ORIGINAL ARTICLE
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UDC: 615.38
DOI: https://doi.org/10.2298/VSP210315051G

Influence of riboflavin and ultraviolet-light treatment on plasma proteins – protein S and alpha 2-antiplasmin – in relation to the time of administration

Uticaj riboflavina i ultravioletnog zračenja na proteine plazme – protein S i alfa 2-antiplazmin – u odnosu na vreme primene

Dragana Gojkov*, Bela Balint[†‡§], Bratislav Dejanović[‖], Dušan Vučetić*[¶]

Military Medical Academy, *Institute for Transfusiology and Haemobiology, [‖]Institute for Medical Biochemistry, Belgrade, Serbia; [†]Serbian Academy of Sciences and Arts, Department of Medical Sciences, Belgrade, Serbia; [‡]Institute for Cardiovascular Diseases "Dedinje", Department of Transfusion Medicine, Belgrade, Serbia; [§]University of Belgrade, Institute for Medical Research, Belgrade, Serbia; [¶]University of Defence, Faculty of Medicine of the Military Medical Academy, Belgrade, Serbia

Abstract

Background/Aim. After the introduction of a careful selection procedure for blood donors and the implementation of highly sensitive screening tests for transfusion-transmitted infections (TTIs), blood has become a very safe product concerning TTIs. However, due to the existence of a "window" period during which these "markers" cannot be detected, as well as the emergence of new pathogens, the risk is still present. Implementation of pathogen reduction technology (PRT) provides a proactive approach to improving blood safety. By damaging nucleic acids, PRT selectively inactivates pathogens and leucocytes. Nevertheless, during the process, plasma proteins are also damaged to some extent. The aim of this study was to conclude whether there is a difference in the effect of PRT on protein S (PS) and alpha 2-antiplasmin (α2AP) regarding the time of inactivation: inactivation immediately after plasma separation from whole blood (before freezing) vs. inactivation after freezing/thawing. **Methods.** The voluntary donors' blood is taken into a quadruple bag system, centrifuged, and separated into blood products. Control group plasma was first inactivated by the Mirasol® PRT system and then frozen. Experimental group plasma was immediately frozen and, after four months, thawed and inactivated. PS and α2AP activity was examined in samples after separation, inactivation, and thawing. **Results.** Analyzing PS and α2AP activity, no statistically significant difference was found between the initial samples. The trend of protein activity reduction after inactivation and freezing/thawing was present in both groups but without a statistically significant intergroup difference. **Conclusion.** No statistically significant difference was found between the activity values of PS and α2AP after immediate inactivation, before freezing, and after freezing/thawing, making stored plasma units suitable for safe and efficient inactivation directly before clinical use and according to the patient's blood type.

Key words:
alpha-2-antiplasmin; plasma; proteins; protein s; riboflavin; safety; time factors; ultraviolet rays.

Apstrakt

Uvod/Cilj. Pažljivim izborom davalaca i korišćenjem visoko osetljivih „skrining" testova za detekciju uzročnika infekcija koje se mogu preneti putem transfuzije (TTI), krv je postala veoma bezbedan produkt u odnosu TTI. Međutim, zbog postojanja perioda „prozora" tokom kojeg se ovi „markeri" ne mogu detektovati, kao i pojave novih patogena, rizik je i dalje prisutan. Uvođenjem tehnologije za redukciju patogena (PRT) ostvaruje se proaktivan pristup u poboljšanju bezbednosti krvi. Oštećenjem nukleinskih kiselina, PRT selektivno inaktivira patogene i leukocite. Nažalost, tokom ovog procesa se u određenom stepenu oštećuju i proteini plazme. Cilj rada je bio da se utvrdi postojanje razlika u efektu PRT na protein S (PS) i α2-antiplazmin (α2AP) u zavisnosti od vremena inaktivacije: ukoliko se plazma inaktivira odmah po izdvajanju iz jedinice krvi (pre zamrzavanja) ili ako se inaktivira naknadno, posle zamrzavanja/odmrzavanja. **Metode.** Krv dobrovoljnih davalaca je bila prikupljena u sistem četvorostrukih kesa, centrifugirana i razdvojena na produkte. Kontrolna plazma je bila najpre inaktivisana

Correspondence to: Dragana Gojkov, Military Medical Academy, Institute for Transfusiology and Haemobiology, Crnotravska 17, 11 000 Belgrade, Serbia. E-mail: jovidrag@gmail.com

Mirasol® PRT sistemom i, potom, zamrznuta. Plazma eksperimentalne grupe je bila odmah zamrznuta, a nakon četiri meseca odmrznuta i inaktivisana. Aktivnost PS i α2AP je bila ispitivana u uzorcima plazme posle separacije, inaktivacije i odmrzavanja. **Rezultati.** Analizom rezultata aktivnosti PS i α2AP utvrđeno je da nije bilo statistički značajne razlike između inicijalnih uzoraka. Nakon inaktivacije i zamrzavanja/odmrzavanja postojao je trend pada aktivnosti ovih proteina u obe grupe, ali statistički značajna razlika između kontrolne i eksperimentalne grupe nije ustanovljena. **Zaključak.** Nije postojala statistički značajna razlika između vrednosti aktivnosti PS i α2AP nakon inaktivacije pre zamrzavanja, odnosno posle zamrzavanja/odmrzavanja, što ukazuje da uskladištene jedinice plazme mogu biti sigurno i efikasno inaktivisane neposredno pre kliničke upotrebe, u skladu sa krvnom grupom primaoca.

Ključne reči:
alfa-2-antiplazmin; plazma; protein; protein s; riboflavin; bezbednost; vreme, faktor; ultravioletni zraci.

Introduction

Plasma is the liquid part of blood that contains pro and anticoagulant factors. Plasma can be defined as fresh frozen plasma (FFP) if frozen within 8 hours from collection, plasma 24 (frozen within 24 hours from collection), or thawed plasma. FFP and plasma 24 contain all coagulation factors. If FFP and plasma 24 are thawed, they become thawed plasma and can be stored at 4 °C for five days.

Plasma is used for treating multiple coagulation deficiencies that occur in patients with liver failure, vitamin K deficiency, warfarin overdose, disseminated intravascular coagulation, or massive transfusion. Sometimes, plasma can be used for treating patients with single factor deficiency, such as factor XI deficiency. FFP is used as a replacement fluid in therapeutic plasma exchange (TPE). In cases of thrombotic thrombocytopenic purpura (TTP), TPE removes inhibitors, and plasma provides a metalloprotease (ADAMTS13), thus reversing the symptoms [1, 2].

Protein S (PS) is a vitamin K-dependent protein that enhances the anticoagulant effect of activated protein C (APC). PS is synthesized in hepatocytes, endothelial cells, megakaryocytes, and brain cells. As a cofactor for APC, PS has a role in the inactivation of the factors Va and VIIIa. Factor Va inactivation happens as an ordered series of peptide bond cleavage in the molecule's heavy chain, first rapid cleavage at Arg 506, then slower cleavage at Arg 306, and then Arg 679. Interaction of PS and APC results in both an increased affinity for negatively charged phospholipids and a 20-fold enhancement of the slower phase of factor Va inactivation. Only 40% of plasma PS is free and available, whereas the rest is bound to C4b-binding protein and cannot interact with APC [3].

The primary inhibitor of plasmin synthesized in the liver is alpha 2-antiplasmin (α2AP). Bound plasmin digests clots and restores blood vessel lumen, and free plasmin in the circulation digests fibrinogen, factor V, factor VIII, and fibronectin which may cause potentially fatal primary fibrinolysis. α2AP rapidly and irreversibly binds free plasmin [4].

Blood for transfusion is extremely safe concerning virus transmission given the improved donor screening method and a range of assays for detecting antibodies, antigens, and genomes [5]. However, the emergence of new pathogens, such as West Nile virus, Severe Acute Respiratory Syndrome (SARS) virus, Chikungunya, Dengue, and many others, make a permanent threat [6, 7].

The other issue is the so-called "window period" during which detecting the presence of the pathogen is impossible, no matter how testing technologies are sensitive. In addition, bacterial contamination, especially of platelet concentrates, and the presence of protozoa transmitted by blood pose significant risks. The safety of the blood is also compromised by the presence of residual leukocytes that can be found even after leukoreduction. For all of the above, the implementation of pathogen reduction technology (PRT) provides a proactive technology approach to blood safety by inactivating pathogens possibly present in blood products [8–14].

The aim of this study was to compare the effects of PRT treatment on PS and α2AP in common prestorage versus post-storage inactivation (after freezing/thawing) setting. We expect that previously frozen plasma units can be inactivated without additional damage to PS and α2AP compared to immediately inactivated plasma, which will allow us to do this procedure before clinical use and according to the blood type needed.

Methods

Whole blood from random healthy donors aged 18 to 65 was collected into a quadruple blood bag system (Terumo, Japan) according to the manufacturer's instructions. Donors were tested for hepatitis B and C virus (HBV and HCV), human immunodeficiency virus (HIV), and lues markers by chemiluminescent immunoassay using Architect 2000 (Abbott, USA), as well as by polymerase chain raection (PCR) test (COBAS AmpliPrep/TaqMan, Roche, Germany).

Primary separation was performed 2–8 hrs after collection by "hard" spin: speed 3,603 rpm (3,890 g) for 10 min (radius: 268 mm, acceleration: 6, brake: 4) at 4 ± 2 °C. After separation, plasma units were: a) inactivated and frozen [prestorage setting or control group (CG); n = 30] or b) immediately frozen [post-storage setting or study group (SG); n = 30] at -80 ± 5 °C and stored at -40 ± 5 °C. After four months, plasma from the SG was thawed and inactivated, as was the plasma from the CG for sampling.

Plasma units were inactivated by the Mirasol® PRT system (Terumo BCT, USA) in the following way: plasma was transferred into an illumination bag, riboflavin (RB) 35 ± 5 mL, was added using the sterile connection (Sterile

Tubing Welder TSCD Terumo, Japan), residual air was extruded to an empty RB bag, and the set (Mirasol® PRT Plasma Illumination/Storage Set) was placed in the illuminator (Mirasol® PRT, Terumo BCT, USA). Plasma was then exposed to UV light (k = 265–370 nm) at the dose of 6.24 J/mL, with constant horizontal shaking (120 cycles/min).

In the prestorage setting (CG), plasma samples (8 mL) were taken immediately after separation (initial sample or autocontrol – AC_{CG}) and following PRT treatment (sample I – S-I_{CG}). Before testing, AC_{CG} samples were held at room temperature for a period equivalent to the illumination time of treated units. Inactivated plasma units were frozen and stored until thawing and testing (sample II – S-II_{CG}).

In the post-storage setting (SG), plasma samples were tested immediately after separation (initial sample or autocontrol – AC_{SG}), after freezing/thawing (sample I – S-I_{SG}), and following PRT treatment (sample II – S-II_{SG}). S-I_{SG} were maintained at room temperature (20 ± 2 °C) for a period of illumination of thawed plasma units.

Natural inhibitors, PS and α2AP, were determined by the BCS XP Coagulation system (Siemens, Germany).

Data for PS and α2AP activity were compared: initial vs. final (AC_{CG} vs. S-II_{CG} and AC_{SG} vs. S-II_{SG}) in both prestorage and post-storage settings, as well as the calculated recovery between the groups.

Descriptive data of plasma research were expressed as mean value ± standard deviation (SD) for each parameter. Statistical analyses were performed by comparing groups using a standard Student *t*-test for paired sample sets. Differences were considered statistically significant if the *p*-value was less than 0.05.

Results

In this study, we examined the influence of time of PRT treatment on PS and α2AP activity by comparing results obtained in prestorage (CG) and post-storage (SG) settings, i.e., immediate inactivation vs. inactivation after four months of cryostorage at -40 ± 4 °C/ thawing PRT application.

The data analysis of the two groups indicated no significant differences between initial samples – autocontrols (AC_{CG} vs. AC_{SG}) for these proteins. Under identical handling fashion, the inactivation process and freezing/thawing conditions of PS and α2AP result in comparable activities in both prestorage and post-storage PRT-treatment settings (Table 1).

The recovery of PS and α2AP was calculated as the ratio, expressed in percent, of the value after PRT treatment and freezing/thawing process compared to the corresponding initial level before *ex vivo* manipulation and was labeled "calculated recovery".

There was a trend toward reduction of protein activity in both prestorage and post-storage PRT-treatment samples (AC vs. S-II; $p < 0.05$).

The rate of recovery of PS was similar in the two groups: 94% recovery in prestorage vs. 90% in post-storage, just as was the recovery of α2AP, which was 69% in prestorage setting compared with 83% in post-storage settings making no significant difference of natural inhibitors activity between the two groups.

Discussion

Protein S is made up of 635 amino acid residues arranged in multiple domains. In human plasma, 60% of PS is bound to the complement regulatory protein C4b-binding protein (C4BP), and the remaining 40% is circulating free.

Protein S is primarily an anticoagulant protein but also has other important roles in immune and vascular systems. Anticoagulant functions of PS are the following: 1) cofactor to APC in the regulation of factor Va in prothrombinase complex and factor VIIIa in tenase complex; 2) direct APC-independent inhibition of prothrombinase and tenase complexes; 3) cofactor to tissue factor pathway inhibitor alpha (TFPIα) in inhibition of factor Xa [15].

Protein S deficiency leads to the risk of venous thrombosis but could be as well associated with arterial thrombotic events [16].

Human α2AP circulates in the blood as a single chain glycoprotein. The protein regulates fibrinolysis in three ways: by forming a complex with plasmin, by inhibiting plasminogen from adsorbing to fibrin, and by making fibrin more resistant to local plasmin (through cross-linking via factor XIIIa). Both thrombus associated and plasma α2AP regulate fibrinolysis, rapidly inactivating plasmin and

Table 1

Pathogen reduction technology (PRT)-treated fresh frozen plasma (FFP) evaluation

Plasma proteins	Prestorage treatment (control group)			Post-storage treatment (study group)		
	AC_{CG}	S-I_{CG}[a]	S-II_{CG}[a]	AC_{SG}	S-I_{SG}	S-II_{SG}[a]
PS	1.30 ± 0.00	1.26 ± 0.16	1.20 ± 0.26	1.27 ± 0.09	1.32 ± 0.24	1.15 ± 0.21[b,c]
α2AP	1.09 ± 0.07	1.02 ± 0.15	0.81 ± 0.15[b,c]	1.06 ± 0.06	1.08 ± 0.16	0.88 ± 0.08[c]

CG – prestorage setting, control group: AC_{CG} – autocontrol, initial sample taken immediately after separation of blood plasma; S-I_{CG} – first sample, taken immediately following the PRT treatment of separated blood plasma; S-II_{CG} – second sample, blood plasma was PRT-treated, and afterward frozen, stored, and then thawed.

SG – post-storage setting, study group; AC_{SG} – autocontrol, initial sample taken immediately after separation of blood plasma; S-I_{SG} – first sample, separated blood plasma was frozen, stored, thawed, and PRT-treated afterward; PS – protein S; α2AP – alpha2-antiplasmin.

[a] – Riboflavin (RB)-associated dilution factor implied.

[b] – AC vs. S-II ($p < 0.05$).

[c] – S-I vs. S-II ($p < 0.05$).

forming stable inactive complex plasmin-α2AP. In α2AP deficiency, bleeding is caused by premature dissolution of hemostatic plugs before tissue and vessel reparation are finished. Therefore, bleeding is often delayed after trauma or invasive procedures [4].

Acquired deficiency of α2AP may occur in patients with severe liver illness when plasma levels fall as low as 8%. Sometimes it is also seen in patients with renal disease, disseminated intravascular coagulation, and patients on thrombolytic therapy [17, 18].

A lot of measures have been introduced to prevent the transmission of infectious agents through blood, so the risk of classical TTI agents (HBV, HCV, and HIV) has been drastically reduced. Unfortunately, blood transfusion still constitutes a risk because of a "window period", new emerging pathogens, parasites, and bacteria [19]. Therefore, a much better and more efficient option would be a preemptive approach, which includes PRT.

Pathogen reduction effectively inactivates most clinically relevant viruses (RNA or DNA, single or double-stranded, enveloped or nonenveloped, intracellular or extracellular). Furthermore, it inactivates gram-positive and gram-negative bacteria, spirochetes, Rickettsia and protozoa, and lymphocytes and probably protects against pathogenic agents that will emerge in the future. Bad sides of PRT are decreased yield for some products (especially platelets), insufficient reduction of some high-titer, nonenveloped agents (hepatitis A virus, parvovirus B-19), concern for potential toxicity, no single pathogen reduction (PR) system for all blood products at present, and anticipated high cost [20].

Mirasol® PRT system uses water-soluble vitamin B2, RB, and UV light. RB is rapidly excreted and cannot be stored in the body. RB is a photosensitizer and mediates selective damage to nucleic acids after exposure to light [21]. RB attaches to nucleic acids and mediates an oxygen-independent electron transfer that causes modification of nucleic acids, mostly guanine, while RB is converted into his photoproduct lumichrome [22]. Damage induced by RB is irreversible because replication and repair processes are diminished due to guanine base modification [23, 24].

Blood safety in terms of TTIs is of particular importance for vulnerable groups of patients who are either exposed to a large number of chemoproducts for a short time or are immunocompromised due to the therapy they receive [patients with thrombotic thrombocytopenic purpura (TTP) or liver transplant recipients]. Due to the aforementioned, it is necessary to have a sufficient amount of PRT-treated FFP at all times, which is not rational, bearing in mind that FFP is given according to the blood type of patients and that universal "AB" FFP is not sufficient since it is the rarest blood type. In addition, it is not realistic to expect that universal inactivation of blood products will soon enter into routine practice due to the cost and complexity of the procedure (but over time, it will prove cost-neutral and possibly cost-saving). The ideal solution would be to inactivate the required amount of stored FFP of appropriate blood groups for a particular patient immediately before administration.

The aim of this study was to show that subsequently - post-storage (after freezing/thawing) treated FFP has the same quality in terms of natural coagulation inhibitors as prestorage, "classically" treated FFP [25].

Data obtained in this study, as those from the previous related study [26], analyzed the plasma hemostatic activity before and after PRT treatment and cryostorage in both prestorage and post-storage settings. As reported, PRT-treated plasma demonstrates a reduction in plasma procoagulant factors [13, 27–31]. This reduction in activity is noted immediately after prestorage PRT treatment and remained relatively constant during cryostorage from 75–79% (for FVIII) to 80–87% (for FII). In our previous study, procoagulant activities are expressed as relative numbers [26] before and after PRT treatment in both prestorage and post-storage settings. The calculated recovery for different procoagulant factors was similar in the two groups: for FII, 79% in CG vs. 81% in SG; for FV, 71% in CG vs. 88% in SG; for FVII, 75% in CG vs. 83% in SG; for FVIII, 70% in CG vs. 71% in SG; for FIX, 77% in CG vs. 72% in SG; and for FX, 75% in CG vs. 65% in SG [26]. Results obtained were comparable with data from the literature [13, 27–31].

For natural inhibitors, activities of PS were similar (no statistical significance) in both groups: the calculated recoveries in prestorage and post-storage groups were 94% and 90%, respectively. In our previous study, the calculated recovery of protein C was 84% in the prestorage and 86% in the post-storage group [26]. Moreover, recovery of α2AP activity of 69% vs. 83% in prestorage and post-storage groups, respectively, was not statistically significant. Similar results were obtained by Singh et al. [32] with amotosalen and UV light, where retention of inhibitors was 78% to 98%, while Smith and Rock [27] had retention between 91% and 100% with Mirasol® PRT.

However, the activity of AT-III was significantly higher ($p < 0.05$) after post-storage PRT treatment [26].

Conclusion

This study, similar to our previous study concerning plasma constituent integrity, confirmed that no clinically relevant intergroup differences (prestorage vs. post-storage PRT treatment) in plasma constituents levels were observed. After post-storage treatment, proteins, quantity, and activity in FFP continue to be satisfying and can be used in clinical practice. Even more, the recovery obtained for AT-III in the post-storage setting was higher. Thus, previously cryostored FFP units could be safely and effectively inactivated just before their clinical application, which is of great importance because only necessary plasma units will be inactivated instead of random ones. In that manner, both significant financial resources and the time for preparation will be saved.

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Received on March 15, 2021
Revised on April 16, 2021
Accepted on April 29, 2021
Online First May 2021