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ORIGINAL ARTICLE

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Dose-dependent inactivation of *Plasmodium falciparum* in red blood cell concentrates by treatment with short-wavelength ultraviolet light

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Abstract

Background and Objectives: *Plasmodium* species are naturally transmitted by *Anopheles* mosquitos. The parasite infects red blood cells (RBCs) and can be transfused with blood products. In non-endemic areas, the main risk of infection arises from travellers coming back and people immigrating from malaria-endemic regions. Endemic countries face a permanent risk of infection from transfusion-transmitted malaria (TTM). TTM may cause life-threatening complications in patients dependent on blood donations. This study aimed to investigate the efficacy of *Plasmodium falciparum* inactivation in RBC units by treatment with short-wavelength ultraviolet C (UVC) light in the absence of photochemical additives.

Materials and Methods: RBC units were spiked with *P. falciparum* to a parasite density of 0.1%–1% and irradiated with up to 4.5 J/cm^2 UVC. The parasite density of UVC-treated dilution series and untreated controls were compared over 3 weeks after irradiation.

Results: The lowest dose of 1.5 J/cm^2 UVC led to a 3.1 log reduction in parasite load compared with the untreated control. The inactivation capacity was dose-dependent. Strikingly, 4.5 J/cm^2 led to ≥5.3 log unit reduction, which was equivalent to a complete inactivation in two out of three experiments.

Conclusion: Pathogen reduction with UVC light was previously shown to be effective for different bacteria and viruses, but the inactivation of parasites in RBC concentrates was not addressed until now. The present study provides evidence for significant inactivation of *P. falciparum*-infected RBCs by UVC light.

Bernd Lepenies and Ute Gravemann are lead contact.

Keywords
blood safety, pathogen inactivation, pathogen reduction, *Plasmodium falciparum*, transfusion-transmitted malaria, UVC inactivation

Highlights

- Red blood cell concentrates were treated with ultraviolet C (UVC) light of 254 nm wavelength.
- UVC treatment dose-dependently inactivated *Plasmodium falciparum*.
- UVC offers a new strategy for preventing the transmission of malaria through transfusion.

INTRODUCTION

Apicomplexan parasites of the genus *Plasmodium* are the causative agents of the blood-borne infectious disease malaria, which are transmitted by female *Anopheles* mosquitos. Severe malaria cases are mostly caused by *Plasmodium falciparum* [1, 2]. The WHO reported 249 million cases for 2022 and a growing disease burden due to the COVID-19 pandemic, humanitarian crisis, climate change and resistance development of *Anopheles* mosquitos to insecticides [3]. Adults living in malaria-endemic regions often remain asymptomatic or develop mild symptoms, while infants, pregnant women and immunologically naïve people carry a high risk for severe disease [1, 2, 4]. Infected blood products can cause transfusion-transmitted malaria (TTM) [5, 6]. TTM facilitates severe disease progression as it directly leads to blood-stage malaria without the prior asymptomatic liver phase [6]. Infectious red blood cell (iRBC) concentrates and whole blood are the main source of TTM [7–10]. Nevertheless, all blood components carrying residual RBCs can be infectious because 10 parasites or less are sufficient for infection, as shown for *Plasmodium vivax* [11]. In the past 20 years, 20 cases of TTM, mostly caused by *P. falciparum*, were registered in Europe and the United States, both of which are non-endemic regions for malaria [6]. Temporal exclusions of donors from blood donations based on recent travel history limit TTM, but rising numbers of travellers and migrants from endemic areas also favour blood shortages [6, 12]. In a meta-analysis from 2010, it was demonstrated that 10% of blood donations were positive for *Plasmodium* in malaria-endemic countries in Sub-Saharan Africa [13]. Consistently, pathogen reduction technologies (PRTs) gained growing attention during the past years. Studies have shown significant inactivation of *P. falciparum* in whole blood using a combination of riboflavin and ultraviolet (UV) light [14, 15]. Both whole blood and RBC units treated with a combination of amustaline and glutathione exhibited robust inactivation as well, but added 24 h of processing time at room temperature [16, 17]. Inactivation using amotosalen with UVA light was efficient, but only tested in platelet concentrates and plasma, which contain residual numbers of erythrocytes only and have a much higher translucency compared with RBC concentrates [18]. Ultraviolet C (UVC) light was previously shown to be effective for *P. falciparum* inactivation in platelet concentrates [19]. The present study investigates the treatment of RBC units with UVC light at a wavelength of 254 nm. UVC light induces single- and double-strand DNA breaks, but also cyclobutane pyrimidine dimerization, the formation of reactive oxygen species and ultimately the loss of replication ability [20, 21]. It was previously shown that a dose of 4.5 J/cm^2 is sufficient to preserve a good in vitro quality of RBCs while facilitating significant pathogen inactivation [22]. In the present study, we describe a substantial and dose-dependent inactivation of *P. falciparum* in erythrocyte concentrates.

MATERIALS AND METHODS

Blood component preparation

Whole blood units of 500 in 70 mL citrate–phosphate-dextrose solution were collected from blood group 0+ donors screened in accordance with the German national guidelines (see Figure 1 for experimental design). Donations were stored at room temperature overnight (RT; 22 ± 2°C). One day after collection, RBC units were separated by high-speed centrifugation (Roto Silenta 630 RS/63 RS; Hettich, Tuttlingen, Germany) and automated blood component separation (Macopress Smarter; Macopharma, Tourcoing, France). Leucocyte depletion (via RCC flexible filter, Fresenius Kabi, Bad Homburg, Germany) was performed after the addition of 300 ± 10 mL additive solution PAGGS-C as previously described, which is used to improve the quality of UVC-treated RBCs [22]. The separation of RBCs was identical to the routine procedure except for the use of the newly developed additive solution PAGGS-C.

Parasite cultivation

Frozen aliquots of ring-stage parasites at 10% infected RBCs were thawed on ice for 10 min, centrifuged at 400 g for 5 min at RT and resuspended in 3.5% sodium chloride solution for washing. After centrifugation, the cell pellet was washed again with culture medium (500 mL RPMI 1640 [RPMI-XA, Capricorn, Ebsdorfergrund, Germany], supplemented with 2 mM L-glutamine [STA-B, Capricorn], 28 µg/mL hypoxanthine [6416.1, Carl Roth, Karlsruhe, Germany], 50 µg/mL gentamycine [G1397, Sigma Aldrich, Taufkirchen, Germany], 0.5% Albumax II [11550376, Thermo Fisher Scientific, Waltham, USA] and 5 mM HEPES [H4034, Sigma Aldrich]). Cultures were first kept in

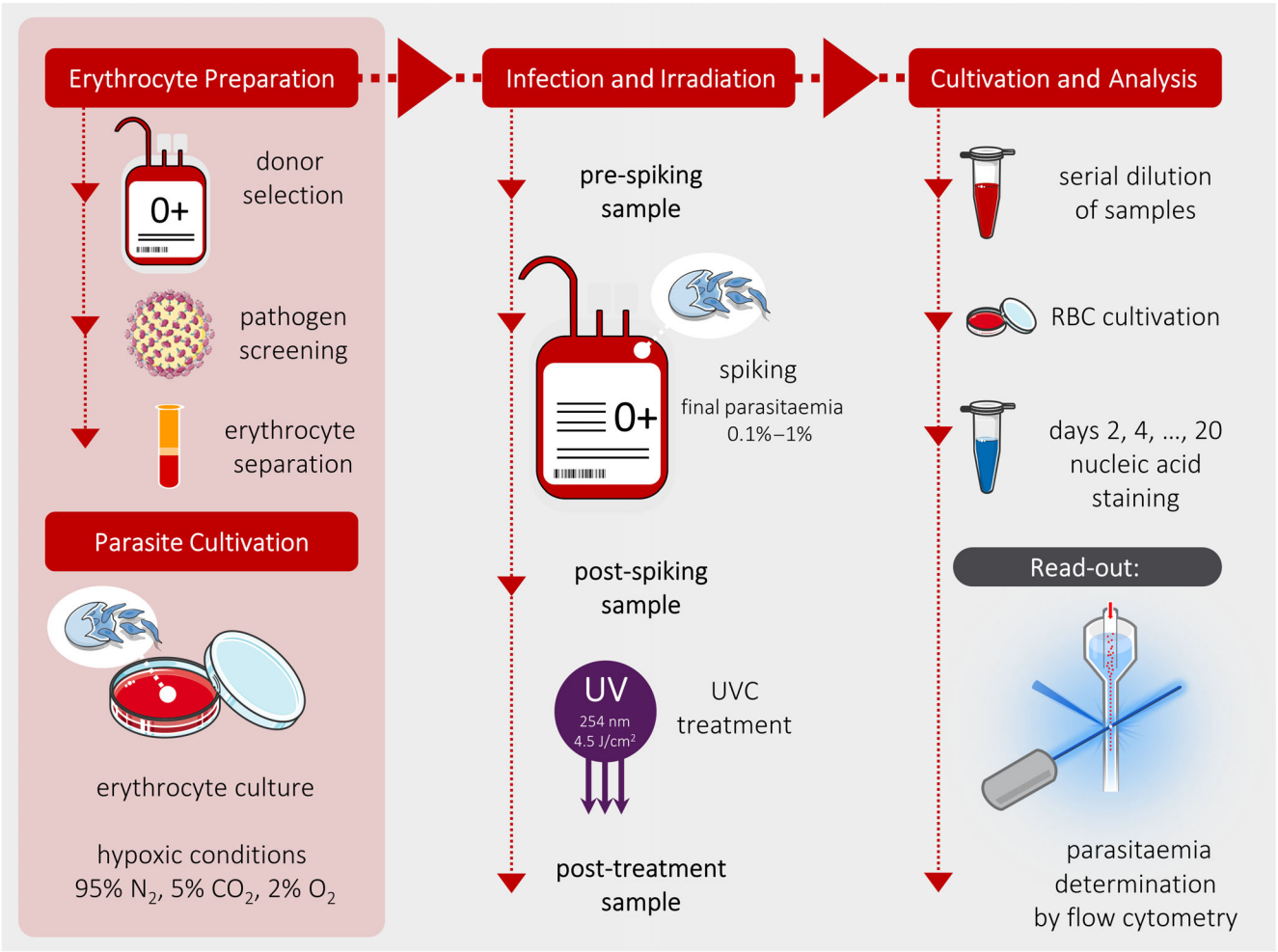

FIGURE 1 Experimental design of the study. Blood donors of blood group 0+ were randomly selected and routinely tested for different infectious diseases based on national guidelines. Red blood cell (RBC) concentrate was obtained by blood component separation and leukocyte depletion. *Plasmodium falciparum* parasites were expanded in an in vitro RBC culture with RPMI 1640 medium. Fresh RBCs were spiked to 0.1%–1% parasite density and sampled as indicated. Spiked RBCs were treated with ultraviolet C (UVC) light of 254 nm and 1.5 J/cm^2 per round with a maximum of 4.5 J/cm^2. Samples were serially diluted (1:10) and cultivated for a minimum of 20 days after inactivation. Every second day, a sample was drawn from each culture and stained for nucleic acids to determine the ratio of *P. falciparum*-infected RBCs among all RBCs.

10-cm cell culture dishes (83.3902, Sarstedt, Nümbrecht, Germany) and for expansion in cell culture flasks (83.3912.002, Sarstedt) and RPMI 1640 medium with 2.8% haematocrit (blood group 0+ to which the parasites are adapted) under hypoxic conditions (93% N_2, 5% CO_2, 2% O_2). Asexual stages of strain 3D7 were used. Parasites became asynchronous during the 1.5-week expansion period and were kept in this state.

Parasite density determination

The parasite density was determined by flow cytometry (Attune NxT Flow Cytometer, Thermo Fisher Scientific), which is an accepted and precise method that allows for a high throughput of samples [23]. A volume of 150 µL of re-suspended culture was centrifuged at 400 g for 2 min at RT. The cell pellet was re-suspended in 200 µL of nucleic acid stain Syto61 (S11343, Thermo Fisher Scientific) at a final concentration of 0.5 µM. Samples were incubated for 10 min before analysis. The mean intensity threshold for Syto61-positive events was always set on the respective day based on a stained *P. falciparum*-negative sample. Analysis of flow cytometric data indicates that heavily UVC-treatment-affected parasites slowly denature within 1–2 weeks, but are not capable of replication anymore (data not shown). Thus, parasites are still positive for Syto61, but the signal decreases over time. Parasites could be unequivocally differentiated from background signals at ≥0.5% parasite density (Figure 2). For definite exclusion of false-positive samples, a threshold of 1% was chosen to define a culture as positive for *P. falciparum* replication upon treatment. The ratio of iRBCs per RBC_{total} multiplied by 100 was defined as the parasite density of a culture.

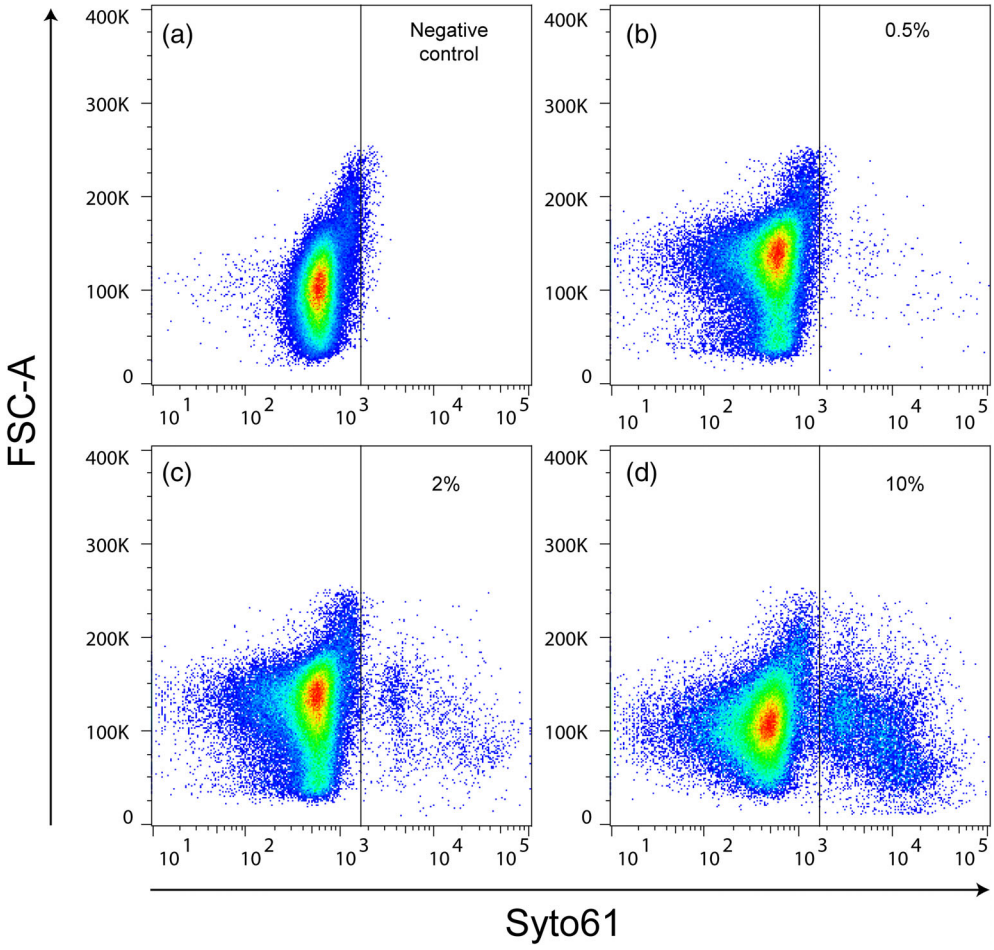

FIGURE 2 Differentiation of infected and uninfected red blood cells by Syto61 staining. Samples of all cultures were taken from D2 onwards every second day until no newly positive cultures were identified. (a)–(d) show example density plots of clearly negative (a) to positive samples with increasing parasite density in ascending order (b: 0.5%, c: 2%, d: 10% parasite density).

Parasite harvest and RBC concentrate spiking

On the day of inactivation (D0), parasite densities of all cultures were individually determined by flow cytometry. The highest in vitro achievable, yet still viable, parasite densities of 8%–14% were used for spiking. The spiking volume was determined based on the volume and haematocrit of the RBC concentrate and the parasite density of the pooled iRBC culture. Parasite cultures were centrifuged at 400 g for 30 min at RT. Cell pellets were re-suspended in 20 mL PAGGS-C and pooled to 10%–12% parasite density before spiking into the inactivation bag. The limit of spiked volume was set to 10% of the RBC unit volume. The final parasite densities of spiked RBC units were therefore 0.1%–1.0%.

Parasite inactivation and sampling

Samples of 4 mL were taken for cultivation at each step (see Figure 1 for experimental design). The negative control (NC) was taken from the inactivation bag after PAGGS-C dilution, but before spiking. The spiking control sample was taken after spiking when iRBCs and uninfected RBCs (uRBCs) had been carefully mixed. The pathogen inactivation treatment by UVC was performed using the MacoTronic illumination device (Macopharma) and the illumination bag from the THERAFLEX UV disposable kit (19 × 38 cm, Ref XUV 4005XU, Macopharma) [22]. Illumination was performed using UVC light at a wavelength of 254 nm, at a final dose of 4.5 J/cm^2. This dose was applied in one step ($n = 1$) or in three consecutive steps of 1.5 J/cm^2 ($n = 2$). During illumination, bags were agitated at 300 rpm to ensure homogeneous exposure of all cells to UVC light. A sample was collected after each illumination step. All samples were kept at RT to ensure comparable conditions during the inactivation procedure.

Sample preparation, cultivation and analysis

All samples were centrifuged at 400 g for 5 min at RT. For the NC sample, 400 µL were directly taken from the cell pellet and taken into culture in a 10 cm cell culture dish with 14-mL RPMI 1640 medium. Spike control and illuminated samples were diluted in a log-fold serial

dilution up to 1:10^8 in fresh uRBCs. A volume of 400 µL of the dilutions was taken into culture as described above. A summary of all samples and dilutions cultivated is given in Table S2. It was determined that a duration of 3 weeks was sufficient to expand a culture from a single parasite to 10% parasite density (data not shown). Therefore, cultures were analysed every 2 days for a minimum of 20 days. Flow cytometric analysis was performed as described above and cultures were considered positive at a parasite density of at least 1%. The titre of iRBC in the spike control was calculated from the parasite density of the spiking material and the spiking volume. For the spike control, the number of iRBCs per plated dilution was correlated with the time to reach 1% parasite density. Based on this correlation, the titre in UVC-treated samples was determined as follows: The number of iRBC in each dilution of UVC-treated samples was calculated from the number of days to reach a parasite density of 1% in the respective sample. The titre in each sample was calculated as a mean from all plated dilutions according to the following equation:

$$c(\text{Sample}) = \sum_{D=n}^{D=0} \frac{\text{iRBC}}{Vn} / n.$$

where c indicates titre of iRBC/mL, iRBC indicates number of infected RBC, D indicates serial dilution, n indicates number of plated dilutions and V indicates volume of plated dilutions (mL).

Samples that remained negative for 21 days were considered to be below the statistical detection limit of 0.007 iRBC/µL, based on a plated volume of 0.4 µL.

Data analysis and statistics

Statistical analysis was performed using commercially available software Microsoft Excel (version 2016 and Professional Plus 2019, Microsoft Office, Redmond, USA) and GraphPad Prism (version 8.4.3 and 9.0.0., Boston, USA) for Windows. Flow cytometric data were analysed with FlowJo (version 10.9.0, Ashland, USA). All data are expressed as mean ± standard deviation (SD). The reduction factors are calculated based on detected parasite density using the following equation:

$$\text{Log reduction} = \text{Log}\,(\text{pre} - \text{treatment titre of iRBC based on spiked parasite density}) - \text{Log}\,(\text{post} - \text{treatment titre of iRBC}).$$

Log reductions at or below the limit of detection are indicated accordingly. The statistical detection limit (95% probability), calculated for a plated volume of 400 µL, was determined according to Rabenau et al. [24].

RESULTS

To analyse the impact of UVC illumination on parasite inactivation in RBC concentrates, illumination was performed in 1.5 J/cm^2 steps and parasite growth was observed for approximately 3 weeks. NC samples were collected before spiking to ensure that RBCs were free of *P. falciparum* or other RBC-infecting parasites containing nucleic acid before spiking. The NC sample was used on all analysis days for flow cytometric gating and differentiating between Syto61-negative and -positive events (Figure 2).

The results of the inactivation study are shown in Table 1. Spiking of the RBC units with a parasite density of 0.1% yielded an average titre of 3 × 10^6 iRBC/mL. UVC treatment resulted in a dose-dependent parasite inactivation. An energy of 1.5 J/cm^2 reduced the infectivity of *P. falciparum* by 3.1 log steps, while 3.0 J/cm^2 reduced it by ≥4.8 log steps. In two out of three experiments, no infectivity was detected after UVC treatment with the standard dose of 4.5 J/cm^2, resulting in a log reduction factor of ≥5.3 ± 0.5 log steps. NC samples remained negative for Syto61 in all three replicates, indicating that the RBC units chosen for spiking were not infected at the time of blood donation (Figure 3, grey). The undiluted spike control reached the threshold of 1% parasite density on Day 2 in all replicates (Figure 3, green). The time to reach 1% parasite density was dependent on the number of iRBCs in the respective sample. Each log dilution took approximately 2 days longer than the previous log dilution to reach a parasite density of 1% (Figure 3, green). The spike control showed no parasite replication in all replicates, highlighting that the critical number of one remaining parasite for re-growth of the culture was exceeded no later than after a 10^8 log-fold dilution, respectively (Figure 3, green).

TABLE 1 Mean log titre of parasitized red blood cells calculated from a parasite density of 0.1%.

	Titre iRBC/mL				Log_{10} reduction factor			
UVC dose (J/cm^2)	**0**	**1.5**	**3**	**4.5**	**0**	**1.5**	**3**	**4.5**
Bag 1	3.3E+06	n.a.	n.a.	≤7.5	0.0	n.a.	n.a.	≥5.6
Bag 2	3.0E+06	5.3E+03	4.0E+02	70.6	0.0	2.8	3.9	4.6
Bag 3	4.0E+06	1.2E+03	≤7.5	≤7.5	0.0	3.5	≥5.7	≥5.7
Mean	**3.4E+06**	**2.2E+03**	**≤1.4E+02**	**28.5**	**0.0**	**3.1**	**≥4.8**	**≥5.3**
SD	**4.2E+05**	**2.3E+03**	**1.9E+02**	**29.8**		**0.4**	**0.9**	**0.5**

Note: Log reduction calculated from log titre pre-treatment–log titre post-treatment. Detection limit of 0.007 iRBC/µL is based on a plated volume of 0.4 mL. ≤Titre and ≥lLog reduction indicate samples with a parasite density below detection limit.
Abbreviations: iRBC, infectious red blood cells; UVC, ultraviolet C.

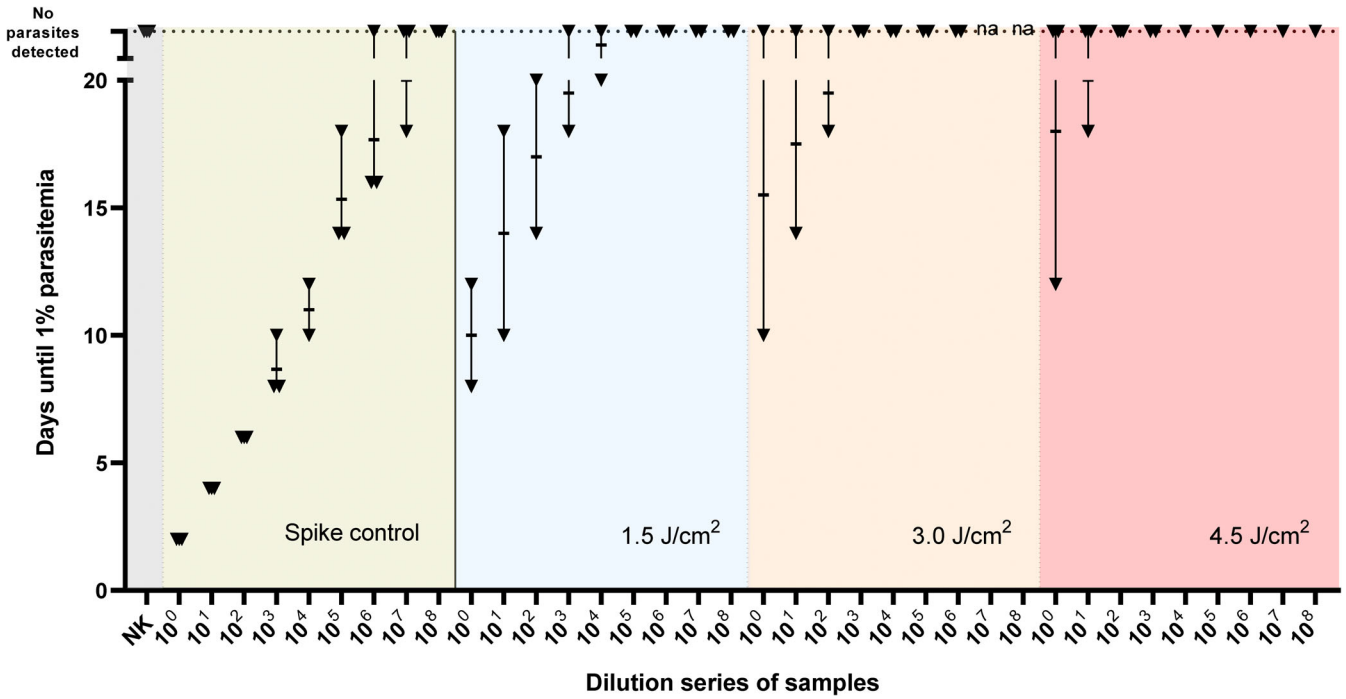

FIGURE 3 Ultraviolet C (UVC) light dose-dependently inactivates *Plasmodium falciparum* in red blood cell (RBC) concentrates. *P. falciparum*-parasitized red blood cell concentrates were illuminated with different doses of UVC light (green—untreated spike control; blue—1.5 J/cm^2; orange—3.0 J/cm^2; red—4.5 J/cm^2). Serial dilutions (1:10) of untreated (green) versus treated samples show dose-dependent inactivation of *P. falciparum* by UVC light. The *x*-axis represents the different dilutions and the *y*-axis days until 1% parasite density was reached by respective samples. Parasite density was determined based on a Syto61 nucleic acid staining. Referring to the undiluted sample, already 1.5 J/cm^2 led to a delay of 6–10 days until 1% parasite density was reached in comparison with the untreated sample that passed this mark on Day 2 already. For 3.0 and 4.5 J/cm^2, this threshold was reached even later or not at all, indicating stronger or complete inactivation of the parasite. Two biological replicates ($n = 2$) were performed for 1.5 and 3.0 J/cm^2, and three biological replicates ($n = 3$) were performed for the negative control, spike control and 4.5 J/cm^2, with each triangle representing one experiment. Error bars show the standard deviation of the two experiments.

DISCUSSION

TTM poses risk of infection, morbidity and mortality. The efficacy of different pathogen inactivation systems has been investigated in the past and may serve as an important means to limit malaria transmission [14–18]. The present study assessed the capacity of the newly developed PI system for RBC concentrates. The system uses short-wavelength UVC light without the addition of photochemicals to eliminate potential side effects, thereby simplifying and accelerating the inactivation procedure. All three replicates showed a highly similar replication rate of *P. falciparum* within the first 2 weeks in cultivation. IRBCs treated with UVC light at 4.5 J/cm^2 were markedly and, in two replicate experiments, even completely inactivated. Differences between replicates were seen, which is not unexpected because inter-recipient differences with respect to parasite replication rate, disease severity and varying host factors are seen in natural infections as well. The inactivation was clearly dose-dependent, with increasing log reduction factors from 1.5 to 4.5 J/cm^2.

The question remains to which extent the UVC-based pathogen reduction method is capable of reducing the risk of TTM and how it can be implemented. Notably, the mean log reduction factor of ≥5.3 ± 0.5 for 4.5 J/cm^2 is insufficient for blood products with high *Plasmodium* loads (e.g., RBC concentrates with 1% parasite density contain millions of parasites per microliter). Thus, the identification of donors with high parasitic loads based on the prevalence of clinical symptoms and by diagnostic methods such as light microscopy is still necessary to prevent transmission through high-density-infected RBC units [7, 25, 26]. However, laboratory screening tests for *Plasmodium* lack the sensitivity for low parasitaemia, which is a risk for TTM [27]. Therefore, UVC treatment could be an additional safety measure and significantly reduce or ideally eliminate the parasitic burden of RBC units derived from asymptomatic donors with low parasitaemia. Furthermore, refrigeration at 4°C, which is already the standard storage condition for RBC units, was described to decrease the number of infective parasites by 86% in 2 weeks [28]. Further studies are needed to estimate the combined inactivation potential of 4°C storage and UVC light treatment. Overall, we propose that a combination of UVC treatment, cold storage and already implemented diagnostics such as blood smears may have a significant impact on TTM eradication.

With 10% of blood products being *Plasmodium*-infected in malaria-endemic areas, UVC treatment could represent a significant advancement, especially in these areas [13]. In non-endemic areas, UVC treatment could be implemented not only to increase the safety of transfusions but also to revise blood donation regulations for

travellers and migrants from malaria-endemic countries to enable a sufficient supply of RBC units. As for all TTID mitigation strategies, there is a remaining risk of incomplete inactivation for high pathogen numbers exceeding the inactivation capacity of the pathogen reduction system [29]. Whether less stringent donor-deferral criteria in non-endemic countries can be applied when implementing UVC treatment will have to be carefully balanced against the paradoxical risk of increased TTM if more semi-immune donors are admitted to blood donation.

In general, the implementation of pathogen reduction processes into routine practice can be a challenge for the production side and may go along with additional costs and a moderate decrease in the quality of the respective blood component. However, it was previously shown that the in vitro quality of UVC-treated RBCs was in an acceptable range and did not affect RBC antigen expression [22].

Notably, the UVC-based pathogen reduction method used in this study was designed to treat RBC units and would require adaptation when applied to whole blood, which is often transfused in endemic countries [30]. However, before clinical implementation of this inactivation approach, additional studies will be necessary for the evaluation of the safety and efficacy of UVC-treated blood products.

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S.F., S.Z., T.J.S., A.S., W.H., B.L. and U.G. conceived and planned the experiments and analysed the data, S.F. and M.R. performed the experiments, B.L. and U.G. supervised the project and S.F. wrote the manuscript, with support from S.Z. All authors read and approved the final manuscript. Open Access funding enabled and organized by Projekt DEAL.

CONFLICT OF INTEREST STATEMENT

S.F., S.Z., M.R., T.J.S., W.H. and B.L. have no conflict of interest to disclose. U.G. and A.S. received grants from the Research Foundation of the German Red Cross Blood Services (Deutsche Forschungsgemeinschaft der Blutspendedienste des Deutschen Roten Kreuzes) and Macopharma for the development of the UVC-based pathogen inactivation technology for platelets. U.G. and A.S. filed a joint patent application for the UVC-based technology for RBCs.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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