Development of a riboflavin and ultraviolet light-based device to treat whole blood

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BACKGROUND: In the United States, blood components are commonly used for patients in need of massive transfusion after blood loss. In combat situations, when severe traumatic injuries occur far from a hospital, fresh whole blood is a valuable transfusion therapy because components may not be available. The risk of infectious or immunological complications from fresh whole blood transfusions could be mitigated by a system that reduces pathogen loads and inactivates white blood cells (WBCs). Such a system is in development and utilizes riboflavin and ultraviolet light to provide pathogen reduction and WBC inactivation.
STUDY DESIGN AND METHODS: The system has been tested with in vitro and in vivo animal studies to evaluate WBC inactivation and pathogen reduction, and with in vitro studies to assess the function of the treated blood products.
RESULTS: Elimination of viable WBCs with the system is equivalent to gamma-irradiation. Results have been reported for reduction of *Babesia microti*, *Trypanosoma cruzi*, HIV, and bacteria, and preliminary results for *Babesia divergens* are available. Treated whole blood, platelets, and plasma maintain coagulation function. Treated red blood cell components exhibit low hemolysis and high adenosine triphosphate levels at the end of storage.
CONCLUSIONS: Treatment with riboflavin and ultraviolet light is a promising alternative to gamma-irradiation. Effectiveness of the system against a variety of pathogens has been established, and further studies are planned. The in vitro studies of function indicate that treated whole blood, as well as components from treated whole blood, will provide acceptable hemostasis and perform well in the next phase of in vivo studies.

BACKGROUND

Hemorrhage is the leading cause of preventable death in combat casualties. Severely injured, bleeding casualties receive lifesaving transfusions as part of resuscitative therapy. Massive transfusion, defined as transfusion of 10 or more units of red blood cells (RBCs) in less than 24 hours, is often required. Per military doctrine, blood transfused to combat casualties is given in the form of standard, Food and Drug Administration (FDA)-licensed blood components when the components are available. When components are not available or when transfusion with components fails to stabilize the patient, the use of fresh whole blood (FWB) under emergency protocol is warranted.

Transfusion-related risks, whether in combat casualties or in civilian patients, include the risk of transmitted infection (viral, bacterial, and parasitic) and the risk of adverse immune responses, primarily due to residual white blood cells (WBCs) from donors. Blood banks in the United States limit the risks of infection through donor screening questionnaires and blood sample testing protocols. To address the risk of complications because of adverse immune responses, many blood centers perform WBC filtration to remove more than 99% of the donor WBCs from RBC components. In addition, blood products can be gamma irradiated to inactivate WBCs and prevent transfusion-associated graft-versus-host disease (TA-GvHD) in susceptible patient populations.

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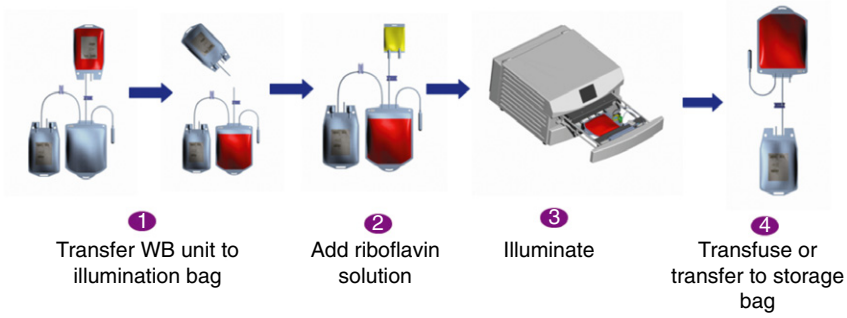

Fig. 1. Treatment of whole blood with riboflavin and UV light.

TABLE 1. Summary of WBC inactivation study results

	γ-irradiation (25 Gy)	Riboflavin and UV light
WBC viability	5 log reduction	5 log reduction
Alloimmunization	Not prevented	No antigen presentation in vitro Animal models in progress
TA-GVHD	Prevented	Prevented in animal model
Cytokine production	Cytokines still produced	Production prevented

When FWB is transfused under emergency conditions, leukoreduction and gamma irradiation are not available, donor screening questions may be limited, and the testing of blood samples with the full panel of tests may not be possible. The donors may also have been exposed to pathogens for which no screening tests are available. To mitigate the risks associated with WBCs and pathogens in donated blood products, a device has been developed to treat whole blood with riboflavin and ultraviolet (UV) light. This device, the Mirasol System for Whole Blood (Terumo BCT, Lakewood, CO), uses the same equipment and disposable kit as the CE-marked Mirasol PRT System for Platelets and Plasma. Figure 1 displays a general process diagram for the treatment of whole blood. The preparation for illumination takes approximately 15 minutes. The illumination step provides the UV energy dose appropriate for whole blood (80 J/mL$_{RBC}$) and can take from 40 to 60 minutes, depending on the size of the unit illuminated. One of the benefits of adapting the CE-marked system is that the data on safety and efficacy for that device[1,2] is relevant to the system for whole blood. Additional studies have been performed, are in progress, or are in the planning phase to ensure that all necessary data is obtained.

RISK MITIGATION—WBC INACTIVATION

WBC inactivation was evaluated in FWB treated with riboflavin and UV light in studies that provided paired comparisons with gamma-irradiated blood. The gamma-irradiation dose used was 25 Gy, the recommended dose of the Council of Europe;[3] the FDA recommends 15 Gy.[4] Inactivation was evaluated with in vitro analyses, as described by Fast and colleagues.[5] The in vitro assays included: proliferation (phytohemagglutinin, anti-CD3/CD28, allogeneic stimulators), antigen presentation, activation (CD69 expression), viability (limiting dilution assay), cytokines (lipopolysaccharide stimulation and CD3/28 stimulation), and phenotype (flow cytometry). The results of these analyses showed that treatment with riboflavin and UV light decreases viability to the same extent as gamma irradiation and provides greater reduction of cytokines and of antigen presentation.

Inactivation of WBC was also evaluated in vivo. The in vivo test was performed in a mouse model of xenogeneic graft-versus-host disease (xGVHD).[5] As with the in vitro study, treated whole blood was compared with paired, gamma-irradiated whole blood. Treatment with riboflavin and UV light was as effective as gamma irradiation at preventing xGVHD (evaluated in a mouse model), whereas untreated blood (the positive control) caused xGVHD in all mice. Table 1 summarizes the results described by Fast and colleagues. Both gamma irradiation and treatment with riboflavin and UV light prevent GVHD, whereas leukoreduction has been shown not to be sufficient in the prevention of disease.

RISK MITIGATION—PATHOGEN TRANSMISSION

Pathogen reduction studies of the device have included studies of parasite, virus, and bacteria reduction. Intraerthrocytic parasites are of concern in the United States (*Babesia* spp.) and in parts of the world where malaria is endemic (*Plasmodium* spp.). Studies with *Babesia microti* yielded reduction values of 5 log.[6] These results correlate with the reduction levels observed with the treatment of platelets and plasma by the Mirasol PRT System.[7] Pilot studies with *Babesia divergens* indicate greater than 6 log reduction (personal communication, Dr Lobo); further investigations with this species are planned. Studies with *Plasmodium* spp. are in progress and include in vitro tests of *Plasmodium falciparum* reduction in human RBCs and in vivo tests in a mouse model of the reduction of *Plasmodium yoelii* (causative agent of murine malaria). Reduction of *P. falciparum* and *P. yoelii* has been evaluated in platelets and plasma, where riboflavin and UV light provided reduction levels of ≥3.2 log[8] and ≥4.4 log,[9] respectively. The clinical relevance of pathogen reduction values is discussed by

Goodrich and colleagues[10] and is related to the amount of infectious pathogen present in a donated blood product.

Nonerythrocytic parasites have also been used to test the effectiveness of the device. A parasite of growing concern in the United States is *Trypanosoma cruzi*, the causative agent of Chagas' disease. This parasite is endemic in parts of Mexico, Central America, and South America[11] and is appearing more frequently in the blood supply in the United States.[12] Reduction of *T. cruzi* by riboflavin and UV light was evaluated with an in vitro assay, and reduction levels of ≥3.5 log (and ≤4.5 log) were observed.[13] The parasites that cause Leishmaniasis (*Leishmania* spp.) are also of concern, particularly because they are endemic in Iraq and Afghanistan as well as other parts of Asia, Africa, South America, Central America, and Mexico. Tests of the reduction of *Leishmania donovani* with the device are in progress. The riboflavin and UV light technology was previously tested with platelets and plasma, where reduction values were ≥5 log for *T. cruzi* and ≥4 log for *L. donovani*.[14]

Virus and bacteria reduction in treated whole blood has also been evaluated. Results of tests of bacterial reduction are described by Goodrich and colleagues.[15] Additional tests are ongoing. Model viruses tested early in development displayed varying levels of reduction.[15] Tests of transfusion-transmitted human viruses are planned. The first of those tests was an evaluation of HIV reduction. In the test, a cell-associated form of the virus was used as a model of early infection or chronic infection, where detection of the virus by testing is more difficult because the virus is primarily in lymphocytes. Reduction levels of 4.5 ± 0.5 log were observed,[16] levels at or above those that would be expected to be present in asymptomatic donors who might yield negative diagnostic test outcomes.

TRANSFUSABLE BLOOD PRODUCT QUALITY—FWB

Mirasol-treated FWB has been evaluated to determine whole blood quality after 24 hours of room temperature storage. Longer storage durations have also been evaluated with respect to coagulation and platelet function.[17,18] The 24-hour storage duration was tested with measurements of the whole blood (weight, free hemoglobin, complete blood count, potassium, glucose, lactate, pH, and blood gases), the RBC fraction (adenosine triphosphate [ATP] and 2,3-DPG), the platelets (CD62P, Annexin V, hypotonic shock response, extent of shape change, and ATP), and the plasma fraction (prothrombin time [PT]; activated partial thromboplastin time [aPTT]; fibrinogen; factors V, VIIa, VIIIc, and XI; von Willebrand factor activity; antithrombin III; and thrombin–antithrombin complex). The RBC, platelet, and plasma measurements were made after isolation of the particular component from the sample. Results for treated units were compared with results for untreated paired controls, collected and stored under the same conditions.

Hemolysis values remained well below 1% at the end of storage for treated whole blood units and for the untreated controls. Methemoglobin was monitored because UV light can convert oxyhemoglobin to methemoglobin. Levels of methemoglobin immediately after treatment ranged from 1.6% to 8.2% and were reduced to background levels (0.9%, equivalent to the untreated controls) during storage overnight. RBCs in untreated controls released potassium during storage (Fig. 2A), although the levels released by treated units were greater in comparison; overall, levels remain below 7 mM at 24 hours. No differences were observed in ATP values between treated and control groups postcollection, or between pretreatment and posttreatment samples (Fig. 2B). The small difference between treated and control groups at 24 hours (4.25 ± 0.65 μmol/gHb vs. 4.43 ± 0.56 μmol/gHb) was significant. The high ATP levels in RBCs from 24-hour stored, treated whole blood indicate that RBCs from treated whole blood should meet and surpass the FDA criteria for radiolabeled RBC recovery.

The platelets in samples removed from the whole blood units were separated for analysis of platelet-specific parameters. No significant differences were observed in platelet ATP and HSR values for test and control units. Figure 2C and 2D display results for Annexin V and CD62P. The CD62P values in postcollection samples (pretreatment for the test units) were higher for Mirasol-treated whole blood, and the figure shows that there was great variability in the postcollection values. Annexin V and CD62P values were higher in the 24-hour samples from Mirasol-treated units, indicating that platelets were more activated. However, the values in 24-hour samples for test and controls are lower than those reported for 5-day stored platelets. ESC values in test samples were lower at 24 hours than in control samples, although the measured values are well within the range where in vivo viability is not affected.[19] These in vitro measurements for platelets isolated from treated whole blood after 24 hours of storage indicate that the in vivo function of the platelets should be similar to that observed for treated platelet components. In vivo studies are the next step in establishing that platelet function is maintained in Mirasol-treated FWB.

Plasma was analyzed after separation from samples of Mirasol-treated and control whole blood; results of all assays except PT and aPTT were corrected for dilution with riboflavin solution. At the end of storage, no significant differences were observed in levels of von Willebrand Factor activity, anti-thrombin III, Protein S, Protein C, or thrombin–antithrombin complex. PT and aPTT were greater in stored treated units than in stored controls. Fibrinogen and factors V, VIIa, VIII, and XI were significantly lower in treated units than in controls. Figure 2E

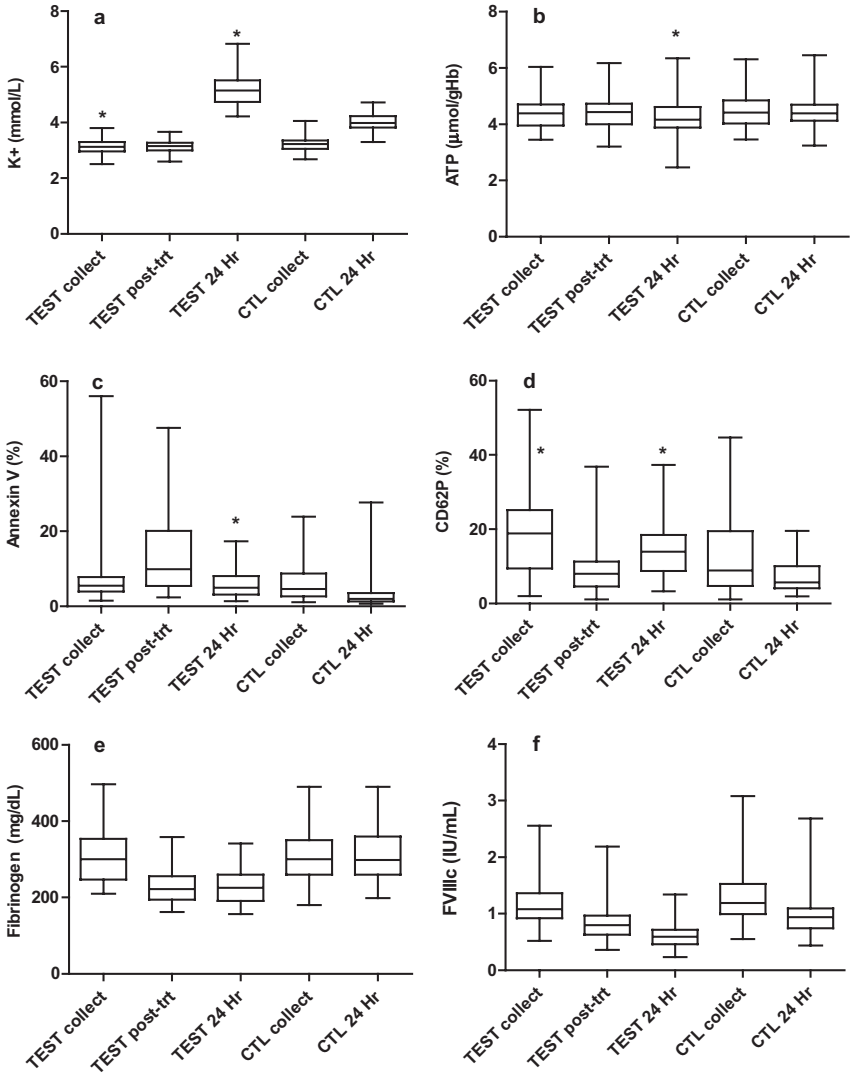

Fig. 2. Measurements for samples removed from test whole blood before treatment (TEST collect), after treatment (TEST post-trt) and after 24 hours of storage (TEST 24 hr), and in samples removed from control whole blood after collection (CTL collect) and after 24 hours of storage (CTL 24 hr). Values shown are for (A) extracellular potassium; (B) RBC ATP; (C) Annexin V; (D) CD62P; (E) fibrinogen; and (F) factor VIIIc. Values for fibrinogen and factor VIIIc are corrected for dilution with riboflavin. Whiskers correspond to maximum and minimum values. Bottom and top of the boxes indicate lower and upper quartiles; the line inside the box indicates the median. An asterisk indicates significant difference ($p > 0.05$) from paired controls.

and 2F display data for fibrinogen and factor VIII and show the variability in the data sets. Fibrinogen and factor VIII both decrease with treatment, and room temperature storage leads to a further decrease in factor VIII. Based on the measurements of protein quality and function, treated plasma (derived from treated whole blood) is expected to support coagulation and hemostasis as well as plasma treated as a component. This will be tested with in vivo studies.

TRANSFUSABLE BLOOD PRODUCT QUALITY—COMPONENTS

All blood components were evaluated in preliminary studies, at varying UV energy doses.[15] The prototype system was evaluated for RBC recovery and survival at UV energy doses of 22, 33, and 44 J/mL$_{RBC}$,[20] and the correlations of ATP and hemolysis with recovery guided further development to a UV energy dose of 80 J/mL$_{RBC}$. For the studies of RBCs derived from treated whole blood (treated RBCs), whole blood was centrifuged after treatment with a hard spin (5000 × *g*), plasma was expressed, and the RBCs were suspended in AS-3 and leukoreduced with a Pall RCM1 filter (Medsep Corporation, Covina, CA) before storage. Leukoreduction is not a requirement of treatment and was incorporated because it is the standard of care at potential clinical trial sites. The leukoreduced, treated RBCs were stored at 4°C for 28 days and sampled on Days 21 and 28. Paired control units of RBCs were prepared with the same steps (without treatment), stored under the same conditions, and sampled on Days 21 and 28.[21]

Hemolysis values for treated units of RBCs remained below 1% throughout storage and were not different from paired controls. The release of potassium by treated RBC is greater than observed for controls (68.8 ± 3.4 mM, compared with 37.6 ± 3.4 mM) (Fig. 3A). The potassium levels in treated RBCs are similar to those observed by Moroff and colleagues for gamma-irradiated RBC in AS-1 stored for 28 days (72.3 ± 6.4 mM) and 42 days (72.0 ± 8.7),[22] and by Wagner and Myrup for gamma-irradiated RBCs in AS-3 stored for 42 days (71.4 ± 4.0).[23] ATP values indicate that the FDA criterion for recovery should be met by treated RBCs (Fig. 3B). Methemoglobin levels postillumination range from 1.6% to 8.2% and are reduced to background levels during storage.

FUTURE WORK

A clinical trial in healthy volunteers, approved by the US FDA, will evaluate the recovery and survival of treated RBCs. This is the first step in the pathway for licensure of

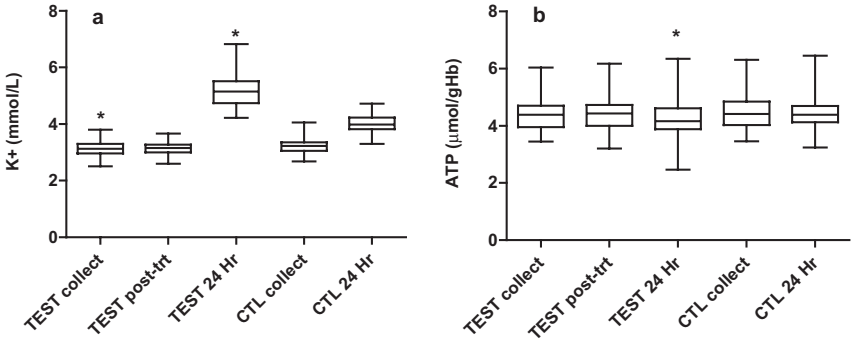

Fig. 3. Values for (A) extracellular potassium and (B) RBC ATP in samples removed from treated RBCs before storage (TRT Day 0), after 21 days of storage (TRT Day 21) and after 28 days of storage (TRT Day 28), and for samples removed from control RBCs at the same intervals (CTL Day 0, CTL Day 21, and CTL Day 28). Whiskers correspond to maximum and minimum values. Bottom and top of the boxes indicate lower and upper quartiles; the line inside the box indicates the median. An asterisk indicates significant difference ($p > 0.05$) from paired controls.

this product by the FDA. A pivotal trial, involving studies in patients requiring transfusion support, will also be necessary before the product can be licensed for use in the routine, clinical setting. In addition to the clinical studies of RBC performance in humans, studies are planned to evaluate the function of treated whole blood in vivo in animal models. The in vivo studies of function will provide information on the effect of the differences observed between treated and control whole blood. Further in vitro studies of platelet and plasma components are also planned, as are in vitro tests of whole blood function with storage at 4 and at 22°C. The tests of blood function at two different storage temperatures follow on the work presented by Pidcoke and colleagues,[18] in which the hemostatic function of Mirasol-treated whole blood was evaluated with in vitro assays with storage out to 21 days at 4 and at 22°C. Tests of parasite, virus, and bacteria reduction will continue. The effects of treatment on the prevention of alloimmunization are also under investigation.

CONCLUSION

Treatment with riboflavin and UV light is as effective as gamma irradiation for the elimination of WBC viability and the prevention of xGvHD and more effective for the prevention of cytokine production and of alloimmunization responses. The device is effective at the reduction of *B. microti*, *B. divergens*, *T. cruzi*, and HIV, and preliminary studies indicate that this will also extend to *Plasmodium* and *Leishmania* species. Treated whole blood is expected to provide acceptable hemostasis upon transfusion; in vivo preclinical studies in animal models will provide information with which to evaluate that expectation. Whole blood treated with the Mirasol System yields platelet and plasma with characteristics similar to components treated with the Mirasol System. Treated RBCs are expected to meet FDA criteria for stored RBCs.

CONFLICT OF INTEREST

All authors are employees of Terumo BCT, Inc. or its affiliates. Terumo BCT is the manufacturer of the Mirasol PRT System for Platelets and Plasma, which is not for sale in the United States.

REFERENCES

1. Reddy HL, Dayan AD, Cavagnaro J, Gad S, Li J, Goodrich RP. Toxicity testing of a novel riboflavin-based technology for pathogen reduction and white cell inactivation (MIRASOL PRT system). Transfus Med Rev 2008;22:133-53.
2. Mirasol Clinical Evaluation Study Group. A randomized controlled clinical trial evaluating the performance and safety of platelets treated with MIRASOL pathogen reduction technology. Transfusion 2010;50:2362-75.
3. Council of Europe. Guide to the preparation, use, and quality assurance of blood components. 16th ed. Strasbourg, France: European Directorate for the Quality of Medicines and Healthcare; 2010.
4. American Association of Blood Banks. Circular of information for the use of human blood and human blood components. Bethesda (MD): American Association of Blood Banks; 2009.
5. Fast LD, Nevola M, Tavares J, Reddy HL, Goodrich RP, Marschner S. Treatment of whole blood with riboflavin plus ultraviolet light, an alternative to gamma irradiation in the prevention of transfusion-associated graft-versus-host disease? Transfusion 2013. doi: 10.1111/j.1537-2995.2012.03715.x.
6. Tonnetti L, Thorp AM, Reddy HL, Keil SD, Goodrich RP, Leiby DA. Riboflavin and ultraviolet light reduce the infectivity of *Babesia microti* in whole blood. Transfusion 2013. doi: 10.1111/j.1537-2995.2012.03791.x.
7. Tonnetti L, Proctor MC, Reddy HL, Goodrich RP, Leiby DA. Evaluation of the Mirasol pathogen [corrected] reduction technology system against *Babesia microti* in apheresis platelets and plasma. Transfusion 2010;50:1019-27. Erratum in: Transfusion 2010;50:1594.
8. Sullivan JJ, Bounngaseng A, Reddy H, Keil S, Goodrich R. Pathogen inactivation of plasmodium falciparum in plasma and platelet concentrates with riboflavin and UV light. 30th International Congress of the ISBT, Macao, SAR, China, June 7-12, 2008. Vox Sang 2008; 95(Suppl. 1):278.

9. Kiser P, Keil S, Marschner S, Avery A. Mirasol pathogen reduction technology (PRT) treatment of *P. yoelii* contaminated plasma neutralized parasite in a murine model for malaria. Vox Sang 2012;103(Suppl. 1):136.
10. Goodrich RP, Custer B, Keil S, Busch M. Defining "adequate" pathogen reduction performance for transfused blood components. Transfusion 2010;50:1827-37.
11. Organización Panamericana de la Salud. Estimación cuantitativa de la enfermedad de Chagas en las Americas. Document OPS/HDM/CD/425-06. Montevideo, Uruguay: Organización Panamericana de la Salud; 2006.
12. Leiby DA, Herron RM Jr, Read EJ, Lenes BA, Stumpf RJ. *Trypanosoma cruzi* in Los Angeles and Miami blood donors: impact of evolving donor demographics on seroprevalence and implications for transfusion transmission. Transfusion 2002;42:549-55.
13. Tonnetti L, Thorp AM, Reddy HL, Keil SD, Goodrich RP, Leiby DA. Evaluating pathogen reduction of *Trypanosoma cruzi* with riboflavin and ultraviolet light for whole blood. Transfusion 2012;52:409-16.
14. Marschner S, Goodrich R. Pathogen reduction technology treatment of platelets, plasma and whole blood using riboflavin and UV light. Transfus Med Hemother 2011;38:8-18.
15. Goodrich RP, Doane S, Reddy HL. Design and development of a method for the reduction of infectious pathogen load and inactivation of white blood cells in whole blood products. Biologicals 2010;38:20-30.
16. Keil S, Rapaport R, Young R, Cambell T, Marschner S. Viral reduction of intracellular HIV using the Mirasol system for whole blood. Vox Sang 2012;103(Suppl. 1):144.
17. Pidcoke HF, Delgado KK, Mora AG, Cap AP. Whole blood stored at four degrees retains platelet function, normal prothrombin time and overall hemostatic function as measured by thromboelastography. Transfusion 2011;51:SP8 (abstract).
18. Pidcoke HF, McFaul SJ, Ramasubramanian AK, Parida BK, Mora AG, Fedyk CG, Valdez-Delgado KK, Montgomery RK, Reddock KM, Rodriguez AC, Aden JK, Jones JA, Bryant RS, Scherer MR, Reddy HL, Goodrich RP, Cap AP. Primary hemostatic capacity of whole blood: a comprehensive analysis of pathogen reduction and refrigeration effects over time. Transfusion 2013;53(Suppl. 1):137S-49S.
19. Holme S, Moroff G, Murphy S. A multi-laboratory evaluation of in vitro platelet assays: the tests for extent of shape change and response to hypotonic shock. Biomedical Excellence for Safer Transfusion Working Party of the International Society of Blood Transfusion. Transfusion 1998;38: 31-40.
20. Cancelas JA, Rugg N, Fletcher D, Pratt PG, Worsham DN, Dunn SK, Marschner S, Reddy HL, Goodrich RP. In vivo viability of stored red blood cells derived from riboflavin plus ultraviolet light-treated whole blood. Transfusion 2011;51:1460-8.
21. Doane SK, Wilkinson S, Hovenga N, Rouse L, Miklauz M, Reddy H, Marschner S, Goodrich R. Storage of packed red blood cells derived from whole blood treated with the Mirasol system. Vox Sang 2012;103(Suppl. 1):143.
22. Moroff G, Holme S, AuBuchon JP, Heaton W, Sweeney JD, Friedman LI. Viability and in vitro properties of AS-1 red cells after gamma irradiation. Transfusion 1999;39:128-34.
23. Wagner SJ, Myrup AC. Prestorage leucoreduction improves several in vitro red cell storage parameters following gamma irradiation. Transfus Med 2006;16:261-5.