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**UTICAJ RIBOFLAVINA I UV ZRAČENJA NA PROTEINE PLAZME PROTEIN S I A2-ANTIPLACEMIN U ODNOSU NA VREME PRIMENE**

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UDC:

DOI: [https://doi.org/10.2298/VSP210315051G](https://doi.org/10.2298/VSP210315051G)

When the final article is assigned to volumes/issues of the Journal, the Article in Press version will be removed and the final version appear in the associated published volumes/issues of the Journal. The date the article was made available online first will be carried over.
INFLUENCE OF RIBOFLAVIN AND UV-LIGHT TREATMENT ON PLASMA’S PROTEIN PROTEIN S AND α2-ANTIPLASMIN; THE MATTER OF TIME
UTICAJ RIBOFLAVINA I UV ZRAČENJA NA PROTEINE PLAZME PROTEIN S I A2-ANTIPLAZMIN U ODNOSU NA VREME PRIMENE

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Abstract.

**Background / Aim.** After introduction of careful selection procedure for blood donors and the implementation of highly sensitive screening tests for transfusion transmissible infections (TTI), blood is very safe product concerning TTI. Nevertheless, because of the „window“ period for the pathogen that are testing and the emergence of new pathogens, the risk still persists. Implementation of pathogen reduction technology (PRT) provides a proactive approach to improve blood safety. By damaging nucleic acids, PRT selectively inactivates pathogens and leucocytes. However, during the process, plasma proteins are, also, damaged in some extent. The goal of this study is to conclude if there is the difference in the effect of PRT on Protein S and α2-antiplasmin regarding the time of inactivation: inactivation immediately after plasma separation from whole blood (before freezing) versus inactivation after freezing/thawing. **Methods.** The voluntary donors’ blood is taken into quadruple bag system, centrifuged and separated into blood products. Control’s group plasma is inactivated by the Mirasol PRT system and frozen. Experimental’s group plasma is frozen and, after four months, thawed and inactivated. Protein S and α2-antiplasmin activity are examined in samples after separation, inactivation and thawing. **Results.** Analysing Protein S and α2-antiplasmin activity, no statistically significant difference is found between the initial samples. The trend of reduction of protein’s activity after inactivation and freezing/thawing is present in both groups but without statistically significant intergroup difference. **Conclusion.** Reduction in Protein S and α2-antiplasmin’s activity after immediate - before freezing and afterwards - after freezing/thawing inactivation does not show a statistically significant difference, making stored plasma units suitable for safe and efficient inactivation directly before clinical use and according to patient’s blood type.

**Key words:** plasma, pathogen inactivation, Protein S, α2-antiplasmin.

**Apstrakt**

**Uvod/Cilj.** Pažljivim izborom davalaca i korišćenjem visoko osetljivih testova za detekciju antitela, antigena i nukleinskih kiselina virusa, značajno je poboljšana sigurnost krvi u odnosu na infekcije koje se prenose transfuzijom. Međutim, zbog postojanja „prozor“ perioda 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
proaktivni pristup u poboljšanju bezbednosti krvi. Oštećenjem nukleinskih kiselina PRT selektivno inaktivise patogene i leukocite. Nažalost, tokom procesa se u određenom stepenu oštećuju i proteini plazme. Cilj rada je da se utvrdi da li postoji razlika u stepenu oštećenja primenom PRT na Protein S i α2-antiplazmin ukoliko se plazma inaktivise odmah po izdvajanju iz jedinice krvi pre zamrzavanja, ili ako se inaktivise naknadno, tj. posle zamrzavanja/odmrzavanja. 

**Metode.** Krv dobrovoljnih davalaca se prikuplja u sistemu četvorostrukih kesa, centrifugira i razdvaja na produkte. Plazma koja čini kontrolnu grupu se prvo inaktivise Mirasol PRT sistemom i zamrzava. Plazma eksperimentalne grupe se odmah zamrzava, a nakon četiri meseca odmrzava i potom inaktivise. Aktivnost Proteina S i α2-antiplazmina se ispituje u uzorcima posle separacije, inaktivacije i odmrzavanja.

**Rezultati.** Analizom rezultata aktivnosti Proteina S i α2-antiplazmina utvrđeno je da nema statistički značajne razlike između inicijalnih uzoraka. Nakom inaktivacije i zamrzavanja/odmrzavanja postoji trend pada aktivnosti ovih proteina u obe grupe ali statistički značajna razlika između kontrolne i eksperimentalne grupe ne postoji.

**Zaključak.** Ne postoji statistički značajna razlika između vrednosti aktivnosti Proteina S i α2-antiplazmina nakon inaktivacije pre zamrzavanja, odnosno posle zamrzavanja/odmrzavanja, tako da usklađene jedinice plazme mogu biti sigurno i efikasno inaktivisane neposredno pre upotrebe, a prema trenutnim potrebama za inaktivisanom plazmom određenih krvnih grupa.

**Ključne reči:** plazma, inaktivacija patogena, Protein S, α2-antiplazmin.

**Introduction**

Plasma is liquid part of blood which contains pro and anticoagulant factors. Plasma can be defined as fresh frozen plasma (FFP) if it is frozen within 8 hours since collection, plasma 24 (frozen within 24 hours) 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 5 days.

Plasma is used for treatment of 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 which enhances the anticoagulant effect of activated Protein C (APC). PS is synthesized in hepatocytes, endothelial cells, megacaryocytes and brain cells. As cofactor for APC, PS has a role in inactivation of factor Va and factor 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 20-fold enhancement of the slower phase of factor Va inactivation. Only 40% of plasma's PS is free and available, whereas the rest is bound to C4b-binding protein and cannot interact with APC \(^3\).

Primary inhibitor of plasmin which is synthesized in the liver is α2-antiplasmin (α2-AP). 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. α2-AP rapidly and irreversibly binds free plasmin \(^4\).

Blood for transfusion is extremely safe concerning virus transmission given improved donor screening method and a range of assays for detection of antibodies, antigens and genoms \(^5\). However, emerging of new pathogens, such as West Nile virus, Sever Acute Respiratory Syndrome (SARS) virus, Chikungunya, Dengue and many others make a permanent threat \(^6\),\(^7\).

The other issue is, so called “window period”, the period during which is impossible to detect the presence of the pathogen, no matter how testing technologies are sensitive. In addition, significant risk is bacterial contamination, especially of platelet concentrates, and the presence of protozoa transmitted by blood. Safety of the blood is, also, compromised by the presence of residual leukocytes that can be found even after leukoreduction. For all numbered, the implementation of pathogen reduction pathology (PRT) provides a proactive approach to blood safety by inactivating pathogens possibly present in blood products \(^8\)-\(^14\).
The aim of this study is to compare the effects of PRT treatment on Protein S and α2-antiplasmin in common prestorage versus poststorage inactivation (after freezing/thawing) setting. We expect that previously frozen plasma units could be inactivated without additional damage to PS and α2-AP compared to immediately inactivated plasma which will allow us to do procedure before clinical use and according to blood type needed.

**Methods**

Whole blood from random healthy donors, age 18 to 65 years, 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 PCR (COBAS AmpliPrep/TaqMan, Roche, Germany) and were non-reactive.

Primary separation was performed 2-8 hours after collection by „hard“ spin: speed 3603rpm (3890g) for 10 minutes (radius: 268mm, 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 (poststorage setting or study group; n=30), at -80±5°C, and stored at -40±5°C. After four months, plasma from study group were thawed and inactivated, as were plasma from control group for sampling.

Plasma units were inactivated by 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 sterile connection (Sterile Tubing Welder TSCD Terumo, Japan), residual air was expressed to an empty RB bag and set (Mirasol PRT Plasma Illumination/Storage Set) was placed in the illuminator (Mirasol PRT, Terumo BCT, USA). There, plasma was exposed to UV light (k = 265–370 nm) in dose 6.24 J/ml, with constant horizontal shaking (120 cycles/min).

In prestorage setting (CG), plasma samples (8ml) 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 unites. Inactivated plasma units were frozen and stored for up to thawing and testing (sample II - S-I_{CG}).

In the poststorage setting (SG), plasma samples were tested immediately after separation (initial sample or autocontrol - A_{CSG}), 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, Protein S and α2-antiplasmin were determined by BCS XP Coagulation system (Siemens, Germany).

Data for PS and α2-AP activity were compared: initial vs. final (A_{CG} vs. S-II_{CG} and A_{SG} vs. S-II_{SG}) in both, prestorage and poststorage settings, as were compared calculated recovery between the groups.

Descriptive data of plasma research were expressed as mean value ± standard deviation (SD) for each of parameters. Statistical analyses were performed by comparisons between groups using a standard Student’s t-test for paired samples sets. Differences were considered statistically significant if p-value was less than 0.05.

**Results**

In this study we examined the influence of time of PRT treatment on Protein S and α2-antiplasmin activity comparing results obtained in prestorage (CG) and poststorage (SG) settings ie. immediate inactivation vs. inactivation after four months cryostorage at -40±4°C/ thawing PRT application.

The data analysis of two group indicated that there were no significant differences between initial samples – autocontrols (A_{CG} vs. A_{SG}) for these proteins. Under identical handling fashion, inactivation process and freezing/thawing conditions PS and α2-AP resulting with comparable activities in both prestorage and poststorage PRT-treatment settings.

Table 1.
The recovery of PS and α2-AP was calculated as the ratio, expressed in percentage, of the value after PRT treatment and freezing/thawing process compared to corresponding initial level before ex vivo manipulation and is labeled „calculated recovery“.

Table 2.

There was a trend toward reduction of proteins activity in both, prestorage and poststorage PRT-treatment samples (AC vs. S-II; p < 0.05).

Rate of recovery of PS is similar in two groups: 94% recovery in prestorage versus 90% in poststorage, just as is recovery of α2-AP which is 69% in prestorage setting compared with 83% in poststorage 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 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: 1) cofactor to APC in regulation of factor Va in prothrombinase complex and factor VIIIa in tenasa complex; 2) direct APC-independent inhibition of prothrombinase and tenasa complexes; 3) cofactor to TFPIα in inhibition of factor Xa.

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

Human α2-AP circulates in blood as a single chain glycoprotein. Regulates fibrinolysis in three ways: by forming a complex with plasmin; by inhibiting plasminogen to adsorb to fibrin and by making fibrin more resistant to local plasmin (through cross linking via factor XIIIa). Both, thrombus associated and plasma α2-AP regulates fibrinolysis rapidly inactivating plasmin and forming stable inactive complex plasmin-α2-AP.
In α2-AP deficiency bleeding is caused by prematures dissolution of haemostatic plugs before tissue and vessel reparation finished. So, bleeding is often delayed after trauma or invasive procedures.

Acquired deficiency of α2-AP may occur in patients with severe liver illness when plasma levels falls as low as 8%. Sometimes it is, also, seen in patient with renal disease, disseminated intravascular coagulation and in patients on thrombolytic therapy 18,19.

A lot of measures have been introduced in order to prevent transmission of infectious agents throug blood, so 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 and parasites and bacteria 20. In that matter, much better and efficient option would be preemptive approach wich includes PRT. Pathogen reduction effectively inactivates most clinically relevant viruses (RNA or DNA, single or double stranded, enveloped or nonenveloped, intracellular or extracelullar). Also it inactivates gram-positive and gram-negative bacteria, spirochets, Rickettsia and protozoa; lymphocytes and probably provides protection of 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 PR system for all blood products at present, and anticipated high cost 21.

Mirasol PRT system uses water soluble vitamin B2, riboflavin and UV light. RB is rapidly excreted and can’t be stored in the body. RB is photosensitizer and mediates selective damage to nucleic acids after exposure to light 22. 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 photoprodut lumichrome 23. Demage induced by RB is irreversible because replication and repair processes is diminished due to guanin base modification 24,25.
Blood safety in terms of TTI is of particular importance for vulnerable groups of patients who are either exposed to a large amount of chemoproducots for a short time and/or are immunocompromised due to the therapy they receive (patients with Thrombotic thrombocitopenic purpura (TTP) or liver transplant recipients). Due to the above, 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 rarest blood type. Also, 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). 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 is to show that subsequently - poststorage (after freezing/thawing) treated FFP has the same quality in terms of natural coagulation inhibitors as prestorage, “classically” treated FFP.

Data obtained in this study as those from previous related study\textsuperscript{15}, analyzed the plasma hemostatic activity before and after PRT-treatment and cryostorage in both, prestorage and poststorage setting. As has been reported, PRT-treated plasma demonstrates reductions in plasma procoagulant factors\textsuperscript{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 study, procoagulant activities are expressed as relative numbers\textsuperscript{15} before and after PRT-treatment in both, prestorage and poststorage setting. The calculated recovery for different procoagulant factors was similar in two groups: for FII 79% in CG vs. 81% in SG; for FV 71% in CG vs. 88% in SG; for FVII in 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\textsuperscript{15}. Results obtained were comparable with data from the literature\textsuperscript{13, 27-31}.

For natural inhibitors, activities of PS were similar (no statistical significance) in both groups: calculated recoveries in prestorage and poststorage groups were 94% and 90%, respectively. Calculated recovery of Protein C was 84% in prestorage and 86% in
poststorage group\textsuperscript{15}. Also, recovery of $\alpha_2$-antiplasmin: 69\% vs. 83\% in prestorage and poststorage groups respectively, was not statistically significant. Similar results were obtained by Singh et al. with amotosalen and UV light, where retention of inhibitors were 78 to 98\% \textsuperscript{32} while Smith et al. had retention between 91 nad 100\% with Mirasol PRT\textsuperscript{27}.

However, activity of AT-III was significantly higher ($p<0.05$) after poststorage PRT treatment \textsuperscript{15}.

In conclusion, this study, as our previous study concerning plasma constituent integrity, confirmed that no clinically relevant intergroup differences (prestorage vs. poststorage PRT treatment) in plasma constituents levels were observed. After poststorage treatment proteins quantity and activity in FFP continues to be satisfying and can be used in clinical practice. Even more, the recovery obtained for AT-III in poststorage 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 unites will be inactivated instead of random ones. In that manner, both significant financial resources will be saved, and also the time for preparation.

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Table 1

|                      | Pre-storage treatment (control group) | Post-storage treatment (study group) |
|----------------------|--------------------------------------|--------------------------------------|
|                      | AC<sub>CG</sub> | S-I<sub>CG</sub><sup>a</sup> | S-II<sub>CG</sub><sup>a</sup> | AC<sub>SG</sub> | S-I<sub>SG</sub> | S-II<sub>SG</sub><sup>a</sup> |
| 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<sup>b,c</sup> |
| A2-AP                | 1.09 ± 0.07 | 1.02 ± 0.15 | 0.81 ± 0.15<sup>b,c</sup> | 1.06 ± 0.06 | 1.08 ± 0.16 | 0.88 ± 0.08<sup>c</sup> |

Control group: AC<sub>CG</sub> = initial sample (autocontrol); S-I<sub>CG</sub> = sample first (PRT-treated); S-II<sub>CG</sub> = sample second (PRT-treated and frozen/thawed). Study group: AC<sub>SG</sub> = initial sample (autocontrol); S-I<sub>SG</sub> = sample first (frozen/thawed); S-II<sub>SG</sub> = sample second (frozen/thawed and PRT-treated).

<sup>a</sup> RB-associated dilution factor implied.

<sup>b</sup> AC vs. S-II (<i>p</i> < 0.05).

<sup>c</sup> S-I vs. S-II (<i>p</i> < 0.05).
Table 2

PRT-treated FFP – recovery of PS and α2-AP.

| Calculated recovery | Prestorage treatment (%) | Poststorage treatment (%) |
|---------------------|--------------------------|---------------------------|
| PS                  | 94                       | 90                        |
| α2-AP               | 69                       | 83                        |

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