Original Article

The Effects of Ultraviolet Light and Riboflavin on Inactivation of Viruses and the Quality of Platelet Concentrates at Laboratory Scale

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Abstract

Background: This study investigated the effects of Riboflavin (RB) combined with different doses of UV on Platelet Concentrate (PC) which was infected by three models of virus. Platelet quality after treatment was also assessed.

Methods: Three models of virus used in this study were Vesicular Stomatitis Virus (VSV), Herpes Simplex Virus (HSV), and Polio virus, which were added to PC. After photochemical treatment with RB and UV light, residual viral infectivity was titrated using 50% Tissue Culture Infective Dose (TCID 50)/ml. This treatment was done with concentration of 50 μM of RB and different doses of UV light (0.24, 0.48, 0.97, 1.29 J/cm 2). Platelet quality was assessed by measuring pH, Lactate Dehydrogenase (LDH), MTT assay and cell count after treatments and during 4 days of storage against control groups.

Results: Concentration of 50 μM RB with combination of 1.29 J/cm 2 dose of UV resulted in the highest titer reduction of VSV (4 log 10) and HSV (4.26 log 10) and lowest titer reduction of Polio virus (2.6 log 10). No significant difference was observed between different doses in comparison with control groups. In all treatment groups, the storage stability of platelets in PC was in the acceptable range in comparison with control group.

Conclusion: This study indicated that RB/UV treatment was a promising pathogen reduction technique in PC and had limited effects on platelet quality. However, further optimization of this method is necessary to deal with blood-borne viruses like non-enveloped viruses.

Key words: Blood platelet, Riboflavin, Ultraviolet light, Virus inactivation

Introduction

Despite various proactive interventional approaches to blood safety which embody a more stringent donor screening, introduction of new exclusion criteria, registries of previously deferred donors, various specific serological and Nucleic Acid based Testing (NAT), post-donation tracking and transfusion haemovigilance, etc., Transfusion Transmitted Infections (TTI) still could occur 1. These residual risks are due to relatively high incidence of bacterial contamination of platelet (PLT) products. Additionally, pathogen levels below the detection limit of a screening test during acute infection (window period) or the presence of newly emerging agents like West Nile, Dengue or Chikungunya viruses and parasites such as Trypanosoma cruzi, Plasmodium falciparum, or Leishmania continue to raise the potential for disease transmission by blood products 1,2.

Several physical, chemical or photochemical processes have been developed to broadly and non-specifically inactivate or reduce pathogens and increase blood supply safety. The principle of this method is based on light treatment of the respective product in the presence of a photoactive substance (a photosensitizer) which absorbs light of defined wavelengths and transfers the absorbed energy to an electron acceptor to inactivate the pathogen contaminating a blood product 3.

One of the main photochemical processes that have been validated for both platelets and plasma components is the use of Riboflavin (RB) combined with UVB (280-360 nm) light. RB acts by intercalation with nucleic acids and, when exposed to light, causes damage to DNA by producing single oxygen, electron transfer, hydrogen peroxide, and hydroxyl radicals, all of

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which lead to nucleic acid basic damages and DNA strand breaks. It seems that the combination of RB and UV selectively enhances damage to the guanine bases in DNA. It was suggested that type and extent of damage to DNA for virus in the presence of RB and light make it less likely to be repaired by normal repair pathways available in host cells. Platelets are anucleated cells derived from megakaryocytes which typically circulate for 10 days. The challenge for all Pathogen Reduction Technologies (PRTs), however, is to maintain biological function of the treated blood cells, while achieving adequate pathogen reduction levels.

Considering the implementation of this technique into routine use, the question arises whether this pathogen reduction method has an influence on the quality and storability of the platelets. Light of shorter wavelength like UVB (280-315 nm) and UVC (200-280 nm) has detrimental effect on the platelets and proteins and C RB25-PC. This control group has been defined by C-PC.

In the first stage, treatment with RB+UV light was accomplished in a petri dish (9 cm in diameter). This result-
microscopically for the cytopathic effects (CPE). Titers were calculated using the method of Reed and Muench and were expressed as log of tissue culture infectious doses (TCID₅₀). In brief, the reduction factors were calculated by the following equation: \( R = \log_{10} A₀ - \log_{10} A_t \)

where \( R \) is the reduction factor, \( A₀ \) is the total virus load after spiking, and \( A_t \) is the total virus load in the treated sample.

**Sampling and cell quality analysis**

To investigate the quality of treated PCs, they were transferred to a storage bag (BEASAT Ind. Co., Iran) using syringe 16G, and stored for 4 days, under standard blood bank conditions, at 22°C under gentle agitation. The samples were drawn aseptically for cell quality analysis on days 2 and 4. The samples were assayed for platelet count (Sysmex, K-1000, Kobe, Japan), pH, LDH (Pars Azmon Co., Iran) and MTT reduction assay (Roche, Germany).

**Platelet enumeration**

The number of platelets was determined using an automated electronic particle counter (Sysmex, K-1000, and Kobe, Japan). For this purpose, 0.1 ml of PC was added to 0.4 ml of phosphate buffer saline (PBS).

**Measurement of lactate dehydrogenase activity**

LDH activity was measured by colorimetric methods using assay kit (Pars Azmon Co., Iran). LDH activity was measured at 340 nm.

**Analysis of the cell viability using MTT assay**

In order to measure the metabolic activity of platelet concentrates, the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT, Roche, Germany) was used. The MTT assay is a colorimetric method to assess cell proliferation and viability. NADPH-dependent mitochondrial oxido-reductase enzymes are capable of reducing the tetrazolium due to insoluble formazan. This enzyme activity is dependent on the survival condition of the cell. The cell viability rate was directly correlated with the amount of purple formazan crystals. This metabolic redox activity of PC was measured to assess platelet viability during platelet storage. For this purpose, PC was diluted in PBS to a concentration of \( 3 \times 10^5 \) platelets/µl. Then, 100 µl of diluted platelets were incubated with 10 µl of MTT (5 mg/ml) for 4 hr at 37°C. After addition of 100 µl of lysis buffer (10% SDS, 0.02 mol/l HCL) and incubation for one night at 37°C, the absorbance was measured at a wavelength of 575 nm by the spectrophotometer.

**Statistical analysis**

Each experiment was done three times, and the average and SD were presented for each sample. Statistical analysis (general linear model analysis of variance [ANOVA]) was carried out using GraphPad Prism® (Version 5.0) software for each experimental condition. According to the standard statistical procedures, a \( p<0.05 \) was considered to indicate a statistically significant difference.

**Results**

**Inactivation of VSV in PCs**

In the primary study, the efficacy of UV for VSV inactivation as an enveloped RNA virus was tested by two concentrations of RB (25 and 50 µM). For both concentrations of RB, viral reduction factor was observed at four doses. As shown in figure 1, a dose of 1.29 J/cm² of UV light for 10 min was able to reduce VSV titer about 3.8, 4.06 and 3.01 log₁₀ for the concentrations of 25 µM, 50 µM of RB and C UV-PC, respectively. Also, in 50 µM concentration of RB, 1.16 logs₁₀ of VSV was inactivated by 0.24 J/cm² for 2 min, but with the same dose, only 0.9 log₁₀ was inactivated for 25 µM and 0.6 log₁₀ for C UV-PC. No Statistically significant difference was found between the two concentrations of RB. C RB-PC was used for the evaluation of the effects of RB alone in the process of virus inactivation and none of RB concentrations were critical factors for virus inactivation (data not shown).

**Inactivation of polio virus and HSV in PLTs**

The optimum concentration of RB was employed to
Reduce Pathogen Load in PC by Photochemical Treatment

Table 1. Effects of RB (50 \( \mu \)M) in combination with different doses of UV light on HSV and Polio virus (The data represent mean±SD)

| Genome          | Lipid envelope | Initial titer (TCID\(_{50}\)/ml) | Logarithmic reduction of virus titer after illumination in comparison with initial titer (TCID\(_{50}\)/ml) |
|-----------------|----------------|----------------------------------|------------------------------------------------------------------------------------------------------|
|                 |                |                                  | 0.24 (J/cm\(^2\)) | 0.48 (J/cm\(^2\)) | 0.97 (J/cm\(^2\)) | 1.29 (J/cm\(^2\)) |
|                 |                |                                  | 2 min | 4 min | 8 min | 10 min | 2 min | 4 min | 8 min | 10 min |
| HSV             | dsDNA          | Yes                              | 7.3±0.28 | 1.4±0.36\(^t\) | 2.23±0.25\(^t\) | 2.8±0.11\(^t\) | 4.27±0.4\(^t\) |
| Polio virus     | ssRNA          | No                               | 5.6±0.35 | 0.74±0.15 | 0.87±0.05 | 1.7±0.41\(^t\) | 2.6±0.32\(^t\) |

\(^t\) \(P<0.0001\)

Table 2. Evaluated parameters of cell quality on day 2 (The data represented mean±SD). Effects of UV alone in different doses on platelet quality (1A). The effects of RB (50 \( \mu \)M) alone and RB in combination with UV light on the platelet quality (1B)

A

| C-PC | C\(_{UV}\)-PC | C\(_{RB}\)-PC |
|------|--------------|--------------|
| Cell count (x10\(^3\)/µl) | 740±7.07 | 707.5±17.67 | 695±63 |
| pH   | 7.9±0.15 | 8.0±0.04 | 8.0±0.04 |
| LDH (U/L) | 134255±188.8 | 147022±543.3 | 131467±340.7 |
| MTT assay (OD 575 nm) | 2.92±0.5 | 2.94±0.5 | 2.4±0.01 |

B

| Treatment with RB (50 \( \mu \)M)+UV | C-PC | C\(_{RB}\)-PC |
|-------------------------------------|------|--------------|
| Cell count (x10\(^3\)/µl) | 727±180.3 | 632±208.6 |
| pH | 7.96±0.007 | 7.91±0.14 |
| LDH (U/L) | 149608±453.3 | 114887±340.3 |
| MTT assay (OD 575) | 3.4±0.27 | 2.9±0.6 |

In vitro PLT quality after treatment and storage

Treated PCs were tested for pH, LDH activity, mitochondrial enzymatic activity (MTT assay), and cell count after treatment during storage on days 2 and 4 at 22°C. The results were compared to control groups. Control groups were collected, processed, and stored in the same condition. In this research, the effect of concentration of RB and UV on the quality of platelets was studied. The results of the quality analysis of PCs after treatment during storage on days 2 and 4 are summarized in tables 2 and 3. As shown in table 2, C\(_{RB}\)-PC and C\(_{UV}\)-PC show that RB or UV alone did not have any significant effect on platelet quality. Also, in parallel experiments, no significant difference was observed between the control and other treated units on days 2 and 4. Direct correlation was observed between quality of platelet and UV dose rate. Increasing doses of UV resulted in clear deterioration in cell count, mitochondrial enzymatic activity (MTT), and LDH. These changes were not statistically significant.

Cell count enumeration during storage of PC after treatments

In parallel with the increasing dose of UV, platelet count declined in all samples, but no significant difference was obtained between the control (C-PC) and treatment groups after treatment and during storage (Tables 2 and 3).

pH changes during storage of PC after treatments

There was no statistically significant difference between the C-PC and treatment groups (Table 2). pH values for all of the samples remained above 7.0 during 4 days of storage (Table 3).

Changes in LDH during storage of PC after treatments

As shown in table 2B, increasing doses of UV resulted in clear deterioration of LDH, but there was no significant difference in LDH level between C-PC and treatment groups (Table 2). LDH levels were raised in all samples during storage (Table 3).

MTT assay during storage of PC after treatments

MTT assay was used for detecting living/dead cells (viability assay). As shown in tables 2A and 2B, different doses of UV resulted in reduction in platelet survival, but the difference was not significant. The greatest decrease in cell survival was observed by RB in combination with UV 0.24 J/cm\(^2\) (this dose was used for 2 min) which was not statistically significant (Table 2B). Cell survival decreased in all treatment groups and control groups during storage, but no statistically sig-
significant difference was observed between the C-PC and treatment groups during storage (Table 3).

Discussion

Platelet transfusion is a mainstream therapy for preventing or treating bleeding episodes in thrombocytopenic patients or patients with a high risk of bleeding. The role of platelets in hemostasis is to migrate to sites of injury or damage, bind to the damaged surface, and release agents that stimulate tissue repair and recruitment of other platelets to the site of injury. Success in platelet transfusion depends on the cellular viability and homeostatic activity of the transfused platelets and on the physiological status of the recipient.

Inactivation of pathogens using photochemical or dynamic procedures is a promising technique to improve blood safety of cellular blood products. These processes may cause damage to the blood components, thereby resulting in the shortening of the lifespan of platelets during storage. For evaluating the success of a PRT for PCs, there are four main factors that must be taken into account. They are ease of use, cell quality, pathogen reduction levels, and toxicity.

Studies showed that 50 μM concentration of RB in combination with UV light at 6.2 J/cm² with an output ranging from 265 to 370 nm can inactivate pathogens (like Porcine parvovirus, cell associated and cell free HIV, WNV, plasmodium spp., Trypanosoma cruzi, Escherichia coli, Staphylococcus epidermidis) in plasma and PC.

The results of this study showed that concentration of 50 μM of RB combined with dose of 1.29 J/cm² (exposure time for this dose was 10 min) in comparison with CUV-PC and the concentrations of 25 μM and 50 μM of RB with combination of different doses of UV, was sufficient to inactivate VSV titer at least about 4.06 log (Figure 1). Our data indicated that with increasing the UV light dose, all parameters tested underwent changes until day 4, but these changes were not significantly different between C-PC and the treatment groups. According to the American Association of Blood Banks (AABB), the pH level of ≥6.2 is an essential requirement for quality control of blood components.

The results of this study showed that increasing exposure of light had no significant effect on the average of pH levels in the PCs tested and pH levels had the accepted range during storage.

Platelet membrane integrity can also be assessed by measuring the amount of the LDH enzyme that leaks from the cell. Although LDH level was raised during the storage in all of the samples, LDH levels were slightly lower in the treated PC with increasing illumination in comparison with the control, except dose 0.24 J/cm² (equal to 2 min). It seems that rate of used doses (0.48, 0.97, 1.29 J/cm²) does not harm the integrity of platelet membrane.

Given the fact that MTT is related to mitochondrial-based dehydrogenase activity, our observation showed that compared to the control, treatment of PCs with RB did not significantly affect their survival and the mitochondrial function (except for dose 0.24 J/cm²). MTT for controls and treated PCs did not significantly change during storage for up to 4 days. Our results on treated PC for MTT assay is also consistent with observations by Li et al (they had used RB (50 μM) plus 5 J/cm² UV light) and they showed that cell viability was lower in the test than that of control on day 4 of storage (Table 3).

This study showed no significant change in cell count after treatment. Post-treatment storage for 4 days did not significantly affect their survival and the mitochondrial function (except for dose 0.24 J/cm²). MTT for controls and treated PCs did not significantly change during storage for up to 4 days. Our results on treated PC for MTT assay is also consistent with observations by Li et al (they had used RB (50 μM) plus 5 J/cm² UV light) and they showed that cell viability was lower in the test than that of control on day 4 of storage (Table 3).
not induce a significant decrease in cell number for either treated PCs. Several studies have been conducted to assess the impact of RB and UV light on PC quality. Previous studies which were designed to use RB at a final concentration of approximately 50 μmol/l and UVB light from a fluorescent lamp with the energy of 18,19. Previous studies which were designed to use RB to assess the impact of RB and UV light on PC quality.

No significant differences in log reduction of VSV titer were observed between different concentrations of RBs. So, pathogen inactivation was carried out with the concentration of 50 μM of RB. Although with raising the dose of illumination, the enveloped viruses, HSV, and VSV (up to 4.26 and 4.06 log10, respectively), were effectively inactivated at 1.29 J/cm2, logs reduction of virus titer for Polio virus that is a non-enveloped virus, was 2.6. This result indicated that the enveloped viruses were more sensitive than non-enveloped viruses.

Consistently, other studies have shown inactivation of enveloped RNA viruses and enveloped DNA viruses and bacteria in platelet concentrates using riboflavin and light. Transfusion 2004;44(6):877-885.

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