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Pathogen inactivation of *Trypanosoma cruzi* in plasma and platelet concentrates using riboflavin and ultraviolet light

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Abstract

Background: Emigration of people infected with *Trypanosoma cruzi* to non-endemic areas has resulted in transfusion transmission to immunocompromised recipients. We studied the feasibility of inactivating *T. cruzi* using a new technology which utilizes riboflavin as a photosensitizer in combination with UV light, Mirasol PRT.

Methods: One billion *T. cruzi* organisms and 30 mL of 500 μM riboflavin were added to each of six units of human plasma and six units of platelets. To determine the level of detection of organism, a sample of each unit was cultured in tenfold serial dilutions beginning with 100 billion/250 mL as the starting culture. After 30 min, each unit was illuminated with 5.9 J/cm^2 of UV light (6.24 J/mL). The units were then cultured again in tenfold serial dilutions post-treatment.

Results: A 6 log reduction of pathogens was demonstrated in 5 of 6 units of plasma, and a 7 log reduction of pathogens was demonstrated in one unit. A 6 log reduction of pathogens was demonstrated in 3 of 6 units of platelets, a 7 log reduction was demonstrated in 2 of 6 units of platelets, and an 8 log reduction of pathogens was demonstrated in 1 of 6 units.

Conclusions: Mirasol PRT treatment demonstrated an ability to inactivate 5–7 logs of *T. cruzi* in plasma and platelets.

1. Introduction

Chagas' disease is caused by the flagellated protozoan parasite *Trypanosoma cruzi* and is transmitted to humans via the bite of the reduvid bug. This disease is endemic in Latin America where efforts have been effective in reducing the number of vector insects, making transfusion the main route of transmission in endemic and non-endemic countries [1–3]. Emigration of people infected with *T. cruzi* to non-endemic countries has resulted in cases of transfusion transmission of Chagas' disease in immunocompromised recipients [1,2,4–7]. Congenital transmission and transmission through organ transplantation have also occurred [1].

Nationwide estimates in the United States indicate that 1 in 25,000 blood donors is infected with *T. cruzi* with local seroprevalence up to 4–5 times higher in areas like Los Angeles and Miami where

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the number of Latin American immigrants is high [7]. In Miami 1 out of 8800 donors was found to be infected with *T. cruzi*, and 1 of 650 donors with Spanish surnames was found to harbor *T. cruzi* [8]. It has been estimated that more than 100 cases of transmission of *T. cruzi* transmission by transfusion occur in the United States each year [8]. Donor testing for *T. cruzi* is conducted in Latin America, but screening is not carried out in the United States, Canada, and Europe where the number of infected donors is rising [8,9]. The US Food and Drug Administration has recently approved a screening test for *T. cruzi*, but this test has not yet been implemented in donor screening.

Several strategies have been developed for the inactivation or removal of *T. cruzi* from blood products. Some of these have been implemented in countries where disease transmission by transfusion is a significant route of transmission. The strategies include the use of chemical and photochemical methods, including gentian violet [10], crystal violet [11], ethyleneimine derivatives [12], phenothiazine dyes [13], psoralens [14], hypochloride and nitrite related radicals [15], phthalocyanines [16], and white cell reduction by filtration [17].

Leukodepletion filters have been shown to remove *T. cruzi* from blood products by mechanical means [5] and by direct adherence to filter fibers [6], but have been only partially effective [7,8]. Levels of 6 log removal have been reported, but the disease was still transmitted in 30–50% of animals given *T. cruzi* infected (2 parasites/mL) filtered blood [17]. The effectiveness was also shown to be dependent on the starting level of organisms in the blood.

Gentian violet addition to blood products has been practiced in endemic areas in an attempt to eliminate transmission [10]. The compound has both a light activated and a dark chemistry associated with adverse effects on pathogen mitochondrial oxidative phosporylation activity. The same mechanism has been associated with crystal violet, phenothiazine dyes, and hypochloride and nitrite related radicals [11,13,15]. Each of these agents inactivates the pathogen primarily by interfering with one or more metabolic pathways associated with normal parasite development and viability. Infusion of residual levels of these compounds thus poses a potential toxicological concern since human cellular systems rely on similar metabolic pathways for maintenance of normal cell function and activity.

Psoralen, pthalocyanine, and ethyleneimine derivatives are believed to prevent replication processes in the parasite by damaging nucleic acids thus preventing pathogen reproduction and disease transmission [12,14,16]. Several chemical modes of action are involved which require activation by photochemical or chemical means, but all primarily result in irreversible and irreparable damage to the parasites' genetic material. These approaches have the benefit of having more specific action since the Chagas parasite is more selectively targeted in blood products such as platelets, plasma and red blood cells where genomic nucleic acids are not present or necessary for normal function and activity. Residual levels of these agents are often removed or quenched prior to re-infusion due to concerns regarding potential mutagenic action in the recipient.

This research was undertaken to study the feasibility of inactivating *T. cruzi* using a new technology which utilizes riboflavin as a photosensitizer in combination with UV light (MIRASOL Pathogen Reduction Technology (PRT) System for Platelets and Plasma, Navigant Biotechnologies, Lakewood, CO). A different procedure is under development for the treatment of erythrocytes. Riboflavin (Vitamin B_2) is a dietary nutrient with known pharmacokinetic and toxicology profiles [18–20]. Riboflavin can rapidly traverse lipid membranes where it non-specifically intercalates with nucleic acids. Upon exposure to light, the riboflavin causes modification of the nucleic acid present in pathogens and white cells through oxidation of guanine residues and generation of reactive oxygen species. Due to these photochemical processes, the nucleic acids of pathogens in blood products undergoing this treatment are rendered unable to replicate [21]. This technology takes advantage of the fact that in contrast to microorganisms, all the components of blood which are advantageous to transfuse do not require intact DNA for efficacy after transfusion. This method has been successful in the inactivation of intracellular and extracellular viral and bacterial pathogens in plasma and platelet concentrates and parasites such as Leishmania and malaria, and has been successful in the presence of high white cell loads or significant red cell contamination [22–28]. This treatment does alter plasma and platelet quality to some extent. The extent of these alterations is undergoing evaluation. This is a proof of concept study which was designed to show activity against an additional transfusion transmitted parasitic agent, *T. cruzi*.

2. Materials and methods

2.1. Collection of platelets and plasma

Within an approved human use protocol, six units of plasma and six units of platelets were collected from normal volunteers by apheresis using a COBE Spectra Cell Separator (Gambro BCT, Lakewood, CO). Methods used were identical to those of the Donor Center at Walter Reed Army Medical Center Blood Bank Donor Center according to American Association of Blood Banks (AABB) and Food and Drug Administration (FDA) guidelines. Each unit consisted of 250 mL of blood product. Platelet units contained 2.5×10^{11}–4.0×10^{11} platelets and less than 1×10^{6} leukocytes per mL, at the time of collection, before spiking with *T. cruzi*.

2.2. Trypanosoma organisms

T. cruzi, WR-2327, was characterized at Walter Reed Army Institute of Research to confirm the species using cellulose acetate electrophoresis of isoenzymes [29].

2.3. Maintenance of Trypanosoma stock cultures

T. cruzi were cultured at 22 °C in Schneider's Drosophila medium (Invitrogen, Carlsbad, CA) supplemented with 30% heat inactivated fetal calf serum (Invitrogen). Parasites were maintained in logarithmic phase by seeding at 2×10^{6}/mL. Stationary phase trypomastigotes were obtained when cultures approached 2×10^{7}/mL. Cell counts were made by counting cell numbers in a hemocytometer. One billion *T. cruzi* parasites contained in 1 mL Schneider's were used to infect each unit of plasma or platelets.

Under an approved animal protocol, parasite cultures of typomastigotes are injected into Balb C mice and Syrian golden hamsters on a monthly basis to assure infectivity and quality control. Upon demonstration of symptoms of infection, or after 30 days, animals are sacrificed. Cultures are obtained and cellulose acetate electrophoresis of isoenzymes is performed to verify species and infectivity.

2.4. Evaluation of T. cruzi cultures

Cultures were read every day for a period of 28 days using an inverted microscope with a 40× objective (Olympus CKX41, Tokyo, Japan). Fields within each flask were examined to identify a moving organism if present. Upon demonstration of any mobile trypomastigotes, the culture was considered positive. If none were detected after 28 days, the culture was considered negative.

2.5. Storage of blood products

Pre-treatment units were stored under blood bank conditions, platelets at 22 °C under gentle agitation prior to treatment. Plasma was stored at −20 °C.

2.6. Reagents

The Mirasol PRT System contains riboflavin (500 μM) in a 0.9% sodium chloride solution with pH adjusted to 4.0–5.0. The solution is non-pyrogenic and is sterilized by autoclaving under GMP manufacturing conditions. The solution is packaged in a disposable polyolefin bag and opaque foil pouch in order to maintain appropriate solution stability through the sterilization process and to protect it from ambient light. The concentrated solution produced in this way is such that when combined with the platelets or plasma, the riboflavin is diluted to the appropriate concentration (50 μM) for pathogen reduction treatment.

2.7. Transfer of blood products

The bag containing platelets or plasma was connected to the pathogen reduction illumination/storage bag [Extended Life Platelet, ELP™] using a Terumo Sterile Tubing Welder. Following sterile connection, 250 mL platelet or plasma product was transferred to the bag containing 30 mL of riboflavin solution (500 μM). Transfer tubing was sealed using a Sebra hand held, radio frequency (RF) tubing sealer. Following connection, the two bags were separated and the original collection bag was discarded. Each platelet or plasma product to be treated contained 280 mL of 90% blood product in a 1-l citrate-plasticized, polyvinyl Chloride (PVC) ELP bag with a surface area of 347 cm^2 per side. The bag is labeled on one side, which results in a total surface area of 590 cm^2 through which light can reach the bag contents.

2.8. Ultraviolet illumination

The MIRASOL PRT System device is composed of an illuminator, driven by a dedicated software

program. UV lamps in the illuminator contain a broad-band phosphor with output ranging from 265 to 370 nm, and are positions above and below the product chamber. A dedicated fan cools both the lamp chambers and the product chamber. The illuminator delivers 6.24 J/mL UV light to the labeled platelet or plasma product (as measured by an optical associated incorporated (OAI) power meter). The platelet or plasma product is placed in the product chamber for mixing (on a motorized platen) and exposure to light. Units were illuminated for 12 min.

2.9. Culture of blood products

Prior to and after illumination 2 mL of blood product was removed from each bag using sterile technique in a laminar flow hood. One hundred microliters of blood product was diluted in 900 μL of Schneider's medium in serial dilutions. Five hundred microliters was added to 10 mL of culture medium which was maintained as described above.

3. Results

3.1. Platelets

Pre-treatment platelets showed parasite detection in 5–7 log dilutions of the starting culture, establishing a lower limit of detection. In treated platelets no growth of organism was detected in 6 of 6 units when cultured. A 6 log reduction of pathogens was demonstrated in 3 of 6 units of platelets, a 7

Table 1
Pathogen inactivation of platelet concentrates infected with 1 billion *Trypanosoma cruzi* per 250 mL

Growth of organism	Pre-treatment						Post-treatment					
Unit	1	2	3	4	5	6	1	2	3	4	5	6
Log dilution												
0	+	+	+	+	+	+	–	–	–	–	–	–
1	+	+	+	+	+	+	–	–	–	–	–	–
2	+	+	+	+	+	+	–	–	–	–	–	–
3	+	+	+	+	+	+	–	–	–	–	–	–
4	+	+	+	+	+	+	–	–	–	–	–	–
5	+	+	+	+	+	+	–	–	–	–	–	–
6	–	+	–	–	+	+	–	–	–	–	–	–
7	–	–	–	–	–	+	–	–	–	–	–	–
8	–	–	–	–	–	–	–	–	–	–	–	–
9	–	–	–	–	–	–	–	–	–	–	–	–

When compared with untreated units treatment with Mirasol PRT for pathogen inactivation of platelet concentrates infected with 1 billion *Trypanosoma cruzi* per 250 mL resulted in a 6 log ten reduction of pathogens in 3 of 6 units of platelets, a 7 log ten reduction in 2 of 6 units of platelets, and an 8 log ten reduction of pathogens in 1 of 6 units.

Table 2
Pathogen inactivation of plasma infected with 1 billion *Trypanosoma cruzi* per 250 mL

Growth of organism	Pre-treatment						Post-treatment					
Unit	1	2	3	4	5	6	1	2	3	4	5	6
Log dilution												
0	+	+	+	+	+	+	–	–	–	–	–	–
1	+	+	+	+	+	+	–	–	–	–	–	–
2	+	+	+	+	+	+	–	–	–	–	–	–
3	+	+	+	+	+	+	–	–	–	–	–	–
4	+	+	+	+	+	+	–	–	–	–	–	–
5	+	+	+	+	+	+	–	–	–	–	–	–
6	–	–	–	+	–	–	–	–	–	–	–	–
7	–	–	–	–	–	–	–	–	–	–	–	–
8	–	–	–	–	–	–	–	–	–	–	–	–
9	–	–	–	–	–	–	–	–	–	–	–	–

When compared with untreated units, treatment with Mirasol PRT for pathogen inactivation of plasma infected with 1 billion *Trypanosoma cruzi* per 250 mL resulted in a 6 log ten reduction of pathogens in 5 of 6 units of plasma, and a 7 log ten reduction of pathogens was in one unit.

log reduction was demonstrated in 2 of 6 units of platelets, and an 8 log reduction of pathogens was demonstrated in 1 of 6 units (Table 1).

3.2. Plasma

Pre-treatment plasma showed parasite detection in 5–7 log dilutions of the starting culture, establishing a lower limit of detection. Treated plasma showed no growth of organism in 6 of 6 units when cultured. A 6 log reduction of pathogens was demonstrated in 5 of 6 units of plasma, and a 7 log reduction of pathogens was demonstrated in one unit (Table 2).

4. Discussion

T. cruzi is prevalent in Latin America, but is not endemic in the United States, Canada, or Europe; however, prevalence is increasing with current immigration patterns. Methods designed to deal with this growing problem need to be developed and implemented. The prevalence of infection among donors in the non-endemic countries is expected to vary based on location due to variations in population origins.

In a review of trypanosomiasis occurring in the United States an estimate of prevalence was made based upon a study of 205 Salvadoran and Nicaraguan immigrants living in Washington, DC in which 5% were found to be infected. The authors stated that if this represents the prevalence of infection among the general population of immigrants from endemic countries, there may be over 50,000–100,000 immigrants with *T. cruzi* chronic infection living in the United States [1]. More recently blood donors in Mexico were tested for *T. cruzi* and found to have a variable prevalence based on location with an overall prevalence of 0.75%. It was estimated that 1800 cases of transfusion transmission of *T. cruzi* occur each year in Mexico [8].

There have been only a few documented cases of transfusion transmission of *T. cruzi* in the United States, and these have all occurred among immunosupressed individuals. It has been estimated that the risk of transmission is 13–23% for each unit of contaminated blood transfused [1,8]. The course of infection with this parasite, involves a long period where the infected individual remains asymptomatic. *T. cruzi* infection is life long and most people who harbor the parasite chronically are asymptomatic and unaware of their being infected [8]. Infective organisms circulate in the blood stream for the entire life of an individual with chronic infection in spite of a lack of symptoms. A long latent period of asymptomatic infection associated with no donor screen produces a risk for transmission by transfusion. This asymptomatic period is followed years to decades later by manifestations of organ damage in 10–30% of infected persons [30]. The extent of transfusion transmission of *T. cruzi* is thus likely to be underestimated in the short term, since infection with *T. cruzi* is more often occult in individuals without immunosuppression. It is very likely that many unrecognized cases of transmission by transfusion have occurred [1]. Transmission of infection by transfusion would be expected to manifest itself only among immunosuppressed individuals in the short term. This is the case in documented transmission by transfusion in the United States. In recipients with healthy immune systems, transmission of *T. cruzi* by transfusion would be expected to manifest itself as organ damage decades after transfusion.

More recently Mexican donors were tested for *T. cruzi* and a look back was performed on the recipients of blood products from these donors. Seventeen recipients of blood from these infected donors were identified. Nine of them were still alive. Those nine were tested, and four were confirmed to have *T. cruzi* infection by RIPA. None were symptomatic for Chagas' disease. The authors state that this is evidence that occult transmission of *T. cruzi* by transfusion is indeed occurring in immunocompetent recipients [8].

Testing for *T. cruzi* is routine in many Latin American countries where the prevalence of infected donors is high. Cross reaction with Leishmania, malaria, syphilis, and collagen vascular disease can occur. There is now an approved test for screening blood donors infected with *T. cruzi* in the United States, but it has not yet been implemented. In the United States there are some areas where the Latin American population is greater than that in other areas, and thus the risk to the blood supply is greater. The availability of pathogen inactivation might be an attractive alternative to testing for pathogens for which the donor prevalence is variable in different regions.

We have demonstrated that treatment of plasma and platelets with riboflavin and ultraviolet light using the Mirasol PRT technology can reduce growth of *T. cruzi* by 5–7 logs in plasma and 7–8 logs in platelets with a starting concentration of 1

billion *T. cruzi* per 250 mL (4 million organisms per milliliter).

As a proof of concept it has been shown that there was a lack of ability to culture organism after treatment of blood products in which a very high number of starting organisms was used to infect units (10^9 organisms per 250 mL unit). A reduction of 5–8 logs demonstrates robust inactivation.

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