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Short Communication

The Impact of Platelet Isolation Protocol on the Release of Extracellular Vesicles

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Abstract

Background: Platelet-derived extracellular vesicles (PEVs) are small vesicles released by activated platelets that are gaining growing interest in the field of vascular biology. The mode of platelet activation is a critical determinant of PEVs release, phenotype and function. However, only very limited information is available concerning the impact of the platelet purification procedure on PEVs release. **Methods**: Washed or isolated platelets were separated by differential centrifugations. For washed platelets, the platelet pellet was washed by resuspension in PIPES buffer and finally resuspended in HEPES buffer. Isolated platelets were obtained by directly resuspending the platelet pellet in HEPES, skipping the washing steps in PIPES buffer. PEVs release was induced in washed or isolated platelets by stimulation with different agonist and analysed by Nanoparticle Tracking Analysis. **Results**: Isolated platelets showed a higher release of PEVs upon adenosine diphosphate (ADP) stimulation compared to washed platelets, whereas PEVs released upon stimulation with strong agonists (thrombin, collagen, A23187, U46619) were similar in the two groups. This different responsiveness to ADP was also observed as a higher α-granules release and protein kinase C activation in isolated platelets compared to washed ones. Residual plasma contamination appeared to be essential for the ability of platelets to release PEVs in response to ADP. **Conclusions**: In conclusion, our study strongly suggests that procedure adopted for platelets preparation is a critical determinant of PEVs release upon ADP stimulation.

Keywords: platelets; platelet-derived extracellular vesicles; platelet preparation procedure

1. Introduction

Platelet-derived extracellular vesicles (PEVs) are small vesicles released by activated platelets, that are gaining growing interest in the field of vascular biology. PEVs are the most abundant EVs in the circulation and they are extensively studied for their roles in a wide range of physiological and pathophysiological processes, including inflammation, cell communication, coagulation, and cancer metastasis [1].

The mechanism supporting platelet activation is a critical determinant of PEVs release, phenotype and function. Several studies have explored this aspect, clearly demonstrating that different stimuli display a different potency in inducing the release of PEVs. Importantly, PEVs release in response to both physiological and pathological stimuli present remarkably distinctive functions, indicating that the mechanisms supporting vesiculation likely influence the composition of PEVs [2]. In this context, only very limited information is available concerning the impact of the platelet purification procedure on PEVs release.

In platelet studies, the isolation protocol is critical since it strongly influences platelet response [3]. The ideal experimental conditions would involve the study of platelets in their physiological environment, however, platelet separation from other blood components is essential to dissect the biology of these cells at molecular and functional levels.

The most common separation procedure involves the washing of platelets. By repeated steps of differential centrifugation and platelet resuspension in specific buffered solutions, platelets are recovered and separated from other blood cells, plasma components and the anticoagulant used for blood withdrawal. Being widely used, the platelet washing procedure is often laboratory customized. Despite some guideline articles and book chapters have been published on this topic to support the authors who approach to the study of platelets, some major differences in the description of the platelet separation protocols adopted are found in literature [4–7]. Although the influence of platelet washing procedure on platelet functionality has been extensively investigated, the information about the impact of platelet separation protocol on the release of PEVs is still limited.

In this work we aimed at investigating this important aspect focusing on two platelet separation procedures used in different published studies.

2. Materials and Methods

2.1 Materials

Thrombin, A23187, prostaglandin E_1 (PGE_1), and apyrase were from Sigma. U46619 was from Enzo. Collagen was from Mascia Brunelli. The antibody against α-2-macroglobulin (H-8) was from Santa Cruz Biotechnology. The antibody against phospho(Ser) PKC substrates was from Cell Signaling Technology. Antibody against CD41 was from Novus Biotechnology. Flow cytometry antibodies, anti-CD62P antibody and anti-PAC-1 antibody were purchased from eBioscience and BD Biosciences, respectively.

2.2 Platelet Preparation

Washed human platelets, as well as isolated platelets, were prepared from buffy-coat bags through a previously described protocol [7]. Briefly, the buffy-coat was diluted with one fourth of its initial volume using a 9:1 solution of HEPES buffer and citric acid/citrate/dextrose (152 mM sodium citrate, 130 mM citric acid and 112 mM glucose) and spun at 120×g for 15 min. A volume corresponding to one third of the upper phase was recovered, added of 1 μM PGE_1 and 0.2 U/mL apyrase and then centrifuged at 750×g for 15 min to recover the platelet pellet. The platelet pellet was washed by resuspension in PIPES buffer (20 mM PIPES and 137 mM NaCl, pH 6.5) and, upon an additional centrifugation at 750×g for 15 min, finally resuspended in HEPES buffer in the presence of 5.5 mM glucose, 1 mM $CaCl_2$, and 0.5 mM $MgCl_2$, at the concentration of 0.5 $\times$ 10^9 platelets/mL. White and red blood cells contaminants in the platelet preparation were monitored using an automated cell counter Sysmex XS-1000i and found to be $<$0.001% and 0.0002% respectively.

Isolated platelets were obtained by directly resuspending the platelet pellet in HEPES buffer plus 5.5 mM glucose, 1 mM $CaCl_2$, and 0.5 mM $MgCl_2$, skipping the washing steps in PIPES buffer.

2.3 Analysis of Platelet Activation

Platelet activation was assessed by flow cytometry analyses. Platelets (at 0.1 $\times$ 10^9/mL) were analysed using anti-CD62P antibody (eBioscience) and anti-PAC-1 antibody (BD Biosciences). Samples were left untreated o stimulated for 15 min at room temperature with ADP (5 μM). The reaction was stopped by eBioscience™ 1-step Fix/Lyse Solution (Invitrogen) 400 μL and samples were analysed on Attune™ NxT Flow Cytometer (Invitrogen). Platelets were identified by forward and side scatter distribution, and by anti-CD61 positivity. Immunoblotting analysis was performed on platelet sample as previously described [8]. Briefly, platelet samples were left untreated or stimulated with ADP (5 min, 37 °C), lysed and separated by SDS-PAGE. Separated proteins were transferred on PVDF membrane and probed using an anti-phospho(Ser) PKC substrates antibody.

2.4 PEVs Generation

Washed and isolated platelets (2 mL at 0.5 $\times$ 10^9 platelets/mL) were incubated with different agonists for 30 min at 37 °C under constant stirring. After stimulation, activation was stopped by addition of 10 mM EDTA and platelets were pelleted by two steps of centrifugation (750 g, 15 min). The supernatant was recovered and ultracentrifuged at 100000 g for 2 h at 10 °C to collect PEVs. PEVs pellets were resuspended in 100 μL of HEPES Buffer and immediately analysed by NTA.

2.5 NTA

Concentration and size distribution of particles in PEVs samples were measured with NanoSight (NS300) (Malvern Panalytical Ltd., Malvern, UK) equipped with NTA software (version 3.4; Malvern Panalytical Ltd., Malvern, UK). All samples were diluted to the appropriate concentration, and five videos of 60 s were recorded for each sample setting camera level to 14, viscosity to "water" (0.909–0.90 cP), at 23 °C. Videos were analysed using NTA software (version 3.4; Malvern Panalytical Ltd., Malvern, UK), with a Detection Threshold of 5. The settings were established according to the manufacturer's software manual (NanoSight NS300 User Manual, MAN0541-01-EN-00, 2017).

2.6 Statistical Analysis

Statistical analysis was performed using the GraphPad Prism 9.2 Software (GraphPad Software Inc., San Diego, CA, USA). Continuous variables were expressed as mean $\pm$ SEM. A $p < 0.05$ was considered to be statistically significant. Statistical analyses were made using paired *t*-test, one and two-way analysis of variance (ANOVA) when appropriated.

3. Results and Discussion

In this study, we hypothesize that the platelet isolation protocol may influence the release of PEVs, since it is known to have a relevant impact on the general functionality of platelets [3]. We have selected two protocols commonly used in literature and described in the materials and methods section. The two platelet preparations were defined as isolated platelets and washed platelets.

To compare the effect of the separation protocol, PEVs were generated from isolated or washed platelets, either under resting conditions or upon stimulation with different physiological platelet agonists. Specifically, platelet agonists known to induce abundant release of PEVs, including thrombin, collagen, the thromboxane A_2 mimetic U46619, and the calcium ionophore A23187, were used. The generated vesicles were characterized by number and size using NTA.

At basal conditions, PEVs released from isolated and washed platelets were similar in terms of both concentration (isolated: 2.8 $\times$ 10^9/mL; washed: 3.3 $\times$ 10^9/mL, $p = 0.29$)

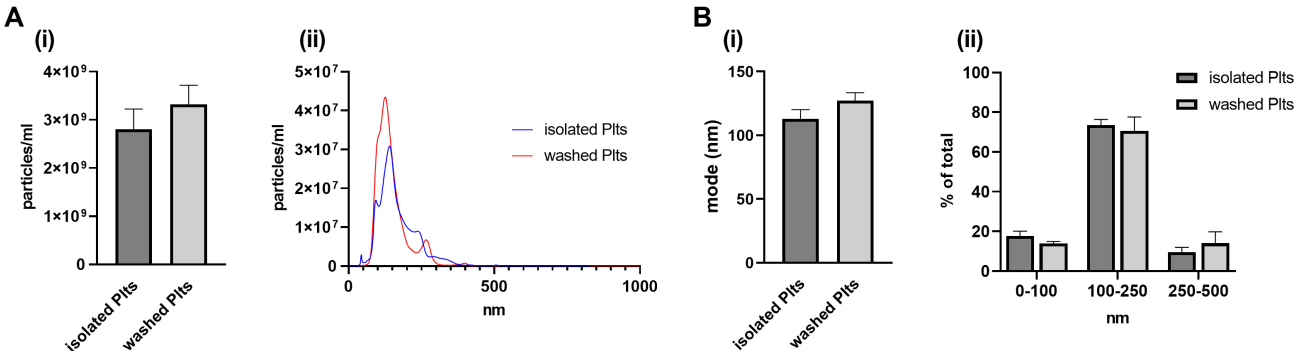

Fig. 1. Release of PEVs in basal conditions. PEVs released from unstimulated (resting) platelets (30 min at 37 °C) were isolated by ultracentrifugation and analysed by NTA. (A) (i) Vesicle concentration, assessed by NTA, is reported as particles/mL and (ii) representative NTA traces. (B) (i) Vesicle average size, measured as mode (nm), and (ii) vesicle size distributions shown as percentages of the total EV populations analysed by NTA. n = 8 independent platelet preparations per group. Data are shown as mean ± SEM.

(Fig. 1A) and dimension (isolated: 111 nm; washed: 113 nm, $p = 0.11$) (Fig. 1B). Most of vesicles ranges from 100 to 250 nm (isolated: 73%; washed: 70%) (Fig. 1B, **Supplementary Fig. 1**).

Upon stimulation with the different agonists (thrombin, collagen, A23187, U46619), an increase of PEVs ranging from 2- to 10-fold compared to respective untreated controls was found in both isolated and washed platelets (Fig. 2A). Under these conditions however, no relevant differences between the two isolation methods were observed in the number of released PEVs ($p = 0.26$; Fig. 2A). In line with previously published results [2], Ca^{2+} ionophore A23187 was the most effective stimulus in inducing the release of PEVs in both isolated (1.93×10^{10} EVs/mL; $p = 0.014$) and washed platelets (2.20×10^{10} EVs/mL; $p = 0.015$). No significant procedure-dependent differences in PEVs size were detected (Fig. 2B), and most of generated PEVs is in the range of 100–250 nm.

Overall, we did not find significant procedure-dependent differences when PEVs were generated upon stimulation with strong inducers of vesiculation. Thus, we next focused our attention on the release of PEVs induced by ADP, which is considered as a weak platelet agonist [9,10]. ADP is a physiological platelet activator mediating its effects via purinergic receptors and playing a central role in thrombus formation. It has been previously reported that responsiveness to ADP is reduced in washed platelets [11]. As shown in Fig. 3, ADP stimulation induced a significant different PEVs release in isolated compared to washed platelets (isolated: 4.99×10^9/mL vs washed: 2.58×10^9/mL, $p = 0.017$) (Fig. 3A). By analyzing size distribution, PEVs released from ADP-stimulated isolated platelets displayed a significantly reduced modal size compared to washed platelets (isolated: 103 nm vs washed: 131 nm, $p = 0.023$) (Fig. 3B), suggesting that ADP-induced activation in isolated platelets was associated to an increased release of smaller vesicles.

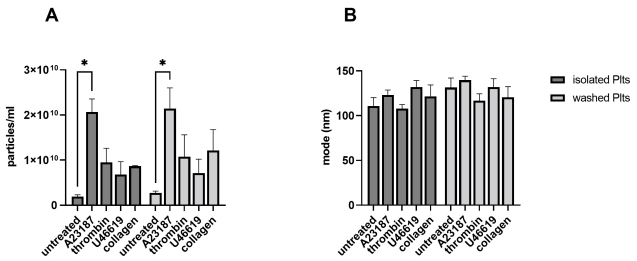

Fig. 2. PEVs generation with different strong agonists. Isolated and washed platelets were left untreated or stimulated with 5 μM A23187, 0.5 U/mL thrombin, 5 μM U46619 and 5 μg/mL collagen for 30 min under stirring. PEVs were isolated by ultracentrifugation and analysed by NTA. (A) concentration and (B) average dimension (measured as mode) of PEVs released in the different conditions. n = 4 independent platelet samples per group. Data are shown as mean ± SEM.

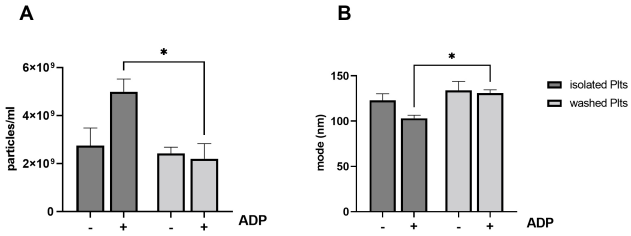

Fig. 3. Release of PEVs from ADP stimulated platelets. Isolated and washed platelets were left untreated or stimulated with 5 μM ADP. PEVs concentration (A) and dimension (B) were analysed by NTA. n = 5 independent platelet preparation per group. Data are shown as mean ± SEM.

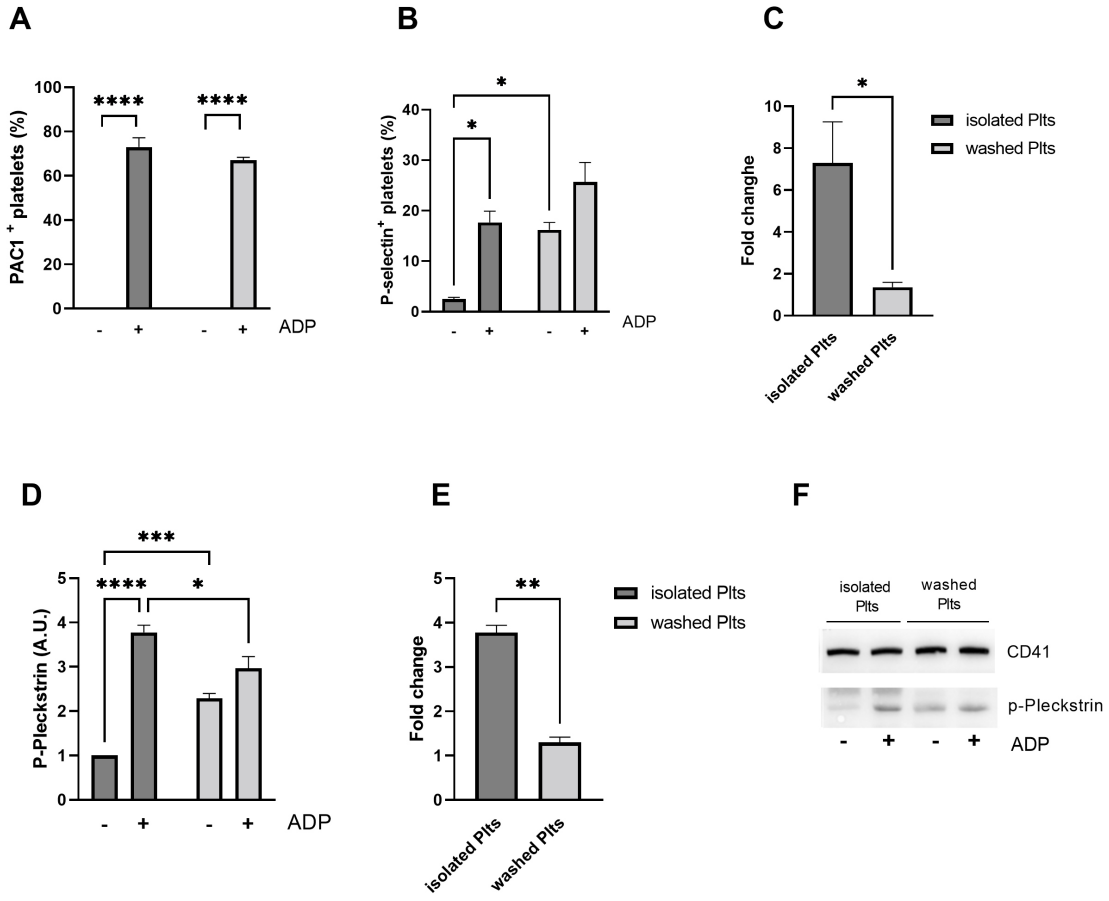

Fig. 4. Platelet activation in response to ADP. Flow cytometric analysis of activation of (A) integrin αIIbβ3 (PAC1) and (B) α-granules release (P-selectin) in isolated or washed platelets left untreated or stimulated with 5μM ADP. (C) P-selectin fold changes of ADP-activated versus matched resting platelets. n = 3 independent platelet preparations per group. (D) quantification, (E) fold changes and (F) representative western blot of PKC activation measured as phosphorylation of Pleckstrin (p-Pleckstrin), the main PKC substrate in platelets. CD41 was used as protein loading control. n = 4 independent platelet preparations per group. Data are shown as mean $\pm$ SEM.

To investigate whether the observed differences resulted from a different general platelet sensitivity to ADP, we assessed agonist-stimulated α-granules release and integrin αIIbβ3 conformational change by flow cytometry analyses. Both isolated and washed platelets showed a strong and similar activation of integrin αIIbβ3 (Fig. 4A), whereas induction of α-granule release, measured as P-selectin exposure, was significantly different in the two groups (Fig. 4B). Interestingly, at basal conditions washed platelets displayed a higher percentage of P-selectin positive cells compared to isolated platelets (p = 0.02), suggesting that washing procedure was associated to a partial α-degranulation. However, a significant induction of P-selectin exposure in response to ADP respect unstimulated control was observed in isolated platelets (p = 0.011), but not in washed platelets (p = 0.13). In particular, as shown in Fig. 4C, the fold changes of activated versus matched resting platelets clearly revealed that ADP induced a 7-fold increase of P-selectin exposure in isolated platelets compared to only a 1.5-fold change in washed platelets (p = 0.042), suggesting a different platelet sensitivity to ADP stimulation.

Interestingly, the activation of Protein Kinase C (PKC), a key player in ADP mediated granules secretion, is in line with α-granules release. ADP induced a significant PKC activation only in isolated platelets ($p < 0.0001$) as shown by the increased phosphorylation of its major substrate Pleckstrin (Fig. 4D–F). Again, resting washed platelets showed a higher PKC activation (p = 0.008) compared to isolated platelets (Fig. 4D). We can then hypothesize that during the washing procedure an incomplete, reversible activation of specific platelet signaling pathways (i.e., PKC-dependent pathways) may occur, leading to a partial α-granule secretion. Indeed, direct stimulation by ADP, failed to induce a strong PKC activation in washed platelets and promoted only a modest increase of P-selectin exposure compared to the unstimulated control (Fig. 4).

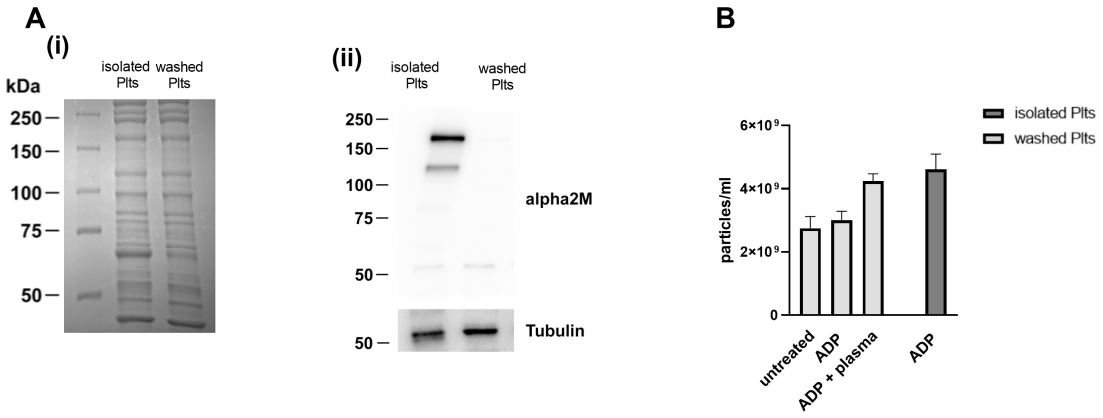

Fig. 5. Analysis of plasma contamination in the two platelets preparations. (A) Lysates of isolated and washed platelets were separated on 7.5% and visualized by (i) Coomassie staining or (ii) by immunoblotting analysis. Representative western blot of α2-macroglobulin (alpha2M) in the two different platelet preparations. Tubulin was used as protein loading control. (B) Quantification of PEVs released from washed platelets left untreated or stimulated with ADP, either in the absence or in the presence of autologous plasma 0.05% (v/v). PEVs released from isolated platelets stimulated with ADP are reported as control. n = 3 independent platelet preparations per group. Data are shown as mean $\pm$ SEM.

So far, we showed that washed and isolated platelets had a similar release of PEVs at basal conditions as well as upon stimulation with a wide range of agonists. By contrast, the two different platelet preparations displayed a clear difference in response to ADP, in terms of PEVs release and platelet activation. To explain these observations, we hypothesized that a residual contamination of plasma components, rather than a limited response of washed platelets due to a preceding undesired platelet activation during the washing procedure, was responsible for the unresponsiveness of washed platelets to ADP in terms of release of PEVs. We have previously demonstrated plasma contamination in the final platelet preparation may have important consequences on platelet functionality in terms of tumor cell-induced platelet aggregation [7]. The analysis of the protein expression pattern, performed by Coomassie blue staining upon SDS-PAGE separation, revealed that isolated and washed platelets display an overall similar composition in terms of the most abundant proteins. However, a strong band between 75 and 50 kDa, likely corresponding to albumin, was detected in isolated platelets, suggesting the presence of a significant plasma contamination. This observation implies that additional contaminant plasma proteins remain in the isolated platelet preparation, although they could not be detected by gel staining. We verified this possibility by immunoblotting analysis, probing the plasma protein α2-macroglobulin, which was actually detected in isolated platelets. As expected, the washing procedure completely eliminated any detectable trace of this plasmatic protein (Fig. 5A).

To investigate whether such plasma contamination could contribute to PEVs release observed in isolated platelets in response to ADP, washed platelets were stimulated with ADP in the presence of small amount (0.05% V/V) of autologous plasma. Traces of autologous plasma were sufficient to rescue the release of PEVs in washed platelets to a level comparable to that observed in isolated platelets (Fig. 5B), suggesting that some plasma components could be responsible for the increased release of PEVs from isolated platelets.

4. Conclusions

Here, we show that platelet responsiveness, in terms of release of PEVs, is strongly influenced by the isolation procedure. The different platelet reactivity is a critical determinant for PEVs release when induced by weak agonist such as ADP, whereas it has no effect upon stimulation with strong agonists. Indeed, any significant difference neither in number of released vesicles, nor in size distribution was observed when isolated and washed platelets were stimulated with thrombin, collagen, thromboxane A2 and calcium ionophore.

It has been previously demonstrated that the composition of PEVs is largely dictated by the stimulus that induce their release [12]. It can be speculated that the platelet purification protocol may influence the mechanism of cargo selection, leading to different vesicle composition in terms of proteins, nucleic acids, and small molecules. Since residual plasma contamination appeared to be essential for the ability of platelets to release PEVs in response to ADP, it is expected that major differences in the cargo composition could be observed when isolated and washed platelets are activated by weak agonists. However, it cannot be excluded that the presence of plasma contaminants could also affect the composition of vesicles released upon stimulation of platelets with stronger agonists. To evaluate this possibility, further investigations exploiting proteome/transcriptome analyses are going to be performed.

Such omics approaches, coupled to bioinformatic analyses, will also allow to predict whether PEVs released by isolated versus washed platelets may also display different functional roles physiological and pathological contexts, such blood coagulation, thrombosis, and cancer. The results collected within these studies will aid the understanding of the complex interplay occurring between platelets, PEVs and the blood microenvironment.

Our results also indicate that the washing procedure may cause a partial preactivation of platelets. Indeed, we detected a higher activation of washed platelets at basal level, implicitly suggesting that using different purification protocols, may lead to different interpretation of the results of α-granules secretion and PKC activation.

In conclusion, our study strongly suggests that procedure by which platelets are isolated is a critical determinant of PEVs release upon ADP stimulation. Future studies are required to in-depth investigate the molecular mechanisms by which plasma influence PEVs release and functionality.

Abbreviations

ADP, Adenosine diphosphate; alpha2M, α2-macroglobulin; NTA, Nanoparticle Tracking Analysis; PEVs, platelet-derived extracellular vesicles; Plts, platelet; PKC, Protein Kinase C.

Author Contributions

SSB and GFG designed the research study. MZ, GDD, MV performed the research. PA, SMGT, LS provided help and advice on manuscript preparation. MZ analysed the data. MZ, GFG, SSB wrote the first version of the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final version of the manuscript.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest. MZ and SSB are serving as the Guest Editors of this journal. We declare that MZ and SSB had no involvement in the peer review of this article and have no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to GP.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2705161.

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