HEMOSTASIS AND THROMBOSIS

Original article

The effect of exosomes released from apheresis platelet concentrates under the impact of gamma irradiation and storage time upon platelet aggregation and hemostasis

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Background - Blood components should be gamma-irradiated (γ-IR) in order to prevent transfusion-associated graft-versus-host disease. The aim of this study is to determine the effect of γ-IR and storage time on the exosomes released from apheresis platelet concentrates (aPC) and to investigate their impact on the maximum platelet aggregation (MPA) and hemostasis.

Materials and methods - Eight units of aPC were included in this study. These were divided into four equal portions. Two portions were irradiated before storage while the other two were not. Thus, irradiated and non-irradiated aPC samples for storage Days 0 (D0) and 5 (D5) were obtained. Exosomes were isolated from these samples using a commercial kit and were evaluated to ascertain their parent cells by flow cytometry. For the following steps, exosomes were pooled according to their features. Pooled exosomes were then used for aggregometry and thromboelastography.

Results - Platelet-derived exosome (PD-EX) levels decreased in D5 compared to D0 in NI-aPC, whereas granulocyte-derived exosome (GD-EX) levels increased. Exosome pools had no effect on MPA compared to saline groups. Exosome pools decreased the time to initial fibrin formation (R), whereas they increased the rate of clot formation (α-angle) and coagulation index (CI) compared to saline groups.

Discussion - Storage time and γ-IR each have almost the opposite effects on PD-EX and GD-EX. Exosomes have no impact on MPA, but enhance the clot strength. The impact of exosomes on aPC quality and effectiveness can be ignored or considered as a positive effect.

Keywords: *aggregation, exosome, gamma irradiation, hemostasis, platelet concentrates.*

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INTRODUCTION

Transfusion can lead to dangerous complications like transfusion-associated graft-*versus*-host disease (TA-GvHD). Donor T lymphocytes can cause this rare complication which can be seen in 0.1-1% of susceptible recipients[1]. TA- GvHD is usually characterised by

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multiple organ failure and pancytopenia, and results in death[2]. Gamma-irradiation (γ-IR) of blood components is the most common process used to prevent TA-GvHD[3]; the lowest γ-IR dose should be around 20-30 Gray (Gy)[4,5]. Some other variables such as the preparation and storage time of blood components may lead to biochemical and functional changes in platelet concentrates (PC) as well as γ-IR[6,7]. Various studies have shown that extracellular vesicles (EV) accumulate[8-10] in PCs during storage. Platelets secrete two types of EV (PEV): microparticles (MP) and exosomes[11]. While γ-IR does not have any impact on total MP[12] release, ionising radiation may have some effects on exosome expression and composition[13]. It has been observed that platelet-derived exosomes (PD-EXs) may suppress platelet aggregation and have the potential to weaken platelet function[14]. In this study, our aim is to determine the effect of γ-IR and storage time on the exosomes released in aPCs and to investigate their impact on maximum platelet aggregation (MPA) and hemostasis.

MATERIALS AND METHODS

This study was carried out with the approval of Clinical Research Ethics Committee of the Faculty of Medicine of Bursa Uludag University: ns. 2019-19/15 and 2021-13/23.

Sample preparation

Based on a similar study conducted by Xie *et al.*[15], the minimum sample size was calculated to be 8 with Type I error 0.05, power 0.95, effect size 1.50. Apheresis PCs were prepared from 8 voluntary blood donors at our blood centre, selected according to national blood donor eligibility criteria. The Trima Accel Apheresis System (Terumo, Lakewood, CO, USA) was used for donation. Acid-citrate-dextrose was used as anticoagulant storage solution (ACD 9%, plasma 91%). One aPC unit from each donor was used for the study. This was divided into four equal portions using a sterile tube connection device (TSCD-T, Terumo) and transferred into platelet storage bags (Teruflex, Terumo). Two of the four portions were immediately gamma-irradiated (Gamma Cell Elan 3000, Nordion, Ottawa, ON, Canada) while the other two portions were not. These four small equal units were used as Day 0 (D0) and Day 5 (D5) storage day samples of non-irradiated and irradiated aPCs (NI-aPC and IR-aPC, respectively). The aPCs were stored in a platelet agitator/incubator (Helmer Scientific, Noblesville, IN, USA) at 20-22°C with continuous agitation. The study steps are summarised in **Figure 1**.

Exosome isolation

Exosome isolation was performed from NI-aPC and IR-aPC samples using the Exosome Purification Kit (Mini kit, Norgen, Thorold, ON, Canada) according to the manufacturer's recommendations. Thus, exosome samples (NI.0-Exo, NI.5-Exo, IR.0-Exo and IR.5-Exo) were obtained and stored at –20°C until use.

BCA protein assay

BCA protein assay was used for colorimetric detection and quantitation of total proteins in the exosome samples. Analyses were performed according to the manufacturer's recommendations (Pierce, Thermo Scientific, Rockford, IL, USA).

Pooling

For pooling, a volume containing 200 µg exosome was taken from each exosome sample (NI.0-Exo, NI.5-Exo, IR.0-Exo and IR.5-Exo) and collected into tubes according to their characteristics. These exosome pools (NI.0-Pool, NI.5-Pool, IR.0-Pool and IR.5-Pool) were stored at –20°C until use.

Characterisation of exosomes

Characterisation of exosome was performed in exosome pools and morphological, size distribution, exosome-specific protein, and surface marker expression analysis were all carried out.

Atomic force microscopy

Atomic force microscopy (AFM) was used for morphological analysis (NanoMagnetics Instruments, Oxford, UK). Purified exosomes were diluted 1:500 in PBS and adsorbed onto mica sheets. Adsorbed samples were air-dried and micrometer scale AFM imaging was conducted in non-contact dynamic mode according to the manufacturer's instructions. Scans were analysed using the NMI Image Analyzer software (NanoMagnetics Instruments).

Tunable resistive pulse sensing

Tunable resistive pulse sensing (TRPS) was used for size distribution analysis and to detect the number of exosomes (qNano-Exoid, IZON, Christchurch, New Zealand). Readings were taken using The Exoid Control Suite Software, and NP150 nanopore was used at 47.01 mm stretch (IZON). Samples were read by diluting 100-fold

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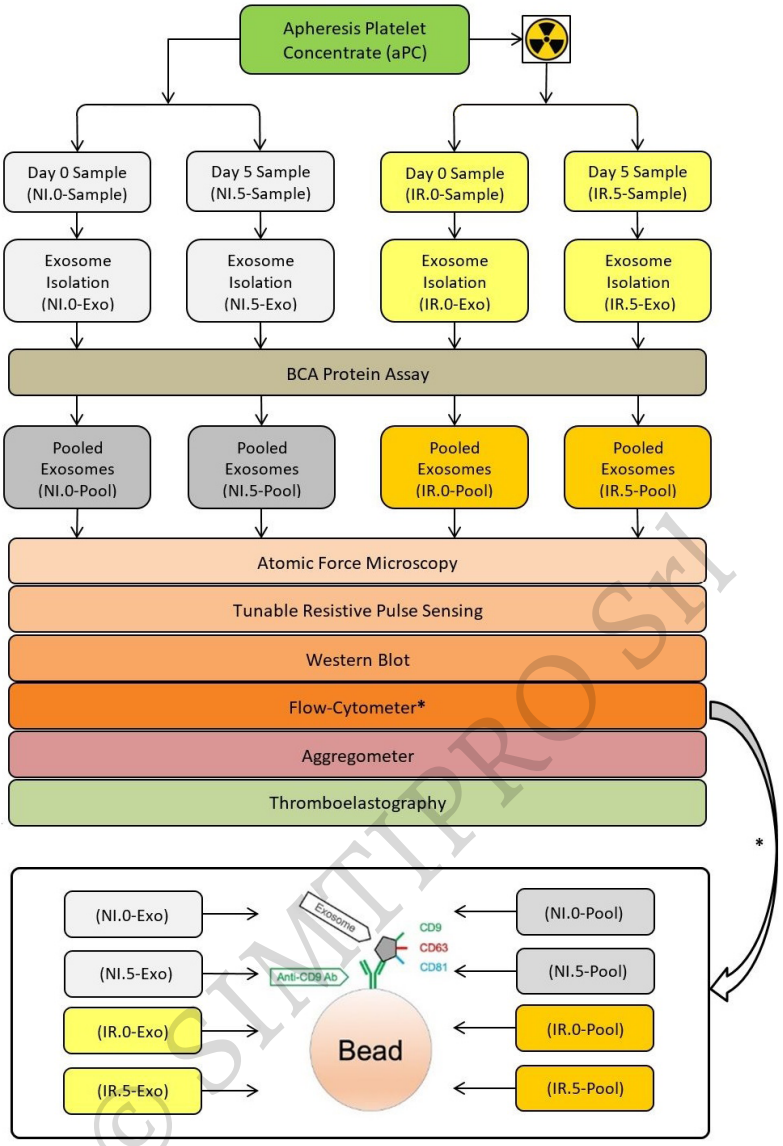


Figure 1 - Study algorithm

with PBS. A minimum of 500 particles were collected for each sample. For the calibration of the samples, standard 120 nm beads (concentration 1.1×10^{13} particles/mL) supplied by the manufacturer were used. The readings were analysed using the Izon Data Suite Software (IZON).

Western blot

Western blot (WB) was used to determine exosome-specific proteins. The protein concentrations of exosome samples were quantified with the BCA Protein Assay kit according to the manufacturer's instructions. For SDS-PAGE, 40 µg of each sample was mixed with reducing 4X Laemmli Buffer and denatured at 95°C for 5 min. Samples were loaded into each well of 4-20% Mini-PROTEAN TGX Stain-Free Protein Gel (Bio-Rad, Dubai, UAE). Gels were transferred into PVDF 0.2 µm membrane using the Mini Trans-Blot Cell System (Bio-Rad) and 1X Tris-glycine buffer with 20% methanol for 90 min at 120 V. The membranes were blocked in 5%BSA-Tween for 2 h at room temperature. As primary antibodies, TSG101 (Abcam, Cambridge, UK), Flotillin-1 (Cell Signaling Technology, Danvers, MA, USA) and GRP94 antibody (Santa Cruz Biotechnology, Dallas, TX, USA) were used and incubated overnight at 4°C. Anti-rabbit, anti-rat and anti-mouse secondary antibodies

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were used and incubated for 1 h at room temperature. The HRP activity was detected with ECL Prime HRP Reagent (Cytiva) and imaged by an Amersham Imager 600 (GE Healthcare, Buckinghamshire, UK).

Flow cytometry

Flow cytometry (FC) was used for surface marker expression and parental analysis of exosomes. Carboxyl latex beads (4% W/V 3.5 µm; Invitrogen, Carlsbad, CA, USA) were used to make these small molecules larger and easily detectable. First, the beads were coated with purified anti-CD9 antibodies (Ab) (Sony Biotechnology, San Jose, CA, USA). For this purpose, 1 µg anti-CD9 Ab was used for each 1 µL of beads. After BCA protein assay, 1 µg exosomes was conjugated with 1 µL of anti-CD9 Ab coated beads. Surface marker expressions (CD9, CD63, and CD81) were evaluated in order to characterise the exosomes in pooled exosome samples. The profiles of exosomes were then evaluated according to their parent cells in each individual exosome sample (NI.0-Exo, NI.5-Exo, IR.0-Exo, IR.5-Exo), not in the pools. These exosome samples were analysed for surface marker expressions specific to the parent cells from which they were derived: CD3, CD4, CD8, CD9, CD11b, CD14, CD15, CD19, CD41, CD56, CD235a and HLA-DR[16]. The fluorochrome-conjugated monoclonal antibodies (mAb) that were used in FC have been summarised in **Table I**. The exosomes were stained with 5 µL mAbs for 1 h in the dark at room temperature. At the end of the incubation period, exosome/bead complexes were centrifuged (450 rcf, 10 min), washed twice with PBS, and analysed by an FC device (Navios, Beckman Coulter, Indianapolis, IN, USA). Analyses were performed in two ways: changes during the storage time in NI-aPC and IR-aPC were compared in the in-group analysis, whereas changes between NI-aPC and IR-aPC were investigated in the intergroup analysis. The gating strategy has been summarised in **Figure 2A**.

Aggregometry

The impact of pooled exosome samples on MPA was evaluated using an aggregometer (Platelet Aggregation Profiler-4; Bio/Data Corporation, Horsham, PA, USA). In addition, the effects of ADP and collagen (Bio/Data Corporation) upon stimulating aggregation were investigated at three different concentrations: 1×, 2×, 5×. Each pooled exosome sample was tested with 8 different platelet-rich plasmas (PRP) taken from different volunteers. To prepare the PRPs, blood samples were obtained from healthy volunteers and were anticoagulated with citrate (3.2%) (citrate/blood ratio: 1/9), centrifuged for 15 min at 250 × *g*, and then transferred to another tube. Platelet counts of PRPs were taken using a hemogram device (Cell-Dyn, Abbott, Abbott Park, IL, USA). The PRP-removed blood sample was centrifuged at 2,000 × *g* for 15 min to obtain platelet-poor plasma (PPP). Aggregation analyses were performed by optical

Table I - *Fluorochrome-conjugated monoclonal antibodies*

Antibodies	Product
Anti-CD9-FITC	Fluorescein isothiocyanate, Beckman Coulter, Indianapolis, IN, USA
Anti-CD81-FITC	Fluorescein isothiocyanate, Beckman Coulter, Indianapolis, IN, USA
Anti-CD63-PE	Phycoerythrin, eBioscience; Waltham, MA, USA
Anti-CD41-PE	Phycoerythrin, eBioscience; Waltham, MA, USA
Anti-CD235a-PE	Phycoerythrin, eBioscience; Waltham, MA, USA
Anti-CD11b-PE	Phycoerythrin, eBioscience; Waltham, MA, USA
Anti-CD3-APC	Allophycocyanin, Beckman Coulter, Indianapolis, IN, USA
Anti-CD4-APC	Allophycocyanin, Beckman Coulter, Indianapolis, IN, USA
Anti-CD14-APC	Allophycocyanin, Beckman Coulter, Indianapolis, IN, USA
Anti-CD8-PC5	Phycoerythrin-cyanin5, Beckman Coulter, Indianapolis, IN, USA
Anti-CD15-PC5	Phycoerythrin-cyanin5, Beckman Coulter, Indianapolis, IN, USA
Anti-CD56-PC5	Phycoerythrin-cyanin5, Beckman Coulter, Indianapolis, IN, USA
Anti-CD3-ECD	Phycoerythrin-TEXas Red-x, Beckman Coulter, Indianapolis, IN, USA
Anti-CD19-ECD	Phycoerythrin-TEXas Red-x, Beckman Coulter, Indianapolis, IN, USA
Anti-HLA-DR-PE/Dazzle	Biolegend, San Diego, CA, USA

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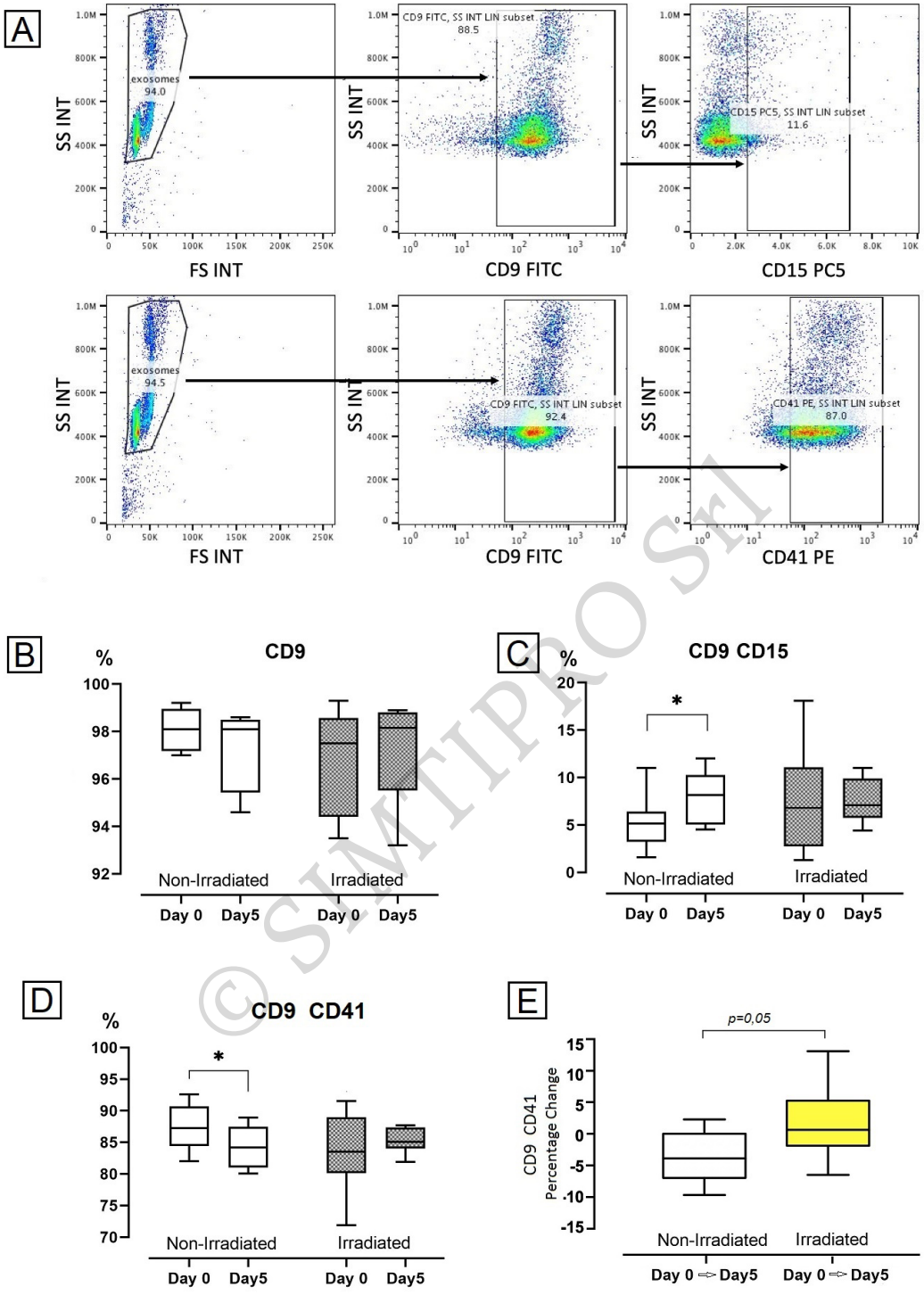


Figure 2 - Flow-cytometry gating strategy and parental analysis results for exosome samples
(A) Exosomes were gated by forward (FS) and side scatter (SS) properties, and also, by SS and surface expression of CD9. Their subgroups were gated and assessed surface expressions (CD41, CD15, etc.) in CD9 positive population. Representative flow-cytometry dot plots showing the granulocyte ($CD9^+CD15^+$) and platelet ($CD9^+CD41^+$) derived exosomes' gating strategies. **(B**, **C** and **D)** Exosome levels in the non-irradiated and irradiated apheresis platelet concentrates samples. Graphs showing the percentage of the total exosomes **(B)** and the exosomes derived from CD15 and CD41 positive cells **(C** and **D**, respectively**)** on day 0 and day 5. n=8 for each column. *p=0.049. **(E)** Percentage changes of $CD9^+$ $CD41^+$ exosomes during the storage time in the non-irradiated and irradiated apheresis platelet concentrates. n=8 for each column.

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aggregometer. Following the calibration via PPP, 490 µL PRP was placed to each channel. Then, saline (one channel) and pooled exosome samples (other channels) were added (4 µL saline or exosome for every 100,000 platelets in the PRP). After 6 min of incubation, 4 µmoL (2×) ADP was added to each channel. The process was followed for 20 min, and the MPA percentages were compared between the exosome and saline groups. This analysis was repeated five times, using four times the exosome volumes in order to evaluate the dose-effect, and nine times with randomly selected samples (NI.0-Exo, NI.5-Exo, IR.0-Exo and IR.5-Exo), to compare the effect of individual and pooled exosomes.

Thromboelastography

The clotting properties of pooled exosome samples were evaluated using viscoelastic hemostatic assay (TEG-5000 Hemostasis Analyzer System; Haemoscope, Niles, IL, USA). Each pooled exosome sample was tested with 7 different blood samples from different volunteers. Blood samples were obtained from healthy volunteers and were anticoagulated with citrate (3.2%; citrate/blood ratio: 1/9). Platelet counts of samples were measured by a hemogram device. Initially, two test tubes that contain 1 mL blood were prepared. Saline and pooled exosome samples were added to these tubes (8.2 µL saline or exosome for every 100,000 platelets in the blood samples). Measurements were carried out after a 30-min incubation period. After incubation, blood samples were transferred to Kaolin tubes (Haemoscope). TEG plain cups were placed into the device and 20 µL of CaCl2 (Haemoscope) was added to each plain cup. Then, 340 µL of blood sample was transferred to plain cups from Kaolin tubes. Analyses of the following parameters were performed simultaneously for the evaluation of hemostasis using reaction time (R) that represents the initiation phase of enzymatic clotting factors, alpha-angle degree (α) that denotes the rate at which the clot strengthens and is most representative of the cleavage of fibrinogen into fibrin by thrombin, maximum amplitude (MA) that reflects the end result of the platelet-fibrin interaction, G that indicates total clot strength, coagulation index (CI) that represents the overall coagulation status, and LY30 that indicates the degree of fibrinolysis at 30 min after MA is reached. Results of the exosome and the saline groups were compared.

Statistical analysis

Continuous variables were evaluated by Shapiro Wilk test. Descriptive statistics are given as median (Q1; Q3) for continuous variables. We used the Mann-Whitney U test to compare the two groups and the Wilcoxon signed test for intragroup comparisons. Percentage change ([last value-initial value]/initial value) was calculated and used for time dependent variables. α=0.05 was considered statistically significant. Data analysis was performed using IBM SPSS Statistics v. 25 software.

RESULTS

BCA protein assay showed that the exosome levels in the product (aPC) were not affected by the storage time or by γ-IR (*Online Supplementary*, **Table SI**).

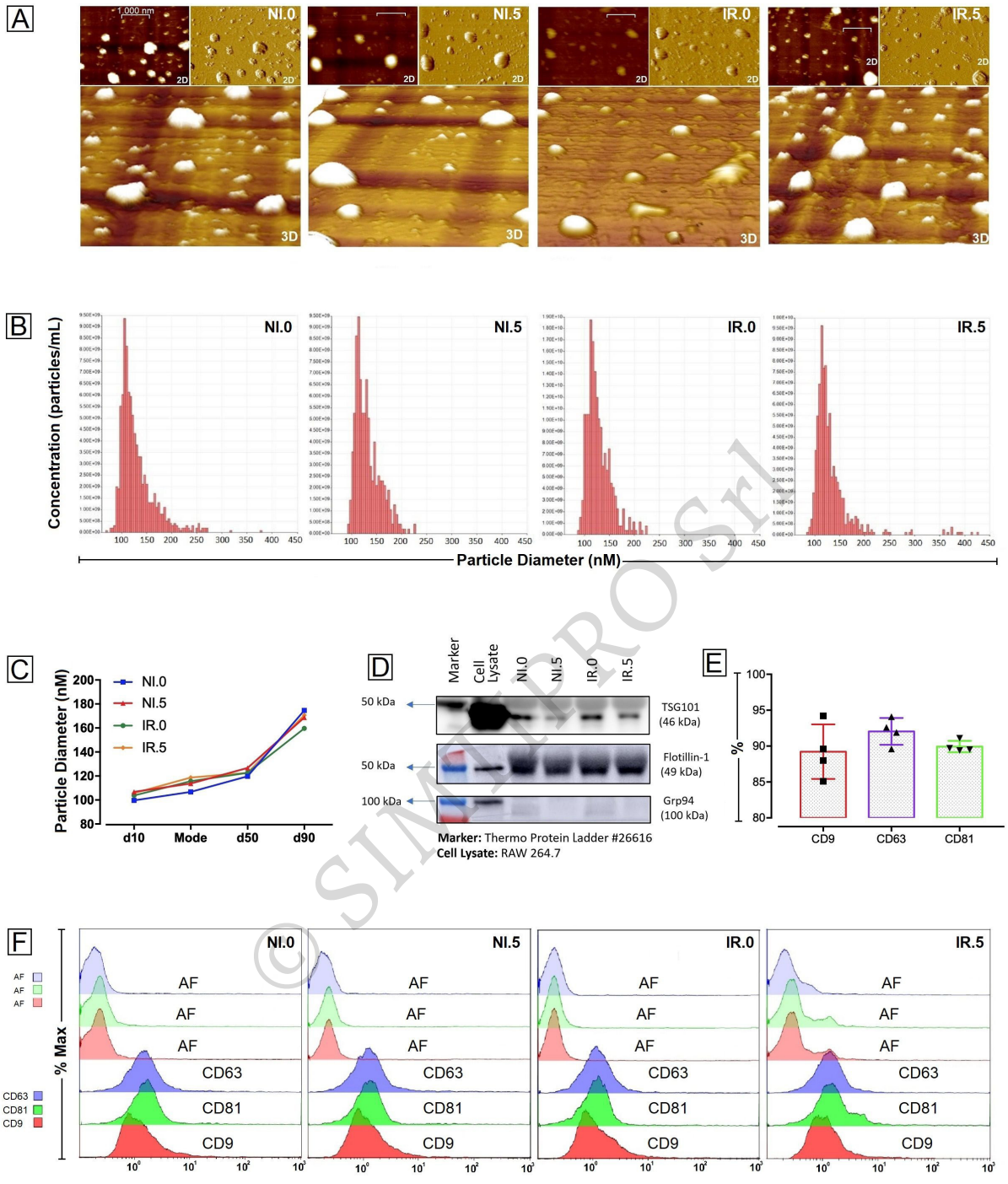
Characterisations of exosomes were carried out in four ways. Exosomes were determined to have typical shape and structure by AFM (**Figure 3A**) and size distributions by TRPS ranging from 99 to 174 nm (**Figure 3B** and **C**). No size difference was detected among the groups. In addition, by WB analysis, exosomes were found to express TSG101 and Flotillin-1 as their specific proteins, but not Grp96 (negative control). However, cell lysate (positive control) expressed all three proteins (**Figure 3D**). Exosomes were also verified according to their CD9, CD63 and CD81 surface marker expression by FC (**Figure 3E** and **F**). These findings show that small EVs derived from NI-aPC and IR-aPC show characteristic exosome features.

Flow cytometry was also used to detect changes in the exosome profile. In-group and intergroup analysis showed no significant differences in total exosome levels ($CD9^+$) ($p>0.05$) (**Figure 2B** and *Online Supplementary* **Table SI**).

Statistically significant changes according to storage time were found in some exosome groups. In the NI-aPC group, the decrease in PD-EX ($CD9^+CD41^+$) levels on storage D5 compared to D0 ($p=0.049$) and the increase in granulocyte-derived exosomes levels (GD-EX) ($CD9^+CD15^+$) ($p=0.049$) were found to be statistically significant (**Figure 2C** and **D**, *Online Supplementary* **Table SI**). Despite the statistical significance, both PD-EXs and GD-EXs levels showed only slight differences during the storage time. In the intergroup analysis, it was only determined that γ-IR affects PD-EXs in aPC. Despite the significant decrease of PD-EXs in NI-aPC samples on D5, their increase in IR-aPC samples was found to have limited statistical significance compared to NI-aPC ($p=0.05$) (**Figure 2E**).

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Figure 3 - Characterization of exosomes

(A) Atomic Force Microscopy (AFM) image of pooled exosome samples. Scale bar represents 1,000 nm. **(B** and **C)** Size distribution of pooled exosome samples. Tunable Resistive Pulse Sensing (TRPS) were used for this purpose and graphs for each analysis are shown in **(B)**. No difference in size was detected among groups **(C)**. **(D)** Western blot analysis of exosomal markers TSG101, Flottilin-1, and GRP94 expression in pooled exosome samples. The cell lysate (RAW264.7 cell line) was used as a positive control, whereas GRP94 was used as a negative control. **(E** and **F)** Surface marker expressions of pooled exosome samples. CD9, CD63, CD81 were analysed using flow cytometry. While percentage values of surface markers were shown in bar graphs **(E)**, flow cytometry graphs for each analysis are given at the bottom of figure **(F)**.

NI.0: non-irradiated Day 0 pooled exosome sample; NI.5: non-irradiated Day 5 pooled exosome sample; IR.0: irradiated Day 0 pooled exosome sample; IR.5: irradiated Day 5 pooled exosome sample; AF: auto-flourescense.

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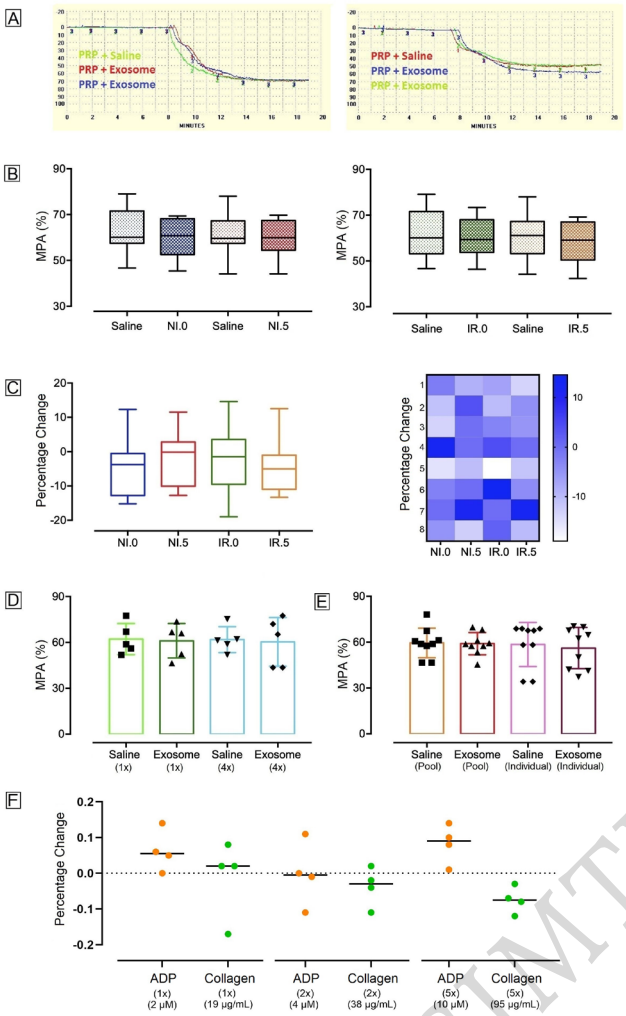


Figure 4 - Impact of pooled exosomes on maximum platelet aggregation (MPA)

(A) Representative aggregometer images from two different experiments. **(B)** Impact of saline and exosomes on MPA. There were no significant changes in the exosome groups compared to saline groups (n=8). **(C)** Percentage change between saline and exosome groups was calculated. The percentage changes between the exosome groups did not show any significance. The percentage change was demonstrated with both a graph and a heat map. The graph presents cumulative effects; the heat map indicates individual results (n=8). **(D)** Dose-related impact of exosomes on MPA. No dose-dependent increase or decrease was found. A similar impact was detected via both (1×) and (4×) exosome volumes (n=5). **(E)** Evaluation of individual or pooled exosomes effects on MPA. Their effect was similar on MPA, a significant difference was not determined (n=9). **(F)** Percentage change between saline and exosome groups for the impacts of ADP and Collagen on the stimulation of aggregation. They both had a similar impact on MPA (n=4).

NI.0: non-irradiated Day 0 pooled exosome sample; NI.5: non-irradiated Day 5 pooled exosome sample; IR.0: irradiated Day 0 pooled exosome sample; IR.5: irradiated Day 5 pooled exosome sample.

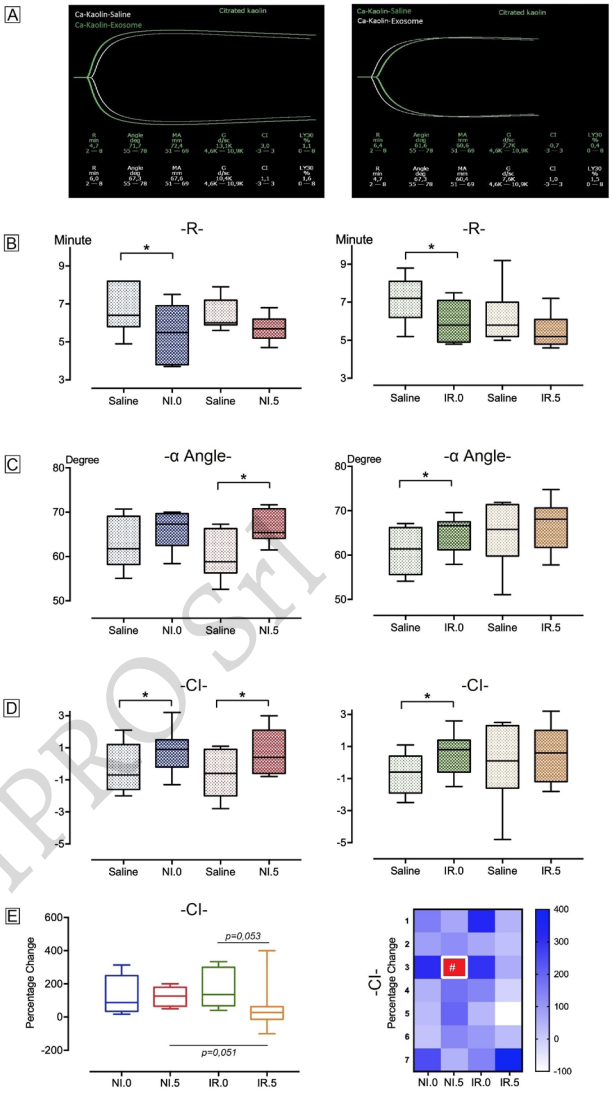


Figure 5 - Impact of pooled exosomes on hemostasis

(A) Representative thromboelastography images from two different experiments. **(B, C** and **D)** The impact of saline and exosomes on hemostasis (n=7). There was a significant decrease in the NI.0 and IR.0 groups compared to saline groups on R-value **(B)**. The significant increases were detected in the NI.5 and IR.0 groups compared to saline groups on α-angle value **(C)**, and in the NI.0, NI.5, IR.0 groups compared to saline groups on CI value **(D)** *p=0.018.

(E) The percentage change between saline and exosome groups was calculated. There were no significant changes in in-group and intergroup analysis via percentage changes. Only CI value in the IR.5-Pool group was close to significance compared to the NI.5-Pool and IR.0-Pool groups (p=0.051 and p=0.053, respectively). This result was demonstrated with both a graph and a heat map. The graph presents cumulative effects; the heat map indicates individual results (n=7). #Percentage change was not calculated, since the related saline group result was "0". NI.0: non-irradiated Day 0 pooled exosome sample; NI.5: non-irradiated Day 5 pooled exosome sample; IR.0: irradiated Day 0 pooled exosome sample; IR.5: irradiated Day 5 pooled exosome sample.

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Results of the aggregometry analyses are summarised in *Online Supplementary* **Table SII**. In the in-group and intergroup analysis for pooled exosomes, no statistical significance was found (p>0.05) (**Figure 4B** and **C**). In addition, no significant difference was seen in the higher exosome dosages. Similarly, no superiority was seen either for the individual or the pooled exosomes (**Figure 4D** and **E**). MPA was found to be affected in almost similar ways by ADP/collagen and their different concentrations (**Figure 4F**, *Online Supplementary* **Table SII**).

Thromboelastography results are summarised in *Online Supplementary* **Table SII**. We found that the R value decreased, while α-angle and CI values increased in pooled exosome groups compared to saline groups (**Figure 5**). The statistical significance of in-group analysis results are shown in **Figure 5**. No changes were observed in MA, G, and LY30 values between the exosome and the saline groups (p>0.05). At the intergroup analysis, no significant differences were observed in R, α-angle, CI, MA, G or LY30 values (p>0.05).

DISCUSSION

This is the first study to investigate the association of exosomes with γ-IR and their effects on maximum aggregation and hemostasis. In our study, it was determined that neither storage time nor γ-IR caused any change in the total exosome level. Most of the exosomes that we identified in aPC were PD-EXs. During the storage time, PD-EX release decreased in the NI group, while it increased in the IR group. The increase in GD-EX release during storage time in the NI group was not observed in the IR group. These results suggest that platelets need stimulation for PD-EX release whereas granulocytes spontaneously express GD-EX, and expression mechanisms can be impaired by some stimuli such as γ-IR. Exosomes released from distinct cells may be affected differently by the storage time and γ-IR. Similarly, it has been reported that while the γ-IR increases the platelet-derived MP release, it decreases the red blood cell-derived MP release[12]. This also suggests that platelets need stimulation for EV release. We think that the PD-EX levels measured on the D0 are probably donor associated. The decrease seen during the storage period in the NI group has been interpreted as PD-EX functioning during the storage time, combining with some target cells, and transmitting their messages. Therefore, the levels may have decreased and not been replaced due to the lack of new exosome release. There seems to be some partial compensation for this decrease in the IR group through the effect of γ-IR. In addition, the increase in GD-EX levels seen in the NI group during storage time has been thought to be related to an acute response of parent granulocytes to storage conditions. Granulocytes are short-lived cells and it is most likely that they release their exosomes and disappear during storage D1.

It is known that γ-IR does not affect PC quality[7,17-19]. Therefore, the slight changes in the exosome profile due to the effect of γ-IR have no impact on PC quality and effectiveness. Nevertheless, they may have some effect on PC recipients owing to their cargo content that can change depending on stimulation because the cargoes that provide their functions are organised according to the physiological state of the parent cell[20], the drugs that are used[21], etc., and therefore the effects of exosomes can be pleiotropic[22]. It is not known whether the cargo contents of exosomes isolated from aPC change during storage, but Huang *et al.* showed that some exosomal miRNAs increase during storage in blood components[23]. This study suggests that exosomes can organise their own cargo contents. We considered that exosomes within aPC might have effects on PC quality and effectiveness, even if there is no significant change in their levels.

We analysed the effects of exosomes released from aPC onto MPA and hemostasis. Srikanthan *et al.* reported that PD-EXs have the potential to weaken the platelet aggregation[14]. We found that exosomes did not have a significant impact on the MPA, even though they slightly decreased the MPA compared to the saline group. In our study, exosomes were isolated from aPC and contained the exosomes released from various cell sources such as PD-EXs and GD-EXs. There may be some discordance between studies because Srikanthan *et al.* only used PD-EXs, or different methods and experimental conditions may have had an impact. Effects of PD-EXs on thrombosis have been found to be suppressive[14] or enhancer[24] in two studies where different isolation methods were used. In our study, exosomes did not show a significant effect on MPA even if most of them consisted of PD-EXs. There was no effect of γ-IR and storage time on MPA. Percentage changes had no significance in

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in-group and intergroup analysis, and there was no relation between exosome quantity and MPA. These results may suggest that γ-IR and/or stored aPC transfusion may not affect the recipient's platelet aggregation via exosomes, and aPC quantity might not change this result.

One study indicated that EVs isolated from PC increased the MA and the α-angle values[25]. Similarly, we demonstrated that the R value decreased in the NI.0-Pool and IR.0-Pool groups, the α-angle value increased in the NI.5-Pool and IR.0-Pool groups, the CI value increased in the NI.0-Pool, NI.5-Pool and IR.0-Pool groups in TEG analysis, but almost all results were within the reference values. These findings show that exosomes increased the clot strength. But there was no significant effect of the IR.5-Pool group on R, α-angle and CI values. Exosomes that were released from the irradiated and 5-day storage aPCs cannot enhance clot strength like other groups. In addition, we detected no impact of γ-IR and storage time on clot strength or hemostasis. There were no significant changes in in-group and intergroup analysis via percentage changes. Only the CI value in the IR.5-Pool group was close to significance compared to the NI.5-Pool and IR.0-Pool groups (**Figure 5E**). This result supports our suggestion related to the irradiated and D5 aPCs. One interpretation of these findings is that the transfusion of exosomes released from aPC (except irradiated and stored aPC) may help improve the clot strength in recipients and their effect can be considered to be harmless.

It was noted that conditions such as bleeding times, hemorrhage, thrombin generation, platelet aggregation, venous thrombus formation, and clot strength can be positively or negatively affected by PD-EVs or PD-EXs[14,24-26]. Although we could not determine the effect of exosomes on MPA, we noted their effects on clot strength. Therefore, we think that exosomes do not have any negative effects on aPC quality.

CONCLUSIONS

Our study shows that storage time and γ-IR each have almost the opposite effect on PD-EX and GD-EX. It was determined that exosomes isolated from aPC had no impact on MPA and enhanced clot strength. No relationship could be found among changes at the exosome levels, the γ-IR and storage time, and their effect on the MPA and hemostasis. These results indicate that the impact of exosomes on aPC quality and effectiveness can be ignored or considered as a positive effect.

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AUTHORSHIP CONTRIBUTIONS

SHB is responsible for hypothesis generation and study design.SHB, ES, MK, MAK, MY, TCY, and DKG performed the experiments and collected data. SHB, ES, FEC, FB, and HBO performed statistical analysis and interpreted data. SHB, ES, LTK, DYE, and YH carried out the literature search and wrote the manuscript. LTK, DYE, YH, IG, and HBO critically reviewed the manuscript.

The Authors declare no conflicts of interest.

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