Ultraviolet C light pathogen inactivation treatment of platelet concentrates preserves integrin activation but affects thrombus formation kinetics on collagen in vitro

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BACKGROUND: Ultraviolet (UV) light illumination in the presence of exogenously added photosensitizers has been used to inactivate pathogens in platelet (PLT) concentrates for some time. The THERAFLEX UV-C system, however, illuminates PLT concentrates with UV-C light without additional photoactive compounds. In this study residual PLT function is measured in a comprehensive paired analysis of UV-C–treated, gamma-irradiated, and untreated control PLT concentrates.

STUDY DESIGN AND METHODS: A pool-and-split design was used with buffy coat–derived PLT concentrates in 65% SSP+ additive solution. Thrombus formation kinetics in microfluidic flow chambers onto immobilized collagen was investigated with real-time video microscopy. PLT aggregation, membrane markers, and cellular metabolism were determined concurrently.

RESULTS: Compared to gamma-treated and untreated controls, UV-C treatment significantly affected thrombus formation rates on Days 5 and 7, not Day 2. PLT degranulation (P-selectin) and PLT apoptosis (annexin V binding) was slightly but significantly increased from Day 2 on. UV-C treatment moreover induced integrin $\alpha_{IIb}\beta_3$ conformational changes reminiscent of activation. However, subsequent integrin activation by either PAR1-activating hexapeptide (PAR1AP) or convulxin was unaffected. This was confirmed by PLT aggregation studies induced with collagen, PAR1AP, and ristocetin at two different agonist concentrations. Finally, UV-C slightly increased lactic acid production rates, resulting in significantly decreased pH on Days 5 and 7, but never dropped below 7.2.

CONCLUSION: UV-C pathogen inactivation treatment slightly but significantly increases PLT activation markers but does not profoundly influence activatability nor aggregation. The treatment does, however, attenuate thrombus formation kinetics in vitro in microfluidic flow chambers, especially after storage.

The increased risk for bacterial growth caused by room temperature storage of platelet (PLT) concentrates and for (emerging) transfusion-transmissible diseases including virus strains with long window periods are genuine reasons for introducing broad-spectrum pathogen inactivation.[1] There are currently three different standardized methods to inactivate pathogens in PLT concentrates. Two marketed systems use ultraviolet (UV) light combined with an exogenously added photoactive reagent, either the psoralen derivative amotosalen with 320- to 400-nm UV-A light[2] (AS-PCT) called the INTERCEPT Blood System (Cerus Corporation, Concord, CA) or riboflavin with 265- to 370-nm broad-spectrum UV light[3] (RF-PRT) called Mirasol Pathogen Reduction Technology System (TerumoBCT, Lakewood, CO). More recently, a third inactivation method has been developed that uses the microbiocidal and virucidal characteristics inherent to short-wavelength (254 nm) UV-C light[4] without exogenously added photosensitizer (UV-C) called THERAFLEX UV-PLTs (Macopharma, Tourcoing, France). This particular technology mitigates concerns about toxicological effects of added

ABBREVIATIONS: GP = glycoprotein; HBS = HEPES-buffered saline.

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photochemicals and/or photoproducts generated during illumination.

The biochemical mechanism involved in UV-C treatment has not been demonstrated as such but is claimed[5] to be similar to the general chemistry[6] underlying UV-C induced damage of nucleic acids. If true, then photochemical formation of cyclobutane pyrimidine dimers, pyrimidine (6-4) pyrimidone dimers as well as abasic sites, strand breaks, and oxidative products prevent elongation of nucleic acid transcripts[7] if not repaired by cellular machinery. A major practical hurdle is to obtain sufficient penetrance of the incident light because proteins as well as particles (including PLTs) will respectively absorb and scatter incoming photons thereby dissipating the energy required for inactivation. Therefore, conditions have been optimized to assure efficient delivery of UV-C light including reducing the plasma content to 30% to 40% with additive solution (AS), exposing both sides of the bag to the light sources, increasing the surface area, and agitating vigorously during illumination.[5,8,9] This way, in vitro pathogen inactivation of several transfusion-transmitted pathogens has been demonstrated.[8,9]

Functional and biochemical studies of UV-C pathogen-inactivated PLTs showed an acceleration of the storage lesion with decreased pH and hypotonic shock response, increased glucose consumption, and lactic acid production. In addition, increased values for degranulation (P-selectin expression), phosphatidylserine expression (annexin V binding), and integrin $\alpha_{IIb}\beta_3$ activation (PAC1 binding) indicate moderate activation of PLTs after UV-C treatment.[8-12] There is a low impact on the PLT proteome[9] and neoantigen formation could not be detected in a dog model.[13] Furthermore, the results of a Phase I study with autologous transfusions in healthy volunteers met the primary safety and tolerance criteria[14] and Bashir and colleagues[12] demonstrated PLT recovery and survival rates within acceptance criteria after autologous transfusions in healthy volunteers.

There are few studies that investigate the influence of pathogen inactivation on integrated PLT function, for example, in aggregometry or microcapillary flow chambers. The latter is a comprehensive functional in vitro test with high sensitivity to perturbations in all steps of hemostasis: adhesion, activation, and aggregation.[15] In vivo studies of thrombus formation in mice have demonstrated that many of the regulatory processes determining arterial thrombosis can well be assessed with these in vitro flow experiments.[16] Furthermore, the technique is used for screening of patients with stent thrombosis[17] and has been validated for drug development[18,19] and for the study of PLT hereditary disorders.[20] In this study we have compared UV-C–treated and untreated paired PLT concentrates using quality markers like pH, metabolic rate, and receptor expression as well as the more comprehensive PLT function assays of light transmission aggregation and microfluidic flow chamber experiments.

MATERIALS AND METHODS

Study design

Whole blood donations from voluntary nonremunerated donors fulfilling Belgian eligibility criteria were used to prepare leukoreduced PLT concentrates from five buffy coats with a plasma content of approximately 35% and the remainder PLT AS (SSP+, Macopharma) following standard operating procedures of the blood service of the Belgian Red Cross Flanders. Three blood group–matched PLT concentrates were pooled and split again to deliver three equivalent PLT concentrates of which one was left untreated to serve as control, one was treated with 25 to 50 Gy gamma irradiation, and one was UV-C treated.

UV-C treatment was according to the THERAFLEX UV-PLTs standard operation protocol only with products fulfilling the inclusion criteria as set by the provider. In brief, the bag content was transferred to an UV-C–permeable ethyl vinyl acetate illumination container which was placed in the Macotronic UV illuminator (Macopharma). A total dose of 0.2 J/cm^2 of 254 nm UV light was delivered under continual agitation in a timespan not exceeding 1 minute. Next, the treated PLT concentrate was transferred to the *n*-butyryl tri-*n*-hexyl citrate plasticized polyvinyl chloride storage bag included in the THERAFLEX UV-C bag combination. The paired control and gamma-irradiated products were stored in the same bag type to ascertain similar storage conditions for all study arms. All products were kept under standard blood bank conditions at 20 to 24°C on a flatbed agitator. The experiments described below were performed on small volumes taken aseptically from the concentrates before (Day 1) and after (Days 2, 5, and 7) treatment. The design included a total of six independent repeat experiments (n = 6) unless where indicated differently.

Blood gases and PLT count

Lactic acid, glucose, and pH were determined immediately after sampling using a point-of-care blood gas analyzer (Rapidpoint 500, Siemens, Munich, Germany). Whole blood counts were performed with an automated hematology analyzer (PocH-100i, Sysmex, Kobe, Japan).

Blood reconstitution

Whole blood was taken from healthy nonmedicated donors in heparin vacutainers (REF 368480, BD Diagnostics, Franklin Lakes, NJ) taking measures for preserving PLT quiescence.[21] A first soft centrifugation (12 min at 250 × *g*) divided the whole blood samples in PLT-rich plasma and concentrated red blood cells (RBCs). PLTs of the PLT-rich plasma were then spun down by hard

centrifugation (20 min at 4500 × *g*) to yield PLT-poor plasma. Reconstitution was by mixing appropriate volumes of these RBCs, PLT-poor plasma, and either of the three subject PLT concentrates (control, gamma irradiation, or UV-C) achieving 40% hematocrit and 250 × 10^9 PLTs/L on average.

Microfluidic flow chamber experiments

Reconstituted blood samples were perfused through microfluidic channels of dimensions 400 μm × 100 μm × 28 mm (Vena8 Fluoro+ Biochips, Cellix Ltd, Dublin, Ireland) at a shear stress of 50 dyn/cm^2 (flow rate of 1100/sec) using a microfluidic pump (Mirus Evo, Cellix Ltd). The channels were coated overnight at 4°C with 50 μg/mL Horm collagen Type I (Takeda, Osaka, Japan). Next, the channels were blocked with 1.0% (wt/vol) bovine serum albumin and 0.1% (wt/vol) glucose in 10 mmol/L HEPES-buffered saline (HBS; 0.9% [wt/vol] NaCl, pH 7.4) at room temperature for 30 minutes. The channels were then rinsed with 1 mL of plain HBS to remove the remaining blocking buffer and unbound collagen. PLTs in the reconstituted blood samples were labeled with 1 μmol/L (final) of the fluorescent dyes 3,3′-dihexyloxacarbocyanine iodide (Sigma-Aldrich, St Louis, MO) or 5 μmol/L calcein-AM (Life Technologies, Carlsbad, CA) where indicated.

Samples were perfused in duplicate at the same time using a channel splitting manifold and an automated x-y stage allowing up to eight simultaneous runs in one experiment. The biochip is mounted on a fluorescent microscope (Z.1 Observer, Carl Zeiss, Oberkochen, Germany) equipped with a fluorescent light source (488 nm; Colibri-LED, Carl Zeiss) and high-resolution CCD camera (Carl Zeiss). A simultaneous recording of three side-by-side images per microcapillary channel was performed in real time for 7 minutes at 100× magnification. The three side-by-side images were digitally stitched to deliver one single compound image per time point. A fixed threshold of 400 to 4096 arbitrary fluorescence units was used to define PLT adherence allowing us to determine the percentage of pixels covered by PLTs in the measurement field (i.e., surface coverage) in function of time (ZEN2012 software, Carl Zeiss). The slopes of these relationships were determined by linear regression (Prism, GraphPad Software Inc., La Jolla, CA) and are a measure for thrombi growth kinetics.

Flow cytometry

Expression of glycoprotein (GP)Ibα (fluorescein-labeled anti-CD42b, Life Technologies), activated integrin $\alpha_{IIb}\beta_3$ (fluorescein-labeled PAC1, BD Biosciences, Erembodegem, Belgium), P-selectin (phycoerythrin–anti-CD62P, BD Biosciences) and annexin V (peridinin chlorophyll-Cy5.5–labeled annexin V, BD Biosciences) of either PLT concentrate were analyzed with an acoustic focusing flow cytometer (Attune, Life Technologies). PLTs were incubated with labeled antibodies or ligand for 10 minutes at room temperature in HBS, supplemented with 1 mmol/L $MgSO_4$, and diluted 1000-fold immediately before readout according to published work.[22] For annexin V measurements, buffers were supplemented with 2 mmol/L $CaCl_2$. For measurements of integrin $\alpha_{IIb}\beta_3$ activation on stimulated PLTs, the PAR1 agonist thrombin-related activating hexapeptide SFLLRN (PAR1AP; Sigma-Aldrich) at 30 μmol/L or the GPVI-FcγRIIa agonist convulxin (Santa Cruz Biotechnology Inc., Dallas, TX) at 6 ng/mL were used. The signals of the respective isotype antibody controls were used to set threshold gates including 0.5% of 10,000 negative events. Mean or median fluorescence intensities and percentage positive events were determined for 10,000 cells staining positive for the PLT marker CD61 (allophycocyanin-labeled anti-CD61, Life Technologies).

PLT aggregation

PLT aggregation was with a light transmission aggregometer (Chrono-Log, Helena Laboratories, Haverton, PA). Three different PLT agonists were used, each at two concentrations: a high agonist concentration to saturate signal transduction and a low concentration to investigate PLT sensitivity. For collagen (American Biochemical & Pharmaceuticals, Epsom, UK) 10 and 4.5 μg/mL were used; for PAR1AP, 10 and 5 μmol/L; and for ristocetin (American Biochemical & Pharmaceuticals), 1.5 and 0.6 mg/mL. These agonist concentrations were determined in separate serial dilution experiments (data not shown). Aggregation cuvettes contained PLTs diluted to 250 × 10^9 PLTs/L with the corresponding autologous PLT-free plasma with AS prepared by centrifugation. Maximal aggregation (amplitude [%]) is reported here; the other variables (slope and area under the curve) were comparable.

Partial pressure reduction of molecular oxygen by degasification

An independent series (n = 5) of pool and split was performed for this experiment. Again, three blood group–matched PLT concentrates were pooled and split; one PLT concentrate was left untreated to serve as a control, one was immediately processed with UV-C, and one was treated to reduce the dissolved molecular oxygen partial pressure as described elsewhere[23] before processing with UV-C pathogen inactivation. To reduce molecular oxygen partial pressures inert nitrogen gas (Air Liquide, Paris, France) was blown into the illumination bag (not bubbled) under continuous gentle agitation to allow reequilibration of dissolved gases following Henry's law. After 7 minutes the residual molecular oxygen partial pressure in the PLT concentrate suspension was 1.8 ± 0.5 kPa compared to 7.5 ± 1.4 kPa in controls as measured by a submerged Clark electrode in real time. Flow cytometric

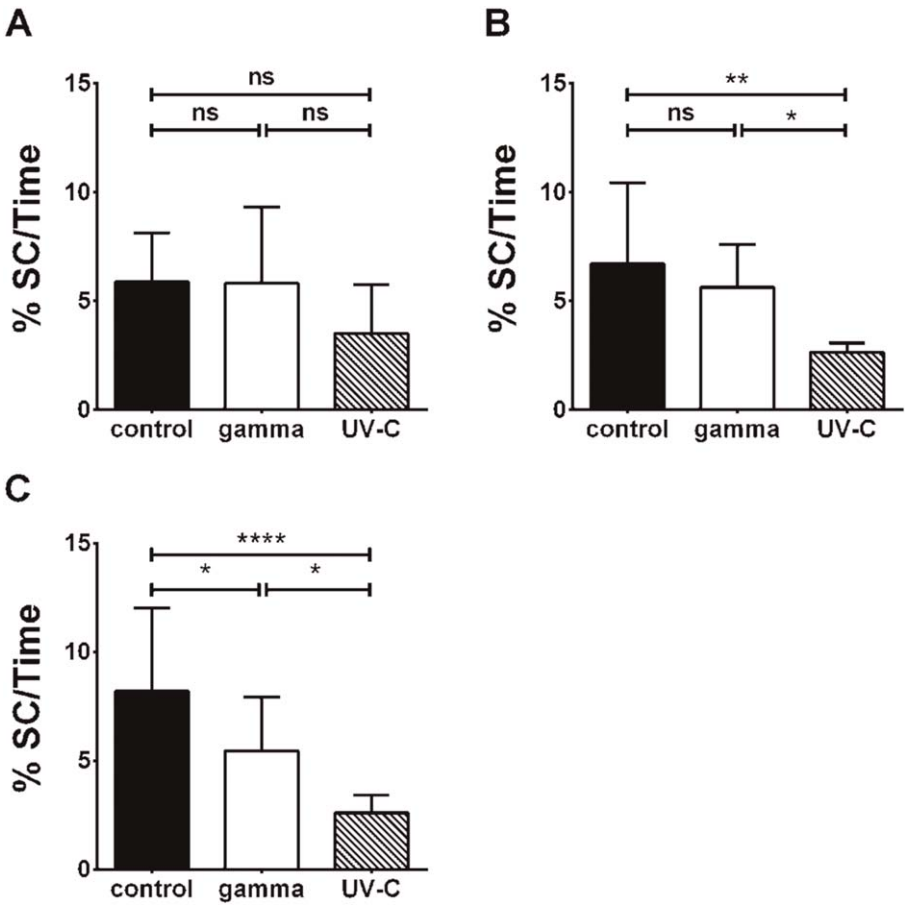

Fig. 1. PLT thrombus formation kinetics are affected by UV-C treatment after storage. Microfluidic flow chamber experiments with collagen-coated microcapillaries were performed in duplicate on Days 2 (A), 5 (B), and 7 (C). Thrombus formation kinetics was followed in real time using video microscopy and software measuring PLT surface coverage. The results are depicted as surface coverage in function of perfusion time (%SC/Time) for control (■), gamma-irradiated (□), and UV-C–treated (▨) PLTs in reconstituted blood. The data are shown as means with SD (n = 8). Results from the statistical analysis are depicted at the top of each panel (ns = not significant; *p < 0.05; **p < 0.01; ****p < 0.0001).

analysis and microfluidic flow chamber experiments of these concentrates were performed as described.

Statistical analysis

Results are reported as mean values with standard deviation (SD) and analyzed with two-way analysis of variance (ANOVA) with Tukey's multiple comparisons algorithm with computer software (Prism, Version 6.01, GraphPad Software, Inc., San Diego, CA) to determine significance ($p \leq 0.05$) between the three subject groups.

RESULTS

UV-C–treated PLTs show impaired thrombus formation kinetics after storage

UV-C–treated PLTs were compared with gamma-irradiated and untreated controls by measuring thrombus formation kinetics onto collagen coated microcapillaries. Figure 1 and Videos S1 through S3 (available as supporting information in the online version of this paper) show a mean decreased surface coverage in function of time onto collagen-coated microcapillary. This was found from Day 2 on, but only significant after storage on Days 5 and 7.

Integrin $\alpha_{IIb}\beta_3$ conformational changes after UV-C treatment

During thrombus formation, PLT integrin $\alpha_{IIb}\beta_3$ becomes activated in a calcium-dependent manner to expose the fibrinogen-binding site and promote PLT cross-linking. The PAC1 monoclonal antibody binds to an epitope exclusively exposed on the active conformation of the receptor and so is a surrogate marker for integrin activation. Figure 2A shows that UV-C

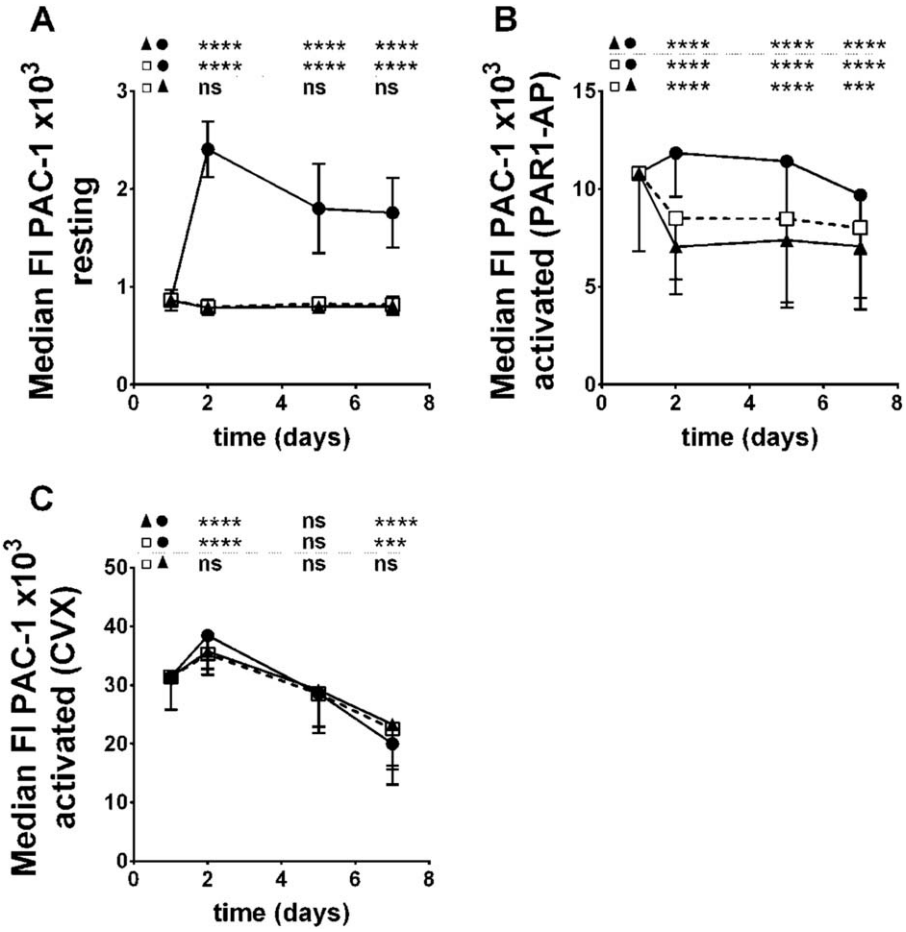

Fig. 2. UV-C treatment induces integrin $\alpha_{IIb}\beta_3$ conformational changes that does not affect further activation through PAR1 or GPVI. Integrin $\alpha_{IIb}\beta_3$ activation was measured by binding of fluorescein-labeled PAC1 to 10,000 CD61-positive events in flow cytometry. Samples were analyzed in resting conditions (A) or after stimulation with 30 μmol/L PAR1AP (B) or 6 ng/mL convulxin (CVX; C) before (Day 1) and after treatment (Days 2, 5, and 7). Data of UV-C–treated (●) , gamma-irradiated (▲), and untreated control (□ dotted line) are shown as mean values with SD. Results from the statistical analysis are depicted at the top of each panel (ns = not significant; ***p < 0.001; ****p < 0.0001).

treatment caused increased PAC1 binding compared to both control and gamma-irradiated PLTs in the absence of any additional agonist. This "activated" receptor state persisted during subsequent 7-day storage. We hypothesized that this would compromise further activation of PLTs by exogenous agonists. However, this was not the case as integrin $\alpha_{IIb}\beta_3$ activation by both PAR1AP and convulxin was not significantly decreased for UV-C–treated PLTs (Figs. 2B and 2C). Instead, a distinct increase was seen in the UV-C–treated group after PAR1AP stimulation, which may be attributed to the subpopulation of receptors that were "preactivated" by UV-C treatment. The overall potency of convulxin to induce and sustain inside-out integrin activation is larger than for PAR1AP and therefore this particular "additive" effect may be masked in this case.

PLT aggregation is mostly unaffected

Unlike PAC1 measurements in highly diluted flow cytometry conditions, aggregation is more inclusive taking into account effects of PLT shape change and signal amplification by vesicle release. The above-mentioned additive effect of integrin $\alpha_{IIb}\beta_3$ activation was also noticed during low-dose collagen aggregations, where UV-C–treated PLTs displayed a slightly increased response on Day 2 (Fig. 3A). However, this observation could not be generalized to the other conditions (Figs. 3B-3F), in particular to low-dose PAR1AP and low-dose ristocetin stimulation where a slightly decreased response was found on Day 2 and after storage, respectively. Overall however, few differences with gamma-irradiated or untreated control PLTs were found.

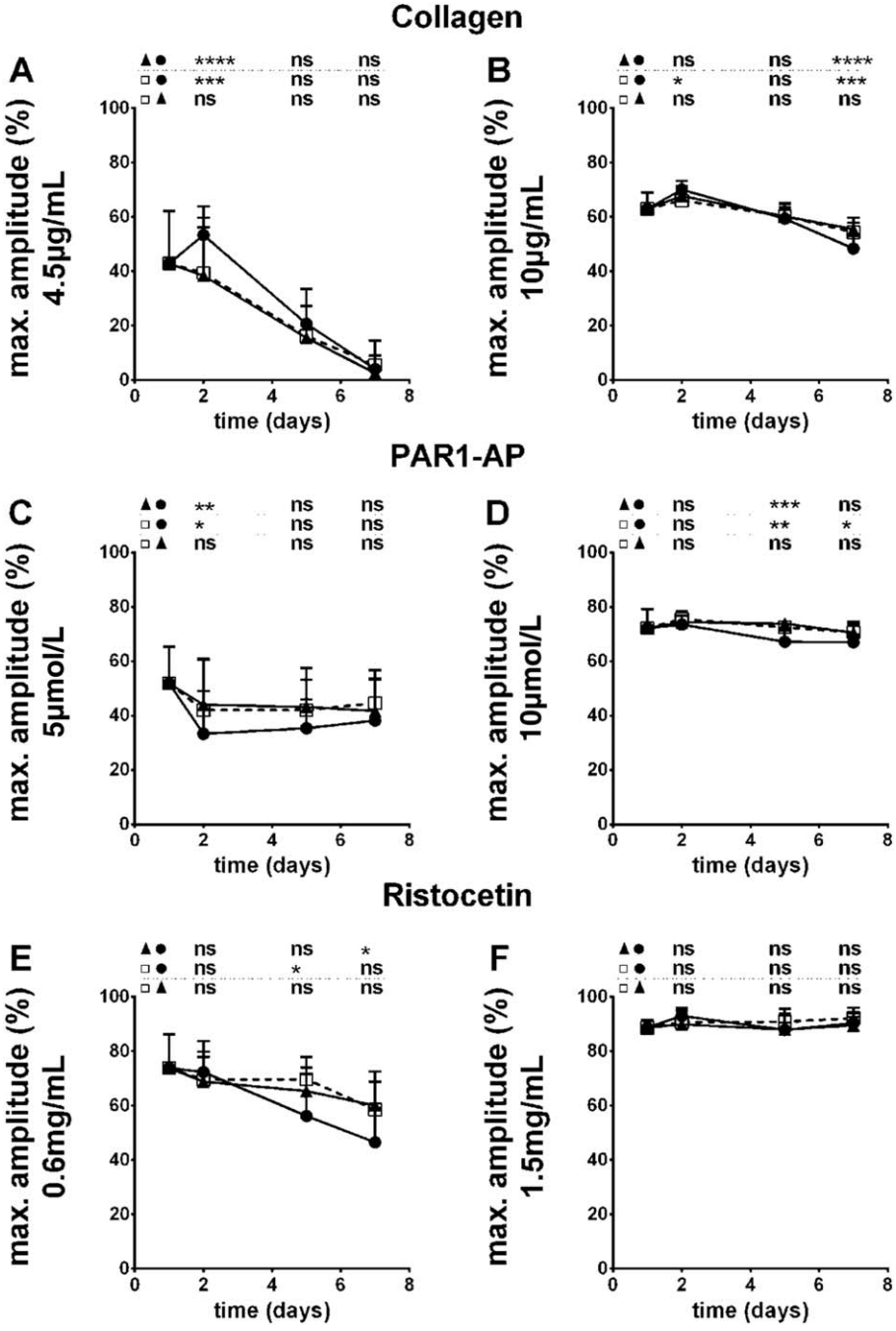

Fig. 3. Light transmission aggregation is not substantially different. PLT light transmission aggregometry was performed at 250 × 10^9 PLTs/L. Three different agonists were used each at low (A, C, and E) and high (B, D, and F) concentrations as indicated on the y-axis. Maximal aggregation (or amplitude %) is shown as means with SD. Untreated control (□ dotted line), gamma-irradiated (▲), and UV-C–treated (●) PLTs are shown before (Day 1) and after treatment (Days 2, 5, and 7). Results from the statistical analysis are depicted at the top of each panel (ns = not significant; *p < 0.05; ****p < 0.0001).

UV-C treatment caused α-degranulation and phosphatidylserine exposure

PLTs contain a number of distinguishable storage granules including α-granules, lysosomes, and dense granules. During activation, PLT degranulation occurs thereby releasing proteins and small molecules that act as modulators of hemostasis by para- and autocrine signaling. P-selectin is a marker of α-granule secretion and showed a significant

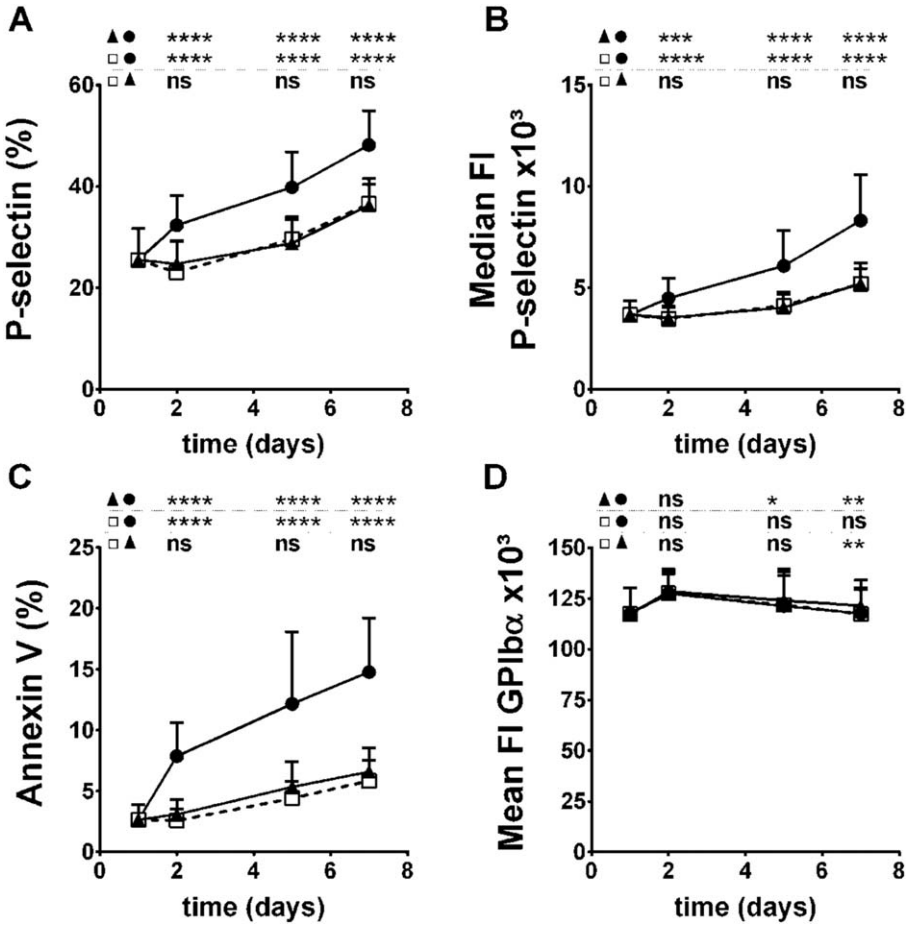

Fig. 4. PLT α-degranulation and expression of negatively charged phospholipids is increased after UV-C treatment. Degranulation was measured by binding of a labeled P-selectin antibody and the percentage above isotype control (A) and median fluorescence intensities (FI; B) are depicted. Externalization of phosphatidylserine/-ethanolamine was measured by binding of labeled annexin V (C) and percentage above control is shown. GPIbα receptor expression is shown as mean fluorescence intensities (D). Untreated control (□ dotted line), gamma-irradiated (▲), and UV-C–treated (●) PLTs are shown before (Day 1) and after treatment (Days 2, 5, and 7). Results from the statistical analysis are depicted at the top of each panel (ns = not significant; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001).

increase after UV-C treatment indicating increased spontaneous degranulation (Figs. 4A and 4B). Furthermore, the expression of phosphatidylserine/-ethanolamine is measured by annexin V binding. Just like P-selectin there was an increased exposure after UV-C treatment (Fig. 4C). GPIbα expression was only marginally affected by UV-C treatment (Fig. 4D).

UV-C treatment slightly increases PLT metabolism

Significantly higher glucose consumption and lactic acid production were seen for UV-C–treated PLTs (Figs. 5A-5C) resulting in a different pH trend (Fig. 5D) with respect to the control conditions. These data indicate a slightly higher metabolic activity, but pH never dropped below 7.2.

Decreased molecular oxygen level cannot rescue PLT thrombus formation kinetics

Previous work from our group showed that experimentally decreased partial pressures of molecular oxygen rescues coagulation factor decreases in RF-PRT–treated plasma by preventing oxidative damage.[24] We hypothesized that the variability in thrombus formation kinetics observed on Day 2 (Fig. 1A) was caused by variable molecular oxygen partial pressures in the primary product thus resulting in variable oxidative damage. To reduce oxidative damage during UV-C treatment, the partial pressure of molecular oxygen was lowered in the PLT concentrate before UV-C treatment by nitrogen gas equilibration. However, Fig. 6 shows that such treatment did not

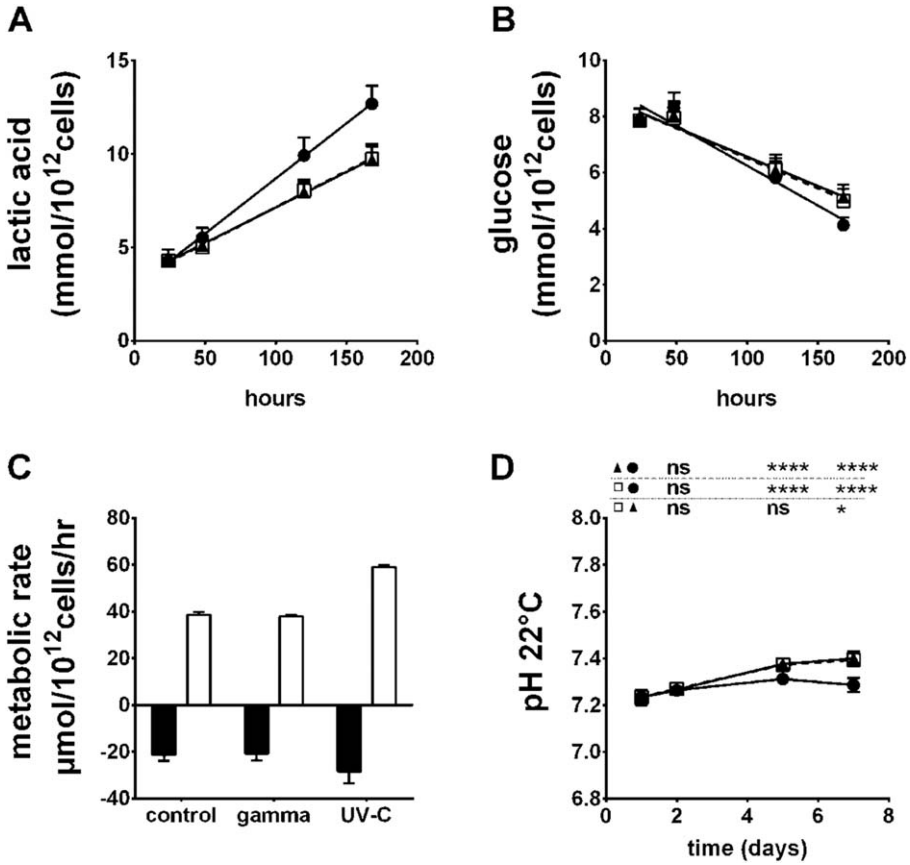

Fig. 5. Increased metabolic rate after UV-C treatment. The lactic acid production (A) and glucose consumption (B) per 10^{12} PLTs of untreated control (□ dotted line), gamma-irradiated (▲), and UV-C–treated (●) PLT concentrates was measured using a point-of-care blood gas analyzer. Linear regression was performed on the lactic acid production and glucose consumption in function of time to model the kinetics of respectively lactic acid (□) and glucose metabolism (■; C). One-way ANOVA of the mean values indicated a significant difference between the lactic acid production ($p < 0.0001$) and glucose consumption ($p < 0.003$) of UV-C and both control and gamma-irradiated PLT concentrates. PLT concentrate pH (D) was determined before (Day 1) and after treatment (Days 2, 5, and 7). Untreated control (□ dotted line), gamma-irradiated (▲), and UV-C–treated (●) PLTs are shown before (Day 1) and after treatment (Days 2, 5, and 7; ns = not significant; *$p < 0.05$; ****$p < 0.0001$).

alter the decreased thrombus formation kinetics onto collagen on Day 2. Of note, this experiment independently replicated the results from Fig. 1 confirming no significant difference on Day 2 for an additional five repeats. As a matter of control for the impact of nitrogen gas equilibration, GPIbα, P-selectin, annexin V, and activated integrin $\alpha_{IIb}\beta_3$ expression were measured but found not to be different between these conditions (Fig. S1, available as supporting information in the online version of this paper).

DISCUSSION

Three pathogen inactivation methods for PLT concentrates have been developed aiming to minimize chances of bacterial growth and/or transmission of virus. The UV-C procedure under study here is operationally straightforward and takes approximately 8 minutes per product. There is no additional workload compared with methods that involve supplementation with a photoactive compound and especially to AS-PCT where an additional photosensitizer removal step is required. On the other hand, criticisms on the UV-C method include its limited inactivation of certain viruses[11] including HIV-1 and its restrictive inclusion criteria.[5]

There are substantial data on the in vitro quality of PLT concentrates generated by AS-PCT and RF-PRT, generally indicating increased rates of storage lesion with a variable range of magnitude.[25-29] Published data on residual quality of PLTs treated with UV-C are less abundant but also indicate a slightly increased anaerobic metabolic rate causing significant differences in lactic acid

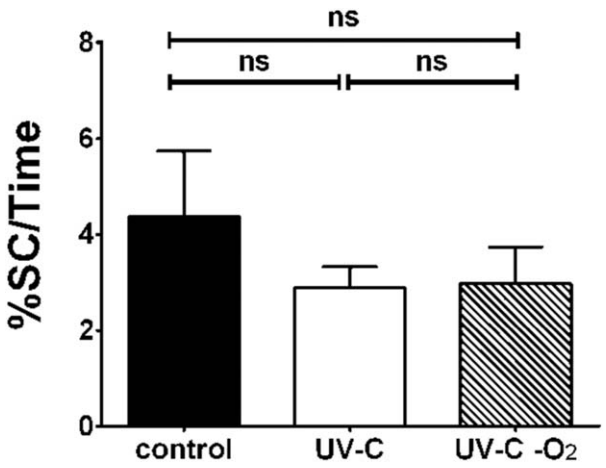

Fig. 6. Lowering the partial pressure of dissolved molecular oxygen does not alter reduced thrombus formation kinetics. PLT surface coverage in function of perfusion time (%SC/Time) is measured in microfluidic flow chambers coated with collagen on Day 2 according to Fig. 1. Paired reconstituted blood samples containing untreated control (■), UV-C–treated normoxic (□), and UV-C–treated hypoxic (UV-C-O_2; ▨) PLTs analyzed in duplicate at a shear stress of 50 dyn/cm^2. Mean values with SD as whiskers (n = 5) are shown. Results from the statistical analysis are depicted at the top of each panel (ns = not significant).

concentrations and pH. However, this is mostly near or beyond expiration dates[8-10,12] which is in line with our findings. Small but significant increases in degranulation (P-selectin)[9,10] and phosphatidylserine/-ethanolamine exposure[12] also support our data and may collectively indicate basal PLT activation or increased apoptosis. Of note, in terms of lesion kinetics our data indicate a small but sudden increase in both degranulation and annexin V binding (Fig. 4) with a subsequent normal rate of additional storage lesion when compared to gamma-irradiated or untreated controls.

PLT functional assays have been used less frequently to evaluate residual PLT quality after pathogen inactivation, also for the other two commercial photochemical methods. We recently published data indicating that both AS-PCT and RF-PRT significantly and irreversibly affected thrombus formation kinetics onto immobilized collagen.[29] The same experimental setup was applied to UV-C here and also showed an irreversible decrease although the impact on Day 2 was variable but not significant. We hypothesized that this is caused by the inherent variability in the partial pressures of dissolved molecular oxygen in the primary products based on our experience with RF-PRT where secondary superoxide anion formation causes biomolecular damage.[24] Therefore, we experimentally lowered the molecular oxygen partial pressures but this could not reverse variability nor the impact on thrombus formation kinetics (Fig. 6).

The increased binding of PAC1 to integrin $\alpha_{IIb}\beta_3$ after UV-C treatment confirms the findings published in the landmark study by Verhaar and coworkers.[30] They showed that short-wavelength light reduces disulfide bonds to free thiols in the fibrinogen receptor core structure. It is plausible that this disulfide reshuffling, which is a fundamental biochemical aspect of integrin activation,[31,32] alters the conformation promoting untimely fibrinogen binding. Even though the overall physical conditions of the study by Verhaar and coworkers differ (they used a lower UV-C dose of 0.15 J/cm^2, delivered to a different primary product with lower plasma content and a fixed penetration depth) from the routine procedure of pathogen inactivation by UV-C, it is unlikely that unrelated biochemical mechanisms cause the same observation.[5] In theory, enforced fibrinogen binding should lead to (some) activation of affected PLTs through outside-in signal transduction,[33] which then subsequently explains the slightly increased metabolic rate,[34] phosphatidylserine exposure, and degranulation. This is the case for RF-PRT, where the observed premature activation actually desensitizes subsequent PLT activation,[29] while in AS-PCT PLTs receptor activation is also affected[27,35] but without premature conformational changes to the receptor. However, in the case of UV-C the PLTs' subsequent reaction to external stimuli acting through G-protein coupled receptors (PAR1AP) or receptor tyrosine kinases (CVX) was not abnormal in light transmission aggregation nor in flow cytometry. It is therefore tempting to ascribe this difference between the methods to the well-known differential effects of UV-B versus UV-C illumination on PLT function; the former acting through direct activation of signal transduction cascades via protein kinases C[36] and oxidative stress while the latter does not.[30]

Of note, one immunogenicity study with autologous UV-C–treated PLTs was conducted in dogs[13] and did not yield measurable antibodies. Hence, either immunogenicity is low or the molecular changes observed are self-epitopes. Furthermore, proteomic analysis has indicated changes in the protein disulfide isomerase ERp72 for UV-C– and not UV-B–treated PLTs,[9] which adds to the data from Verhaar and colleagues[30] a role for redox regulation of integrin $\alpha_{IIb}\beta_3$ in the context of UV-C illumination–induced biochemical alterations.

We argue that the hemostatic sequence of PLT adhesion, activation, aggregation, and retraction or stability as measured comprehensively in perfusion chambers is affected by all three pathogen inactivation methods albeit not equally in manner and/or magnitude. RF-PRT treatment prematurely activates PLTs[26,37] while AS-PCT specifically affects integrin $\alpha_{IIb}\beta_3$ receptor activation after stimulation with agonists.[29,35] It is less clear from the current study what aspect of UV-C contributes to its reduced

flow chamber thrombus formation, but the redox induced changes in integrin $\alpha_{IIb}\beta_3$ conformation are a likely culprit. We performed two separate but similar experimental series in the flow chambers (Figs. 1 and 6) and confirmed that despite an average decrease in surface coverage kinetics, the statistical difference between UV-C and gamma-treated or untreated control was not reached on Day 2. Bashir and coworkers[12] previously demonstrated that UV-C pathogen inactivation reduces the posttransfusion recovery of PLTs after storage. Whether the effect of storage of UV-C–treated PLTs on both posttransfusion recovery and in vitro flow chambers reflect a related phenomenon is worth addressing.

In our hands, all pathogen inactivation techniques have more or less of an impact on PLT thrombus formation rates under hydrodynamic flow and therefore our findings raise questions about the exact contribution of pathogen-inactivated transfusion PLTs in arterial hemostasis. Randomized clinical trials with sufficient statistical power and standardized approaches[38] that allow assessment of hemostasis efficacy should help in elucidating the correlation with in vitro findings.

ACKNOWLEDGMENTS

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CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Fig. S1. Lowering molecular oxygen partial pressures has no effect on platelets. (A) P-selectin, (B) phosphatidylserine/-ethanolamine by annexin V binding, (C) GPIbα and (D) integrin $\alpha_{IIb}\beta_3$ activation was not different in normoxic (*open bars*) versus hypoxic (*shaded bars*) conditions. (E) Integrin activation in response to 30 μM PAR1AP or (F) 6 ng/mL convulxin was also not different. The differences with untreated controls (*closed bars*) were consequently comparable. The results from statistical analyses are indicated on top of each panel (n = 5). ns = not significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Video S1. THERAFLEX Day 2.

Video S2. THERAFLEX Day 5.

Video S3. THERAFLEX Day 7.