VoxSanguinis
The International Journal of Transfusion Medicine
ISBT International Society of Blood Transfusion
Vox Sanguinis (2013)

DOI: 10.1111/vox.12106

ORIGINAL PAPER

Oxygen removal during pathogen inactivation with riboflavin and UV light preserves protein function in plasma for transfusion

H. B. Feys,[1] B. Van Aelst,[1] K. Devreese,[2] R. Devloo,[1] J. Coene,[3] P. Vandekerckhove[3] & V. Compernolle[1,3]

[1]*Transfusion Research Center, Belgian Red Cross-Flanders, Ghent, Belgium*
[2]*Coagulation Laboratory, Ghent University Hospital, Ghent, Belgium*
[3]*Blood Service of the Belgian Red Cross-Flanders, Mechelen, Belgium*

Vox Sanguinis

Background and Objective Photochemical pathogen inactivation technologies (PCT) for individual transfusion products act by inhibition of replication through irreversibly damaging nucleic acids. Concern on the collateral impact of PCT on the blood component's integrity has caused reluctance to introduce this technology in routine practice. This work aims to uncover the mechanism of damage to plasma constituents by riboflavin pathogen reduction technology (RF-PRT).

Methods Activity and antigen of plasma components were determined following RF-PRT in the presence or absence of dissolved molecular oxygen.

Results Employing ADAMTS13 as a sentinel molecule in plasma, our data show that its activity and antigen are reduced by 23 ± 8% and 29 ± 9% (n = 24), respectively, which corroborates with a mean decrease of 25% observed for other coagulation factors. Western blotting of ADAMTS13 shows decreased molecular integrity, with no obvious indication of additional proteolysis nor is riboflavin able to directly inhibit the enzyme. However, physical removal of dissolved oxygen prior to RF-PRT protects ADAMTS13 as well as FVIII and fibrinogen from damage, indicating a direct role for reactive oxygen species. Redox dye measurements indicate that superoxide anions are specifically generated during RF-PRT. Protein carbonyl content as a marker of disseminated irreversible biomolecular damage was significantly increased (3·1 ± 0·8 vs. 1·6 ± 0·5 nmol/mg protein) following RF-PRT, but not in the absence of dissolved molecular oxygen (1·8 ± 0·4 nmol/mg).

Conclusions RF-PRT of single plasma units generates reactive oxygen species that adversely affect biomolecular integrity of relevant plasma constituents, a side-effect, which can be bypassed by applying hypoxic conditions during the pathogen inactivation process.

Received: 23 July 2013, revised 25 September 2013, accepted 1 October 2013

Key words: pathogen inactivation, plasma, reactive oxygen species.

Introduction

Fresh-frozen plasma (FFP) is used clinically to replenish isolated coagulation factor deficiencies, to reverse the effect of warfarin and to treat the dilution coagulopathy that occurs with massive bleeding [1]. Plasma exchange, requiring large quantities of donor plasma, has become the treatment of choice in patients suffering from thrombotic thrombocytopenic purpura (TTP). Like other blood components for transfusion, FFP inherently carries a risk of pathogen transmission [2, 3], which can be reduced by various treatment methods. Solvent/detergent (S/D) treatment efficiently kills enveloped viruses and was licensed in Europe in 1991 and recently in the United Sates. The

Correspondence: Hendrik B. Feys, Ottergemsesteenweg 413, Ghent 9000, Belgium
E-mail: hendrik.feys@rodekruis.be

process includes pooling of multiple donations increasing patient exposure to donor material [4]. In the last couple of decades, bench-top alternatives have been developed allowing treatment of single donations and effectiveness against non-enveloped viruses. These methods are commonly based on light energy treatment of individual component bags in the presence of a dissolved chemical photosensitizer.

Photosensitizers are exogenously added molecules, which interact with nucleic acids to various extents and induce strand breakage or cross-linking upon photoexcitation, thereby irreversibly preventing replication [4]. Methylene Blue [5] (Macopharma, Tourcoing, France or Baxter International Inc, Deerfield, IL)-treated plasma is used in some European countries but requires freezing or additional filtering to, respectively, release or remove cytoplasm-borne pathogens. 'Newer' photosensitizers include amotosalen [6, 7] (or S-59, Cerus Corporation, Concord, CA), which does not require a freezing step, and riboflavin [8, 9] (or vitamin B2, Terumo BCT, Lakewood, CO), which is a naturally occurring vitamin, and therefore does not require cumbersome removal from plasma products following treatment. Both amotosalen and riboflavin initially were described for inactivation of pathogens in platelet concentrates, but have since also been validated for treatment of single plasma units [6, 10].

Photochemical pathogen inactivation treatment (PCT) of plasma involves risks of losing valuable material through processing and/or affecting the constituents required for efficient transfusion [11]. There are few sufficiently powered studies that have evaluated clinical efficacy of pathogen reduced plasma [12, 13]; therefore, evaluation of plasma quality is principally based on *in vitro* analysis of (labile) coagulation factors. From our previous paired analysis of all three photosensitizer-based methods [14], we concluded that coagulation factor loss is most pronounced in riboflavin treatment, though compensated to some extent by its low volume loss. Inversely, the methylene blue and amotosalen methods affect the constituent proteins less but are associated with larger volume losses.

The present work reveals the mechanism underlying the protein loss in riboflavin-based PCT (Mirasol® pathogen reduction, Terumo BCT, Lakwood, CO), showing a significant role for reactive oxygen species (ROS) and suggesting significant improvement by removal of dissolved oxygen prior to treatment.

Methods

Pathogen inactivation by riboflavin and UV light

ADAMTS13 was measured in paired plasma samples treated either by methylene blue (MB) (Macotronic®, Macopharma, Tourcoing, France), riboflavin pathogen reduction (RF-PRT) or amotosalen (AS) (Intercept®, Cerus, Concord, CA). Sample preparation and study design were as reported [14].

Plasma for riboflavin RF-PRT treatment [15] is mixed with 35 ml riboflavin solution (final concentration approximately 50 μM) and transferred to an illumination bag, which is subsequently illuminated with a UV (265–370 nm) light dose of 6·24 J/ml. Finally, the treated plasma is transferred to the storage bag.

ADAMTS13 activity and antigen determination

ADAMTS13 enzyme activity was measured using the fluorogenic substrate FRETS-VWF73 (AnaSpec, Fremont, CA, USA) [16] with modifications [17]. Normal human pooled plasma (NHP) (n = 21) was used as a reference and EDTA at 10 mM as a negative control. The final mixture contained 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, pH 7·4 supplemented with 1 mM $CaCl_2$, 1 μM $ZnCl_2$, 4·2 mM 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (Sigma-Aldrich) and 2 μM FRETS-VWF73. Fluorescence intensity was measured every 150 s for 90 min at 25°C in a microplate reader (Infinite F200 Pro, Tecan, Männedorf, Switzerland) equipped with a 340 nm excitation and 448 nm emission filter. Product formation rates were determined relative to NHP.

A sandwich enzyme-linked immunosorbent assay (ELISA) (Imubind®, American Diagnostica, Greenwich, CT, USA) was used to measure ADAMTS13 antigen, following the instructions of the provider. Detection of bound antigen is through a goat anti-human IgG antibody labelled with a streptavidin-horseradish peroxidase (HRP), which reacts with the perborate-3,3′-5,5′-tetramethylbenzidine (TMB) substrate to generate a blue coloured solution. After acid addition to stop colorimetric development, absorbance was measured at 450 nm in a microplate reader.

Electrophoresis and Western blotting of ADAMTS13

Sodium dodecyl sulfate–polyAcrylamide gel electrophoresis (SDS–PAGE) was performed on stain-free precast TGX gel, 7·5% (Bio-rad, Hercules, CA, USA) in a 2-amino-2-hydroxymethyl-propane-1,3-diol Tris-glycine buffered system (25 mM Tris, 192 mM Glycine, 0·1% (w/v) SDS). Plasma samples were diluted 20-fold in phosphate-buffered saline (PBS) containing 137 mM NaCl, 2·7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7·4 and non-reducing sample buffer containing 60 mM Tris–HCl, 10% (v/v) glycerol, 2% (w/v) SDS and 0·01% (w/v) bromophenolblue. NHP was

used as a negative control. As a positive control, NHP was incubated with 250 U/ml urokinase-type plasminogen activator (Sigma-Aldrich, St Louis, MO, USA) overnight at 37°C, followed by addition of 100 μM amiloride hydrochloride hydrate (Sigma-Aldrich, St Louis, MO, USA) to stop proteolysis. Transfer was on nitrocellulose blotting sandwiches for Turbo™ (Bio-rad, Hercules, CA, USA). After incubation with anti-ADAMTS-13 primary antibody[18] (kind gift of Dr. JTB Crawley, Imperial College London), HRP-labelled goat anti-rabbit (Jackson ImmunoResearch, West Grove, PA, USA) and chemiluminescence (Thermo Fisher Scientific, Rockford, IL, USA) were used to detect bound antibody. Gel images were developed on a digital imager (ChemiDoc MP System, Bio-rad, Hercules, CA, USA) and analysed by Image Lab (Bio-rad, Hercules, CA, USA).

Superoxide anion measurements

As a reporter for oxidative stress[19] 170 μM nitroblue tetrazolium (NBT) (Merck, Darmstadt, Germany) was mixed with 5 μM riboflavin (RF) and 0·5 mM ethylenediaminetetraacetic acid (EDTA) (both Acros Organics, Geel, Belgium) in phosphate-buffered saline (PBS, pH 7·4) in a polyolefin platelet illumination and storage bag (Terumo BCT). To specifically scavenge O_2^- , superoxide dismutase (SOD) (Sigma-Aldrich, St Louis, MO) was added to 100 U/ml. Following PRT according to standard operating protocols in a Mirasol® illuminator (Terumo BCT), formazan formation was measured by absorbance at 550 nm in a regular spectrophotometer (UV-3100PC, VWR International, Radnor, PA). For dynamic range purposes, a lead-up experiment using a serial dilution of RF in the same assay conditions (Fig. S1) indicated that 5 μM of RF resides in the linear portion of the dose–response relationship. All samples, bags and equipment were kept shaded from ambient light at all times, and air bubbles were removed manually by applying physical pressure.

Degasification procedure

Molecular oxygen gas dissolves in aqueous solutions according to Henry's law depending on partial gas pressure and solubility. To remove dissolved oxygen from buffer solutions or plasma, a small tube carrying pressurized inert nitrogen gas (Air Liquide, Paris, France) at 40 kPa was introduced in polyolefin storage bags and mixed for 20 min at room temperature in the dark. Following this procedure, oxygen concentrations were decreased to levels unable to oxidize resorufin (Fig. S2) in the alkaline 'blue bottle' oxygen reporter system containing 0·5 M KOH and 0·2 M D-glucose (all Acros Organics). Measurements by micro-Clark electrode potentiometry (Lazar Laboratories, Los Angeles, CA) indicated that the residual oxygen pressure in the bags was 540 ± 169 Pa (mean ± SEM, $n = 3$) (Fig. S3).

Factor VIII and fibrinogen

FVIII activity (%) was measured in a one-stage activated partial thromboplastin time-based clotting assay with factor deficient plasma. Fibrinogen activity was measured by the Clauss method, and both analyses were performed in duplicate on a STA-R Evolution apparatus with tools and reagents from Diagnostica Stago (Asnières, France).

Carbonylation as a measure of protein oxidation

Protein carbonylation is an irreversible biochemical modification resulting from protein oxidation and serving as an indirect marker of elevated ROS in biological systems. Carbonyl content was determined by spectrophotometric analysis of dinitrophenylhydrazone as described by Dalle-Donne *et al.* [20]. In brief, samples containing 2 mg protein were supplemented with 10 mM 2,4-dinitrophenylhydrazine (DNPH) (Sigma-Aldrich) in 2 M HCl (final concentrations) and incubated at room temperature with shaking for 1 h in shaded vials. Parallel samples only contained vehicle (2 M HCl) to determine background. Next, trichloroacetic acid (VWR International) in water was added to a final 10% (w/v) followed by gentle mixing and 10 min incubation on ice. Precipitated proteins were centrifuged at 2000 **g** for 10 min at 4°C followed by resuspension of the pelleted fraction in 6 M guanidine hydrochloride (GuHCl) in water. Excess unreacted DNPH was removed by desalting column chromatography over a 6 M GuHCl pre-equilibrated spin column (Zeba™, Thermo Fisher Scientific). Absorbance of dinitrophenylhydrazone in the flow-through fraction was determined by spectrophotometry at 370 nm, and values were corrected for background. An extinction coefficient of 22 000 $M^{-1}cm^{-1}$ was used for determination of carbonyl quantity. Finally, the samples were analysed for protein concentration by bicinchoninic acid assay (Fisher Scientific) to enable expression of carbonylation as mole per milligram protein.

Statistical analysis

Statistical analysis used standard algorithms from PRISM 5.04 software (GraphPad, La Jolla, CA) including one-way repeated measures ANOVA with Bonferroni or Dunn's post-tests, *t*-test and Mann–Whitney *U*-tests for comparison of means. Estimation of Gaussian distribution used Shapiro–Wilk or Kolmogorov–Smirnov tests.

Results

ADAMTS13 differentially affected by three photochemical pathogen inactivation methods

Our group recently investigated secondary hemostasis coagulation factors in a paired analysis of all three PCT methods [14]. It concluded that RF-PRT affects these factors more than MB and AS. To investigate whether this also is the case for ADAMTS13, this follow-up study measured activity and antigen in these samples. From the raw data, the recovery percentage was calculated, which relates final to initial ADAMTS13 absolute values and is a measure for retention of protein over the entire pathogen inactivation procedure. This recovery percentage hence takes into account all procedural steps including volume loss. The result is in line with the effects of PCT on the other coagulation factors [14] (Fig. 1) showing that RF-PRT treatment is not superior in retaining ADAMTS13 more than MB or AS despite the methodological asset of RF-PRT to avoid volume loss. Fig. 2 shows why this is so by plotting ADAMTS13 activity and antigen concentration before and after PCT. A significant decrease in ADAMTS13 antigen and function of 29% and 23%, respectively, can be observed for RF-PRT. Such decrease is less prominent for MB and least for AS. Taken together, significant ADAMTS13 (functional) enzyme is lost in both MB and RF, but volume loss accounts for decreases in the former, while molecular changes contribute most to the latter. Recovery is most sustained by AS treatment.

ADAMTS13 is not cleaved nor directly inhibited by riboflavin

To explain the ADAMTS13 molecular changes in RF-PRT, we hypothesized that the plasma treatment causes collateral serine protease activation. It is known that ADAMTS13 is variably susceptible to proteolytic degradation and subsequent inactivation by serine proteases, including factor Xa, thrombin or plasmin [18, 21]. However, despite substantial loss of the intact full length ADAMTS13 molecule in Western blot analysis (Fig. 3), no increase in smaller immunoreactive fragments characteristic for proteolytic breakdown is seen.

In addition, VWF73 proteolysis is not directly inhibited by RF at concentrations used in pathogen inactivation (Fig. S4) indicating no direct binding of RF to ADAMTS13.

Superoxide anions are generated during RF-PRT

Next, we hypothesized that the molecular changes in ADAMTS13 are caused by oxidation promoted by newly generated ROS. Oxidative stress during RF-PRT was directly measured by (colourless) NBT reduction to blue formazan. In buffer, significant formazan formation is seen upon illumination of 5 μM RF in a Mirasol® apparatus (Fig. 4). To dissect the nature of the reactive species involved, the specific superoxide scavenging enzyme SOD was added. A significantly lower signal is seen in the presence of 100 U/ml SOD indicating that a considerable portion of NBT reduction is caused by O_2^-, which can be scavenged by SOD. Superoxide anion radicals are formed when dissolved molecular oxygen ($O_{2,aq}$) accepts the excess energy from photochemically excited sensitizers. Indeed, when the $O_{2,aq}$ concentration is experimentally lowered by nitrogen gas sparging, the O_2^- -related NBT reduction can no longer be diverted using SOD (Fig. 4). Instead, NBT reduction is now established through non-oxygen and most probably direct electron transfer from excited photosensitizer molecules to the reporter. These data show that photosensitized RF transfers its excitation energy to $O_{2,aq}$ when present, generating O_2^- in the exact conditions used for RF-PRT of plasma.

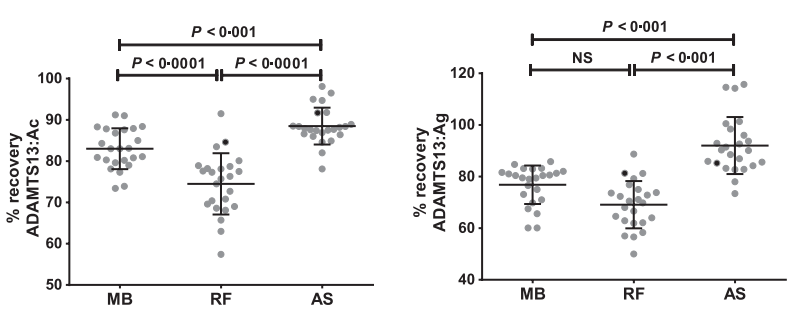

Fig. 1 Percentage recovery of ADAMTS13 in three pathogen reduction methods. The percentage recovery of ADAMTS13 activity (left) or antigen (right) is the absolute amount in the final bag expressed relative to the absolute starting amount. It is depicted here in function of each PCT method. The percentage recovery takes into account several procedural steps including molecular degradation as well as volume loss. Individual data are shown as dots, dataset mean as a horizontal line, and standard deviation as whiskers. Statistics indicated on top were generated by repeated measures one-way ANOVA with Bonferroni multiple comparison testing ($n = 24$).

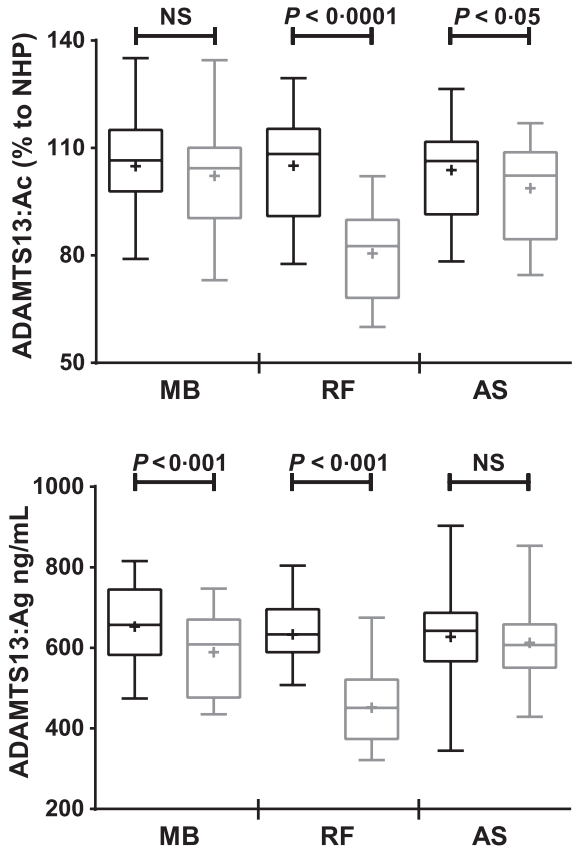

Fig. 2 ADAMTS13 activity and antigen are affected most by RF-based pathogen reduction. ADAMTS13 activity (top) and antigen (bottom) absolute concentrations were determined in plasma sampled from paired bags before (black boxes) and after (grey boxes) PCT treatment with either MB, RF or AS. Box edges represent standard deviations, median (horizontal line) and mean (+). Whiskers indicate data range (n = 24). Statistical results of repeated measures one-way ANOVA with Bonferroni multiple comparison testing are indicated above each dataset.

Oxidative damage can be prevented by lowering dissolved molecular oxygen

Superoxide anions and its derived radicals are highly reactive and not selective therefore not restricting its effects to ADAMTS13. To assess disseminated oxidative damage on plasma proteins, total carbonylation levels were determined (Fig. 5). Carbonyl groups (both aldehydes and ketones) are irreversibly added to protein by many different oxidative mechanisms [22] including reactions with advanced glycation and lipid end-products as well as metal-catalysed (Fenton-like chemistry) reactions. The data show that in the presence of normal $O_{2,aq}$ concentrations generic oxidation of proteins is prominent following RF-PRT. However, when $O_{2,aq}$ is experimentally lowered by inert gas sparging this damage is prevented (Fig. 5). This shows a direct role of $O_{2,aq}$ in modification of proteins present in plasma.

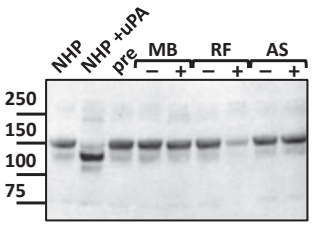

Fig. 3 ADAMTS13 is not proteolysed but partially degraded in RF-PRT. Plasma samples before (-) or after (+) RF-PRT were analysed in SDS–PAGE with Western blotting for ADAMTS13 protein. An equal amount of protein was loaded in each lane. As negative and positive controls for enzymatic proteolysis, untreated (NHP) or urokinase (NHP+u-PA) incubated, paired plasma samples were loaded. The sample prior to thawing for RF-PRT (pre) is also included. Molecular weight references are indicated to the left in kilodaltons.

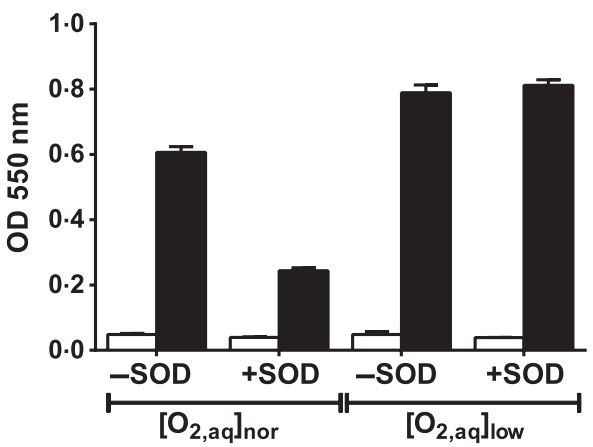

Fig. 4 Riboflavin and light pathogen reduction conditions generate O_2^- depending on $O_{2,aq}$. The nitroblue tetrazolium redox indicator is converted to formazan, which absorbs at 550 nm upon reduction by superoxide or its derivatives. The absorbance in blood component storage bags before (open bars) and after (closed bars) RF-PRT with 5 μM RF in a Mirasol® illumination apparatus is shown. Addition of 100 U/ml superoxide dismutase (SOD) indicates the specific presence of O_2^- molecules. These are generated in function of $O_{2,aq}$ availability as is shown when ambient partial pressures (nor) of $O_{2,aq}$ are experimentally lowered (low) by nitrogen gas sparging. Means (n = 5) with standard error of the mean as error bars are depicted.

Because some proteins in donorplasma are specifically relevant in the context of coagulopathies, these were investigated in more detail with respect to RF-PRT in the presence or absence of $O_{2,aq}$. Figure 6 shows that ADAMTS13 activity, fibrinogen concentration and FVIII:C are significantly better retained when the donorplasmas are pretreated with nitrogen gas to reduce $O_{2,aq}$.

Discussion

Photochemical treatment of biological material affects the (functional) integrity of the constituents to various

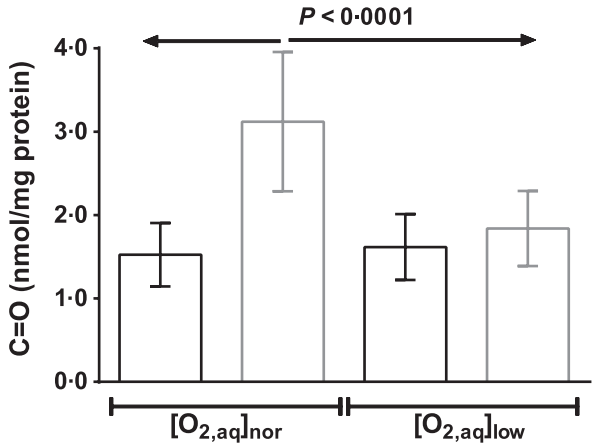

Fig. 5 Disseminated oxidative damage of plasma proteins following RF-PRT. Carbonyl content of protein molecules is determined by chromogenic derivatization on paired samples (n = 7) taken from plasma bags before (black bars) and after (grey bars) RF-PRT without plasma pretreatment ($[O_{2,aq}]_{nor}$) or following oxygen reduction ($[O_{2,aq}]_{low}$) by inert gas purging. Data are expressed as mean molar carbonyl content per unit of mass protein and standard deviations are indicated by whiskers. Statistics compared means by repeated measures one-way ANOVA using Bonferroni multiple comparison posttesting.

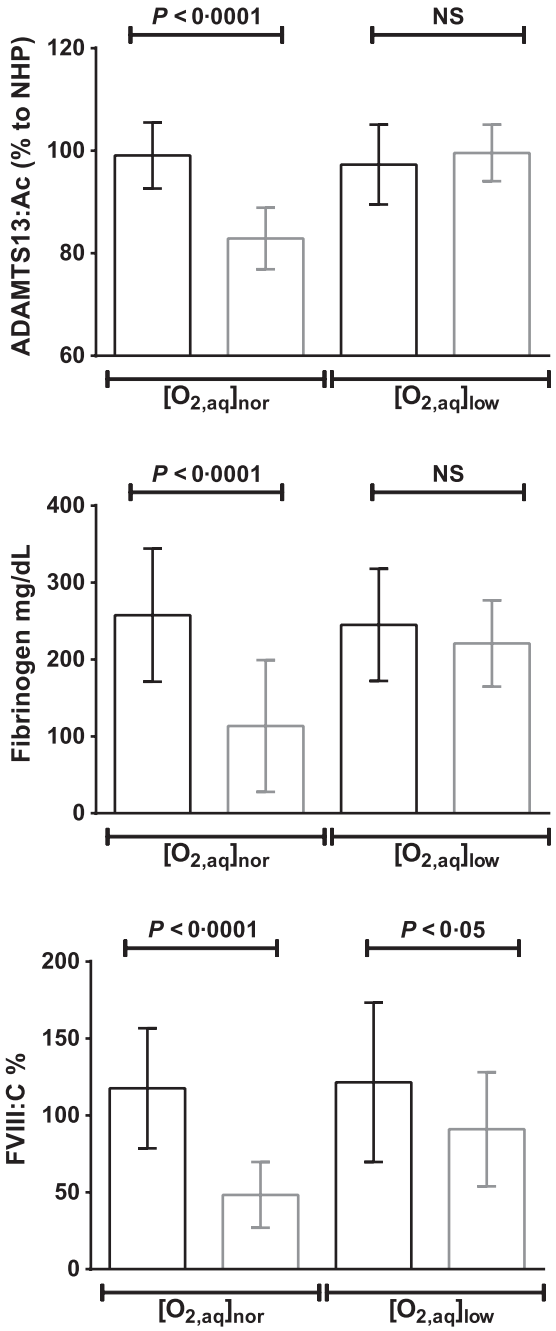

Fig. 6 Dissolved molecular oxygen underlies damage to ADAMTS13, fibrinogen and FVIII during RF-PRT. ADAMTS13 activity, fibrinogen and FVIII levels were determined in paired samples taken from plasma bags before (black bars) and after (grey bars) RF-PRT without plasma pretreatment ($[O_{2,aq}]_{nor}$) or with oxygen reduction ($[O_{2,aq}]_{low}$) by inert gas purging. Bars represent means and whiskers standard deviations (n = 7). Statistics compared means by repeated measures one-way ANOVA using Bonferroni multiple comparison posttesting.

degrees. This has been demonstrated for plasma with all three available methods for blood product processing and most specifically using intrinsically labile factors like FVIII [11]. However, the molecular basis for the observed effect is unclear and novel insights should allow optimization of current methods to deliver better plasma products to patients.

From our experiments measuring ADAMTS13 in treated plasma, it is clear that the largest biomolecular damage is seen with RF-PRT when compared to MB and AS. These specific data corroborate well with the observed changes in other unrelated coagulation factors during PCT [14]. Plasma products containing fewer active ADAMTS13 may in theory be less efficient to treat TTP. Remission might be achieved more slowly [23] or only following administration of a larger volume, which may in turn increase typical risks of plasma exchange or plasma infusion like fluid overload or citrate-induced hypocalcemia. Some might argue that the difference in ADAMTS13 concentration is not that relevant when taking into account the seemingly low threshold of ADAMTS13 activity required for achieving remission of TTP [24], but adequately powered clinical studies are lacking to confirm either statement.

Although adverse effects of photochemistry on coagulation factors have been reported before, there are no data describing the underlying phenomenon that damages plasma biomolecules. Using the ADAMTS13 susceptibility to RF-PRT as a readout, a series of experiments sought to identify the cause. First, direct riboflavin inhibition of ADAMTS13 function was investigated as a potential bias in the readout. Using the FRETS-VWF73-based

ADAMTS13 activity assay, no direct effect of RF on the enzyme function could be detected. Next, random activation of zymogens was hypothesized relating the observed functional and effective decrease in ADAMTS13 to its previously reported susceptibility to serine proteases [21]. In a Western blotting experiment known to be sensitive to proteolytic bands of ADAMTS13 [18], no increased breakdown could be detected following RF-PRT despite a significantly weaker intensity of the full length band of ADAMTS13.

Next, the role of increased oxidative stress was investigated using the redox reporter NBT [19]. Riboflavin and ultraviolet light have been used for decades in chemistry laboratories as an easy and inexpensive model system for the generation and investigation of superoxide anions and singlet oxygen. However, in the specific context of PCT, it is unknown whether and to what extent ROS are produced. To investigate this, blood product bags that would otherwise contain donor plasma were filled with buffered NBT redox reporter and treated with RF-PRT. An oxidative response in function of RF dose could be detected, indicating dose dependent increase in redox potential in the exact setting of bench-top plasma pathogen inactivation. By addition of the specific superoxide scavenger SOD, the reporter was significantly less reduced demonstrating that substantial amounts of superoxide anion were driving the NBT redox reaction during the process of RF-PRT.

Because ROS react with multiple targets in an unspecific fashion, generic damage to the plasma content was investigated by measurement of carbonyl modifications to plasma protein. Carbonylation is irreversible and can be detected and quantified by chemical derivatization in solution. Indeed, following RF-PRT, a significant increase in protein carbonyl content is seen. This might also explain the observed decrease in anti-ADAMTS13 binding in Western blot (Fig. 3) as epitopes may be directly affected by these or other molecular modifications induced by ROS.

Nonetheless, our data show that the damage to the product can be bypassed through performing the RF-PRT procedure in a hypoxic environment. First, when the NBT redox reporter experiment with and without SOD was repeated in hypoxic conditions, we found that the RF-mediated NBT redox reaction was no longer driven by superoxide. This shows that in the absence of dissolved oxygen photo-excited RF intermediates are still able to reduce NBT and possibly other targets (like pathogen nucleic acids) but that the superoxide production is severely hampered. Second, in such hypoxic conditions, the disseminated carbonylation of protein also was efficiently rescued as shown in Fig. 5. Finally, the earlier reported specific damage to FVIII, fibrinogen and ADAMTS13 can be rescued eventually resulting in higher yields of these clinically significant factors.

Taken together, our data show that UV light excitation of RF in the practical context of PCT transfers energy to dissolved molecular oxygen, generating superoxide anion. This and other reactive species not only cause protein carbonylation [25, 26] but may potentially also induce fatty acid peroxidation chain reactions in lipids [27], auxiliary modifications in proteins [28] (e.g. nitrosylation) and glycation of carbohydrates [29]. Therefore, superoxide and other ROS generated during RF-PRT likely contribute to the biostatic action of PCT by effectively damaging pathogen biomolecules [30] as an indirect effect of RF excitation. However, this is not the sole pathogen inactivation mechanism [9] because direct damage of nucleic acids by excited RF is also likely to be involved because direct irreversible oxidative chemistry by photo-excited RF has been demonstrated [31] as well as direct (non-covalent) binding of RF to nucleic acids [32]. Therefore, performing the RF–PRT procedure in hypoxic conditions may still adequately affect pathogenic nucleic acids with the important advantage of avoiding random damage in the product caused by ROS, but this requires further testing.

The same phenomenon may apply to platelet concentrates treated with Mirasol® technology because recent work by the Australian Red Cross [33] indicates that platelet lysates from concentrates treated with RF-PRT have increased carbonylation levels as well as a rise in the advanced lipid end product 4-hydroxy-2-nonenal. It is unclear whether performing PCT of platelets in the absence of dissolved molecular oxygen can rescue the observed biomolecular damage without compromising the cell's metabolic housekeeping. Anoxia causes metabolic damage in platelets [34], and therefore, the beneficial effects of such pretreatment in the setting of PCT may be balanced out. Despite this, the entire process of illumination only takes a couple of minutes, which may allow to effectively remove and resupply oxygen without significantly disturbing platelet metabolism and gas exchange. If feasible, this would prove a major technological step forward in safekeeping blood products during pathogen inactivation.

Pathogen inactivation is an important step forward in the aim to reduce transmission of disease by transfusion of blood products. Our experimental work on RF-PRT indicates that the delicate balance to conserve plasma product integrity while ensuring a high safety level may benefit from working in hypoxic conditions.

Acknowledgements

This work has been made possible through the generous support from the Foundation for Research and Development of the Belgian Red Cross-Flanders Blood Service.

We thank Dr. James TB Crawley (Imperial College, London) for providing the polyclonal anti-ADAMTS13 antibody. We thank Bea Sabot for her contribution on the comparison of the three different PRT methods.

Authorship contributions

HBF and VC designed research; VC, JC and PV contributed critical analytical tools, reagents, samples or data; BVA, RD, KD and HBF performed research and collected data; BVA, RD, VC, KD and HBF analysed and interpreted data; BVA and HBF performed statistical analyses; HBF wrote the manuscript; all authors critically reviewed and amended the manuscript. VC has primary responsibility for final content.

Conflicts of interest

The authors declare that they have no conflict of interests.

References

1 O'Shaughnessy DF, Atterbury C, Bolton Maggs P, *et al.*: Guidelines for the use of fresh-frozen plasma, cryoprecipitate and cryosupernatant. *Br J Haematol* 2004; **126**:11–28
2 Dodd RY: Emerging pathogens and their implications for the blood supply and transfusion transmitted infections. *Br J Haematol* 2012; **159**:135–142
3 Allain JP, Stramer SL, Carneiro-Proietti AB, *et al.*: Transfusion-transmitted infectious diseases. *Biologicals* 2009; **37**:71–77
4 Pelletier JP, Transue S, Snyder EL: Pathogen inactivation techniques. *Best Pract Res Clin Haematol* 2006; **19**:205–242
5 Hornsey VS, Drummond O, Young D, *et al.*: A potentially improved approach to methylene blue virus inactivation of plasma: the Maco Pharma Maco-Tronic system. *Transfus Med* 2001; **11**:31–36
6 Ciaravino V, McCullough T, Cimino G, *et al.*: Preclinical safety profile of plasma prepared using the INTERCEPT Blood System. *Vox Sang* 2003; **85**: 171–182
7 Lin L, Cook DN, Wiesehahn GP, *et al.*: Photochemical inactivation of viruses and bacteria in platelet concentrates by use of a novel psoralen and long-wavelength ultraviolet light. *Transfusion* 1997; **37**:423–435
8 Ruane PH, Edrich R, Gampp D, *et al.*: Photochemical inactivation of selected viruses and bacteria in platelet concentrates using riboflavin and light. *Transfusion* 2004; **44**:877–885
9 Kumar V, Lockerbie O, Keil SD, *et al.*: Riboflavin and UV-light based pathogen reduction: extent and consequence of DNA damage at the molecular level. *Photochem Photobiol* 2004; **80**:15–21
10 Smith J, Rock G: Protein quality in Mirasol pathogen reduction technology-treated, apheresis-derived fresh-frozen plasma. *Transfusion* 2010; **50**:926–931
11 Benjamin RJ, McLaughlin LS: Plasma components: properties, differences, and uses. *Transfusion* 2012; **52**(Suppl. 1):9S–19S
12 Mintz PD, Bass NM, Petz LD, *et al.*: Photochemically treated fresh frozen plasma for transfusion of patients with acquired coagulopathy of liver disease. *Blood* 2006; **107**:3753–3760
13 Bartelmaos T, Chabanel A, Leger J, *et al.*: Plasma transfusion in liver transplantation: a randomized, double-blind, multicenter clinical comparison of three virally secured plasmas. *Transfusion* 2013; **53**:1335–1345
14 Coene J, Devreese K, Sabot B, *et al.*: Paired analysis of plasma proteins and coagulant capacity following treatment with three methods of pathogen reduction. *Transfusion* 2013. (in press)
15 Bihm DJ, Ettinger A, Buytaert-Hoefen KA, *et al.*: Characterization of plasma protein activity in riboflavin and UV light-treated fresh frozen plasma during 2 years of storage at -30 degrees C. *Vox Sang* 2010; **98**:108–115
16 Kokame K, Nobe Y, Kokubo Y, *et al.*: FRETS-VWF73, a first fluorogenic substrate for ADAMTS13 assay. *Br J Haematol* 2005; **129**:93–100
17 Anderson PJ, Kokame K, Sadler JE: Zinc and calcium ions cooperatively modulate ADAMTS13 activity. *J Biol Chem* 2006; **281**:850–857
18 Crawley JT, Lam JK, Rance JB, *et al.*: Proteolytic inactivation of ADAMTS13 by thrombin and plasmin. *Blood* 2005; **105**:1085–1093
19 Gibson SL, Cohen HJ, Hilf R: Evidence against the production of superoxide by photoirradiation of hematoporphyrin derivative. *Photochem Photobiol* 1984; **40**:441–448
20 Dalle-Donne I, Rossi R, Giustarini D, *et al.*: Protein carbonyl groups as biomarkers of oxidative stress. *Clin Chim Acta* 2003; **329**:23–38
21 Feys HB, Vandeputte N, Palla R, *et al.*: Inactivation of ADAMTS13 by plasmin as a potential cause of thrombotic thrombocytopenic purpura. *J Thromb Haemost* 2010; **8**:2053–2062
22 Levine RL, Wehr N, Williams JA, *et al.*: Determination of carbonyl groups in oxidized proteins. *Methods Mol Biol* 2000; **99**:15–24
23 Del Río-Garma J, Alvarez-Larrán A, Martínez C, *et al.*: Methylene blue-photoinactivated plasma versus quarantine fresh frozen plasma in thrombotic thrombocytopenic purpura: a multicentric, prospective cohort study. *Br J Haematol* 2008; **143**:39–45
24 Moake JL: Thrombotic thrombocytopenic purpura: the systemic clumping "plague". *Annu Rev Med* 2002; **53**:75–88
25 Speck WT, Chen CC, Rosenkranz HS: In vitro studies of effects of light and riboflavin on DNA and HeLa cells. *Pediatr Res* 1975; **9**:150–153
26 Joshi PC: Comparison of the DNA-damaging property of photosensitised riboflavin via singlet oxygen ($1O_2$) and superoxide radical (O_2^-) mechanisms. *Toxicol Lett* 1985; **26**:211–217
27 Niki E: Lipid peroxidation: physiological levels and dual biological effects. *Free Radic Biol Med* 2009; **47**:469–484
28 Suzuki YJ, Carini M, Butterfield DA: Protein carbonylation. *Antioxid Redox Signal* 2010; **12**:323–325

29 Singh R, Barden A, Mori T, *et al.*: Advanced glycation end-products: a review. *Diabetologia* 2001; **44**:129–146
30 Maisch T, Baier J, Franz B, *et al.*: The role of singlet oxygen and oxygen concentration in photodynamic inactivation of bacteria. *Proc Natl Acad Sci USA* 2007; **104**:7223–7228
31 Dardare N, Platz MS: Binding affinities of commonly employed sensitizers of viral inactivation. *Photochem Photobiol* 2002; **75**:561–564
32 Kasai H, Yamaizumi Z, Berger M, *et al.*: Photosensitized formation of 7,8-dihydro-8-oxo-2'-deoxyguanosine (8-hydroxy-2'-deoxyguanosine) in DNA by riboflavin: a nonsinglet oxygen-mediated reaction. *J Am Chem Soc* 1992; **114**:9692–9694
33 Johnson L, Marks D: Mirasol pathogen reduction technology modulates platelet oxidative stress [abstract]. *Vox Sang* 2013; **105**:65–299 Abstract P-195.
34 Kilkson H, Holme S, Murphy S: Platelet metabolism during storage of platelet concentrates at 22 degrees C. *Blood* 1984; **64**:406–414

Supporting Information

Additional Supporting Information may be found in the online version of this article:
Fig. S1 Reduction of nitrobluetetrazolium (NBT) by Mirasol™ illumination of various RF concentrations.
Fig. S2 Nitrogen gas sparging effectively decreases dissolved oxygen in illumination bags.
Fig. S3 Partial pressure of dissolved oxygen in plasma decreases in function of N2 sparging time.
Fig. S4 ADAMTS13 is not directly inhibited by RF.