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ORIGINAL PAPER

In vitro effects on platelets irradiated with short-wave ultraviolet light without any additional photoactive reagent using the THERAFLEX UV-Platelets method

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Vox Sanguinis

Background A novel short-wave ultraviolet light (UVC) pathogen reduction technology (THERAFLEX UV-Platelets; MacoPharma, Mouvaux, France) without the need of any additional photoactive reagent has recently been evaluated for various bacteria and virus infectivity assays. The use of UVC alone has on the one hand been shown to reduce pathogens but may, on the other hand, have some impact on the platelet (PLT) quality. The purpose of this study was to determine the potential effects on PLT quality of pathogen inactivation treatment using the novel UVC method for PLT concentrates.

Study Design and Methods Buffy-coat-derived PLTs suspended in SSP+ were irradiated with UVC light in plastic bags (MacoPharma) made of ethyl vinyl acetate, considered to be highly permeable to UVC light. The UVC-treated (test, $n = 8$) as well as the untreated (reference, $n = 8$) PLT units were stored in PLT storage bags composed of n-butyryl, tri n-hexyl citrate–plasticized polyvinyl chloride (MacoPharma) on a flat bed agitator for *in vitro* testing during 7 days of storage.

Results No significant difference in PLT counts and lactate dehydrogenase between the groups was detected. During storage, glucose decreased more and lactate increased more in the test units. Statistically significant differences were found for glucose ($P < 0{\cdot}01$) and lactate ($P < 0{\cdot}05$) on day 7. ATP levels were higher ($P < 0{\cdot}01$ from day 5) in the reference units. With exception of day 7 ($P < 0{\cdot}01$ reference vs. test), hypotonic shock response reactivity was not different between groups. Extent of shape change was lower ($P < 0{\cdot}01$), and CD62P ($P < 0{\cdot}05$ day 5) was higher in the test units. CD42b and CD41/61 showed similar trends throughout storage, without any significant difference between the units. pH was maintained at >6·8 (day 7) and swirling remained at the highest level (score = 2) for all units throughout storage.

Conclusion Our results suggest that irradiation with UVC light has a slight impact on PLT *in vitro* quality and appears to be insignificant with regard to current *in vitro* standards.

Key words: additive solutions, platelets, storage.

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Introduction

Many remarkable advances in the prevention of transfusion-transmitted infections (TTI) have been made in the past decades. Improved screening and testing strategies

considerably reduced the risk of known transfusion-transmissible viruses, e.g. HIV, HBV and HCV. However, TTI continue to be associated with ongoing morbidity and mortality in transfusion recipients. This is partially due to the potential microbiological risks to the blood supply caused by emerging pathogens. Another transfusion-associated event, transfusion-associated bacteria sepsis, remains the highest risk factor of platelet (PLT) transfusion [1]. The rate of bacterial contamination of PLTs is estimated at 1 in 2000 units. The risk of a severe septic reaction after a PLT transfusion is estimated at 1 in 50 000 units [2].

Several methods have been used to screen for contamination in PLT units in order to prevent bacteria sepsis in transfusion recipients. These methods include microscopic investigation [3], measurement of glucose and pH levels, visual inspection of swirling [4, 5], different cultures methods [6, 7] as well as post-transfusion bacteria detection systems [8]. An additional system indicates the presence of bacteria in case of fall in the oxygen tension, as measured in PLT samples after incubation for 24 h at 35°C [9, 10]. Storage at 4°C is another approach to dealing with bacterial contamination [11, 12]. However, such PLTs must be demonstrated to circulate and survive *in vivo* [13, 14].

A current alternative to sterility testing are different pathogen inactivation procedures, including either the presence of the psoralen compound 'amotosalen' [15] or the presence of the photosensitizer riboflavin (vitamin B2) [16] followed by ultraviolet irradiation. Although no toxicologically relevant effects of photochemically treated PLTs have been observed [17, 18], the use of pathogen reduction techniques without addition of any photoactive reagent may exclude the possibility of such effects.

In contrast to the current methods of pathogen reduction, novel short-wave ultraviolet light (UVC) pathogen reduction technology (THERAFLEX UV-Platelets; MacoPharma, Mouvaux, France) without the need of any additional photoactive reagent was recently evaluated for various bacteria and virus infectivity assays. The Theraflex UV-Platelets system uses short-wave UV light (UVC, 254 nm) in combination with strong agitation, which leads to the formation of areas with thin layers within the PLT irradiation bag and to effective mixing, thus providing sufficient penetration of UVC light. The inactivation principle is based mainly on UVC light absorbance by nucleic acids in pathogens. This results in formation of cyclobutane pyrimidine and pyrimidine pyrimidone dimers that block nucleic acid replication as outlined in Fig. 1. The use of UVC alone has, on one the hand, been shown to reduce pathogens [19–21] but may, on the other hand, have some impact on the PLT quality [19, 20, 22]. It has also been reported that the irradiation of PLTs with UVC led to the disruption in S–S bonds of the integrin αIIbβ3 (GPIIb/IIIa) [23].

Platelets do contain numerous mRNAs, undergo signal-dependent translational regulation [24] and can respond to physiological stimuli that are regulated at the level of protein translation [25], signifying a functional role for PLT mRNA [26, 27]. Therefore, impact on the molecular regulatory mechanisms may play a crucial role in PLT biology during storage [28]. Taken together, these data emphasize the need of further studies on UVC-exposed PLTs with subsequent storage. The purpose of this study was to determine the potential effects on PLTs using the THERAFLEX UV-Platelets system for pathogen inactivation of PLT units, as measured by *in vitro* parameters during a 7-day storage period.

Materials and methods

Preparation and storage of platelets

In this study, outlined in Fig. 2, PLTs were collected (day 0) from normal blood donors who met standard donation

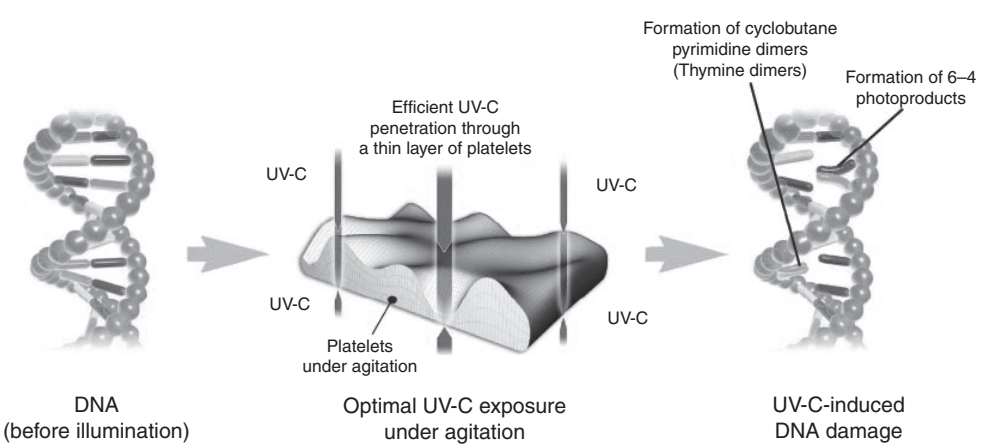


Fig. 1 Mechanism of action.

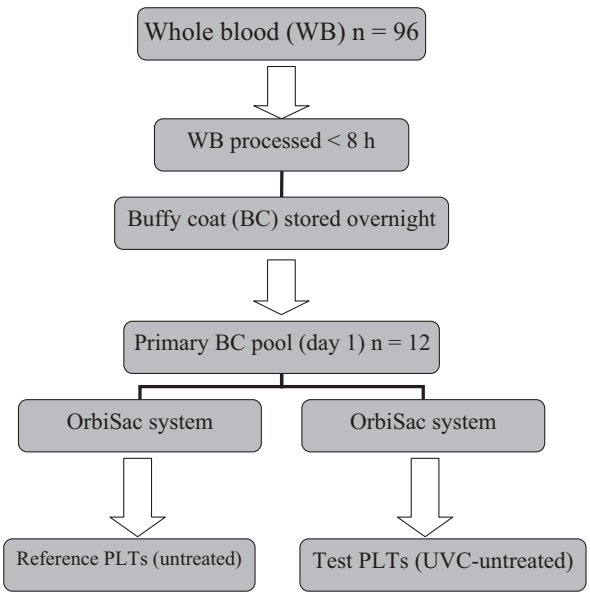


Fig. 2 Study design.

criteria and gave written, informed consent according to institutional guidelines. A total of approximately 450 ml of whole blood (WB) was drawn either into the CPD/SAG-M quadruple-bag blood container system (Fenwal, La Châtre, France) or the top-and-top system (Imuflex-CRC, Terumo, Tokyo, Japan). After storage at 22 ± 2°C for 2–6 h, WB units were centrifuged (2700 *g*) for 10 min at 22°C. Automatic equipment was used for the preparation of blood components (either Optipress, Fenwal or T-ACE, Terumo), including buffy coat (BC) (day 0). BCs (12 units) were stored overnight and combined in a large-volume container to create a primary pool (in total, eight primary pools from 96 BC units). The primary pools were split into two equivalent parts for the preparation of PLT units. A well-established automated technique, the OrbiSac System [29], was used to prepare leucodepleted PLTs from six pooled *ABO*-identical BCs (day 1). The air and foam were then excluded, and the PLT units were stored in approximately 70% PLT additive solution (PAS) (SSP+; MacoPharma) and 30% plasma for storage on a flat bed agitator (60 cycles a minute, model LPR-3; Melco, Glendale, CA, USA) in a temperature-controlled cabinet at 22 ± 2°C for a minimum of 2 h preceding UVC treatment.

UVC treatment

After sterile connection (TSCD-II; Terumo), PLT units (test) were then transferred and further processed using the integrated set (MacoPharma, Ref.XUV4005XU) consisting of an irradiation bag and a PLT storage bag. UVC treatment of PLTs was performed in the irradiation bag made of ethyl vinyl acetate [30] using the irradiation device (Macotronic UV, MacoPharma) equipped with three UVC bulbs mounted above and three mounted below a quartz plate. During irradiation, the PLTs were agitated and exposed to UVC light (0·2 J/cm^2) through <1 min. The wavelength used is 254 nm, UVC dose is controlled by four calibrated sensors integrated in the Macotronic UV irradiation device, and the agitation frequency is 1·8 Hz. The process is outlined in Fig. 3. The air and foam were then excluded, and the treated (test) as well as the untreated (reference) PLTs were transferred to and stored in PLT storage bags composed of n-butyryl, tri n-hexyl citrate plasticized polyvinyl chloride (MacoPharma) on the flat bed agitator for *in vitro* testing. Due to further development, the UV transparency of the irradiation bag has been improved, and 0·2 J/cm^2 is the target dose in the THERAFLEX UV-Platelets process using the integrated bag system Ref. XUV4005XU, enabling an inactivation performance comparable to previously reported results at 0·3 J/cm^2 [19].

Analysis of metabolic and cellular parameters

The samples were drawn aseptically on days 2, 5 and 7, being careful to avoid artificial activation. All sampling was done by sterile connection (Terumo) of sampling bags to the respective containers.

Cellular, metabolic and flow cytometry *in vitro* parameters were evaluated in a PLT storage study, including measurements of PLT counts (10^9/l) and mean PLT volume (MPV) using CA 620 Cellguard (Boule Medical, Stockholm, Sweden). The volume (ml) was calculated by weighing the contents of the storage bag, in grams, on a scale (Mettler PB 2000; Mettler-Toledo, Albstadt, Switzerland), and the result, in grams, was divided by 1·01 (1·01 g/ml is the density of the storage medium composed of approximately 70% SSP+ and 30% plasma). The PLT content (10^9/unit) was calculated by multiplication of the volume (ml) with the PLT count (10^9/l)/1000. Using routine blood gas equipments (ABL 800; Radiometer, Copenhagen, Denmark), we also measured the pH (at 37°C), pO_2, pCO_2 (kPa at 37°C), glucose (mM) and lactate (mM). Bicarbonate (mM) was calculated based on other measured variables. The pH of all samples was measured at 37°C. Therefore, Rosenthal's factor of 0·0147 unit/1°C was used to correct pH to the temperature of sampling (22°C). This factor gives an approximation to the change in pH of the sample per degree centigrade when it is warmed anaerobically from the collecting temperature 22–37°C.

The assessment of swirling was done by inspection and grading according to Bertolini [31]. The white-blood-cell count (WBC) on day 1 was determined with a Nageotte chamber and a microscope (Zeiss, standard, Zeiss, Chester, VA, US) [32]. Hypotonic shock response reactivity (HSR)

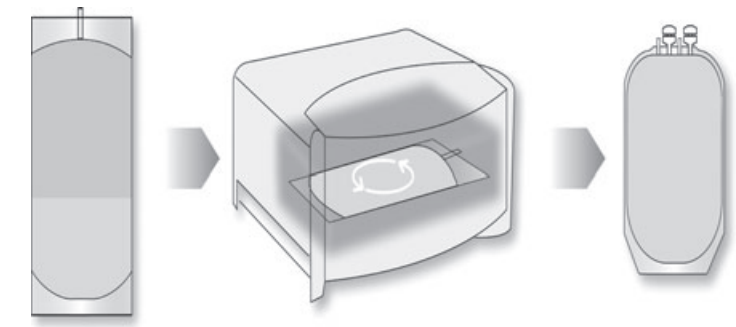


Fig. 3 THERAFLEX UV-Platelets process.

and the extent of shape change (ESC) measurements were performed using a dedicated microprocessor based instrument (SPA 2000; Chronolog, Havertown, PA, US) with the modifications of these tests described by VandenBroeke *et al.* [33]. The total adenosine triphosphate (ATP) concentration, (μmol/10^{11} PLTs), was determined with a Luminometer (Orion Microplate Luminometer, Berthold Detection Systems GmbH, Pforzheim, Germany) based on the principles described by Lundin [34]. The extracellular lactate dehydrogenase (LDH) activity (% of total), a marker for disintegration of PLTs, was measured with a spectrophotometric method (Sigma Aldrich kit 063K6003; St Louis, MO, US; Spectrophotometer Jenway 6500; Staffordshire, UK) [35].

Monoclonal antibody labelling

Platelet concentrate samples, fixed by adding an equal volume of 1% paraformaldehyde, PFA-PBS (pH 7·2–7·4) at 22°C for 10 min, were then stained for 20 min at the same temperature in the dark by incubating with 20 μl of fluorochrome-labelled monoclonal antibodies (MAbs) per approximately 20×10^6 PLTs. Phycoerythrin (PE)-conjugated (IgG1 isotype) CD62P (P-selectin/GMP-140/PADGEM; clone CLB Thromb/6) and fluorescein isothiocyanate (FITC)-conjugated (IgG1 isotype) CD41 (clone P2), CD42b (clone SZ2) and CD61 (clone SZ21) MAbs were used for single-colour staining purchased from Immunotech (Beckman Coulter, Marseilles, France). Control specimens were processed as above, but incubated with a PE- or FITC-conjugated monoclonal antibody (IgG1 isotype) with irrelevant specificity, purchased from Immunotech (Beckman Coulter). After incubation with fluorochrome-conjugated antibodies, the samples were washed twice by adding 2·0 ml filtered phosphate-buffered saline-ethylenediamine tetraacetic acid (PBS-EDTA, 0·33%, pH 6·9) with 0·1% Na-azid and centrifuged at 2760 *g* (Eppendorf 5810R; Eppendorf AG, Hamburg, Germany) for 10 min at +18°C.

Flow cytometry analysis

A total of 100 000 PLT events were acquired on a FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, US) equipped with 15-mW argon ion lasers and Cellquest pro software. Daily controls of optics and fluorescence intensity were performed using standardized beads (Calibrite, BD). The flow cytometer settings were optimized for the acquisition of PLTs by logarithmic signal amplification in all four detectors (forward and side scatter channels and fluorescence channels FL1 and FL2). For analysis, the gate was set around intact PLT population as defined by forward and side scatter characteristics. The percentage of positive PLTs of total PLT expressing activation markers CD62P and surface membrane glycoproteins (CD41, CD42b and CD61) above that of background (negative control) as well as the mean fluorescence intensity (MFI) was recorded.

Bacterial cultures

Bacterial cultures were performed at day 7 with the routine methods of the bacteriologic laboratory at Karolinska University Hospital/Huddinge, including aerobic and anaerobic cultures performed on Columbia blood agar plates, with 48 h to final report.

Statistical analyses

The mean values and standard deviations (n = 8) are usually given. Repeated measures ANOVA were performed including 'Post Hoc Test', Fischer's LSD. Two different groups (reference and test) were studied over time (Days). 'Days' was the repeated factor and 'Group' was a between factor. The results of Fisher's LSD are presented in subsequent figures and in the result section. The P-value represents the differences between groups at specific time-points and was considered statistically significant at $P < 0{\cdot}05^*$ and $P < 0{\cdot}01^{**}$. The analyses were carried out using the

Statistica software, version 9 StatSoft, Inc 1984–2007 (SPSS, Chicago, IL, USA).

Results

In this paired-study, *in vitro* parameters of PLTs irradiated with UVC light were studied during a 7-day storage period. Effects on different cellular, metabolic and flow cytometry parameters are all listed in Fig. 4.

Cellular assays

The PLT and leucocyte counts on day 1 are given in Table 1. No significant difference in PLT counts and contents between the groups was detected throughout storage (data not shown). MPV (Fig. 4a) increased more in the test units ($P < 0{\cdot}05$ day 7), and the extracellular LDH (Fig. 4b) in % of total remained stable at low levels in all units, without any significant difference between the groups.

Metabolic assays

The glucose concentration (Fig. 4c) was lower ($P < 0{\cdot}01$), and the production of lactate (Fig. 4d) was higher ($P < 0{\cdot}01$ day 7) in UVC-treated PLTs when compared with the reference units. From day 5, pH (Fig. 4e) was significantly higher in the reference units ($P < 0{\cdot}05$). Carbon dioxide (pCO_2) decreased in all units throughout storage (Fig. 4f), without any significant difference between the units (NS). The calculated bicarbonate (Fig. 4g) level and the ATP (Fig. 4h) levels were higher from day 5 ($P < 0{\cdot}01$) in the reference units.

HSR, ESC and swirling

With the exception of day 7 ($P < 0{\cdot}01$ reference vs. test), the HSR reactivity (Fig. 4i) of PLTs over 7 days was not different between groups. During storage, ESC (Fig. 4j) was lower in the UVC-treated PLTs ($P < 0{\cdot}01$). Swirling remained at the highest level (score = 2) for all units throughout storage (data not shown).

Flow cytometry analysis

The percentage of PLTs expressing the activation marker CD62P (Fig. 4k) as well as the MFI (data not shown) showed slight increase during storage in all units. The mean expression in all preparations was below 20% in PLTs (day 2) and below 25% in 7-day-old PLT units. From day 2, a slight decrease in the CD62P expression was seen in the reference units, while the activation level of PLTs in UVC-treated units increased and became significantly higher from day 5 ($P < 0{\cdot}05$). The percentage of PLTs expressing the surface membrane glycoproteins (CD41, CD42b and CD61) showed similar trends throughout storage, without any significant difference between the units (Fig. 4l–n).

Bacterial cultures

All bacterial cultures were negative.

Discussion

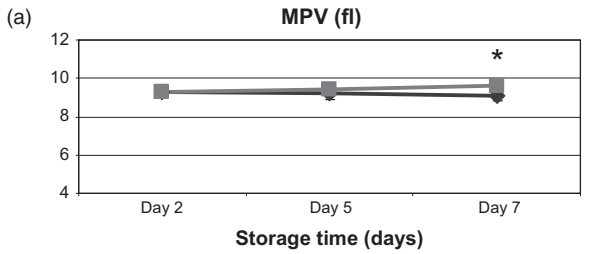
This study describes the *in vitro* quality of PLTs irradiated with UV light using novel short-wave ultraviolet light (UVC) pathogen reduction method (THERAFLEX UV-Platelets; MacoPharma). In contrast to the current methods of pathogen reduction used, this technique does not require any additional photoactive reagent.

The majority of *in vitro* parameters tested until day 5 were not significantly different between the two groups. The storage stability of the PLTs was not impaired throughout 7-day storage, and differences observed between reference and test PLTs appeared negligible, with regard to current *in vitro* standards. Our results are in accordance with previously published data by Mohr *et al.* [19, 20].

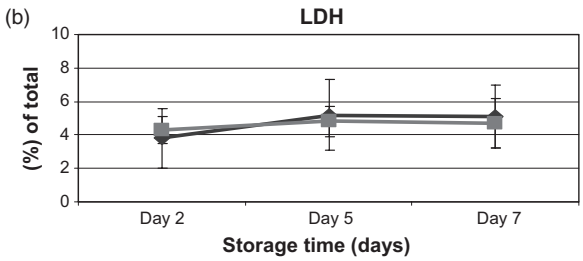
In this study, UVC irradiation as a source for disintegration of PLTs seems to be insignificant, as evidenced by LDH release comparable to untreated PLT units. Thus, all units showed satisfactory PLT content (Table 1) according to current European standards ($>60 \times 10^9$/single unit equivalent). In agreement with recent published data [19, 20], markers of PLT metabolism were only moderately influenced by UVC irradiation. Glucose consumption was enhanced, lactate accumulation was increased, and pH decreased during storage in the test units when compared with the reference units. The lower pH in the test units is probably related to the higher concentration of lactate in these units.

In order to maintain viability, PLTs must continuously generate new ATP to meet their energy needs [36]. Although all units were well above the levels suggested being associated with a loss of *in vivo* viability [37], the decreased ATP levels in the test units may be associated with decreased mitochondrial function and/or increased ATP demand [38]. Apparently, this situation and the increase in the metabolic rate seem to be associated with decreased response to HSR, a reduction in ESC values and increased PLT activation, as evidenced by a higher expression of CD62P.

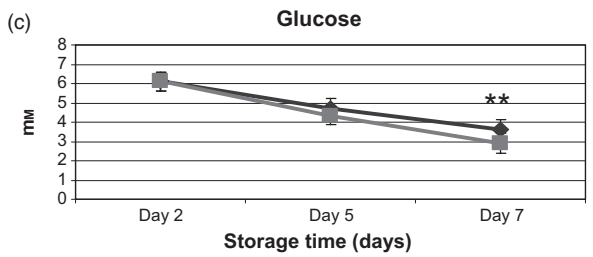
HSR and ESC have been widely used to characterize the *in vitro* function of PLTs and have been shown to correlate with *in vivo* viability [39]. On the one hand, we found that all HSR scores were above the level in a



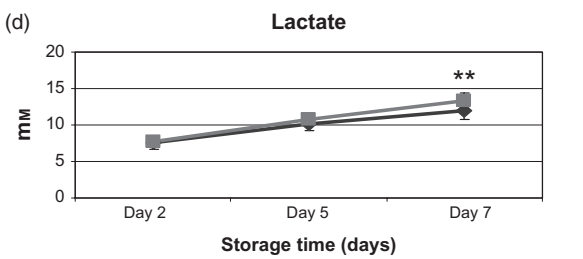
Mean platelet volume (MPV) during 7 days of storage in reference ♦ Test □ platelet (PLT) units (P<0·05 day 7). Values given as mean ± SD, n = 8



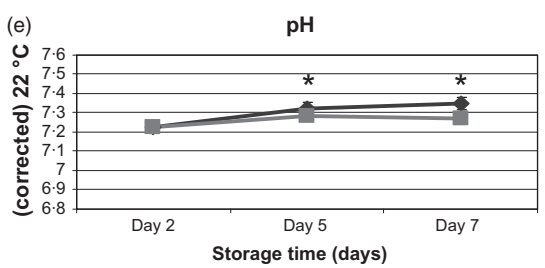
Lactate dehydrogenase activity (%) of total during 7 days of storage in reference ♦ Test □ platelet (PLT) units (NS). Values given as mean ± SD, n = 8.



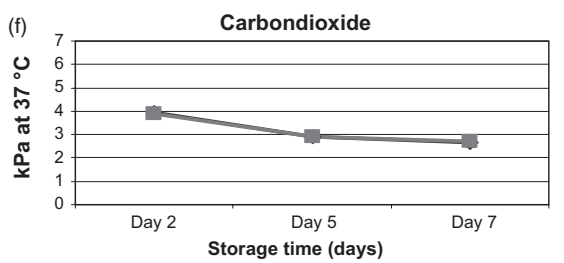
Glucose concentration (mM) during 7 days of storage in reference ♦ Test □ platelet (PLT) units (P<0·01 day 7). Values given as mean ± SD, n = 8



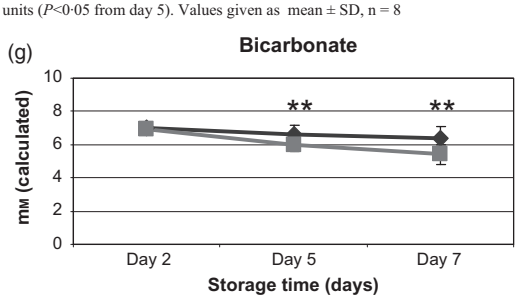
Lactate concentration (mM) during 7 days of storage in reference ♦ Test □ platelet (PLT) units (P<0·01 day 7). Values given as mean ± SD, n = 8



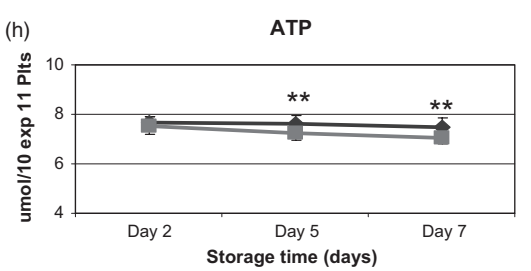
pH (corrected to 22 °C) during 7 days of storage in reference ♦ Test □ platelet (PLT) units (P<0·05 from day 5). Values given as mean ± SD, n = 8



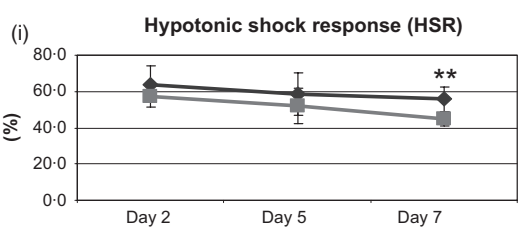
Carbondioxide (kPa at 37 °C) during 7 days of storage in reference ♦ Test □ platelet (PLT) units (NS). Values given as mean ± SD, n = 8



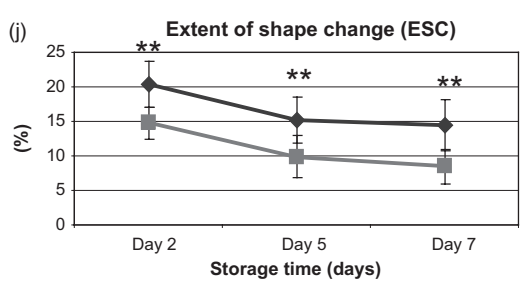
Bicarbonate calculated (kPa at 37 °C) during 7 days of storage in reference ♦ Test □ platelet (PLT) units (P<0·01 from day 5). Values given as mean ± SD, n = 8



Adenosinetriphosfate (μmol/10^{11}PLTs) during 7 days of storage in reference ♦ Test □ platelet (PLT) units (P<0·01 from day 5). Values given as mean ± SD, n = 8

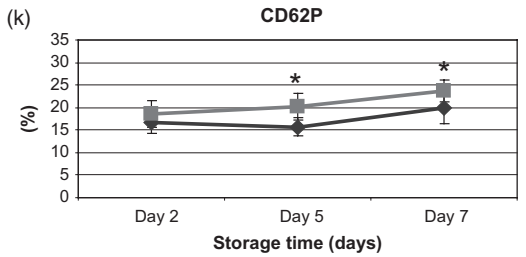


Hypotonic shock response (%) during 7 days of storage in reference ♦ Test □ platelet (PLT) units (P<0·01 day 7). Values given as mean ± SD, n = 8

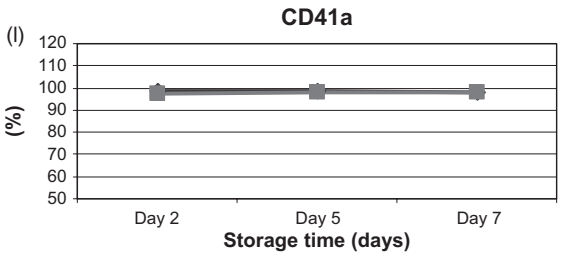


Extent of shape change (%) during 7 days of storage in reference ♦ Test □ platelet (PLT) units (P<0·01 from day 2). Values given as mean ± SD, n = 8

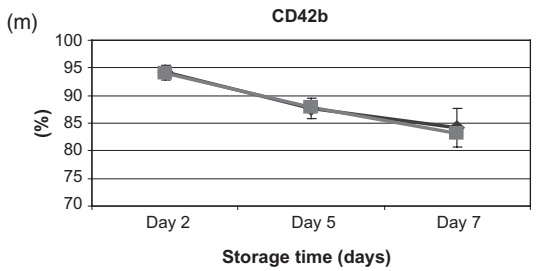
Fig. 4 Diagram results.



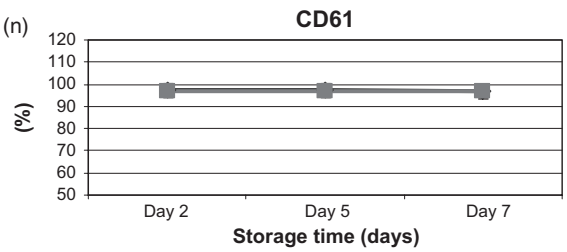
Changes in platelet (PLT) expression of CD62P (%) during 7 days of storage in reference ♦ Test □ platelet (PLT) units (P<0·05 from day 5). Values given as mean ± SD, n = 8



Changes in platelet (PLT) expression of CD41a (%) during 7 days of storage in reference ♦ Test □ platelet (PLT) units (NS). Values given as mean ± SD, n = 8



Changes in platelet (PLT) expression of CD42b (%) during 7 days of storage in reference ♦ Test □ platelet (PLT) units (NS). Values given as mean ± SD, n = 8



Changes in platelet (PLT) expression of CD61 (%) during 7 days of storage in reference ♦ Test □ platelet (PLT) units (NS). Values given as mean ± SD, n = 8

Fig. 4 (Continued)

Table 1 Comparison of platelets (PLTs) irradiated with short-wave ultraviolet light, Test (UVC-treated) and Reference PLTs (untreated) on day 2*

Platelet units	Volume (ml)	Platelet count (10^9/l)	Platelet content (10^9/unit)	Leucocyte content (10^6/unit)
Reference (untreated)	343 ± 5	1060 ± 107	364 ± 33	<0·2
Test (UVC-treated)	340 ± 6	1025 ± 98	348 ± 28	<0·2

*Results are expressed as mean ± SD (n = 8).

plasma storage environment, for which poor *in vivo* viability has been predicted to occur [39]. On the other hand, all ESC values were below that level. One explanation for this situation may be that storage in PAS effects PLT responsiveness to ADP [40].

Glycoprotein (GP) Ibα is the receptor for von Willebrand factor (vWF) and a high-affinity receptor for thrombin [41]. We found no difference in the CD42b expression between the groups, and our findings correspond with those of others who have shown that storage of PLTs reduces the expression of GP1bα on the surface of the PLTs [42, 43]. This reduction may impair the ability of PLTs to become activated by thrombin as storage time increases [43].

In this study, the impact of THERAFLEX UV treatment on PLT membrane protein αIIbβ3 is only partly described. The mAbs used (CD41/61) recognizes the intact complex of αIIbβ3 [44] and was found to bind equally well to test as well as reference PLTs. Recently, Verhaar *et al.* [23] reported on αIIbβ3-dependent PLT aggregation due to UVC treatment. However, that group used experimental conditions different from ours: 250 µl aliquots of a plasma-free PLT suspension at 5× 10^7 cells/ml were irradiated. Therefore, the impact of potentially detrimental changes involving the regulatory conformations within the complex needs to be further analysed using mAbs that binds preferentially to activated PLTs under the conditions employed in this study [45].

To summarize, this study has shown that UVC treatment have a slight effect on the PLT parameters tested *in vitro*. The majority of *in vitro* parameters tested were not significantly different after UVC treatment until day 5, and the storage stability of the PLTs was not impaired throughout 7-day storage. However, there is still little insight into the impact on the hemostatic behaviour after PLT transfusion. Therefore, the impact of these findings needs to be examined performing *in vivo* studies of recovery and survival, as well as/or increments in thrombocytopenic patients to confirm the haemostatic effect of PLTs irradiated with UVC light.

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