Inactivation of model viruses and bacteria in human fresh frozen plasma using riboflavin and long wave ultraviolet rays

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ABSTRACT

Background and Objectives: Pathogen reduction technologies are among methods to eliminate transfusion transmitted infections. Mirasol method using riboflavin in combination with ultraviolet rays is one of them. The aims of this study were to investigate the effectiveness of Mirasol method to inactivate some model pathogens as well as examination of the sensitivity of plasma proteins after treatment.

Materials and Methods: Riboflavin in 50µM concentration and ultraviolet (365 nm) in three different energy doses (3.6, 7.2, and 10.8 J/cm²) were employed to inactivate model pathogens. Four standard viruses were used in this study including Vesicular Stomatitis Virus (VSV), Herpes Simplex Virus1 (HSV-1), Bovine Viral Diarrhea Virus (BVDV) and Polio Virus. 50% Tissue Culture Infectious Dose (TCID₅₀) and Reed–Muench Methods were used to estimate viruses’ titers. E. coli and Staphylococcus aureus were used as bacterial models. Four plasma proteins including factor V, VIII, fibrinogen and antithrombin were used to determine their sensitivity to pathogen inactivation treatment.

Results: The most pathogen reduction titre was determined for 15 minutes irradiation period equal to 10.8 J/cm² that is corresponding to Log 6.10 for BVDV, Log 6.09 for HSV-1, Log 6.62 for VSV and Log 3.36 for Polio. Bacterial reduction titer was Log 6.94 for E. coli and Log 7.00 for S. aureus. Indicator proteins for plasma activity were determined to be 75% for factor V, 88% for factor VIII, 52% for fibrinogen and 94% for antithrombin.

Conclusion: Results showed that the employed method can inactivate most of the pathogens in fresh frozen plasma. The acceptable activities of selected plasma proteins remained after treatment.

Keywords: Viruses, Bacteria, Fresh frozen plasma, Riboflavin, Ultraviolet rays, Inactivation
man Immunodeficiency Virus (HIV) and Hepatitis C Virus (HCV) and emerging or re-emerging viruses such as West Nile Virus or Chikungunya Virus which there is no method to detect them (2, 3).

Several pathogen reduction technologies (PRT), have been developed to prevent transfusion transmitted infections. Some methods using photosensitizers as a key character, which absorb light energy and, in the ensuing electronically excited species, undergo redox reaction with surrounding molecules. Most current methods target nucleic acids and lead to pathogen inactivation (4).

Riboflavin in combination with long wave Ultra Violet (UV) is one of the effective PRTs. Riboflavin as Vitamin B2, is an essential nutrient in humans and it is generally recognized as Safe (GRS) (5). The safety of it has been demonstrated for oral, subcutaneous, intra-peritoneal and intravenous routes of administration (6-8). Mirasol system using riboflavin in combination with UV inactivates pathogens in blood components. Treatment of blood components employing this method has reduced significantly the risk of pathogen transmission (2, 5, 9-11). Its effectiveness for some pathogens has not been investigated. In this study, we used it as a new source for UV and investigate its effectiveness on four model viruses and two model bacterial pathogens. The impacts of this treatment on some proteins in Fresh Frozen Plasma (FFP) were also investigated.

MATERIALS AND METHODS

Viruses and cell lines. Four model viruses, Bovine Viral Diarrhea Virus (BVDV), Herpes Simplex Virus 1 (HSV-1), Vesicular Stomatitis Virus (VSV) and Poliomyelitis Virus (Polio), to represent enveloped, non-enveloped and both RNA and DNA V Viruses were selected for this study. All four strains obtained from virus bank of Iranian Blood Transfusion Organization (IBTO) (12).

Madin-Darby Bovine Kidney (MDBK), African Green Monkey Kidney (Vero), and Cervix Carcinoma (HeLa) cell lines were used to propagate and quantify model viruses. All cell lines were obtained from National Cell Bank of Iran (NCBI), Pasteur Institute of Iran. The cells were grown in monolayers in Dulbecco Modified Eagle Medium (DMEM) (Gibco, UK) with 5% Fetal Bovine Serum (FBS) (Gibco) and 1% Pen-Strep (Sigma) at 37°C in a humid atmosphere of 5% CO₂.

Virus propagation and quantification. 50% Tissue Culture Infectious Dose (TCID₅₀), were used to quantify viruses. Model viruses propagated in monolayer cell lines and their infectivity titer were determined by Reed and Muench method employing 10 fold dilution series four replicate culture per dilution (13). Each virus titre should be reached at least 10⁶ according to WHO guidelines for PRT (Pathogen Reduction Technologies) (14). Then virus stock has been frozen at -70°C until used.

Bacteria. With respect to the most common bacterial contamination in blood transfusion two Gram-positive and Gram-negative bacteria were selected for this study: E. coli (ATCC:25922) and S. aureus (ATCC: 25923). They cultured in nutrient agar for 24 hours at 37°C. Then a suspension of each organism was prepared in sterile phosphate buffer saline (PBS) (pH=7.4) to reach 10⁶ CFU/ml.

Fresh frozen plasma (FFP). FFP samples have been obtained from IBTO. They were negative for HIV (Human Immunodeficiency Virus), HCV (Hepatitis C Virus) and HBV (Hepatitis B Virus) and stored at -20°C until used. All of them were O positive.

Plasma proteins. To determine the quality of FFP before and after riboflavin and UV treatment, 4 plasma proteins including factor V, factor VIII, antithrombin and fibrinogen were selected as indicators to examine their concentration before and after treatment.

Riboflavin. Riboflavin was obtained from Darou Pakhsh Holding Co and stored at 25°C in dark before use. After that it was suspended in PBS and used in 50 µM final concentration in FFP samples.

Irradiation source. The source of irradiation was a UV Irradiator from HAMAMATSU (lighting lure series LC5 model). It could irradiate UV in 200-400 nm and its optimum irradiation was in 365nm. The energy dose in samples depends on their distance from the source as 12cm distance designed to reach optimum energy and also 3 different exposure times of 5, 10 and 15 minutes were employed to obtain different energy dose to identify optimum dose for in-
activation of pathogens. The final calculated energy dose for each exposure time were 3.6, 7.2, and 10.8 J/cm² respectively.

**Pathogen inactivation procedure.** Nine milliliter of FFP samples were added to 9cm petri-dishes and 1ml of bacteria and virus stocks were suspended in FFP to reach at least 10⁷ TCID₅₀ for virus and 10⁶ CFU for bacteria per ml. Then riboflavin in a final concentration of 50µM was added to each sample (5). The irradiation procedure was done in microbiological safety cabinet level II to prevent cross contamination. Virus and bacteria titers before and after irradiation was examined using TCID₅₀ and Reed and Muench method for viruses and serial 10- fold dilutions in nutrient agar for bacteria.

**Controls.** Negative controls were designed to be FFP without any pathogen spiking and also relative cell lines and bacterial media without pathogen spiked them. Positive controls were FFP contained pathogens without treatments.

**Statistical methods.** All experiments repeated 3 times. SPSS (version 20.Inc) was used for data analysis.

**RESULTS**

**Viruses.** The original titer of BVDV, HSV, VSV and Polio Viruses were 6.54, 6.82, 7.07 and 6.66 respectively. Using riboflavin in 50µM final concentration in associated with UV in 365nm and in three different times reduced BVDV log in FFP samples to 6.10 (Table 1).

For HSV-1 best log reduction was 6.09 for 15 minutes irradiation, and VSV with the same exposure times showed a 6.62 log reduction. Polio virus was the most resistance virus that showed only a 3.36 log reduction after 15 minutes irradiation in presence of the riboflavin. The results were compared to the controls.

**Bacteria.** The original titer for *E. coli* was 10⁷ CFU/ml and for *S. aureus* was 10⁶ CFU/ml. *E. coli* showed Log 6.94 CFU/ml reduction after 15 minutes treatment and *S. aureus* h owed more sensitivity to riboflavin and UV treatment that log 7 CFU/ml reduction has been shown after 10 minutes (Table 2).

**Plasma proteins.** The results showed selected plasma proteins remained in normal range after riboflavin in combination with UV treatment except fibrinogen which was the most sensitive factor to this method. Antithrombin was the most resistance one. Table 3 shows the average activity of the proteins before and after treatment.

**DISCUSSION**

Blood-borne pathogens removal or reduction to the acceptable level always considered as a critical point in blood transfusion process. Although the efficiency of present methods, including screening blood donor and testing blood samples are useful to improve blood component quality, but still remain some risks, espec-

| Times of irradiation | Log reduction for E. coli, CFU/ml | Log reduction for S. aureus, CFU/ml |
|----------------------|----------------------------------|-----------------------------------|
|                      | Mean± SD                          | Mean± SD                          |
| 5 min                | 2.7±0.01                          | 3.64±0.11                         |
| 10 min               | 5.67±0.06                         | 7.00±0.09                         |
| 15 min               | 6.94±0.12                         | 7.00±0.15                         |

| Times of irradiation | BVDV (Mean± SD) | HSV (Mean± SD) | VSV (Mean± SD) | Polio (Mean± SD) |
|----------------------|-----------------|----------------|----------------|------------------|
| 5 min                | 3.07±0.24       | 3.52±0.343     | 4.6±0.25       | 1.06±0.10        |
| 10 min               | 5.02±0.03       | 4.44±0.28      | 5.46±0.12      | 2.11±0.05        |
| 15 min               | 6.10± 0.09      | 6.09±0.14      | 6.62±0.09      | 3.36±0.17        |
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Table 3. Impact of 50 µM riboflavin concentration in association with 10 minutes ultraviolet treatment on FFP proteins.

| Protein               | Average of activity before irradiation | Average of activity after 10 m irradiation | Normal range | Percent of remaining activity after treatment |
|-----------------------|----------------------------------------|--------------------------------------------|--------------|-----------------------------------------------|
| Factor V %            | 110                                    | 82.3                                       | 60-130       | 75%                                           |
| Factor VIII %         | 89                                     | 78.5                                       | 60-150       | 88%                                           |
| Fibrinogen mg/ml      | 323                                    | 170.5                                      | 200-400      | 52%                                           |
| Antithrombin %        | 114                                    | 108                                        | 80-120       | 94%                                           |

The bacterial selection in this study was based on their high contamination rate in blood transfusion. Previous studies showed *Staphylococcus epidermis*, *S. aureus* and *E. coli* sensitivity to Mirasol method. In current study the same results for *E. coli* and *S. aureus* was observed. However, *E. coli* showed high sensitivity to treatment, as more than 6 Log reduction after 15 minutes was observed (5, 10, 11).

Marsh and et al. showed antitrombin can save 90% of its activity after using the Mirasol method and is the most resistant protein and fibrinogen lost 30% of its activity and is the most sensitive one. The current results were near to it and all plasma factors remained in normal range after pathogen inactivation (5, 17).

The results showed the PRT treatment could inactivate most pathogens including both bacterial and viral contamination at the much closed level to WHO guideline, and is a safe and acceptable method for pathogen reduction in blood transfusion centers. A 10.8 J/cm² is the most effective energy dose, although, 7.2 J/cm² still is remaining as an alternative to save more plasma proteins’ activity.

REFERENCES

1. Busch M, Kleinman S, Nemo G. Current and emerging infectious risks of blood transfusion. *J Am Med Assoc* 2003; 289:959-962.
2. Goodrich R, Edrich R, Li J, Seghatchian J. The Mirasol PRT system for pathogen reduction of platelets and plasma: an overview of current status and future trends. *Transfus Apher Sci* 2006; 35:5-17.
3. Vanlandingham D, KeilSh, McElroy K, Pyles R, Goodrich R, Higgs S. Photochemical inactivation of chikungunya virus in plasma and platelets using the Mirasol pathogen reduction technology system. *Transfusion* 2013; 53:284-290.
4. Wainwright M, Baptista M. The application of photosensitisers to tropical pathogens in the blood supply. *Photodiagnosis Photodyn Ther* 2011; 8: 240-248.
5. Marschner S, Goodrich R. Pathogen reduction technology treatment of platelets, plasma and whole blood using riboflavin and UV light. *Transfus Med Hemother* 2011; 38:38-18.
6. Hayashi M, Kishi M, Sofuni T, Ishidate M. Micronucleus tests in mice on 39 food additive and eight miscellaneous chemicals. *Food Chem Toxicol* 1988; 26:487-500.
7. Munoz N, Hayashi M, Bang LJ, Wahrendorf J, Crespi M, Bosch FX. Effect of riboflavin, retinol and Zinc on micronuclei of buccal mucosa and of esophagus: a randomized double – blind intervention study in China. *J Natl Cancer Inst* 1987; 79:687-691.
8. Unna K, Greslin JG. Studies on the toxicity and pharmacology of riboflavin. *J Pharmacol* 1942; 76:75-80.
9. Li J, de Korte D, Woolum MD, Ruane PH, Keil SD, Lockerbie O, et al. Pathogen reduction of buffy coat platelet concentrates using riboflavin and light: comparisons with pathogen-reduction technology- treated apheresis platelet products. *Vox Sang* 2004; 87:82-90.
10. Ruane PH, Edrich R, Gampp D, Keil SD, Leonard RL, Goodrich RP. Photochemical inactivating of selected viruses and bacteria in platelet concentrates using riboflavin and light. *Transfusion* 2004; 44: 877-885.
11. Goodrich RP, Gilmour D, Hovenga N, Keil SD. A laboratory comparison of pathogen reduction technology treatment and concerns. *Transfusion* 2009; 49:1205-1216.
12. Aghaie A, Pourfatollah A, Bathaei S.Z, Moazzeni S.M, Khorsand Mohammad pour H, Sharifi Z. Inactivation of virus in intravenous immunoglobulin G using solvent/detergent treatment and pasteurization. *Hum Antibodies* 2008; 17: 79-84.
13. Reed L.J, Muench H. A simple method of estimating fifty per cent endpoints. *Am J Hyg* 1938; 27:493-497.
14. Goodrich R, Custer B, Keil SH, Bussch M. Defining adequate pathogen performance for transfused blood components. *Transfusion* 2010; 50: 1827-1837.
15. Aubuchon JP. Update on the status of pathogen inactivation methods. *ISBT Science Series* 2011; 6:181-188.
16. Goodrich R, Doane S, Heather R. Design and development of a method for the reduction of infectious pathogen load an inactivation of white blood cells in whole blood products. *Biologicals* 2010;38:20-30.
17. Antic A, Stanojkovic Z, Macukanovic-Golubovic L, Jelic M. Evaluation of coagulation factors in fresh frozen plasma treated with riboflavin and ultra violet light. *Vojnosanit Pregl* 2012; 69: 22-26.