INACTIVATION OF PLASMODIUM FALCIPARUM
in whole body by riboflavin plus irradiation

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ABBREVIATIONS
qPCR = real-time polymerase chain reaction.

ABSTRACT

BACKGROUND
Malaria parasites are frequently trans- mitted by unscreened blood transfusions in Africa. Pathogen reduction methods in whole blood would thus greatly improve blood safety. We aimed to determine the efficacy of riboflavin plus irradiation for treatment of whole blood infected with *Plasmodium falciparum*.

STUDY DESIGN AND METHODS
Blood was inocu- lated with 10^4 or 10^5 parasites/mL and riboflavin treated with or without ultraviolet (UV) irradiation (40-160 J/mL red blood cells [mL_{RBC}s]). Parasite genome integrity was assessed by quantitative amplification inhibition assays, and *P. falciparum* viability was monitored in vitro.

RESULTS
Riboflavin alone did not affect parasite genome integrity or parasite viability. Application of UV after riboflavin treatment disrupted parasite genome integrity, reducing polymerase-dependent amplification by up to 2 logs (99%). At 80 J/mL_{RBC}s, riboflavin plus irradiation prevented recovery of viable parasites in vitro for 2 weeks, whereas untreated controls typically recovered to approximately 2% parasitemia after 4 days of in vitro culture. Exposure of blood to 160 J/mL_{RBC}s was not associated with significant hemolysis.
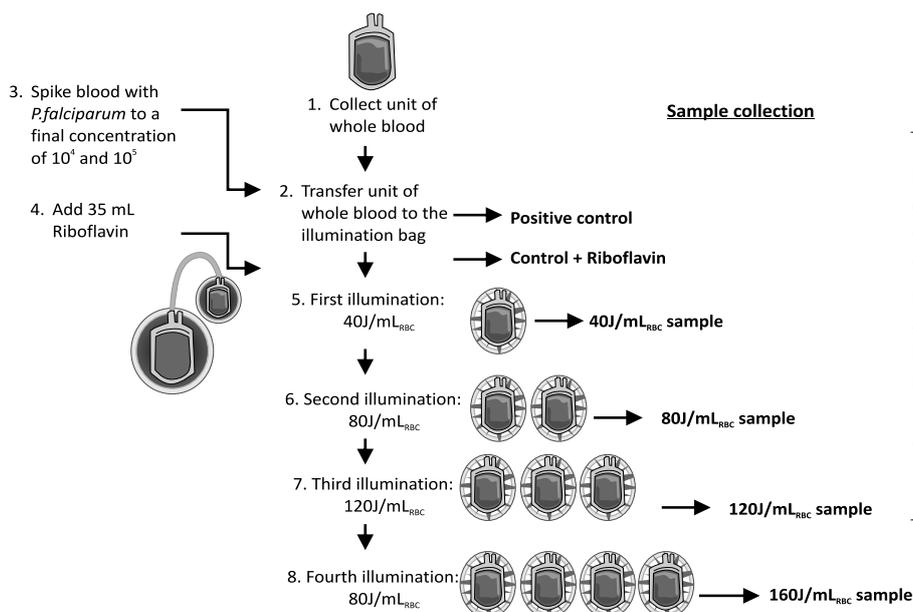
CONCLUSIONS
Riboflavin plus irradiation treatment of whole blood damages parasite genomes and drastically reduces *P. falciparum* viability in vitro. In the absence of suitable malaria screening assays, parasite inactivation should be investigated for prevention of transfusion-transmitted malaria in highly endemic areas.

Malaria is a major disease accounting for a high number of deaths annually in tropical regions of sub-Saharan Africa, Asia, and Latin America.[1-3]
In Africa, most severe malaria and deaths occur in children younger than 5 years and in pregnant women.[4,5] Malaria cases also occur in nonendemic areas as a result of travel from malaria endemic countries. Six species in the genus *Plasmodium* are identified as human malaria parasites: *P. falciparum*, *P. vivax*, *P. ovale curtisi*, *P. ovale wallikeri*, *P. malariae*, and *P. knowlesi*.

Infections with *P. falciparum* can be much more severe than those caused by other species, with complications including cerebral malaria, severe anemia, respiratory distress, and renal failure. Malaria is transmitted by mosquitoes of the genus *Anopheles*, but may also be transmitted person to person through direct inoculation of infected blood due to needle sharing among drug users, organ transplantation, and blood transfusion.[6,7]

3. Spike blood with *P.falciparum* to a final concentration of 10^4 and 10^5

4. Add 35 mL Riboflavin

Sample collection

1. Collect unit of whole blood

2. Transfer unit of whole blood to the illumination bag → **Positive control**

→ **Control + Riboflavin**

5. First illumination: $40J/mL_{RBC}$ → **$40J/mL_{RBC}$ sample**

6. Second illumination: $80J/mL_{RBC}$ → **$80J/mL_{RBC}$ sample**

7. Third illumination: $120J/mL_{RBC}$ → **$120J/mL_{RBC}$ sample**

8. Fourth illumination: $80J/mL_{RBC}$ → **$160J/mL_{RBC}$ sample**

FIGURE 1. **Experimental design.**
One unit of whole blood was inoculated with *P. falciparum*. A control sample was collected before and after adding riboflavin, before UV exposure. Four additional samples were collected after illumination at 40, 80, 120 and J/mL_{RBC}s, respectively. All collected samples were tested for inhibition by qPCR, and an aliquot was returned to in vitro culture for viability testing.

Although recommended by the World Health Organization (WHO), blood testing for malaria parasites is rarely implemented in developing countries because microscopy has low sensitivity and takes too long to be useful for high-throughput screening.[8]
Other assays detecting parasite antigens or nucleic acids either lack sensitivity or are too expensive, given the constraints of an already short blood supply.[8] Inactivation of *Plasmodium* in whole blood units, before transfusion, would be much more suitable in endemic countries provided that it was effective and affordable. The riboflavin plus irradiation pathogen reduction system uses a combination of riboflavin, a non- toxic substance (vitamin B2), and ultraviolet (UV) irradiation to induce damage in nucleic acid-containing infectious agents.[9] The system is known to inactivate bacteria, viruses, and the protozoan *Babesia* in platelets (PLTs) and plasma,[10-12] but has not been proven as a pathogen inactivation strategy in whole blood. In this study, whole blood units spiked with *P. falciparum* were treated with the riboflavin plus irradiation system. Parasite inactivation was quantified by measuring amplification inhibition of *P. falciparum* genomic sequences and by assessing *P. falciparum* viability in culture.

MATERIALS AND METHODS
Parasite culture and enrichment
Asexual blood stage parasites of *P. falciparum* Clone 3D7 were cultured in human A+ blood and complete medium according to established protocols,[13,14] except that human serum was replaced by bovine serum albumin (Albumax II, Sigma, Gillingham, UK) to a final concentration of 50%. Cultures were incubated at 37°C under a gas phase of 3% CO2/1% O2/96% N2 with daily media changes. Ring- enriched culture at 7% hematocrit (Hct) with parasitemia between 5 and 9% were prepared for initial experiments. To obtain higher levels of parasitemia, magnet-activated cell sorting (MACS, Miltenyi BioTec, Bergisch Gladbach, Germany) was used to enrich and synchronize mixed stage asexual parasites through a 21-gauge flow resistor as previously described.[13,15] Late-stage parasites retained in the column were collected into a 50-mL tube, pelleted by centrifugation as above, and returned to culture with fresh blood and medium. These late stages were incubated with shaking overnight, so that ring-stage trophozoites were present next day, typically with a parasitemia of 10% to 14%. All parasite preparations were sent at room temperature to the Department of Haematology, University of Cambridge, by courier.

Treatment with the riboflavin plus irradiation system
Blood units (blood group A+) were obtained from the German Red Cross blood service in Frankfurt, Germany, by courtesy of Dr M. Schmidt. Before treatment, the blood units were equilibrated at 37°C and transferred into the illumination bag using a tubing welder (TerumoBCT, Denver, CO). Infected red blood cells (RBCs) resuspended in RPMI, brought by courier from London, were pelleted by centrifugation (600 ¥ g for 5 min). The pellet was resus- pended in 20 mL of whole blood withdrawn from the initial blood unit. After being mixed, the infected blood was spiked into the respective blood units at a final con- centration of 104 or 105 *P. falciparum* parasites/mL. A quantity of 35 mL of riboflavin was added to the illumination bag, which was irradiated using the riboflavin plus irradiation UV system at 40, 80, 120, and 160 J/mL_{RBC}s. Samples were collected at four different time points, (summarized in Fig.1). Ten-milliliter samples were sent by courier to the London School of Hygiene and Tropical Medicine at room temperature for approximately 2 hours for viability testing.

Subculturing and monitoring of treated parasites
Aliquots of riboflavin plus irradiation-treated parasite-infected blood units, and uninfected control units were handled simultaneously and transported from Cambridge to the Category 3 laboratory at London School of Hygiene and Tropical Medicine by courier, typically in volumes of 10 to 20 mL per treatment, and were returned to in vitro culture. After two pilot experiments in which evidence of growth retardation in riboflavin plus irradiation-treated samples was observed, the following procedure was adopted. Treated material was centrifuged to remove residual white blood cells (WBCs) and washed once in RPMI, and then 2 mL of RBCs was removed into 25 mL of fresh complete culture medium in a flat-bottomed vent-capped flask and supplemented with 0.5 mL of uninfected RBCs freshly washed in RPMI without serum. These "recovery" cultures were monitored for parasite growth at 48-hour intervals by blood slide microscopy for at least 14 days, and up to 24 days in some experiments, or until parasitemia reached 2%. In five of the six experiments, the volume of fresh medium added every 48 hours was increased in 2-mL increments; RBCs were also added to maintain Hct if needed.

P. falciparum genomic amplification inhibition

For the preamplification inhibition and real-time polymerase chain reaction (qPCR) assay, DNA was extracted from 500 mL of whole blood using the a viral nucleic acid kit (High Pure, Roche Diagnostics, Burgess Hill, UK) in accordance with the manufacturer's instructions. The principles of the assays have been previously reported.[16]

In brief, riboflavin plus UV irradiation randomly damages the nucleic acids by forming adducts or breakage at intervals depending on concentration of riboflavin and intensity of irradiation. It is hypothesized that these damages would affect amplification by PCR proportionally to the length of amplicons targeted: the longer the higher likelihood of amplification inhibition. Quantitative estimation of genomic replication inhibition by riboflavin plus irradiation treatment was derived as previously described for treatment of virus-infected blood products.[16] Suitable targets for the assay were identified in the 18S rRNA genes and the mitochondrial genome of *P. falciparum*. For 18S rRNA target, four nested genomic amplicons were amplified: 1654, 1092, 676, and 317 bp (Fig. 2). A 50-mL PCR mixture was prepared containing 1¥ NH$_4$ buffer, 4 mmol/L MgCl$_2$, 0.8 mmol/L dNTPs, 0.4 mmol/L of each primer, and 5 U of DNA polymerase (BIOTAQ, Bioline, London, UK). Five microliters of template DNA, estimated to comprise 10^4 to 10^5 copies/mL, was used for each reaction. Primer sequences and PCR conditions for the initial template preamplification step are given in Table 1. The 1654-bp PCR procedure, after an initial incubation for 5 minutes at 94°C, 10 cycles of touchdown PCR of 30 seconds at 94°C, 45 seconds at 65 to 55°C, and 3 minutes at 72°C were followed by nine cycles of 30 seconds at 94°C, 45 seconds at 55°C, and 3 minutes at 72°C. For PCR generating a 1092-bp amplicon, 20 cycles of 30 seconds at 94°C, 45 seconds at 55°C, and 2.5 minutes at 72°C, and for those generating 676- and 317-bp amplicons, 17 cycles of 30 seconds at 94°C, 45 seconds at 55°C, and 2 minutes at 72°C were performed. All PCR procedures were followed by an incubation for 7 minutes at 72°C.

For the mitochondrial genes, primers were designed to five nested regions including portions of the *cox1* and *cytb* genes, generating amplicons of 2316, 1308, 934, 577, 240, and 134 bp in length. Specific primers and a single probe were designed to quantify each amplicon from the two genes (Fig. 2). For the 2316-bp PCR procedure, after an initial incubation for 5 minutes at 94°C, 20 cycles of touchdown PCR of 30 seconds at 94°C, 45 seconds at 60 to 50°C, and 2 minutes at 68°C were performed. For PCR generating 1308-bp amplicon, 12 cycles of touchdown of 30 seconds at 94°C, 45 seconds at 60 to 50°C, and 1 minute at 68°C, and for PCR generating a 934-bp amplicon, 11 cycles of touchdown of 30 seconds at 94°C, 45 seconds at 60 to 50°C, and 1 minute at 68°C were performed. For those generating 577- and 240-bp amplicons, 10 cycles of 30 seconds at 94°C, 45 seconds at 50°C, and 30 seconds at 68°C were performed. All PCR procedures were followed by an incubation for 7 minutes at 68°C.

Quantification of preamplified products

Preamplified products were quantified by qPCR using a multiplex qPCR system (MX3000, Stratagene, La Jolla, CA). Primers and probes (5' labeled with Cy5 and 3' labeled with BHQ2) for qPCR of both 18S rRNA and mitochondrial gene, and amplification cycling conditions, are given in Table 1. Amplification was performed in duplicate using a PCR kit (Brilliant III Ultra-Fast Q, Stratagene) according to the manufacturer's instruction. The small nonpreamplified qPCR was used as reference and log amplification inhibition of preamplified amplicon was determined as previously described.[16]
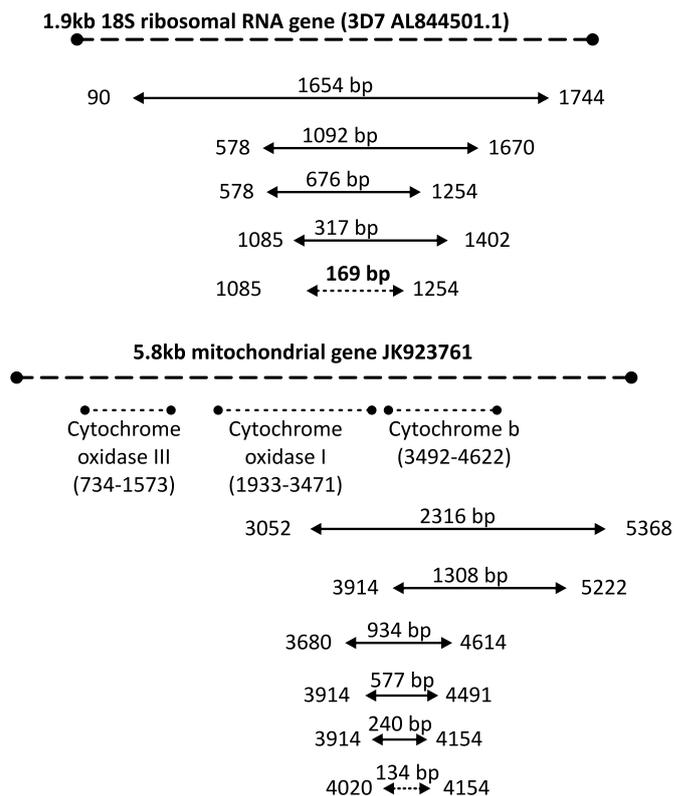

FIGURE 2. Primers and probes design for the preamplification of *P. falciparum* genome. Two conserved regions of *Plasmodium* genome were used to design primers and probes; the 18S rRNA gene and the mitochondrial gene. Four overlapping regions of 18S rRNA were amplified (1654, 1092, 676, and 317 bp) and quantified by qPCR of a region common to all segments (169 bp). For the mitochondrial gene, five overlapping regions were amplified. Each primary amplicon was then quantified by qPCR using common primers and probe (134 bp).

Statistical analysis

Correlation between continuous variables was evaluated by calculating Spearman's rho and associated significance. Associations between binary categorical variables were explored by estimation of odds ratios (ORs), and significance was tested using the chi-squared distribution. All statistical analysis was performed in a software package (STATA, Version 10, StataCorp, College Station, TX).

RESULTS

Optimization of the *Plasmodium* amplification inhibition assay

The number of preamplification cycles of each amplicon was optimized against the *P. falciparum* nucleic acid test standard[17] to generate similar amount of final PCR product followed by qPCR measurement using the reference short qPCR amplicon (169 bp for the 18S rRNA and 134 bp for the mitochondrial gene) irrespective of preamplified amplicon length. The qPCR standard curve for each amplicon length for the same gene reproducibly overlapped, hence demonstrating accurate normalization of the preamplification conditions (Fig. 3). A 4-log range of preamplification for 18S rRNA gene and 3 log for the mitochondrial gene compared with the baseline control (serially diluted sample quantified samples without a preamplification step) was obtained.

TABLE 1. Primers and probes for quantification of P. falciparum 18S rRNA gene and mitochondrial genome amplicons

| Name	Sequence 5'-3'	Amplicon size (bp)
18S rRNA gene amplicons		
Plasmo 1 (F)	GTTAAGGGAGTGAAGACGATCAGA	169
Plasmo 2 (R)	AACCCAAAGACTTTGATTTCTCATAA	
Malprobe	(Cy5) ACCGTCGTAATCTTAACCATAAACTATACCGACTAG (BHQ2)	
Mitochondrial genome amplicons		
cyt 135 (F)	CCATTTATTGGATTATGTATTGTATTTATAC	135
cyt 240 (R)	CCTTTAACATCAAGACTTAATAGATTTGGA	
Probe cyt 1	(Cy5) TACATTTACATGGTAGCACAAATCCTTTAGGGTATGA (BHQ2)	
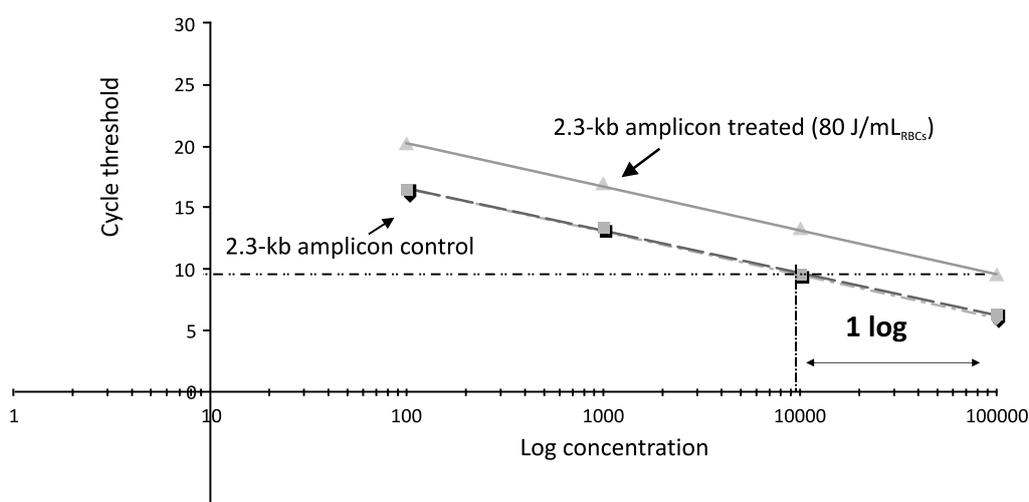
FIGURE 3. Quantification of log inhibition of P. falciparum genome amplification. Example of log inhibition quantification comparing before and after riboflavin plus irradiation treatment. A whole blood unit was treated with riboflavin and UV and results were com- pared to an untreated control unit. Three samples of parasite-infected whole blood were tested for amplification of the 2.3-kb mitochondrial genome target: the first received no treatment (◆), the second riboflavin only (■), and the third was treated with riboflavin and UV (80 J/mL; ▲). All samples were preamplified and quantified by qPCR. The sample treated with riboflavin and UV showed 1-log inhibition when compared with both non–UV-treated samples.

P. falciparum amplification inhibition
with riboflavin plus irradiation treatment of whole blood

For the measurement of PCR inhibition by riboflavin plus irradiation, 25 paired blood units spiked with *P. falciparum* asexual parasites were treated or not treated and relative genome inhibition was measured by the qPCR method described above. The log inhibition after treatment was estimated by comparing the C_t of four log dilutions before and after treatment (Fig. 3) in all amplicons. There was no detectable amplification inhibition when riboflavin was added without UV treatment. We explored two dose-response relationships in these data. First, as shown in Fig. 4A, log inhibition increased with increasing amplicon size when both riboflavin and UV illumination were present. With the smaller amplicons (577, 317, and 240 bp), inhibition was less than 0.7 log. The observed level of inhibition was greater with longer amplicons, but this relationship reached a plateau at 676 and 935 bp for 18S and mitochondrial targets, respectively, when treated with 80 J/mL$_{RBCs}$ (1-log inhibition). Inhibition continued to increase with longer amplicons when infected blood was treated at 160 J/mL$_{RBC}$s reaching 2-log inhibition with both targeted regions (Figs. 4A and 4B). Second, inhibition of genome amplification was also positively associated with increased illumination energy. Amplification of parasite genomic targets was inhibited by approximately 1 log at 80 J/m$_{LRBCs}$ and by 2 logs at 120 J/mL$_{RBCs}$, reaching a plateau above that level (Fig. 4C). When results with the two genes were compared, no significant difference in the estimates of genome amplification inhibition was observed. These results confirm that the combination of riboflavin and irradiation generate measurable DNA damage to malaria parasites in whole blood.

In vitro viability testing

To test whether *P. falciparum* viability was impaired by the observed genome damage inflicted by riboflavin and irradiation we performed studies of posttreatment parasite viability in in vitro culture. Two pilot experiments were performed to establish appropriate starting parasitemia and growth monitoring procedures. Using starting parasitemia in whole blood of 8.9 ¥ 10^4 and 1.2 ¥ 10^5 parasites/ mL, respectively, parasites in untreated blood were detected by Day 4 in both experiments and recovered to 2% parasitemia by Days 11 and 7, respectively. In contrast, addition of riboflavin and irradiation for 80 J/mL$_{RBCs}$ delayed full parasite recovery to Days 13 and 15, respectively, whereas riboflavin with 160 J/mL$_{RBCs}$ of irradiation delayed parasite recovery to Day 17 in the first experiment and prevented parasite recovery in the second.
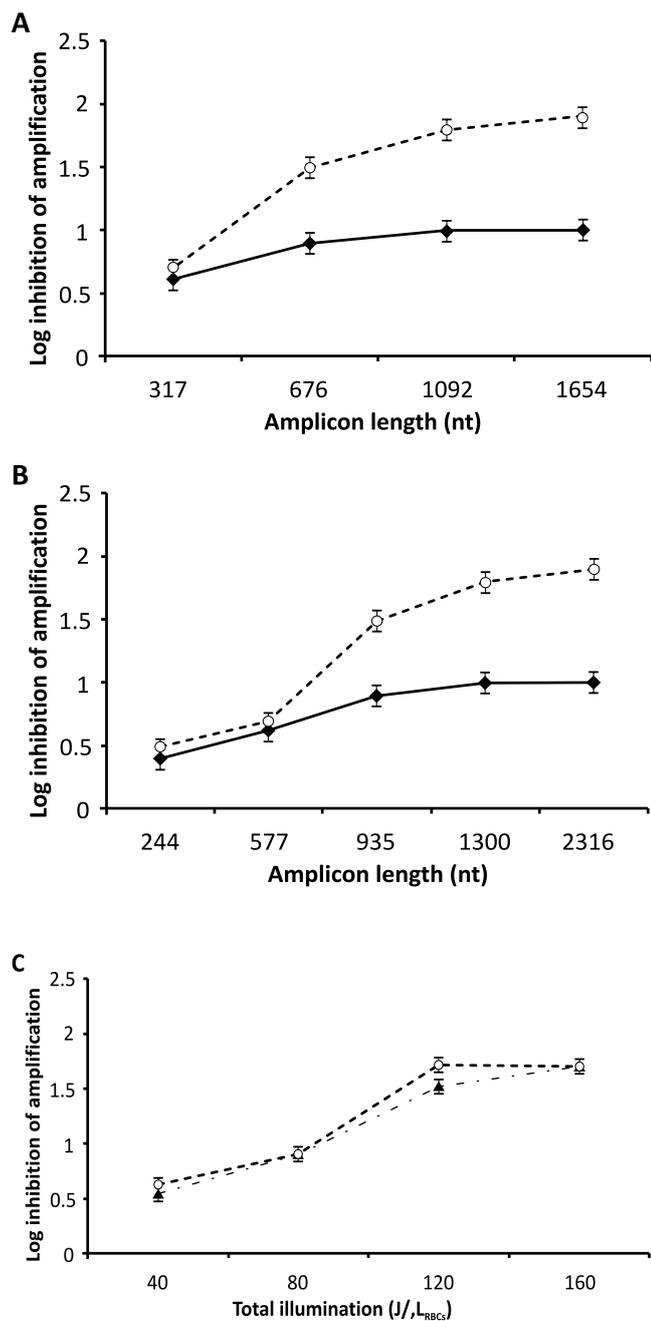
A

B

C

FIGURE 4. Log inhibition of amplification of different lengths of mitochondrial DNA and rRNA with riboflavin plus irradiation at 80 (◆) and 160 (O) J/cm$_{RBCs}$. (A and B) Impact of riboflavin and UV treatment on plasmodium genes: 18S rRNA and mitochondrial genes, respectively. Inhibition was measured using four amplicons for the 18S rRNA and five amplicons for the mitochondrial gene, at two different exposure levels: 80 (◆) and 160 (O) J/mL$_{RBCs}$. (C) Summary of the effect of UV exposure on two P. falciparum genes, using the longest target amplicons for each, at increasing level of UV exposure: 40, 80, 120, and 160 J/mL$_{RBCs}$. (▲) Mitochondrial gene; (O) 18S rRNA

To measure the effect of treatment and investigate whether there was a dose–response relationship between irradiation dose and parasite growth inhibition, a series of six experiments was performed, each in duplicate. Table 2 sets out the different treatments tested and notes minor variations among experiments. In one duplicate each from Experiments 1, 3, and 4, control cultures, which were not treated with riboflavin and received no irradiation, failed to recover to 2% parasitemia. One of these odd cases was due to an error of culture dispatch and the other two to bacterial contamination overgrowing the parasite; all data from these experiments were excluded from the analysis. Results from all cultures left a total of nine replicate experiments, each of which included two control cultures. Irradiation treatments of 80 and 160 J/mL$_{RBC}$s were also included in each of these nine experiments, which altogether provided a total of 46 evaluable cultures. There was no significant correlation between spiking parasitemia and day of recovery to 2% parasitemia in either the 80 or the 160 J/mL$_{RBC}$s irradiation groups (n = 9 in both cases; Spearman rho = 0.017, p = 0.965; and rho = -0.567, p = 0.112, respectively). We therefore pooled identical treatments across experiments for the remaining analyses.

To easily compare the rate of parasite recovery among the 46 cultures, four categories were generated from the data. The first category represents the fastest growing quartile of culture growth (recovery to 2% parasitemia by Day 8 or sooner), the second category represented the interquartile group (recovery between Day 9 and Day 18), the third category the slowest quartile (recovery between Day 19 and Day 24), and Category 4 those cultures in which no growth was observed. Figure 5 shows the proportion of cultures in each of these recovery categories, grouped by irradiation exposure. A clear dose-response relationship is observed, with all 22 cultures receiving riboflavin plus at least 80 J/mL$_{RBC}$s of irradiation showing either substantially delayed parasite growth recovery or no growth at all. Indeed, for these 22 cultures there was a very strong likelihood of complete inhibition of parasite growth compared to the other 24 cultures (OR, 27.6; 95% confidence interval, 3.07-1230; p = 0.0002). The occasional discrepancy between level of spiking and inactivation of parasite was related to the relatively small difference in parasitemia after spiking that was not different. In vivo, when examined microscopically, the range of parasite density is approximately 5 logs starting at five parasites/mL of blood.

DISCUSSION

Photochemical pathogen reduction for parasites in human blood and blood products has been examined using several compounds such as Inactine, amotosalen, and riboflavin and successfully demonstrated for L*eishmania donovani, Trypanosoma cruzi,* and *Plasmodium*.[18-21]   However, except for *T. cruzi*[19] and *Babesia microti,*[20] these experiments have been conducted in PLT concentrates or fresh frozen plasma, rather than whole blood, as poor UV light absorption considerably limits the potential for photochemical inactivation of pathogens in the presence of RBCs.[19,21,22] Previous studies of *Plasmodium* have measured efficacy of photochemical inactivation in either animals such as hamsters[18] or in fractionated blood products spiked with cultured *P. falciparum*.[19]

This study has significantly extended this approach by deployment of both a quantitative molecular assay of genome damage and parasite viability testing after photochemical treatment of whole human blood infected with *P. falciparum*.
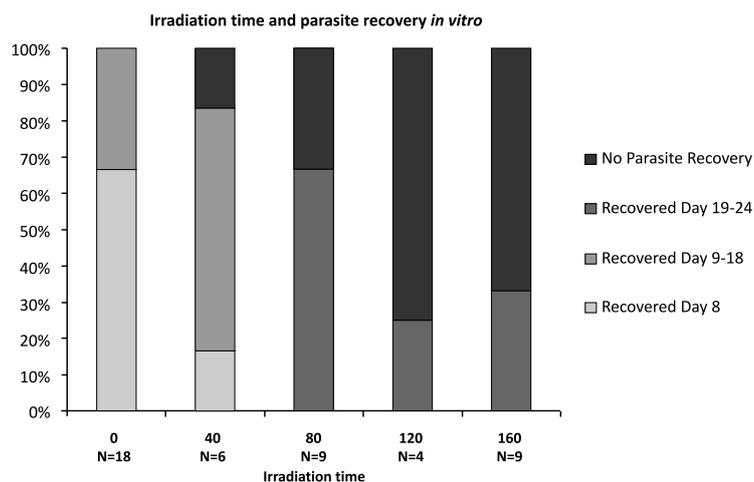
Irradiation time and parasite recovery *in vitro*

FIGURE 5. Relationship between duration of UV irradiation and time taken for recovery to 2% parasitemia in riboflavin plus irradiation–treated cultures of P. *falciparum*. Forty-six different parasite cultures contribute to the data presented. Recovery cat- egories were derived as described in the text. Y-axis depicts the proportion of the total number of cultures in each test category. Nine of the cultures receiving 0 minutes irradiation did not have riboflavin added to the treated blood; the other nine cultures were supplemented with riboflavin.

TABLE 2. Summary of *P. falciparum* viability testing experiments*

Experiment	Parasitemia treated (¥10^5 parasites/mL-1)	No treatment	Riboflavin (J/mL$_{RBC}$s)				
			Alone	40	80	120	160
1 (C)	1.71	X†	X‡		X		X
2 (D)	1.25; 1.87	X	X		X		X
3 (E)	2.19	X§	X	X	X	X	X
4 (F)	1.75	X	X	X‖	X	X‖	X‖
5 (G)	2.80; 3.75	X	X	X	X		X
6 (J¶)	2.50; 2.50	X	X	X	X	X	X

* Deviations from the standard protocol are indicated in the footnotes. All experiments were performed in duplicate unless indicated.
† X = treatment was included in this experiment.
‡ Only a single culture treated with riboflavin without irradiation was tested.
§ Only a single untreated control culture was received for testing.
‖ One replicate failed due to bacterial contamination.
¶ Lower culture volumes used in this experiment only.

We deployed a specifically designed quantitative molecular assay based on the rationale that damage inflicted upon the parasite genome through adducts or breakage should be reflected by an inhibition of amplification which increased with the length of the amplicons targeted.[16] As expected, it was found that the odds of a given strand of *P. falciparum* DNA being damaged increased with its length (Fig. 2) and the nested set of amplicons of different length provided good sensitivity for detection of genome damage under various conditions. We provide strong evidence of parasite genome amplification inhibition and showed dose–response relationships between the degree of genome damage and both amplicon length and total illumination energy. These data are compatible with the previous estimation that riboflavin plus irradiation treatment causes a DNA adduct insertion event every 245 to 1850 bp in human WBCs.[23] Similarly, in Jurkat lymphocytes or bacteria, the percentage of DNA strands with damage increases with illumination energy, such that 90% of strands are damaged at 20 J/cm² for bacteria.[24] In the data presented here, Plasmodium proliferation was reduced but not completely ablated except at the highest levels of energy used (120 and 160 J/cm²) while for WBCs, complete inactivation was achieved at these energy levels.[22,25] This difference might be related to the smaller size of the *Plasmodium* genome compared to lymphocytes and its lower degree of biologic complexity. Incomplete inactivation of *Plasmodium* in PLT preparations has previously been reported for another photochemical inactivation system (amotosalen).[23]

It is difficult to compare the molecular and functional methods used in this work to determine the potential efficacy of riboflavin plus illumination inactivation process. The two are more complementary than comparable. The amplification inhibition approach clearly provides a molecular explanation for the functional culture results. The 1- to 2-log inhibition reflects the extent of the damage inflicted to the parasite genome but the complete blockade of parasite growth in vitro with 160 J/cm² suggests 4- to 5-log functional inhibition. The apparent correlation between the two methods suggests a relation between them but extrapolating a direct relationship would require a considerable amount of experimental repetitions.

The results presented here suggest that a higher level of energy is required to inactivate malaria parasites in whole blood than the 80 J/mL$_{RBCs}$ shown to effectively inactivate mononuclear cells and bacteria. Irradiation of 120 J/mL$_{RBCs}$ would provide maximum *P. falciparum* inactivation according to the data presented here. However, such higher energy might increase the risk of damage to RBCs and be detrimental to their functionality after transfusion. Crude measurement of hemoglobin levels in plasma before and after riboflavin plus irradiation treatment did not show significantly different levels of RBC lysis,[26] but further investigation of the viability of RBCs after treatment is warranted. *P. falciparum* in whole blood treated with riboflavin but without exposure to UV light grew normally and showed no evidence of genome damage, suggesting that although riboflavin may attach to the DNA it does not form adducts at high frequency without exposure to UV light.

One weakness in our study design is that, due to the large number of parallel parasite cultures being generated, we did not also test the effect on parasite viability of irradiation alone, without riboflavin addition. This should be explored in future work. The amplification inhibition methods developed here, particularly assays of the longest amplicons from the 16S RNA gene, would provide a sensitive and rapid efficacy endpoint for future clinical trials of photochemical inactivation in blood donations in malaria endemic settings.

Blood in endemic countries is usually not tested for malaria before transfusion[3] and many of the diagnostic tests available, including microscopy and rapid antigen detection tests, are not sensitive enough to detect low levels of parasites that will still present a threat to blood recipients.[7,27] In addition, should a more sensitive PCR assay be used for screening, a high prevalence of sub-patent parasitemia is likely to be detected in endemic countries. This would cause deferral of a substantial percentage of units, which would threaten maintenance of a lifesaving supply of blood, which is already in shortage in most African countries.[6,27] Whole blood is the most frequently indicated and utilized blood product in Africa for treatment of massive bleeding in obstetrics and surgery.[28]

In such emergency circumstances, lack of transfusion is a clear cause of mortality[29] that depends not only on the immediate availability of blood but also on its freshness and on the retention of essential clotting factors. The second most frequent indication for transfusion is acute malaria, particularly in children.[30] Although WHO still indicates whole blood as adequate therapy in such circumstances, clinicians are likely prescribing concentrated RBCs to limit circulatory overload.[31] Preliminary data indicate that safe RBCs can be prepared following photo-chemical treatment of whole blood to inactivate viruses,[9] bacteria,[10] *T. cruzi,*[19] *Leishmania,*[32] and WBCs.[9,10] The data presented in this study show that *Plasmodium* inactivation can be added to this list, offering a possible solution to transfusion-transmitted malaria, an often neglected transfusion side effect.[3]

However, one weakness of our study was that an irradiation alone control was not tested here. Alternative strategies previously deployed in the past include presumptive addition of antimalarial drugs to whole blood units, although current combination drugs, which are effective against chloroquine-resistant parasites, such as artemether-lumefantrine and artesunate-amodiaquine would require administration directly into blood units of the active metabolite of each component, rather than the parent drugs, rendering this option expensive. It would be useful to compare the respective costs of pathogen reduction and antimalarial blood treatment. Systematic antimalarial prophylaxis, particularly in young children has been widely used in Africa as long as chloroquine was effective because of its low cost. Reducing the transfusion transmission of malaria in this way with combination antimalarials is widely considered economically unmanageable except in children below age 3 or 5 where the largest benefit is seen.[21,30,31]

In summary, we have shown using a quantitative molecular assay that photochemical treatment of *P. falciparum*–infected whole blood using the riboflavin plus irradiation system induced parasite genome damage and that inactivation of genome replication was more marked with longer target amplicons and with higher total irradiation energies. This dose response was reflected in viability testing in vitro, with the highest irradiation energies being significantly more effective at preventing parasite growth. This approach could be developed as a means to render whole blood donations safer in malaria endemic countries.

CONFLICT OF INTEREST
None of the authors have any conflict of interest regarding this work.

REFERENCES
1. Alexandre MA, Ferreira CO, Siqueira AM, Magalhães BL, Mourão MP, Lacerda MV, Alecrim M. Severe Plasmodium vivax malaria, Brazilian Amazon. Emerg Infect Dis 2010; 16:1611-4.
2. Rijken MJ, McGready R, Boel ME, Poespoprodjo R, Singh N, Syafruddin D, Rogerson S, Nosten F. Malaria in pregnancy in the Asia-Pacific region. Lancet Infect Dis 2012; 12:75-88.
3. Owusu-Ofori AK, Parry C, Bates I. Transfusion-transmitted malaria in countries where malaria is endemic: a review of the literature from sub-Saharan Africa. Clin Infect Dis 2010; 51:1192-8.
4. Bardaji A, Sigauque B, Sanz S, Maixenchs M, Ordi J, Aponte JJ, Mabunda S, Alonso PL, Menéndez C. Impact of malaria at the end of pregnancy on infant mortality and morbidity. J Infect Dis 2011; 203:691-9.
5. Nnaji GA, Ikechebelu JI, Okafor CI. A comparison of the prevalence of malaria parasitaemia in pregnant and non pregnant women. Niger J Med 2009; 18:47-51.
6. Allain JP. Malaria and transfusion: a neglected subject coming back to the forefront. Clin Infect Dis 2010; 51:1199-200.
7. Hassanpour G, Mohebali M, Raeisi A, Abolghasemi H, Zeraati H, Alipour M, Azizi E, Keshavarz H. Detection of malaria infection in blood transfusion: a comparative study among real-time PCR, rapid diagnostic test and microscopy: sensitivity of malaria detection methods in blood transfusion. Parasitol Res 2011; 108:1519-23.
8. Kitchen AD, Chiodini PL. Malaria and blood transfusion. Vox Sang 2006; 90:77-84.
9. 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.
10. 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.
11. 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 2012; 50:1019-27.
12. Vanlandingham DL, Keil SD, Horne KM, Pyles R, Goodrich RP, Higgs S. Photochemical inactivation of chikungunya virus in plasma and platelets using the Mirasol pathogen reduction technology system. Transfusion 2012; 53:284-90.
13. Miltenyi S, Muller W, Weichel W, Radbruch A. High gradient magnetic cell separation with MACS. Cytometry 1990; 11:231-8.
14. Saeed M, Roeffen W, Alexander N, Drakeley CJ, Targett GA, Sutherland CJ. Plasmodium falciparum antigens on the surface of the gametocyte-infected erythrocyte. Plos ONE 2008; 3:e2280.
15. Staalsoe T, Giha HA, Dodoo D, Theander TG, Hviid L. Detection of antibodies to variant antigens on Plasmodium falciparum-infected erythrocytes by flow cytometry. Cytometry 1999; 35:329-36.
16. Allain JP, Hsu J, Pranmeth M, Hanson D, Stassinopoulos A, Fischetti L, Corash L, Lin L. Quantification of viral inactivation by photochemical treatment with amotosalen and UV A light, using a novel polymerase chain reaction inhibition method with preamplification. J Infect Dis 2006;194:1737- 44.

17. Padley DJ, Heath AB, Sutherland C, Chiodini PL, Baylis SA. Establishment of the 1st World Health Organization International Standard for Plasmodium falciparum DNA for nucleic acid amplification technique (NAT)-based assays. Malar J 2008; 7:139.

18. Zavizion B, Pereira M, de Melo Jorge M, Serebryanik D, Mather TN, Chapman J, Miller NJ, Alford B, Bzik DJ, Purmal A. Inactivation of protozoan parasites in red blood cells using INACTINE PEN110 chemistry. Transfusion 2004; 44:731-8.

19. 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.

20. 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; 53:860-7.

21. Grellier P, Benach J, Labaied M, Charneau S, Gil H, Mon- salve G, Alfonso R, Sawyer L, Lin L, Steiert M, Dupuis K. Photochemical inactivation with amotosalen and long- wavelength ultraviolet light of Plasmodium and Babesia in platelet and plasma components. Transfusion 2008; 48:1676-84.

22. Cardo LJ, Salata J, Mendez J, Reddy H, Goodrich R. Pathogen inactivation of Trypanosoma cruzi in plasma and platelet concentrates using riboflavin and ultraviolet light. Transfus Apher Sci 2007; 37:131-7.

23. Marschner S, Fast LD, Baldwin WM 3rd, Slichter SJ, Goodrich RP. White blood cell inactivation after treatment with riboflavin and ultraviolet light. Transfusion 2010; 50:2489-98.

24. Kumar V, Lockerbie O, Keil SD, Ruane PH, Platz MS, Martin CB, Ravanat JL, Cadet J, Goodrich RP. Riboflavin and UV-light based pathogen reduction: extent and consequence of DNA damage at the molecular level. Photochem Photobiol 2004; 80:15-21.

25. Fast LD, Nevola M, Tavares J, Reddy HL, Goodrich RP, Marchner 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 2012; 53:373-81.

26. 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.

27. Freimanis G, Sedegah M, Owusu-Ofori S, Kumar S, Allain JP. Investigating the prevalence of transfusion transmission of Plasmodium within a hyperendemic blood donation system. Transfusion 2013; 53:1429-41.

28. Natukunda B, Schonewille H, Smit Sibinga CT. Assessment of the clinical transfusion practice at a regional referral hospital in Uganda. Transfus Med 2010;20:134-9.

29. Bates I, Chapotera GK, McKew S, van den Broek N. Maternal mortality in sub-Saharan Africa: the contribution of ineffective blood transfusion services. BJOG 2008;115:1331-9.

30. Lackritz EM, Hightower AW, Zucker JR, Ruebush TK 2nd, Onudi CO, Steketee RW, Were JB, Patrick E, Campbell CC. Longitudinal evaluation of severely anemic children in Kenya: the effect of transfusion on mortality and hematologic recovery. AIDS 1997;11:1487-94.

31. Maitland K, Pamba A, English M, Peshu N, Levin M, Marsh K, Newton CR. Pre-transfusion management of children with severe malarial anaemia: a randomised controlled trial of intravascular volume expansion. Br J Haematol 2005;128:393-400.

32. Jimenez-Marco T, Riera C, Fisa R, Fisa R, Girona-Llobera E, Sedeño M, Goodrich RP, Pujol A, Guillen C, Muncunill J. The utility of pathogen inactivation technology: a real-life example of Leishmania infantum inactivation in platelets from a donor with an asymptomatic infection. Blood Transfus 2012;10:536-41.