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ORIGINAL PAPER

Functional characteristics and clinical effectiveness of platelet concentrates treated with riboflavin and ultraviolet light in plasma and in platelet additive solution

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Background and Objectives Pathogen reduction technologies may affect platelet quality during storage. We studied functional characteristics and clinical effectiveness of platelet concentrates (PCs) treated with Mirasol in plasma and in platelet-additive solution SSP+.

Materials and Methods Mirasol-treated, gamma-irradiated and untreated apheresis PCs were examined on days 0, 1, 3 and 5 of storage. Phosphatidylserine, P-selectin and active glycoprotein IIb/IIIa were analysed using flow cytometry before and after platelet stimulation. Platelet count increments, the numbers of inefficient transfusions and post-transfusion reactions were analysed to estimate clinical effectiveness.

Results A significant increase in all platelet activation markers occurred during storage in all PC groups. Activation markers in Mirasol-treated samples were already significantly higher compared with the control ones on the day of harvesting, and continued to grow during the storage. Mirasol treatment increased the number of platelets with a mitochondrial membrane potential loss. On the 3rd day of storage, 50% of Mirasol-treated platelets did not respond to activation; on the 5th day, none did. This agreed well with a decrease (approximately twofold) in the effectiveness of Mirasol-treated PC transfusions. Transfusions of PCs stored in SSP+ were accompanied by fewer inefficient transfusions and post-transfusion reactions than of PCs stored in plasma.

Conclusion Treatment with Mirasol decreased platelet function, particularly profoundly on the 5th day of storage, and led to a decrease in the effectiveness of transfusions. SSP+ did not affect laboratory parameters significantly compared with plasma, but decreased the percentage of transfusion complications.

Key words: pathogen inactivation, platelet concentrates, platelet function, platelet transfusion, SSP+.

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Introduction

Allogenic platelet transfusions are important in clinical practice as an efficient treatment and prevention of thrombocytopenic haemorrhagic syndrome. Development of the syndrome is associated with a reduction in platelet number in the peripheral blood (thrombocytopenia) or platelet dysfunction as a result of depression of blood, associated with various diseases of the haematopoietic system, myelotoxic effect of cytotoxic drugs and/or radiation therapy [1, 2]. The main and most difficult task of transfusion medicine is to improve methods of storage and processing of blood components to ensure its high quality, as well as maximal efficiency and safety of blood transfusions.

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The problems of infectious and immunological safety remain urgent for transfusion therapy. Established and emerging infections such as human immunodeficiency virus, hepatitis B and C viruses, West Nile virus, dengue virus, chikungunya virus, etc. can be transmitted by transfusion [3, 4]. Immunological transfusion complications (after effects) are associated with the presence of viable allogeneic leucocytes in blood components, despite the blood filtering. These include febrile nonhaemolytic reactions, acute lung injury associated with transfusion, alloimmunization to leucocyte antigens, forming refractory to PC transfusions and transfusion-associated disease 'graft-versus-host disease' [5]. Standard processing of blood components with gamma radiation (25–30 Gy) results in a reduction in viable leucocytes by 5–6 $\log_{10}$ [6].

To minimize the risk of transfusion-transmitted infections, introduction of various technologies is advisable for inactivation of pathogens and donor leucocytes in blood components. There are three most commonly used techniques for pathogen inactivation of PCs: riboflavin/UVA–UVB (Mirasol PRT, pathogen reduction technology system, Caridian-BCT Biotechnologies, USA), amotosalen/UVA (Intercept Blood System, Cerus Concord, CA, USA) and UVC (Theraflex-UV, Macopharma, France) [7, 8]. All these techniques use UV light; Mirasol and Intercept require a photosensitizer (riboflavin or amotosalen, respectively). Photochemical action leads to inactivation of pathogens by damaging their nucleic acids. All of these technologies allow to quickly obtain PCs free of pathogens (viruses, bacteria, protozoa) and can effectively inactivate residual donor leucocytes [9, 10]. The additional convenience of Mirasol is that no need for removal of the photosensitizer after treatment [11].

However, it is known that pathogen inactivation may result in a reduced platelet quality, increased markers of activation during storage and reduced agonist-induced platelet activation. Several studies specifically investigated the quality of Mirasol-treated platelets [12–17]. However, their conclusions do not completely agree with each other, and there is little information about the mechanisms underlying changes in platelets, or about correlation of laboratory studies with clinical effectiveness. As activator, TRAP is usually used that is known to be a very poor inducer of phosphatidylserine (PS) externalization [18, 19].

Here, we attempted to investigate the changes of Mirasol-treated platelets during storage by (1) using a comprehensive panel of platelet activation markers; (2) stimulating platelets with collagen-related peptide (CRP) that acts via glycoprotein VI, which is a main PS-inducing pathway; (3) correlating our findings with clinical data; (4) evaluating the effect of additive solution SSP+; (5) investigating mitochondrial function in order to get insight into the mechanisms of platelet function loss.

Materials and methods

Materials

The sources of the following materials are as follows: Alexa Fluor 647-conjugated annexin V (Biolegend, San Diego, CA, USA); fluorescein isothiocyanate (FITC) – conjugated PAC-1 antibody (BD Biosciences, San Jose, CA, USA), phycoerythrin (PE) – conjugated CD62P antibody (eBioscience, San Diego, CA, USA); tetramethylrhodamine methyl ester (TMRM), HEPES, bovine serum albumin (Sigma-Aldrich, St Louis, MO, USA). Collagen-related peptide (CRP) was kindly provided by Prof R.W. Farndale (University of Cambridge, Cambridge, UK).

Study design

Every PC was collected into autologous plasma and/or additional solution SSP+ and was divided into three parts – untreated control, X-ray irradiated and Mirasol treated. All products were stored at 22°C with agitation for a maximum of 5 days. About 10 ml sample was removed from the storage bags on days 0, 1, 3 and 5 for cell quality analysis.

PCs collection and treatment

PCs were obtained from healthy donors. All donors were informed of the nature of the evaluation and gave written consent. PCs were collected using Trima Accel apheresis system (Caridian BCT) running version 5.1 software into autologous plasma (n = 10) or into additional solution (n = 10) SSP+ (MacoPharma, Mouvaux, France). SSP+ solution was added instantly at the end of apheresis procedure not less than 40 ml for $6{\cdot}0 \times 10^9$ cells. The ratio of plasma and SSP+ was 2:3 (40% donor plasma, and 60% SSP+). Volume was 570 ml for PCs collected into plasma or 610 ml for PCs collected into SSP+, with yield of $8{\cdot}5 \times 10^{11}$ platelets per bag. All PCs were leucoreduced, and residual WBC content was below 10^6/product. PCs were divided into three parts as follows: 360 ml of PC in plasma or 450 ml of PC in SSP+ was used for Mirasol treatment, 80 ml of plasma or SSP+ PCs was gamma-irradiated and 105 ml of each PC was left as untreated control.

X-ray irradiation was performed on X-ray machine ARDOK-1 (VELIT, Istra, Russia) no later than 2 h after harvesting. The Mirasol-treated PCs were prepared as follows. After the 2-h rest period, the PC was transferred into an illumination/storage bag and riboflavin solution

was added (500 μmol/l, 35 ± 5 ml). Then, the bag was sealed using the MIRASOL PRT Welder, placed in the illuminator and exposed to light at 6·24 J/ml. Mirasol treatment was the same for *in vitro* and clinical studies. The dilution by adding riboflavin was by 10% and here we neglected this.

In vitro testing

Platelet count and morphometric parameters were performed on an automated haematology analyser Sysmex KX-21 (Sysmex Corp., Kobe, Japan). For flow cytometry, PCs were diluted 1:20 with buffer A (150 mmol/l NaCl, 2·7 mmol/l KCl, 1 mmol/l $MgCl_2$, 0·4 mmol/l Na_2HPO_4, 20 mmol/l HEPES, 5 mmol/l glucose, 0·5% bovine serum albumin (pH 7·4)). Platelet activation marker P-selectin (PE–CD62P), activated form of the glycoprotein GPIIb/IIIa (FITC–PAC-1), and PS (Alexa Fluor 647-annexin V) expression on the surface of platelets were measured by flow cytometry on Accuri C6 (BD Biosciences) before and after platelet stimulation with CRP (20 μg/ml CRP, 2·5 mM $CaCl_2$, 15 min). Activated and non-activated platelets were incubated with 250 ng/ml FITC–PAC-1, 70 ng/ml PE–CD62P and 400 ng/ml Alexa Fluor 647-annexin V for 10 min. The platelet population was defined by forward- vs. side scatter characteristics. Isotype controls were used to estimate non-specific binding.

Changes in platelet mitochondrial transmembrane potential were determined using TMRM. Platelets were preincubated at 4×10^5 μl^{-1} with 10 μM TMRM for 15 min, followed by incubation at 2×10^5 μl^{-1} with Alexa Fluor 647-conjugated annexin V in buffer A in the presence of 2·5 mM $CaCl_2$.

Interventions

Four groups of PCs transfusion were analysed:

Group 1 (PCs stored in plasma): n = 387 (132 f, 255 m), median age of 11 years (0·3–21) with diagnoses acute lymphoblastic leukaemia (ALL) n = 223, acute myelogenous leukaemia (AML) n = 164.

Group 2 (PCs stored in plasma and treated with Mirasol): n = 70 (32 f, 38 m), median age of 15 years (2·4–18) with diagnoses ALL n = 30, AML n = 40.

Group 3 (PCs stored in SSP+): n = 257 (77 f, 180 m), median age of 10 years (0·3–21) with diagnoses ALL n = 152, AML n = 105.

Group 4 (PCs stored in SSP+ and treated with Mirasol): n = 100 (38 f, 62 m), median age of 12 years (0·3–21) with diagnoses ALL n = 56, AML n = 44.

Information collected with each transfusion included transfused platelet count, ABO group, date and method (plasma or SSP+) of harvesting of PC and date of transfusion. Patient information for each transfusion was collected before and 24 h post-transfusion and included age, sex, diagnosis, weight, height, ABO group, vital signs and platelet count.

The study included transfusions of PCs for patients with thrombocytopenia less than 20×10^9/l, haemorrhagic syndrome of less than 2 points on the WHO scale, who does not require transfusions of red blood cells, and that there were no conditions associated with increased platelet consumption (DIC, infection, fever, splenomegaly etc). We compared the absolute count increment (ACI) and corrected count increment (CCI) at 24 h after PLT transfusion, the duration of the interval between transfusions, the number of inefficient transfusions and the number of post-transfusion reactions for four groups of PCs transfusions. Inefficient transfusions were those PCs transfusions when the ACI was equal to or below zero, there was no haemostatic effect and retransfusion of platelets was required for the patient to relief of haemorrhagic syndrome.

ACI ($\times 10^9$/l) was determined as the difference between platelet count at 24 h after transfusion and platelet count before transfusion. CCI was calculated as follows: CCI = ACI ($\times 10^9$/l) × BSA (m^2) /platelet transfused ($\times 10^{11}$), where BSA (body surface area) = (height × weight/3600)^0·5. Therapeutic dose was determined as $0{\cdot}55–0{\cdot}6 \times 10^{11}$ platelets per 10 kg body weight of the recipient.

Statistical analysis

The results were analysed using Origin 8.0 software (OriginLabCorp., Northampton, MA, USA). The data are presented as mean ± standard deviations. Normal distribution was not observed in all groups of parameters as determined by the Kolmogorov–Smirnov test. Therefore, Mann–Whitney test was used for statistical comparison of means. F-test was used to compare the clinical efficacy differences between patient groups. P values <0·05 were considered significant.

Results

Platelet count and morphological parameters change during storage

We compared Mirasol-treated PCs with X-ray-treated and untreated PCs. Platelet count, morphological parameters and activation markers before and after activation with CRP were analysed on days 0, 1, 3 and 5 of storage. Changes in platelet count and such parameters as mean platelet volume (MPV), platelet distribution width (PDW) and platelet large cell ratio (P-LCR) over 5 days of storage are presented in Fig. 1. Mean platelet count within all investigated groups of PCs did not change throughout the period of storage. The platelet count in Mirasol-treated

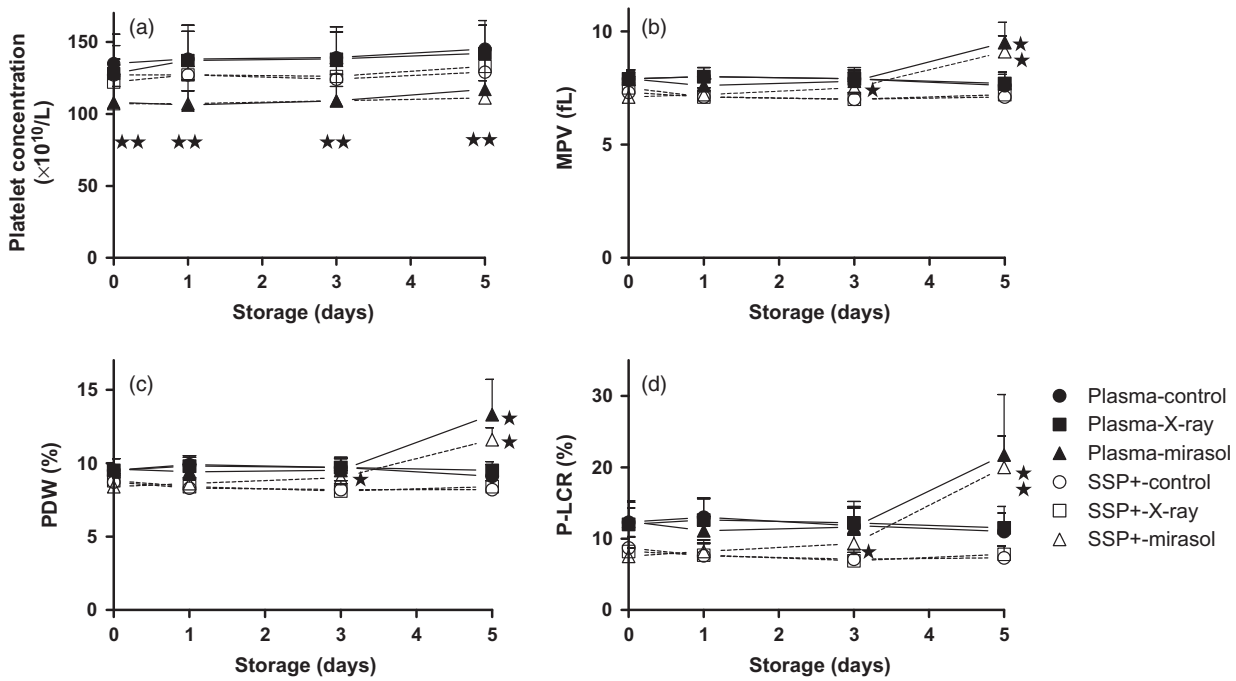

Fig. 1 Morphological characteristics of PCs during storage. (a) platelet count, (b) mean platelet volume (MPV), (c) platelet distribution width (PDW) and (d) platelet large cell ratio (P-LCR). ★$P < 0.05$, Mann–Whitney test was used for statistical comparison between Mirasol-treated samples and untreated control samples stored in plasma ($n = 10$) or SSP+ ($n = 10$).

PCs was about 1·2 times less than in control group, which corresponds to the dilution by adding a solution of riboflavin. This ratio was maintained throughout the storage period for both samples stored in plasma and in SSP+.

Morphological parameters were similar for all investigated groups of PCs stored in plasma or in SSP+ during the first 3 days of storage; on the 5th day, the values of PDW, MPV and P-LCR were increased significantly in Mirasol-treated PCs samples compared with the control, both in plasma and in SSP+.

Spontaneous and CRP-induced platelet activation during storage

Spontaneous P-selectin (Fig. 2a), PS (Fig. 2b) and active form of integrin GP IIbIIIa (Fig. 2c) expression were similar for control and X-ray-irradiated groups of PCs stored in plasma or in SSP+ throughout the storage period. In contrast, in the Mirasol-treated samples, P-selectin and PS expression were significantly higher compared with control already on the day of harvesting. The difference continued to grow during the storage. On the day 5 of storage, P-selectin expression was sixfold greater and PS expression was sixfold to 10-fold greater in Mirasol-treated samples than in the control ones. This was true both for samples harvested in plasma and in SSP+. Therefore, P-selectin and PS appear to be sensitive markers of spontaneous platelet activation during storage.

The number of PAC-1-positive platelets was less than 3% during storage for all groups studied. On the day 5, a slight increase in the PAC-1-positive platelets was observed in Mirasol-treated samples compared with the control.

On the contrary, Mirasol-treated platelets responded to CRP activation increasingly less readily over storage (Fig. 2d–f). By the 5th day, P-selectin expression dropped to the level of spontaneous activation (Fig. 2d), the same was observed for PS exposure (Fig. 2e) and GPIIb-IIIa activation (Fig. 2f). Dynamics of the interrelated changes of spontaneous activation and CRP-induced response are shown in Fig. 2g for PS externalization.

Interestingly, a two-colour analysis reveals that these changes are not correlating between the markers (Fig. 2h). It can be observed that a vast majority of platelets crossing the P-selecting activation boundary remain completely annexin V negative. In contrast, half of the PS-expressing cells are P-selectin-negative. This indicates that there are several mechanisms at work and that the development of activation markers in a given cell does not occur simultaneously.

Changes in mitochondrial membrane potential during storage

To get better insight into the mechanisms of platelet function loss during storage, we investigated the

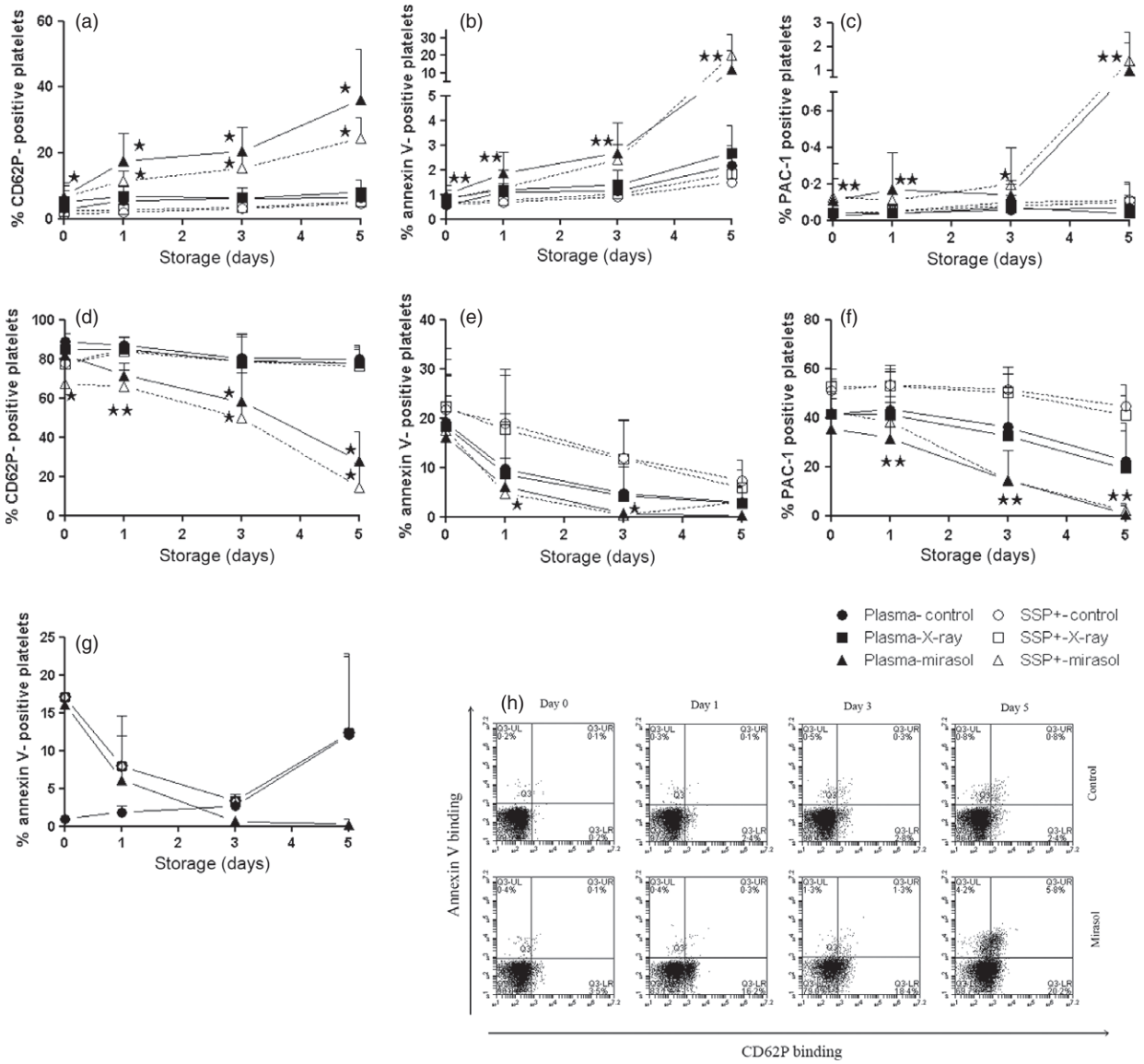

Fig. 2 Spontaneous and CRP-induced expression of platelet activation markers. Graphs (a–f) showing percentage of positive platelets for P-selectin (a, d), PS (b, e) and active GP IIbIIIa (c, f) before (a–c) and after (d–f) CRP activation during 5 days of storage. ★$P < 0.05$, Mann–Whitney test was used for statistical comparison between Mirasol-treated samples and untreated control samples stored in plasma ($n = 10$) or SSP+ ($n = 10$). Graph (g) representing percentage of PS-positive platelets in Mirasol-treated PCs during storage: spontaneous (●), induced (○) and substraction (▲). (h) The typical dot plots show P-selectin and annexin V exposure during storage for Mirasol-treated and untreated PC.

mitochondrial membrane potential using a potential-sensitive dye TMRM in combination with annexin V staining (Fig. 3). Little PS exposure or membrane potential loss was observed in control platelets. In contrast, mitochondrial membrane potential was significantly decreased in Mirasol-treated samples already on day 0, and this progressed with time of storage together with PS exposure. Interestingly, although most TMRM-negative platelets were PS-positive, there were some TMRM-positive PS-positive events; this might indicate another mechanism of cell death or, more likely, formation of aggregates.

Interventions

To evaluate the clinical efficacy of transfusions of PCs harvested in plasma or in SSP+ and treated or untreated with Mirasol, four groups of transfusions were analysed: (1) PCs stored in plasma ($n = 387$), (2) PCs stored in plasma and treated with Mirasol ($n = 70$), (3) PCs stored

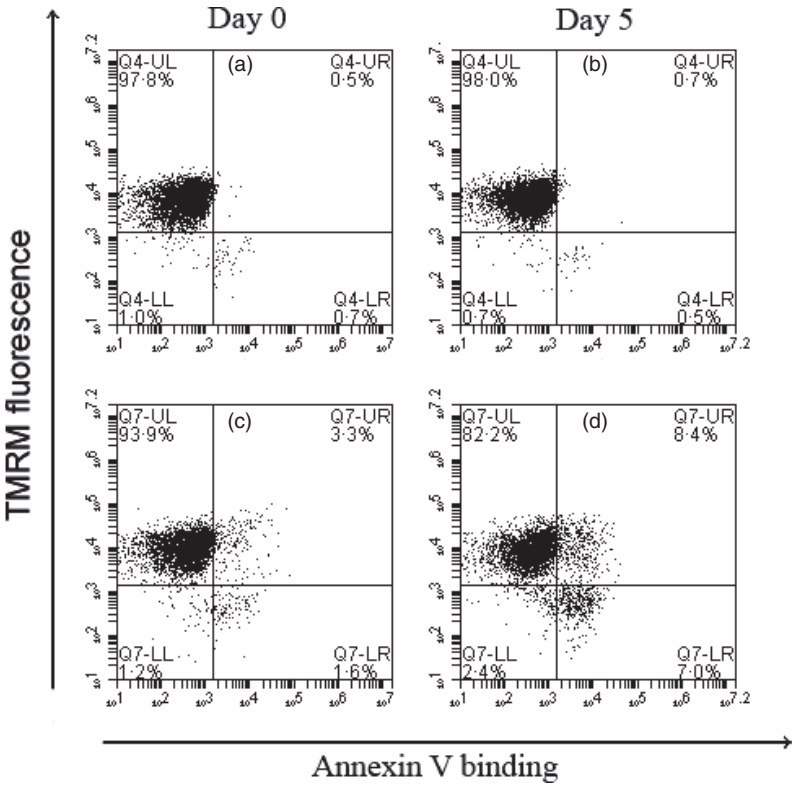

Fig. 3 Changes in mitochondrial membrane potential of platelets during storage. Typical dot plots showing mitochondrial membrane potential decrease and PS externalization in control (a, b) and in Mirasol-treated (c, d) samples stored in plasma.

in SSP+ (n = 257) and (4) PCs stored in SSP+ and treated with Mirasol (n = 101). The parameters of clinical efficacy are presented in Fig. 4.

Mean platelet counts before transfusion were (14·2 ± 6·9) × 10^9/l, (14·6 ± 7·0) × 10^9/l, (13·5 ± 6·5) × 10^9/l and (13·8 ± 6·3) × 10^9/l for 1, 2, 3 and 4 groups,

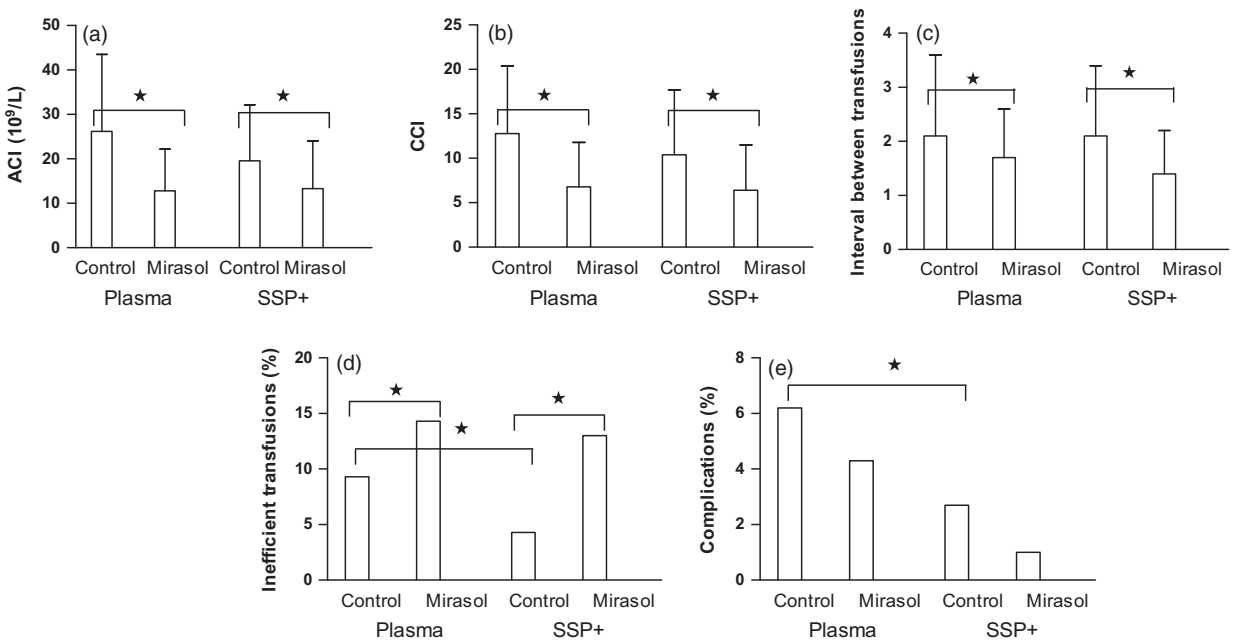

Fig. 4 Effectiveness of transfusion of PCs stored in plasma or in SSP+ and treated or untreated with Mirasol. (a) absolute count increment (ACI) and (b) corrected count increment (CCI) at 24 h after PC transfusion, (c) duration of the interval between transfusions, (d) number of inefficient transfusions and (e) number of post-transfusion reactions (non-haemolytic febrile reactions). $P < 0.05$, F-test was used to compare the clinical efficacy differences between patient groups.

respectively. Mean therapeutic doses were $(2{\cdot}3 \pm 1{\cdot}1) \times 10^{11}$, $(2{\cdot}7 \pm 1{\cdot}1) \times 10^{11}$, $(2{\cdot}3 \pm 1{\cdot}3) \times 10^{11}$ and $(2{\cdot}3 \pm 1{\cdot}0) \times 10^{11}$ for 1, 2, 3 and 4 groups, respectively. The majority of transfusions were performed on days 2 and 3 of PC storage.

Mean platelet count at 24 h after transfusion was $(40{\cdot}3 \pm 17{\cdot}8) \times 10^9$/l, $(27{\cdot}4 \pm 12{\cdot}0) \times 10^9$/l, $(33{\cdot}1 \pm 13{\cdot}5) \times 10^9$/l and $(27{\cdot}2 \pm 12{\cdot}6) \times 10^9$/l for 1, 2, 3 and 4 groups, respectively. In all groups, the platelet number at 24 h after transfusion exceeds the original value, which indicated that satisfactory performance replacement therapy. PC transfusion can be considered successful if minimum 24 h CCI values were more than 4·5 [20].

However, a clear difference (in most cases, a 1·5- to twofold one) between control and Mirasol-treated samples was observed for all parameters of clinical efficacy (Fig. 4a–d). This agrees well with the twofold decrease in platelet responsiveness on Day 3 judging from flow cytometry parameters (Fig. 2). Interestingly, a beneficial statistically significant effect of SSP+ on the number of transfusion complications was observed for control samples (Fig. 4e). In this study, all the transfusion complications were non-haemolytic febrile reactions. There were also less complications with SSP+ for Mirasol-treated samples; and the frequency of complications was also in all cases smaller with Mirasol than without Mirasol (Fig. 4e). However, these changes were not statistically significant, as absolute number of complications with Mirasol was too small, so that it was not possible to determine the reliability of the Mirasol effects within the limits of this study.

Discussion

In the present study, we aimed to investigate functional and clinical significance of changes in PC quality during storage upon Mirasol treatment and use of the SSP+ additive solution. The main conclusions were as follows: (1) Mirasol treatment significantly impaired laboratory parameters of PCs (by the 3rd day, a twofold decrease in platelet responsiveness compared with control or gamma-irradiated platelets; at the 5th day, all platelets were either pre-activated or non-responding); (2) clinical efficacy indicators were also 1·5- to twofold decreased upon Mirasol treatment; (3) PS exposure and P-selectin externalization occurred in different platelet subpopulations, and massive PS externalization was accompanied by the mitochondrial membrane potential loss; and (4) SSP+ did not affect laboratory parameters significantly compared with plasma, but decreased the percentage of transfusion complications.

The overall unfavourable effect of Mirasol treatment on platelet pre-activation and responsiveness *in vitro* agrees well with the majority of previous reports. The results for spontaneous platelet activation in the Mirasol-treated PCs compared with controls are in good agreement with [12–17, 21–23]. An increase in CD62P and annexin V-positive platelets during storage was observed for Mirasol-treated PC both stored in plasma [12, 13, 24] or in platelet-additive solution [13–17, 21–23]. Significant increase in platelet size (MPV) at days 5–6 in Mirasol-treated platelets was noted in [17, 21]. Platelet concentration remained within the initial range throughout storage in most studies [13, 14, 16, 17, 22].

Adverse impact of Mirasol on platelet responsiveness was also reported by several papers. Decreased platelet aggregation response to collagen [22, 25, 26], TRAP [22, 25, 26] and ristocetin [24, 26], spontaneous platelet aggregation due to hyperreactivity [24], adhesive properties deterioration [24] were observed. In contrast, studies [13, 27] reported that Mirasol PRT-treated platelets preserved adhesive and cohesive functions during 5 days; probably, the difference is due to the use of different platelet agonists. Significant decrease in the expression of P-selectin as a result of platelet activation by ADP, CRP or TRAP on 6- to 8-day storage after Mirasol treatment also described [12] and is consistent with our data, although the degree of changes in our study was particularly great. We also observed spontaneous glycoprotein IIb/IIIa activation during storage and significant decrease in agonist-stimulated glycoprotein IIb/IIIa activation described in the [28].

Although activation markers obtained with flow cytometry cannot be used as definitive criterion of quality assessment, the degree of Mirasol-treated platelet deterioration by the 5th day is clearly obvious. All markers used (P-selectin, PAC-1 and PS exposure) agree well in this respect, although P-selectin seems to be the most sensitive one in our work. It is also of substantial interest that, despite this agreement, activation of different parameters occurs in different subpopulations.

Several conflicting conclusions were reported for Mirasol influence on mitochondrial membrane potential. In [14], Mirasol treatment and subsequent storage did not significantly alter mitochondrial membrane polarization. Our experiments are more in line with [20, 28] where Mirasol treatment was accompanied by decrease in mitochondrial membrane potential.

Evaluation of the clinical efficacy in the present study should be interpreted with caution: although the number of patients was significant, they were diverse, and we did not have an opportunity to compare PCs transfused at different days of storage; almost all transfusions were done by the 2nd day. However, the changes in the parameters of clinical efficacy were pro-

nounced and correlated well with impairment observed in the laboratory parameters. It is interesting that SSP+ did not have clear effects on the laboratory parameters of the stored PCs, but significantly affected clinical parameters.

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Conflict of interest

The authors have no conflict of interest to disclose.

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