The effectiveness of riboflavin and ultraviolet light pathogen reduction technology in eliminating *Trypanosoma cruzi* from leukoreduced whole blood

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BACKGROUND: The parasitic Chagas disease is caused by the protozoan *Trypanosoma cruzi*, which is mainly transmitted by insect vectors. Other infection routes, both in endemic and in nonendemic areas, include organ and marrow transplantation, congenital transmission, and blood transfusion. Asymptomatic chronic chagasic individuals may have a low and transient parasitemia in peripheral blood and, consequently, they can unknowingly transmit the disease via blood transfusion. Riboflavin and ultraviolet (UV) light pathogen reduction is a method to reduce pathogen transfusion transmission risk based on damage to the pathogen nucleic acids.

STUDY DESIGN AND METHODS: In this study, we tested the effectiveness of this technology for the elimination of *T. cruzi* parasites in artificially contaminated whole blood units (WBUs) and thus for decreasing the risk of *T. cruzi* transfusion transmission. The contaminated WBUs were leukoreduced by filtration and treated with riboflavin and UV light. The level of pathogen reduction was quantified by a real-time polymerase chain reaction (qPCR) and a real-time reverse transcription–polymerase chain reaction (RT-qPCR) as a viability assay.

RESULTS: The RNA (cDNA) quantification of the parasites showed a more than 99% reduction of viable *T. cruzi* parasites after leukoreduction and a complete reduction (100%) after the riboflavin and UV light treatment.

CONCLUSION: Riboflavin and UV light treatment and leukoreduction used in conjunction appears to eliminate significant amounts of viable *T. cruzi* in whole blood. Both strategies could complement other blood bank measures already implemented to prevent the transmission of *T. cruzi* via blood transfusion.

Chagas disease, or American trypanosomiasis, is a parasitic disease caused by the flagellate protozoan *Trypanosoma cruzi*. Considered a neglected tropical disease or a disease of poverty, Chagas is endemic in 21 countries of the Americas. In endemic areas the parasite is mostly transmitted to humans and other mammals by the blood-sucking bugs of the subfamily Triatominae, when the infected feces of the vector are inoculated through a bite site or through an

ABBREVIATIONS: ENC = extraction negative control; PRT = pathogen reduction technology; qPCR = real-time polymerase chain reaction; RT-qPCR = real-time reverse transcription–polymerase chain reaction; ULR = universal leukoreduction; WBU(s) = whole blood unit(s).

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intact mucous membrane of the mammalian host.[1,2] However, in both endemic and nonendemic areas, *T. cruzi* can also be acquired through other routes, including blood transfusion,[3] organ and bone transplantation,[4] and congenital transmission from mother to child.[5]

Chagas disease has two clinical forms or phases: acute and chronic. All patients infected with *T. cruzi* enter the acute phase, characterized by flu-like symptoms that are often mild and may even go unnoticed. During the acute phase, a high number of parasites circulate in the blood. The subsequent chronic phase usually takes an indeterminate asymptomatic form in 70% to 80% of infected individuals, while in 20% to 30% it causes damage in the tissue of the heart, digestive system, and/or nervous system.[1,2] Due to a low and transient parasitemia in the peripheral blood of asymptomatic chronic individuals,[6,7] the infectious status can go undetected even decades after the infection, thereby introducing a risk of parasite transmission by blood transfusion.[3] Moreover, studies have shown that *T. cruzi* is able to survive under blood bank component storage conditions (4°C, 22°C), as well as in fresh-frozen plasma and cryopreserved blood components, making *T. cruzi* a potential threat for blood safety.[8,9]

In this study, we tested the ability of the riboflavin and UV light pathogen reduction technology (riboflavin-UV-PRT; Mirasol Pathogen Reduction Technology System, Terumo BCT), which prevents pathogen replication by irreversible nucleic acid damage, to reduce the viable and infectious *T. cruzi* load in leukoreduced whole blood and consequently lessen the risk of *T. cruzi* transfusion transmission.

It has been previously shown that riboflavin and UV light treatment can decrease *T. cruzi* growth in plasma and platelets (PLTs)[10] and in nonleukoreduced whole blood.[11] However, until now, the effectiveness of riboflavin-UV-PRT in eliminating *T. cruzi* from leukoreduced whole blood has not been demonstrated.

The evaluation was carried out in in vitro experiments in which *T. cruzi* epimastigotes were spiked into whole blood units (WBUs). The artificially contaminated blood products were leukoreduced by filtration and treated with the riboflavin-UV-PRT.

The combined effect of leukoreduction by filtration and riboflavin-UV-PRT treatment on the *T. cruzi* load in artificially spiked whole blood was investigated by applying two methods for parasite detection: a real-time polymerase chain reaction (qPCR) for DNA detection and a real-time reverse transcription–polymerase chain reaction (RT-qPCR) for RNA identification.

Since DNA can be detected after cell death, its presence does not distinguish between live and dead pathogens. As RNA degrades more rapidly than DNA after cell death, assays targeting RNA, such as RT-qPCR and nucleic acid sequence–based amplification, have been used to assess the viability and potential infectivity of microbial pathogens, including protozoa kinetoplastids.[12,13] Thus, in this study, in addition to a qPCR for DNA identification, an RT-qPCR for RNA detection was used as an indicator of the viability of these parasites after filtration and riboflavin-UV-PRT treatment.

MATERIALS AND METHODS

Blood selection

Two WBUs were obtained from healthy blood donors seronegative for Chagas disease, selected after a serologic study with two different enzyme-linked immunosorbent assays (Bio-ELISA Chagas assay, Biokit, Werfen Group; or Ortho Clinical Diagnostics). This study was approved by the ethical committees of the participating institutions, the Balearic Islands Ethics Committee, and the Research Ethics Committee of the University of Barcelona. Written informed consent was obtained from the participating donors.

Culture of *T. cruzi*

A culture of *T. cruzi* epimastigotes of the Maracay (strain provided by the Cryobank of the Universitat de Barcelona) in liver infusion tryptose medium was maintained at 28°C until the logarithmic growth phase. The culture was centrifuged at 1800 × *g* for 10 minutes; the pellet was suspended in the same medium and viable parasites were counted in a Rosenthal hemocytometer chamber with trypan blue dye. The stock prepared (1×10^6 parasites/mL) in liver infusion tryptose was equally divided to make two sets of parasite suspension. One set of parasites was used for live parasite studies and the other set was subjected to inactivation by exposure to 80°C for 30 minutes in a water bath to kill the parasites.

The experiment is outlined in Fig. 1. Whole blood infection was performed as follows: two WBUs obtained from two healthy blood donors, both seronegative for Chagas disease, were used for the study. Both WBUs were stored overnight at 22 to 24°C. At 18 hours after collection, the WBUs (537 mL) were experimentally infected using a sterile connection with 4.5 mL (1×10^6 parasites/mL) of the aforementioned *T. cruzi* stock, one WBU with live and the other with dead epimastigotes. The WBUs were leukoreduced before storage by filtration 1 hour after spiking (CompoFlow CQ 31451 with whole blood filter, Fresenius-Kabi AG). The treatment with riboflavin-UV-PRT was performed immediately after filtration according to the manufacturer's instructions:[12] the blood from the two leukoreduced WBUs was transferred to two illumination bags. After addition of 35 mL of riboflavin and removal of the residual air, the WB was gently mixed and a 20-mL sample was removed for testing and measurement of hematocrit (Hct) to calculate the UV illumination time according to the manufacturer's instructions. WBUs were

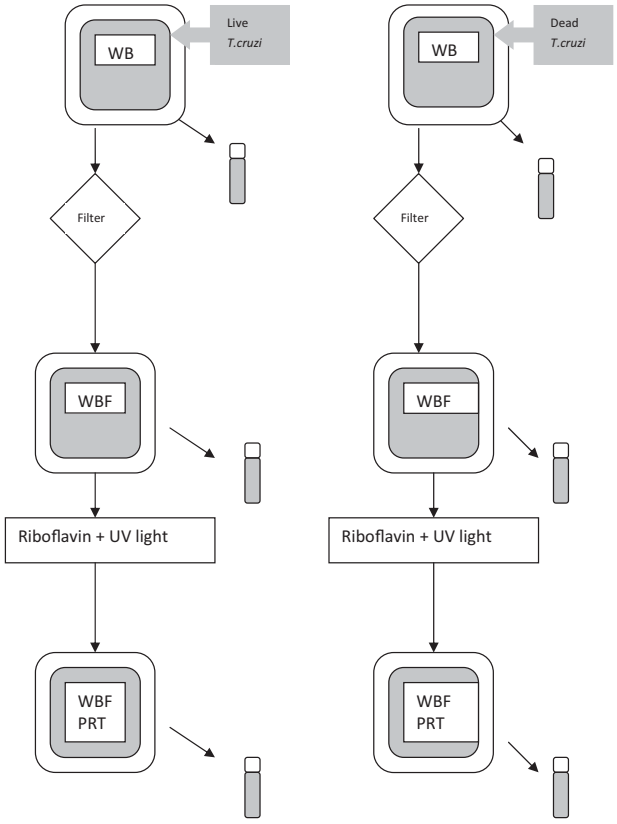

Fig. 1. Experiment design. A 20-mL sample was collected from the two WBUs—one with living and the other with dead epimastigotes—for DNA and RNA extraction after every step of the experiment: spiking, leukoreduction, and riboflavin-UV-PRT treatment.

illuminated with 80 J/mL of red blood cells (RBCs), of UV light (280-400 nm) for at least 45 minutes. An aliquot of 20 mL blood was kept for RNA and DNA extraction from each WBU, after every step of the experiment: spiking, leukoreduction, and riboflavin-UV-PRT treatment of the artificially infected WBUs, one with living and the other with dead epimastigotes.

DNA extraction

The DNA of the infected WBU, the infected and leukoreduced WBU, and the infected leukoreduced and riboflavin-UV-PRT–treated WBU was extracted in triplicate with a PCR template preparation kit (High Pure, Roche); eluted in 200 μL of elution buffer (10 mmol/L Tris-HCl, pH 8.5) according to the manufacturer's instructions; and stored at −20°C for qPCR analysis. For the extraction negative control (ENC), a blood sample was used without a template. To build the standard curve for the quantification of parasite loads, DNA from a culture of *T. cruzi* epimastigotes (Maracay strain, 1 10^5 parasites/mL) was extracted as described above.

RNA extraction

In parallel with DNA extraction, 200 μL of the same samples was submitted to RNA extraction in triplicate. The extraction was performed with an RNA isolation kit (RNeasy mini kit, Qiagen); DNase I was used in the protocol for removal of the genomic DNA and the RNA was eluted in 30 μL of water (RNase free) according to the manufacturer's instructions for the purification of total RNA of animal cells. The eluate was stored at −80°C for RT-qPCR analysis. For the ENC, a blood sample was used without a template. To build the standard curve for quantification of parasite loads, RNA from a culture of *T. cruzi* epimastigotes (Maracay strain, 1×10^5 parasites/mL) was extracted as described above.

qPCR

Five microliters of extracted DNA was amplified by qPCR in a thermocycler (LightCycler 480, Roche) in triplicate. The primers, probes, and conditions of the technique, which amplifies a fragment of 166 bp in the satellite DNA of *T. cruzi,* were performed as described by Piron and colleagues[13] with some modifications. Briefly, the following were used: Cruzi 1 and Cruzi 2 primers and a Cruzi 3 probe labeled with 6-carboxyfluorescein and a minor groove binder. The final concentrations in the PCR mixture were as follows: 1× high-performance, medium- to high-throughput PCR platform (LightCycler 480 Probes Master, Roche), 750 nmol/L of each *T. cruzi* primer, and 250 nmol/L of the *T. cruzi* probe in a 20-μL reaction volume. The amplification was run in 45 cycles and the annealing temperature was 58°C. A standard curve was constructed with 1-in-10 serial dilutions, in elution buffer (10 mmol/L Tris-HCl, pH 8.5), of total DNA extracted from the Maracay strain from 1×10^5 to 1 parasite/mL. Molecular biology-grade water (Sigma-Aldrich) and ENC were used as negative controls. The parasitic load of every sample was calculated using the qPCR software (LightCycler 480, Roche) by the second derivative maximum method.

RT-qPCR

Five microliters of RNA was submitted to RT before qPCR to obtain cDNA by a first-strand cDNA synthesis kit (Transcriptor, Roche). Random hexamer primer 600 pmol/μL was used in the reaction in a final volume of 15 μL. The reaction was carried out in a thermocycler (PTC-200, MJ Research). The cDNA obtained was stored at −40°C. Five microliters of extracted cDNA was amplified by qPCR in a thermocycler (LightCycler 480, Roche) in triplicate. The primers D71 and D72 described by Souto and Zingales[14] were used and the protocol and conditions were briefly as follows: the final concentrations in the PCR mixture were 1× high-performance reagent (SensiMix SYBR No-ROX kit, Bioline Reagents) and 750 nmol/L of each *T. cruzi* primer in a 20-μL reaction volume. The optimal cycling

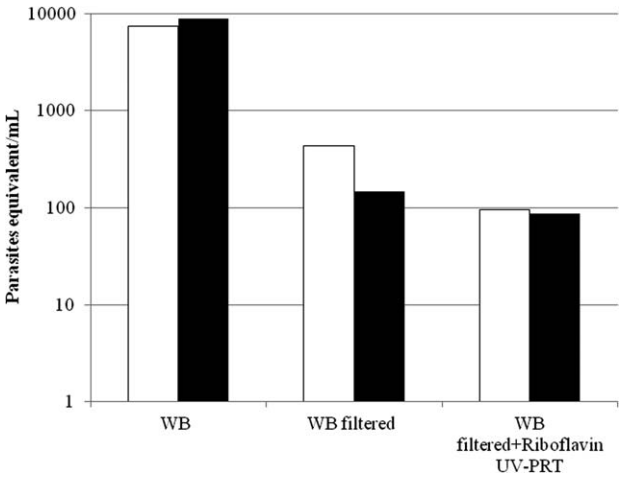

Fig. 2. Quantification of *T. cruzi* DNA (logarithmic values of parasite equivalents/mL) in the different WB samples of WBUs inoculated with live and dead parasites before and after leukoreduction by filtration and the riboflavin pathogen reduction system (riboflavin UV-PRT) procedures were applied. (□) WBUs inoculated with live parasites; (■) WBUs inoculated with dead parasites.

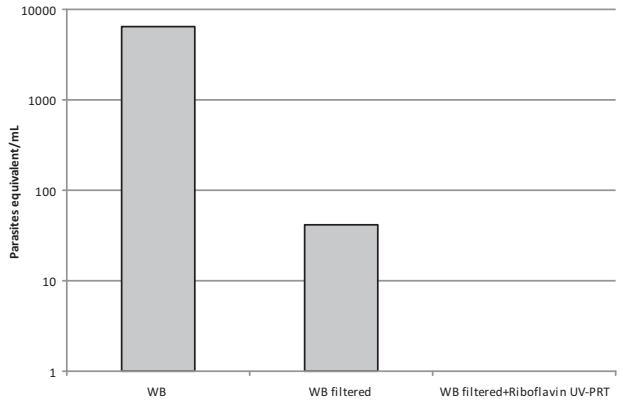

Fig. 3. Quantification of *T. cruzi* cDNA (logarithmic values of parasite equivalents/mL) in the different WB samples of WBUs inoculated with live parasites before and after leukoreduction by filtration and riboflavin pathogen reduction (riboflavin UV-PRT) procedures were applied. (▨) WBUs inoculated with live parasites.

conditions were a first step of 2 minutes at 50°C and 10 minutes at 95°C followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. A melting curve was done for 5 seconds at 95°C, 65°C for 1 minute, and 97°C continuous. A sample was considered positive when the quantification cycle was less than 35 and a melting temperature peak was between 79 and 80°C. A standard curve was constructed with 1-in-10 serial dilutions, in water (RNase free) of total RNA extracted from the Maracay strain from 1×10^5 to 1 parasite/mL. The parasitic load of every sample was calculated using LightCycler 480 software by the second derivative maximum method. Molecular biology-grade water (Sigma-Aldrich) and an ENC were used as a negative control. Also, to control the complete digestion of DNA by DNase I, the RNA was studied by qPCR as described. The quantification of cDNA of dead parasites was used as a control of the RT-qPCR technique for measuring parasite viability.

RESULTS

The parasitic load of the infected WBU (live parasites, mean ± SD parasite equivalent/mL, 7583.3 ± 481.7; dead parasites, 9052.2 ± 879.2), the infected and leukoreduced WBU, and the infected leukoreduced and riboflavin-UV-PRT–treated WBU was analyzed by qPCR; each value represents a mean of nine analyses and is expressed in parasite equivalents/mL of blood. The concentration of live and dead parasite DNA was similar in both infected WBUs, and both decreased by more than 94% when a leukoreduction filter was applied (436.1 ± 91.2 parasite equivalents/mL remained in the filtered WBU spiked with live parasites and 146.8 ± 12.9 parasite equivalents/mL remained in the filtered WBU spiked with dead parasites). After application of the riboflavin-UV-PRT to both filtered WBUs, the concentration of both live and dead parasite DNA was reduced by more than 98% (94.7 ± 21.9 parasite equivalents/mL remained in the riboflavin-UV-PRT-treated WBU spiked with live parasites and 86.9 ± 9.1 parasite equivalents/mL remained in the riboflavin-UV-PRT–treated WBU spiked with dead parasites). These values reflect the amount of DNA present in the samples and not necessarily the live parasites (Fig. 2). However, the cDNA quantification, measured by RT-qPCR, revealed that the concentration of viable parasites was reduced by more than 99.3% after leukoreduction (41 ± 21.1 parasite equivalents/mL remained in the filtered blood) and by 100% after the riboflavin-UV-PRT procedure (0 parasite equivalents/mL was detected). The quantification of cDNA of dead parasites allowed the technique to be controlled, and no RT-qPCR signal was observed (Fig. 3 and Table 1). No DNA contamination was observed in any of the RNA samples studied.

DISCUSSION

Several methods, including increasingly stringent donor selection criteria and donor screening, have been implemented over the years to reduce pathogen transfusion transmission risk. However, asymptomatic yet infectious blood donors continue to challenge blood transfusion safety. *T. cruzi*, the causal agent of Chagas disease, is a representative example of an emerging pathogen that is gaining importance in nonendemic countries, where one of the major transmission routes is via blood transfusion.[3]

TABLE 1. Quantification of *T. cruzi* by qPCR and RT-qPCR in the different WB samples of WBU inoculated with live and dead parasites, after every step of the experiment: spiking, filtration, and riboflavin-UV-PRT treatment

	WBU spiked with live parasites				WBU spiked with dead parasites*	
	qPCR for DNA detection		RT-qPCR for RNA identification		qPCR for DNA detection	
Results	Mean parasite equivalents/mL	% Reduction	Mean parasite equivalents/mL	% Reduction	Mean parasite equivalents/mL	% Reduction
WBUs	7583.3				9052.2	
Filtered WBUs	436.1	94.3	41	99.5	146.8	98.4
Filtered and PRT-treated WBUs	94.7	98.8	0	100	86.9	99.3

* The quantification of cDNA of dead *T. cruzi*, measured by RT-qPCR, showed no signal.

Human migration is a potential factor for increased Chagas disease risk in nonendemic areas, and the disease, no longer confined to Latin America, has become a global problem.[15] Strategies applied until now to prevent *T. cruzi* transfusion transmission have certain limitations: screening blood donors by risk factor questions for *T. cruzi* infection is vulnerable to intentional or inadvertent misreporting, and selective donor screening does not capture all incident cases (seroconversion) or new autochthonous cases.[16] Hence the importance of studying other strategies such as universal leukoreduction (ULR) and PRT, which may complement existing screening methods and therefore further minimize the possible risk of infections by blood transfusion.

ULR refers to the process of removing white blood cells (WBCs) from all units of whole blood, RBCs, or PLTs before storage to a standardized degree of purity, that is, down to 5 $\times$ 10^6 per unit as required by the AABB[17] or 1 $\times$ 10^6 according to European Union recommendations.[18] Leukoreduction is recognized as useful for the prevention of three complications arising from blood transfusion: febrile nonhemolytic transfusion reactions, PLT refractoriness due to HLA alloimmunization, and transmission of cytomegalovirus. Patients at risk of these complications have traditionally been provided with leukoreduced blood. Then in the late 1990s, accumulating evidence of transfusion-related immunomodulation, a potential effect of the transfused WBCs on the recipient's immune system, and the suggestion that leukoreduction might decrease the transmission of a new variant of Creutzfeldt-Jakob disease, led to the implementation of ULR in the United Kingdom, together with other European countries and Canada, in 1999.[19]

In addition to the aforementioned advantages of leukoreduction, the removal of WBCs can contribute to the elimination of several intracellular pathogens, including certain viruses (i.e., CMV, HHV-8, and HTLV-I/II), rickettsial species,[20,21] trypanosomatids such as *Leishmania,*[22-24] and the extracellular protozoan *T. cruzi*.[25] The removal of infected WBCs is not the only mechanism explaining the efficacy of leukoreduction filters in reducing the risk of protozoa transmission by transfusion. It has been proven that both intracellular[20-24,26] and extracellular[25] protozoan parasites can be retained by leukoreduction filters via several mechanisms. In the case of *Leishmania*, its life cycle involves two stages: an intracellular form or amastigote reproducing in macrophages and an extracellular form or promastigote, which is the initial form injected into the skin of the human host. It has been described that the amastigote form contained within monocytes is removed as WBCs are trapped within the filters, while free extracellular amastigotes and promastigotes are removed by adhering directly to the filter fibers.[25] Alternative mechanisms to explain parasite removal by leukoreduction filters have been proposed in other parasites, including the adherence of *Plasmodium falciparum*–infected RBCs to filters, PLTs, and other RBCs.[26]

In the case of *T. cruzi*, removal by blood leukoreduction has been described via direct adherence of the trypomastigotes to filter fibers.[25] Indeed, in this work we observed that the leukoreduction filters retained up to 99% of the live parasites in the infected blood, which would constitute an important reduction of the transmission risk of this extracellular parasite. Likewise, Moraes-Souza and colleagues[27] reported a reduction of 6 logs in *T. cruzi* concentration by means of leukoreduction filtration in experimentally infected blood. However, despite this considerable reduction, the infection was still transmitted to 30% to 50% of the animals infected with this filtered blood. Similarly, our study has demonstrated that even though filtration can substantially reduce the *T. cruzi* load in artificially spiked whole blood, it is not completely eliminated, because some of these parasites remained in the filtered blood. The most common blood component involved in Chagas disease blood transfusion transmission is PLT concentrates, including leukoreduced PLTs from donors with serologic evidence of Chagas infection.[3,7] This is something to consider, as the cases of transmission by transfusion reported in the literature involve chronic chagasic patients with a low parasite load, usually lower than 1 parasite equivalent/mL in peripheral blood.[6,7]

Riboflavin-UV-PRT is based on the ability of riboflavin to easily cross lipid membranes of the parasite and nonspecifically interacts with nucleic acids. Upon UV (265- 370 nm) light exposure, the riboflavin modifies the pathogen nucleic acids through the oxidation of guanine residues and production of reactive oxygen species to an extent that pathogens in blood can no longer replicate. This method benefits from the lack of DNA in RBCs and PLTs (components that are beneficial in transfusion) and its presence in parasites and other microorganisms.[28] Various studies, both in vivo and in vitro, have investigated the effectiveness of treating whole blood with riboflavin-UV-PRT to reduce infectivity of several intracellular parasites, such as *Babesia microti*,[12,29] *Plasmodium*,[30] and *Leishmania*,[31] and extracellular parasites such as *T. cruzi*.[10,11] Although leukoreduction filtration[25] or riboflavin and UV light treatment[10,11] have been studied separately for their efficacy in reducing the *T. cruzi* load in whole blood, to our knowledge, the combined effect of both methods on the transfusion transmission risk of *T. cruzi* has not been investigated until now.

Parasite viability methods such as hemocultures have been used to prove the effectiveness of reducing the infectious parasite by techniques like leukoreduction[21] and riboflavin-UV-PRT.[24] However, the well-known drawbacks associated with hemoculture, which is time-consuming and gives results with low sensitivity,[32,33] raises the question of what results might be obtained when applying more sensitive and efficient assays for parasite detection such as PCR.

Nevertheless, although PCR can detect and quantify parasitic DNA, it cannot differentiate between live and dead parasites, as shown in Fig. 2. Additionally, parasite genome amplification was not inhibited by PRT due to the small length of genome amplified (116 bp), as other authors have indicated.[34] Previously published data on the impact of whole blood riboflavin-UV-PRT treatment on the *Plasmodium* genome show that its amplification was inhibited by PRT in proportion to the length of genome amplified; that is, small amplicons are scarcely inhibited by PRT (577-, 317-, and 240-bp amplicons).[34] These data are compatible with the previous estimation that riboflavin-UV-PRT treatment causes a DNA adduct insertion event every 245 to 1850 bp in human WBCs.[35]

Therefore, the short length of genome amplified in our experiment (166 bp) was small enough to persist intact in nonviable cells, demonstrating that the qPCR used did not allow differentiation between live and dead parasites. This limitation can be resolved by the quantification of 24Sα ribosomal RNA specific to *T. cruzi* by RT-qPCR, which effectively detects and quantifies RNA of viable parasites. The viability of parasites other than *T. cruzi* (*Leishmania* spp.,[36] *Giardia lamblia*,[37] and *Plasmodium vivax*[38]) has been evaluated previously using the RT-qPCR technique.

Interestingly, despite the large number of organisms with which the WBUs were infected, we observed a complete elimination of the *T. cruzi* load in the samples taken from the infected WBUs by the combined use of leukoreduction filtration and riboflavin-UV-PRT. A far greater parasite load was used than would be expected in a unit collected from a donor in the asymptomatic chronic phase of Chagas disease, which is characterized by an intermittent circulation and low burden of *T. cruzi*.[6,7] However, it is known that parasite loads of newly infected children range from 0.01 to 640 parasites/mL,[5] values that could be similar to those in newly infected adults. This very high amount of parasite may not be completely eliminated by filtration but viable parasites could certainly be eliminated by subsequent riboflavin-UV-PRT treatment.

In conclusion, although the currently implemented donor screening measures such as assessment of donor risk through questionnaires and selective testing may significantly lower the incidence of transfusion transmission of *T. cruzi*, the risk of donating blood products with infectious parasites remains. While leukoreduction by filtration lessens the risk, without eliminating it, the riboflavin-UV-PRT treatment could entirely eradicate viable parasites remaining in the filtered WBUs, as has been proven in vitro by this study and the cases of *T. cruzi* transmission by leukoreduced blood component transfusion.[3] In addition, while such cases are rare, the selective serologic testing approach presently in use in some endemic and nonendemic countries may fail to detect newly infected donors with high parasitemia levels insufficiently removed by leukoreduction filters. Although this is a preliminary study and further investigations are needed, the data presented here show that riboflavin-UV-PRT in conjunction with leukoreduction filtration can eliminate significant amounts of *T. cruzi* from whole blood and could complement other blood bank measures already implemented to prevent the risk of *T. cruzi* transfusion transmission. Additionally, this approach would avoid the need for multiple individual screening tests for other agents such as *Plasmodium* spp.[30] and *Leishmania* spp.,[31] also prevalent in Chagas endemic areas.

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CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

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