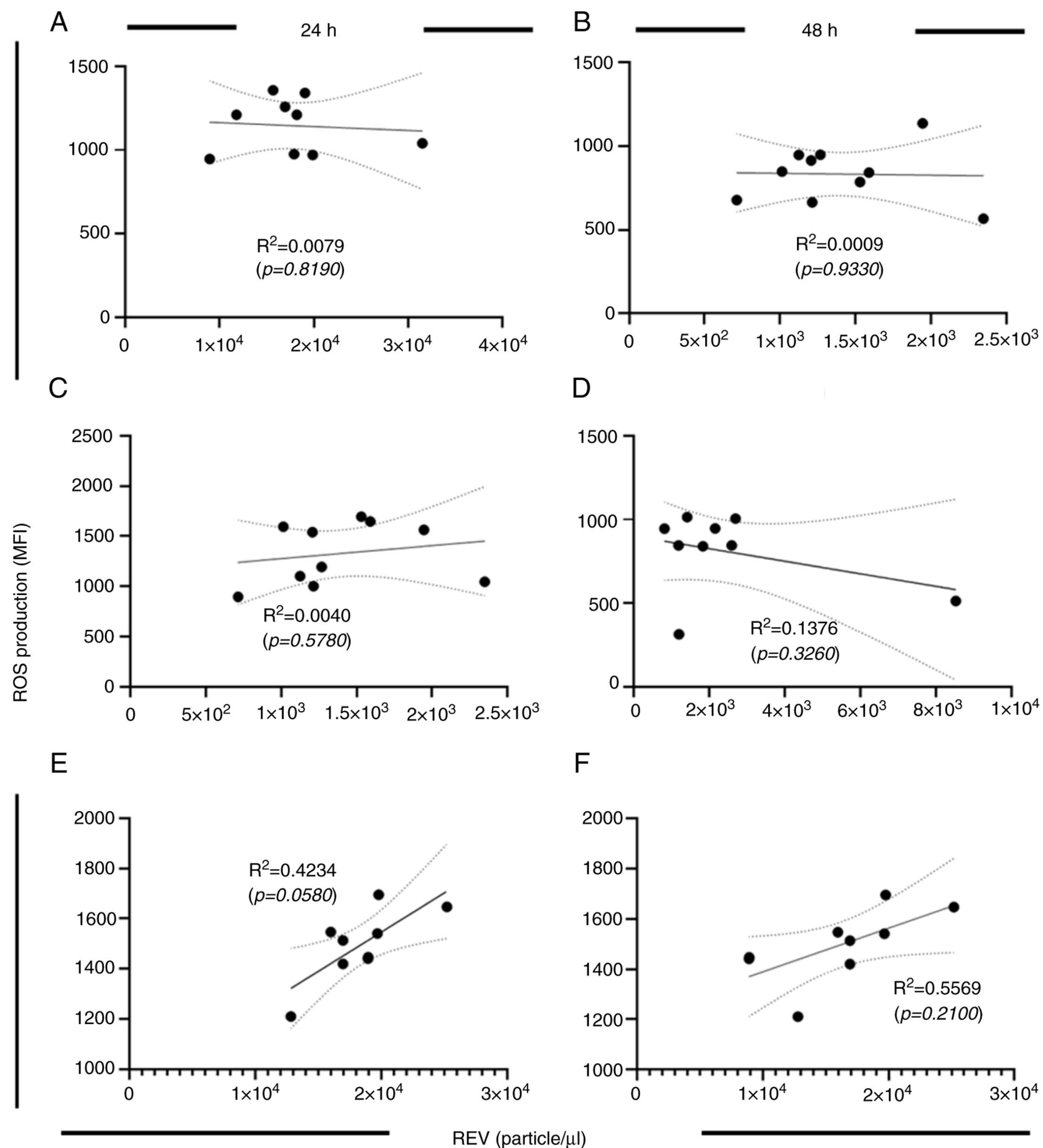

Figure 6. Comparison of ROS and REV production at different incubation periods and glucose concentrations. The association between ROS production and REV numbers were tested for correlation at (A C and E) 24 h and (B D and F) 48 h. (E and F) The results showed positive correlation in ROS and REV production observed at 24 or 48 h of incubation after treatment with 100 mM glucose. Statistical significance was assessed using the Spearman rank test and regression analysis. ROS, reactive oxygen species; REV, red blood cell-derived extracellular vesicle.

REV production observed in NAC-pretreated RBC at 45- and 100-mM glucose levels (P=0.0039 and P=0.0039, respectively, whereas no significant difference of REV production following NAC treatment at 5 mM glucose culture (Fig. 5E-H).

Increased REV production is associated with elevated ROS production and decreased CD47 expression. Elevated ROS production is a common feature of hyperglycemic conditions (23). However, there is no evidence of the factors associated with ROS production on REV biogenesis, to the best of our knowledge. Therefore, the association between ROS production and REV formation was assessed using correlation analysis. The data represented in Fig. 2 (ROS production) and Fig. 4 (REV production) were used for calculation. The results revealed that there was no statistically significant correlation between ROS production and REV production in the study (Fig. 6A-D). These findings suggested that elevated ROS levels may contribute to increased REV formation under hyperglycemic conditions at either 24 or 48 h of incubation (Fig. 6E and F), highlighting the potential impact of oxidative stress on RBC integrity.

Furthermore, the findings of the present study demonstrated a downregulation of CD47 expression in glucose-treated RBCs. This emphasizes the role of CD47 in REV physiology. Therefore, to further evaluate this relationship, correlation analysis was performed to assess the association between CD47 expression and REV production under distinct culture conditions. The data represented in Fig. 3 (CD47 expression) and Fig. 4 (REV production) were used for calculation. The results revealed that there was no significant correlation between CD47 expression and REV production after 24 h of

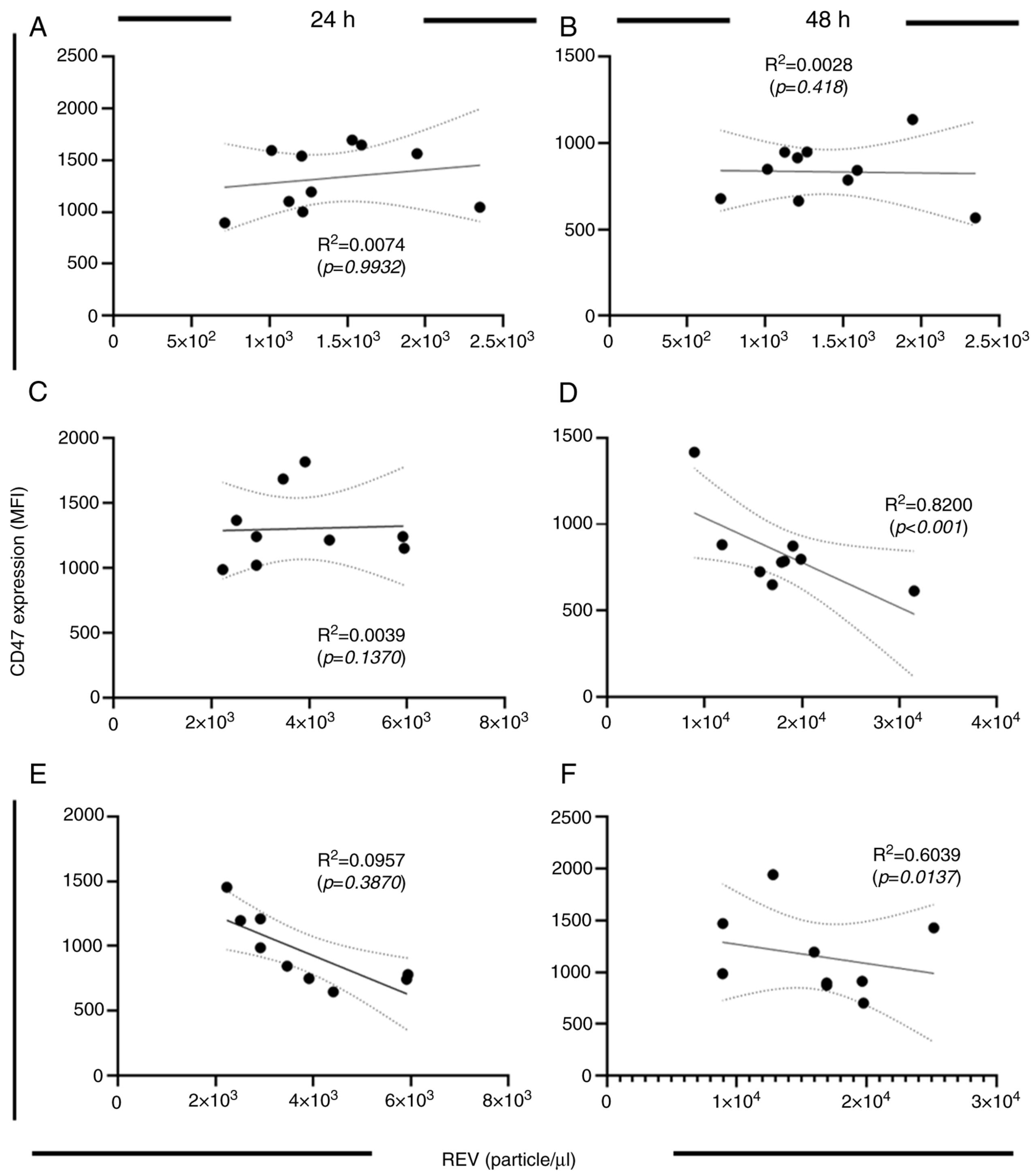

Figure 7. Comparison between CD47 expression and REV production at different incubation periods and glucose concentrations. No significance correlation between CD47 expression and REV production were observed after (A C and E) 24 h and (B and F) 48 h of glucose culture. (D) However, the significant decrease in CD47 expression observed in the intermediate hyperglycemic condition at 48 h of culture. Statistical significance was assessed using the Spearman rank test and regression analysis. REV, red blood cell-derived extracellular vesicle.

glucose culture (Fig. 7A, C and E). At 48 h of glucose culture, there was no significant correlation between CD47 expression and REV production at euglycemic condition (Fig. 7B). By contrast, a significant decrease in CD47 expression was observed in the intermediate hyperglycemic condition at 48 h of culture (Fig. 7D). Moreover, an inverse correlation between CD47 expression and REV production was demonstrated in the severe glucose concentration condition at 48 h of culture (Fig. 7F). These results indicate the physiologic alterations of REV and RBCs in different glucose treatments. ROS increased in a dose-related pattern, which was observed at the early phase of glucose exposure. By contrast to CD47 expression, which was altered later at 48 h of glucose treatment. These results suggest that increased ROS production is associated with REV production in a dose-related manner, whereas CD47 expression is associated with a time-dependent effect on REV.

Discussion

Hyperglycemia is a common feature of DM which is a major concern and non-communicable disease globally. Chronic inflammation is one of the proposed mechanisms that drive the progression of the disease (3,4). Therefore, prolonged and uncontrolled hyperglycemia are considered factors that drive multi-organ dysfunctions which are the major complications of DM. Previous research has reported that increased pro-inflammatory responses are observed in patients with both prediabetic conditions and DM (15,25). Chronic inflammation is closely associated with cellular and tissue damage, particularly affecting the endothelial structures, which are commonly involved in microvascular complications in patients with DM (1-3). The shedding of cell membrane fragments, known as EVs, is implicated in several pathological processes. This has attracted significant attention regarding the characteristics and biogenesis of EVs in several disease conditions (6,7).

EVs are small particles shed from cells, and the mechanisms underlying EV biogenesis and characteristics are of increasing interest in understanding disease pathogenesis, particularly in malignancies, autoimmune diseases and hematologic disorders (10). The present study assessed the effects of hyperglycemia on RBC membrane microvesiculation and the shedding of EVs. EVs are involved in modulating inflammatory responses by carrying bioactive molecules such as proteins, lipids and nucleic acids to distant sites (25). EVs, previously known as MPs, are small membrane-bound particles characterized by PS expression, commonly used as a marker for EV identification (8). Whereas MPs were traditionally associated with functions especially thrombosis. Recently, it was found that EVs exhibit broader biological roles. Therefore, the Minimal Information for Studies of EVs 2023 guidelines recommended using the term 'EVs' instead of 'MPs' (8,26).

The present study demonstrated an increase in intracellular ROS and REVs under hyperglycemic conditions. The glucose concentrations used in the present study were selected based on prior research that demonstrated significant alterations in RBC membrane properties under hyperglycemic conditions (17-19). Our results showed that the increased PS-expressing REV and downregulation of CD235 align with the increased percentage of eryptosis (17,19) and decreased cell viability (18). These results reinforcing the notion that prolonged glucose exposure contributes to RBC membrane alterations observed by the elevated REV production. These comparisons support the validity of our *in vitro* model and highlight the relevance of our observations in the context of diabetes-related RBC dysfunction.

The present findings showed elevated REV production following oxidative stress induction (Fig. S1). Oxidative stress is a well-established driver of EV release, and prior studies have reported that ROS overproduction in RBCs can trigger this process (26,27). In particular, ROS overproduction has been shown to promote oxidative stress that leads to the modification and internalization of membrane proteins such as CD33 (28). Moreover, oxidative stress can activate inflammatory pathways, leading to the release of cytokines such as TNF-α, which further alter protein expression and compromise membrane stability (28). Compared with the current findings, a significant increase ROS production was observed at the 100-mM glucose concentration compared with control, indicating a dose-related effect at higher concentrations. Whereas no significant difference was found between medium dose (45 mM) and control at 24 h of culture. However, at 48 h of culture, a time-dependent trend was observed with increased ROS production at 45- and 100-mM glucose (P=0.0426 and P0.0034, respectively) (Fig. 2A). These results suggested that prolonged hyperglycemia elevates the risk of EV release. These findings further confirm that prolonged glucose exposure enhances REV production.

The results of the present study are important from the perspective of their clinical implications, particularly for patients with poorly controlled DM, who often experience prolonged hyperglycemic episodes (6). This is associated with serious complications, including diabetic nephropathy, diabetic retinopathy and microvascular damage (9,11). Vascular injuries have been proposed as characteristics of chronic inflammation in DM. The expression of PS on endothelial cells activates platelets, thereby facilitating the activation of primary hemostasis. Previous studies have reported that PS-expressing REVs contribute to thrombogenicity and procoagulant activity (29-31). Moreover, PS provides binding sites for certain coagulation factors, such as factor Xa and factor Va, which are essential for prothrombin formation (29). Aberrant expression of PS in these conditions is commonly observed in patients with DM (30). However, evidence of PS expression on RBCs under these conditions remains limited.

Alongside with the alteration of RBC membrane integrity, which can be explained by the increased REV production and aberrant RBC membrane phenotypes. In the present study, CD235 was selected as surrogate markers for RBCs. CD235a (CD235) (Glycophorin A) is a glycosylated sialoglycoprotein expressed abundantly on the surface of mature red blood cells (RBCs). It plays a crucial role in maintaining the structural integrity of the RBC membrane, enabling flexibility and stability. Functionally, CD235 is also involved in the regulation of RBC shape and the modification of membrane protein complexes during RBC aging (32). This makes it a valuable marker for identifying erythroid cells in experimental studies, including flow cytometry assays, due to its high and stable expression throughout the life cycle of the RBC (33). CD47, another key protein present on RBCs membrane complex, plays a significant role in regulating immune interactions. The role of CD47 and its interactions in diabetes and inflammation have been proposed in several ways. For example, CD47-SIRP signaling promotes the release of proinflammatory cytokines, such as TNF-α and IL-6, contributing to systemic inflammation (15).

The present study explored the potential relationship between CD235a and CD47 in the context of RBC membrane dynamics. Changes in the expression of either CD47 or CD235a could affect RBC survival and contribute to pathological conditions. The present study demonstrated that CD47 dysregulation and downregulation of CD235 were associated with aberrant ROS production. The downregulation of CD47 expression at higher glucose concentrations suggests a glucose-induced disruption in RBC membrane integrity, which could have critical implications for RBC survival, particularly in hyperglycemic conditions (13). Increased glucose levels enhance the production of ROS, leading to oxidative damage. Oxidative stress can affect membrane proteins such as CD47, resulting in their degradation or reduced surface

expression. One potential mechanism underlying this decrease is glucose-induced oxidative stress, which is known to be elevated in high-glucose environments. Elevated glucose levels promote the generation of ROS, which contribute to oxidative damage of membrane lipids and proteins in several cell types, including RBCs (27,28). This oxidative damage alters membrane integrity and increases membrane rigidity, both of which are known to facilitate vesiculation (34,35). This is in line with the findings of the present study, where RBCs exposed to 100 mM glucose exhibited significantly lower CD47 levels, likely due to oxidative damage.

Although CD235 and CD47 expressed distinct localization on RBC membrane. Both CD235a and CD47 are expressed on the outer surface of RBCs, they do not directly interact in terms of membrane linkage. However, indirect interactions may occur through their involvement in membrane remodeling and vesiculation. A previous study suggested that in response to oxidative stress or membrane damage, both proteins may be internalized or redistributed within the membrane. For instance, oxidative stress might lead to vesiculation, where both CD235a and CD47 are incorporated into EVs, potentially influencing cellular communication and immune modulation (36).

Reduced CD47 levels typically exhibited in senescent RBCs, making them more susceptible to clearance by macrophages (37,38). A decrease in CD47 expression may indicate early-stage membrane remodeling associated with vesicle shedding and immune recognition, rather than terminal RBC senescence.

The present results are thus more indicative of acute membrane injury caused by oxidative stress rather than the gradual deterioration seen in physiological RBC aging. However, further investigations are needed to directly confirm the functional consequences of CD47 downregulation, including the potential mechanisms on immune activation and RBC clearance.Additionally, glucose-induced modifications in key membrane proteins, such as CD47, may disrupt the balance of intracellular signaling pathways regulating vesicle shedding (37). Previous studies have shown that oxidative stress can lead to cytoskeletal destabilization and PS externalization, both of which promote vesicle formation and release (39,40). In the present study, the observed reduction in CD47 expression following glucose exposure may reflect membrane remodeling events that precede vesiculation. Therefore, the significant downregulation of CD47 in the 100 μM glucose treatment group could reflect an accelerated aging process, which may lead to increased RBC turnover in hyperglycemic conditions.

Hyperglycemia can disrupt protein glycosylation processes. Glycosylation is critical for maintaining protein stability and function, and hyperglycemia has been reported to cause abnormal glycosylation patterns in several proteins, leading to compromised cellular function (37). In the case of CD47, altered glycosylation in a high-glucose environment may impair its proper localization to the RBC membrane, thereby reducing its expression (38). This mechanism has been supported by previous research, which emphasizes the role of glycosylation in regulating membrane protein stability (35).

Considering the findings related to REV formation, ROS production and CD47 expression, the results of the present study demonstrated a significant association between REV production and ROS levels during the early phase of glucose treatment. However, further investigation is required to fully elucidate the interplay between these markers in EV genesis, as well as to assess their potential complications, sources of EV generation and functions in DM. The alteration in CD47 expression suggests a connection to senescent phenotypes associated with prolonged hyperglycemic conditions, which could potentially serve as novel biomarkers for monitoring DM.

A key finding of the present study is the significant change in membrane integrity observed under hyperglycemic conditions, demonstrated by the downregulation of CD47 and CD235. The reduction of these markers was associated with increased REV counts. However, the patterns of decreased CD235 are different to the CD47 downregulations. This aligns with a previous study reporting that chronic high-glucose exposure increased RBC membrane rigidity and decreased deformability-both of which are critical factors contributing to microvascular dysfunction (41). To our observation, CD47 significantly decreased at 100 mM only observed at 48 h of incubation ($P=0.0003$) whereas CD235 significantly decreased expression were observed both 24 h and 48 h of culture at different glucose concentrations. Suggesting that the underlying mechanisms of membrane destabilization under hyperglycemic stress between these membrane proteins possibly different, requires further investigations.

Additionally, the findings of the present study highlight the importance of maintaining glucose homeostasis in preserving RBC integrity. Chronic hyperglycemia, as observed in diabetes, could exacerbate the downregulation of CD47, contributing to increased RBC clearance and potentially leading to anemia (42). The differential response to glucose concentrations suggests that even moderate increases in glucose levels can disrupt RBC function, emphasizing the need for tight glycemic control to prevent such effects. However, the precise molecular pathways through which glucose mediates CD47 downregulation should be investigated further.

A complex relationship was also demonstrated in the present study, involving elevated intracellular ROS production, decreased CD47 expression and REV production. However, there are certain limitations of the present study that need to be considered. The first limitation is the specific markers of EVs characterization using flow cytometry. In the present study, PS was used as a marker for EVs due to its well-established role in identifying membrane vesicles shed from cells under stress conditions. While PS exposure is commonly utilized for detecting and isolating EVs, it is not exclusive to specific EV subtypes, as apoptotic bodies can also externalize PS (8). However, in the context of the present study, the use of PS alone as a marker was considered sufficient for identifying EV populations of interest, as the primary objective was to assess changes in vesiculation under hyperglycemic conditions rather than to distinguish between different EV subtypes. The observed alterations in PS-expressing EVs provide meaningful insights into RBC membrane dynamics in response to glucose exposure. Nevertheless, for enhanced specificity, future studies should incorporate additional markers, such as tetraspanins (CD9, CD63 and CD81) for exosomes or other membrane proteins characteristic of different EV subpopulations (43). Another limitation is the *in vitro* cell culture models were used, which offer controlled conditions for evaluating specific

biological mechanisms. However, there several limitations to this approach, especially when compared with cross-sectional studies performed in real-world populations. The *in vitro* cell culture systems lack the complexity of whole organisms: Whilst they provide valuable insights into cellular processes in a controlled environment, they do not fully replicate the physiological interactions found in living organisms. Factors such as immune responses, cell signaling between different tissues and systemic metabolic influences are absent *in vitro*. The applicability of these findings to physiological conditions may be limited. Therefore, further investigation and characterization of these findings in diabetic patients are recommended to enhance clinical relevance and understanding.

The role of ROS production in this context points to the potential benefits of incorporating antioxidants into treatment regimens. Previous studies have reported the potential benefits of several antioxidant-rich foods in managing DM and its complications, such as vitamin C in combination with vitamin E and CoQ10 (44,45). Additionally, exploring affordable nutritional supplements in combination with current treatments could be a promising approach. Preventing cellular damage through antioxidants and identifying effective types of antioxidants could enhance the quality of life for patients with DM and reduce the risk of serious complications in patients with uncontrolled hyperglycemia.

In summary, understanding the role of EVs in DM provides benefits in several ways, such as identifying biomarkers for monitoring the complications of DM and developing novel therapeutic strategies aimed at modulating EV production, release and uptake. Future research is expected to further elucidate the molecular mechanisms underlying EV-mediated effects in DM, paving the way for innovative treatments that can mitigate the burden of this chronic disease.

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Availability of data and materials

The data generated in the present study are not publicly available due to the ethical consideration but may be requested from the corresponding author.

Authors' contributions

SS conceived, designed and supervised the study, conducted experiments, acquired funding, interpreted data, drafted and critically revised the manuscript for important intellectual content. WC contributed to research design, laboratory work and data analysis. TN contributed to research design, data acquisition, laboratory experiments and data analysis. TB performed statistical analysis and data interpretation. SJ was involved in laboratory work, data collection and analysis. SYC assisted with data acquisition and statistical analysis. NWo contributed to research design and data interpretation. NWa was involved in literature review, data analysis and manuscript preparation. DP contributed to laboratory work, and data analysis. IP supervised the study, conducted project administration, acquired funding and provided final approval of the manuscript. All authors contributed to the revision, read and approved the final version of the manuscript and agree to be accountable for all aspects of the work. SS and IP confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The protocol was approved (approval no. WUEC-23-143-01) by the Walailak University Institutional Review Board (Tha Sala, Thailand). Written informed consents were obtained from all participants prior to the commencement of the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Xie D, Shen Z, Yang L, Zhou D, Li C and Liu F: Global, regional, and national burden of type 2 diabetes mellitus attributable to particulate matter pollution from 1990 to 2021: An analysis of the Global Burden of Disease Study 2021. Diabetes Res Clin Pract 218: 111934, 2024.
2. Lu X, Xie Q, Pan X, Zhang R, Zhang X, Peng G, Zhang Y, Shen S and Tong N. Type 2 diabetes mellitus in adults: pathogenesis, prevention and therapy. Signal Transduct Target Ther 9: 262, 2024.
3. Lima JEBF, Moreira NCS and Sakamoto-Hojo ET: Mechanisms underlying the pathophysiology of type 2 diabetes: From risk factors to oxidative stress, metabolic dysfunction, and hyperglycemia. Mutat Res Genet Toxicol Environ Mutagen 874-875: 503437, 2022.
4. Jia G, Bai H, Mather B, Hill MA, Jia G and Sowers JR: Diabetic vasculopathy: Molecular mechanisms and clinical insights. Int J Mol Sci 25: 804, 2024.
5. Zhang M, Wang L and Chen Z: Research progress of extracellular vesicles in type 2 diabetes and its complications. Diabet Med 39: e14865, 2022.
6. Gauthier BR, Cobo-Vuilleumier N and López-Noriega L: Roles of extracellular vesicles associated non-coding RNAs in diabetes mellitus. Front Endocrinol (Lausanne) 13: 1057407, 2022.
7. Gustafson D, DiStefano PV, Wang XF, Wu R, Ghaffari S, Ching C, Rathnakumar K, Alibhai F, Syonov M, Fitzpatrick J, *et al*: Circulating small extracellular vesicles mediate vascular hyperpermeability in diabetes. Diabetologia 67: 1138-1154, 2024.
8. Welsh JA, Goberdhan DCI, O'Driscoll L, Buzas EI, Blenkiron C, Bussolati B, Cai H, Di Vizio D, Driedonks TAP, Erdbrügger U, *et al*: Minimal information for studies of extracellular vesicles (MISEV2023): From basic to advanced approaches. J Extracell Vesicles 13: e12404, 2024.
9. Wei J, Wang Z, Han T, Chen J, Ou Y, Wei L, Zhu X, Wang K, Yan Z, Han YP and Zheng X: Extracellular vesicle-mediated intercellular and interorgan crosstalk of pancreatic islet in health and diabetes. Front Endocrinol (Lausanne) 14: 1170237, 2023.
10. Sun Y, Tao Q, Wu X, Zhang L, Liu Q and Wang L: The utility of exosomes in diagnosis and therapy of diabetes mellitus and associated complications. Front Endocrinol (Lausanne) 12: 756581, 2021.

11. Pretorius L, Thomson GJA, Adams RCM, Nell TA, Laubscher WA and Pretorius E: Platelet activity and hypercoagulation in type 2 diabetes. Cardiovasc Diabetol 17: 141, 2018.
12. Gustafson D, Veitch S and Fish JE: Extracellular vesicles as protagonists of diabetic cardiovascular pathology. Front Cardiovasc Med 4: 71, 2017.
13. Collado A, Humoud R, Kontidou E, Eldh M, Swaich J, Zhao A, Yang J, Jiao T, Domingo E, Carlestål E, *et al*: Erythrocyte-derived extracellular vesicles induce endothelial dysfunction through arginase-1 and oxidative stress in type 2 diabetes. J Clin Invest 135: e180900, 2025.
14. Garcia-Herreros A, Yeh YT, Peng Z and Del Álamo JC: Cyclic mechanical stresses alter erythrocyte membrane composition and microstructure and trigger macrophage phagocytosis. Adv Sci (Weinh) 9: e2201481, 2022.
15. Catan A, Turpin C, Diotel N, Patche J, Guerin-Dubourg A, Debussche X, Bourdon E, Ah-You N, Le Moullec N, Besnard M, *et al*: Aging and glycation promote erythrocyte phagocytosis by human endothelial cells: Potential impact in atherothrombosis under diabetic conditions. Atherosclerosis 291: 8798, 2019.
16. Sudnitsyna, J, Skverchinskaya E, Dobrylko I, Nikitina E, Gambaryan S and Mindukshev I: Microvesicle formation induced by oxidative stress in human erythrocytes. Antioxidants (Basel) 9: 929, 2020.
17. Viskupicova J, Blaskovic D, Galiniak S, Soszyński M, Bartosz G, Horakova L and Sadowska-Bartosz I: Effect of high glucose concentrations on human erythrocytes in vitro. Redox Biol 5: 381-387, 2015.
18. Loyola-Leyva A, Alcántara-Quintana LE, Terán-Figueroa Y and González FJ: The in vitro effect of high glucose concentrations on erythrocyte morphology assessed by scanning electron microscopy. Micron 154: 103179, 2022.
19. Batista da Silva MV, Alet AI, Castellini HV and Riquelme BD: A new protocol for in vitro red blood cell glycation. Comp Biochem Physiol A Mol Integr Physiol 264: 111109, 2022.
20. Piwkham D, Pattanapanyasat K, Noulsri E, Klaihmon P, Bhoophong P and Prachongsai I: The in vitro red blood cell microvesiculation exerts procoagulant activity of blood cell storage in Southeast Asian ovalocytosis. Heliyon 9: e12714, 2022.
21. Bass DA, Parce JW, Dechatelet LR, Szejda P, Seeds MC and Thomas M: Flow cytometric studies of oxidative product formation by neutrophils: a graded response to membrane stimulation. J Immunol 130: 1910-1917, 1983.
22. Tenório MCDS, Graciliano NG, Moura FA, Oliveira ACM and Goulart MOF: N-acetylcysteine (NAC): Impacts on human health. Antioxidants (Basel) 10: 967, 2021.
23. Fiorentino TV, Prioletta A, Zuo P and Folli F: Hyperglycemia-induced oxidative stress and its role in diabetes mellitus related cardiovascular diseases. Curr Pharm Des 19: 5695-5703, 2013.
24. Amer J, Goldfarb A and Fibach E: Flow cytometric measurement of reactive oxygen species production by normal and thalassaemic red blood cells. Eur J Haematol 70: 84-90, 2003.
25. Bazzoni R, Takam Kamga P, Tanasi I and Krampera M: Extracellular vesicle-dependent communication between mesenchymal stromal cells and immune effector cells. Front Cell Dev Biol 8: 596079, 2020.
26. Doyle LM and Wang MZ: Overview of extracellular vesicles, their origin, composition, purpose, and methods for exosome isolation and analysis. Cells 8: 727, 2019.
27. Zhuge Z, McCann Haworth S, Nihlén C, Carvalho LRRA, Heuser SK, Kleschyov AL, Nasiell J, Cortese-Krott MM, Weitzberg E, Lundberg JO and Carlström M: Red blood cells from endothelial nitric oxide synthase-deficient mice induce vascular dysfunction involving oxidative stress and endothelial arginase I. Redox Biol 60: 102612, 2023.
28. Gonzalez Y, Herrera MT, Soldevila G, Garcia-Garcia L, Fabián G, Pérez-Armendariz EM, Bobadilla K, Guzmán-Beltrán S, Sada E and Torres M: High glucose concentrations induce TNF-α production through the down-regulation of CD33 in primary human monocytes. BMC Immunol 13: 19, 2012.
29. Reddy EC and Rand ML: Procoagulant phosphatidylserine-exposing platelets *in vitro* and *in vivo*. Front Cardiovasc Med 7: 15, 2020.
30. Su Y, Chen J, Dong Z, Zhang Y, Ma R, Kou J, Wang F and Shi J: Procoagulant activity of blood and endothelial cells via phosphatidylserine exposure and microparticle delivery in patients with diabetic retinopathy. Cell Physiol Biochem 45: 2411-2420, 2018.
31. Nagata S, Sakuragi T and Segawa K: Flippase and scramblase for phosphatidylserine exposure. Curr Opin Immunol 62: 31-38, 2020.
32. Brugnara C, Williams A and Van Hove J: Hematologic disorders and the biology of glycophorin A. Blood Reviews 35: 1-15, 2019.
33. Liu J, Guo X, Mohandas N, Chasis JA and An X: Membrane remodeling during reticulocyte maturation. Blood 115: 2021-2027, 2010.
34. Bartosz G: Reactive oxygen species: Destroyers or messengers? Biochem Pharmacol 77: 1303-1315, 2004.
35. Asaro RJ and Cabrales P: Red blood cells: Tethering, vesiculation, and disease in micro-vascular flow. Diagnostics (Basel) 11: 971, 2021.
36. Tian X, Yu H and Zhao Y: Oxidative stress and red blood cell vesiculation: A link to immune modulation. Free Radical Biol Med 167: 122-131, 2021.
37. Willekens FL, Werre JM, Groenen-Döpp YA, Were JM, Groenen-Döpp YA, Roerdinkholder-Stoelwinder B, de Pauw B and Bosman GJ: Erythrocyte vesiculation: A self-protective mechanism? Br J Haematol 141: 549-556, 2008.
38. Burger P, Hilarius-Stokman P, de Korte D, van den Berg TK and van Bruggen R: CD47 functions as a molecular switch for erythrocyte phagocytosis. Blood 119: 5512-5521, 2012.
39. Bosman GJ, Were JM, Willekens FL and Novotný VM: Erythrocyte aging in vivo and in vitro: Structural aspects and implications for transfusion. Transfus Med 18: 335-347, 2008.
40. Oldenborg PA, Zheleznyak A, Fang YF, Lagenaur CF, Gresham HD and Lindberg FP: Role of CD47 as a marker of self on red blood cells. Science 288: 2051-2054, 2000.
41. Zhu H, Liao SD, Shi JJ, Chang LL, Tong YG, Cao J, Fu YY, Chen XP, Ying MD, Yang B, *et al*: DJ-1 mediates the resistance of cancer cells to dihydroartemisinin through reactive oxygen species removal. Free Radical Biology and Medicine 71: 121-132, 2014.
42. Arkew M, Asmerom H, Gemechu K and Tesfa T: Global prevalence of anemia among type 2 diabetic adult patients: A systematic review and meta-analysis. Diabetes Metab Syndr Obes 16: 2243-2254, 2023.
43. Kobayashi H., Shiba T, Yoshida T, Bolidong D, Kato K, Sato Y, Mochizuki M, Seto T, Kawashiri S and Hanayama R: Precise analysis of single small extracellular vesicles using flow cytometry. Sci Rep 14: 7465, 2024.
44. Balbi ME, Tonin FS, Mendes AM, Borba HH, Wiens A and Fernandez-Llimos F: Antioxidant effects of vitamins in type 2 diabetes: A meta-analysis of randomized controlled trials. Diabetol Metab Syndr 10: 18, 2018.
45. Kolahdouz Mohammadi R Hosseinzadeh-Attar MJ, Eshraghian MR, Nakhjavani M, Khorami E and Esteghamati A: The effects of coenzyme Q10 supplementation on glycemic control and lipid profile. J Diabetes Metab Disord 19: 99-109, 2019.

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