Treatment of Platelet Concentrates with the Mirasol Pathogen Inactivation System Modulates Platelet Oxidative Stress and NF- κ B Activation

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Keywords

Pathogen inactivation · Riboflavin · Ultraviolet light · Platelet · Platelet-additive solution · Reactive oxygen species

Summary

Background: Pathogen inactivation (PI) technologies for platelets aim to improve transfusion safety by preventing the replication of contaminating pathogens. However, as a consequence of treatment, aspects of the platelet storage lesion are amplified. Mirasol treatment also affects platelet signal transduction and apoptotic protein expression. The aim of this study was to examine the effect of Mirasol treatment on the generation of reactive oxygen species (ROS) and subsequent oxidative stress. **Methods:** Pooled platelet concentrates were prepared in platelet-additive solution (70% SSP+ / 30% plasma). ABO-matched platelets were pooled and split, and treated with the Mirasol system (TerumoBCT) or left untreated as a control. Platelet samples were tested on day 1, 5, and 7 post-collection. **Results:** Mirasol-treated platelets had increased formation of ROS by day 5 of storage. Oxidative damage, in the form of protein carbonylation, was higher in Mirasol-treated platelets, whilst no effect on nitrotyrosine formation or lipid peroxidation was detected. The NF- κ B signaling pathway was also activated in Mirasol-treated platelets, with increased expression and phosphorylation of NF- κ B p65 and I κ B α . **Conclusion:** These data demonstrate that Mirasol-treated platelets produce more ROS and display protein alterations consistent with oxidative damage.

Introduction

The Mirasol pathogen inactivation (PI) system for platelets utilizes a combination of broad-spectrum UV light and riboflavin (vitamin B2) to prevent the replication of dividing cells. This system has been shown to effectively inactivate a broad range of bacterial, viral and parasitic contaminants from platelet components prepared in both plasma and platelet-additive solution [1], thereby improving their safety profile.

Although the introduction of PI may improve transfusion safety, with the potential to negate the need for platelet irradiation, bacterial testing and additional donor screening tests [1], several aspects of in vitro platelet quality or function are affected by treatment. A number of studies have concluded that Mirasol treatment exacerbates the effects of the platelet storage lesion, resulting in increased glucose consumption, lactate production, and increased markers of platelet activation [2–6]. Further, we and others have previously demonstrated that Mirasol treatment affects platelet signal transduction and apoptotic protein expression [7–10].

It is well established that both riboflavin and UV light can independently lead to the formation of reactive oxygen species (ROS) [11–14]. Further, Mirasol treatment of plasma has recently been shown to result in oxidative damage of key plasma proteins [15]. As platelets generate ROS during activation [16, 17] and ROS are capable of selectively modulating platelet activation [18, 19], it is imperative to understand the role of Mirasol treatment in this context to ensure a complete understanding of the toxicological profile of this component [20]. Persistent high levels of ROS may overwhelm endogenous antioxidant systems, resulting in damage to proteins, lipids, and nucleic acids leading to apoptosis [21, 22].

Both UV light and ROS are known to cause activation of many early transcription factors, including nuclear factor- κ B (NF- κ B) [11, 23–26]. The activation of this family of proteins can result in a

myriad of cellular responses, including cell cycle activation, inflammation, and apoptosis. However, the effect of PI on this pathway has not been investigated. Therefore, the aim of this study was to examine whether Mirasol treatment and subsequent storage enhanced the formation of platelet ROS, resulting in activation of signal transduction pathways and platelet oxidative damage.

Material and Methods

Preparation of Platelet Concentrates

This study was approved by the Australian Red Cross Blood Service Human Research Ethics Committee. Whole blood units (450 ± 45 ml) were collected (day 0) into bottom-and-top bags containing 63 ml citrate phosphate dextrose (Fresenius Kabi, Bad Homburg, Germany) and stored at 22°C between collection and processing. Buffy coat-derived platelets were prepared from four ABO/RhD compatible units with 300 ml SSP+ (MacoPharma, Mouvoux, France), as previously described [27]. The platelet concentrates had a final composition of approximately 70% SSP+ and 30% plasma.

Treatment of Platelet Concentrates with the Mirasol System

ABO/RhD matched platelet concentrates were pooled and split to obtain paired control and test units ($n = 8$). Mirasol treatment was performed on day 1 post-collection. Briefly, the test units (Mirasol) were transferred to an illumination/storage bag (TerumoBCT, Lakewood, CO, USA), and 35 ml riboflavin ($500 \mu\text{mol/l}$ in 0.9% sodium chloride) was added to give a final concentration of approximately $50 \mu\text{mol/l}$. The products were then illuminated in the Mirasol system[®] (TerumoBCT) to $9.1 \text{ J/ml}_{\text{PLASMA}}$. The light dose was calculated using the weight of the treated platelet component, based on a plasma content of approximately 30%. The average illumination time was approximately 3 min. A saline surrogate (35 ml 0.9% sodium chloride; Baxter Healthcare Pty Ltd, Toongabbie, NSW, Australia) was added to the control units. These units remained untreated and were stored in the original bag (TerumoBCT).

In vitro Platelet Quality Analysis

All products were stored on a flat-bed agitator under constant agitation inside an enclosed incubator maintained at $20\text{--}24^\circ\text{C}$ (Helmer Inc., Noblesville, IN, USA). The enclosed incubator was sufficient to protect the treated units from prolonged exposure to light following treatment, as recommended by the manufacturer (TerumoBCT). Platelets were sampled on day 1, 5, and 7 post-treatment. At each time point, platelet concentrates were weighed, and unit volumes were calculated by dividing the unit weight by the specific gravity of 1.01 for platelets in SSP+. Platelet samples (10 ml) were removed under sterile conditions, using a plasma transfer set (Charter Medical, Winston-Salem, NC, USA). The platelet count was measured using an automated hematology analyzer (CellDyn Ruby, Abbott Diagnostics, Abbott Park, IL, USA). The pH was measured using a BASIC pH meter (at $20\text{--}24^\circ\text{C}$; Denver Instrument, Göttingen, Germany). Plasma carryover was determined using a bicinechonic acid (BCA) assay kit according to the manufacturer's instructions (Pierce Biotechnology, Rockford, IL, USA).

The exposure of phosphatidylserine on the outer plasma membrane of the platelets was determined from binding of annexin V-fluorescein isothiocyanate (FITC; BioLegend, San Diego, CA, USA) by flow cytometry (FACSCanto II, Becton Dickinson, Franklin Lakes, NJ, USA). Platelets were stained with annexin V-FITC in calcium-containing annexin-V binding buffer (BioLegend), as previously described [28].

Changes in platelet mitochondrial transmembrane potential ($\Delta\psi$) were determined using JC-1 (Biotium Inc., Hayward, CA, USA), as previously described [27]. Briefly, platelets (3×10^4) were incubated with $5 \mu\text{l}$ JC-1 dye in PBS for 20 min at 37°C . The cells were then washed before analysis by flow cytometry. The percentages of platelets displaying a polarized (red fluorescence) and depolarized mitochondrial membrane (green fluorescence) were reported.

Intracellular ROS generation was measured using the non-fluorescent 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H₂DCFDA; Invitrogen, Eugene, OR, USA). CM-H₂DCFDA is taken up and cleaved by intracellular esterases, preventing egress of the probe from the cell. With subsequent oxidation, the probe forms a fluorescent compound, carboxydichlorofluorescein (DCF). Superoxide anion production was determined using dihydroethidium (DHE; Invitrogen), which is oxidized by superoxide to fluorescent ethidium. For analysis, platelets were diluted to 1×10^6 cells/ml in phosphate-buffered saline (PBS) supplemented with 0.1% human plasma and stained with 5 mmol/l CM-H₂DCFDA or 5 mmol/l DHE for 20 min at 37°C , as previously described [29]. Fluorescence was detected by flow cytometry.

Protein lysates were prepared by centrifuging 5 ml of the platelet concentrate at $1,000 \times g$ for 15 min at 22°C . The pellet was then resuspended in $300 \mu\text{l}$ radioimmunoprecipitation assay buffer (RIPA: 20 mmol/l Tris-HCl (pH 7.5), 150 mmol/l NaCl, 1 mmol/l Na₂EDTA, 1 mmol/l EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mmol/l sodium pyrophosphate, 1 mmol/l b-glycerophosphate, 1 mmol/l sodium orthovanadate, $1 \mu\text{g/ml}$ leupeptin) (Cell Signaling Technology, Beverly, MA, USA) supplemented with $1\times$ complete protease inhibitor cocktail (Roche Applied Science, Basel, Switzerland) and 1 mmol/l phenylmethylsulfonyl fluoride (Sigma Aldrich Co., St Louis, MO, USA). The lysates were then cleared by centrifugation, and aliquots of the supernatant were stored at -80°C until required. Prior to use, the protein concentration of all samples was determined using a BCA assay as described above.

Protein carbonyl groups were detected using an OxyBlot[™] protein oxidation kit (Millipore Corporation, Billerica, MA, USA), according to the manufacturer's instructions. Platelet lysates were prepared in RIPA buffer (Cell Signaling Technology), supplemented with protease inhibitors and 2% 2-mercaptoethanol (Sigma Aldrich Co.), as described above. Derivatization reactions were carried out with $20 \mu\text{g}$ protein from day-5 untreated and Mirasol-treated platelet lysates. The proteins were separated by electrophoresis in a 4–20% polyacrylamide gel (BioRad, Hercules, CA, USA) and transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked and then incubated with the primary and secondary antibodies included in the kit. Enhanced chemiluminescent reagent (BioRad) and a digital imager (LAS4000; GE Life Sciences, Uppsala, Sweden) were used for detection. A representative blot from four separate experiments is presented. Protein carbonyl groups were also quantitated using an ELISA kit, according to the manufacturer's instructions (Cell Biolabs Inc., San Diego, CA, USA). Platelet lysates ($20 \mu\text{g/ml}$) were tested in triplicate, and the concentrations were calculated from a protein carbonyl BSA standard curve.

Detection of 3-nitrotyrosine-containing proteins was performed by a competitive ELISA (Cell Biolabs Inc.), according to the manufacturer's instructions. Platelet lysates ($20 \mu\text{g/ml}$) from untreated and Mirasol-treated platelets were tested in triplicate, and concentrations were calculated using a nitrated BSA protein standard curve.

4-hydroxynonenal-histidine (HNE-His) adduct formation was measured with a commercially available ELISA kit, according to the manufacturer's instructions (Cell Biolabs Inc.). Platelet lysates ($20 \mu\text{g/ml}$) were tested in triplicate, and concentrations were calculated using a HNE-BSA standard curve.

For western blotting, platelet lysates ($30 \mu\text{g}$) were separated by electrophoresis in a 4–20% polyacrylamide gel (Bio-Rad) and transferred to a PVDF membrane (Bio-Rad). Membranes were blocked with 5% skim milk powder in phosphate-buffered saline plus 0.05% Tween 20 (PBS-T) and then incubated with the following primary antibodies: anti-NF- κB -65, anti-phospho-NF- κB -65 (Ser 536), anti-I $\kappa\text{B}\alpha$, anti-phospho-I $\kappa\text{B}\alpha$ (Ser32) from Cell Signaling Technology and anti- β -actin (Sigma Aldrich Co.), followed by an appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (Cell Signaling Technology). Enhanced chemiluminescent reagent (Bio-Rad) was used for detection on a digital imager (ImageQuant LAS4000, GE Healthcare Life Sciences, Uppsala, Sweden).

Statistical Analysis

Results are expressed as mean $\pm$ standard deviation (SD). Data were analyzed using GraphPad Prism software (version 5.04; La Jolla, CA, USA). Un-

Table 1. Starting parameters of platelet concentrates

	Untreated (n = 8)	Mirasol (n = 8)	Treatment specifications
Volume, ml	355.00 ± 13.46	348.49 ± 8.61	250–450
Platelets × 10 ⁶ /μl	0.89 ± 0.08	0.94 ± 0.07	0.8–1.5
Plasma carryover, %	28.3 ± 1.6		30–45
pH (22 °C)	7.28 ± 0.08	7.26 ± 0.11	n/a

Fig. 1. Platelet membrane symmetry is altered but the mitochondrial membrane is not affected by Mirasol treatment. Platelets were treated with the Mirasol system or left untreated and stored at 22 °C for 7 days. At each time point, platelets were stained with **A** Annexin V-FITC (n = 8) and **B** JC-1 dye (n = 6) and analyzed immediately by flow cytometry. The data represent mean ± SD (error bars). *Indicates $p < 0.05$ compared to same time point.

treated and Mirasol-treated samples were compared over storage using a two-way repeated measures analysis of variance (ANOVA), with post-hoc comparisons performed using Bonferroni's test for multiple comparisons. Significance was defined as $p < 0.05$.

Results

On day 1 following treatment, the untreated and Mirasol-treated platelet groups were comparable to each other (table 1). The components met the manufacturer's specifications for platelet concentration and volume, although the plasma carryover was slightly below the manufacturer's recommendation for treatment, which may exacerbate some aspects of platelet metabolism.

Externalization of phosphatidylserine was increased in the Mirasol-treated platelets by day 5 of storage, and continued to increase during subsequent storage (fig. 1A). However, PI treatment and subsequent storage did not significantly alter mitochondrial membrane polarization (fig. 1B).

The production of ROS, measured using DCF and DHE staining, was examined in untreated and Mirasol-treated units during platelet storage. Due to the fluorescent properties of riboflavin, background fluorescence was monitored to ensure that it did not interfere with the data interpretation. Autofluorescence was higher in the Mirasol-treated platelets on day 1, but no differences were detected between untreated and Mirasol-treated platelets from day 2 onwards (data not shown). Mirasol-treated platelets exhibited significantly higher DCF fluorescence compared to untreated

platelets from day 5 of storage (fig. 2A, B), and was more pronounced by day 7 of storage, indicating increased generation of ROS. Similar results were seen for the percentage of cells positive for DHE, although these differences were not statistically significant (fig. 2C, D).

Protein carbonyl groups can be produced on protein side chains following oxidation [30]. Protein carbonylation was significantly higher in the Mirasol-treated platelets at all points during storage, compared to untreated platelets (fig. 3A, B). ROS can interact with nitrogen to form reactive nitrites, such as peroxynitrite (ONOO^-), which can modify proteins resulting in 3-nitro-tyrosine [31]. Mirasol treatment and storage did not appear to influence nitrotyrosine formation (fig. 3C). By-products of lipid peroxidation, such as 4-hydroxynonenal (4-HNE), are able to covalently bind to proteins via histidine (His) and may also be formed following oxidative damage [32]. The formation of HNE-His adducts was not affected by Mirasol treatment (fig. 3D).

Activation of proteins of the NF- κ B family is known to occur as a consequence of ROS generation and in response to treatment with UV light [24, 26]. In the Mirasol-treated platelets, NF- κ B was activated, as indicated by phosphorylation, by day 5 of storage, whereas phosphorylated NF- κ B was not detected in untreated platelets over storage (fig. 4A). The expression of total NF- κ B was also higher in the Mirasol-treated platelets at the same time points. Further, I κ B, the inhibitor of NF- κ B, was phosphorylated in Mirasol-treated platelets at the same points of storage (fig. 4B). However, degradation of I κ B was not detected.

Fig. 2. Mirasol treatment and storage results in increased intracellular hydrogen peroxide production, but not superoxide generation. Platelets were treated with the Mirasol system or left untreated and stored at 22 °C for 7 days. At each time point, platelets (1×10^6) were stained with **A, B** CM-H2DCFDA and the oxidation to fluorescent DCF ($n = 8$) or **C, D** Dihydroethidium (DHE) and the formation of fluorescent ethidium ($n = 6$) was analyzed by flow cytometry. The data represent mean $\pm$ SD (error bars). *Indicates $p < 0.05$ compared to same time point. Flow cytometry histograms demonstrate **A** DCF- and **C** DHE-positive cells from a representative sample on day 7 of storage.

Discussion

The platelet oxidative status was assessed in order to elucidate a mechanism for the enhanced platelet activation and altered signal transduction previously observed in Mirasol-treated platelets. The data presented here demonstrate that Mirasol treatment and subsequent ex vivo storage results in increased oxidative stress, resulting in protein carbonylation and phosphorylation of NF- κ B and I κ B.

The formation of ROS is capable of modulating several aspects of normal platelet function. ROS are intimately involved in the process of oxidative metabolism, the process by which platelets obtain the majority of their energy supply when stored under ideal ex vivo conditions [33]. Hydrogen peroxide has also been shown to trigger platelet aggregation [17, 19, 34, 35], as well as inhibit aggregation, depending on the presence of other mediators, such as NO and the type of agonists used [36]. ROS may also play a role in the generation of platelet microparticles and subsequent procoagulant effects [37] as well as in regulating tyrosine phosphorylation of many key proteins involved in platelet signal transduction, including vasodilator-stimulated phosphoprotein (VASP) [34, 38]. Similarly, increased metabolism, a reduced ability to aggregate in response to agonists, and increased expression of platelet signaling

proteins, including VASP, have been observed in Mirasol-treated platelets [5, 9, 39, 40]. The similarities between the effects of ROS and PI on platelet function are striking.

Oxidative stress occurs where excess ROS overwhelm endogenous antioxidant systems, resulting in damage to many cellular components, including proteins, lipids and nucleic acids, and subsequent apoptosis [21, 22]. Hydrogen peroxide is capable of causing oxidative damage to platelet proteins [41], and a recent study demonstrates that riboflavin/UV treatment of platelets induces oxidative modification of selected peptides, such as fibronectin [42], and Mirasol treatment of plasma increases protein carbonylation [15]. Protein carbonylation is widely used as a marker of protein oxidation. Our results demonstrate an increase in the formation of carbonylated proteins immediately following Mirasol treatment. This is interesting given that the increase in DCF-positive cells was not detected until day 5 following Mirasol treatment and storage, but this may be explained by the role of riboflavin as a photosensitizer, which may induce oxidation by multiple mechanisms [43]. Firstly, direct binding of riboflavin may occur, resulting in photoadduct formation and subsequent damage [44]. Alternatively, oxidative molecules, in addition to those measured in this study, may result in protein carbonylation [42].

Fig. 3. Mirasol treatment results in increased protein carbonylation, but not nitrotyrosine formation or lipid peroxidation. Platelets were treated with the Mirasol system or left untreated and stored at 22 °C for 7 days. At each time point, platelet lysates (20 µg/ml) were analyzed for **A** carbonyl concentration **B** carbonyl formation by Oxy-Blot **C** 3-Nitrotyrosine concentration and **D** HNE-His adduct concentration by ELISA at 450 nm, according to the manufacturer's instructions (Cell Biolabs Inc.). The data represent mean $\pm$ SD (error bars) from 8 replicates at the indicated time points. *Indicates $p < 0.05$ compared to same time point.

The result of oxidative damage can also be seen in the form of protein nitration and peroxidation of lipids. Under the experimental conditions used in this study, Mirasol treatment and storage did not induce detectable 3-nitro-tyrosine formation or overt signs of oxidative lipid damage, measured as HNE-His adduct formation. However, there are known pitfalls in the measurement of these outputs using ELISAs, particularly 3-nitro-tyrosine [45]. Further, the measurement of other endproducts, such as malondialdehyde as a marker of lipid peroxidation, could yield different results.

NF- κ B is one of the major transcription factors that responds directly to oxidative stress and induces the production of pro-inflammatory cytokines in various nucleated cell types [46]. In nucleated cells, NF- κ B activation proceeds via phosphorylation of its inhibitor, I κ B, which leads to its ubiquitination and degradation, thus allowing phosphorylation of the p65 NF- κ B subunit which then translocates to the nucleus to induce specific gene expression [23]. Whilst platelets lack DNA, the NF- κ B/I κ B complex has a role in signal transduction and activation in platelets in the absence of nuclear translocation [47], although the precise mechanisms modu-

Fig. 4. Activation of the NFκB pathway occurs following Mirasol treatment and storage. Platelets were treated with the Mirasol system or left untreated and stored at 22 °C for 7 days. At each time point, platelet lysates (30 μg) were separated by electrophoresis on SDS-polyacrylamide gels and transferred to a PVDF membrane. Blots were incubated with **A** NFκB p65, phospho-NFκB and actin or **B** IκBα, phospho-IκBα and actin. A representative blot is presented from three separate experiments. Molecular weight standards are shown on the left (kDa). *Indicates complex formation.

lating this process are not as clearly defined as for nucleated cells. It is known that platelets express NF-κB and that IκB phosphorylation and degradation occur in response to agonist-induced platelet activation [47, 48].

Our results demonstrate that NF-κB is phosphorylated in Mirasol-treated platelets after 5 days of storage, with concurrent phosphorylation of IκB. However, IκB degradation was not evident, perhaps due to the fact that the samples were not 'stimulated' prior to sample collection or because treatment with UV light can result in delayed IκB degradation [23]. Also evident was the formation of higher molecular weight NF-κB- and IκB-containing complexes.

NF-κB and IκB have been shown to form complexes with many proteins, including each other. Of particular interest, NF-κB and IκB have been found to form complexes with the catalytic subunit of cAMP-independent protein kinase A (PKAc) [49]. Activation of platelets with agonists such as collagen leads to disruption of IκB-PKAc complexes. Release of free, active PKA in turn leads to VASP phosphorylation as well as other signaling events [49]. Increased VASP phosphorylation has been observed in response to Mirasol treatment of platelets and was correlated with increased platelet activation [9]. These results are suggestive that the NF-κB complex formation observed in Mirasol-treated platelets may have several functional consequences. Identification of the protein partners that are complexed with NF-κB may assist in understanding the elaborate signal transduction role of this protein family in platelets.

It is evident that Mirasol treatment and the accumulation of ROS result in a similar outcome of platelet activation and apoptosis. As such, it would be easy to hypothesize that Mirasol treatment induces ROS formation, which in turn results in the changes associated with PI (i.e., reduced aggregation, altered metabolism, and induction of an apoptotic phenotype). However, an alternate proposition is that Mirasol treatment may simply accelerate platelet activation, and the excess ROS generation is consequently produced by the activated platelets. Studies using ROS scavengers, such as N-acetyl-L-cysteine (NAC), prior to treatment may assist in answering this question and will be the focus of future investigations. However, as has been noted previously [7], the addition of inhibitors to platelet products to prevent these changes may not be ideally suited for a platelet product destined for transfusion.

The combination of Mirasol treatment and subsequent storage modulates several aspects of the oxidative process. This study presents novel findings in relation to the redox biology of Mirasol-treated platelet components. Moreover, the complexity of the interactions between riboflavin, UV light and endogenous platelet ROS processes are also highlighted. By understanding the complex mechanisms by which these platelet changes occur, the possibility to mitigate some of these effects may be identified.

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Disclosure Statement

The authors have no conflict of interest to disclose.

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